THE ATPase COMPLEX OF *Escherichia coli*: STUDIES ON THE DCCD-BINDING PROTEIN

by

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B.Sc., The University of British Columbia, 1977

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTORATE OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Biochemistry

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 1983

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Date 10 June 1983

ABSTRACT

The ATPase complex of E. coli consists of two functional units. ECF_1 is an extrinsic membrane protein having the active site(s) for ATP synthesis and hydrolysis. F_0 is intrinsic and catalyzes the reversible transfer of protons across the membrane. ECF_1 consists of five polypeptides (α-ε) ranging in molecular weight from 13 000 - 57 000. F_0 has three polypeptides (9 000, 18 000, 24 000), the smallest of which is the dicyclohexylcarbodiimide (DCCD)-binding protein postulated to be a transmembrane pathway for proton translocation. An ECF_1F_0 complex was solubilized from the membranes of E. coli with N-lauroyl sarcosine and purified by chromatography on Phenyl-Sepharose CL-4B followed by sedimentation of the enzyme at 250 000 xg for 16-17 h. The purified ECF_1F_0 complex consisted of the eight polypeptides described above, as well as associated polypeptides of molecular weights 30 000, 28 000 and 14 000.

Removal of ECF_1 from the membranes of the wild-type E. coli resulted in the membranes becoming leaky to protons so that they could not be energized. The unc mutants, E. coli AN382, CBT-302 and N_{I44} could maintain a proton gradient across the membrane in the absence of ECF_1. A normal DCCD-binding protein was present in the F_0 complex of each mutant. However, the 18 000 dalton polypeptide of F_0 was absent in the membranes of E. coli N_{I44}, suggesting that it was required for a functional F_0. The involvement of the 18 000 dalton polypeptide in the proton-translocating activity was also suggested by the observation that this polypeptide was absent in the ECF_1F_0 complex immunoprecipitated from trypsin-treated "stripped" vesicles, which had been reconstituted with

ECF_1. Although these trypsin-treated "stripped" vesicles could rebind ECF_1, the membranes could not be energized during ATP hydrolysis.

Leakiness of the membranes to protons could be repaired by the reaction of the ECF_1-stripped membranes with DCCD or ECF_1. Similarly, antibody raised against the DCCD-binding protein prevented this leakage of protons. The antibody also inhibited the rebinding of ECF_1 to the "stripped" everted membrane vesicles. These results indicated that the DCCD-binding protein was exposed on the cytoplasmic surface of the cell. Attempts to show whether the DCCD-binding protein was transmembranous were not successful. Radioimmunoassay techniques were used to show in vitro, the involvement of the arginyl residue(s) of the DCCD-binding protein in the binding of ECF_1. Binding of ECF_1 to the DCCD-binding protein appeared to involve the α and/or ß subunits of ECF_1. Chemical modification of the methionyl residue(s) of the DCCD-binding protein did not alter its capacity to bind ECF_1, but destroyed the antigenic site(s) of the polypeptide. In summary, these results are consistent with the proposed "loop" arrangement of the DCCD-binding protein in which the polar central region of this molecule is at the cytoplasmic surface of the cell membrane.

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ABBREVIATIONS

ADP	- Adenosine-5'-diphosphate.
AH-Sepharose 4B	- Amino Hexyl Sepharose 4B.
Aminoxid WS-35	- Acyl (C_{11}-C_{17}) aminopropyldimethylaminoxide.
ATP	- Adenosine-5'-triphosphate.
Ammonyx Lo	- Lauryl dimethylaminoxide.
Brij 35	- Polyoxyethylene (23) lauryl ether.
BSA	- Bovine serum albumin.
Chloramine T	- N-chloro-4-methylbenzenesulphonamide, sodium salt.
CMC	- Critical micellar concentration.
CM-cellulose	- Carboxymethyl cellulose.
DCCD	- N,N'-dicyclohexylcarbodiimide.
DEAE-Sepharose CL-6B	- Diethylaminoethyl-Sepharose CL-6B.
DNase	- Deoxyribonuclease.
DTT	- Dithiothreitol.
EDTA	- (Ethylenedinitrilo)-tetraacetic acid.
EGTA	- [Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid.
F_1-ATPase	- ATP phosphohydrolase (catalytic portion of the proton-translocating adenosine triphosphatase); CF_1, chloroplast F_1-ATPase; ECF_1, E. coli F_1-ATPase; MF_1, mitochondrial F_1-ATPase; TF_1, F_1-ATPase from the thermophile, PS3.
F_1F_0-ATPase complex	- Proton-translocating adenosine triphosphatase; ECF_1F_0, E. coli F_1F_0 complex; TF_1F_0, F_1F_0 complex from the thermophile, PS3.
HEPES	- N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid.
HPLC	- High performance (pressure) liquid chromatography.

Lubrol 17A-10	- Polyoxyethyleneglycol (n=?) cetyl-stearyl alcohol.
Lubrol PX	- Polyoxyethyleneglycol (n=?) cetyl-stearyl alcohol.
Lubrol WX	- Polyoxyethyleneglycol (17) cetyl-stearyl alcohol.
MOPS	- Morpholinopropanesulphonic acid
NADH	- Reduced nicotinamide adenine dinucleotide.
NAP_4-ADP	- 3'-O-(4-[N-(4-azido-2-nitrophenyl) amino] butyryl) ADP.
NAP_4-ATP	- 3'-O-(4-[N-(4-azido-2-nitrophenyl) amino] butyryl) ATP.
Nbf-Cl	- 4-chloro-7-nitrobenzofuran.
NEM-Hg	- Mercuriated N-pyrrolo-isomaleinimide.
Ninhydrin	- 1,2,3-triketohydrindene hydrate.
Nonidet P-40	- Polyoxyethyleneglycol (9) p-t-octylphenol.
Pi	- inorganic phosphate.
PBS	- Phosphate-buffered saline (0.137 M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4, pH 7.5).
PEG	- Polyethyleneglycol.
PMS	- Phenazine methosulphate.
PMSF	- Phenylmethysulphonylfluoride.
psi	- Pounds per square inch.
RNase	- Ribonuclease.
SDS	- Sodium dodecyl sulphate.
Sephadex LH-20	- Hydroxypropyl Sephadex G-25.
Sephadex LH-60	- Hydroxypropyl Sephadex G-50.
TAMM	- Tetrakis (acetoxymercuri) methane.
TCA	- Trichloroacetic acid.

TEMED	- N,N,N',N'-tetramethylethylenediamine.
TPCK-trypsin	- Trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone.
Tris	- Tris (hydroxymethyl)-aminoethane.
Triton X-100	- Polyoxyethyleneglycol (9-10) p-t-octylphenol.
Triton X-114	- Polyoxyethyleneglycol (7-8) p-t-octylphenol.
Tween 60	- Polyoxyethyleneglycol (20) sorbitol monostearate.
Tween 80	- Polyoxyethyleneglycol (20) sorbitol monooleate.
$\Delta \psi$	- Membrane potential.
Δ pH	- Difference in pH across the membrane.
$\Delta \tilde{\mu}_{H^+}$	- Electrochemical potential difference of protons across the membrane, proton-motive force.

ACKNOWLEDGEMENTS

I thank Dr. P.D. Bragg for his advice, patience and understanding throughout my graduate studies and during the writing of this thesis.

The completion of this thesis would not have been possible without the help of the many members of the Department of Biochemistry. In particular, I thank Drs. R.S. Molday, R. Barton and J.C. Brown (Physiology) for access to the equipment in their laboratories. I especially thank Mr. D.J. Mackenzie for help in setting up the radioimmunoassay experiments and for the photography. I am very grateful to my colleagues, Mr. David M. Clarke, Ms. Cynthia Hou and Dr. Helga Stan-Lotter for providing a pleasant atmosphere which was conducive to research and collaboration. I am also grateful to the Medical Research Council of Canada for supporting this work. The typing of this manuscript by Ms. Judith Smith is greatly appreciated.

Finally, I would like to acknowledge the support of my family during this project.

INTRODUCTION

The reversible, proton-translocating adenosine triphosphatase (F_1F_0-ATPase complex) plays a major role in the energy-transduction reactions of the cell. Structurally similar forms of the enzyme are found in the membranes of eukaryotes and prokaryotes (1-4). The enzyme catalyzes the synthesis of ATP by oxidative or photo-phosphorylation, and the hydrolysis of ATP (5) according to the reaction.

$$nH^+_{in} + ATP + H_2O \quad ADP + P_i + nH^+_{out}$$

The ATPase complex consists of two functional units, F_1 and F_0. F_1 is a complex of extrinsic membrane proteins containing the active site(s) of ATP synthesis and hydrolysis (6-8). The F_1 interacts with F_0, which is a complex of integral membrane proteins not having ATPase activity. The F_0 complex is thought to extend through the membrane and functions as a pathway for the reversible translocation of protons through the membrane (9,10,14).

The existence of reversible, proton-translocating pumps is an important postulate of Mitchell's chemiosmotic hypothesis on the mechanism of oxidative phosphorylation (11). According to this hypothesis, the components of the electron transport chain are arranged in the membrane such that there is vectorial translocation of protons across the membrane during respiration or light-dependent electron flow (Fig 1) (5,12,13). The vectorial translocation of protons generates a proton-motive force ($\Delta\mu_H+$), which consists of two components: the membrane potential ($\Delta\psi$), generated as a result of charge separation, and the chemical concentration gradient, ($Z\Delta pH$). The relation is given by the following equation:

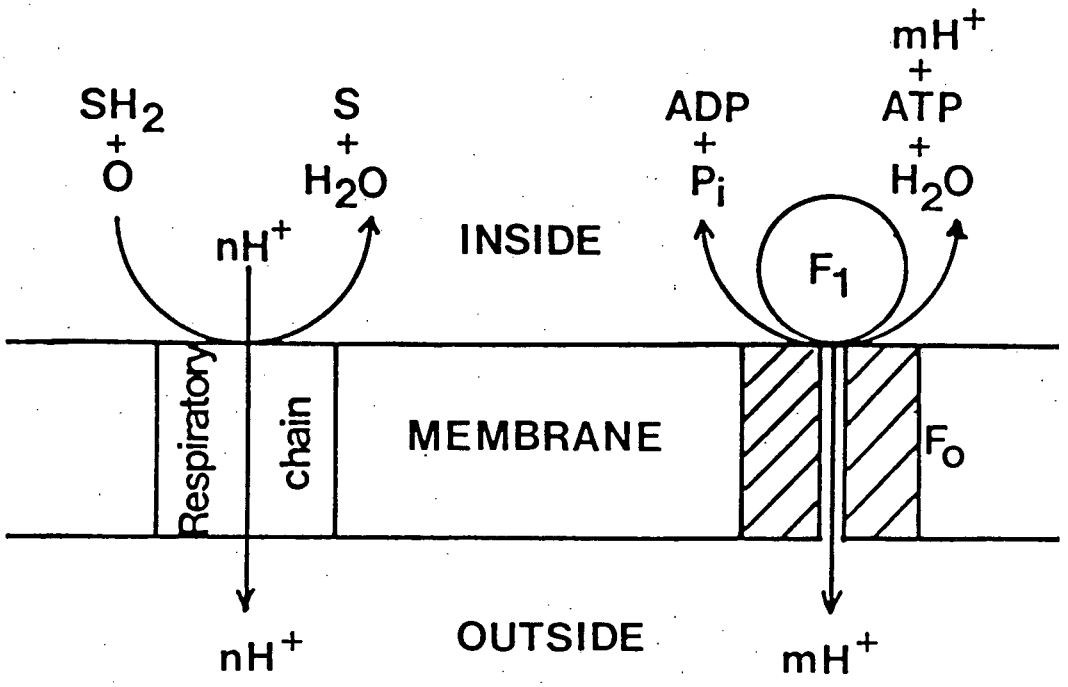


FIG 1: Schematic representation of oxidative phosphorylation and generation of a proton gradient. "mH^+" and "nH^+" represent protons which are translocated, but whose exact number has not been agreed on.

$$\Delta\tilde{\mu}_{H^+} = \Delta\psi - Z\Delta pH \quad (\text{where } Z = \frac{2.3\ RT}{F} = 59\ \text{mV at } 25°C)$$

The energy stored within this proton gradient is used to drive other energy-requiring processes. For example, the synthesis of ATP is coupled to the return of protons down the gradient through the F_1F_0-ATPase complex. Similarly, the proton gradient can be regenerated through the hydrolysis of ATP. A stoichiometry of two or three protons translocated per molecule of ATP hydrolyzed or synthesized has been measured (5,15).

Some microorganisms such as Streptococcus faecalis, Streptococcus lactis (2) and the strict anaerobe Clostridium pasteurianum (16), growing in the presence of a limiting amount of glucose or in the absence of oxygen or other terminal electron acceptors, cannot carry out oxidative phosphorylation. This is because their respiratory chains are either non-functional or non-existent. These organisms rely solely on the ATP produced by substrate-level phosphorylation (glycolysis) and the F_1F_0 complex to generate a proton-motive force, which is capable of driving other energy-requiring processes.

LOCATION OF THE F_1F_0 COMPLEX

ATPase activity was shown to be localized in the plasma membrane of Streptococcus faecalis (17), Bacillus cereus and Escherichia coli (18). Ferritin-labelled antibody against purified F_1-ATPase was used to show that the F_1 was located on the inner surface of the bacterial plasma membrane (19). Negatively-stained preparations of everted membrane vesicles of E. coli revealed the presence of "knobs", each with a diameter of about 100 A, attached to the inner surface of the plasma membrane via a "stalk". A similar morphology exists in the submitochondrial particles and

in the thylakoid membranes (6,20).

ROLE OF THE F_1F_0 COMPLEX IN ENERGY TRANSDUCTION

The role of the F_1F_0 complex of E. coli in energy-transduction reactions has been demonstrated through reconstitution studies and the isolation of mutants.

The F_1 component of the F_1F_0 complex can readily be released from the everted membrane vesicles of E. coli by washing them in buffers of low ionic strength containing EDTA (21-23,33). The ECF_1-depleted membranes no longer exhibit energy-transducing properties such as respiration-induced proton uptake, ATP-driven and respiration-driven transhydrogenation of $NADP^+$ by NADH, or oxidative phosphorylation. Restoration of these activities to normal levels can be achieved by the addition of purified ECF_1-ATPase to the depleted membranes in the presence of Mg^{2+} or high ionic strength buffer (21-25,33). These results suggest that the loss of the energy-transducing properties of ECF_1-depleted membranes is due to an increased permeability of the membranes to protons upon the removal of ECF_1, such that the proton-gradient cannot be built up or maintained by respiration.

Proton impermeability can also be restored in the ECF_1-depleted membranes by treatment with either ECF_1, isolated from an unc A mutant, which has no ATP hydrolytic activity or with DCCD. DCCD is a potent inhibitor of the ATPase activity of the F_1F_0 complex and under appropriate conditions it reacts with a specific component of F_0 and inhibits proton-translocating activity (discussed under "The DCCD-binding protein").

In the E. coli mutants, DL-54 and NR-70, the F_1 is either defective

or absent (26,27). Membrane vesicles or whole cell preparations of these mutants show decreased levels of respiration-driven transhydrogenation and transport of proline than in the corresponding parent-strains. K^+-loaded spheroplasts of E. coli DL-54 also show a larger change in the pH of the external medium upon addition of valinomycin, than is seen with the wild-type strain (28). The increased permeability of these mutant membranes to protons is due to an exposed F_0 since the addition of DCCD or ECF_1 causes the membranes to become less permeable to protons.

These results suggest (24) that (i) the ECF_1 is responsible not only for the energization of the membrane through ATP hydrolysis, but also for maintaining the impermeability of the membranes to protons by interacting with F_0, and (ii) the F_0 acts as a proton-specific channel or pore.

The concept of F_0 being a proton-specific pore is complicated by the observations that its proton-conducting properties in E. coli DL-54 and NR-70 varies significantly when the growth conditions are varied in aerobic culture (29-32).

PROPERTIES OF F_1

Subunit Composition of F_1

The molecular weight of ECF_1 has been determined by a variety of methods, with the following values: gel filtration, 360 000 - 390 000 (33); sedimentation equilibrium, 360 000 - 390 000 (34); laser light scattering, 362 000 (35); small angle X-ray scattering, 35 8000 (36); sedimentation coefficient, diffusion coefficient and partial specific volume, 350 000 (36); light scattering, 345 000 (36) and small angle neutron scattering, 315 000 (37).

Three problems exist which make it difficult to determine the correct molecular weight of ECF_1 (6). First, there is the tendency of the enzyme to dissociate, resulting in the loss of some subunits of ECF_1. Secondly, the extent of the loss of the δ subunit during purification of ECF_1 is variable and this is dependent to some extent on the choice of the purification procedure. Finally, the amount of δ subunit in ECF_1 preparations is also dependent on the source, for the amount of δ subunit in F_1 preparations isolated from E. coli K_{12} strains is more variable than that from the E. coli ML strains. These factors cause the molecular weight of the ECF_1 to be underestimated. The actual molecular weight of ECF_1 may be closer to the molecular weight of the more stable TF_1, which has been reported to be 380 000 (38).

The F_1 from all sources (with the exception of Clostridium pasteurianum and Lactobacillus casei) generally contain five different polypeptides (α, ß, γ, δ and ε) (4). MF_1 has an additional subunit (Table 1) which is a natural inhibitor of the ATPase activity, but is unnecessary for ATP synthesis (24). The F_1 from C. pasteurianum (16) consists of only three different subunits with molecular weights of 65 000, 57 000 and 43 000, while that from L. casei (39) contains only one polypeptide of 43 000 daltons.

The molecular weights of each of the subunits of ECF_1, TF_1, CF_1 and MF_1 are very similar (40), with those of ECF_1 reported to be α, 56 800; ß, 51 800; γ, 32 000; δ, 20 700; and ε, 13 200 (7,41). These are in good agreement with the molecular weights determined from the DNA sequence (42) of each polypeptide: α, 55 264; ß, 50 157; γ, 34 100; δ, 19 310; and ε, 14 194.

The ratio of these subunits in F_1 remains controversial. Most of

the data on F_1 from bacteria and yeast mitochondria support an $\alpha_3\beta_3\gamma\delta\epsilon$ stoichoimetry (8,41,43-44,72,155). Although the data on the F_1 from mammalian mitochondria and chloroplast suggest $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ and $\alpha_2\beta_2\gamma\delta\epsilon_{(1-2)}$ stoichiometries, respectively (45-48); more recent estimates support the $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry (49).

Tightly-Bound Nucleotides

CF_1, ECF_1, MF_1 and TF_1 all contain non-covalently bound nucleotides, which cannot be removed even after extensive purification of the enzyme (50,51,73). In E. coli, three molecules of tightly-bound nucleotides per molecule of F_1 were detected. The only nucleotides found to be present were ATP and ADP. Different molar ratios of these nucleotides were reported, but this could be attributed to the different procedures used for the preparation of ECF_1 and for nucleotide detection.

The function(s) of these tightly-bound nucleotides has not been established. The turnover rate of the tightly-bound ATP is too slow for it to be involved as an intermediate in oxidative phosphorylation (52). It is more likely that these tightly-bound nucleotides are present in the "regulatory" site rather than in the active site. At present, there is very little information about the regulation of the enzyme in vivo.

FUNCTION OF THE SUBUNITS OF ECF_1

Understanding the function and arrangement of the subunits of the ECF_1 is essential to the determination of the mechanism of oxidative phosphorylation. Several approaches to the study of the function and properties of the subunits of ECF_1 have been used. These have included chemical modification of specific residues, affinity labelling,

immunological techniques, genetics, and subunit isolation and holoenzyme reconstitution.

Recently, methods have been developed for the dissociation and isolation of the subunits of ECF_1 in non-denatured form such that a functional ATPase could be reconstituted from its subunits (6).

The Delta (δ) Subunit

ECF_1 preparations can be obtained which are deficient in the δ subunit (53-57). This δ-deficient, four-subunit enzyme (i.e. $\alpha_3\beta_3\gamma\varepsilon$), has an ATPase activity equal to that of the native, five-subunit enzyme. However, the former is incapable of reconstituting respiration-driven and ATP-driven transhydrogenase activity in F_1-depleted membranes. Also, ATPase activity was not detected in the depleted membranes, which had been reconstituted with the δ-deficient ATPase.

Smith and Sternweiss (58,59) have purified the δ subunit, by treatment of ECF_1 with 50% pyridine followed by chromatography of the fraction containing the δ and ε subunit on Sephadex G-75. Addition of the purified δ subunit to the δ-deficient enzyme restored the membrane-binding capability of the enzyme. Besides rebinding, ATP-driven transhydrogenase activity and the formation of ATP by oxidative phosphorylation were restored in these depleted membranes (57). These results suggest that the δ subunit is involved in the binding of ECF_1 to the membrane and that the binding of δ restores the ATPase to its native state. The role of the δ subunit in attaching the F_1 to the membrane has also been confirmed for CF_1 (60), TF_1 (61) and F_1 from Streptococcus faecalis (62).

The Epsilon (ε) Subunit

By passing the δ-deficient, four-subunit enzyme (i.e. $\alpha_3\beta_3\gamma\varepsilon$) through an affinity column containing immobilized antibodies to the ε subunit, a three-subunit enzyme (i.e. $\alpha_3\beta_3\gamma$) which is deficient in the ε subunit has been obtained (63). This three-subunit enzyme had an ATPase activity which was 10-15% greater than that of the native, five-subunit enzyme. The purified δ or ε subunit could bind to the three-subunit enzyme to form four-subunit complexes (i.e. $\alpha_3\beta_3\gamma\delta$ or $\alpha_3\beta_3\gamma\varepsilon$). Neither of these complexes could bind ECF_1-depleted membranes and reconstitute oxidative phosphorylation activity unless both δ and ε were present. This suggests that both δ and ε are involved in binding ECF_1 to the membrane and that the "stalks" seen in negatively-stained preparations of everted E. coli membrane vesicles may be composed of the δ and ε subunits (6).

Thus, it might be expected that the δ and ε subunits should interact directly with the F_0 component in the membrane. However, the binding of either purified δ or ε subunit to ECF_1-depleted membranes could not be detected by using antibodies raised against either of these subunits. Furthermore, a mixture of purified γ, δ and ε subunits did not reduce the proton permeability of the depleted membranes as measured by respiration-driven transhydrogenase (6,63). By contrast, both the δ and ε subunits of TF_1 could bind to the TF_0 which had been reconstituted into liposomes (61) and to this TF_0 - $\delta\varepsilon$ complex could be bound either the purified γ subunit or a 3 subunit enzyme of TF_1, containing $\alpha_3\beta_3\gamma$.

A second function has been assigned to the ε subunit. It is a non-competitive inhibitor of the ATPase activity of purified ECF_1.

Addition of the purified ε subunit to either the five-subunit enzyme ($\alpha_3\beta_3\gamma\delta\varepsilon$), the four-subunit enzyme ($\alpha_3\beta_3\gamma\delta$) or the three-subunit enzyme ($\alpha_3\beta_3\gamma$) resulted in 70-90% inhibition of the ATPase activity (58,64). The inhibition of the activity of the five-subunit enzyme can be explained by the dissociable nature of the ε subunit. The ATPase activity of an ECF_1 preparation was increased by more than four-fold following dilution of the enzyme preparation. This suggested that by diluting the ECF_1 preparation, the endogenous ε subunit dissociated from the enzyme and was in equilibrium with the ECF_1-ε complex. Addition of anti-ε-serum to an ECF_1 preparation also stimulated the activity of the enzyme by more than two-fold. Immunoprecipitation of a reconstituted five-subunit enzyme, in which the ε subunit was labelled with ^{125}I, with anti-ε-serum, resulted in quantitative precipitation of radioactivity (65). However, no ATPase activity was detected in the precipitate. The anti-ε-serum, therefore promoted the dissociation of ε from the F_1 either directly or by the removal of free ε which was in equilibrium with the ECF_1-ε complex.

It appears that the relationship of the ε subunit to ECF_1 is similar to that of the mitochondrial ATPase inhibitor protein to MF_1, in that both may be involved in determining whether the ATPase is operating in the direction of ATP hydrolysis or synthesis. It has been suggested (66,67) that substrate oxidation or a low molar ratio of ATP/ADP tends to decrease the interaction between the inhibitor and MF_1. The kinetic data (64) on ECF_1 also suggest that ATP accelerates the release of the ε subunit from ECF_1, whereas ADP prevents this activation, perhaps by stabilizing the interaction of ε with ECF_1.

The ε subunit of ECF_1 differs from the mitochondrial inhibitor protein (6) in that (i) the mitochondrial ATPase inhibitor protein is not

necessary for attaching MF_1 to the membrane, and (ii) the mitochondrial ATPase inhibitor protein inhibits the ATPase activity of the membrane-bound enzyme, whereas an excess of ε subunit has no effect on this activity in E. coli membranes.

The Gamma (γ) Subunit

The activity of purified ECF_1 is stimulated by up to 100% by brief treatment of the enzyme with trypsin (41). Examination of the trypsin-treated enzyme on SDS-polyacrylamide gels revealed that the δ and ε subunits, and to a small extent the γ subunit, were completely destroyed (53,63). The resulting enzyme following trypsin-treatment is essentially a two subunit enzyme (i.e. $\alpha_3\beta_3$) with three copies of each subunit. Dunn and coworkers (68) have shown that trypsin-treatment removes the first 15 residues from the NH_2 terminus of each of the α subunits and that a fragment of the γ subunit of 10 000 dalton remained bound to the enzyme.

Although the purified ε subunit inhibited the ATPase activity of the three-subunit enzyme (i.e. $\alpha_3\beta_3\gamma$), it had no effect on the activity of the trypsin-treated enzyme. Incubation of the trypsin-treated enzyme following cold-dissociation with a γ-rich fraction prepared from native ECF_1 restored the sensitivity of the trypsin-treated enzyme to inhibition by the ε subunit (69). These results suggest that the γ subunit is required to bind the ε subunit to ECF_1 and is supported by the observation (70) that there is a high affinity interaction between purified γ and ε subunits, in a 1:1 ratio. This interaction is specific since interactions of the purified ε with either purified α or ß subunits was not detected. Also, the addition of purified γ subunit to a purified ε preparation prevented the latter from inhibiting the ATPase activity of a three-subunit

enzyme ($\alpha_3\beta_3\gamma$).

Thus the γ subunit not only binds the ε subunit but it, or a portion of it, also holds the α and ß subunits together.

The Alpha (α) and Beta (ß) Subunits

ECF_1 has the property of being cold-labile and cold-dissociable. Taking advantage of this, Vogel and Steinhart (77) dissociated the enzyme by freezing it in a solution of high ionic strength. Through ion-exchange chromatography, the dissociated enzyme was separated into three fractions, none of which possessed any catalytic activity. Fraction 1 contained the α, γ and ε subunits, fraction 2, the α, γ, δ and ε subunits, and fraction 3, only the ß-subunits. ATPase activity could be reconstituted by combining either fractions 1 and 3 or 2 and 3 but not 1 and 2. This was the first indication that the active site(s) for ATP hydrolysis was probably on the α and ß subunits.

Recently, Dunn and Futai (72) have been able to purify the individual subunits from the cold-dissociated enzyme. The purified subunits could be reconstituted to give enzyme activity. The highest specific activity was obtained when the molar ratio of the α, ß and γ subunits was 3:3:1 (i.e. $\alpha_3\beta_3\gamma$). The γ subunit was essential for expression of ATPase activity since a reconstituted α and ß (1:1) preparation had only 0-10% of the maximum activity.

ECF_1 of the unc A mutant has no hydrolytic activity, but is indistinguishable from the parent enzyme in subunit composition, bound nucleotides and possession of an inhibitor-sensitive (Nbf-Cl) tyrosine residue on the ß subunit (24,73). The activity of the mutant ECF_1 could be restored by replacing the cold-dissociated enzyme fraction containing

the α, γ and ε subunits with the corresponding fraction obtained from the wild-type strain (74). Similarly, the ATPase activity could be restored in the F_1 from the unc A mutant, E. coli AN120, by dialyzing the total cold-dissociated enzyme together with an excess of purified α-subunit from the wild-type strain. The ß and γ subunits, purified from the wild-type ECF_1 did not restore ATPase activity in the mutant enzyme. These results indicate that the lesion responsible for the lack of ATPase activity in the unc A mutant resides in the α subunit (75,76).

STUDIES ON THE ACTIVE SITE OF ECF_1

It was previously mentioned that the tightly-bound nucleotides of ECF_1 are unlikely to be involved as intermediates of oxidative phosphorylation and are probably involved in the regulation of enzyme activity. Therefore, the intermediates of oxidative phosphorylation must be at other nucleotide-binding sites. Detection of these binding sites have involved the use of photo-affinity labels as well as compounds which label specific amino acids and inhibit the ATPase activity of the isolated ECF_1 (6).

Under slightly acidic conditions (pH 6.5), the compound DCCD reacts with a carboxyl residue on the ß subunit of ECF_1 and inhibits the ATPase activity (77,78). The reaction with DCCD also affected the ability of the ECF_1 to bind to ATP, suggesting that there is a loss of an ATP-binding site.

The compound Nbf-Cl, which can react with cysteine and tyrosine residues, inhibits the ATPase activity of ECF_1. Prolonged incubation of ECF_1 (24 h) with [^{14}C]Nbf-Cl (53), resulted in the ß subunit being preferentially labelled, but after a shorter period of incubation (30 min) (79), most of the label was associated with the α subunit. The binding

of Nbf-Cl to ECF_1 also resulted in the loss of a nucleotide-binding site (80). Photo-affinity labelling of ECF_1 with arylazido analogs of ATP and ADP (NAP_4-ATP and NAP_4-ADP) showed that the inactivation of ATPase activity was associated with the incorporation of 2 mol NAP_4-ADP per mol ECF_1 (80). At low concentrations (5 μM) of either analog, the α subunit was preferentially labelled, but at high concentrations (75 μM), both α and ß subunits were labelled to the same extent. This suggests the presence of a high affinity nucleotide-binding site(s) on the α subunit and a lower affinity binding site on the ß subunit and that the interaction of both sites may be essential for expression of activity. Other labels which also bind to the α subunit are [2,8-^{3}H]-ATP, [2,8-^{3}H]-ADP (72) and 8-azido ATP (79). In the ECF_1 of the unc A mutant, E. coli AN120, the α subunit was not labelled with 8-azido ATP.

Bragg et al. (81,82) have used the 2',3'-dialdehyde derivates of ADP(oADP) and ATP(oATP) to probe for nucleotide-binding sites involved in ATP hydrolysis in ECF_1. These compounds react covalently at their respective binding sites. Only the binding of oADP resulted in the inhibition of ATPase activity. Both the oADP and oATP binding sites were located on the α subunit. The α subunit of the unc A mutant, E. coli AN120, did not possess the oADP binding site(s). These results suggest that the oADP-binding site on the α subunit may be the active site(s) for ATP hydrolysis, while the oATP-binding site(s) may be regulatory sites.

The compounds DCCD and Nbf-Cl also affected the binding of oADP to the α subunit. The binding of oATP was influenced to a lesser extent. The inhibition of binding of oADP to the active site may be due to a conformational change induced in the α subunit as a result of reaction of the ß subunit with DCCD or Nbf-Cl. However, it is also possible that the

presence of these substituents on the ß subunit could sterically prevent the binding of oADP. The results suggest that the oADP-binding site on the α subunit must likely be adjacent to the active carboxyl, tyrosyl or cysteinyl residue on the ß subunit. Therefore, the active site would likely be at the interface of the α and ß subunits. Such an arrangement would explain why individual α or ß or both subunits have low ATPase activity and that the maximum activity is obtained when the α, ß and γ subunits are present. Presumably the γ subunit is necessary to hold the α and ß subunits in the proper conformation, such that the active site is preserved.

CROSS-RECONSTITUTION STUDIES

Reconstitution of energy-transducing reactions in F_1-depleted membranes with F_1 is not restricted to ATPases from the same species. Schatz et al. (83) first reported hybrid reconstitution between F_1 from Baker's yeast and F_1-depleted submitochondrial particles of beef heart, with the restoration of oxidative phosphorylation activity. Respiration and ATP-driven transhydrogenase activity was also demonstrated in F_1-depleted membranes of *E. coli*, which were reconstituted with F_1 from *Salmonella typhimurium* (41). Similarly, hybrid reconstitution between rat liver MF_1 and F_1-depleted submitochondrial membranes from human liver carcinoma has also been demonstrated (84). More recently (85), ATP-driven proton uptake was demonstrated in the membranes by cross-hybrid reconstitution between F_1 and F_1-depleted membranes of either *E. coli* or rat liver mitochondria.

Hybrid F_1 has also been reconstituted by using subunits of F_1 isolated from different sources (86,87). The following combinations, in

the molar ratios indicated were found to contain ATPase activity: $\alpha_3^E\beta_3^E\gamma^T$ (i.e. α and ß subunits from ECF_1 and the γ subunit from TF_1), $\alpha_3^T\beta_3^T\gamma^E$ and $\alpha_3^E\beta_3^T\gamma^E$. The γ subunit seems to be mutually interchangeable. These reconstituted hybrid enzymes had physical properties, such as thermostability and optimum pH, which were different from either ECF_1 or TF_1.

ATPase activity could not be reconstituted by randomly combining the subunits. For example, the combinations: $\alpha_3^T\beta_3^E\gamma^E$ and $\alpha_3^E\beta_3^T\gamma^T$ or $\alpha_3^T\beta_3^E\gamma^T$ did not reconstitute ATPase activity.

THE ARRANGEMENT OF THE SUBUNITS OF ECF_1

The arrangement of the subunits of ECF_1 has been examined by two general methods. The first method has involved the reconstitution with the isolated subunits, or various forms of ECF_1, and their binding to ECF_1-depleted membranes as was discussed earlier. In this respect, one has to be fairly cautious in interpreting the data because these reconstitution experiments do not take into consideration the possible conformational change induced in the enzyme, when a purified subunit is added.

The second approach has been through the use of cleavable, cross-linking reagents, such as dithiobis (succinimidyl propionate) and cupric 1,10-phenanthroline, which will cross-link suitably placed amino and sulphydryl groups, respectively. The results of such experiments (88,89) indicated cross-linking between αα, αß, αδ, ßß, ßγ, ßδ, ßε, γε and possibly αγ. There was no formation of γδ or δε pairs, but it has been pointed out that the absence of cross-linked products does not exclude the possibility of other subunits being in close proximity. The results of these cross-linking experiments are often compatible with those obtained

through reconstitution studies.

αß, αγ and ßγ interaction are suggested by the observations that the reconstitution of ATPase activity from the individual subunits require the γ subunit. A γε interaction has been demonstrated by Dunn (70) using purified γ and ε subunits. The αδ interaction is also suggested in the experiments of Dunn et al. (68) in which they have shown that the purified δ subunit does not bind to trypsin-treated ECF_1 because of the removal of 15 amino acids from the NH_2-terminal region of the α subunits.

Electron micrographs of purified ECF_1 which has been negatively-stained with uranyl acetate suggest that the enzyme is a planar hexagon of six subunits about a central core (7). The presence of three subunits of both α and ß suggest that the six peripheral subunits of the hexagon are likely to be composed of α and ß subunits.

It is difficult to propose a unique arrangement of the subunits of ECF_1 because the data do not completely support any one particular model. Three models (Fig. 2) have been proposed (7,89): i) Model 1 consists of a slightly puckered hexagon of alternating α and ß subunits around the central γ subunit. The ε and δ subunits are placed on opposite sides of the hexagon. This arrangement of the δ and ε subunits contradict the results from the reconstitution experiments in which both δ and ε were required to bind ECF_1 to the membrane. In support of this arrangement is the finding that anti-δ-serum detached the entire ECF_1 from the membrane without affecting the ATPase activity, whereas anti-ε-serum selectively removed only the ε subunit (65). ii) Model 2 is similar to Model 1 except that the α and ß subunits are stacked as pairs. iii) Model 3 is different from models 1 and 2 in that the α and ß do not alternate and the δ and ε subunits are on the same side of the

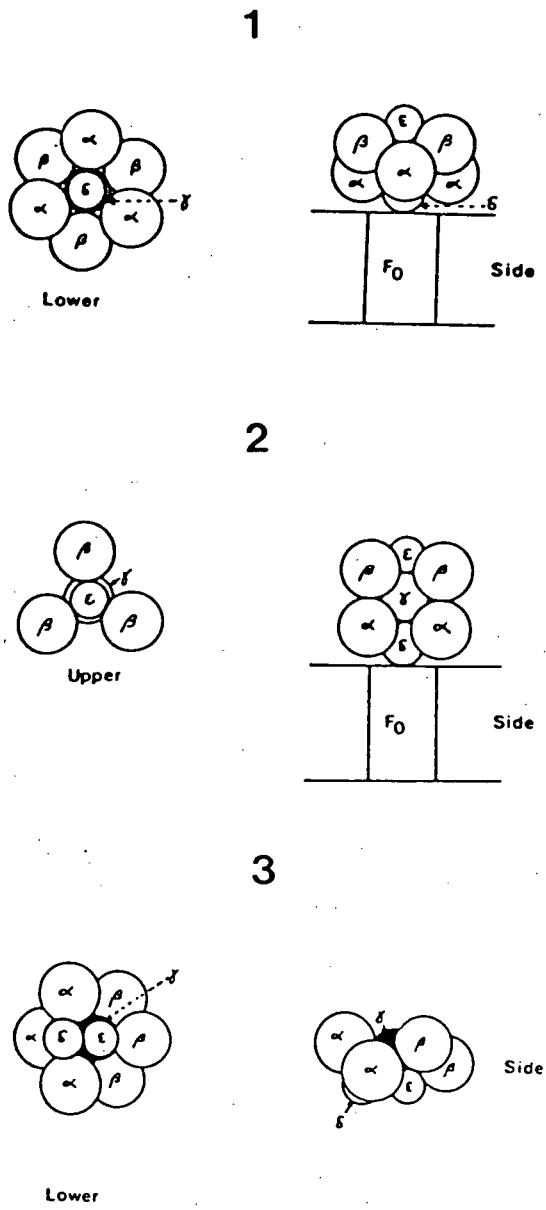


FIG 2: Three models for the arrangement of the subunits in the F_1-ATPase of E. coli (7, 89).

hexagon. This arrangement of the subunits is supported by the reconstitution experiments discussed for Model 1 and by results of cross-linking experiments (89). More recently, it was shown that the δ and ε subunits contain additional antigenic sites in the isolated ECF_1 which are inaccessible when the ECF_1 is bound to the membrane (65).

THE F_0 COMPLEX

Solubilization of the F_1F_0 Complex

One approach to the study of the F_0 polypeptides has been through the purification of the F_1F_0 complex. This requires the presence of high ionic strength buffers and the use of detergents to solubilize the F_1F_0 complex (4,24,25). Buffers of high ionic strength are needed during solubilization and purification of the F_1F_0 complex to keep the F_1 attached to the F_0, as the activity of the F_1 provides a means of following the presence of the F_1F_0 complex. Non-ionic and ionic detergents, such as Triton X-100, sodium cholate and deoxycholate are most commonly used to solubilize membrane proteins (90,91). Zwitterionic detergents such as Ammonyx Lo and Aminoxid WS 35 have recently become available and these are becoming the detergents of choice, since they have powerful solubilizing properties and often do not possess the denaturing effects shown by ionic detergents (92).

Criteria for Determining the Intactness and Purity of the F_1F_0 Complex

The first criterion often used to determine whether the F_1 and the F_0 have remained associated during the purification is the retention of sensitivity to inhibitors of ATPase activity (5). Membrane-bound ATPase activity is sensitive to DCCD in prokaryotes, while in eukaryotes, it is

sensitive to both DCCD and oligomycin. Both inhibitors bind to a specific polypeptide of F_0 (discussed under DCCD-binding protein) to inhibit the ATPase activity when F_1 is coupled to F_0. Ryrie (93) has shown that the sensitivity of the purified F_1F_0 to these inhibitors is often a poor guide to determine its intactness. The sensitivity of the F_1F_0 complex from yeast mitochondria to these inhibitors varied considerably and depended on the type of activity being measured (e.g. ATPase or ATP-^{32}Pi exchange) and whether the complex was first reconstituted into phospholipid vesicles. Similar results were also found by Foster and Fillingame (94) with the ECF_1F_0 complex.

The second criterion used is to reconstitute the purified F_1F_0 complex into phospholipid vesicles and to demonstrate energy-transducing reactions such as ATP-^{32}Pi exchange activity, ATP-driven proton-uptake, and $\Delta\tilde{\mu}_{H^+}$ -driven ATP synthesis. These are properties shown by the membrane-bound ATPase (5,25).

The criterion for purity of the functional F_1F_0 complex is through SDS-polyacrylamide gel electrophoresis. However, this method of determining the purity of a F_1F_0 preparation can also be misleading, since the number of polypeptides present in the gel will depend on the resolving power of the gel system used and on the choice of the protein-detecting reagents. For example, the yeast mitochondrial F_1F_0 complex which was originally determined to contain 9 different subunits, was resolved into 12 bands on a more resolving gel system (95).

The F_1F_0 Complex

The F_1F_0 complexes have been purified from various organisms. The numbers of polypeptides in each of these preparations are summarized in

Table 1. The mitochondrial F_1F_0 preparations contain more polypeptides either because the enzyme is more complex than in other organisms or because these preparations are contaminated with other proteins or protease digestion has occurred. The polypeptides listed in Table 1 are those which the authors have determined to be the major components of the F_1F_0 complex. In many of these preparations, varying numbers of minor bands were present to which no function was assigned.

The least complex preparation of the F_1F_0 complex was obtained from the thermophilic bacterium, PS3 (Tables 1 and 2). Densitometric tracing of the purified F_1F_0 complex, analyzed on SDS-polyacrylamide gels which had been stained with Amido Black, revealed the presence of eight major polypeptides. Five of these polypeptides were from TF_1. The remaining three polypeptides with molecular weights of 19 000, 13 500 and 5 400 were designated as TF_0 polypeptides. The TF_1F_0 complex was capable of the energy-transducing reactions summarized in Table 2. $\Delta\tilde{\mu}_{H^+}$ -driven ATP synthesis could only be demonstrated using phospholipids from PS3 for reconstitution. Soybean phospholipids were ineffective. Furthermore, a $\Delta\tilde{\mu}_{H^+}$ of greater than 170 mV was necessary for the demonstration of appreciable formation of ATP (112,113).

The ECF_1F_0 complex also appears to consist of eight subunits. Foster and Fillingame (94) solubilized the F_1F_0 complex from the membrane of E. coli with deoxycholate in the presence of 1M KCl. The solubilized fraction was subjected to ammonium sulphate precipitation and the F_1F_0 complex was purified by sucrose density gradient centrifugation. The purified complex was capable of the energy-transducing reactions summarized in Table 2. Analysis of the F_1F_0 preparation on SDS-polyacrylamide gels, revealed the presence of eight major subunits,

TABLE 1 Polypeptide Composition of F_1F_0-ATPase Complexes from Various Sources

Source	Beef Heart Mitochondria	Rat Liver Mitochondria	*S. cerevisiae*	*N. crassa*	Chloroplast	*Bacillus* PS3	*E. coli*
Reference	(96)	(97)	(95)	(98)	(99)	(102)	(94)
F_1							
α	54 000	62 500	52 000	59 000	59 000	56 000	55 000
ß	48 000	57 000 48 000	48 000	56 000	55 000	53 000	50 000
γ	33 000	33 800	31 000	36 000	37 000	32 000	37 000
δ	19 500	12 500	14 500	15 000	17 500	15 500	20 000
ε	7 700	7 000	10 700	12 000	13 500	11 000	12 000
I.P*	12 300	13 000	7 000	a	-	-	-
OSCP**	20 800	22 000	23 000	20 000	-	-	-
F_0	25 000	46 800	28 500	21 000	17 500[b]	19 000[b]	24 000
	20 500	31 600	24 500	19 000	15 500	13 500	19 000
	20 000	26 000	21 500	16 000	13 500	5 400	8 400
	14 700	9 200	16 700	8 000	7 500		
	12 300		12 700				
	11 800		9 000				
	8 500						
	6 500						

* Inhibitor polypeptide
** Oligomycin-sensitivity conferring protein

a Inhibitor polypeptide did not precipitate with purified enzyme
b Recent studies suggest that this component may be an impurity (100, 101, 103)

five of which corresponded to the subunits of ECF_1. The F_0 polypeptides had molecular weights of 24 000, 19 000 and 8 400. The smallest subunit of F_0 was identified as the DCCD-binding protein (discussed under "The DCCD-binding protein"). When the ECF_1F_0 complex was isolated from cells grown in a medium containing succinate, malate and acetate, rather than glucose as the carbon source, additional bands with molecular weights of 76 000, 68 000, 34 000, 26 000, 15 000 and 14 000 were also present in the preparation. Of these, only the 14 000 dalton polypeptide copurified with an invariant stoichiometry when different fractions of the sucrose gradient were analyzed on SDS-polyacrylamide gels. Thus, the 14 000 dalton polypeptide cannot be excluded as a possible subunit of the F_1F_0 complex when the cells are grown on a mixture of succinate, malate and acetate. It has been observed in E. coli that some characteristics of the F_1F_0 complex do change depending on the growth conditions (29-32).

Rosen and Hasan (105) have also purified the F_1F_0 complex from E. coli. Following solubilization of the membrane with deoxycholate in the presence of 140 mM KCl, the F_1F_0 was purified by chromatography on DEAE-cellulose and glycerol gradient centrifugation. This preparation consisted of six subunits and was deficient in the δ subunit. Although the ATPase activity of this preparation was inhibited by DCCD, other energy-transducing activities could not be demonstrated with this preparation (Table 2). The polypeptides with molecular weights of 10000 and 8300 were designated as the subunits of F_0.

Recently, the membranes of E. coli were solubilized with the zwitterionic detergent, Aminoxid WS 35, and the F_1F_0 complex purified by chromatography on DEAE-Sepharose CL-6B (106). The purified complex was capable of the energy-transducing reactions summarized in Table 2 and

consisted of eight different polypeptides. Five of these polypeptides were subunits of F_1 and the F_0 polypeptides had molecular weights of 28 000, 19 000 and 8 500. About 4% of the total protein of the F_1F_0 preparation was residual contamination, the majority of which had spectral characteristics of cytochrome b_1 and a molecular weight of 65 000. Minor components with molecular weights of 24 000 and 14 000 were also present and these were concluded to be possible degradation products of the larger subunits. A similar subunit composition for the ECF_1F_0 complex was obtained by Schneider and Altendorf (107) who used the same method of purification, but included centrifugation of the active ATPase fractions from the DEAE-Sepharose CL-6B column at 220 000 xg for 15 h (Table 2). However, differences were still present. It was observed that the ECF_1F_0 preparation obtained after chromatography on DEAE-Sepharose CL-6B contained the 28 000 and 19 000 dalton polypeptides in approximately equal amounts, and the 8 500 dalton subunit was present in only a third of the amount of the ϵ subunit. By including the centrifugation step, the 28 000 dalton subunit was present in about twice the level as the 19 000 dalton subunit, and the 8 500 dalton and ϵ subunits were present in about equal amounts. Further modification of this purification procedure (108) has included the precipitation of the ECF_1F_0 complex, obtained after DEAE-Sepharose CL-6B, with polyethylene glycol 6000 and 400. Differences which were observed (Table 2) in this ECF_1F_0 preparation were: i) the 28 000 dalton subunit so prominent in the previous preparations was present in almost negligible amounts, ii) the 24 000 dalton subunit was one of the major polypeptides of F_0, and iii) the complete absence of the 14 000 dalton polypeptide.

Table 2 lists only the major subunits of F_1F_0 which were present

TABLE 2 Properties of Various Preparations of Bacterial F_1F_0 Complexes

Source	Detergent	Method of Purification of		Subunit composition of F_0 ($M_r \times 10^{-3}$).	F_1F_0 Activity[b]			Ref.
		F_1F_0	F_0		Sensitivity of ATPase activity to DCCD.	ATP - $^{32}P_i$ Exchange.	ATP - driven proton uptake.	
E. coli	Deoxycholate	Sucrose gradient	n.d.[e]	29; 9[a]	s[f]	n.d.[e]	n.d.	104
E. coli	Deoxycholate	DEAE-Cellulose and sucrose gradient	n.d.	10; 8.3	s	n.d.	-	105
E. coli	Deoxycholate	Sucrose gradient	n.d.	24; 19; 8.4[a]	s	+[g]	+	94
			Treatment of F_1F_0 with EDTA	24; 19; 8.4[a]	N.A.[d]	N.A.	N.A.	9
E. coli	Aminoxid WS-35	DEAE-Sepharose CL-6B and centrifugation	n.d.	28; 19; 8.3[a]	s	+	+	106, 107
			Treatment of F_1F_0 with 7M Urea	19; 14; 8.3	N.A.	N.A.	N.A.	107
E. coli	Aminoxid WS-35	DEAE-Sepharose CL-6B and PEG precipitation	n.d	24; 19; 8.3	s	+	+	108
			Treatment of F_1F_0 with EDTA and KSCN.	24; 19; 8.3	N.A.	N.A.	+[c]	108
PS3	Triton X-100	DEAE-Cellulose and Sepharose 6B	n.d.	19; 13.5; 5.4[a]	s	+	+	102
			Treatment of F_1F_0 with 7M Urea	19; 13.5; 5.4[a]	s[c]	+[c]	+[c]	109
			Treatment of F_0 with 4M Urea and CM-cellulose	13.5; 5.4[a]	s[c]	+[c]	+[c]	103
M. phlei	Triton X-100	Sucrose gradient	n.d.	24; 18; 8	s	n.d.	n.d.	110
S. faecalis	Deoxycholate	Ammonium sulphate precipitation and non-denaturing gel.	n.d.	27; 15; 6[a]	s	n.d.	n.d.	111
C. pasteurianum	Triton X-100	DEAE-Sepharose CL-6B and Sephadex LH-60	Treatment of F_1F_0 with EDTA	15[a]	s	n.d.	+	16

a. This subunit identified as the DCCD-binding protein using [^{14}C]DCCD
b. Measured after reconstitution into phospholipid vesicles.
c. Demonstrated when the appropriate F_1 - ATPase was added.
d. N.A., not applicable - see Table 3.
e. n.d., not done.
f. s, sensitive.
g. +, detected, -, not detected.

on the SDS-acrylamide gels. In all cases, small amounts of minor contaminants were also present and these could have been proteolytic degradation products. Ryrie and Gallagher (95) have demonstrated the existence of proteases in the F_1F_0 complex from yeast mitochondria. Thus, it would do well to keep in mind the presence of proteases during the purification of the F_1F_0 complex as this could affect the identification of the F_0 subunits. It is also clear from Table 2 that, although it is possible to purify the ECF_1F_0 complex and reconstitute energy-transducing reactions, the authenticity and the minimum number of subunits of F_0 required to reconstitute these reactions are still controversial.

The Isolation of the F_0 Complex

The second approach to the study of the polypeptides of F_0 has been through the isolation of the intact F_0 complex from the purified or partially purified F_1F_0 complex. Isolation of the F_0 complex has involved the extraction of the F_1F_0 complex with either urea, EDTA, or EDTA and KSCN in order to remove the F_1 subunits. As with the F_1F_0 complex, the intactness of the F_0 complex can be determined by demonstrating proton-translocating activity in reconstituted vesicles. These results are summarized in Table 3.

The F_0 complex was first isolated in bacterial systems from the thermophile, PS3 (109). It consisted of three subunits with molecular weights of 19 000, 13 500 and 5 400. Treatment of the F_0 complex with urea and subsequent purification on CM-cellulose (103) resulted in an F_0 preparation containing two subunits with molecular weights of 13 500 and 5 400. This F_0 complex was capable of mediating proton conduction in reconstituted vesicles. Energy-transducing reactions could also be

Table 3 Properties of Various Preparations of Bacterial F_0 Complexes

Source	Detergent	Method of Purification of		Subunit composition of F_0 ($M_r \times 10^{-3}$)	F_0 Activity[b]		Ref.
		F_1F_0	F_0		Proton translocation	Sensitivity of Proton Conduction to DCCD	
E. coli	Deoxycholate	Sucrose gradient	n.d.[e]	29; 9[a]	N.A.[d]	N.A.	104
E. coli	Deoxycholate	DEAE-Cellulose and sucrose gradient	n.d.	10; 8.3	N.A.	N.A.	105
E. coli	Deoxycholate	Sucrose gradient	n.d.	24; 19; 8.4[a]	N.A.	N.A.	94
			Treatment of F_1F_0 with EDTA	24; 19; 8.4[a]	+[e]	s[f]	9
E. coli	Aminoxid WS-35	DEAE - Sepharose CL-6B and centrifugation	n.d.	28; 19; 8.3[a]	N.A.	N.A.	106,107
			Treatment of F_1F_0 with 7M Urea	19; 14; 8.3	+	s	107
E. coli	Aminoxid WS-35	DEAE - Sepharose CL-6B and PEG precipitation	n.d.	24; 19; 8.3	N.A.	N.A.	108
			Treatment of F_1F_0 with EDTA and KSCN	24; 19; 8.3	+	s	108
PS3	Triton X-100	DEAE - Cellulose and Sepharose 6B	n.d.	19; 13.5; 5.4[a]	N.A.	N.A.	102
			Treatment of F_1F_0 with 7M Urea	19; 13.5; 5.4[a]	+	s	109
			Treatment of F_0 with 4M Urea and CM-cellulose	13.5; 5.4[a]	+	s	103
M. phlei	Triton X-100	Sucrose gradient	n.d.	24; 18; 8	N.A.	N.A.	110
S. faecalis	Deoxycholate	Ammonium sulphate precipitation and non-denaturing gel.	n.d.	27; 15; 6[a]	N.A.	N.A.	111
C. pasteurianum	Triton X-100	DEAE-Sepharose CL-6B and Sephadex LH-60	Treatment of F_1F_0 with EDTA	15[a]	+	s	16

a. This subunit identified as the DCCD - binding protein using [^{14}C]DCCD
b. Measured after reconstitution into phospholipid vesicles
c. n.d., not done
d. N.A., not applicable - see Table 2
e. +, detected; -, not detected.
f. s, sensitive

reconstituted by the addition of purified TF_1 to the TF_0-reconstituted vesicles. It was concluded that the TF_0 consisted of two subunits and that the 19 000 dalton polypeptide was most likely a contaminant.

When the ECF_1F_0 complex, which contained the 28 000, 19 000 and 8 300 dalton polypeptides as the major subunits of F_0, and the 24 000 and 14 000 dalton polypeptides as the minor contaminants, was extracted with urea, an F_0 preparation containing essentially the 19 000, 14 000 and 8 300 dalton polypeptides was obtained (106,107). This F_0 complex could reconstitute proton translocating activity in reconstituted vesicles (Table 3). It was suggested that the 28 000 dalton subunit was probably a dimer, such that conversion to the monomer was obtained after urea treatment.

In contrast, treatment of the ECF_1F_0 complex, which contained the 24 000, 19 000 and 8 300 dalton polypeptides as the major subunits of F_0, with EDTA or EDTA and KSCN resulted in an F_0 preparation consisting of the same three polypeptides (9,108). The 24 000, 19 000 and 8 300 dalton polypeptides were shown to be genuine subunits of F_0, through the use of the defective transducing phage, λ_{asn-5}, which carries the genes for the ECF_1F_0 polypeptides (114,115). This is also confirmed by the results obtained from the genetic studies.

Biochemical Genetics

The third approach to the study of the subunits of F_0 and therefore F_1F_0, has been through the isolation and generation of mutants of *E. coli* which are defective in oxidative phosphorylation (25,116,117). These mutants can be generated by the use of mutagens such as N-methyl-N'-nitrosoguanidine, ethyl methyl sulphonate, hydroxylamine, ultra-violet irradiation and the bacteriophage, Mu. The mutants defective in oxidative

phosphorylation cannot grow on non-fermentable substrates such as acetate, malate or succinate, and have low yields when grown on a limiting amount of glucose. The membranes of these mutants also cannot be energized with ATP and are therefore referred to as "unc" ("uncoupled") mutants.

Initially, two classes of mutants were described. The first, designated unc A (e.g. unc A401) did not possess ATPase activity, whereas the second group, which retained activity was designated, unc B (e.g. unc B402). The lesions in these mutants were further characterized as affecting either the F_1 or F_0 portion of the ECF_1F_0 complex, through reconstitution experiments. Oxidative phosphorylation activity could be reconstituted in ECF_1-depleted membranes from the unc A strain with purified ECF_1 either from the wild-type strain or from the unc B strain. The ECF_1-depleted membranes from the unc B strain were also relatively impermeable to protons upon energization of the membrane through substrate oxidation. Thus it was established that the unc A mutation affected the subunit(s) of ECF_1, whilst the unc B mutation affected the F_0 portion of the ECF_1F_0 complex (116,118-120).

All of the known unc mutations map at the 83.5 min region in the E. coli chromosome (25,121). Since the ECF_1F_0 complex probably consists of 7-9 different polypeptides, and all the mutations map at the same locus, it was not possible to distinguish mutations in the different genes by simple genetic-mapping.

The exact composition of the F_0 complex and the characterization of the order and number of genes responsible for oxidative phosphorylation required more refined biochemical and genetic experiments. A genetic complementation system was developed to characterize the defective gene in the unc mutants. This involved the construction of partial diploids which

contained two different unc alleles: one on the host chromosome and the other on a plasmid (F-plasmid) (25,121-124). When the mutation affected the same gene on the plasmid as on the host chromosome, the resulting partial diploid remained defective in oxidative phosphorylation and expressed the phenotype of being unable to grow in the presence of non-fermentable carbon sources. On the other hand, when the mutation affected different genes, there was a normal copy of each of the affected genes present in the cell. The resulting partial diploid was similar to the wild-type strain. Biochemical tests, such as ATP-driven membrane energization and two-dimensional isoelectric focusing gels were used to confirm the presence or absence of genetic complementation.

Seven distinct complementation groups were identified (121) in the unc region of the chromosome and these were designated unc A, B, C, D, G, F and G coding for the polypeptides shown in Table 4.

Since the unc genes all mapped in the same region of the E. coli chromosome, the bacteriophage Mu was used to determine the gene inter-relationship and whether these genes formed an operon (125). The bacteriophage Mu has a polar effect on an operon, in that the insertion of the phage in an early gene prevents the transcription of all subsequent genes. Genetic complementation tests on these Mu-induced mutants demonstrated that the unc genes did form an operon. Together with the information obtained from cloning experiments, the gene order was postulated by Downie et al. (126,127) to be unc BFEAGDC. However, the subsequent DNA sequencing of the unc operon not only showed that the order unc BFE was incorrect, but also that the gene coding for the δ subunit (unc H) was located between unc F and unc A (42, 128-133). Therefore, the correct order of the genes in the unc operon is unc BEFHAGDC, with the unc

TABLE 4 Polypeptides Coded by the "unc" Genes

Gene	Polypeptides of F_1F_0 [a]	Molecular Weight [b]	Molecular Weight [c]
Gene 1	?	14 183	?
unc B	a	30 258	24 000
E	c	8 365	8 300
F	b	17 233	19 000
H	δ	19 310	20 700
A	α	55 264	56 800
G	γ	34 100	32 000
D	ß	50 157	51 800
C	ε	14 194	13 200

a The general nomenclature for the F_0 polypeptides are a, b and c with c being the DCCD-binding protein

b Determined from the DNA sequence

c Determined by SDS-polyacrylamide gel electrophoresis

B gene being closest to the promoter (Table 4).

In vitro protein synthesis experiments with plasmids (126,127,134) or specialized λ transducing phages (135) carrying the *unc* genes confirmed that the *unc* B, E and F genes code for polypeptides of F_0 with molecular weights of 24 000, 8-9 000 and 18 000, respectively.

The molecular weight of the *unc* B gene product determined by SDS-polyacrylamide gel electrophoresis has been underestimated. This appears to be common to integral membrane proteins (136,137). Steffens et al. (138) have purified the 24 000 and the 18 000 dalton polypeptides from the purified ECF_1F_0 complex and found that the amino acid compositions agreed with those deduced from the corresponding DNA sequences.

The DNA sequence of the *unc* operon also indicates the existence of another gene (Gene 1) coding for a polypeptide of molecular weight 14 183 (128). This gene lies between the promoter and the *unc* B gene. Whether gene 1 is transcribed *in vivo* is not clear, but the presence of a 14 000 dalton polypeptide in some ECF_1F_0 preparations may suggest that it is (94,106,107). *In vitro* coupled transcription-translation experiments with plasmids containing the whole *unc* operon also resulted in the production of a 14 000 dalton polypeptide (126). The function of this polypeptide is unknown, although it has been postulated that it may function in regulating the assembly of the F_0 polypeptides (128). *In vitro* studies (139) with plasmids containing the *unc* genes, but not the promoter or Gene 1, showed that the membrane-association of the F_0 polypeptides occurred via the insertion of the proteins into the membranes and this process was independent of the synthesis of each F_0 polypeptide or of other F_1 polypeptides. Whether the polypeptides of F_0 are inserted into the membrane in the correct orientation, or whether a functional F_0 complex

was formed, was not clear.

By contrast, Cox et al. (140) have shown that the insertion of some of the F_0 polypeptides into the membrane, and therefore the assembly of a functional F_0 complex, required the presence of α and ß subunits of ECF_1.

THE DCCD-BINDING PROTEIN

The function of each subunit of F_0 has not been characterized because of the difficulties encountered in the isolation of the individual subunits in non-denatured form. However, there is considerable evidence in prokaryotic and eukaryotic systems suggesting that the smallest subunit of F_0 is intimately involved in the proton-translocating properties of the F_0 complex. This polypeptide is the best characterized subunit of F_0 in mitochondria, chloroplasts, and bacteria with respect to its structure and function.

Identification and Isolation

The compound DCCD is able to react non-specifically with amino, carboxyl, hydroxyl and sulfhydryl residues to form stable adducts (141). But under basic conditions (pH 8.5), it reacts specifically with a carboxyl residue of a subunit of F_0 and inhibits the ATPase activity of either the membrane-bound ATPase or of the purified ECF_1F_0 complex (78,142). The reaction of DCCD with this subunit of F_0 also decreases the permeability of the F_1-depleted membranes to protons, as discussed before. Beechey et al. (143) were the first to demonstrate that the inhibition of the ATPase activity in beef-heart mitochondria was associated with the specific incorporation of [^{14}C]DCCD into a 9 000 dalton polypeptide. In E. coli

(141,144), the label was also associated exclusively with a 8 000 - 9 000 dalton polypeptide. This polypeptide was termed the "DCCD-binding protein".

DCCD also inhibits the energy-transducing reactions of the F_1F_0 and F_0 complexes which have been reconstituted into liposomes (Table 2). Inhibition of these reactions was associated with the labelling of the smallest polypeptide of F_0 with [^{14}C]DCCD.

The DCCD-binding protein is unique in that it can be extracted from the membrane with organic solvents (145,146). It is due to this property that it was originally called a "proteolipid". The DCCD-binding protein from the mitochondria of Neurospora crassa and yeast can be extracted in almost pure form with a mixture of chloroform-methanol (2:1) if the mitochondrial membranes are prewashed several times with a mixture of chloroform:methanol:diethylether (2:1:12). Similarly, the DCCD-binding protein isolated from beef heart and rat liver mitochondria, lettuce or pea chloroplast and the sarcoplasmic reticulum, by extraction with n-butanol followed by precipitation with diethyl ether, is also in pure form (146).

By contrast, the extraction of membranes or whole cells preparations with a mixture of chloroform:methanol (2:1) without prior washing with solvent, also extracts together with the DCCD-binding protein, several other hydrophobic proteins and phospholipids. Purification of the DCCD-binding protein is achieved by repeated precipitation of the extract with diethyl ether followed by either: thin layer chromatography (147), adsorption chromatography on Sephadex LH-20 or LH-60 (145), reversed-phase HPLC (148) or in the case of E. coli (149,150), by ion-exchange chromatography on either DEAE or CM-cellulose followed by adsorption chromatography on Sephadex LH-60.

The purified DCCD-binding protein of E. coli (149,150) migrates with

an apparent molecular weight between 8 000 and 9 000 on SDS-polyacrylamide gels.

Reconstitution of Proton Translocating Activity

The DCCD-binding protein, isolated from chloroplasts (151) or beef heart mitochondria (152) by extraction with n-butanol was capable of proton-translocating activity when reconstituted into liposomes. With the DCCD-binding protein from beef-heart mitochondria, the rate of proton influx was dependent on the amount of the protein incorporated into the liposome. In both cases, proton-translocating activity was sensitive to DCCD but oligomycin-sensitivity was not observed with the protein from beef heart mitochondria. Similarly, the protein isolated by extraction of yeast mitochondria with a mixture of chloroform:methanol (2:1) was also capable of proton-translocating activity when reconstituted into liposomes (153,154). The rate of proton influx was proportional to the amount of protein incorporated. Oligomycin, which is a specific inhibitor of oxidative phosphorylation in eukaryotes and which also binds to the DCCD-binding protein, inhibited proton conduction. The proton-translocating activity was insensitive to oligomycin when the DCCD-binding protein from an oligomycin-resistant strain was used.

Similar types of reconstitution experiments with the DCCD-binding protein from bacterial systems, did not result in any proton-translocating activity (5). This may be due to the method of isolation and purification of this protein, which may result in the loss of the native oligomeric structure and subsequent loss of function. Evidence for the existence of this protein in an oligomeric form in E. coli or PS3 comes from the determination of the stoichiometry of the subunits of the F_1F_0 complex.

The F_1F_0 complex isolated from cells grown in the presence of either $^{35}SO_4^{2-}$, [U-^{14}C]D-glucose (155) or L-[U-^{14}C] amino acid mixtures (156) result in stoichiometries of $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10}$ and $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_5$ for ECF_1F_0 and TF_1F_0, respectively. In addition, the inhibition of membrane-bound ATPase activity of E. coli was complete when only one third of the total DCCD-binding protein was labelled with [^{14}C]DCCD, suggesting that the protein existed as a trimer (144).

The second reason for the lack of protein conduction upon reconstitution may be due to the requirement of additional polypeptides to direct the proper insertion of the DCCD-binding protein into the membrane (103,140).

The Amino Acid Composition

The DCCD-binding protein has been purified from various sources and the amino acid composition of each is listed in Table 5. In all cases, the amino acid composition is derived from the amino acid analysis and confirmed by either complete or partial sequence analysis (146).

The DCCD-binding proteins from the various sources contain unusually high amounts of nonpolar amino acids and therefore are very hydrophobic in nature. The protein from E. coli is the most hydrophobic of those which have been studied (Table 4). Only 16.5% of its amino acids are polar. The predominating nonpolar amino acids are alanine (15%), leucine (16%), glycine (13%) and methionine (10%).

The DCCD-binding protein from all sources except Aspergillus nidulans (157) lack histidine. Similarly, tryptophan is present only in the protein from the chromatophores of Rhodospirillum rubrum (158). In addition to histidine and tryptophan, the protein from E. coli (149) also lacks the

Table 5 Amino Acid Composition of the DCCD-Binding Protein from Mitochondria, Chloroplast and Bacteria [a] (146)

Amino acid	*Aspergillus nidulans*[b]	*Neurospora*	*Saccharomyces*	Bovine heart	Spinach	*Mastigocladus*	*Escherichia coli*	PS-3	*Bacillus acidocaldarius*	*Halobacterium*	*Rhodospirillum*
Lysine	2	2	2	2	1	1	1	-	3	1	2
Histidine	1	-	-	-	-	-	-	-	-	Trace	-
Arginine	3	2	1	1	2	2	2	4	2	1	1
Aspartic acid	6	4	3	3	2	3	5	1	3	4	4
Threonine	1	2	3	3	3	3	1	3	1	7	3
Serine	7	5	5	5	3	4	-	3	4	3	3
Glutamic acid	6	5	2	3	7	7	4	5	5	5	3
Proline	5	1	2	1	4	4	3	3	2	6	1
Glycine	12	11	10	11	11	10	10	11	12	10	10
Alanine	12	14	10	13	17	16	13	9	14	18	16
Valine	5	6	6	4	7	4	6	8	9	6	6
Methionine	4	4	3	3	2	2	8	2	4	1	3
Isoleucine	5	6	9	7	6	8	8	9	5	5	9
Leucine	8	11	12	9	12	13	12	10	9	10	8
Tyrosine	3	2	1	2	1	1	2	1	2	1	1
Phenylalanine	4	6	6	7	3	3	4	3	4	3	3
Cysteine	-	-	1	1	-	-	-	-	-	-	-
Tryptophan	-	-	-	-	-	-	-	-	-	ND[c]	2
Total residues	83	81	76	75	81	81	79	72	79	81	75
End group	f-Met	Tyr	f-Met	Asp	f-Met	f-Met	f-Met	f-Met	f-Met	ND	f-Met
Polarity	30	24.7	21.7	22.7	22.2	24.7	16.5	22.2	22.8	25.9	25

[c] ND, not done. Values are given in units of moles per mole

[b] from (157)

amino acids serine and cysteine.

In most cases, the amino acid content is in agreement with the apparent molecular weight determined by SDS-polyacrylamide gel electrophoresis. The exception is the DCCD-binding protein from PS3, whose molecular weight of 5 400 determined by SDS-gel electrophoresis was underestimated (102). The results from the amino analysis suggests that the molecular weight is 7 300.

The Amino Acid Sequence

If the native DCCD-binding protein is indeed a proton channel, then the knowledge of its amino acid sequence is a pre-requisite for the understanding of its structure and function (146). The advantages of knowing its primary structure are: i) theoretical calculations can be used to predict the arrangement of the polypeptide in the membrane and ii) a better understanding of how mutations in the polypeptide can affect its reactivity with DCCD (discussed under DCCD-resistent mutants) and oligomycin, or affect the proton permeability of the membrane, can be obtained.

The complete amino acid sequence of the DCCD-binding protein has been determined from a variety of organisms and these are summarized in Fig. 3. The amino acid sequence of the protein from *E. coli* has been confirmed by its DNA sequence (128).

In almost all organisms [^{14}C]DCCD reacted with a specific glutamyl residue (Fig. 3). But in *E. coli*, it reacted with an aspartyl residue (asp^{61}). The proteins from distantly related organisms have a high degree of homology in their amino acid sequence and this reflects their being involved in a similar function in the cell.

```
                                                10                                  20
N. cr.   Tyr-Ser-Ser-Glu-Ile-Ala-Gln-Ala-Met-Val-Glu-Val-Ser-Lys-Asn-Leu-Gly-Met-Gly-Ser-Ala-Ala-Ile-Gly-Leu-
Bovine                                  Asp-Ile-Asp-Thr-Ala-Ala-Lys-Phe-Ile-Gly-Ala-Gly-Ala-Ala-Thr-Val-Gly-Val-
S. cer.                          f-Met-Gln-Leu-Val-Leu-Ala-Ala-Lys-Tyr-Ile-Gly-Ala-Gly-Ile-Ser-Thr-Ile-Gly-Leu-
Spinach                f-Met-Asn-Pro-Leu-Ile-Ala-Ala-Ala-Ser-Val-Ile-Ala-Ala-Gly-Leu-Ala-Val-Gly-Leu-Ala-Ser-
M. lam.                f-Met-Asp-Pro-Leu-Ile-Ser-Ala-Ala-Ser-Val-Leu-Ala-Ala-Ala-Leu-Ala-Ile-Gly-Leu-Ala-Ala-
E. coli                f-Met-Glu-Asn-Leu-Asn-Met-Asp-Leu-Leu-Tyr-Met-Ala-Ala-Ala-Val-Met-Met-Gly-Leu-Ala-Ala-
PS-3                                    f-Met-Ser-Leu-Gly-Val-Leu-Ala-Ala-Ala-Ile-Ala-Val-Gly-Leu-Gly-Ala-

                                30                                  40                                  50
N. cr.   Thr-Gly-Ala-Gly-Ile-Gly-Ile-Gly-Leu-Val-Phe-Ala-Ala-Leu-Leu-Asn-Gly-Val-Ala-Arg-Asn-Pro-Ala-Leu-Arg-
Bovine   Ala-Gly-Ser-Gly-Ala-Gly-Ile-Gly-Thr-Val-Phe-Gly-Ser-Leu-Ile-Ile-Gly-Tyr-Ala-Arg-Asn-Pro-Ser-Leu-Lys-
S. cer.  Leu-Gly-Ala-Gly-Ile-Gly-Ile-Ala-Ile-Val-Phe-Ala-Ala-Leu-Ile-Asn-Gly-Val-Ser-Arg-Asn-Pro-Ser-Ile-Lys-
Spinach  Ile-Gly-Pro-Gly-Val-Gly-Gln-Gly-Thr-Ala-Ala-Gly-Gln-Ala-Val-Glu-Gly-Ile-Ara-Arg-Gln-Pro-Glu-Ala-Glu-
M. lam.  Ile-Gly-Pro-Gly-Ile-Gly-Gln-Gly-Asn-Ala-Ala-Gly-Gln-Ala-Val-Glu-Gly-Ile-Ala-Arg-Gln-Pro-Glu-Ala-Glu-
E. coli  Ile-Gly-Ala-Ala-Ile-Gly-Ile-Gly-Ile-Leu-Gly-Gly-Lys-Phe-Leu-Gln-Gly-Ala-Ala-Arg-Gln-Pro-Asp-Leu-Ile-
PS-3     Leu-Gly-Ala-Gly-Ile-Gly-Asn-Gly-Leu-Ile-Val-Ser-Arg-Thr-Ile-Glu-Gly-Ile-Ala-Arg-Gln-Pro-Glu-Leu-Arg-

                                60                                  70
N. cr.   Gly-Gln-Leu-Phe-Ser-Tyr-Ala-Ile-Leu-Gly-Phe-Ala-Phe-Val-Glu-Ala-Ile-Gly-Leu-Phe-Asp-Leu-Met-Val-Ala-
Bovine   Gln-Gln-Leu-Phe-Ser-Tyr-Ala-Ile-Leu-Gly-Phe-Ala-Leu-Ser-Glu-Ala-Met-Gly-Leu-Phe-Cys-Leu-Met-Val-Ala-
S. cer.  Asp-Thr-Val-Phe-Pro-Met-Ala-Ile-Leu-Gly-Phe-Ala-Leu-Ser-Glu-Ala-Thr-Gly-Leu-Phe-Cys-Leu-Met-Val-Ser-
Spinach  Gly-Lys-Ile-Arg-Gly-Thr-Leu-Leu-Leu-Ser-Leu-Ala-Phe-Met-Glu-Ala-Leu-Thr-Ile-Tyr-Gly-Leu-Val-Val-Ala-
M. lam.  Gly-Lys-Ile-Arg-Gly-Thr-Leu-Leu-Leu-Thr-Leu-Ala-Phe-Met-Glu-Ser-Leu-Thr-Ile-Tyr-Gly-Leu-Val-Ile-Ala-
E. coli  Pro-Leu-Leu-Arg-Thr-Gln-Phe-Phe-Ile-Val-Met-Gly-Leu-Val-Asp-Ala-Ile-Pro-Met-Ile-Ala-Val-Gly-Leu-Gly-
PS-3     Pro-Val-Leu-Gln-Thr-Thr-Met-Phe-Ile-Gly-Val-Ala-Leu-Val-Glu-Ala-Leu-Pro-Ile-Ile-Gly-Val-Val-Phe-Ser-

                    80
N. cr.   Leu-Met-Ala-Lys-Phe-Thr
Bovine   Phe-Leu-Ile-Leu-Phe-Ala-Met
S. cer.  Phe-Leu-Leu-Leu-Phe-Gly-Val
Spinach  Leu-Ala-Leu-Leu-Phe-Ala-Asn-Pro-Phe-Val
M. lam.  Leu-Val-Leu-Leu-Phe-Ala-Asn-Pro-Phe-Ser
E. coli  Leu-Tyr-Val-Met-Phe-Ala-Val-Ala
PS-3     Phe-Ile-Tyr-Leu-Gly-Arg
```

FIG. 3 Amino acid sequences of the DCCD-binding protein from Neurospora crassa (N. cr.) bovine heart (Bovine), Saccharomyces cerevisiae, (S. cer.), spinach chloroplasts (Spinach), Mastigocladus laminosus (M. lam.), Escherichia coli (E. coli), and the thermophilic bacterium PS-3. The numbering is according to the Neurospora sequence. Reprinted from Sebald and Hoppe (146).

DCCD-RESISTANT MUTANTS

DCCD-resistant mutants have been isolated from E. coli. Two types of these mutants (25) have been identified: i) Mutants of the unc B phenotype. These mutants have a functional ECF_1, with activities comparable to those of the wild-type strain, but the activity of the membrane-bound ATPase is insensitive to DCCD. Also, the membranes of these mutants cannot be energized through ATP hydrolysis suggesting a defect in the F_0 component. ii) Mutants in which the alteration causes an insensitivity of the membrane-bound ATPase activity to DCCD. These mutants can further be divided into two classes: class I and class II (159). The wild-type membrane-bound ATPase activity is inhibitied half-maximally at 3-5 nmol DCCD per mg membrane protein, but the mutants of class I and II are inhibited half-maximally, at 30 and 200 nmol DCCD per mg membrane protein, respectively. These mutants are distinct from the unc B phenotypes, in that the removal of ECF_1 from the membrane results in the membrane becoming leaky to protons.

Sequencing studies on the DCCD-binding protein isolated from these DCCD-resistant mutants, revealed that point-mutations in the polypeptide are responsible for DCCD-insensitivity and proton-impermeability. These results are summarized in Table 6.

In the unc B phenotypes, it is observed that the replacement of the residue responsible for reacting with DCCD (i.e. asp) with either glycine or asparagine caused the loss of DCCD-binding and the membrane became impermeable to protons. The membrane-bound ATPase activity was not affected. This suggests that ATP hydrolysis and proton-translocation are uncoupled and that the mutation causes a conformational change in F_0 or in the DCCD-binding protein, which does not favour proton conduction. The

TABLE 6 Properties of DCCD-Resistant Mutants of *E. coli*

Strain	Sensitivity of the Membrane-bound ATPase Activity to DCCD	Proton-Permeability of F_1-depleted Membranes	Binding of [^{14}C]DCCD to DCCD-binding Protein	Amino Acid affected in the DCCD-binding Protein	References
Control	sensitive	permeable	binds	none	159
"Unc B"					
DG 7/1; DG 7/10	insensitive	impermeable	does not bind	$Asp^{61} \rightarrow Gly$	159, 160
DG 18/3; DG 3/2	insensitive	impermeable	does not bind	$Asp^{61} \rightarrow Gln$	161
Class I					
DC 1; DC 13 DC 19; DC 24	sensitive[a]	permeable	binds[a]	$Ile^{28} \rightarrow Val$	160, 162
Class II					
DC 25; DC 54	sensitive[a]	permeable	binds[a]	$Ile^{28} \rightarrow Thr$	162

a only at very high concentrations of DCCD

importance of this acidic residue (aspartic acid) in proton translocation is also seen in the DCCD-binding proteins from other organisms in which this acidic residue is conserved (glutamic acid). Also, the mutants of classes I and II, in which the aspartic acid at position 61 is conserved, have membranes which are permeable to protons upon removal of ECF_1.

In the mutants of classes I and II, DCCD-resistance is due to the replacement of isoleucine at position 28 with either value or threonine. Since a change at ile^{28} can cause such a large change in DCCD-sensitivity, it has been proposed that this residue interacts at the DCCD-binding site (asp^{61}). Although the mutations are 33 residues away from the DCCD-binding site, these two residues could be in close proximity to each other if the DCCD-binding protein exists as a "hairpin" structure (150), similar to that proposed for bacteriorhodopsin (163).

OBJECTIVES OF THIS STUDY

From the information presented in the INTRODUCTION, it is evident that more is known about ECF_1 than about the F_0 complex. The elucidation of the subunit composition of F_0, of the mechanism of proton translocation through F_0, and of the interaction of F_0 with ECF_1 has been hindered by the lack of a purified, intact ECF_1F_0 complex. Therefore, one of the aims of this thesis was to purify the ECF_1F_0 complex in order to identify the subunits of the F_0 complex. Secondly, mutants of *E. coli* were available in which the membranes were relatively impermeable to protons. Since, an ECF_1F_0 complex with an altered or missing subunit(s) in the F_0 complex could give an insight into the function of the individual subunits of the F_0 complex, these mutants were characterized by a variety of biochemical methods. Finally, understanding the orientation

and/or organization of the F_0 polypeptide(s) in the membrane is a prerequisite for studying the mechanism of proton-translocation. The DCCD-binding protein of the F_0 complex has been implicated in the protonophoric activity of F_0. Therefore, the orientation and/or organization of this polypeptide in the membrane, as well as its interaction with ECF_1 were studied by immunological techniques.

MATERIALS AND METHODS

CHEMICALS

All chemicals were of reagent-grade purity and were purchased from the following sources:

Aldrich Chemical Company: 9-aminoacridine hydrochloride.

Amersham Corporation: ACS (Aqueous counting scintillant), NCS tissue solubilizer, [7-^{14}C]phenylglyoxal.

BioRad Laboratories: Acrylamide, agarose, ammonium persulphate, Bio-Gel P6DG, Bio-Gel HTP (hydroxylapatite), Cellex-CM, 2-mercaptoethanol, N,N'-methylene-bis-acrylamide, SDS, TEMED, urea.

Calbiochem Corporation: Complete and incomplete Freund's adjuvant, α-chymotrypsin (bovine pancreas), octyl ß-D-glucopyranoside, PMS, pronase, valinomycin.

Chemical Dynamics Corporation: ε-aminocaproic acid, p-aminobenzamidine dihydrochloride.

Difco Laboratories: Bacto-agar, Bacto-Penassay broth, Bacto-peptone, Bacto-tryptone, casamino acids, nutrient broth, yeast extract.

E. Merck. AG (Germany): Amido Black 10B.

Eastman Kodak Company: Chloramine T, DCCD, Merthiolate.

Fisher Scientific Company: Silicotungstic acid.

Goldschmidt (Essen): Aminoxid WS-35.

LKB-Produkter AB (Sweden): Ampholytes.

Mann Research Laboratories: Tween 60, Tween 80, thyroglobulin (Pig).

Mandel Scientific Company: Whatman CM-32.

Matheson, Coleman and Bell Manufacturing Chemists: Cyanogen bromide, sodium dithionite.

Miles-Yeda Ltd.: ω-Amino Butyl Agarose, Butyl Agarose, Decyl Agarose.

Miles Laboratories: Staphylococcus aureus V_8 protease.

Onyx Chemical Company: Ammonyx Lo.

Particle Data Laboratories Ltd.: Nonidet P-40.

Pharmacia Fine Chemicals, Inc.: AH-Sepharose 4B, Blue Dextran, DEAE-Sepharose CL-6B, Octyl-Sepharose CL-4B, Phenyl-Sepharose CL-4B, Sepharose CL-6B, Sephadex G-50, Sephadex LH-60, Molecular weight markers for gel electrophoresis.

Pierce Chemical Company: Amino acid standards for analyzer, ninhydrin.

Research Products International Corporation: [^{14}C]DCCD.

Sigma Chemical Company: ATP, bovine serum albumin, Brig 35, 2,3-butanedione, Coomassie Brilliant Blue G, Coomassie Brilliant Blue R, catalase (bovine), DNase 1 (bovine pancreas), DTT, guanidine hydrochloride, HEPES, L-amino acids, lysozyme (egg), Lubrol PX, Lubrol WX, Lubrol 17A-10, L-α-phosphatidylcholine (egg), MOPS, NADH, phenylglyoxal, poly-L-lysine (M_r, 400 000), PMSF, phosphatidylcholine (soybean, 22%), RNase (bovine pancreas), sodium cholate, sodium deoxycholate, sodium N-lauroyl sarcosine, sodium succinate, sucrose, Tris base, Triton X-100, Triton X-114.

Worthington Biochemical Corporation: TPCK-trypsin, soybean trypsin inhibitor.

Rabbit γ-globulin and Na(^{125}I) were generous gifts from Dr. R.S. Molday. TPCK-trypsin treated ECF_1 was a kind gift from Cynthia Hou.

MAINTENANCE OF BACTERIAL STRAINS

Table 7 lists the strains of bacteria used in this study. These strains were maintained as slants and stabs containing Penassay broth-agar.

The slants were prepared by boiling 1.75 g Bacto-Penassay broth and 1.5 g agar in 100 ml distilled water. The mixture was then dispensed in 5 ml portions into screw-cap tubes and autoclaved for 20 min at a pressure of 15 psi. The tubes were allowed to cool such that the nutrient broth solidified as slants. Exponentially growing bacterial cultures were streaked onto the slants, incubated overnight at 37°C and then stored at 4°C until needed. These strains were subcultured every 4-6 months after checking for nutritional markers and then transferred to fresh slants.

Cultures to be stored for longer periods of time (9 months) were inoculated into nutrient broth prepared as for slants, but containing 0.7% (w/v) agar and dispensed in 2.5 ml aliquots into screw-cap vials.

GROWTH OF CELLS

A 10 ml volume of Penassay broth was inoculated with a culture from a slant and grown overnight at 37°C. This was then transferred to 400 ml of the appropriate medium in a 2 l conical flask and grown overnight at 37°C with shaking (at 250 rpm) in a New Brunswick Rotary Incubator. The cells were either harvested and used immediately or the growing culture was used as 10% (v/v) innocula for growing larger quantities of cells (4.5 l).

Batches of 4.5 l were grown at 37°C (except for PS3 which was grown at 60°C) with vigorous aeration (at 25 l/min) in a Lab-Line/S.M.S. Hi-Density Fermentor. Cell growth was monitored by measuring the absorbance of the culture at 420 nm.

The cells were harvested at the late exponential phase of growth by

TABLE 7 Bacterial Strains used in this Study

Strain	Genotype	Origin
E. coli ML 308-225	i^-, Z^-, y^+, a^+	H.R. Kaback
DL-54	i^-, Z^-, y^+, a^+, *unc*	R.D. Simoni
AN 180	F^-, *arg*, *thi*, *mtl*, *xyl*, str^R	F. Gibson
AN 120	F^-, *arg*, *thi*, *mtl*, *xyl*, str^R, *unc* A401	F. Gibson
AN 382	F^-, *arg*, *thi*, *mtl*, *xyl*, str^R, *tfr-3*, λ^-, *unc* B402	B.J. Bachmann
WS1	F^-, *pro*, *lac*, T_6^R, *gal*, *ara*, *his*, *xyl*, *man*, *thi*, str^R	D.L. Gutnick
N_{I44}	same as WS1 but neo^R and *unc*	D.L. Gutnick
CBT-1	*thi*	T.C.Y. Lo
CBT-302	*thi*, *unc*	T.C.Y. Lo
Thermophile PS3		Y. Kagawa

centrifugation at 4 500 xg for 15 min, then washed twice in 0.9% (w/v) NaCl and sedimented by centrifugation at 17 600 xg for 10 min. The cells were stored at -20°C.

MEDIA

All the strains listed in Table 7, except PS3, were grown on a minimal salts-glucose media (164) containing 0.7% (w/v) $K_2HPO_4.3H_2O$, 0.3% (w/v) KH_2PO_4, 0.05 % (w/v) sodium citrate $.2H_2O$, 0.02% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) $(NH_4)_2SO_4$, 0.4% (w/v) glucose and supplemented with 0.1% (w/v) casamino acids. In some experiments, the glucose concentration was increased to 0.8% (w/v). Ferric citrate (12 µM) was added to media of volumes exceeding 1 litre. Where required, amino acids and thiamine were added at 50 µg per ml and 1 µg per ml, respectively.

In order to obtain a higher yield of cell mass when growing mutants, the media was also supplemented with 0.1% (w/v) yeast extract, 0.1% (w/v) Bacto-tryptone, 3.3 µM $CaCl_2$, 6 µM $CaSO_4$, 6 µM $MnCl_2$, 7 µM $CoCl_2$, 0.6 µM $ZnCl_2$ and 64 µM EDTA.

In experiments requiring large quantities of E. coli ML 308-225, the cells were purchased from a commercial source (University of Alberta). These cells were grown to the late exponential phase on minimal -salts glucose (0.4% (w/v)).

PS3 was grown on media (165) containing 13.3% (w/v) Nutrient broth, 6.7% (w/v) Bacto-peptone, 5% (w/v) NaCl and 5% (w/v) $K_2HPO_4.3H_2O$ at pH 7.4.

PREPARATION OF MEMBRANES

The method described by Bragg et al. (164) was used. Cells were suspended in buffer containing 50 mM Tris-H_2SO_4, 10 mM $MgCl_2$ pH 8.0

(TM buffer) at a ratio of 1 g wet weight/3 ml. About 5-10 µg of DNase was added and the cells broken by one or, in some cases, two passages through an Aminco French Pressure cell precooled to 0°C, at a pressure of 1400 Kg/cm^2. Cell debris and unbroken cells were removed by centrifugation at 12 000 xg for 10 min and the membranes sedimented by centrifugation at 250 000 xg for 2 to 2.5 h. Unless indicated otherwise, all centrifugation steps were carried out at 0-5°C. The membranes were washed twice by resuspension in TM buffer and resedimented as before. They were processed in a number of ways to be described below.

PREPARATION OF EDTA-LYSOZYME SPHEROPLASTS

These were prepared by the method of Singh and Bragg (166). When possible, freshly-grown cells were used.

E. coli cells were grown to the late exponential phase and harvested by centrifugation at 4 500 xg for 15 min. The cells were washed three times by resuspension in 50 mM Tris-HCl buffer, pH 8.0 and centrifugation at 12 000 xg for 10 min. The washed cells (2.75 g) were suspended at 20°C in 37 volumes (w/v) of 50 mM Tris-HCl buffer, pH 8.0 containing 0.5 M sucrose. EDTA and lysozyme were added to final concentrations of 2.5 mM and 0.1 mg/ml, respectively.

The rate of spheroplast formation was followed by removing a 50 µl sample of the cell suspension at various intervals, diluting it to 2 ml with distilled water, and then measuring the absorbance at 420 nm.

After 30 min of incubation, the suspension was centrifuged at 12 000 xg for 10 min. The pellet was suspended in 275 ml of 20 mM potassium phosphate buffer, pH 8.0 and $MgSO_4$ was added to a final concentration of 1 mM. This was followed by addition of RNase and DNase at 20 µg/ml and

the suspension stirred for 20 min and then centrifuged at 6 400 xg for 15 min. The supernatant was centrifuged at 125 000 xg for 45 min. When K^+-loaded vesicles were not required, the spheroplasts were washed twice by resuspension in a buffer containing 0.4 M Sucrose and 10 mM $MgCl_2$, and resedimented as before. The spheroplasts were finally taken up in the same buffer, stored at 4°C and were used within 12 h.

PREPARATION OF K^+-LOADED SPHEROPLASTS

The pellet containing the spheroplasts was taken up in 10 volumes (w/v) of 0.5 M potassium phosphate buffer, pH 7.0 and gently heated at 40°C for 0.5 h (28,167). The suspension was then rapidly chilled in an ice-bath and after 10 min of incubation, $MgSO_4$ was added to a final concentration of 10 mM. After a further 10 min incubation, the spheroplasts were sedimented by centrifugation at 125 000 xg for 45 min. The spheroplasts were washed twice by resuspension in 0.4 M Sucrose, 10 mM $MgCl_2$ and centrifuged as before. The pellet containing K^+-loaded spheroplasts was finally taken up in the same buffer, and stored at 0°C. It was used within 12 h.

ISOLATION OF ECF_1

ECF_1 was isolated from 60 g of cells as described by Bragg and Hou (89). Membrane vesicles from 60 g of E. coli ML 308-225 were prepared in TM buffer as previously described. They were washed twice by suspension in a buffer (200 ml) containing 5 mM Tris-HCl, pH 7.3, 10% (v/v) glycerol, 20 mM ε-aminocaproic acid, 6 mM p-aminobenzamidine, and 0.5 mM DTT, followed by centrifugation at 250 000 xg for 2 h. The washed vesicles were suspended in 60 ml of 1 mM Tris-CHl buffer pH 7.5 containing 0.5 mM EDTA, 0.1

mM DTT and 10% (v/v) glycerol ("dialysis buffer") and dialyzed against 3 l dialysis buffer for 16 h at 4°C. The dialyzed material was diluted to 200 ml with dialysis buffer and then centrifuged as above. The supernatant was recentrifuged at 250 000 xg for 3 h. After this step, the supernatant was removed and concentrated to 18 ml by ultrafiltration using an Amicon XM-100A filter. Methanol (4.5 ml) was then added drop-wise to the concentrated solution followed by 0.46 ml of 1M $CaCl_2$. After incubation of the mixture for 20 min at 20°C, it was centrifuged at 12 000 xg for 20 min and the supernatant, containing ECF_1, was applied to a column of AH-Sepharose 4B.

PURIFICATION OF ECF_1 ON AH-SEPHAROSE 4B

AH-Sepharose 4B was suspended in an excess of 0.5 M NaCl and equilibrated for 4 h at 20°C. The suspension was degassed and then poured into a 2 cm diameter column to a height of 7.5 cm. The column was washed with several volumes of 0.5 M NaCl, distilled water and finally equilibrated with dialysis buffer containing 1 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.5 mM EDTA and 0.1 mM DTT. The sample containing ECF_1 was adsorbed onto the column at 20°C which was then washed with 2-3 volumes of dialysis buffer. Elution of the enzyme was carried out with a linear gradient (total volume, 200 ml) of 0.25 to 0.75 M KCl in dialysis buffer. The fractions containing ATPase activity were pooled and concentrated to one or two ml by passage through an Amicon XM-100A filter. The concentrated material was either used immediately or divided into small aliquots, rapidly frozen, and stored at -70°C.

The column was regenerated between experiments by washing with several volumes of 1 M KCl in dialysis buffer, re-equilibrated with dialysis buffer, and stored at 4°C.

In some cases, the ECF_1 obtained after AH-Sepharose 4B was further purified by sucrose density gradient centrifugation.

PURIFICATION OF ECF_1 AND TPCK-TRYPSIN TREATED ECF_1 BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

A discontinuous gradient (23) consisting of successive 1 ml layers of 25%, 22.5%, 20%, 17.5% and 15% (w/v) sucrose in 50 mM Tris-H_2SO_4 buffer, pH 7.8 containing 0.5 mM EDTA and 0.1 mM DTT was poured. The sample (0.4 ml, 3-5 mg) protein was loaded and the gradient centrifuged at 260 000 xg for 16 h. Twenty drop fractions were collected by using a Beckman fractionator attached to a fraction collector.

PREPARATION OF ECF_1-DEPLETED MEMBRANES

Several methods were used to strip the membranes of ECF_1.

a) The membrane fraction remaining after dialysis against 1 mM Tris-HCl buffer, pH 7.5 containing 0.5 mM EDTA, 0.1 mM DTT and 10% (v/v) glycerol ("dialysis buffer") as described under "Isolation of ECF_1", was essentially devoid of ECF_1. The membranes were washed once by resuspension in dialysis buffer and centrifuged as before, and then stored at 4°C.

b) More drastic treatments with urea, guanidine hydrochloride, silicotungstic acid, or proteases were also used.

Dialyzed membranes (described in (a)), or membranes prepared in TM buffer, pH 8.0, suspended in dialysis buffer, pH 7.5 at a protein concentration of 10 mg/ml were incubated at 20°C for 30 min with either 2 M urea, 2 M guanidine hydrochloride, 1% or 2% (w/v) silicotungstic acid, *Staphylococcus aureus* V_8 protease or TPCK-trypsin at an enzyme to protein ratio of 1:40 and 1:15, respectively. In the case of TPCK-trypsin, the reaction

was stopped by the addition of soybean trypsin inhibitor at a ratio to trypsin of 3:5 with incubation for another 10 min. The incubated mixtures were diluted 4-fold with buffer and then centrifuged at 250 000 xg for 2.5 h. The sedimented vesicles were washed twice by resuspension in dialysis buffer followed by resedimentation as above.

The ECF_1-depleted membranes prepared by either method (a) or (b) were finally suspended in the appropriate buffer at 4°C. The pH of the vesicle suspension was readjusted to pH 7.5 with dilute KOH when silicotungstic acid was used.

PREPARATION OF RAT-LIVER MITOCHONDRIAL MEMBRANES

Phosphate-washed purified inner mitochondrial membranes were prepared from rat-liver mitochondria as described by Soper and Pedersen (168). The purified membranes at 5 mg protein/ml of PBS-10 mM $MgCl_2$ were sonicated at a power of 50 W in a Branson W185D sonifier for 15 s periods for a total of 2.5 min. The temperature was kept at 0°. Large fragments were removed by centrifugation at 12 000 xg for 10 min. The supernatant was either centrifuged at 250 000 xg for 2 h to sediment "sonicated washed mitochondrial membranes" or incubated with 4 M urea for 30 min at 22°C prior to centrifugation as above. The "urea-treated washed mitochondrial membranes" were washed by recentrifugation of a suspension in PBS-10 mM $MgCl_2$. All membranes were resuspended in PBS-10 mM $MgCl_2$.

PREPARATION OF THE SUBUNITS OF ECF_1

TPCK-Trypsin Treated ECF_1

ECF_1 lacking the δ and ϵ, and perhaps part of the γ subunit, was

prepared by controlled treatment of the F_1 with TPCK-trypsin, as described by Bragg and Hou (89).

1.25 mg of sucrose gradient-purified ECF_1 in 0.385 ml of 22.5% (w/v) sucrose in 20 mM triethanolamine-HCl buffer, pH 7.5 containing 0.5 mM EDTA was treated with 37 µg TPCK-trypsin in 10 µl buffer at 37°C for 10 min. Bovine pancreas trypsin inhibitor (146 µg in 10 µl buffer) was added and after 5 min at 20°C, the solution was diluted with an equal volume of buffer and the trypsin-treated ATPase reisolated by sucrose density gradient centrifugation.

α and ß Subunits of ECF_1

These were prepared by Dr. Helga Stan-Lotter (University of British Columbia). ECF_1 obtained after purification on AH-Sepharose 4B was dissociated into its subunits with high ionic strength buffer as described by Dunn and Futai (72). The dissociated ECF_1 subunits were applied to a freshly made column of hydroxylapatite. The subsequent separation of the α and ß subunits was carried out on a column of DEAE-Sepharose CL-6B as described by Bragg et al. (82).

SOLUBILIZATION OF MEMBRANE VESICLES WITH DETERGENTS

The solubilization characteristics of various detergents were determined as follows.

Membranes of E. coli ML 308-225 were prepared in TM buffer as described previously. They were suspended in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol at a membrane protein concentration of 20-25 mg/ml. The suspension was divided into several aliquots and to each was added, drop-wise, different amounts of

detergents from either a 10% (w/v) or 20% (w/v) stock solution in distilled water, or in some instances added neat. After incubation for 30 min at 20°C, they were centrifuged at 250 000 xg for 2.5 h and the ATPase activity and protein content determined in the supernatant and pellet fractions.

PURIFICATION OF THE ECF_1F_0 COMPLEX SOLUBILIZED WITH N-LAUROYL SARCOSINE

Gel Filtration on Sepharose 6B

Membranes of E. coli ML 308-225 were prepared in TM buffer as described previously. They were suspended in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol at a protein concentration of 20-25 mg/ml. Detergent was added drop-wise, from a 20% (w/v) stock solution in distilled water, to give a detergent (mg) to protein (mg) ratio of 0.5. After incubation at 20°C for 30 min, the mixture was centrifuged at 250 000 xg for 2 h. The supernatant was recentrifuged, then concentrated to about one quarter of its original volume (20-25 mg protein per ml) by ultrafiltration using an Amicon XM-100 A filter. The concentrated material was loaded onto a column of Sepharose 6B.

Pre-swollen Sepharose 6B was suspended in 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 10% (v/v) glycerol at 4°C. A slurry was poured into a 2.5 cm diameter column to a height of 37 cm, and the column equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 0.25 M Na_2SO_4, 10% (v/v) glycerol and 0.5% (w/v) of detergent. The sample (2-5% (v/v) of total bed volume) was loaded onto the column and eluted with the appropriate buffer. The column was regenerated between experiments by washing with 2-3 bed volumes of the apropriate buffer.

Hydrophobic-Interaction Chromatography

Membranes of E. coli were prepared in TM buffer and then washed twice by resedimentation at 250 000 xg for 2 h from 5 mM Tris-HCl buffer, pH 7.3 containing 10% (v/v) glycerol, 20 mM ε-aminocaproic acid, 6 mM p-aminobenzamidine and 0.5 mM DTT. The washed membranes were suspended in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol at a protein concentration of 20-25 mg/ml. Detergent was added drop-wise, from a 20% (w/v) stock solution in distilled water, to give a detergent (mg) to protein (mg) ratio of 0.25-0.5. After incubation at 20°C for 30 min, the mixture was centrifuged at 250 000 xg for 2 h. The supernatant was recentrifuged and then subjected to ammonium sulphate precipitation. Saturated ammonium sulphate (adjusted to pH 7.5 with NH_4OH) was added to the supernatant at 0°C to give 35% of saturation. After 30 min incubation, the solution was centrifuged at 30 000 xg for 20 min. The supernatant was next brought to 50% of saturation. The pellet precipitating at 35-50% (0.35-0.5 P) saturation of $(NH_4)_2SO_4$ was taken up at 4°C, in solubilization buffer at a protein concentration of 4-6 mg/ml and N-lauroyl sarcosine added to give a detergent:protein ratio of 0.8-1.0. This was applied to a column of Phenyl-Sepharose CL-4B.

Phenyl-Sepharose CL-4B (or other hydrophobic-exchange resins) in 50 mM Tris-H_2SO_4 buffer, pH 8.0 was poured into a 1.8 cm diameter column to a height of 25 cm and the column equilibrated at 4°C with 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 20% $(NH_4)_2SO_4$ (by saturation), 10 mM $MgCl_2$ and 10% (v/v) glycerol. The solubilized fraction was adsorbed onto the column which was then washed with several volumes of the same buffer. The enzyme was eluted finally with the appropriate buffer as described in the "RESULTS" section.

The resin was regenerated by washing with 2-3 bed volumes of 2% (w/v) Triton X-100 followed in succession by

i) 2 bed volumes of distilled water

ii) 2 bed volumes of ethanol

iii) 2 bed volumes of n-butanol

iv) 2 bed volumes of ethanol

v) 2 bed volumes of distilled water and

vi) equilibration with starting buffer.

With Phenyl-Sepharose CL-4B, an additional washing with 2-3 bed volumes of 2% (w/v) SDS after the Triton X-100 wash was found to be necessary to remove tightly-bound proteins.

PURIFICATION OF THE ECF_1F_0 COMPLEX BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

The enzyme obtained after chromatography on Phenyl-Sepharose CL-4B was concentrated by ultrafiltration using an Amicon PM-10 filter, and purified by sucrose density gradient centrifugation. A linear 17.5% to 25% (w/v) sucrose gradient (12 ml, total volume) was poured. The sucrose was dissolved in 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 0.5% (w/v) sodium cholate, 5 mM $MgCl_2$ and 0.25 mM DTT. In some experiments, 12 μM p-aminobenzamidine was included in the gradients as a protease inhibitor and, on occasion, 0.1% (w/v) L-α-phosphatidylcholine (soybean) was also added.

The sample (1-1.2 ml, 5-10 mg protein per ml) was loaded and the gradients centrifuged at 280 000 xg for 23 h and ten-twenty drop fractions collected by using a Beckman fractionator connected to a fraction collector.

DEAE ION-EXCHANGE CHROMATOGRAPHY

This was based on the method of Friedl et al. (106). A slurry of pre-swollen gel in 50 mM Tris-HCl buffer, pH 8.0 at 4°C was poured into a 1.5 cm diameter column to a height of 13 cm and the column equilibrated with 50 mM Tris-HCl buffer, pH 8.0 containing 1 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM EGTA, 0.1 mM PMSF, 100 mM KCl, 20% (v/v) methanol, 50 mg/ml soybean phospholipid, 0.9% (w/v) Aminoxid WS-35, until the conductivity of the effluent was the same as the buffer.

The sample was adsorbed onto the column and washed with equilibration buffer. The enzyme was eluted with a linear gradient of 100-300 mM KCl in equilibration buffer. The resin was regenerated by washing with 1 M KCl in 50 mM Tris-HCl buffer, pH 8.0 and equilibrated with the appropriate buffer.

PREPARATION OF DCCD-BINDING PROTEIN

The method described by Fillingame (149) was used. Membrane vesicles or ECF_1-depleted membranes at a protein concentration of 40-50 mg/ml in either TM buffer, pH 8.0 or in distilled water, were mixed with 20 volumes of chloroform:methanol (2:1) and stored at 4°C for 24 h. The suspension was passed through a fritted glass funnel of medium porosity. To the filtrate, in a glass-stoppered bottle, was added 0.20 volume of distilled water and the contents mixed by gently inverting the bottle. After standing at 4°C overnight, the contents of the bottle were warmed to 20°C to completely resolve the two phases. The lower phase was washed twice with 0.05 volume (of original filtrate) of a mixture of chloroform: methanol:water (3:47:48). Finally, the washed lower phase was clarified with a small amount of methanol and the mixture diluted with one volume of chloroform. The chloroform was added in small amounts and a requisite

amount of methanol was added whenever the mixture started to turn cloudy. It was then carefully taken to dryness by rotoevaporation at 20°C using the vacuum from a water-aspirator. On occasion, chloroform:methanol (10:1) was added to the evaporation flask whenever the contents started to turn cloudy. The dried contents of the flask were taken up in a small volume of chloroform:methanol (2:1) and any traces in insoluble material removed by passage through a medium or fine porosity fritted glass funnel. The filtrate was chilled to -20°C and 4 volumes of precooled (-20°C) diethyl ether added slowly with stirring. The mixture was stored at -20°C for 24 h and the precipitate removed by centrifugation for 45 min at 4 000 xg and -20°C in a capped stainless steel tube. The pellet was again taken up in the original volume of chloroform:methanol (2:1) and precipitation with 4 volumes of pre-cooled diethyl ether was repeated. The precipitate was removed by centrifugation and dried at 20°C with a stream of nitrogen gas. It was dissolved in a small volume of chloroform:methanol (2:1) and insoluble material removed by filtration through a fritted glass funnel. The filtrate was then applied to a thin-layer plate or adsorbed onto a column of CM-cellulose as described below.

For the isolation of large amounts of DCCD-binding protein, the method of Altendorf et al. (150) was followed. In this procedure, the use of whole cells as starting material was more convenient than using membrane vesicles.

400 to 500 g (wet weight) of frozen E. coli cell was mixed with 20 volumes (w/v) of chloroform:methanol (2:1) at 20°C and the suspension mixed in a Waring blender at top speed for 1 min. It was stirred at 4°C for 24 h and then filtered through a medium porosity fritted glass funnel. The filtrate was treated as described above.

PURIFICATION OF DCCD-BINDING PROTEIN

Thin Layer Chromatography

A 20 x 20 cm glass plate, precoated with silica gel G (1500 µm, Analtech Inc.) was activated by placing it into an oven at 100°C for 1 h and then allowed to cool to 20°C. The isolated DCCD-binding protein (2 mg protein) in chloroform:methanol (2:1) was applied as a 0.5 cm wide streak, 9 cm in length and 2.5 cm from the bottom edge of the plate. The streak was thoroughly air-dried and the chromatogram developed in a covered tank at 20°C in a mixture of chloroform:methanol:water (65:25:4) containing 20 mM HCl (147). The solvent was allowed to ascend to within 1 cm from the top of the plate (0.5-0.75 h). The plate was air-dried and placed in a tank equilibrated with iodine crystals for 3-5 min. The stained spots were scraped from the plate and treated with enough water to completely wet the powder. After 30 min at 20°C, the powder was extracted with 5-10 ml of chloroform:methanol (2:1) containig 20 mM HCl for 30 min. The suspension was centrifuged at 2 500 xg for 15 min. The silica gel was re-extracted in the same manner, three times. The combined extracts were passed through a fine-porosity fritted glass funnel and the filtrate dried under a stream of nitrogen. The residue was taken up in a small volume of chloroform:methanol (2:1) and chilled to -20°C. To this was added 4 volumes of precooled (-20°C) diethyl ether and the mixture stored at -20° C for 24 h. The precipitate containing the DCCD-binding protein was removed by centrifugation at 4 000 xg and -20°C for 45 min and then dried under nitrogen at 20°C.

Chromatography on CM-Cellulose

The methods described by Rouser and coworkers (169) and Altendorf and coworkers (150) were modified for our purposes.

CM cellulose (Whatman CM-32 or BioRad Cellex CM) was suspended in 15 volumes (w/v) of glacial acetic acid for 1.5 h at 20°C. The fines were removed and the resin washed with distilled water until the pH of eluent was between 5 and 7. Next, the resin was suspended in 10 volumes (v/v) of 25% (v/v) ammonium hydroxide. After 1.5 h at 20°C, the suspension was diluted several fold with distilled water and centrifuged at 4 500 xg for 10 min. The sedimented resin was washed repeatedly with distilled water until the pH of the supernatant was between 7 and 9. Finally, the resin was washed sequentially with 3 volumes (v/v) each of methanol, chloroform: methanol (1:1), and chloroform:methanol (2:1).

A slury of the resin in chloroform:methanol (2:1) was poured into a solvent-resistant column and the column bed protected with glass wool held down with marbles. Two volumes (v/v) of chloroform:methanol (2:1) was passed through the column and the sample (in chloroform:methanol (2:1)) was then applied. The column was washed sequentially with 5-10 volumes of chloroform:methanol (2:1), 3-5 volumes of chloroform:methanol (1:1), and the DCCD-binding protein eluted with 5 volumes of chloroform:methanol:water (5:5:1). In some experiments, the column was washed with 5 volumes of chloroform:methanol:water (10:10:1) prior to elution of the DCCD-binding protein. The fractions containing the DCCD-binding protein were pooled and chloroform and water added so that the final ratio of chloroform:methanol: water was 8:4:3 and a two-phase system was obtained (149). The lower phase was diluted with 1 volume of chloroform and the requisite amount of methanol was added to keep the solution clear. The mixture was then taken to dryness by rotoevaporation at 20°C. The dried contents were immediately taken up in a small volume of chloroform:methanol (2:1) containing 20 mM ammonium acetate and applied to a Sephadex LH-60 column.

Chromatography on Sephadex LH-60

This was based on the method of Fillingame (149). Sephadex LH-60 was stirred into 25 volumes (w/v) of chloroform:methanol (2:1) containing 20 mM ammonium acetate and left at 20°C for 6 h. A slurry of this suspension was poured into a solvent-resistant column and a small amount of washed reagent-grade sea sand carefully applied to the top of the bed to facilitate sample application. The column was equilibrated with the same solvent at 20°C and the sample applied. The DCCD-binding protein was eluted with the same solvent system at a flow rate of 0.1 to 0.15 ml per min. The fractions containing the purified DCCD-binding protein were pooled and stored at -20°C. When the protein was needed, samples were removed and taken to dryness under reduced pressure at 16-18°C in a rotary evaporator (Buchler Instruments, Rotary Evapo-mix).

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sample Preparation

Salts and detergents were removed from the samples in one of two ways:

(i) Samples from fractions obtained after column chromatography were dialyzed at 4°C for 20-48 h against 100 volumes of distilled water (containing 0.01% (v/v) hibitaine) and which was changed at 8 h intervals. The dialyzed fractions were lyophilized and stored at -70°C.

(ii) A more rapid method of removal of salt and detergent from the samples was by the centrifugation-column chromatography procedure of Penefsky (170).

A slurry of Sephadex G-50 equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 10 mM $MgCl_2$ was poured into the barrel of a 1 ml disposable tuberculin syringe (Becton-Dickinson) which had been fitted

with a porous polyethylene disk. It was placed in a 15 x 125 mm test tube and allowed to drain. The volume of the gel was 1 cm^3. Excess buffer was removed by centrifuging at 900 xg for 2 min at 20°C (International Equipment Company, Model CL45436M). A 100 µl sample was carefully loaded onto the column which was then recentrifuged. The eluent was depolymerized before gel electrophoresis.

Depolymerization of Samples

The eluent was depolymerized by adding an equal volume of 125 mM Tris-HCl buffer, pH 6.8 containing 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue, and the mixture heated at 100°C for 5 min. The sample was cooled to 20°C.

Lyophilized samples were dissolved in a solution containing 62.5 mM Tris-HCl buffer, pH 6.8, 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue and heated at 100°C as above.

The DCCD-binding polypeptide was depolymerized in the same buffer as described previously except that the final Tris concentration was 50 mM and the sample was heated at 45°C for 18 h.

The protein concentration of the depolymerized sample was in the range of 0.25-1 mg/ml.

In some instances, centrifugation of the depolymerized sample in a desk-top centrifuge (International Equipment Company, model CL45436M) at full speed for 5 min was needed to remove any insoluble material prior to gel electrophoresis.

Slab-Gel Electrophoresis

(i) Preparation of Separating Gel

SDS-polyacrylamide gels were run using the discontinuous buffer system of Laemmli (171). A 13% (w/v) acrylamide gel was prepared as follows:

15 ml of separating gel containing 0.375 M Tris-HCl buffer, pH 8.8, 0.1% (w/v) SDS, 13% (w/v) acrylamide and 0.35% (w/v) N,N'-methylene-bis-acrylamide was prepared (the acrylamide and N,N'-methylene-bis-acrylamide were made up as a stock solution of 30% (w/v) and 0.8% (w/v), respectively, in distilled water, filtered through a Whatman 1 MM filter and stored in the dark at 4°C). 75 µl of freshly-prepared 10% (w/v) ammonium persulphate was added to the mixture and the solution degassed by aspiration. Polymerization of the solution was initiated by addition of 15 µl of TEMED and the solution was quickly poured between two glass plates of a BioRad slab-gel former (172) separated by 0.75 mm spacers. A pocket-former consisting of a teflon comb, 0.75 mm thick, and with a row of ten or twenty teeth, was inserted between the upper edges of the plates and the separating gel was poured to within 1.0 - 1.5 cm from the bottom of the comb. A small amount of tertiary butanol was gently layered over the gel so as to obtain a straight boundary during polymerization. The gel was allowed to polymerize at 20°C for 1-2 h or in some cases, overnight, after which the tertiary butanol was removed by means of a filter paper. The surface of the gel was rinsed with distilled water and the excess water removed with a filter paper. The pocket-former was reinserted between the plates and the stacking gel poured to the top of the comb. In some experiments, the separating gel consisted of 10% (w/v), 15% (w/v) or a linear gradient of 7.5 to 16.5% (w/v) acrylamide.

(ii) Preparation of Stacking Gel

A 5 ml stacking gel solution containing 0.125 M Tris-HCl buffer pH 6.8, 0.1% (w/v) SDS, 4% (w/v) acrylamide and 0.105% (w/v) N,N'-methylene-bis-acrylamide was prepared. To this was added 15 µl of a freshly-prepared solution of 10% (w/v) ammonium persulphate and the solution deaerated. Polymerization was initiated by adding 10 µl of TEMED and the solution layered over the separating gel. The gel was allowed to polymerize for 1.5-2 h after which the pocket former was carefully removed and the pockets rinsed and filled with electrode buffer, pH 8.4 consisting of 25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS. The depolymerized samples (25-100 µg protein) were applied to the gel beneath the electrode buffer by means of a Hamilton syringe.

Gel Electrophoresis in Tubes

Two types of gels were used.

(i) A separating gel solution was prepared as described before under "Preparation of separating gel". After initiation of polymerization, the gel solution was poured into a glass tube to a height of 10 cm. The tube had a length of 13 cm with an internal diameter of 6 mm. The lower end was sealed with a piece of parafilm. The surface of the gel was overlayed with tertiary butanol and the gel allowed to polymerize for 1.5-2 h. Following removal of the tertiary butanol, a 4% (w/v) acrylamide stacking gel, prepared as described above was layered on top of the separating gel to a height of 1.0-1.5 cm. Tertiary butanol was again layered on top of the stacking gel solution which was allowed to stand at 20°C for 2-3 h. The gel surface was then rinsed with distilled water, then with electrode buffer. The tube was filled to the surface with electrode buffer, pH 8.4

and the sample applied to the surface of the gel beneath the buffer as before.

(ii) The second method, described by Fillingame (144), is a modification of that of Laemmli (171) and includes 8 M urea. A gel solution consisting of 0.375 M Tris-HCl buffer, pH 8.8, 0.5% (w/v) SDS, 8 M urea, 10% (w/v) acrylamide, 0.5% (w/v) N,N'-methylene-bis-acrylamide and 0.075% (w/v) ammonium persulphate was deaerated and polymerization initiated by addition of TEMED at a concentration of 125 µl per ml of gel solution. The solution was immediately poured into 13 cm x 0.6 cm (internal diameter) glass tubes, to a height of 10 cm. Tertiary butanol was carefully layered on top of the gel solution and allowed to stand at 20°C for 1.5-2 h. The surface of the gel was rinsed with distilled water, and then with electrode buffer, pH 8.4 which contained 25 mM Tris base, 192 mM glycine and 0.2% (w/v) SDS. The stacking gel was omitted. Samples were loaded onto the gel surface as before.

Gradient Gel Electrophoresis

One of two procedures was used:

(i) The first method used was developed by Cox and coworkers (173) and is a modification of the Laemmli method (171).

Two gel solutions were prepared simultaneously. 6 ml of a gel solution containing 0.375 M Tris-HCl buffer, pH 8.8, 0.1% (w/v) SDS, 7.5% (w/v) acrylamide, 0.2% (w/v) N,N'-methylene-bis-acrylamide and 0.033% (w/v) ammonium persulphate and a second 6 ml solution containing 0.375 M Tris-HCl buffer, pH 8.8, 10% (v/v) glycerol, 0.1% (w/v) SDS, 16.5% (w/v) acrylamide, 0.44% (w/v) N,N'-methylene-bis-acrylamide and 0.01% (w/v) ammonium persulphate were deaerated. Polymerization was initiated by addition of 6 µl

and 3 µl of TEMED to the solution, respectively. Both solutions were immediately transferred to a Buchler Instruments gradient-maker attached to a Buchler Instruments polystaltic pump by means of a Tygon tubing (2 mm internal diameter). The outlet of the tubing was placed between the two plates of a slab-gel former separated by two 0.75 mm thick lucite spacers.

The gel mixture was allowed to drip between the glass plates such that a linear gradient of 7.5% (w/v) acrylamide on the top to 16.5% (w/v) acrylamide at the bottom was obtained. Tertiary butanol was used to give a straight boundary during polymerization, after which a stacking gel (4% (w/v) acrylamide) was cast. The pockets were washed and filled with electode buffer pH 8.4 and sample loaded onto the gel surface as previously described.

(ii) The second method was a modification of the procedure of Weber and Osborn (174) and involved a phosphate-buffered system. Two gel solutions were prepared simultaneously. A 6 ml solution consisting of 0.1 M sodium phosphate buffer, pH 7.2, 0.1% (w/v) SDS, 7.5% (w/v) acrylamide, 0.2% (w/v) N,N'-methylene-bis-acrylamide and 0.033% (w/v) ammonium persulphate and a second 6 ml solution containing 0.1 M sodium phosphate buffer, pH 7.2, 10% (v/v) glycerol, 0.1% (w/v) SDS, 16.5% (w/v) acrylamide, 0.45% (w/v) N,N'-methylene-bis-acrylamide and 0.01% (w/v) ammonium persulphate were degassed. Polymerization was initiated by addition of 6 µl and 3 µl of TEMED, respectively. A 0.75 mm thick gradient slab gel was cast as in the discontinuous buffer system. The stacking gel consisted of 0.1 M sodium phosphate buffer, pH 7.2, 0.1% (w/v) SDS, 4% acrylamide, 0.11% (w/v) N,N'-methylene-bis-acrylamide and 0.03% (w/v) ammonium persulphate. Polymerization was initiated by addition of 2 µl TEMED per ml of gel solution. The sample pockets were rinsed and filled with an electrode

buffer consisting of 0.1 M sodium phosphate, pH 7.2 and 0.1% (w/v) SDS. Samples were then loaded onto the gel.

Electrophoresis

(i) Slab Gels

The two glass plates containing the gel were attached to a gel electrophoresis cell (BioRad, Model 220, dual vertical slab gel electrophoresis cell) and the samples loaded onto the gel surface. The cathode and the anode reservoirs were then filled with the appropriate electrode buffer and electrophoresis was carried out at 20°C at a constant current of 30 mA and 40 mA for 0.75 mm and 1.5 mm thick gels, respectively. Electrophoresis was stopped when the bromophenol blue dye was within 0.5-1 cm from the anodic edge of the gel.

(ii) Tube Gels

The tube containing the gel was placed in a BioRad Model 150A gel electrophoresis cell and sample loaded onto the gel surface. The appropriate electrode buffer was placed in the cathode and anode chambers and electrophoresis was carried out at 20°C and at a constant current of 5 mA until the dye front was within 0.5-1 cm from the anodic edge of the gel.

TWO-DIMENSIONAL ISOELECTRIC FOCUSING GEL ELECTROPHORESIS

Preparation of Sample

(i) This was based on the method of Cox and coworkers (140).

10 ml of precooled (4°C) acetone was added to 15 mg of membrane protein (10 mg/ml). After 30 min at 4°C, the mixture was centrifuged in a desk-top centrifuge at maximum speed for 10 min. The pellet was again extracted with 10 ml of acetone and then dried under a stream of nitrogen gas at

20°C. The dried material was taken up in lysis buffer containing 9.5 M urea, 2% (w/v) Nonidet P-40, 1.6% (w/v) pH 5-7 ampholytes, 0.8% (w/v) pH 3.5-10 ampholytes and 5% (v/v) 2-mercaptoethanol, and centrifuged at 15 600 xg for 10 min to remove any insoluble material. Extraction with acetone was omitted when either immunoprecipitated or purified ATPase complex preparations were analyzed.

(ii) The second method was that developed by Merril et al. (175).

To 1 ml of membrane protein (5 mg/ml) was added 1 volume of denaturation solution containing 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, 4.5% (w/v) Nonidet P-40, 3.2% (w/v) pH 5-7 ampholytes and 0.16% (w/v) pH 3.5-10 ampholytes. The mixture was sonicated at 50 W and 0°C in a Branson W185D Sonifier, for 15 second periods for a total of 1 min. It was then heated at 95°C for 5 min, after which it was cooled to 20°C and centrifuged at 15 600 xg for 10 min. To the supernatant was added urea at a concentration of 56 mg per 100 µl (6.6 M urea) and then applied to the gel.

First-Dimension Isoelectric Focusing

The first-dimension gel was prepared as described by O'Farrel (176) with the following modifications.

The first-dimension gel contained 9.5 urea, 3.77% (w/v) acrylamide, 0.215% (w/v) N,N'-methylene-bis-acrylamide, 2% (w/v) Nonidet P-40, 1.6% (w/v) pH 5-7 ampholytes, 0.8% (w/v) pH 3.5-10 ampholytes and 0.03% (w/v) ammonium persulphate. Polymerization was initiated by addition of 1 µl TEMED per ml of gel solution and the gel cast in tubes of 2 mm internal diameter and to a height of 10 cm. Tertiary butanol was layered over the gel which was allowed to stand at 20°C for 6-8 h. The surface of the gel

was then rinsed with lysis buffer. 50-250 µg of the prepared sample was loaded onto the gel and overlayed with a solution of 8 M urea in water. The gels were placed in a BioRad Model 150A gel electrophoresis cell and the anode and cathode chambers filled with freshly prepared 0.01 M H_3PO_4 and deaerated 0.02 M NaOH, respectively. The first-dimension was run at 20°C and 400-450 V for 16-18 h, followed by 800 V for 1-2 h, to give a total of 9000 to 9500 volt-hours.

Determination of pH Gradient

In the first-dimension, a gel loaded with sample preparation buffer rather than the prepared sample was run simultaneously. After electrophoresis, the acidic or basic end of the gel was identified and the gel cut into 1 cm segments. Each segment was incubated with 1.5 ml of deaerated distilled water for 1.5-2 h at 20°C after which the pH was measured.

Second-Dimension SDS-Polyacrylamide Gel

The second-dimension gel was either a 13% (w/v) or a linear 7.5% to 16.5% (w/v) acrylamide gel prepared in the discontinuous buffer system of Laemmli (171). The slab gels were cast as described before but modified as follows.

The gel was 1.5 mm thick rather than 0.75 mm and the pocket former was not used with the stacking gel. Instead, the stacking gel was cast to a height of 1.5-2 cm and was about 3 mm from the upper edge of the glass plate.

Prior to running the second dimension, the acidic or basic end of the gel from the first dimension was identified with India ink. The gel was incubated for 3 h in 5 ml of 125 mM Tris-HCl buffer, pH 6.8 containing 10%

(v/v) glycerol, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue. When the gel from the first-dimension was not to be run immediately in the second dimension, the gel in the above solution was frozen rapidly in a dry-ice ethanol mixture and stored at -70°C.

The gel from the first dimension was mounted above the second-dimension gel in 1% (w/v) agarose in 62.5 mM Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue.

Electrophoresis was carried out as previously described except that it was continued for 10-15 min after the dye front had run off the lower (anode) end of the gel. The gel could then be stained immediately without removal of ampholytes.

CROSSED IMMUNOELECTROPHORESIS

This was based on the methods of Nowotny (177), Bjerrum and Lundhal (178) and Mayer and Walker (179).

First-Dimension Gel Electrophoresis

2.4 ml of 1% (w/v) agarose in Bjerrum buffer, pH 8.8 containing 100 mM glycine, 38 mM Tris and 1% (w/v) Triton X-100 at 55°C was poured onto a 50 x 50 mm glass plate surrounded by a plastic mold. The agarose was allowed to cool to 20°C and the mold carefully removed such that a 40 x 40 x 1.5 mm gel was obtained. Agarose in the same buffer was layered around the periphery of the cast gel to the edges of the plate (agarose bridges) to give a more uniform conduction of the current. A row of four wells (3 mm diameter), about 1 cm from the cathodic edge of the gel, and about 1 cm apart, was cut out with a BioRad gel-puncher. The wells were filled with

various levels of antigen and the gel placed in a Pharmacia flat-bed electrophoresis unit at 4°C. Wicks, 4 cm wide (Ultrawicks, BioRad) were placed onto the agarose bridges in such a manner that they did not overlap onto the wells. The chambers were filled with Bjerrum buffer, pH 8.8, without any detergent and electrophoresis was carried out for 1.5-2 h at 4°C and 100 V. After electrophoresis, the gel was cut into strips (5 mm width) such that each contained a sample well at one end. The individual strips were stained immediately or run in the second dimension.

Second-Dimension Gel Electrophoresis

1% (w/v) agarose in Bjerrum buffer, pH 8.8 and various levels of antiserum (final volume, 2.4 ml) were mixed at 55°C and cast into a 40 x 40 x 1.5 mm gel as above. A strip of the gel from the first-dimension was placed at the cathodic end of the gel and agarose bridges constructed around the gel. The electrode buffer was the same as in the first dimension. Electrophoresis was carried out perpendicular to the direction of the first dimension for 16 to 18 h at 10 V and 4°C.

STAINING AND DRYING GELS

SDS-Polyacrylamide Gels

After electrophoresis, these gels were either stained immediately or fixed prior to staining. Gels were fixed in either a solution of:

(i) 5% (w/v) TCA, 5% (w/v) sulfosalicylic acid and 10% (v/v) methanol for 30 min at 60°C (173) or

(ii) 50% (w/v) TCA, overnight at 20°C (171).

The staining solutions were filtered through a Whatman 1 MM filer before use. The gels were then stained in one of the following staining

systems for different periods at 20°C.

(i) 0.12% (w/v) Coomassie Blue (Brilliant Blue R) in ethanol:acetic acid:water (25:8:67) (171)

(ii) 0.05% or 0.1% (w/v) Coomassie Blue in isopropanol:acetic acid:water (25:10:65) (180)

(iii) 0.25% (w/v) Coomassie Blue in methanol:acetic acid:water (45:10:45) (174)

(iv) 1% (w/v) Amido Black 10B in 7% (v/v) acetic acid (165).

Gels were then destained in 10% (v/v) acetic acid until a fairly clear background was obtained. The tube gels were scanned at 545 nm and 620 nm when Coomassie Blue and Amido Black were used, respectively. Slab gels were incubated in a solution of 10% (v/v) acetic acid and 2% (v/v) glycerol for 4-6 h and then dried onto a piece of Whatman 3 MM paper in a Gel Slab Dryer, Model 224 (BioRad), equipped with a heating unit and connected to a vacuum pump.

Crossed Immunoelectrophoresis Gels

The gels were processed by the methods of Weeke (188) and Mayer and Walker (179). After electrophoresis, the glass plate containing the gel was taken and the sample well filled with 0.9% (w/v) NaCl. A 5 x 5 cm piece of Whatman 3 MM paper was carefully placed on top of the gel, followed by a pad of absorbent paper towels, a glass plate and finally a weight of about 0.5 Kg. After 10 min at 20°C, the pressed gel was washed thrice by incubating each time with 0.9% (w/v) NaCl for 20 min. The gel was then soaked in distilled water for 20 min and then a piece of 5 x 5 cm Whatman 3 MM paper gently placed on top of the gel. This was placed in an oven at 70°C and allowed to dry. After the gel had dried onto the glass

plate, the paper was gently removed and the cooled (20°C) plate was incubated for 30 min in a solution of 0.25% (w/v) Coomassie Blue in methanol:acetic acid:water (45:10:45). The plate was rinsed with distilled water and destained in a solution containing 10% (v/v) acetic acid and 45% (v/v) methanol for 5-10 min or until the stained proteins (rockets) could be seen against a sufficiently colourless background. Finally, the plate was rinsed with distilled water and dried with a hair-dryer. It was kept in the dark.

PURIFICATION OF GOAT ANTI-RABBIT IMMUNOGLOBULIN BY AFFINITY CHROMATOGRAPHY

Preparation of Affinity Column

The affinity column was prepared by L. Molday (University of British Columbia) using the method of Cuatrecasas (181).

750 mg of CNBr was dissolved in 10 ml of distilled water and mixed with 20 ml of washed Sepharose 4B (equal volumes of resin and water) which had previously been adjusted to pH 11 with NaOH. The suspension was stirred at 0°C for 25 min and the pH kept between 10 and 11 by addition of 10 M NaOH. The gel was filtered and washed with 200 ml of distilled water, then with 0.1 M sodium borate buffer pH 9.0. The resin was washed for 2-3 min since CNBr-activated agarose is unstable.

80 mg of rabbit γ-globulin in 4 ml of 0.1 M sodium borate buffer pH 9.0 was added to 20 ml of the CNBr-activated gel (equal volumes of gel and buffer) in borate buffer and then stirred at 20°C for 16-18 h. The amount of protein bound to the resin was determined from the amount still remaining unbound in the supernatant following sedimentation of the gel. This was calculated from absorbance at 280 nm and using the extinction of $\varepsilon_{280}^{0.1\%} = 1.35$.

The resin was then washed with 0.1 M sodium borate buffer pH 9.0 and the residual active sites quenched by incubating the resin in several volumes of 0.1 M glycine for 6-8 h at 20°C. Finally, the resin was washed with phosphate-buffered saline, pH 7.5 (PBS: 0.137 M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4) and stored in the same buffer in the presence of 20 mM NaN_3 at 4°C.

Purification of Goat Anti-Rabbit Immunoglobulin

The rabbit γ-globulin-Sepharose 4B affinity column in PBS was poured into a 10 x 1 cm BioRad column to a height of 4 cm and equilibrated with PBS. 5 ml of goat anti-rabbit serum was applied to the column at 20°C and washed with several volumes of PBS. Goat anti-rabbit immunoglobulin was eluted from the column with 3M NaSCN in distilled water (pH 7.5). Fractions containing goat anti-rabbit immunoglobulin, as determined by SDS-polyacrylamide gel electrophoresis, were pooled and dialyzed against 50 volumes of 0.1 M NH_4HCO_3 buffer pH 7.4 containing 20 mM NaN_3 for 24 h at 4°C with exchanges of the external buffer at intervals of 6 h. The dialyzed material was lyophilized and stored at -70°C.

RADIOIODINATION OF GOAT ANTI-RABBIT IMMUNOGLOBULIN

To 1 mg of affinity-purified goat anti-rabbit immunoglobulin (1 mg/ml) in phosphate buffered saline, pH 7.4 (PBS) was added 1 mCi of Na(^{125}I) followed by 100 µl of a 0.4% (w/v) Chloramine T solution. The mixture was incubated in the fume-hood at 20°C for 20 min, after which, it was loaded onto a 19 x 1.5 cm column of BioGel P6DG which had been equilibrated with PBS. The column was eluted with the same buffer at a flow rate of 0.1-0.15 ml per min and 0.9 ml fractions collected. Fractions containing

protein (determined by absorbance at 280 nm) and the highest radioactivity were pooled, made up to 20 mM NaN_3, and stored at 4°C. A specific radioactivity of 0.5 to 2 x 10^6 dpm/ug was obtained

PEPTIDE MAPPING OF CNBr-CLEAVED DCCD-BINDING PROTEIN

One-Dimensional Thin Layer Separation

A 20 x 20 glass plate precoated with silica gel G (250 µm, Analtech Inc.) was activated by placing it in an oven at 100°C for 1 h. The plate was cooled to 20°C and then the sample (in 80% (v/v) formic acid) was applied as a spot of 2-3 mm in diameter. The sample was applied repeatedly on the same spot and finally air-dried at 20°C. The plate was placed in a covered glass tank, which had been equilibrated with the appropriate solvent, and developed at 20°C. The solvent system consisted of one of the following:

(1) chloroform:methanol (9:1 (v/v)). Run time, 40 min.

(2) n-butanol:acetic acid:water (4:1:5). Run time, 3 h.

(3) chloroform:methanol:water (65:25:4) containing 20 mM HCl. Run time, 65 min.

(4) n-butanol:pyridine:acetic acid:water (60:40:12:48). Run time 4.5-5 h.

After the plates were developed, they were dried overnight at 20°C and the positions of the peptides determined.

Two-Dimensional Thin Layer Separation

The first dimension was thin-layer electrophoresis. Prior to electrophoresis, a 20 x 20 cm cellulose MN300 plate (CEL 300-10, 0.1 mm,

Macherey-Nagel Co.) was developed in n-butanol:pyridine:acetic acid:water (60:40:12:48) perpendicular to the direction of electrophoresis, for 6 h at 20°C and then thoroughly air-dried. The plate was then sprayed with thin-layer electrophoresis (TLE) buffer, pH 6.45 consisting of a mixture of pyridine:acetic acid:water (20:0.7:180) and electrophoresis carried out at 400 V for 1 h in a Camag TLE apparatus. The sample (in 80% (v/v) formic acid) was spotted (2-3 mm diameter) onto the dried plate at the midpoint between the electrodes and 2.5 cm from the edge of the plate. The spot was air-dried and protected by gently inverting a clean vial over the spot. The plate was resprayed with TLE buffer and through diffusion, the area under the vial was dampened with buffer. Electrophoresis was carried out at 400 V for 65 min, after which the plate was thoroughly dried at 22°C, overnight.

Separation in the second dimension was by ascending chromatography. A covered glass chromatography tank was equilibrated at 20°C with a solvent mixture of n-butanol:pyridine:acetic acid:water (60:40:12:48) by lining the tank with sheets of Whatman 3 MM paper soaked with the solvent mixture. The plate from the first dimension was then developed in the solvent system such that the direction of migration of the solvent front was perpendicular to the direction of electrophoresis. After 5.5-6 h, at which point the solvent front was 2-3 cm from the top edge of the plate, the plate was removed, thoroughly dried at 20°C, and the peptides detected.

Detection of Peptides

The dried thin-layer plate was sprayed with a solution containing 1% (w/v) ninhydrin and 4% (v/v) acetic acid in acetone, and then developed in an oven at 100°C for 15 min. An outline of the peptide spots was made

immediately. In some cases, the spots were fixed by spraying the plate with solution containing 1 ml saturated $Cu(NO_3)_2$, 0.2 ml 10% (v/v) HNO_3 and 100 ml 96% (v/v) ethanol. The plate was air-dried and stored in the dark.

CHEMICAL MODIFICATION OF MEMBRANES

Labelling of Membrane Vesicles of E. coli with [^{14}C]DCCD

[^{14}C]DCCD (50 mCi/mMol) was purchased in ether in a sealed ampoule. It was opened and the contents transferred to a vial. The ether was evaporated at 20°C in the fume-hood and the dried contents taken up in absolute ethanol and stored at -20°C.

The method of labelling was based on that described by Fillingame (144). Membranes prepared in TM buffer, pH 8.0 were suspended in 10 mM Tris-H_2SO_4 buffer, pH 7.6 containing 0.25 M sucrose, 5 mM $MgSO_4$ and 0.2 mM DTT at a protein concentration of 20 mg/ml. To 0.5 ml of membrane suspension was added 0.02 volumes (10 µl) of 5 mM [^{14}C]DCCD in absolute ethanol and the suspension stirred at 4°C for 24 h. The labelled membranes were washed three times by resuspension in buffer following centrifugation at 250 000 xg for 2 h. The membranes were used immediately or stored at -20°C.

Treatment of Membrane Vesicles with Phenylglyoxal

A 200 mM phenylglyoxal solution was prepared by dissolving 53.8 mg in 1.8 ml of 50 mM HEPES-KOH buffer, pH 7.5 containing 10 mM $MgCl_2$ and 10% (v/v) glycerol. The pH was readjusted to 7.5 by addition of KOH and the final volume made up to 2 ml with buffer.

Two ml of urea-stripped membrane vesicles in 50 mM HEPES-KOH buffer,

pH 7.5 containing 10 mM $MgCl_2$ and 10% (v/v) glycerol at a protein concentration of 12.5 mg/ml was incubated with 1 volume of 200 mM phenylglyoxal in the dark at 20°C for 3 h. The suspension was then diluted 8-10 fold with buffer and centrifuged at 250 000 xg for 2.5 h. The membranes were washed once by resedimentation in buffer and finaly suspended at a protein concentration of 10 mg/ml. They were used immediately or rapidly frozen and stored at -70°C.

CHEMICAL MODIFICATION OF THE DCCD-BINDING PROTEIN

Hydrolysis of the DCCD-Binding Protein

This was based on the method of Moore and Stein (182).

Heavy-walled 13 x 100 mm Pyrex culture tubes were washed sequentially in chromic acid, alcoholic KOH and 1 M HCl. They were rinsed with distilled water after each step and finally dried at 100°C. 100-150 µg of purified DCCD-binding protein in chloroform:methanol (2:1) containing 20 mM ammonium acetate, was taken to dryness in a 13 x 100 mm Pyrex tube at 16-18°C by using a rotary evaporator (Buchler Instruments, Rotary Evapo-mix). The protein in the tube was taken up in 1.5-2 ml of 6 M HCl. With an oxygen flame, a section of the tube about 3 cm from the top was partially constricted. The lower half of the tube was immersed in a dry ice-ethanol mixture for 5-10 min, at which point the solution in the tube started to become viscous. The tube was then connected to a vacuum pump through a piece of Tygon tubing and the system evacuated to a pressure of less than 50 microns. During this period, gently warming the lower half of the tube by hand caused bubbles to rise from the viscous solution. Re-immersing the tube into the dry ice mixture broke the bubbles. The tube was sealed at the constriction, when bubble formation had almost ceased. The vacuum seal

was checked for leaks with an ionizing gun. Hydrolysis was then conducted at 110°C ± 2°C for 24, 31, 42 and 60 h after which the tubes were cooled to 20°C. Any liquid on the inner walls of the tube was spun down by centrifugation in a desk-top centrifuge (International Equipment Company, model CL 45436 M) at 900 xg for a few minutes. The tube was scored with a file near the constriction and cracked open by means of a hot glass rod. Hydrochloric acid was removed under reduced pressure at 70-80°C by placing the sample tube in an 18 x 150 mm Pyrex ignition tube and attaching it to the rotary evaporator. Almost all of the HCl was removed in 45-60 min. The dried contents were taken up in the appropriate buffer for analysis of its amino acid composition. The amino acid composition was determined by using the Durram (model D-500) amino acid analyzer.

Treatment of the DCCD-Binding Protein with Cyanogen Bromide

The method of Sebald et al. (183) was followed.

2 mg of purified DCCD-binding protein was taken to dryness and the residue dissolved in 0.8 ml of 1 M cyanogen bromide in 98% (v/v) formic acid. 0.2 ml distilled water was added to the mixture which was incubated in the dark at 20°C for 17-18 h. The solvent was then removed by rotary evaporation under reduced pressure at 30-40°C as previously described. The dried residues were dissolved in the appropriate buffer. Control experiments were simultaneously carried out with DCCD-binding protein, which were treated in the same manner but in the absence of cyanogen bromide.

Treatment of the DCCD-Binding Protein with Performic Acid

This was done according to the method of Hirs (184).

Performic acid was prepared by mixing 9 ml of 90% (v/v) formic acid

with 1 ml of 30% (v/v) H_2O_2 and incubating the mixture at 20°C for 1 h. The solution was cooled to 0°C and used immediately.

DCCD-binding protein in chloroform:methanol (2:1) containing 20 mM ammonium acetate was taken to dryness and performic acid added to the dry protein such that the protein concentration was 1-2% (w/v). The tubes were capped and incubated in the dark at 0°C for 20 h, after which the performic acid was removed by evaporation under reduced pressure at 35-40°C. Evaporation to dryness took about 45 min. The residue was either subjected to hydrolysis in the same tube to determine its amino acid composition as described before or taken up in the appropriate buffer. In some cases, control experiments were simultaneously carried out with DCCD-binding protein, which were treated in the same manner with 80% (v/v) formic acid only.

Treatment of the DCCD-Binding Protein with 2,3-Butanedione or Phenylglyoxal

A 200 mM solution of 2,3-butanedione and a 150 mM solution of phenylglyoxal were made up in 100 mM sodium borate buffer containing 2% (w/v) Triton X-100, and the pH adjusted to 8.8 with NaOH.

0.3 mg of dried DCCD-binding protein was dissolved in 1 ml of borate-Triton buffer, pH 8.8 and incubated with an equal volume of either 200 mM 2,3-butanedione or 150 mM phenylglyoxal in the dark at 20°C for 3 h. 1 ml of 330 mM L-arginine hydrochloride in 100 mM sodium borate buffer, pH 8.8 was then added. The final mixture was incubated for another 30 min and then lyophilized. The dried residue was dissolved in borate-Triton buffer at a protein concentration of 0.15 mg per ml. Control experiments were simultaneously carried out with DCCD-binding protein, which were treated in the same manner but in the absence of either 2,3-butanedione or phenylglyoxal.

TREATMENT WITH PROTEASES

ECF_1 and DCCD-Binding Protein

ECF_1 obtained after purification on AH-Sepharose CL-4B was suspended in 50 mM HEPES-KOH buffer, pH 7.5 containing 10 mM $MgCl_2$ and 10% (v/v) glycerol and treated with one of the following: α-chymotrypsin, Staphylococcus aureus V_8 protease or pronase at a protein:protease ratio of 10:1. The final protein concentration of ECF_1 was 0.75 mg/ml.

Similarly, DCCD-binding protein in 100 mM sodium borate buffer, pH 8.8 containing 2% (w/v) Triton X-100 was treated with one of the above proteases at a protein:protease ratio of 10:1. The DCCD-binding protein was also treated with TPCK-trypsin at a protein:protease ratio of 10:1.

The reaction mixtures were incubated at 37°C for 3 h after which TPCK-trypsin was inhibited by addition of an equivalent amount of soybean trypsin inhibitor followed by incubation for 15 min at 37°C. The final concentration of DCCD-binding protein in the reaction mixtures was 0.13 mg protein/ml. 25 µl of these mixtures were incubated in flex-vinyl microtitre wells, which had been previously coated with 0.1% (w/v) poly-L-lysine. For controls, 25 µl of these mixtures containing no DCCD-binding protein was also incubated in polylysine-treated microtitre wells. The appropriate radioimmune assays were then carried out as described in the legends to the Figures in the RESULTS section.

PREPARATION OF ANTIGENS FOR IMMUNIZATION

ECF_1

ECF_1 was prepared for injection by Helga Stan-Lotter (University of British Columbia) as follows:

0.7 mg of ECF_1 (obtained after AH-Sepharose 4B chromatography) at

1.4 mg/ml was mixed with 1 volume of complete Freund's adjuvant and the mixture emulsified by repeated passing between two syringes connected through a 22 gauge needle. The mixture was ready for injecting into the rabbit when a drop of the emulsion placed on the surface of the water did not disperse when the beaker was slightly agitated.

DCCD-Binding Protein

The DCCD-binding protein was dissolved in a solution containing 0.9% (w/v) NaCl and 2% (w/v) SDS at a protein concentration of 2 mg/ml. It was mixed with two volumes of complete Freund's adjuvant and emulsified. In some preparations, incomplete Freund's adjuvant was used.

IMMUNIZATION OF THE RABBIT

A female, New Zealand rabbit of about 3 Kg was obtained from the University of British Columbia Animal Care Unit, several months prior to immunization and maintained as recommended (185). A month after its arrival, the rabbit was bled at 4-week intervals until enough preimmune serum was obtained. Three weeks after the last bleeding, the animal was immunized a follows. The rabbit was wrapped in a blanket leaving only the head and the site of injection exposed. A small amount of hair was clipped off at the area to be injected and the injection site cleaned with 70% (v/v) ethanol. A tent of skin was raised with the thumb and index finger and with the other hand, the needle (22 gauge) was inserted underneath the skin and parallel to the underlying muscle. The antigen was injected immediately, the needle withdrawn, and the site of injection wiped with ethanol. The rabbit was generally injected at two sites on the thigh of the hind legs with a total of either 0.6 mg ECF_1 or 0.3 mg DCCD-binding

protein. Subsequent injections were at 4-week intervals, with a total of either 0.3 mg ECF_1 or 0.2 mg DCCD-binding protein and incomplete Freund's adjuvant was substituted for the complete Freund's adjuvant in the case of ECF_1. For the DCCD-binding protein, incomplete Freund's adjuvant was used after the fifth injection as continued use of the complete adjuvant resulted in the development of hard pea-size lumps at the sites of injection.

BLEEDING THE RABBIT

The method of Herbert (186) was used.

The rabbit was wrapped in a blanket, leaving only the head exposed. A small area over the outer marginal vein of the ear was shaved and cleaned with soap and water. The shaved area was dried and lightly smeared with petroleum jelly. A gauze soaked in xylene was pressed onto the shaved area for a few seconds, until the marginal ear vein was visibly dilated. With a sterile razor blade, an incision of about 2 mm was made into the vein in the longitudinal direction and about 2 inches from its distal end. The ear was held in the horizontal position and the thumb and index finger was used to occlude the venous return. A centrifuge tube was placed under the ear to collect the blood. When the flow of blood stopped prematurely, the wound was wiped with a piece of gauze to open the wound. Usually 25 to 30 ml of blood was collected at each bleeding.

The bleeding was stopped by placing a dry gauze on the wound and securing it with a paper clip. On occasion, the bleeding was persistent and a piece of Gelfoam sponge (The Upjohn Co., Michigan) was used to stop the bleeding. After the bleeding had stopped, any xylene remaining was removed with 70% (v/v) ethanol and the ear wiped with soap and water. The rabbit was bled alternately from each ear at 7-10 days post-injection.

SEPARATION OF SERUM

This was based on the method of Garvey (187).

The blood on collection clotted to some degree. The clot was separated from the wall of the centrifuge tube with a wooden applicator stick and the blood allowed to stand at 20°C for 2 h, then at 4°C for 18-24 h. The serum was removed and centrifuged at 2 000 xg for 30 min. Centrifugation was repeated when the serum was not clear. On occasion, the serum was turbid due to the presence of lipids (or haemoglobin) and could not be cleared by centrifugation at 2 000 xg. In these cases, the serum was centrifuged at 25 000 xg for 30 min. A film of lipid formed on the surface which was easily removed with a tissue paper. The serum was passed through a sterile 0.22 µm Millipore filter and stored at -70°C.

PARTIAL PURIFICATION OF IMMUNOGLOBULINS

This was based on the methods of Nowotny (177) and Mayer and Walker (179).

3 ml of saturated $(NH_4)_2SO_4$ (neutralized to pH 7.0 with NH_4OH) was added dropwise to 5 ml of serum and the mixture stirred at 20°C. The pH of the mixture was monitored during the addition of $(NH_4)_2SO_4$. After 30 min of incubation, the mixture was centrifuged at 17 600 xg for 30 min. The pellet was resuspended to the original volume (5 ml) in 10 mM potassium phosphate buffer, pH 7.0 and to this was added 2.6 ml of saturated $(NH_4)_2SO_4$ and the precipitate removed by centrifugation. It was then taken up in 5 ml of 10 mM potassium phosphate buffer, pH 7.0 and dialyzed at 4°C against 500 ml of distilled water for 20 h followed by dialysis against 250 ml of 0.4 M Sucrose and 10 mM $MgSO_4$ for 12 h. The external medium was changed at 6 h intervals. The dialyzed material was centrifuged to remove

insoluble material and the supernatant concentrated to half its original volume (2.5 ml) by ultrafiltration using an Amicon XM-50 filter. It was stored at -70°C.

BINDING OF ECF_1 TO MEMBRANE VESICLES

The measurement of the extent of rebinding of ECF_1 to urea-treated membrane vesicles which had been treated with proteases or phenylglyoxal was carried out as follows.

(i) 2-4 mg of the treated membrane vesicles in 50 mM HEPES-KOH buffer, pH 7.5 containing 10 mM $MgCl_2$ and 10% (v/v) glycerol were incubated with various levels of ECF_1 at 4°C for 45 min in a final volume of 0.5 ml. The mixture was then diluted 8-10 fold with buffer and the membranes sedimented at 250 000 xg for 2.5 h. The membranes were resuspended in buffer and the ATPase activity measured.

(ii) Urea-treated membrane vesicles were also treated with antiserum prior to rebinding of ECF_1.

Urea-treated membranes (15 mg/ml) in 50 mM HEPES-KOH buffer, pH 7.5 containing 10 mM $MgCl_2$ and 10% (v/v) glycerol were incubated at 4°C for 20 h with different amounts of antiserum in a final volume of 4.4-4.5 ml. After a 4-fold dilution in buffer, the mixture was centrifuged at 250 000 xg for 2.5 h. The sedimented vesicles were washed once by suspension in buffer followed by resedimentation as before. The washed vesicles were suspended in buffer at 6.5 mg protein per ml and reconstituted with ECF_1 as described above.

ASSAYS

Determination of Protein

One of several procedures was used.

(i) The protein in most experiments was determined by the method of Lowry et al. (189). Bovine serum albumin was used as the standard over a range of 0-300 µg. Interference by components in the sample buffer was corrected for by incorporating in the standard curve the appropriate volume of sample buffer equivalent to that assayed.

Triton X-100 in high concentrations gave a greenish-yellow precipitate. In these cases, the precipitate was removed by centrifugation at 12 000 xg for 10 min and the absorbance of the supernatant measured at 500 nm.

(ii) The protein content in samples obtained during the isolation and purification of the DCCD-binding protein was determined by a modified procedure of Lowry et al. (189), as done by Fillingame (144).

Membrane samples were dissolved in 0.5 M NaOH whereas DCCD-binding protein samples were taken to dryness under reduced pressure to remove organic solvents and then solubilized by adding 2.5% (w/v) SDS in 0.5 M NaOH. Both were incubated at 37°C for 2 h prior to assay.

In addition, 1% (w/v) SDS was included in the 2% (w/v) Na_2CO_3 Lowry reagent used in these assays. Bovine serum albumin was used as the standard protein over a range of 0-300 µg.

(iii) The third method was that of Bradford (190). This method was used for soluble enzyme preparations (e.g. ECF_1 and its subunits) where interfering substances in the buffer were minimal and the amount of protein to be assayed was low. Bovine serum albumin was used as the standard over a range of 0-50 µg.

If an interfering substance was present in the sample buffer, an

equivalent volume was incorporated in the samples used for the standard curve. 3 ml of filtered reagent containing 0.01% (w/v) Coomassie Brilliant Blue G-250, 5% (v/v) 95% (v/v) ethanol and 0.1% (v/v) 85% (v/v) H_3PO_4 was added to 0.1 ml of sample or standard protein and the absorbance at 595 nm measured after 15 min at 20°C.

Determination of ATPase Activity

Measurement of ATPase activity was done according to the method of Davies and Bragg (21). The inorganic phosphate released during the reaction was determined by a modified method of Ames (122).

(i) Rapid Assay

This assay was used when interfering substances such as phospholipids and detergents were not present in the sample and was generally used to determine the activity of the ECF_1 preparations.

The sample was incubated with 0.5 ml of 100 mM Tris-HCl buffer, pH 8.3 containing 5 mM ATP and 2.5 mM $CaCl_2$ at 37°C. After a period of incubation, 2.5 ml of "inorganic phosphate assay mixture" containing 1.8 ml ammonium molybdate (0.42% (w/v) ammonium molybdate in 2.86% (v/v) H_2SO_4), 0.3 ml of 10% (w/v) ascorbic acid, 0.25 ml of 10% (w/v) TCA and 0.15 ml water, was added.

The reaction mixture was incubated at 37°C for 15 min and the reaction stopped by placing the mixture at 0°C. The absorbance at 660 nm was measured against a reagent blank and the amount of inorganic phosphate liberated was calculated from a standard curve. $MgCl_2$ was substituted for $CaCl_2$ when the activity of TPCK-trypsin-treated ECF_1 was measured.

(ii) Slow Assay

This assay was used when interfering substances in the samples were

present. The procedure was essentially the same as described for the "rapid" assay except that after incubation of the sample with ATP, the reaction was stopped by addition of 0.5 ml of 10% (w/v) TCA and the reaction mixture centrifuged at 12 000 xg for 10 min 0.5 ml of the supernatant was removed and the inorganic phosphate which had been released was determined by addition of 2.5 ml of "inorganic phosphate assay mix" as before.

One unit of ATPase activity was defined as the amount of enzyme which liberated 1 µmol of phophate per min at 37°C.

Substrate Oxidation-Dependent Quenching of Fluorescence of 9-Aminoacridine

This was based on the method of Singh and Bragg (191).

0.1 ml of everted membrane vesicles (10 mg/ml) in 50 mM HEPES-KOH buffer, pH 7.5 containing 5 mM $MgCl_2$ was mixed in a fluorescence cuvette in a final volume of 2 ml with 10 mM HEPES-KOH buffer pH 7.5 containing 300 mM KCl, 5 mM $MgCl_2$ and 2-10 µM 9-aminoacridine. The energy sources were NADH, ascorbate in the presence of PMS or ATP. Each was prepared fresh in 10 mM HEPES-KOH buffer, pH 7.5 containing 300 mM KCl and 5 mM $MgCl_2$. In the case of ascorbate, PMS was added to the membrane vesicles before 9-aminoacridine. DCCD, when required, was prepared as a stock solution in absolute ethanol and stored at -20°C. The maximum level of ethanol allowed in the reaction cuvette was 2% (v/v).

Fluorescence was excited by light at 420 nm and emission was measured at 500 nm (192). At these wavelengths, there was no interference from the fluorescence of NADH. Fluorescence was measured at 20°C with a Turner Model 420 Spectrofluorometer connected to a Varian strip chart recorder.

Measurement of Proton Conduction in K^+-loaded Membrane Vesicles

K^+-loaded vesicles in 0.4 M sucrose, 10 mM $MgSO_4$ (0.85 ml) were stirred in a glass cuvette at 20°C. Valinomycin was added to the vesicle and the pH change which followed was measured with a glass pH electrode connected to a Fisher Accumet Model 325 expanded scale pH meter. The output of the meter was amplified so that 0.2 pH units gave a full-scale deflection on the recorder (John's Scientific Linear Co.). In some experiments, the vesicles were preincubated with DCCD (in ethanol) or with ammonium sulphate purified antiserum for 45 min. at 20°C. Each assay was internally calibrated by addition of an aliquot of known concentration of acid (H_2SO_4 or HCl).

Determination of Cytochrome Content

This was used in a semi-quantitative manner to detemine whether any form of separation was achieved between the ATPase complex and the cytochromes during ion-exchange, hydrophobic-exchange or gel exclusion chromatography.

1.5 ml of sample at 20°C was placed in each of two cuvettes of 1 cm light path. 25-50 µl of 0.3% (v/v) H_2O_2 was added to the reference cuvette, while the other sample was reduced with an excess of sodium dithionite. The reduced versus the oxidized (difference) spectrum was then scanned between 500 and 670 nm in a Perkin Elmer model 356 Double Beam Spectrophotometer equipped with a recorder. Baseline readings were obtained by scanning two cuvettes of untreated samples in the same range.

Cytochome d content was calculated by measuring one-half the height in absorbance units from the trough at 648 nm to the peak at 628 nm in the difference spectra and using the extinction coefficient of 8.51 $mM^{-1}cm^{-1}$

(193). The content of cytochrome a_1 was determined from the absorbance above the baseline at 594 nm and using the extinction coefficient of cytochrome a_3 of 4.6 $mM^{-1}cm^{-1}$ (193). Cytochrome b_1 content was determined from the height of the peak above the baseline at 558 nm and using the extinction coefficient of 16.0 $mM^{-1}cm^{-1}$ (193).

Determination of Catalase Activity

Catalase was used as a standard of molecular weight 232 000 for calibrating gel-filtration columns. Activity was determined by the method of Beers and Sizer (194).

1.5 ml of 4% (v/v) H_2O_2 was mixed with 25 ml of 50 mM potassium phosphate buffer, pH 7.0. To 1.99 ml of this mixture in a cuvette was added 10 µl of the enzyme solution to be measured. The change in absorbance at 240 nm was measured with a spectrophotometer equipped with a recorder.

One unit of activity was expressed as the rate of change in absorbance at 240 nm per min at 37°C.

Determination of Radioactivity in Gel Slices

The tube gel was frozen at -70°C for 30-45 min and then sliced into 1 mm thick disks. Each slice was incubated with 0.5 ml of a mixture of NCS:water (9:1) at 50°C for 2 h, after which it was cooled to 4°C and 5-10 ml of ACS added. These were chilled to 4°C in the dark and the radioactivity determined in a Packard Model 3000 scintillation counter. The samples were corrected for quenching by using standard ^{14}C-quenched series under identical conditions. In some experiments, the gel was sliced into 2 mm thick disks, in which case the volume of NCS:water (9:1) used was

increased to 1.0 ml.

Solid Phase Radioimmune Assays

The procedures of Mackenzie and Molday (195) were used. In the "competitive inhibition assay" flex vinyl microtitre plate wells were pretreated with 25 µl portions of 0.1% (w/v) polylysine at 22°C for 4 to 6 hours. The wells were subsequently washed with water to remove unbound polylysine. DCCD-binding protein (25 µl) at a concentration of 0.13 mg/ml in 100 mM sodium borate buffer, pH 8.8, containing 2% (w/v) Triton X-100 was incubated in the wells for 8 to 10 hours at 22°C. Unbound polypeptide was removed by extensive washing with phosphate-buffered saline (PBS) (0.13 M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4, pH 7.5) containing 10 mM $MgCl_2$. Nonspecific binding sites were then quenched by incubation overnight at 4°C with radioimmune assay (RIA) buffer consisting of 2% (w/v) bovine serum albumin, 2% (v/v) fetal calf serum and 0.1% (w/v) NaN_3 in PBS-10 mM $MgCl_2$. The assay wells were then incubated with 25 µl portions taken from a mixture of varying concentrations of the free antigen (vesicles, modified DCCD-binding protein, etc.) which had been preincubated with appropriately diluted antiserum to the DCCD-binding protein (1:500 in RIA buffer) for 60 min. After 1.5 h at 22°C, the wells were washed with PBS-10 mM $MgCl_2$, and then incubated with 25 µl of affinity purified ^{125}I-labelled goat anti-rabbit immunoglobulin (15-40 µg/ml RIA buffer: 1-2 x 10^6 dpm/µg) for 60 min. The wells were rinsed extensively with PBS-10 mM $MgCl_2$, cut out, and the bound radioactivity determined in a Beckman Gamma 8000 counter. Two basic versions of the "binding assay" were used which differed in the nature of the free antigen. This was either *E. coli* F_1 or the DCCD-binding protein. Binding of the

free antigen to the fixed antigen was then detected by using an antiserum to the free antigen. For example, polylysine-treated micro-titer wells were incubated with 25 µl portions of the antigen to be fixed (ECF_1, 0.75 mg protein/ml in 50 mM HEPES-KOH buffer, pH 7.5 containing 10 mM $MgCl_2$ and 10% (v/v) glycerol; DCCD-binding protein, 0.13 mg protein/ml in 100 mM sodium borate buffer, pH 8.8, containing 10 mM $MgCl_2$ and 2% (w/v) Triton X-100). Following quenching of non-specific binding sites as in the competitive inhibition assay, the wells were incubated with ECF_1 (0.75 mg/ml in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$, 10% (v/v) glycerol and 3% (w/v) bovine serum albumin) or DCCD-binding protein (0.13 mg/ml in 100 mM sodium borate buffer, pH 8.8, containing 10 mM $MgCl_2$, 3% (w/v) bovine serum albumin and 2% (w/v) Triton X-100) depending on the nature of the fixed antigen. The wells were washed with PBS-10 mM $MgCl_2$ and then reacted with 25 µl of serial dilutions in RIA buffer of the antiserum to the free antigen. The extent of binding of the rabbit antiserum was measured with ^{125}I-labelled goat anti-rabbit immunoglobulin as described above. Controls for the non-specific binding of ECF_1 or DCCD-binding protein to the wells were run by omitting the fixed antigen in the procedure. Controls for potential cross-reactivity between ECF_1 and the antiserum to the DCCD-binding protein, or between the DCCD-binding protein and the antiserum to ECF_1, were run by omitting the free antigen in the procedure.

For experiments in which the fixed antigen (ECF_1, chemically-modified or normal DCCD-binding protein, subunits of ECF_1) was titrated with the free antigen (DCCD-binding protein or ECF_1), the experiments were carried out as described above except that the concentration of free antigen was varied with a constant amount of fixed antigen. (The amounts are given in

the legends to the Figures in the "Results" section). The antisera against ECF_1 and the DCCD-binding protein were diluted 1:300 in RIA buffer. 25 µl was used in each well. "Net binding" of free to fixed antigen was always corrected for any non-specific binding of the free antigen or for cross-reactivity with heterologous antiserum.

RESULTS

PART I PURIFICATION OF THE ECF$_1$F$_0$ COMPLEX

SELECTION OF AN E. coli STRAIN

The ATPase activity of the membrane-bound enzyme from several strains of bacteria was measured at 37°C and pH 8.3 using the "slow ATPase" assay procedure as described in MATERIALS AND METHODS. Table 8 shows the average specific activities for the ATPase enzyme in the membrane vesicles of E. coli AN180, WS1 and ML308-225. Under these conditions, E. coli ML308-225 had a higher average specific activity (560 nmol per min per mg protein) than either E. coli AN180 (250 nmol per min per mg protein) or E. coli WS1 (370 nmol per min per mg protein).

From the results in Table 8, it was not known if the different specific activities were due to each strain of E. coli having a different cation to ATP ratio for optimal activity. In order to determine this, membrane vesicles were prepared from each strain of E. coli listed in Table 8 and suspended in 50 mM Tris-H_2SO_4 buffer, pH 8.0. ATPase activity was then measured as previously described, except that $CaCl_2$ was replaced with different amounts of one of the following cations: $MgCl_2$, $CaCl_2$, $MnCl_2$ or $ZnCl_2$.

The results of such an experiment are shown in Fig. 4. Mg^{2+} had the highest capacity for stimulating the membrane-bound ATPase activity. For each strain, maximum activity was obtained at a [Mg^{2+}] to [ATP] ratio of 0.3 to 0.5; above and below which, the activity decreased significantly. These results are consistent with the findings of others (196,197) in which the maximum activity was obtained at a [Mg^{2+}] to [ATP] ratio of 0.5.

In the presence of Mg^{2+}, the maximum activity of E. coli ML308-225

Table 8 Specific Activity of the Membrane-Bound ATPase of Different Bacterial Strains

Strain	Specific Activity (units/mg protein)	
	Mean ± S.D*	Range
E. coli AN 180 (6)**	0.25 ± 0.07	0.12 - 0.31
E. coli WS1 (16)	0.37 ± 0.095	0.14 - 0.56
E. coli ML 308-225 (40)	0.56 ± 0.117	0.39 - 0.85

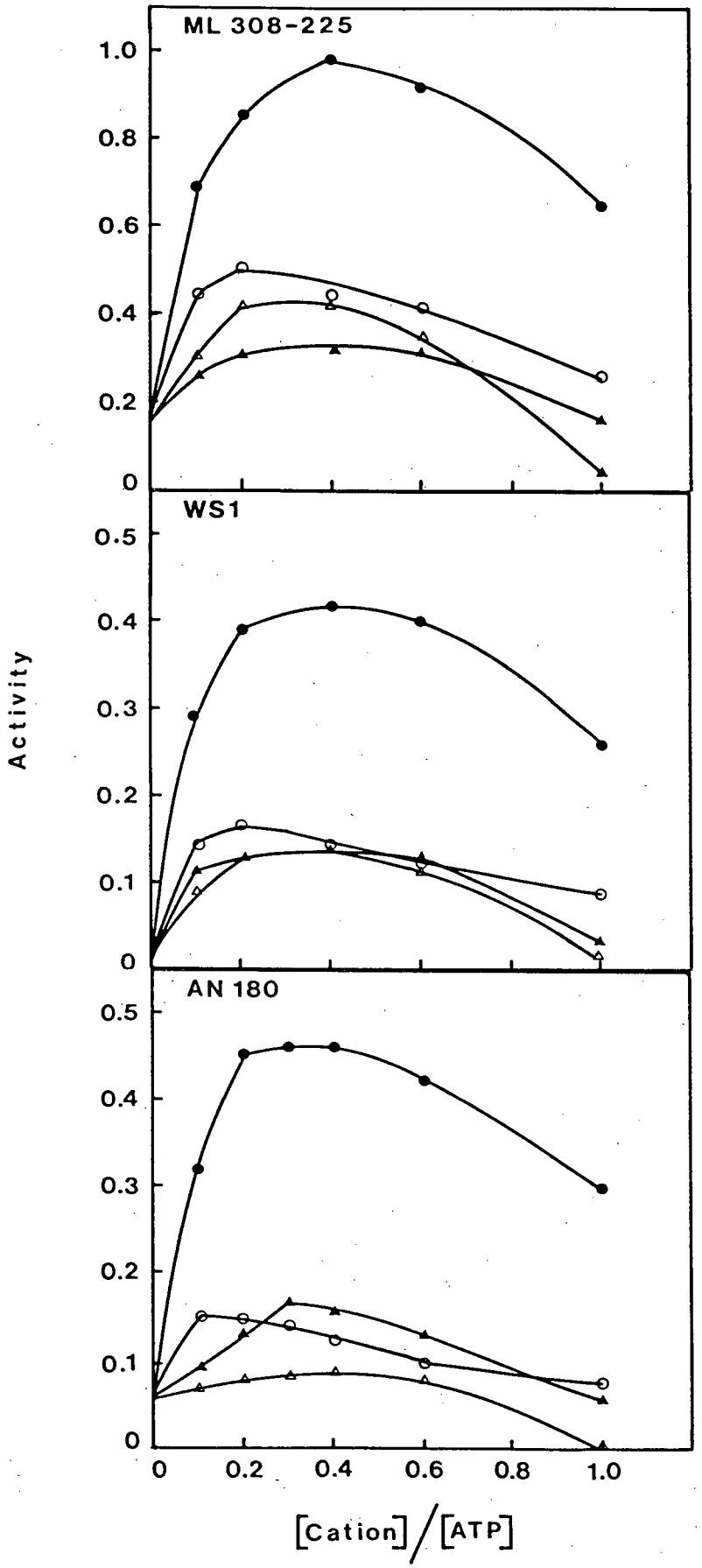
* Standard deviation

** Figures in parenthesis indicate the number of membrane vesicle preparations assayed.

Membrane vesicles from each strain of E. coli were suspended in either 50 mM Tris- H_2SO_4 buffer, pH 8.0 containing 10 mM $MgCl_2$ or in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25M Na_2SO_4 and 10% (v/v) glycerol. ATPase activity was assayed by the "slow assay" as described in MATERIALS AND METHODS.

Fig. 4 Effect of cations on the membrane-bound ATPase activity of different strains of E. coli.

Membranes from E. coli ML308-225, WS1 and AN180 were prepared in 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 1 mM $MgCl_2$. The membrane vesicles were suspended at a protein concentration of 3.3 mg/ml in 50 mM Tris-H_2SO_4 buffer, pH 8.0. ATPase activity was measured by the "slow ATPase" assay as described in MATERIALS AND METHODS, except that $CaCl_2$ was replaced with various levels of either $MgCl_2$ (●-●), $CaCl_2$ (○-○), $MnCl_2$ (▲-▲) or $ZnCl_2$ (△-△). The concentration of ATP in the assay mixture was 5 mM and the amount of membrane protein per assay was 16.5 µg. Enzyme activity is expressed as units per mg protein.


ML 308-225
WS1
AN 180
Activity
[Cation]/[ATP]
0
0.1
0.2
0.3
0.4
0.5
0.6
0.8
1.0

(980 nmol per min per mg protein) was present in about twice the amount of that found in E. coli AN180 (460 nmol per min per mg protein) and E. coli WS1 (420 nmol per min per mg protein).

The results suggested that the ATPase enzyme was likely to be present in a higher amount in E. coli ML308-225 than in E. coli AN180 and WS1 and thus this strain would be a good source of the ECF_1F_0 complex.

SOLUBILIZATION OF THE ECF_1F_0 COMPLEX

Selection of Detergent

In contrast to water-soluble proteins, membrane-bound proteins must be released from the membrane prior to purification. Numerous detergents have been used for this purpose (90,91). An effective detergent should satisfy certain conditions. It should not inactivate the enzyme. As some purification procedures were carried out over a period of 3 to 5 days, it was important to select a detergent which would preserve the enzyme activity. Second, the detergent should selectively solubilize a significant amount of the enzyme without solubilizing all other membrane-bound proteins.

On this basis, a number of detergents were tested, so that a suitable detergent could be selected for the extraction of the ATPase complex from the membranes of E. coli.

High ionic strength buffers have often been used to keep the F_1-ATPase associated with the F_0. Sone et al. (102) used a buffer containing 0.5 M Tris-H_2SO_4, pH 8.0, 0.25 M Na_2SO_4 and 10% (v/v) glycerol to keep the TF_1F_0 complex together during solubilization of the membrane of PS3. The same buffer was used in my experiments for studying the solubilization characteristics of different detergents.

Membrane vesicles of E. coli ML308-225 were suspended in high ionic strength buffer and treated with various levels of detergents as described in MATERIALS AND METHODS. Membrane proteins and ATPase activity were considered as being "solubilized" if they were not sedimented following centrifugation at 250 000 xg for 2 h.

The results of the treatment with various detergents are illustrated in Fig. 5. Optimal solubilization of the ATPase activity was obtained with the detergent, N-lauroyl sarcosine. At a detergent to protein ratio of 0.25, 80% of the activity was solubilized by N-lauroyl sarcosine, whilst less than 60% was solubilized by the other detergents. N-lauroyl sarcosine also exhibited some selectivity, in that only 38% of the membrane protein was solubilized at this detergent:protein ratio. This selectivity was lost at higher ratios. At a detergent:protein ratio of 0.96, almost all the activity was solubilized (96%), while at the same time, the amount of protein solubilized increased to 80%.

Ammonyx Lo and sodium deoxycholate also solubilized significant amounts of ATPase activity at higher detergent to protein ratios. At at ratio of 0.4, Ammonyx Lo and deoxycholate solubilized 80% and 50% of the activity, respectively. With sodium cholate, only 50% of the activity was solubilized at a ratio of 0.8.

Sodium cholate and deoxycholate required the presence of high ionic strength buffers, in order for significant amounts of membrane proteins to be solubilized (94,198). As shown in Table 9, sodium cholate at a detergent:protein ratio of 0.69 in the presence of low ionic buffer (50 mM Tris-H_2SO_4, pH 8.0, 10 mM $MgCl_2$ and 10% (v/v) glycerol), solubilized 3.9% of the activity and 8.4% of the membrane proteins. But at the same ratio, in the presence of high ionic strength buffer, sodium cholate

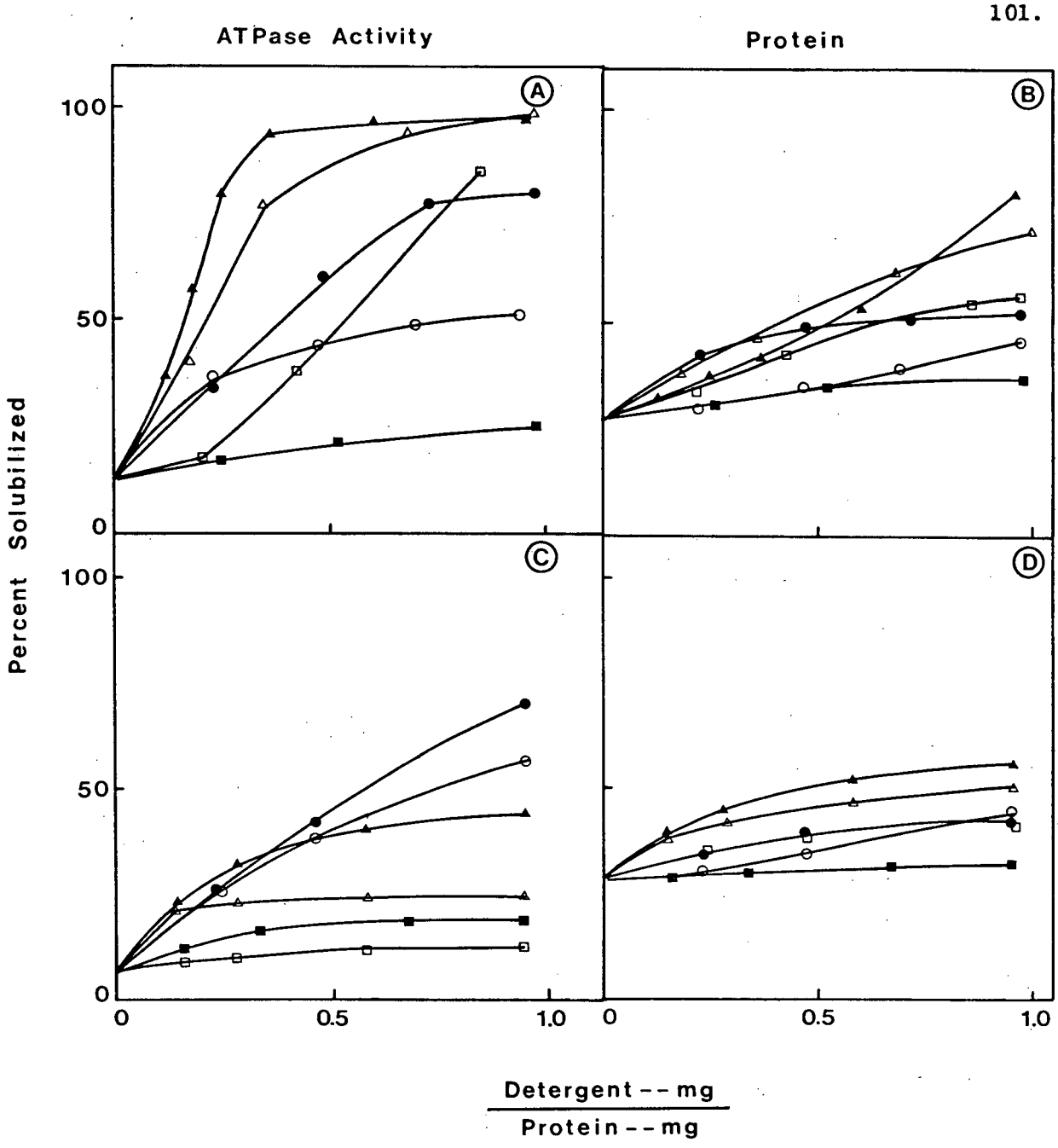


Fig. 5 Solubilization of the membrane-bound ATPase activity of E. coli by various detergents.

Membrane vesicles of E. coli ML308-225 were suspended at a protein concentration of 15-20 mg/ml in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol, and solubilized, as described in MATERIALS AND METHODS, with one of the following detergents: PANELS A and B: N-lauroyl sarcosine (▲-▲), Ammonyx Lo (△-△), sodium deoxycholate (●-●), sodium cholate (○-○), octyl-ß-D-glucopyranoside (□-□), Brij 35 (■-■). PANELS C and D: Triton X-100 (●-●), Triton X-114 (○-○), Lubrol WX (▲-▲), Lubrol 17A-10 (△-△), Tween 60 or Tween 80 (■-■), Lubrol PX (□-□).

Table 9 Solubilization of the Membrane-Bound ATPase Activity of E. coli with Sodium Cholate

Detergent: Protein	Total Protein (mg)	Total Units	Percent Solubilized	
			ATPase Activity	Protein
A				
0	37	33.5	1.6	2.7
0.34		34.2	2.1	4.5
0.69		37.2	3.9	8.4
1.42		39.7	11.3	13.1
B				
0	35.5	37.2	11.8	30
0.22		37.2	34.8	32.9
0.46		37.6	43	36.5
0.69		36.9	45	40.5
0.94		37.7	50.8	48

Membranes vesicles of E. coli ML308-225 were suspended at a protein concentration of 15-20 mg/ml in buffer containing either (A) 50mM Tris-H_2SO_4 pH 8.0, 10mM $MgCl_2$ and 10% (v/v) glycerol or (B) 0.5 Tris-H_2SO_4 pH 8.0, 0.25M Na_2SO_4 and 10% (v/v) glycerol. The vesicles were solubilized with various amounts of sodium cholate as described in the MATERIALS AND METHODS section.

solubilized 45% and 40.5% of the activity and protein, respectively.

The non-ionic detergents, Triton X-100, Triton X-114 and Lubrol WX showed some potential in extracting the membrane-bound ATPase activity (Fig. 5). At a detergent to protein ratio of 0.96, Triton X-100, Triton X-114 and Lubrol WX solubilized 70%, 55% and 45% of the ATPase activity, while the corresponding amounts of protein solubilized were 42%, 45% and 55%, respectively. At the same detergent:protein ratio, Brij 35, Tween 60, Tween 80, Lubrol PX and Lubrol 17A-10 extracted less than 30% of the activity and protein from the membrane.

Effect of Detergents on the Membrane-Bound ATPase Activity

The detergents also stimulated the membrane-bound ATPase activity (Table 10). In general, greater stimulation of the activity was observed with higher detergent to protein ratios. N-lauroyl sarcosine, at ratios of 0.12 and 0.48, stimulated the ATPase activity by 1.3- and 4.5-fold, respectively.

There also appeared to be some correlation between the extent of stimulation and the solubilizing capacity of the detergent. Comparing the ionic detergents, sodium cholate and deoxycholate, it was found that at a ratio of 0.98, the activities were increased by 1.1- and 2.45- folds, respectively. Similarly, the non-ionic detergent, Triton X-100, at a ratio of 0.96 stimulated the activity by 2.5 fold whereas Lubrol PX at a ratio of 2.48 stimulated the activity by only 1.1 times.

The activities of the solubilized fractions could also be stimulated by up to 100%, when assayed in the presence of L-α-lysolecithin (data not shown). It was not known if the increase in activity in the presence of detergent lyso-phospholipid was due to activation of latent ATPase enzymes

Table 10 Effect of Detergent on the Membrane-Bound ATPase Activity of *E. coli*

Ionic Detergents			Non-Ionic Detergents		
Detergent	D:P*	Percent Control Value	Detergent	D:P	Percent Control Value
-	0	100	-	0	100
Ammonyx Lo	0.34	340	Brij 35	0.52	120
	0.68	410		1.06	125
	1.4	425			
			Lubrol PX	0.59	105
N-lauroyl	0.12	130		2.48	110
sarcosine	0.24	270			
	0.48	450	Lubrol WX	0.14	160
				0.28	165
Octyl-β-D-	0.21	115			
glucopyrano-	0.42	170	Lubrol 17A-10	0.14	150
side	0.84	260		0.28	180
Sodium	0.48	105	Triton X-100	0.47	200
cholate	0.98	110		0.96	250
	2.08	170			
			Triton X-114	0.47	280
Sodium	0.48	145		0.96	325
deoxycholate	0.98	245			
	2.08	350	Tween 60	0.33	135
				0.67	140
			Tween 80	0.33	140
				0.67	155

* Detergent to protein ratio

Membrane vesicles of *E. coli* ML308-225 were suspended at a protein level concentration of 15 - 20 mg/ml in 0.5M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol. Different levels of various detergents were added to aliquots of the membrane vesicle suspension. After 30 min. at 20°C, samples were withdrawn and assayed for ATPase activity by the "slow assay" as described in MATERIALS AND METHODS.

as in Mycobacterium phlei (199) and Micrococcus lysodeikticus (200) or to proteolytic digestion of ECF_1 (6,7,24).

Stability of the Solubilized Enzyme

Since the detergents stimulated the ATPase activity, it was of interest to determine whether the activity of the solubilized fraction was stable over the period of time spanned by some of the purification procedures used in this study.

The solubilized fractions of membrane vesicles of E. coli ML308-225, solubilized with either Ammonyx Lo, N-lauroyl sarcosine or sodium cholate (at detergent to protein ratios of 0.68, 0.48 and 0.48, respectively) were stored at 4°C. Samples were removed at timed intervals and the ATPase activity measured.

In the presence of Ammonyx Lo, the activity decreased by 30% after 140 h (Fig. 6). This decrease in activity could be due to denaturation or solubilization of the other membrane components, necessary for the functional conformation of the enzyme. By contrast, the activity in the presence of sodium cholate had increased to 112% forty hours after solubilization, and decreased to its original level (100%) only after another 200 h. Similarly, the activity in the presence of N-lauroyl sarcosine or in control membranes decreased by only 5-10% over 10 days.

MOLECULAR SIZE OF THE DETERGENT-SOLUBILIZED ENZYME

Gel Filtration on Sepharose 6B or Bio-Gel A-0.5m

The solubilized enzyme was chromatographed on gel filtration columns in order to estimate the molecular weight of the solubilized ATPase enzyme. In addition, gel filtration was thought to be suitable as an

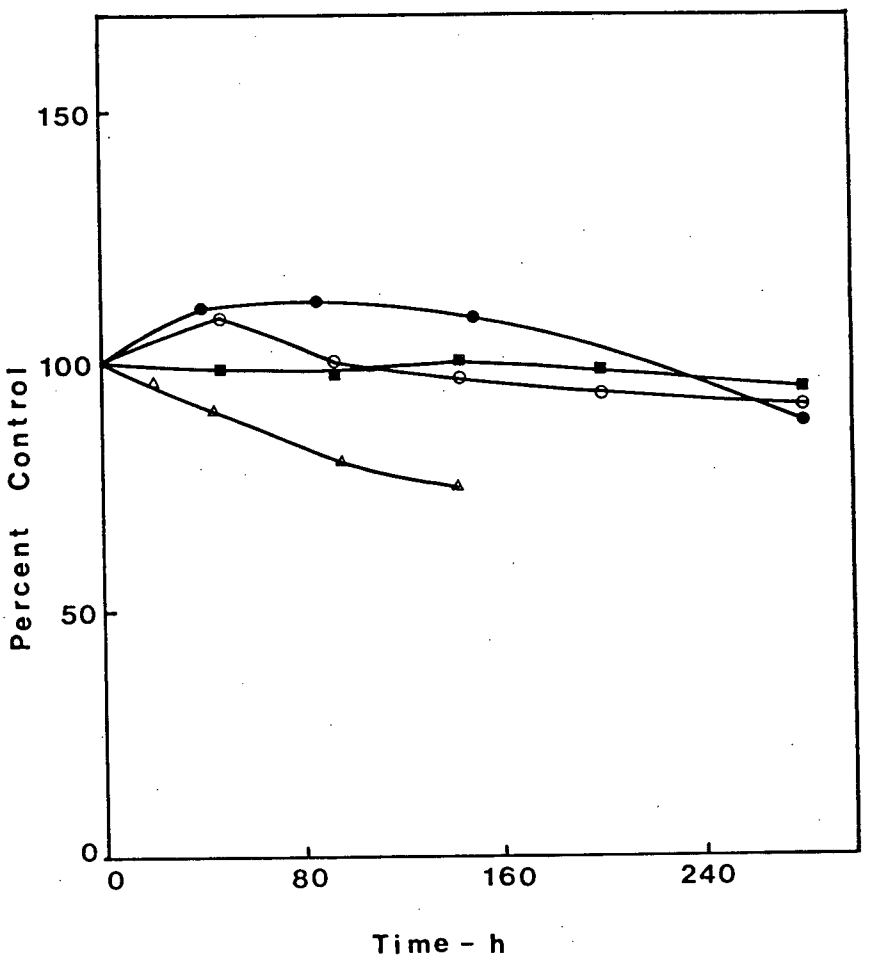


Fig. 6 Stability of solubilized ATPase activity on storage at 4°C.

Membrane vesicles of E. coli ML308-225 were suspended in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol at a protein concentration of 15-20 mg/ml. The vesicles were solubilized with either Ammonyx Lo (△-△), N-lauroyl sarcosine (○-○), or sodium cholate (●-●) at detergent:protein ratios of 0.68, 0.48 and 0.48, respectively. The conditions of solubilization are described in MATERIALS AND METHODS. The ATPase activity of the solubilized fraction was measured immediately and at various times following storage at 4°C. A control, consisting of a suspension of untreated membrane vesicles of E. coli ML308-225 (■-■), was also stored at 4°C and the activity measured at timed intervals.

initial purification step. Since the ATPase complex (molecular weight 380 000) is much larger than most other membrane-bound proteins in E. coli, it should be separated from the proteins with smaller molecular weights on a gel filtration column.

Membrane particles of E. coli ML308-225 in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol were solubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.5. The solubilized material was concentrated and then applied to a Sepharose 6B column, which had been equilibrated with 0.5%, (w/v) of various detergents.

Profiles of the separations on Sepharose 6B in the presence of 0.5% (w/v) Lubrol WX and 0.5% (w/v) N-lauroyl sarcosine are illustrated in Fig. 7, panels A and B. Fig. 7C is the separation profile on Sepharose 6B, in the presence of 0.5% (w/v) N-lauroyl sarcosine, of the solubilized material which was made up to 3% (w/v) N-lauroyl sarcosine and 2% (w/v) sodium cholate, prior to loading onto the column (201).

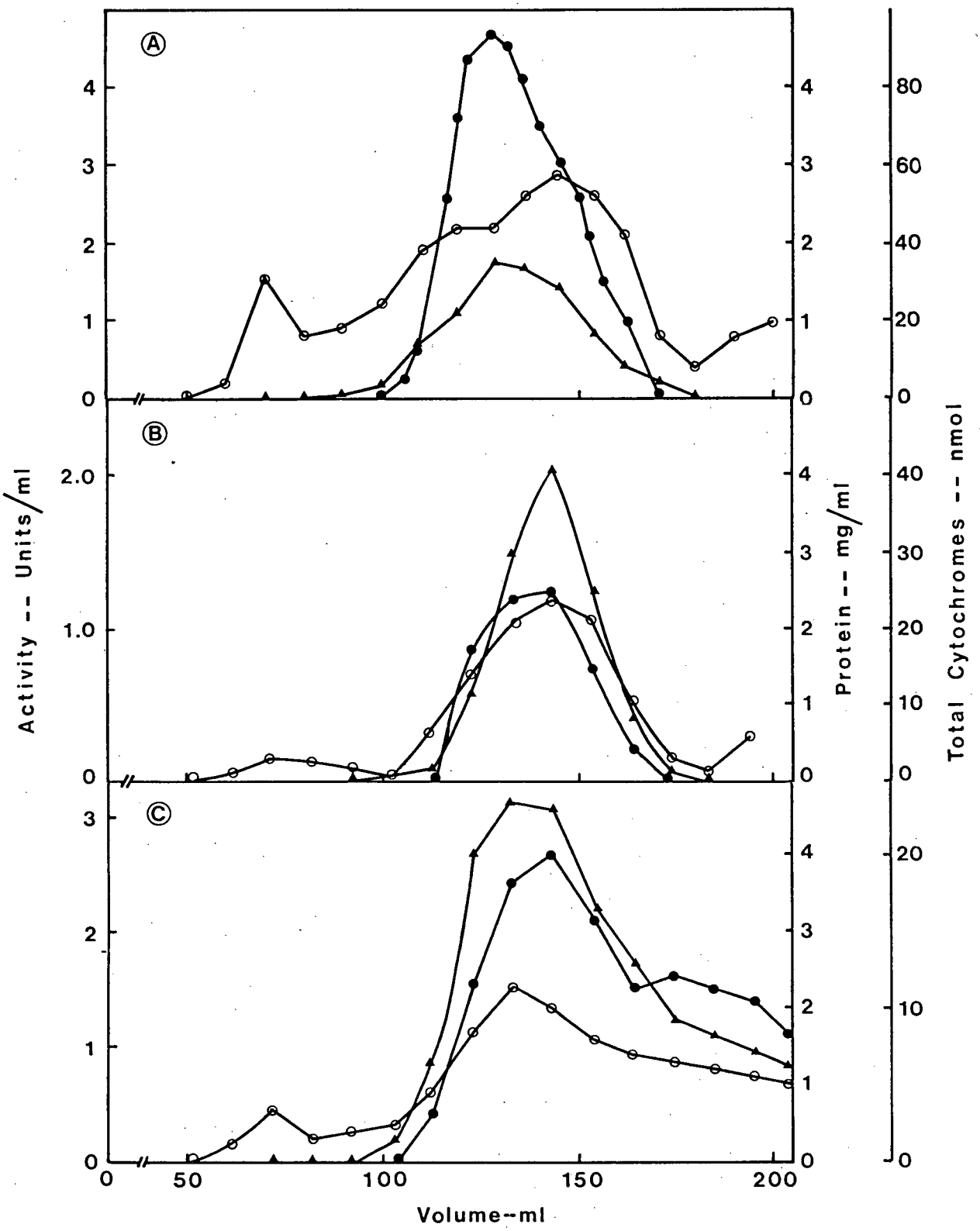
In all three cases, the ATPase activity migrated as a single peak, close to the void volume (V_o = 75 ml). Cytochromes a_1, b_1 and d comigrated with the ATPase activity peak as did the majority of the protein which was applied to the column. Similar elution profiles were obtained in the presence of the other detergents listed in Table 11.

When the solubilized fraction was passed through a column of Bio-Gel A-0.5 m, which was equilibrated with 0.5% (w/v) N-lauroyl sarcosine, the enzyme activity also co-eluted with all the cytochromes and the majority of the protein close to the void volume (V_o = 85 ml).

The recovery of activity depended on the detergent used during chromatography (Table 11). Recoveries ranged from 55 - 60% with N-lauroyl sarcosine to 125% with sodium cholate. Although the recovery was low in

Fig. 7 Chromatography of the detergent-solubilized ATPase complex on Sepharose 6B in the presence of various detergents.

Membrane vesicles of E. coli ML308-225 were solubilized with N-lauroyl sarcosine at a detergent:protein ratio of 0.5. The solubilized fraction was concentrated by ultrafiltration through an Amicon XM-100A filter as described in MATERIALS AND METHODS. The concentrated material (100-150 mg protein) was applied to a column of Sepharose 6B (2.5 x 37 cm) equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 0.25 M Na_2SO_4, 10% (v/v) glycerol and 0.5% (w/v) of one of the following detergents: PANEL A, Lubrol WX; PANELS B and C, N-lauroyl sarcosine. In PANEL C, the concentrated material was made up to 3% (w/v) N-lauroyl sarcosine and 2% (w/v) sodium cholate prior to loading onto the column. The columns were then eluted with the same buffer containing the appropriate detergent. Fractions (10 ml) were collected and assayed for ATPase activity (●-●), protein (○-○) and total cytochromes (a_1, b_1 and d) (▲-▲) as previously described.


Ⓐ
Ⓑ
Ⓒ
Activity -- Units/ml
Protein -- mg/ml
Total Cytochromes -- nmol
Volume--ml
0
50
100
150
200
0
1
2
3
4
0
1.0
2.0
0
10
20
30
40
0
20
40
60
80

the presence of N-lauroyl sarcosine, it was higher (105%) if the solubilized material was made up to 3% (w/v) N-lauroyl sarcosine and 2% (w/v) sodium cholate before applying to the column.

The profiles in all cases showed that the enzyme activity eluted as a broad peak. This suggested that the solubilized enzyme most likely existed in different aggregated forms.

The molecular weight of the solubilized enzyme was estimated in the presence of different detergents. These values are summarized in Table 11. The molecular weights were calculated to be in the range of 450 000 to 890 000. In general, chromatography in the presence of non-ionic detergents resulted in higher molecular weights for the ATPase enzyme than in the presence of ionic detergents. With the non-ionic detergents, the highest molecular weight for the enzyme was obtained with either Lubrol WX or Triton X-114 (890 000 daltons), and the lowest with Triton X-100 (640 000 daltons). With the ionic detergents, the highest molecular weight was obtained in the presence of Ammonyx Lo (580 000 daltons) and the lowest with N-lauroyl sarcosine (450 000 to 510 000 daltons).

The molecular weights estimated by gel filtration suggested that the ECF_1 and F_0 remained associated during solubilization of the membrane and subsequent chromatography. In order to test for this, the sensitivity to inhibition by DCCD of the fraction from the Sepharose 6B column containing the highest ATPase activity was determined for two preparations of the enzyme. The results are shown in Fig. 8. The ATPase activity in the presence of either 0.01% (w/v) Brij 35 or 0.025% (w/v) N-lauroyl sarcosine was inhibited by 50% at 0.4 mM and 1 mM DCCD, respectively. By contrast, ECF_1 was inhibited by 5-10% at 1 000 μM DCCD. These results suggested that the solubilized ATPase enzyme was the intact ECF_1F_0 complex.

Table 11 Estimation of the Molecular Weight of the Solubilized ECF_1F_0 Complex by Gel Filtration Chromatography

Gel filtration resin	Detergent in equilibration buffer	Detergent Concentration [% (w/v)]	Estimated Molecular Weight ($M_r \times 10^{-5}$)	Recovery of activity (%)
Sepharose 6B	Brij 35	0.5	8.5	110
Sepharose 6B	Lubrol WX	0.5	8.9	80
Sepharose 6B	Triton X-100	0.5	6.4	64
Sepharose 6B	Triton X-114	0.5	8.9	69
Sepharose 6B	Ammonyx Lo	0.5	5.8	73
Sepharose 6B	Sodium cholate	0.5	5.2	125
Sepharose 6B	N-lauroyl sarcosine	0.5	5.1	61
Sepharose 6B[a]	N-lauroyl sarcosine	0.5	4.7	107
Bio-Gel A - 0.5m	N-lauroyl sarcosine	0.5	4.5 - 4.8	55

[a]The solubilized material was made up to 3% (w/v) N-lauroyl sarcosine and 2% (w/v) sodium cholate before loading onto the column.

The details of the experiment are described in the legend to Fig. 7. Thyroglobulin (669 00 daltons), catalase (232 000 daltons) and haemoglobin (64 500 daltons) were used to calibrate the gel filtration columns under the various conditions.

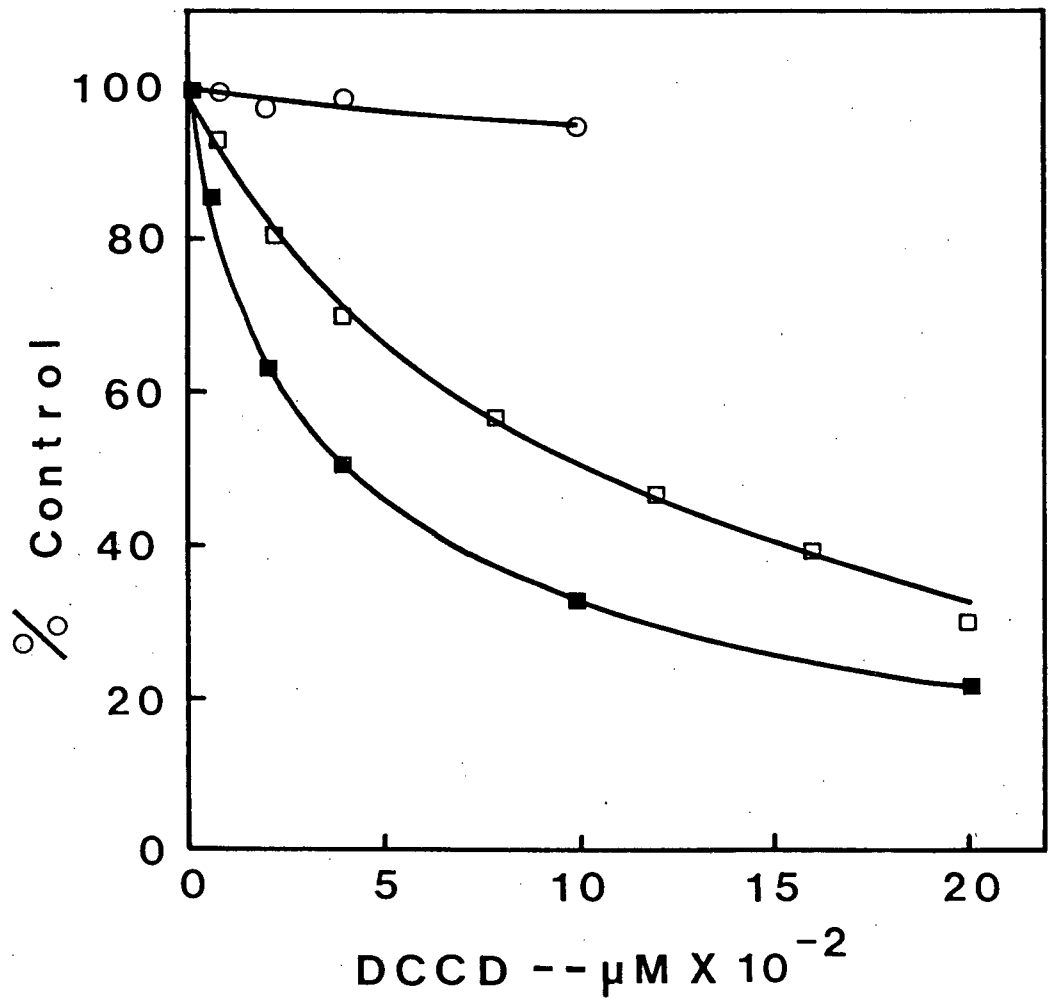


Fig. 8 Effect of DCCD on the detergent-solubilized ATPase activity.

Membrane vesicles of E. coli ML308-225 were solubilized with N-lauroyl sarcosine at a detergent:protein ratio of 0.5. The solubilized fraction was chromatographed on a column of Sepharose 6B in the presence of 0.5% (w/v) detergent, as described in the legend to Fig. 7, PANELS A and B. Samples of the most active fraction from the Sepharose 6B column, in the presence of 0.5% (w/v) Brij 35 (□-□) or N-lauroyl sarcosine (■-■) were incubated with various levels of DCCD in 0.1 M Tris-HCl, pH 8.0 for 45 min at 37°C. ECF_1 of E. coli ML308-225 (○-○), obtained after dialysis of the membranes in low ionic strength buffer, was also treated with DCCD. Following incubation with DCCD, ATPase activity was measured as described in MATERIALS AND METHODS. The specific activities of the ATPase enzyme in the presence of Brij 35 (final concentration, 0.01% (w/v)), N-lauroyl sarcosine (final concentration, 0.025% (w/v)) and of ECF_1 were 2.06, 2.52 and 3.04 units per mg protein and the amount of protein per assay was 3, 13.5 and 14 μg, respectively.

Since, the ATPase activity was not separated from other solubilized proteins (cytochromes) by chromatography on Sepharose 6B, other methods of purifying the ECF_1F_0 complex were investigated. These are discussed in the following sections.

Purification of the ECF_1F_0 Complex by Hydrophobic-Interaction Chromatography

Hydrophobic-interaction chromatography has been used mainly for the purification of water-soluble proteins (202). Membrane-bound proteins interact very strongly with these resins and can only be released from the resin with the use of detergents. Usually little separation is achieved. However, hydrophobic-interaction chromatography on Phenyl Sepharose CL-4B was used successfully to purify the membrane-bound enzyme, fumarate reductase (203). This enzyme consists of a membrane-bound and a water-soluble component and was eluted from the resin by lowering the ionic strength. The enzyme obtained was essentially in a pure form. Since the ECF_1F_0 complex resembles fumarate reductase in having a water-soluble (ECF_1) and a membrane-bound (F_0) component, experiments were carried out to test the feasibility of using Phenyl-Sepharose CL-4B as a means of purifying the ECF_1F_0 complex.

Membrane vesicles of *E. coli* ML308-225 were solubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.5, as described earlier. The solubilized material was applied to a column of Phenyl-Sepharose CL-4B, which was equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4, 10 mM $MgCl_2$, 10% (v/v) glycerol and 0.5% (w/v) sodium cholate. The column was washed with the same buffer and then a linear decreasing gradient of 0.25 M to 0 M Na_2SO_4 applied. Finally, the

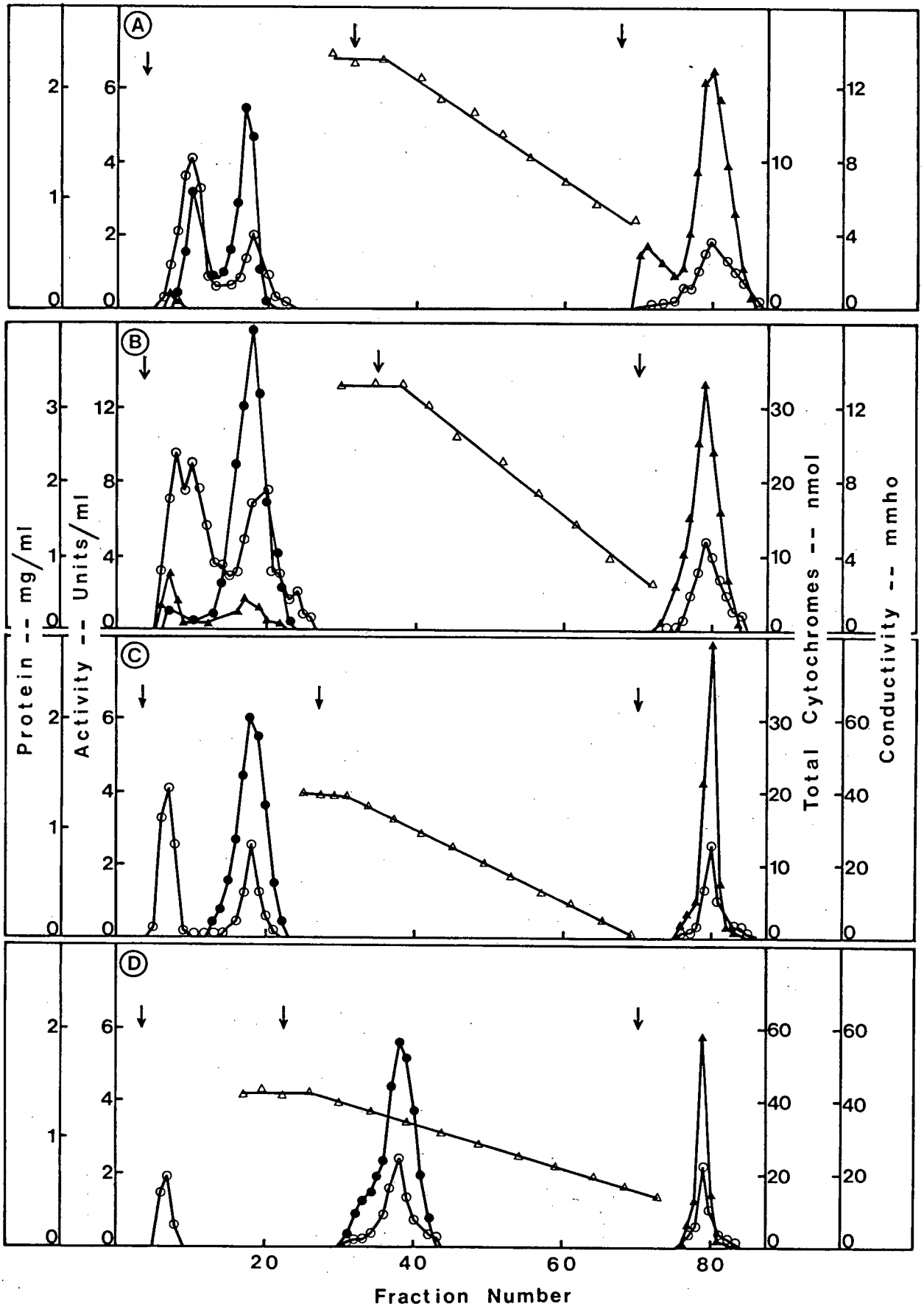
column was washed with 2%, (w/v) Triton X-100. The elution profile is shown in Fig. 9A. The ATPase activity eluted as two peaks during the initial washing of the column. The first activity peak was eluted during the application of the first two column volumes of wash buffer and represented 37% of the total activity recovered. The second activity peak was eluted during the last two column volumes of wash buffer and 63% of the total activity was recovered in this peak. No enzyme activity was detected in the fractions from the gradient elution. Only a small amount of cytochrome d co-eluted with the ATPase activity. Most of the cytochromes (a_1, b_1 and d) were tightly bound to the resin and were eluted with 2% (w/v) Triton X-100.

An attempt was made to increase the interaction of the ATPase enzyme with the resin, by omitting sodium cholate from the equilibration buffer only. The experiment was repeated as described above and the elution profile is shown in Fig. 9B. Again, the ATPase activity was detected only in the fractions obtained during the initial washing. As before, the enzyme activity eluted as two peaks. However, in contrast to Fig. 9A, 96% of the total activity was associated with the second peak. Although most of the cytochromes (a_1, b_1 and d) bound very tightly to the resin, some co-eluted with the two ATPase activity peaks.

Further attempts to improve the interaction of the ATPase enzyme with the resin was achieved by using ions which had "salting-out" properties. This was achieved by replacing the Na_2SO_4 with 20% ammonium sulphate (by saturation) in the buffers described in the legend to Fig. 9B. The gradient consisted of a linear decreasing concentration of 20% to 0% ammonium sulphate. The experiment was repeated as described above and the elution profile is shown in Fig. 9C. All the cytochromes bound very

Fig. 9 Chromatography of the detergent-solubilized ATPase complex on Phenyl-Sepharose CL-4B.

Membrane vesicles of E. coli ML308-225 were solubilized with N-lauroyl sarcosine at a detergent:protein ratio of 0.5. The solubilized fraction (100-125 mg protein) was applied to a column of Phenyl-Sepharose CL-4B (1.8 x 25 cm) equilibrated with 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 10 mM $MgCl_2$, 10% (v/v) glycerol and the following: PANEL A, 0.25 M Na_2SO_4 and 0.5% (w/v) sodium cholate; PANEL B, 0.25 M Na_2SO_4; PANELS C and D, 20% $(NH_4)_2SO_4$ (by saturation). The columns were then washed with 4-5 column volumes of equilibration buffer but with the inclusion of 0.5% (w/v) sodium cholate in PANELS B and C. The columns were then developed with a decreasing linear gradient (8-10 column volumes) of either 0.25 M to 0 M Na_2SO_4 (PANELS A and B) or 20% to 0% $(NH_4)_2SO_4$ (by saturation) (PANELS C and D) in 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 10 mM $MgCl_2$, 10% (v/v) glycerol and 0.5% (w/v) sodium cholate. Following elution with the gradient, the columns were washed with 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 2% (w/v) Triton X-100. The arrows indicate the position of the buffer changes. Fractions (10 ml) were collected and assayed for ATPase activity (●-●), protein (○-○), total cytochromes (a_1, b_1 and d) (▲-▲), and conductivity (△-△) as described in MATERIALS AND METHODS.


A
B
C
D
Protein -- mg/ml
Activity -- Units/ml
Total Cytochromes -- nmol
Conductivity -- mmho
Fraction Number
20
40
60
80

tightly to the resin and were eluted with 2% (w/v) Triton X-100. ATPase activity was again detected only in the fractions from the wash. However, the activity eluted as a single peak during elution with the last two column volumes of buffer. It appeared that the presence of sodium cholate in the buffer was causing the enzyme to be released from the resin. To test for this possibility, sodium cholate was omitted in the wash buffer and the experiment performed as described for Fig. 9C. The elution profile is shown in Fig. 9D. Again, all the cytochromes were completely separated from the ATPase activity. The activity eluted as a single peak during the gradient elution. The fraction with the highest activity eluted at an ammonium sulphate concentration of 15-16% (34-36 mmho).

Thus, the presence of sodium cholate decreased the interaction of the ATPase enzyme with the resin, whilst ions with "salting-out" properties caused greater binding of the protein to the resin. The latter was also suggested by the elution profile of the cytochromes. When bound in the presence of Na_2SO_4, the cytochromes were subsequently eluted as a broad peak (Figs. 9A and 9B). In contrast, they were eluted as a sharp peak (Figs. 9C and 9D) when bound in the presence of ammonium sulphate.

As it was possible to bind the solubilized enzyme to the Phenyl Sepharose CL-4B resin, it was of interest to determine how ECF_1 behaved under identical conditions. ECF_1 of E. coli ML308-225 obtained after purification on a sucrose gradient or after dialysis of membranes in low ionic strength buffer, as described in MATERIALS AND METHODS, was chromatographed onto a Phenyl-Sepharose CL-4B column under conditions identical to those in Fig. 9D. In both cases, the ATPase activity eluted as a single peak during the elution with the gradient. The elution profiles were similar to Fig. 9D. However, the fractions containing the

highest activities eluted at an ammonium sulphate concentration different to that required to elute the detergent-solubilized enzyme. Purified ECF_1 or ECF_1 obtained after dialysis of the membranes were eluted at ammonium sulphate concentrations of 9.5-10% (22-24 mmho) and 10.5-11.5% (24-26 mmho), respectively. Therefore, it appeared that the detergent-solubilized ATPase enzyme was of a different composition than soluble ECF_1. The elution of the solubilized ATPase at a higher ionic concentration than purified ECF_1 suggested that the former must have a different conformation. This might be expected of an intact ECF_1F_0 complex.

The presence of an intact ECF_1F_0 complex was confirmed when fractions from the Phenyl-Sepharose CL-4B column were found to be sensitive to DCCD. The activity was inhibited by 50% at a DCCD concentration of 600 μM (not shown).

Other Hydrophobic-Interaction Resins

In addition to Phenyl-Sepharose CL-4B, a number of other hydrophobic resins were tested for their potential use in the purification of the ECF_1F_0 complex. The resins, ω-Amino Butyl Agarose, ω-Amino Hexyl agarose (AH-Sepharose CL-4B), Butyl Agarose, Octyl Sepharose CL-4B and Decyl Agarose were selected for this purpose.

Membrane vesicles of *E. coli* ML308-225 in 0.5 M Tris-H_2SO_4 buffer, pH 8.0 containing 0.25 M Na_2SO_4 and 10% (v/v) glycerol were solubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.5. The solubilized fraction was applied to a column of hydrophobic-interaction resin, under conditions identical to those described in the legend to Fig. 9D.

Under these conditions, only a small amount (15%) of the protein was

bound to either ω-Amino Butyl Agarose or ω-Amino Hexyl Agarose (AH-Sepharose CL-4B). In both cases, the ATPase activity co-eluted as a single peak with the cytochromes (a_1, b_1 and d) during the initial column wash step. Therefore, both of these resins were considered to be unsuitable for the purification of the ATPase complex. It is probable that both functioned predominantly as ion-exchange resins.

The identical experiment was repeated with Butyl Agarose, Octyl Sepharose CL-4B and Decyl Agarose. In all three cases, the ATPase enzyme bound very tightly to the resin and was only released during elution with the gradient. The elution profiles were almost identical to that in Fig. 9D. The ATPase activity eluted as a single peak at an ammonium sulphate concentration of 15-16%. With Butyl Agarose and Octyl Sepharose CL-4B, the cytochromes (a_1, b_1 and d) could be eluted from the resin with 2% (w/v) Triton X-100. However, cytochromes were not detected in the fractions when Decyl Agarose was eluted with buffer containing either 2% (w/v) or 5% (w/v) Triton X-100. It was not known if the cytochromes were eluted in a denatured form or were still tightly bound to the resin.

The specific activity of the eluted ATPase enzyme was determined in each case. The highest specific activities in the fractions from Butyl Agarose, Decyl Agarose and Octyl Sepharose CL-4B were 2.25, 4.7 and 5.2 units per mg protein, and represented 4.7-, 6.1- and and 8.7-fold purifications, respectively. Although some purification was observed in each case, it was not as high as that obtained in the fraction eluted from Phenyl-Sepharose CL-4B (24.8-fold purification). When the most active fractions from these columns were analyzed on SDS-polyacrylamide gels, the fewest number of polypeptide bands was observed in the fraction from the Phenyl-Sepharose CL-4B column. Therefore, Phenyl-Sepharose CL-4B was

chosen as the resin for the purification of the ECF_1F_0 complex.

Further Purification of the ECF_1F_0 Complex

The ATPase complex was partially purified on Phenyl-Sepharose CL-4B as described earlier. Analysis of the fraction with the highest specific activity on SDS-polyacrylamide gel revealed the presence of approximately 14 major protein-staining bands (including the subunits of ECF_1) and many minor bands (the gel was similar to Fig. 11A, lane c). The ATPase complex of the thermophile, PS3, was purified by Sone et al. (102) and shown to consist of eight different polypeptides. It was thought that the composition of the ECF_1F_0 complex should be similar. Therefore, an attempt was made to remove some of the polypeptides (contaminants) of the partially purified ECF_1F_0 complex by sucrose gradient centrifugation.

Membrane vesicles of E. coli ML308-225 were solubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.5 as described in MATERIALS AND METHODS. The solubilized fraction was applied to a column of Phenyl-Sepharose CL-4B and chromatography carried out as described in the legend to Fig. 9D. The active fractions eluted during the gradient were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane. The concentrated material was applied on a 15 to 25% (w/v) sucrose density gradient and centrifuged at 280 000 xg for 23 h. The results of the separation are shown in Fig. 10. The ATPase activity peak was well separated from the majority of the protein, which remained at the top of the gradient. Approximately 10% of the total activity applied to the sucrose gradient was recovered. Attempts to stimulate the ATPase enzyme by addition of L-α-lysolecithin, prior to assaying for activity, were not successful. As shown in Fig. 10, the added lyso-phospholipid inhibited the

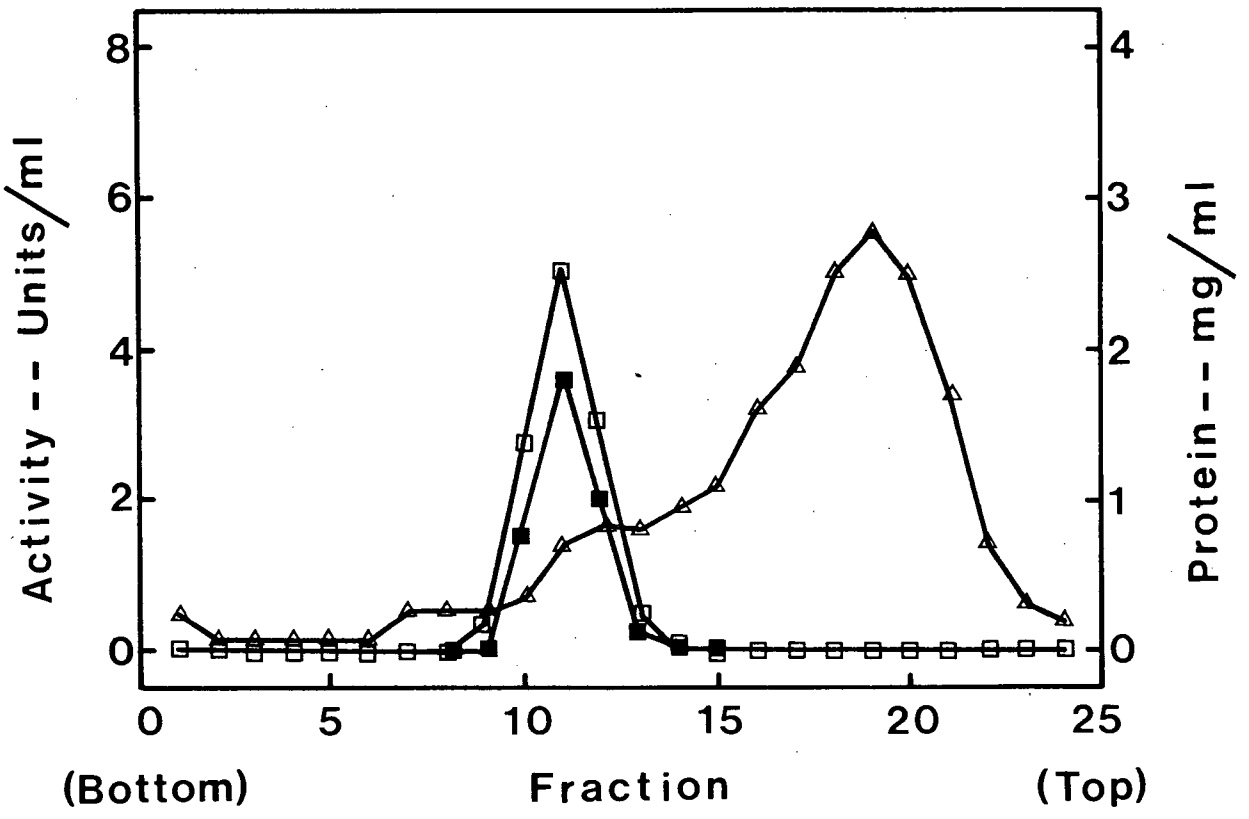


Fig. 10 Purification of the ECF_1F_0 complex by sucrose gradient centrifugation.

The ECF_1F_0 complex obtained after chromatography on Phenyl-Sepharose CL-4B was concentrated by ultrafiltration using an Amicon PM-10 filter. The concentrated material (13 mg protein in a volume of 1.2 ml) was applied to a linear gradient of 15% - 25% (w/v) sucrose in 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 0.5% (w/v) sodium cholate, 5 mM $MgCl_2$ and 0.25 mM DTT. The gradient was centrifuged at 280 000 xg for 23 h and ten drop fractions collected. Protein (Δ-Δ), and ATPase activity determined in the absence (□-□) or presence (■-■) of 0.02% (w/v) L-α-lyso-phosphatidylcholine were measured as described in MATERIALS AND METHODS.

enzyme activity. Similarly, the inclusion of 0.1% (w/v) soybean phosphatidylcholine in the sucrose gradients did not prevent the loss of activity. When the concentrated fraction, which was applied to the sucrose gradient, was stored at 4°C for the same length of time spanned by the sucrose gradient centrifugation step (24-28 h), about 70% of the ATPase activity was lost. These results indicated that the enzyme was unstable at 4°C. The highest specific activity in the sucrose gradient fractions was 7.7 units per mg protein and this represented a 22-fold purification over the intact membranes.

Because of the different activating properties of the detergents as well as the unstable nature of the ATPase enzyme during purification, a comparison of the purification with respect to specific activities is meaningless. A better method for comparing the purification procedures is the analysis of the fractions on SDS-polyacrylamide gels.

The active fractions from the Phenyl-Sepharose CL-4B column, when analyzed on SDS-polyacrylamide gels, revealed that the fractions from the leading edge (the gel was again similar to Fig. 11A, lanes b to d) of the activity peak appeared to contain many more minor bands than those from the trailing edge. Many of these contaminants were subsequently removed by sucrose gradient centrifugation. In addition to the subunits of ECF_1 (α-ε), polypeptides with molecular weights of 48 000, 35 000, 24 000, 18 000, 14 000 and 9000 were also present in invariant stoichiometry. Although the sucrose gradient removed many of the minor bands with molecular weight less than 30 000 as well as a major contaminant of 70 000 daltons, many minor bands of less than 50 000 daltons were still present. Many more polypeptides than those shown in Fig. 11, were present in this preparation. In order to remove some of these minor bands, an ammonium

sulphate precipitation step was included prior to chromatography on Phenyl-Sepharose CL-4B.

Membrane vesicles of E. coli ML308-225 were solubilized at a detergent to protein ratio of 0.25 rather than 0.5, as described earlier. The fraction precipitating between 0.35 and 0.5 saturation of ammonium sulphate (0.35-0.5 P fraction) was suspended in the buffer used for solubilization at a protein concentration of 5-7 mg/ml. N-lauroyl sarcosine was added to give a detergent to protein ratio of 0.8 to 1.0. This fraction was chromatographed on a column of Phenyl-Sepharose CL-4B under conditions identical to those described in the legend to Fig. 9D except that the column was eluted with a linear decreasing gradient of 20% to 12.5% ammonium sulphate.

On occasion, the 0.35-0.5 P fraction did not pellet following centrifugation at 30 000 xg for 20 min, but floated to the surface. This problem was alleviated by using 10% (v/v) methanol rather than glycerol in the solubilization buffer. The presence of 10% (v/v) methanol did not appear to affect the purification and was subsequently used in the other experiments.

Applying a shallower gradient (20-12.5%) did not result in a change in the elution profile of the enzyme from that seen in Fig. 9D. The activity eluted as a single peak at an ammonium sulphate concentration of 15-16%. The active fractions were concentrated and applied to a linear 17.5 to 25% (w/v) sucrose gradient as described in MATERIALS AND METHODS. As before, approximately 15% of the activity was recovered following sucrose gradient centrifugation. The separation on the sucrose density gradient was similar to Fig. 10. The majority of the protein (contaminants) remained at the top of the gradient, whereas the ATPase activity was associated with a smaller

protein peak close to the bottom of the tube.

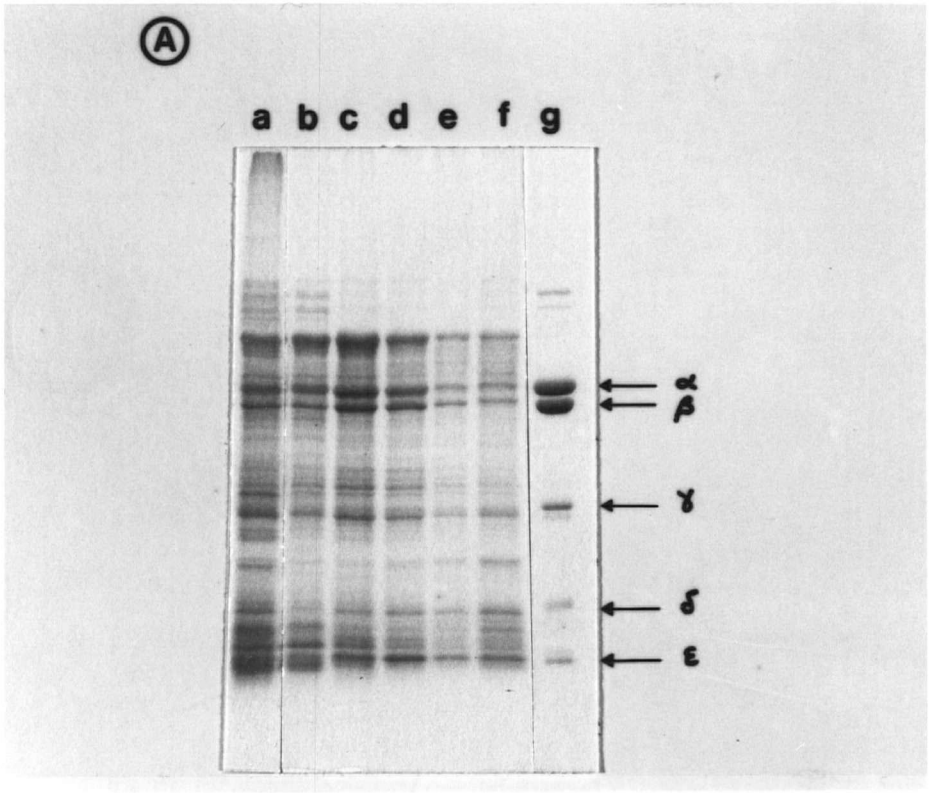
Inclusion of the ammonium sulphate fractionation step reduced the number of minor bands of less than 50 000 daltons in the fractions from the Phenyl-Sepharose CL-4B column and subsequently in the fractions from the sucrose gradient (Fig. 11). In the fractions from the sucrose gradient, the subunits of ECF_1 (α - ε) as well as major-protein staining bands with molecular weights of 48 000, 35 000, 24 000, 18 000, 14 000 and 9 000 were present in invariant stoichiometry. Relative to the other subunits of ECF_1, the δ subunit (molecular weight, 21 000) was present in fairly low amounts in the active fractions (Fig. 11B) from the sucrose gradient. It has been reported that this subunit is very susceptible to protease digestion (6,24).

Foster and Fillingame (94) have also reported the purification of the ECF_1F_0 complex. The F_0 complex consisted of three subunits of molecular weight 24 000, 18 000 and 8 500. They reported that dialysis of the detergent-solubilized fraction followed by resolubilization with detergent removed many of the minor protein-staining bands.

Membrane vesicles of E. coli ML308-225 were solubilized at a detergent to protein ratio of 0.25 a described earlier. The solubilized fraction was dialyzed against buffer for 24-30 h. The reaggregated material in the dialysate was collected by centrifugation and resolubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.8 to 1.0. Only 60% of the activity was recovered following dialysis. The resolubilized material was subjected to ammonium sulphate precipitation, chromatography on Phenyl-Sepharose CL-4B and sucrose gradient centrifugation as described in the previous experiment. Again, only 15% of the total activity applied to the sucrose gradient was recovered. However, the activity peak was well

Fig. 11 SDS-polyacrylamide gel electrophoresis of the ECF_1F_0 complex purified by chromatography on Phenyl-Sepharose CL-4B and sucrose gradient centrifugation.

Membrane vesicles of E. coli ML308-225 were solubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.25. The solubilized fraction was subjected to ammonium sulphate precipitation and the fraction precipitating between 35 and 50% of saturation was suspended in the solubilizing buffer at a protein concentration of 5-7 mg/ml. N-lauroyl sarcosine was added to give a detergent to protein ratio of 0.8-1.0, as described in MATERIALS AND METHODS. The resolubilized material was applied to a column of Phenyl-Sepharose CL-4B (1.8 x 25 cm) under conditions identical to those described in the legend to Fig. 9D. The active fractions from the Phenyl-Sepharose CL-4B column were concentrated and applied to a linear 17.5 to 25% (w/v) sucrose gradient and centrifuged at 280 000 xg for 23 h as described in the MATERIALS AND METHODS section. The active fractions from the Phenyl-Sepharose CL-4B column and the sucrose gradient were analyzed by SDS-gel electrophoresis. The separating gel (Tris-buffered system) consisted of a linear 7.5 - 16.5% (w/v) acrylamide gradient. The stacking gel consisted of 4% (w/v) acrylamide. Following electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Blue. PANEL A: Fractions from the Phenyl-Sepharose CL-4B column. Lane a represents the material applied to the column. Lanes b-f represent the fractions eluted from the column, with the fraction highest in ATPase activity in lane c. Lane g is purified ECF_1. The position of migration of the subunits of ECF_1 (α-ε) are indicated. PANEL B: Fractions from the sucrose gradient. Lane a represents the material loaded on the sucrose gradient. Lanes b-i represent the fractions from the sucrose gradient. The fraction containing the highest ATPase activity is in lane e. Lane j is purified ECF_1. The position of migration of the subunits of ECF_1 (α-ε) are also indicated.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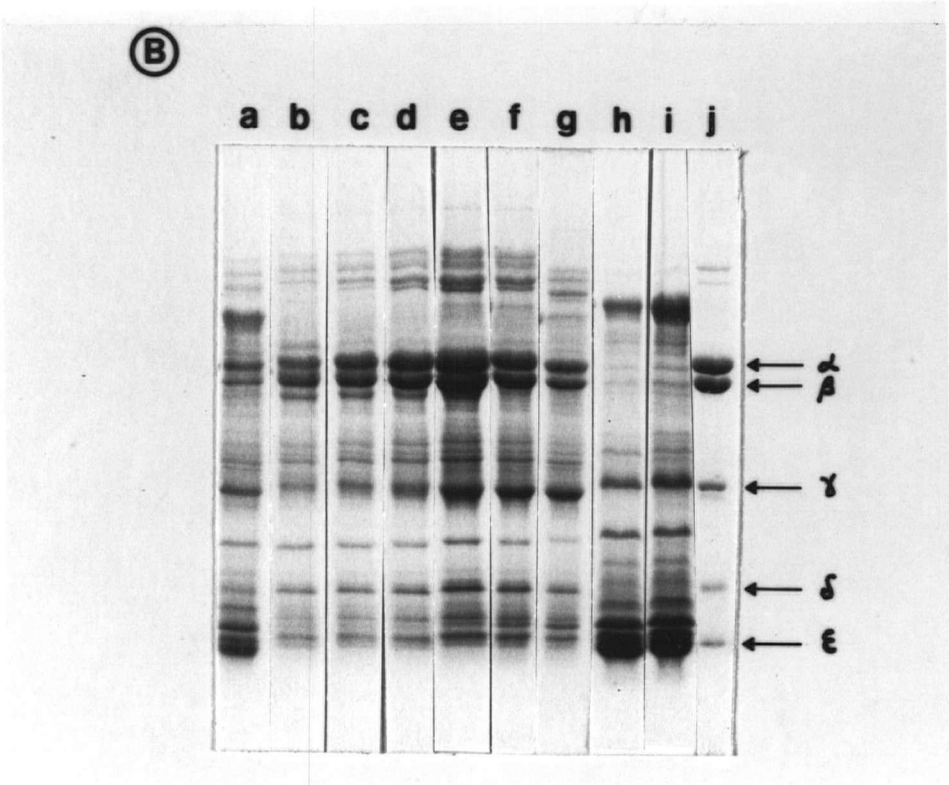
B
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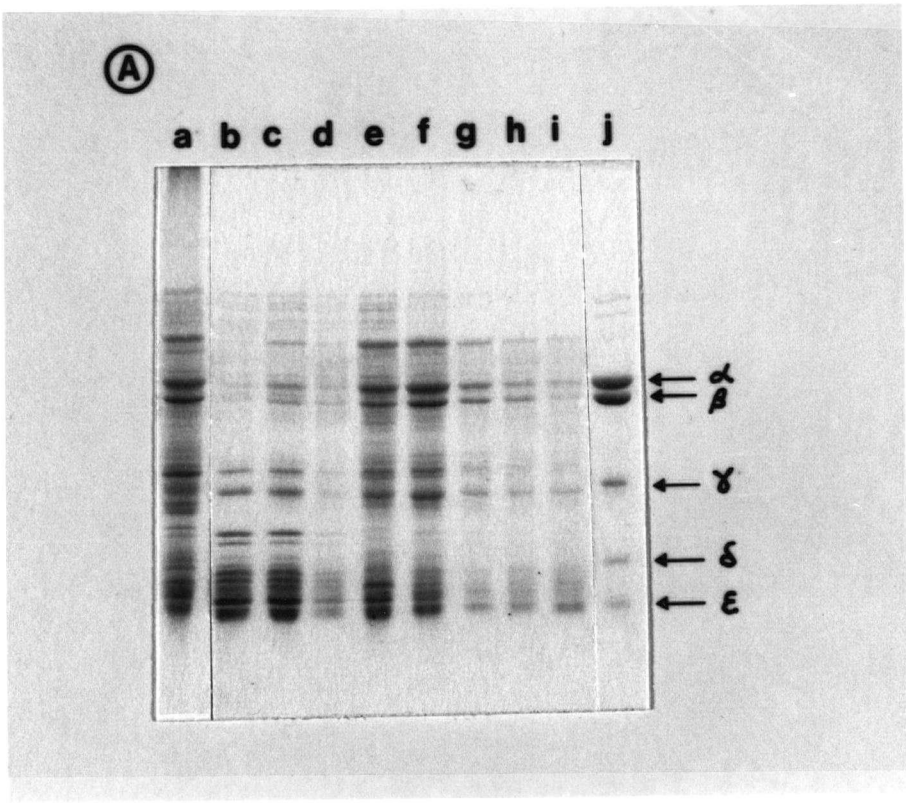
separated from the majority of the protein (as in Fig. 10).

Inclusion of the dialysis step appeared to decrease the number and intensity of the minor protein-staining bands with molecular weights greater than 20 000, in the fractions from the Phenyl-Sepharose CL-4B column (Fig. 12A), when compared to those in Fig. 11A. However, those minor bands with molecular weights less than 20 000 were increased. Nevertheless, the inclusion of the dialysis step did improve the overall purification as seen in the active fractions from the sucrose gradient (Fig. 12B). The subunits of ECF_1 (α - ε), as well as major protein-staining bands with molecular weights of 35 000, 24 000, 18 000, 14 000 and 9 000, appeared to be present in invariant stoichiometry. In contrast to the results in Fig. 11B, the 48 000 dalton polypeptide was almost completely absent in this preparation. In addition, the staining-intensity of the 18 000 and 14 000 dalton polypeptides were greater in this preparation. Furthermore, the δ subunit was more distinguishable, suggesting that this preparation was perhaps less contaminated with proteases. The inclusion of the protease inhibitors PMSF (0.1 mM) and p-aminobenzamidine (6 mM) during the concentration step, as well as in the sucrose gradient, did not result in any change in the pattern of bands seen in Fig. 12B. It was not known if these protease inhibitors were capable of inhibiting the activity of the protease(s) which may have been present in the preparation. The fraction from the sucrose gradient which was richest in ATPase activity was found to be sensitive to DCCD. The ATPase activity was inhibited by 30% at a DCCD concentration of 200 µM.

More recently, Schneider and Altendorf (107) have also purified the ECF_1F_0 complex by chromatography on DEAE-Sepharose CL-6B in the presence of Aminoxid WS-35, followed by centrifugation of the active fractions at

Fig. 12 SDS-polyacrylamide gel electrophoresis of the ECF_1F_0 complex obtained after chromatography on Phenyl-Sepharose CL-4B and sucrose gradient centrifugation.

Membrane vesicles from E. coli ML308-225 (25-30 g wet weight) were solubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.25. The solubilized material was dialyzed (1:100) against 50 mM Tris-H_2SO_4 buffer, pH 8.0 containing 1 mM DTT, 0.1 mM EGTA, 25 mM Na_2SO_4 and 10% (v/v) methanol for 24-30 h at 4°C, with changes of the external buffer at 6-8 h intervals. The reaggregated material was collected by centrifugation at 250 000 xg for 3 h and resolubilized with N-lauroyl sarcosine at a detergent to protein ratio of 0.8-1.0. The resolubilized fraction was then subjected to ammonium sulphate precipitation, as described in MATERIALS AND METHODS. The fraction precipitating between 35 and 50% of saturation was applied to a column of Phenyl-Sepharose CL-4B (1.8 x 25 cm) under conditions identical to those described in the legend to Fig. 9D, except that the enzyme was eluted with a linear decreasing gradient of 20% - 12.5% ammonium sulphate (by saturation). The active fractions from the column were concentrated and applied to a linear 17.5 to 25% (w/v) sucrose gradient and centrifuged at 280 000 xg for 23 h as described previously. The active fractions were analyzed by SDS-gel electrophoresis. The separating gel (Tris-buffered system) consisted of a linear 7.5-16.5% (w/v) acrylamide while the stacking gel was 4% (w/v) acrylamide. The gels were fixed in a solution containing 5% (w/v) TCA, 5% (w/v) sulfosalicylic acid and 10% (v/v) methanol, and then stained with 0.1% (w/v) Coomassie Blue. PANEL A: Lane a represents the material applied to the column of Phenyl-Sepharose CL-4B. Lanes b-i represent the active fractions eluted from the column, with the material in lane e containing the highest activity. Lane j is purified ECF_1. The migration positions of the subunits of ECF_1 (α-ε) are indicated. PANEL B: Lane a represents the material loaded onto the sucrose gradient. Lanes b-j represent the active fractions from the sucrose gradient, with the material in lane d containing the highest activity. Lane k is purified ECF_1. The migration positions of the subunits of ECF_1 (α-ε) are also shown.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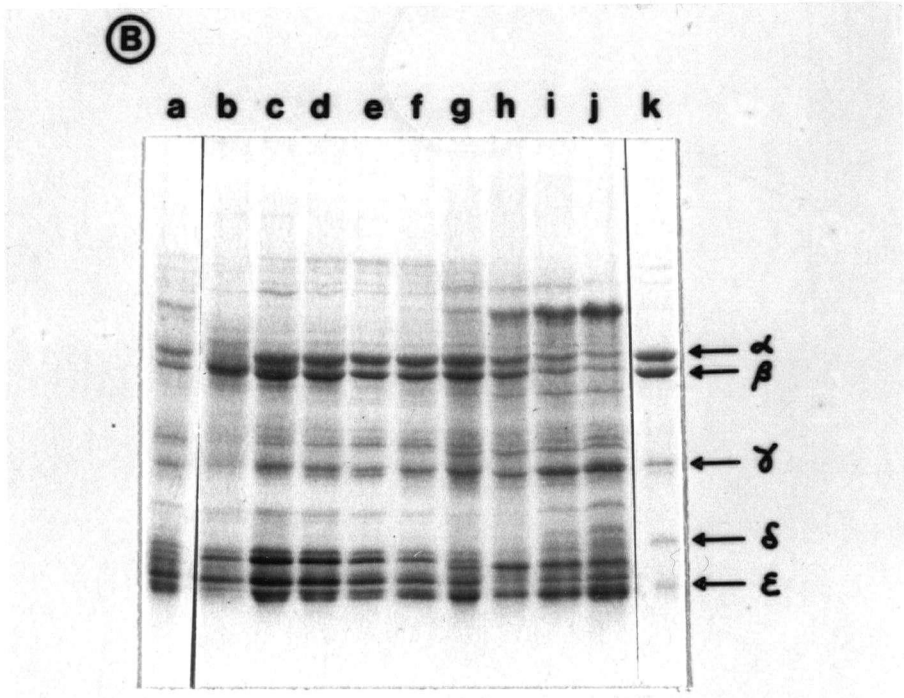
B
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220 000 xg for 15 h. Therefore, centrifugation of the active fractions from the Phenyl-Sepharose CL-4B column was used to overcome two of the problems associated with the purification on a sucrose gradient: the poor recovery of activity and the small quantity of the purified enzyme which could be obtained with a sucrose gradient.

The experiment described previously (legend to Fig. 12) was repeated except that the sucrose gradient step was omitted. Instead, the active fractions from the Phenyl-Sepharose CL-4B column were made up to 0.1 mM PMSF and 6 mM p-aminobenzamidine prior to centrifugation at 250 000 xg for 16-17 h. The pellet was taken up in 50 mM MOPS-KOH buffer, pH 7.5 containing 10 mM $MgCl_2$, 20% (v/v) glycerol and 0.2% (w/v) Triton X-100. Approximately 40% and 10% of the total activity centrifuged was recovered in the pellet and supernatant fractions, respectively. A recovery of 40% was a substantial improvement over the 8-10% yield usually obtained following sucrose gradient centrifugation.

The preparation of the samples for SDS-polyacrylamide gel electrophoresis was modified at this stage. Detergent and salts, which interfered with the migration of the polypeptides in the gels were removed by the column chromatography-method of Penefsky (170) as described in MATERIALS AND METHODS. This is in contrast to the previous preparations, in which the samples were dialyzed against water for 1-2 days and then lyophilized.

The fraction from Phenyl-Sepharose CL-4B richest in ATPase activity revealed the presence of at least 15 major protein-staining bands and several minor ones on SDS-polyacrylamide gels. The gel resembled that in Fig. 12A. Analysis of the sedimented ATPase enzyme on SDS-polyacrylamide gels is shown in Fig. 13 (lane a). Centrifugation of the active fractions resulted in the enrichment of 11 polypeptides. Of these, five polypeptides

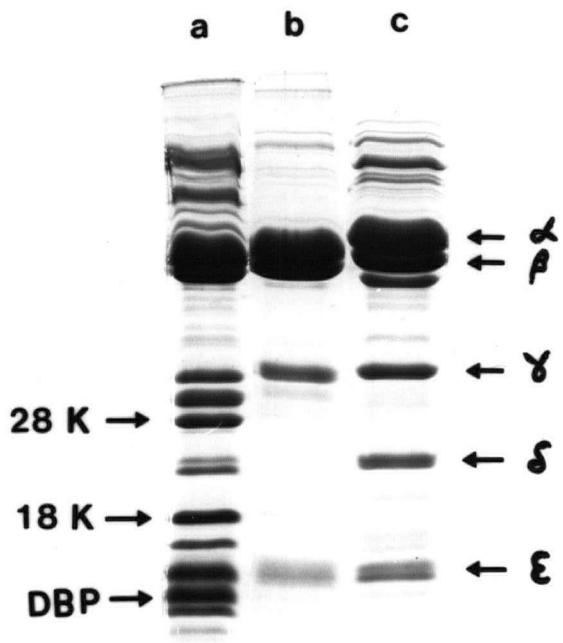


Fig. 13 SDS-polyacrylamide gel electrophoresis of the ECF_1F_0 complex obtained by chromatography on Phenyl Sepharose CL-4B and sedimentation at 250 000 xg for 16-17 h.

The experiment described in the legend to Fig. 12 was repeated except that purification of the ECF_1F_0 complex by sucrose gradient centrifugation was omitted. Instead, the active fractions from the Phenyl-Sepharose CL-4B column were pooled and made up to 0.1 mM PMSF and 6 mM p-aminobenzamidine, and centrifuged at 250 000 xg for 16-17 h. The sedimented enzyme (lane a) was subjected to further purification on a column of DEAE-Sepharose CL-6B, as described in MATERIALS AND METHODS. It was taken up in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM EGTA, 0.1 mM PMSF, 100 mM KCl, 20% (v/v) methanol, 50 mg/ml soybean phospholipid and 0.9% (w/v) Aminoxid WS-35 prior to loading onto the column. The fraction containing the highest activity was analyzed by SDS-gel electrophoresis (lane b). The composition of the SDS-gel was the same as that described in the legend to Fig. 12 except that the concentration of SDS in the gel was 0.5% (w/v). Lane c represents purified ECF_1. The migration positions of the subunits of ECF_1 (α-ε) as well as of the DCCD-binding protein (identified using [^{14}C]DCCD, see Fig. 15), the 28 000 and 18 000 dalton polypeptides of F_0, are also indicated.

were the subunits of ECF_1 and the remaining had molecular weights of 30 000, 28 000, 18 000, 14 000, 9 000 and 7 500. Minor bands of 85 000, 71 000 and 24 000 daltons were also present: Some of these polypeptides were thought to be contaminants. Attempts were made to remove these contaminating polypeptides by subjecting the sedimented ATPase enzyme to further fractionation.

The first method involved the treatment of the enzyme with PEG 6 000 and 400, as described by Friedl et al. (108). This was not successful. Precipitation of the ATPase complex with PEG 6000 and 400 resulted in almost complete (98%) inactivation of the enzyme activity. In addition to being γ-deficient, the precipitated enzyme also contained very low amounts of the α and ß subunits of ECF_1.

The second method involved ion-exchange chromatography on DEAE-Sepharose CL-6B in the presence of Aminoxid WS-35, as described by Friedl and Schairer (106). The sedimented ATPase complex dissociated during ion-exchange chromatography. The active fractions from the DEAE-Sepharose CL-6B column were analyzed by SDS-polyacrylamide gel electrophoresis and found to contain only δ-deficient ECF_1 (Fig. 13, lane b). The absence of intact ECF_1F_0 complex was confirmed, by determining the sensitivity of the ATPase activity of this preparation to DCCD (Fig. 14).

INTACTNESS OF THE ECF_1F_0 COMPLEX

In this study, centrifugation of the active fractions from the Phenyl-Sepharose CL-4B column, at 250 000 xg for 16-17 h resulted in optimal purification of the ATPase enzyme. The intactness of the purified ECF_1F_0 complex was determined by using DCCD. The results are shown in Fig. 14.

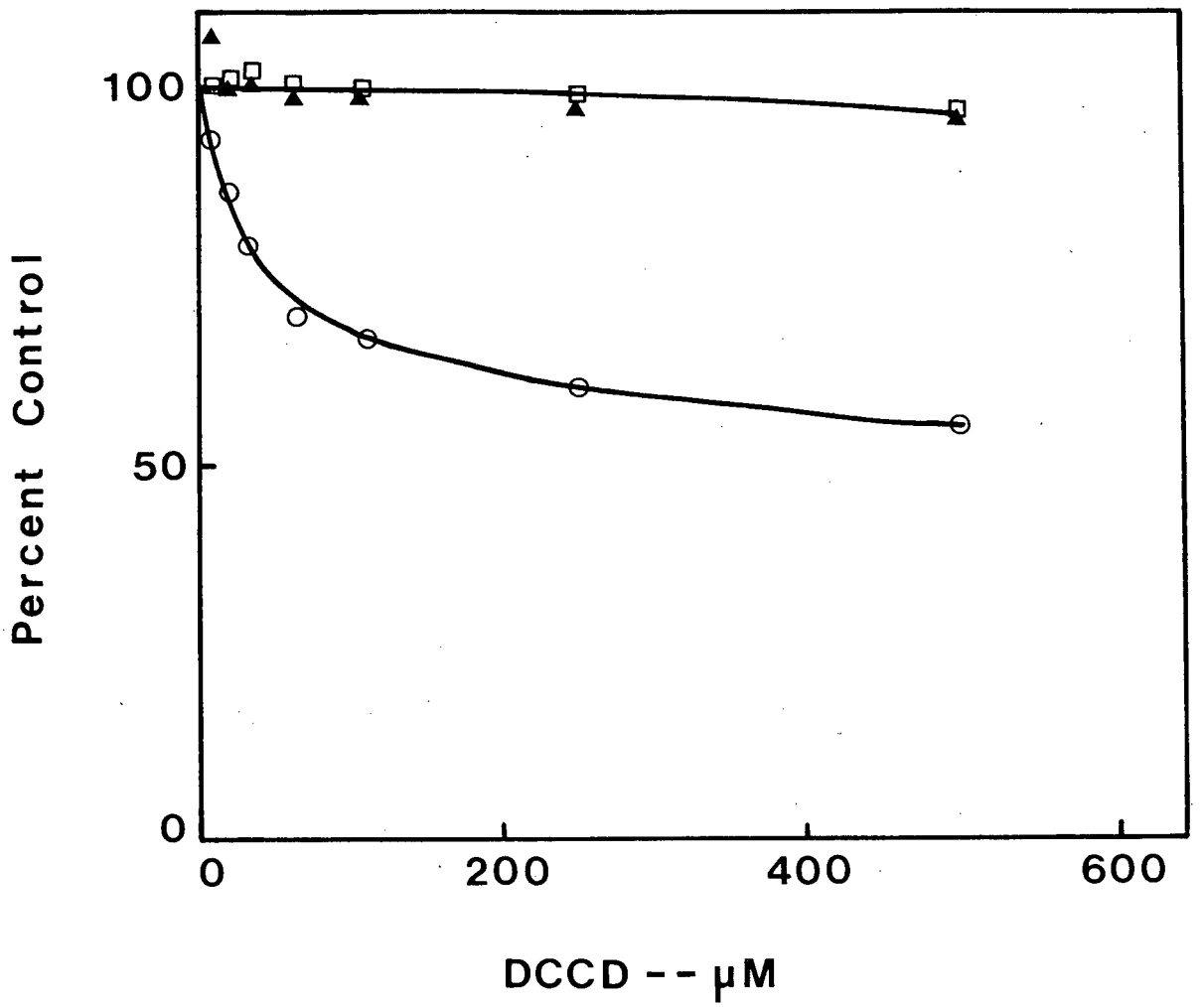


Fig. 14 Effect of DCCD on the ATPase activity of the ECF_1F_0 complex.

The ATPase enzymes obtained after chromatography on Phenyl-Sepharose CL-4B and sedimentation at 250 000 xg for 16-17 h (Fig. 13, lane a), or after further purification of the sedimented enzyme on DEAE-Sepharose CL-6B (Fig. 13, lane b), were incubated with various levels of DCCD in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$ (final volume, 0.1 ml) at 37°C for 45 min. ECF_1 obtained after AH-Sepharose 4B chromatography was also treated with DCCD. Following incubation with DCCD, a sample of the mixture was removed and the ATPase activity measured as described in MATERIALS AND METHODS. The specific activities of the sedimented enzyme (○-○), DEAE-Sepharose CL-6B-purified enzyme (□-□) and ECF_1 (▲-▲) were 18, 31.4 and 26.3 units per mg protein and the amount of protein per assay was 1.75, 0.85 and 0.8 μg, respectively.

Over a DCCD concentration range of 500 μM, the activity of the purified ECF_1 was inhibited by only 5-10%. Similar results were obtained with the DEAE-Sepharose CL-6B-purified enzyme. This was expected since this preparation appeared to be δ-deficient ECF_1 and not the ECF_1F_0 complex.

By contrast, the enzyme sedimented by centrifugation at 250 000 xg for 16-17 h, was sensitive to DCCD. The ATPase activity was inhibited by 30% and 45% at DCCD concentrations of 100 μM and 500 μM, respectively.

Treatment of all three enzyme preparations with [^{14}C]DCCD (136 μM) at pH 8.0 in the presence of 5 mM $MgCl_2$, under conditions identical to those described in the legend to Fig. 14, resulted in the labelling of the ß subunit (52 000 daltons). In addition, a polypeptide of about 9 000 daltons was labelled in the enzyme sedimented by centrifugation at 250 000 xg for 16-17 h (Fig. 15).

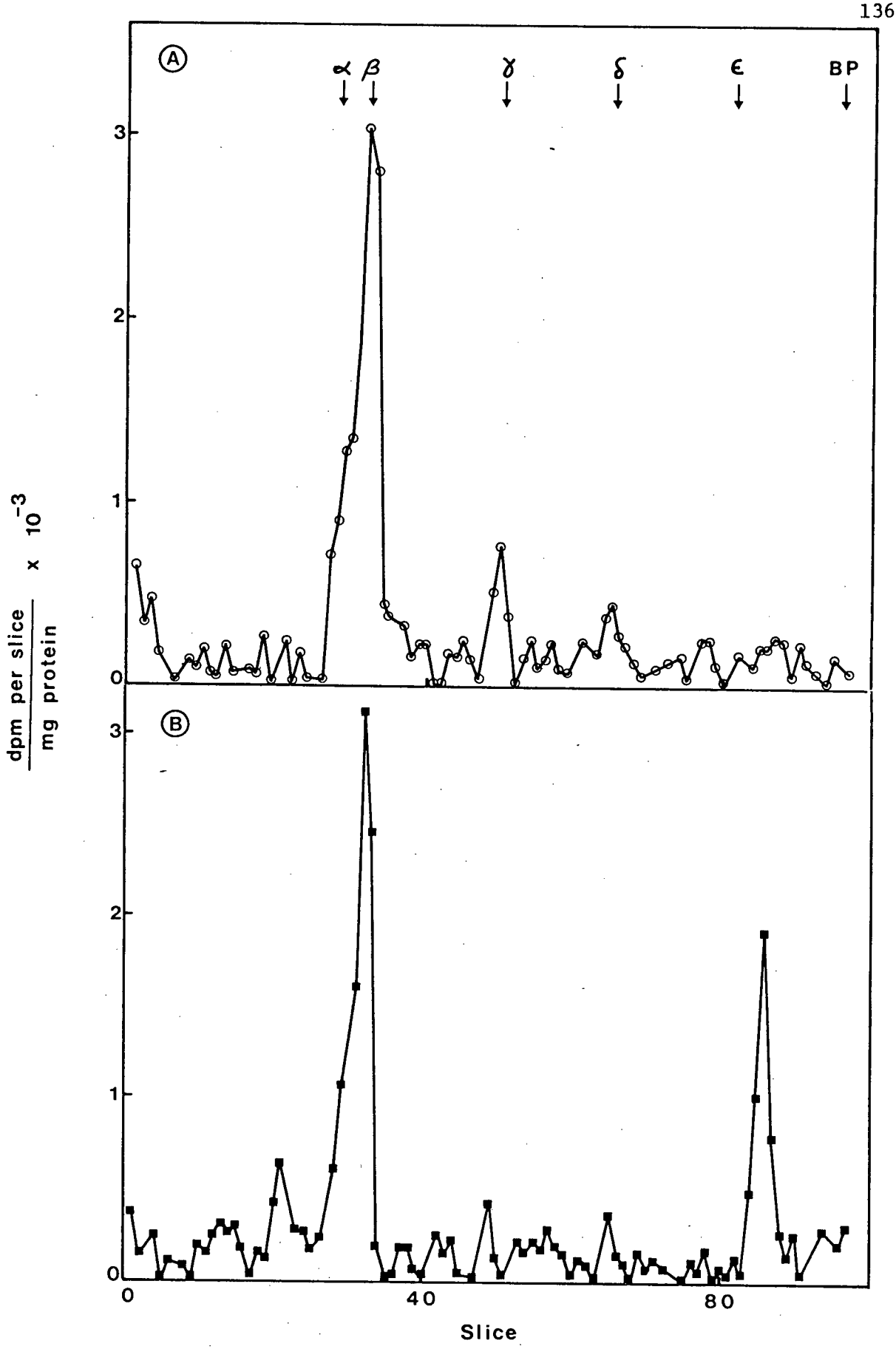
Other evidence that the sedimented ATPase enzyme was present in intact form was otained by using [1-^{14}C]iodoacetamide and the fluorescent label, 5-iodoacetamidofluorescein. Recently, Paradies et al. (35) have shown that the compounds TAMM and NEM-Hg labelled only the ß subunit of purified ECF_1. However, the labels were associated only with the α subunit when the F_1F_0 complex was used.

[^{14}C]- iodoacetamide was found to label both the α and ß subunits of the sedimented ATPase complex but only the α of the purified ECF_1. With the fluorescent probe 5-iodoacetamidofluorescein, both the α and ß subunits of the intact ATPase complex were labelled, with a higher amount of the label on the α subunit. But with purified ECF_1, only the ß subunit was labelled (these experiments were done in collaboration with Drs. Helga Stan-Lotter and P.D. Bragg).

These results suggested that the ECF_1 remained associated with F_0

Fig. 15 SDS-polyacrylamide gel electrophoresis of ECF_1 and ECF_1F_0 complex labelled with [^{14}C]DCCD.

ECF_1 (0.34 mg), obtained after chromatography on AH-Sepharose 4B, and the purified ECF_1F_0 complex (0.35 mg, Fig. 13, lane a) were suspended in 0.25 ml of 50 mM Tris-HCl buffer, pH 8.0 containing 5 mM $MgCl_2$. To each was added 7 µl of 5 mM [^{14}C]DCCD (Specific activity, 50 mCi/nmol) and the mixtures incubated at 37°C for 45 min. The unreacted label was removed by the centrifugation column-chromatography method described by Penefsky (170). The eluents were depolymerized and 82.5 µg of the ECF_1 (PANEL A) and 70 µg of the ECF_1F_0 complex (PANEL B) were applied to the SDS-gel. The slab gel (Tris-buffered system) consisted of a separating gel of 13% (w/v) acrylamide and a stacking gel of 4% (w/v) acrylamide with the SDS concentration in the gel being 0.5% (w/v). After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Blue, the separating gel sliced into 1 mm segments, and the radioactivity of the slices determined as described in MATERIALS AND METHODS. α - ε refer to the migration positions of the subunits of ECF_1 in the gel. The position of the tracking dye, bromophenol blue (BP) is also indicated.


A
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β
γ
δ
ε
BP
3
2
1
0
B
3
2
1
0
0
40
80
Slice
dpm per slice / mg protein x 10^-3

following centrifugation at 250 000 xg for 16-17 h, but dissociated during subsequent ion-exchange chromatography.

REPRODUCIBILITY OF PURIFICATION ON PHENYL SEPHAROSE CL-4B

Problems were encountered when attempts were made to use Phenyl-Sepharose more than once. Although the position of elution of the activity (15-16% ammonium sulphate concentration) was almost identical when the resin was re-used, analysis of the active fractions from the column on SDS-polyacrylamide gels revealed that a different pattern of polypeptide bands was obtained each time.

The experiment was repeated as described for Fig. 12 except that the fraction was applied to a column of Phenyl-Sepharose CL-4B which had been used and regenerated under different conditions. The fractions richest in ATPase activity were analyzed on SDS-polyacrylamide gels.

About 14-16 protein straining bands were found when the ATPase enzyme was chromatographed on a column of freshly-prepared resin (Fig. 16, lane a). When the enzyme was chromatographed on a column of resin which had been used several times and regenerated as recommended by the manufacturer (Pharmacia), more than 22 different polypeptides were found to be present. These extra protein bands ranged in molecular weight from 10 000 to 100 000 (Fig. 16, lane b).

A more complete regeneration of the resin was obtained when the previously used resin was washed with 2% (w/v) SDS and then regenerated as recommended. The most active fraction contained 13-15 major polypeptides (Fig. 16, lane c). However, the resin continued to deteriorate with repeated use, even with inclusion of a 2% (w/v) SDS washing step (Fig. 16, lane d).

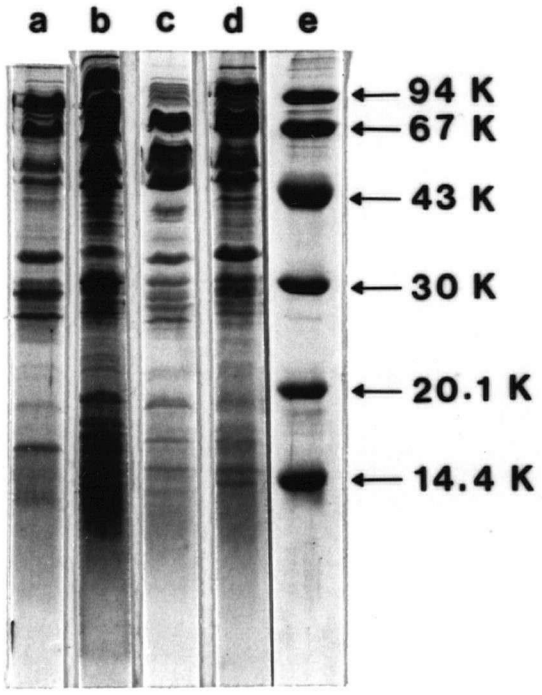


Fig. 16 SDS-polyacrylamide gel electrophoresis of the ECF_1F_0 complex obtained after chromatography on Phenyl-Sepharose CL-4B: Reproducibility of the purification.

The experiment described in the legend to Fig. 12 (PANEL A) was repeated except that the fraction was applied to a column of Phenyl-Sepharose CL-4B (1.8 x 25 cm) which had been regenerated under several different conditions. The most active fraction from the column was analyzed on SDS-gels. The gel consisted of 13% (w/v) acrylamide with a 4% (w/v) acrylamide stacking gel, as described by Laemmli (171). Protein bands were visualized with 0.1% (w/v) Coomassie Blue as described in MATERIALS AND METHODS. Lane a, freshly-prepared Phenyl-Sepharose CL-4B; lane b, resin was used and regenerated according to the manufacturer's (Pharmacia) recommendations; lane c, resin used for experiment in lane b was regenerated as before but an extra wash with 2% (w/v) SDS was included; lane d, the resin used for the experiment in lane c was regenerated as for the experiment in lane c; lane e consists of molecular weight marker proteins (M_r, 94 000 - 14 400).

Fairly reproducible purifications could be obtained each time with a column of freshly-prepared resin.

COMPARISON OF THE GEL ELECTROPHORESIS AND PROTEIN-DETECTION SYSTEMS

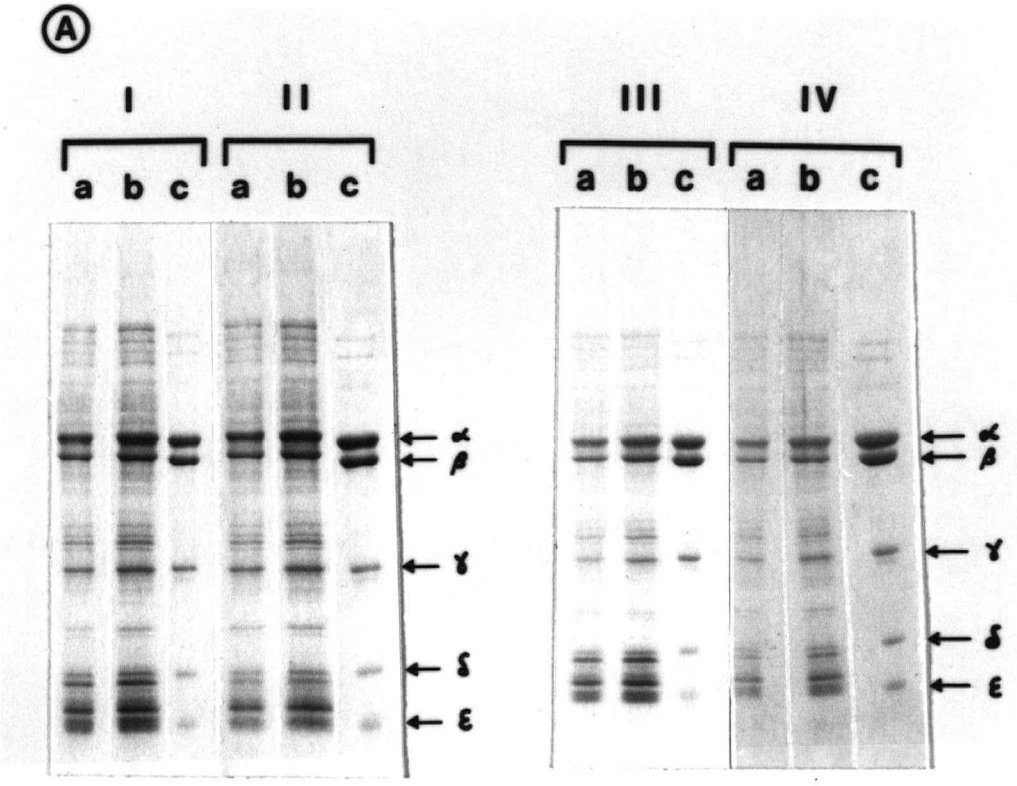
The main determinant in assessing the purity of the ATPase complex was by SDS-polyacrylamide gel electrophoresis. The number of polypeptide bands observed has been reported to depend on the resolving power of the gel electrophoresis system (5,95). In this study, a comparison of the resolving power of the phosphate-buffered (174) and the Tris-buffered (171,173) gel electrophoresis systems as well as the protein-detection systems was made. A preparation of the ATPase complex purified on a sucrose gradient was subjected to the different electrophoresis systems. The results are shown in Fig. 17. In each case, the gels were stained with one of the staining systems described in MATERIALS AND METHODS.

The Tris-buffered system (Fig. 17A) was more resolving than the phosphate-buffered system (Fig. 17B). In the former, the subunits of ECF_1 (α - ε) were highly resolved, whereas in the latter, the α and β subunits overlapped. The ε subunit could barely be identified with the phosphate system. In addition, many of the minor protein bands seen in the Tris system were not resolved in the phosphate system. In general, the polypeptide bands were more distinct in the Tris-buffered system.

The number of protein-staining bands seen on the gel also depended on the staining properties of the dye used. A comparison of four different staining systems is shown in Fig. 17. The most intensely-stained preparation and the greatest number of bands was obtained with 0.05% (w/v) Coomassie Blue in 25% (v/v) isopropanol and 10% (v/v) acetic acid (Fig. 17A, I). A slightly weaker staining-intensity, but equally as resolving,

Fig. 17 Comparison of the SDS-gel electrophoresis and protein-detection systems.

Partially purified ECF_1F_0 complex obtained after chromatography on Phenyl-Sepharose CL-4B and sucrose gradient centrifugation, and sucrose gradient-purified ECF_1, were subjected to SDS-gel electrophoresis on a linear 7.5% - 16.5% (w/v) acrylamide gradient gel in the Tris-buffered system (PANEL A), or in the phosphate-buffered system (PANEL B), as described in MATERIALS AND METHODS. Following electrophoresis, the gels were fixed with a solution containing 5% (w/v) TCA, 5% (w/v) sulfosalicylic acid and 10% (v/v) methanol for 30 min at 60°C. The gels were then stained for 9 h at 20°C with (I), 0.05% (w/v) Coomassie Blue in isopropanol:acetic acid:water (25:10:65); (II), 0.25% (w/v) Coomassie Blue in methanol:acetic acid:water (45:10:45); (III), 0.12% (w/v) Coomassie Blue in ethanol:acetic acid:water (25:8:67); (IV), 1% (w/v) Amido Black in 7% (v/v) acetic acid. Lanes a and b are partially purified ECF_1F_0 complex, with lane b containing twice the amount of ECF_1F_0 complex of that in lane a. Lane c is purified ECF_1. The migration position of the subunits of ECF_1 (α - ϵ) are indicated.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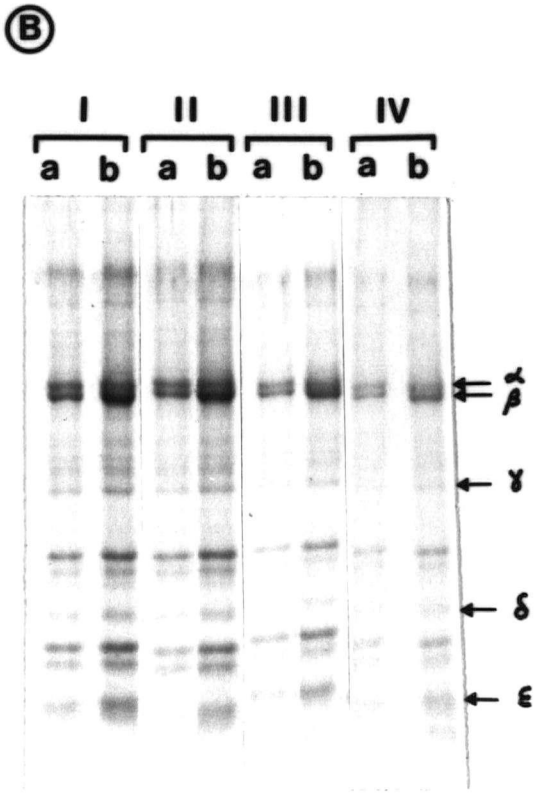
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was obtained with 0.25% (w/v) Coomassie Blue in 45% (v/v) methanol and 10% (v/v) Acetic acid (Fig. 17A, II).

The number of minor bands observed decreased considerably when the gel was stained with either 0.12% (w/v) Coomassie Blue in 25% (v/v) ethanol and 8% (v/v) acetic acid or 1% (w/v) Amido Black in 7% (v/v) acetic acid. The least number of polypeptide bands as well as the weakest staining was observed with Amido Black.

PART II STUDIES ON MUTANTS OF E. coli DEFECTIVE IN PROTON-TRANSLOCATING ACTIVITY

The ECF_1F_0 complex purified in the preceding section consisted of eleven major polypeptides. Other workers have shown that the complex consists of eight subunits (Tables 2 and 3). Five of these polypeptides were subunits of ECF_1 (α - ε). The remaining polypeptides of 24 000, 18 000 and 8 400 daltons were considered to be subunits of F_0. However, the mechanism of proton conduction through F_0 and the relationship of these polypeptides to each other are not known.

One approach to this problem has been through the isolation of mutants of E. coli which are defective in oxidative phosphorylation (unc mutants). It is conceivable that a defect in any of the three subunits of F_0 would alter its proton-translocating properties.

Several of these mutants have been isolated and the properties of those studied in this thesis are listed in Table 12. E. coli WS1 is a wild-type strain. Both of the unc mutants, E. coli N_{I44} and CBT-302 did not exhibit ATPase activity. Analysis of membrane preparation from these two mutants on O'Farrell gels (176) revealed the absence of any subunits of ECF_1. ATPase activity was detected in the mutant, E. coli AN382. In contrast to the wild-type strain, the activity of E. coli AN382 was relatively insensitive to DCCD. At a DCCD concentration of 63 μM, the ATPase activities of E. coli WS1 and AN382 were inhibited by 60% and 8%, respectively. The ECF_1 from both strains were analyzed on O'Farrell gels and found to be identical. Thus, it is unlikely that the difference in the sensitivity of the ATPase activity to DCCD was due to differences in the ECF_1 portion of the ECF_1F_0 complex.

Table 12 Some Properties of the unc Mutants of E. coli used in this Thesis

Strain	ATPase activity[a]		% Inhibition	Presence of ECF_1[c]
	-DCCD	+DCCD[b]		
WSI	0.294	0.116	60	+
N_{I44}	0	0	-	0
CBT-302	0	0	-	0
AN382	0.199	0.183	8	+

a. µmol/min/mg protein.

b. 63 µM DCCD

c. Determined by two-dimensional gel electrophoresis of the membranes +, present; -, absent.

Membrane vesicles of E. coli WSI, N_{I44}, CBT-302 and AN382 were suspended in 50 mM Tris-HCI buffer, pH 8.0, containing 10 mM $MgCI_2$ and 10% (v/v) glycerol at a protein concentration of 4 mg/ml. The membrane suspensions were incubated with DCCD for 30 min. at 37°C after which the ATPase activity was determined as described in MATERIALS AND METHODS.

SENSITIVITY OF THE MEMBRANE-BOUND ATPase ACTIVITY TO INHIBITION BY DCCD IN THE PRESENCE OF CATIONS

The effect of Ca^{2+} and Mg^{2+} on the sensitivity of ATPase activity to DCCD in membrane vesicles of E. coli ML308-225, AN180, AN382 and WS1 was also examined. In all four strains, maximum inhibition of activity by DCCD was observed in the presence of Mg^{2+} (Fig. 18). In the wild-type strains, E. coli WS1, AN180 and ML308-225, the enzyme activity was inhibited half-maximally at 3-6 nmol DCCD per mg protein. By contrast, in E. coli AN382, the enzyme was inhibited by only 35% at 100 nmol DCCD per mg protein. Therefore, the resistance of the activity to DCCD appeared to be due to defect(s) in the F_0 portion of the ECF_1F_0 complex. Other methods were then used to characterize this mutant as well as the unc mutants E. coli N_{I44} and CBT-302.

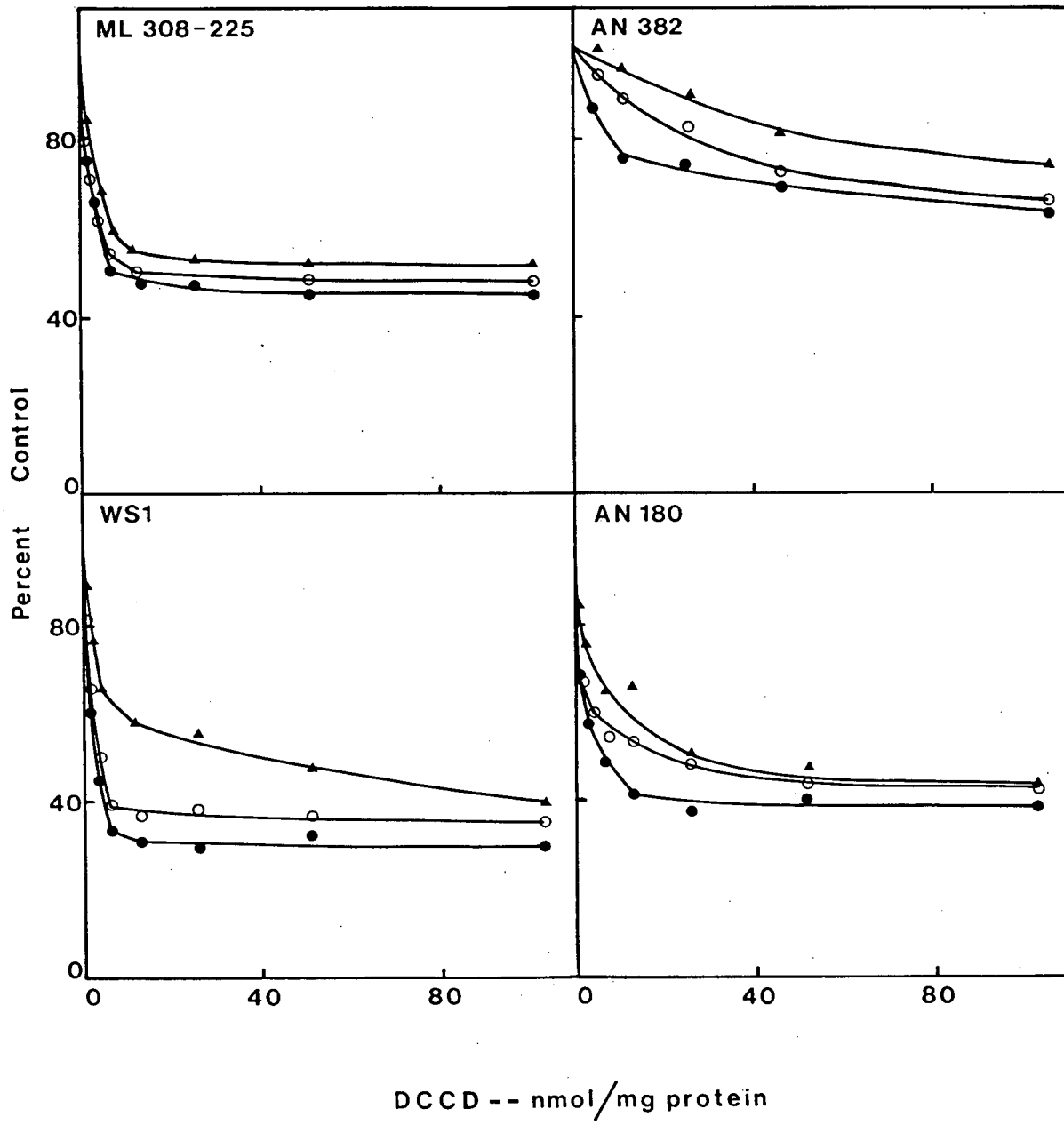
MEASUREMENT OF THE PROTON GRADIENT USING 9-AMINOACRIDINE

Everted membrane vesicles of E. coli have the ECF_1 portion of the ATPase complex exposed to the external medium such that during ATP hydrolysis or substrate oxidation a proton gradient is generated in these vesicles.

The proton gradient within these everted vesicles can be detected by using the fluorescent, lipophilic weak base, 9-aminoacridine. As shown in Fig. 19 (trace 1), the fluorescent dye equilibrated across the membrane of these everted vesicles in response to a proton gradient generated by oxidation of substrate (NADH). This resulted in a decrease in fluorescence. When the system went anaerobic, the respiratory chain became non-functional and the fluorescence returned to its original level as a result of re-equilibration of the proton gradient across the membrane. The addition

Fig. 18 Sensitivity of the membrane-bound ATPase activity to inhibition by DCCD in the presence of cations.

Membrane vesicles from E. coli AN382, AN180, ML308-225, and WS1 were prepared in 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 1 mM $MgCl_2$ as described in MATERIALS AND METHODS. The membrane vesicles were suspended at a protein concentration of 4 mg/ml in 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10% (v/v) glycerol (▲-▲) and either 10 mM $CaCl_2$ (○-○) or 10 mM $MgCl_2$ (●-●). Various concentrations of DCCD in ethanol (10 µl) were added to 1 ml of membrane vesicles. After 45 min at 37°C, the ATPase activity of the membrane vesicles was determined as previously described. The specific activities of the membrane-bound ATPase of E. coli AN382, AN180, ML308-225 and WS1 in the absence of cations were 0.09, 0.30, 0.67 and 0.30 units per mg protein; in the presence of Ca^{2+} were 0.11, 0.28, 0.67 and 0.26 units per mg protein and in the presence of Mg^{2+} were 0.16, 0.36, 0.66 and 0.38 units per mg protein, respectively.


ML 308-225
AN 382
WS1
AN 180
Percent Control
0
40
80
0
40
80
DCCD -- nmol/mg protein

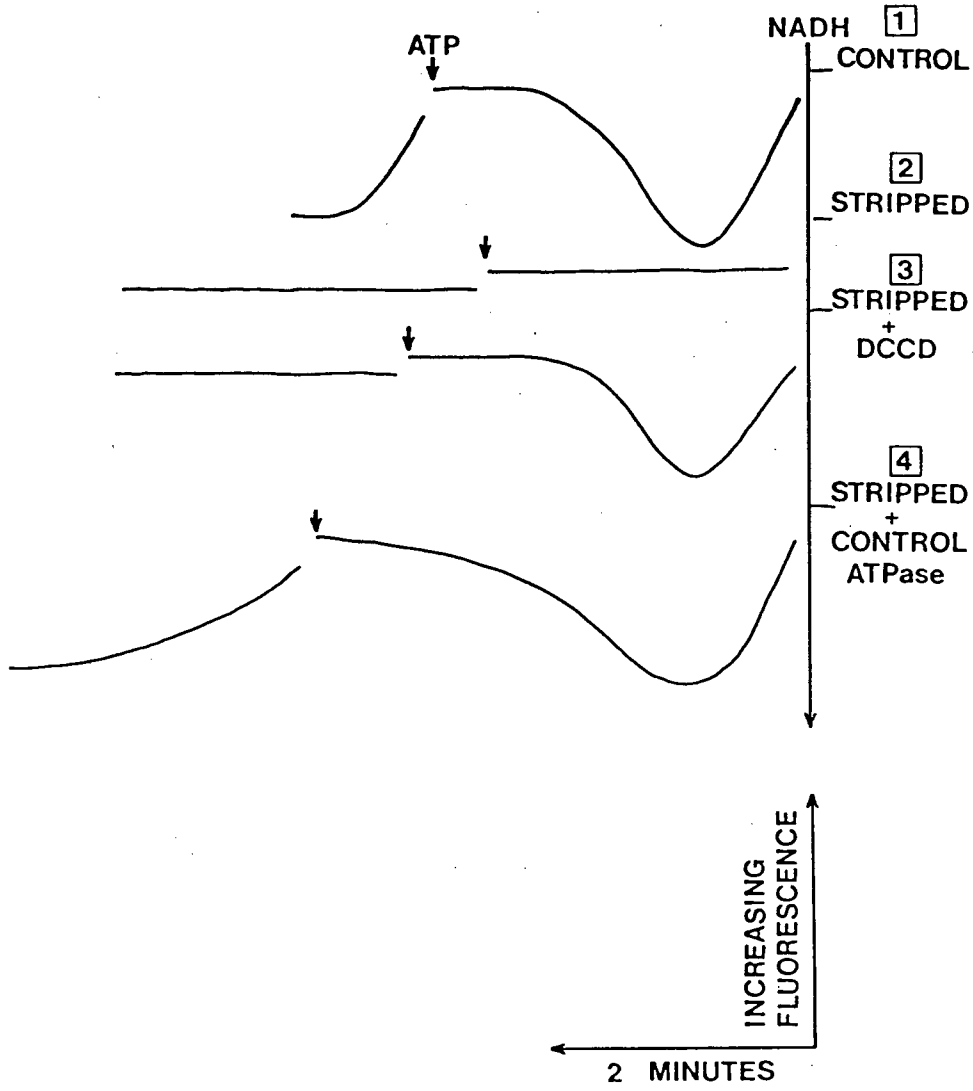


Fig. 19 Measurement of the proton gradient in everted membrane vesicles using the fluorescent dye, 9-aminoacridine.

Everted membrane vesicles of E. coli WS1 were dialyzed against a low ionic strength buffer to remove ECF_1 from the membranes, as described in MATERIALS AND METHODS. The ECF_1-stripped membrane vesicles were suspended in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$ at a protein concentration of 10 mg/ml. Samples (0.1 ml) were removed and assayed for the ability to quench the fluorescence of 9-aminoacridine during substrate (NADH) oxidation, or during hydrolysis of ATP, as described in the MATERIALS AND METHODS section. Trace 1, untreated everted membrane vesicles; Trace 2, ECF_1-stripped membrane vesicles; Trace 3, ECF_1-stripped everted membrane vesicles incubated with (375 µM)DCCD for 5 min at 20°C prior to assay; Trace 4, ECF_1-stripped everted membrane vesicles incubated with ECF_1 (90 µg protein) for 5 min at 20°C prior to assay.

of ATP at this point again energized the membrane and a decrease in fluorescence was observed. Quenching of fluorescence was not observed in everted vesicles which had been stripped of ECF_1 (Fig. 19, trace 2). However, addition of DCCD or purified ECF_1 to these stripped membranes restored its ability to be energized during substrate oxidation. Stripped membranes which had been reconstituted with ECF_1 could also be energized by ATP hydrolysis (Fig. 19, trace 4).

These results suggested that the F_0 portion of the ATPase complex is a proton pore and that ECF_1 and DCCD could block the passive proton leakage through F_0.

The mutants, E. coli N_{I44}, CBT-302 and AN382, as well as the wild-type strain, E. coli WS1, were investigated in a similar manner.

Normal and mutant membrane vesicles were treated with 2M urea, 2M guanidine hydrochloride or 2% (w/v) silicotungstic acid to remove any subunits of ECF_1 which might be retained by the membrane. In this respect, the mutant membrane vesicles were also treated with trypsin. The results shown in Fig. 20 are typical of all these treatments. Oxidation of ascorbate (in the presence of PMS to introduce electrons into the cytochrome region of the respiratory chain) by untreated ("washed") membranes resulted in the quenching of fluorescence of 9-aminoacridine indicating that a proton gradient had been established across the membrane vesicle (Fig. 20, traces 1 and 3). When the system became anaerobic, addition of ATP resulted in the restoration of the proton gradient only in the wild-type membranes (Fig. 20, trace 1).

Treatment of the membranes of the wild-type strain with urea ("stripping") resulted in the removal of ECF_1 with the loss of the ability to set-up and maintain a proton gradient (Fig. 20, trace 2). By

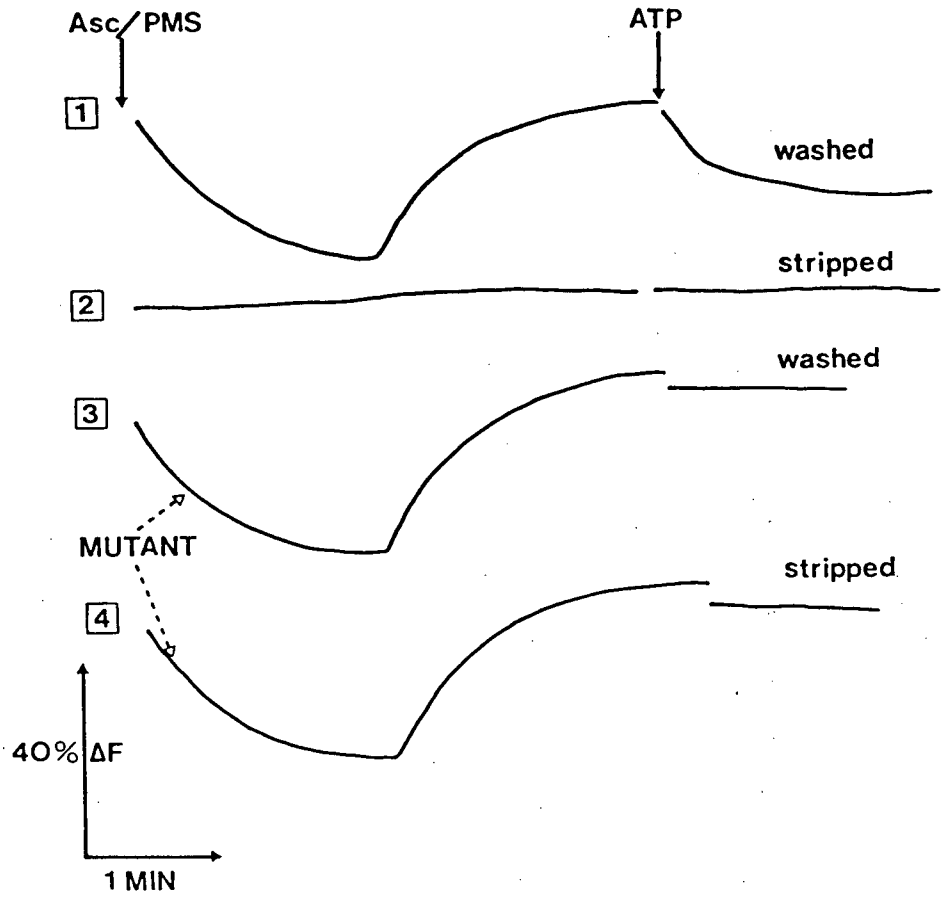


Fig. 20 Effect of stripping everted membrane vesicles from the parent (E. coli WS1) and mutant (E. coli N_{I44}) on the energization of the membrane.

Membrane vesicles of E. coli WS1 and N_{I44} were treated with 2 M urea to remove ECF_1 from the membrane. The membrane vesicles were suspended at a protein concentration of 10 mg/ml in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$. Samples (0.1 ml) were removed and assayed for the ability to quench the fluorescence of 9-aminoacridine during oxidation of ascorbate (Asc) in the presence of phenazine methosulphate (PMS) or during ATP hydrolysis, as described in MATERIALS AND METHODS. 1 and 3 are traces of the quenching observed with the untreated everted vesicles of the parent (WS1) and the mutant (N_{I44}), respectively. 2 and 4 are traces of the quenching observed with urea-treated ("ECF_1-stripped") everted vesicles of the parent (WS1) and mutant (N_{I44}), respectively. Similar results (trace 4) were obtained if the membrane vesicles from the mutant (N_{I44}) were treated with 2 M guanidine hydrochloride, 2% (w/v) silicotungstic acid or TPCK-trypsin.

contrast, none of the stripping procedures described earlier destroyed the capacity of the mutant membranes to generate a proton gradient during substrate oxidation (Fig. 20, trace 4). Thus, it is unlikely that retention of individual subunits on the membrane was responsible for the relative impermeability of these membranes to protons.

Although ATPase activity was detected in the membranes of the mutant, E. coli AN382 (Table 12), and the ECF_1 was identical to that of the wild-type strain, ATP-dependent fluorescence quenching was not observed in this mutant.

These results indicated that the lesion(s) responsible for the relative proton-impermeability of the membranes of the unc mutants, E. coli N_{I44}, CBT-302 and AN382 resided in the F_0 portion of the ECF_1F_0 complex. This was then investigated.

LABELLING OF MEMBRANES OF E. coli WITH [^{14}C]DCCD

It has been shown that the inhibition of ATPase activity in E. coli vesicles by DCCD was associated with the labelling of a 8-9 000 dalton polypeptide (141,144). Similarly, the addition of DCCD to "stripped" everted membranes of E. coli blocked proton-translocation, as shown in Fig. 19 (trace 3). Therefore, it was postulated that the 9 000 dalton polypeptide (DCCD-binding protein) must be involved in proton conduction (144,145). The relative impermeability of the stripped membranes of the mutant cells suggested that the lesion responsible for this might reside in this polypeptide. The presence of this polypeptide in the mutant membranes was investigated by labelling the membranes of the normal and mutant E. coli with [^{14}C]DCCD followed by separation of the polypeptides by SDS-polyacrylamide gel electrophoresis. A typical result of the labelling

experiment of the membranes of the wild-type (E. coli WS1) and the mutant (E. coli N_{I44}) strains is shown in Fig. 21 (traces 1 and 2). Besides material at the top of the gel, two major peaks of radioactivity were observed both with the normal and mutant membranes. The larger of these peaks coincided with a Coomassie Blue staining polypeptide of 9 000 daltons (data not shown). The smaller peak was not associated with a Coomassie Blue staining polypeptide.

A characteristic property of the DCCD-binding protein of F_0 is its ability to be extracted into chloroform-methanol (2:1) (144,145,150). [^{14}C]-labelled membrane vesicles were extracted with chloroform-methanol and the proteins precipitated with ether. Gel electrophoresis of this material from both normal and mutant membranes gave mainly a single polypeptide band of molecular weight 9 000 (data not shown) coincident with the main peak of radioactivity (Fig. 21, traces 3 and 4). Similar results were obtained with the unc mutants, E. coli CBT-302 and AN382 as well as with the wild-type strain E. coli ML308-225. These results indicated that the DCCD-binding protein occurs in the mutants and that the aspartyl residue, which reacts with DCCD and is involved in proton translocation, is present (159,160).

PURIFICATION OF THE DCCD-BINDING PROTEIN

Although the DCCD-binding protein was found to be present in the mutants, E. coli AN382 and CBT-302 and N_{I44}, there remained the possibility that the amino acid composition of the protein was altered in these mutants. Therefore, attempts were made to purify this polypeptide.

[^{14}C]DCCD labelled membrane vesicles of E. coli WS1 or ML308-225 were extracted with chloroform-methanol (2:1) and the proteins precipitated

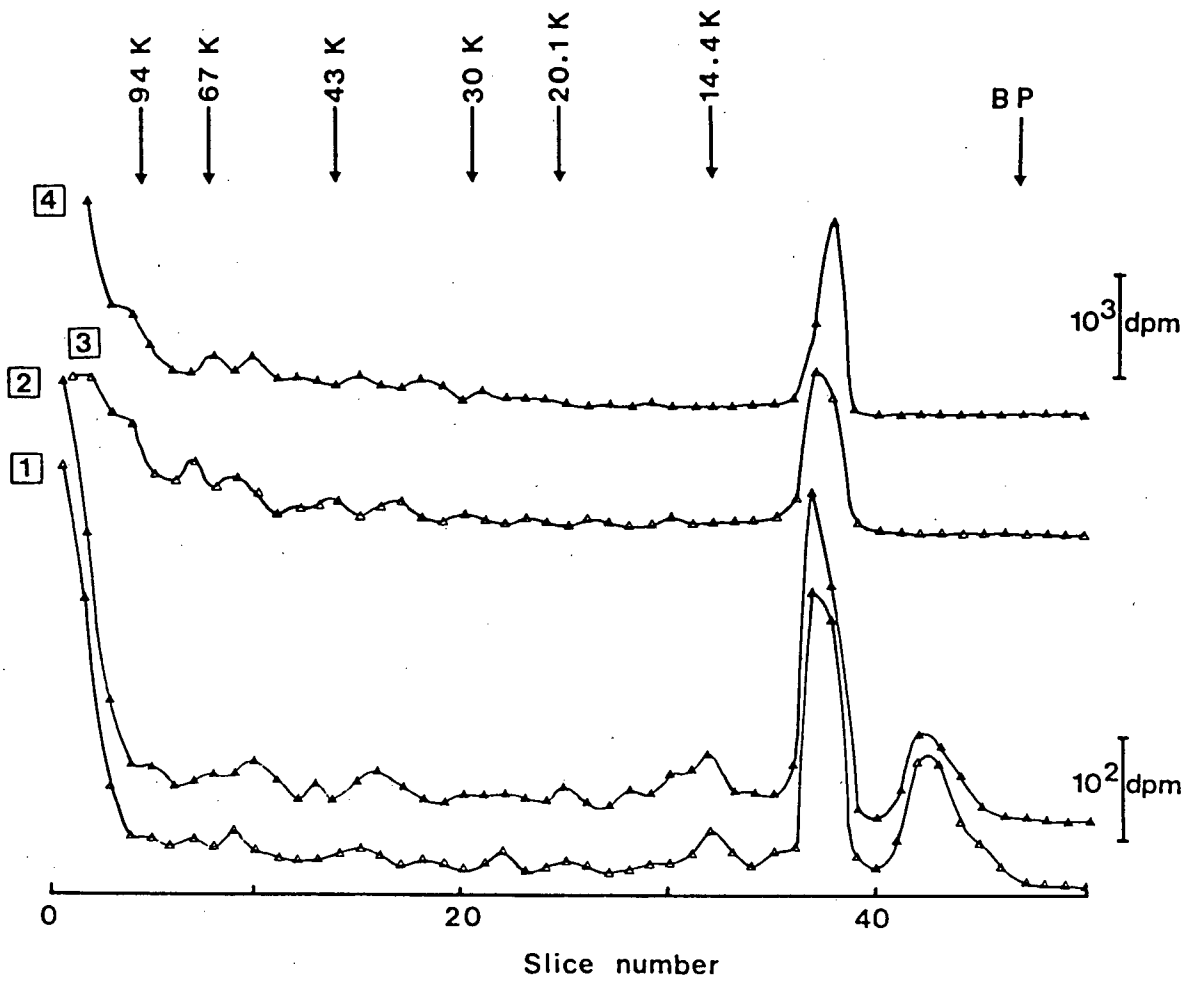


Fig. 21 SDS-polyacrylamide gel electrophoresis of [^{14}C]DCCD-labelled membranes and of ether-precipitated proteins of chloroform-methanol extracts of the labelled membranes of E. coli.

Membrane vesicles of the parent (WS1) and mutant (N_{I44}) strains of E. coli were labelled with [^{14}C]DCCD. The DCCD-binding proteins were isolated from the labelled membrane vesicles by extraction with chloroform: methanol (2:1) followed by precipitation with ether, as described in MATERIALS AND METHODS. The labelled membrane vesicles (150-200 µg protein) and the isolated DCCD-binding proteins (12-15 µg protein) were subjected to electrophoresis on SDS-urea (8 M) gels. Following electrophoresis, the gels were fixed with 50% (w/v) TCA, stained with 0.25% (w/v) Coomassie Blue, cut into 2 mm slices and the radioactivity of each slice determined as described previously. Traces 1 and 2 show the distribution of the radioactivity in the [^{14}C]DCCD-labelled membranes of the parent (WS1) and the mutant (N_{I44}) strains, respectively. Traces 3 and 4 show the distribution of the radioactivity of the isolated DCCD-binding protein from the parent (WS1) and mutant (N_{I44}) strains, respectively. The migration positions of the molecular weight marker proteins (M_r, 94 000 - 14 400) and the bromophenol blue (BP) tracking dye are shown.

with ether. The DCCD-binding protein was further purified by thin-layer chromatography. Several bands were visible on the chromatogram (Fig. 22A). About 80% of the total radioactivity applied was associated with band 4 (R_f = 0.9) and 10% with band 1 (R_f = 0.05). The remaining radioactivity was distributed between bands 2 (R_f = 0.45) and 3 (R_f = 0.68). The material eluted from the chromatogram was analyzed by SDS-polyacrylamide gel electrophoresis. A peak of radioactivity was observed with both bands 1 and 4 (Fig. 22B), but only the radioactivity peak of band 1 coincided with a Coomassie Blue staining polypeptide of 9 000 daltons (not shown). The peak of band 4 was associated with an opaque region on the gel, which migrated with a molecular weight of less than 9 000 daltons.

The amino acid composition of the material eluted from band 1 revealed the presence of the amino acids, serine, histidine and cysteine. This was diagnostic of a contaminated preparation of DCCD-binding protein since these amino acids are not present in this polypeptide (146). Therefore, another procedure for purifying the DCCD-binding protein was needed.

The [^{14}C]DCCD-binding protein isolated from E. coli WS1 by extraction of the membranes with chloroform-methanol (2:1) was chromatographed on a column of Whatman CM-32 or BioRad Cellex CM, in the presence of organic solvents, as described in MATERIALS AND METHODS. The elution profiles were quite different (Fig. 23). Most of the radioactive material applied to the column of BioRad Cellex CM did not bind to the resin (Fig. 23A). The unbound fraction essentially consisted of phospholipids. Protein was not detected in this fraction. By contrast, most of the radioactivity applied to the column of Whatman CM-32 was bound to the resin and eluted in the subsequent washing steps (Fig. 23B). Fractions A and B (Fig. 23B)

Fig. 22 Thin layer chromatography of the DCCD-binding protein.

The DCCD-binding protein was isolated from [^{14}C]DCCD-labelled membranes of E. coli WS1 by extraction with chloroform:methanol (2:1) and precipitation with ether. The ether-precipitated protein(s) in chloroform: methanol (2:1) was applied to a silica gel G plate. The chromatogram was developed by ascending chromatography in a mixture of chloroform:methanol: water (65:25:4) containing 20 mM HCl, as described in MATERIALS AND METHODS. The positions of the bands were visualized with iodine (PANEL A). The stained spots were scraped off the silica plate and extracted with chloroform:methanol (2:1) containing 20 mM HCl. The extracts were dried, precipitated with ether and the precipitate subjected to electrophoresis on SDS-urea (8 M) gels. The gels were fixed with 50% (w/v) TCA, stained with 0.25% (w/v) Coomassie Blue, cut into 2 mm slices and the radioactivity of each slice determined (PANEL B). The migration positions of the bromophenol blue (BP) tracking dye and of the molecular weight marker proteins (M_r, 94 000 - 14 400) are also shown.

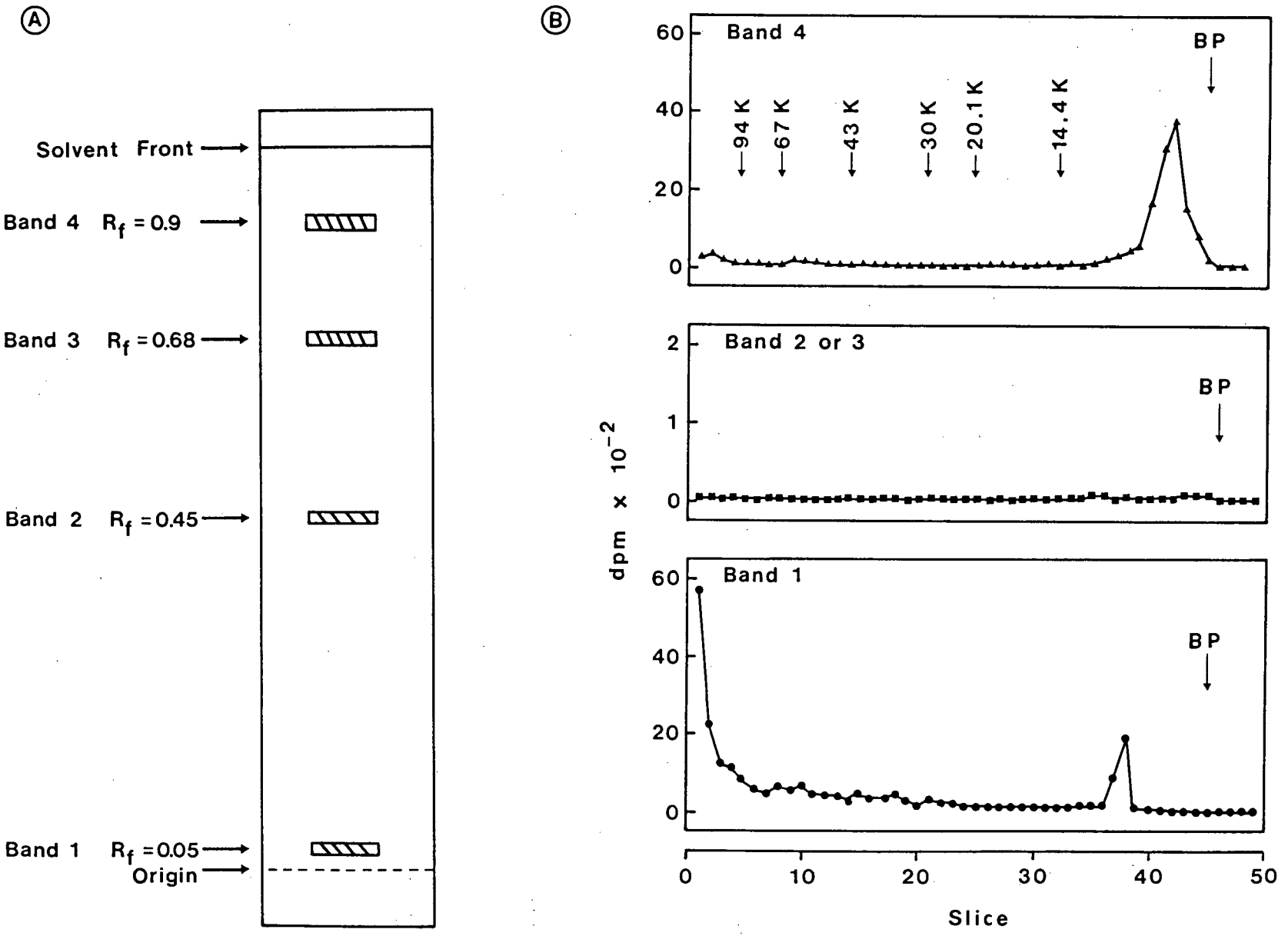
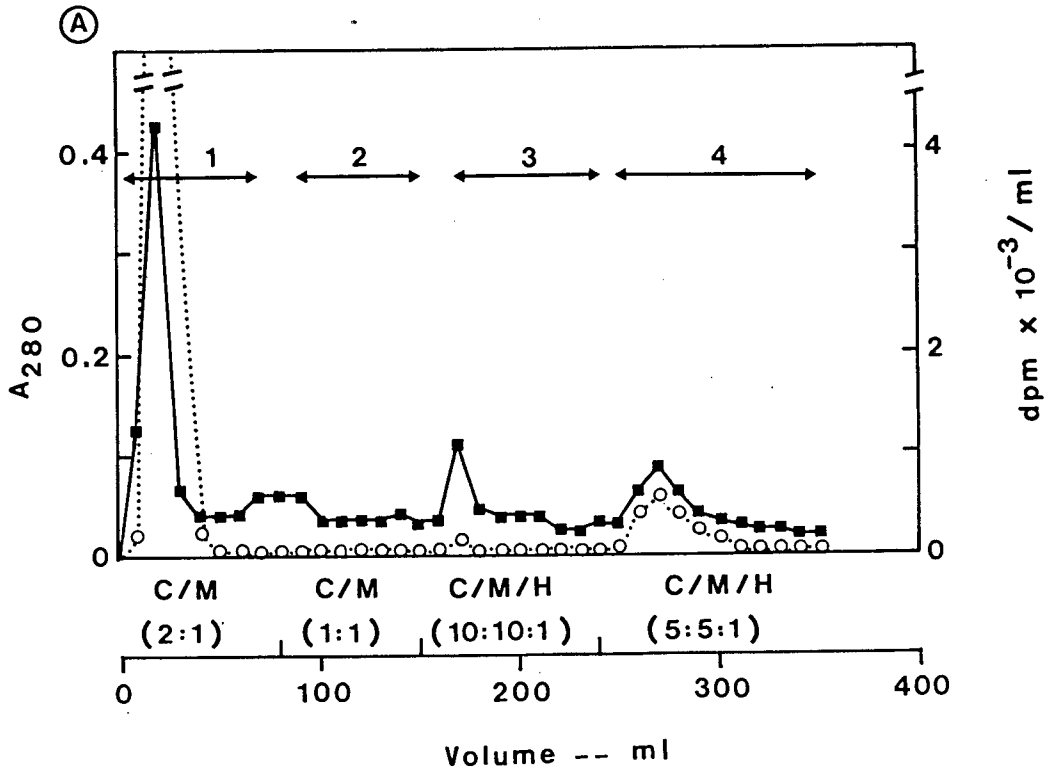
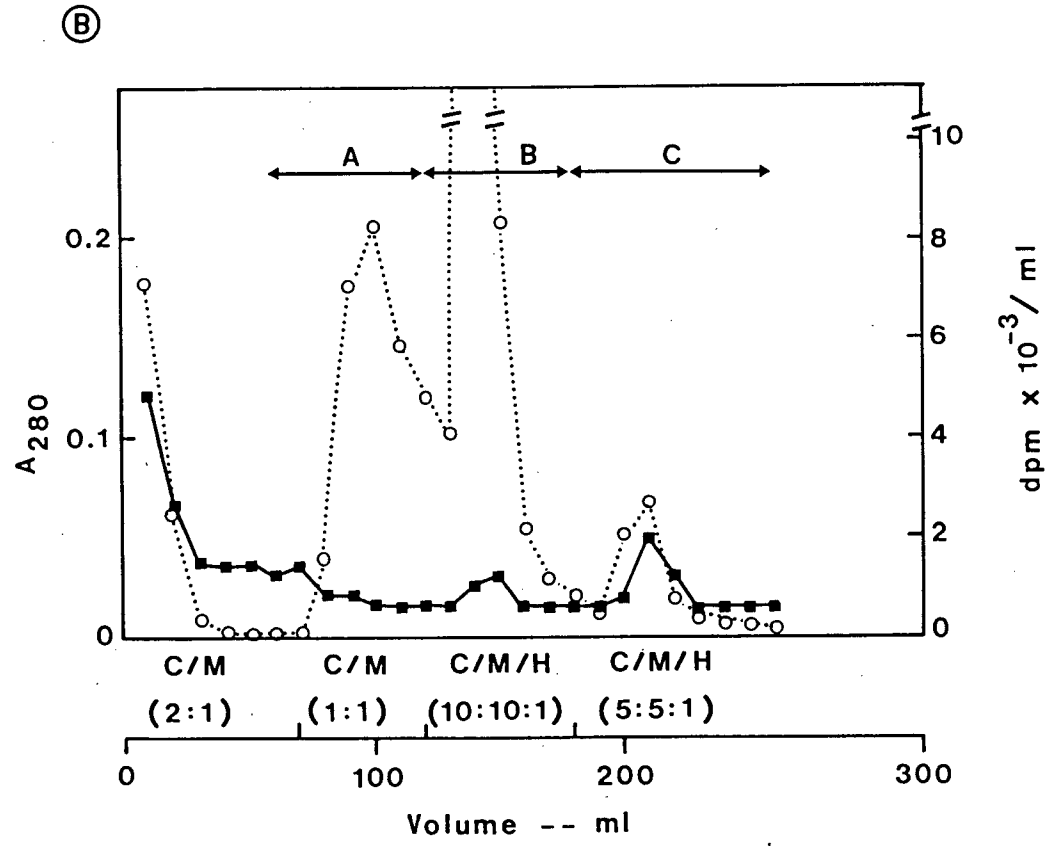

A
Solvent Front
Band 4 R_f = 0.9
Band 3 R_f = 0.68
Band 2 R_f = 0.45
Band 1 R_f = 0.05
Origin
B
Band 4
BP
94 K
67 K
43 K
30 K
20.1 K
14.4 K
Band 2 or 3
Band 1
dpm x 10^{-2}
Slice

Fig. 23 Chromatography of the DCCD-binding protein on CM-cellulose.

DCCD-binding protein was extracted from [^{14}C]DCCD-labelled membranes of E. coli WS1 with chloroform:methanol (2:1) and precipitated with ether. The ether-precipitated proteins were taken up in chloroform:methanol (2:1) and applied to a column of CM-cellulose, as described in MATERIALS AND METHODS. PANEL A: The isolated [^{14}C]DCCD-binding protein (4-5 mg protein) was applied to a column of BioRad Cellex CM (0.9 x 14 cm) equilibrated with chloroform:ethanol (2:1). The column was washed sequentially with 10 column volumes of chloroform:methanol (2:1) and 5 column volumes each of chloroform:methanol (1:1), chloroform:methanol:water (10:10:1) and chloroform:methanol:water (5:5:1). 10 ml fractions were collected and the absorbance at 280 nm (A_{280}) (■-■) and the radioactivity (O-O) determined. 1-4 refer to the fractions which were pooled and subjected to SDS-gel electrophoresis (see text). PANEL B: The experiment described for Panel A was repeated, except that the resin was Whatman CM-32 cellulose. A_{280}, (■-■); radioactivity, (O-O). 1-4 and A, B and C refer to the fractions which were pooled and subjected to SDS-gel electrophoresis (see Fig. 24). C, chloroform; M, methanol; H, water.


A
0.4
0.2
0
A_{280}
1
2
3
4
4
2
0
dpm x 10^{-3}/ ml
C/M (2:1)
C/M (1:1)
C/M/H (10:10:1)
C/M/H (5:5:1)
0
100
200
300
400
Volume -- ml


B
0.2
0.1
0
A_{280}
A
B
C
10
8
6
4
2
0
dpm x 10^{-3}/ ml
C/M (2:1)
C/M (1:1)
C/M/H (10:10:1)
C/M/H (5:5:1)
0
100
200
300
Volume -- ml

contained high amounts of phospholipids.

Analysis of the pooled fractions (A, B and C) obtained from the column of Whatman CM-32 on SDS-polyacrylamide gels revealed that the radioactivity was distributed throughout the length of the gel in fractions A and B (Fig. 24, panels A and B). However, only the radioactivity at 9 000 daltons was coincident with a Coomassie Blue staining polypeptide (not shown). The other broad radioactivity peaks were associated with an opaque region extending throughout most of the gel. By contrast, fraction C contained essentially one peak of radioactivity (Fig. 24, panel C), which coincided with a single protein-staining band of 9 000 daltons.

The fractions eluted from the column of BioRad Cellex CM were also analyzed in the same manner. Fractions 2 and 3 (Fig. 23A) contained large amounts of phospholipids, but a protein-staining band of 9 000 daltons was present only in fraction 3. The radioactivity profile of fraction 3 on SDS-polyacrylamide gel was similar to that in Fig. 24 (panel B). However, the fractions (fraction 4) eluted with chloroform-methanol-water (5:5:1), contained very little phospholipid. On SDS-gels, essentially one radioactivity peak coincident with a Coomassie Blue staining polypeptide was observed, as in Fig. 24 (panel C).

Therefore, BioRad Cellex CM was subsequently used for the purification of the DCCD-binding protein. Elution with chloroform-methanol-water (10:10:1) was omitted from the purification procedure (150). The amino acid composition of the material eluted with chloroform-methanol-water (5:5:1) revealed that the preparation of DCCD-binding protein was still contaminated with a polypeptide(s) containing the amino acids serine, histidine and cysteine. The contaminant(s) could be removed by adsorption chromatography on Sephadex LH-60 in the presence of chloroform-methanol

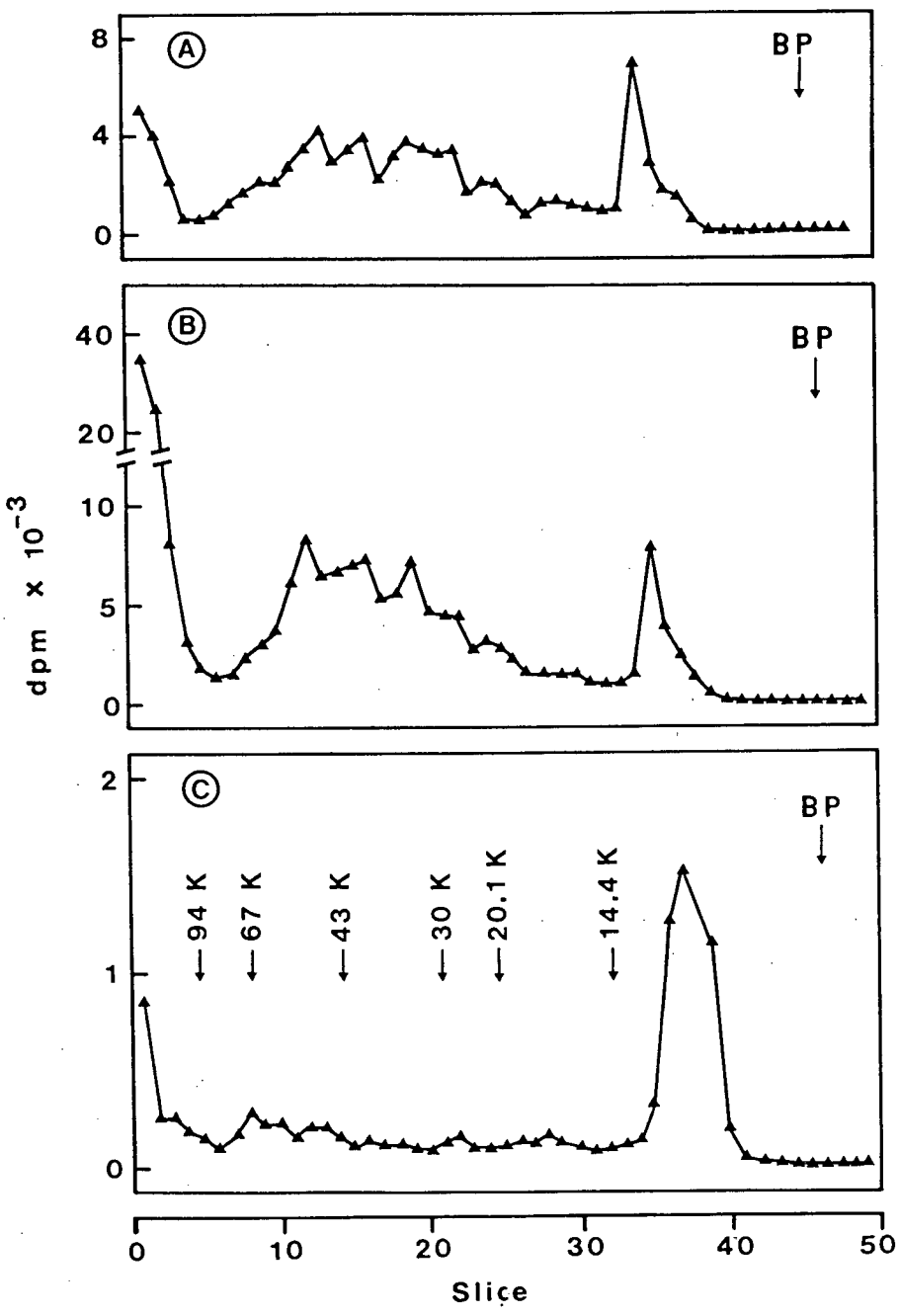


Fig. 24 SDS-polyacrylamide gel electrophoresis of DCCD-binding protein obtained by chromatography on CM-cellulose.

The fractions eluted from the column of Whatman CM-32 (Fig. 23, PANEL B) were pooled (A, B and C), dried and subjected to electrophoresis on SDS-urea (8 M) gels as described in MATERIALS AND METHODS. The gels were fixed with 50% (w/v) TCA, stained with 0.25% (w/v) Coomassie Blue, cut into 2 mm slices and the radioactivity of each slice determined as described previously. BP indicates the position of the bromophenol blue tracking dye. The migration positions of the molecular weight marker proteins (M_r, 94 000 - 14 400) are also shown. PANELS A, B and C correspond to the pooled fractions in Fig. 23, PANEL B.

(2:1) containing 20 mM ammonium acetate (149). Two major protein peaks were obtained (Fig. 25). The DCCD-binding protein eluted as the larger of the two peaks immediately after the void volume. Judicious pooling of the fractions from this peak was required for obtaining pure DCCD-binding protein (149).

AMINO ACID COMPOSITION OF THE DCCD-BINDING PROTEIN

The DCCD-binding protein from normal and mutant cells were extracted with chloroform-methanol (2:1) and purified to homogeneity as described in MATERIALS AND METHODS. Their amino acid compositions were similar, suggesting that the polypeptides from the wild-type and mutant strains were identical (Table 13). Other evidence for identity was obtained by peptide-mapping of the cyanogen-bromide cleaved fragments of the DCCD-binding protein. Separation of the cleaved fragments by only one-dimensional thin-layer chromatography was not adequate to resolve all the fragments. Better resolution of the fragments was obtained with two-dimensional thin-layer chromatography. The pattern of migration of the peptides of the DCCD-binding protein from the normal and mutant cells were identical (Fig. 26). Therefore, the lesion responsible for the relative impermeability of the mutant membranes to protons did not appear to be present on the DCCD-binding protein.

ANALYSIS OF THE MEMBRANES OF *E. coli* BY TWO-DIMENSIONAL ISOELECTRIC FOCUSING GEL ELECTROPHORESIS

The previous results showed that the DCCD-binding protein from the wild-type (WS1 and ML308-225) and mutant strains of *E. coli* (N_{I44}, AN382 CBT-302) were identical. In order to determine if the defect responsible

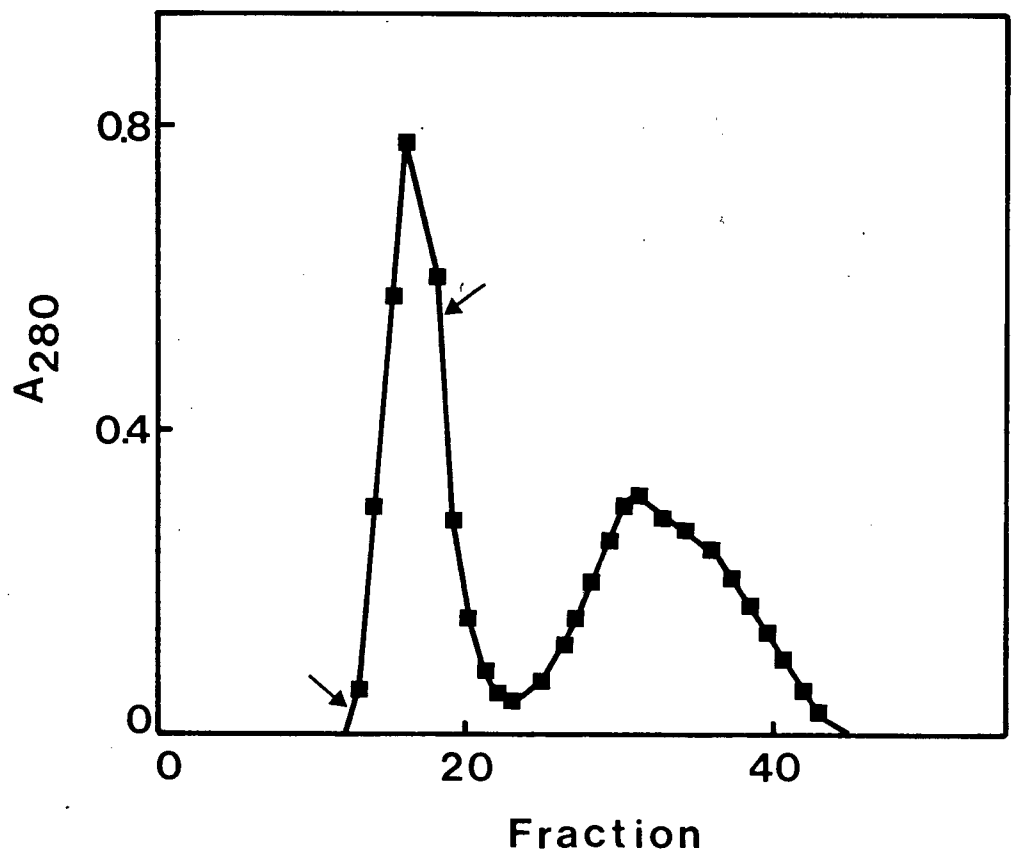


Fig. 25 Chromatography of the CM-cellulose-purified DCCD-binding protein on Sephadex LH-60.

The DCCD-binding protein (6 mg protein) purified by chromatography on CM-cellulose was applied to a column of Sephadex LH-60 (1.2 x 40 cm) which was equilibrated with chloroform:methanol ((2:1) containing 20 mM ammonium acetate. The column was eluted with this solvent and 1 ml fractions collected as described in MATERIALS AND METHODS. The fractions between the arrows were pooled and stored at -20°C.

Table 13 Amino Acid Composition of the DCCD-Binding Protein from Different Strains of *E. coli*[a]

Amino Acids	ML 308-225	WS1	N_{144}	AN382	CBT-302	DNA sequence[b]
Asp	5.0	4.80	4.67	4.95	5.0	5
Thr	1.06	1.03	1.20	1.04	1.02	1
Ser	0.09	0	0.10	0.04	0.03	0
Glu	4.71	4.47	4.53	4.48	4.34	4
Pro	3.30	2.13	2.64	2.77	2.2	3
Gly	10.29	10.23	10.40	10.23	10.26	10
Ala	12.99	13.20	13.24	13.40	13.30	13
Cys	0	0	0	0	0	0
Val	5.83	5.90	5.84	5.95	5.84	6
Met	6.95(8.0)*	7.09(8.0)	6.61(8.2)	6.90(8.1)	7.05(8.3)	8
Ile	7.81	7.82	7.88	7.78	7.88	8
Leu	12.41	12.39	12.50	12.21	12.37	12
Tyr	1.14	1.30	1.23	1.32	1.33	2
Phe	4.19	4.41	4.68	4.45	4.24	4
His	0	0	0	0	0	0
Lys	1.39	1.26	1.23	1.31	1.44	1
Arg	2.01	2.14	2.15	2.13	2.09	2

* values in parentheses were obtained after performic acid oxidation of the DCCD-binding protein and are relative to alanine = 13 and glycine = 10.

a. Mean of four determinations; values are in mol amino acid per mol polypeptide.

b. from (128, 131, 132).

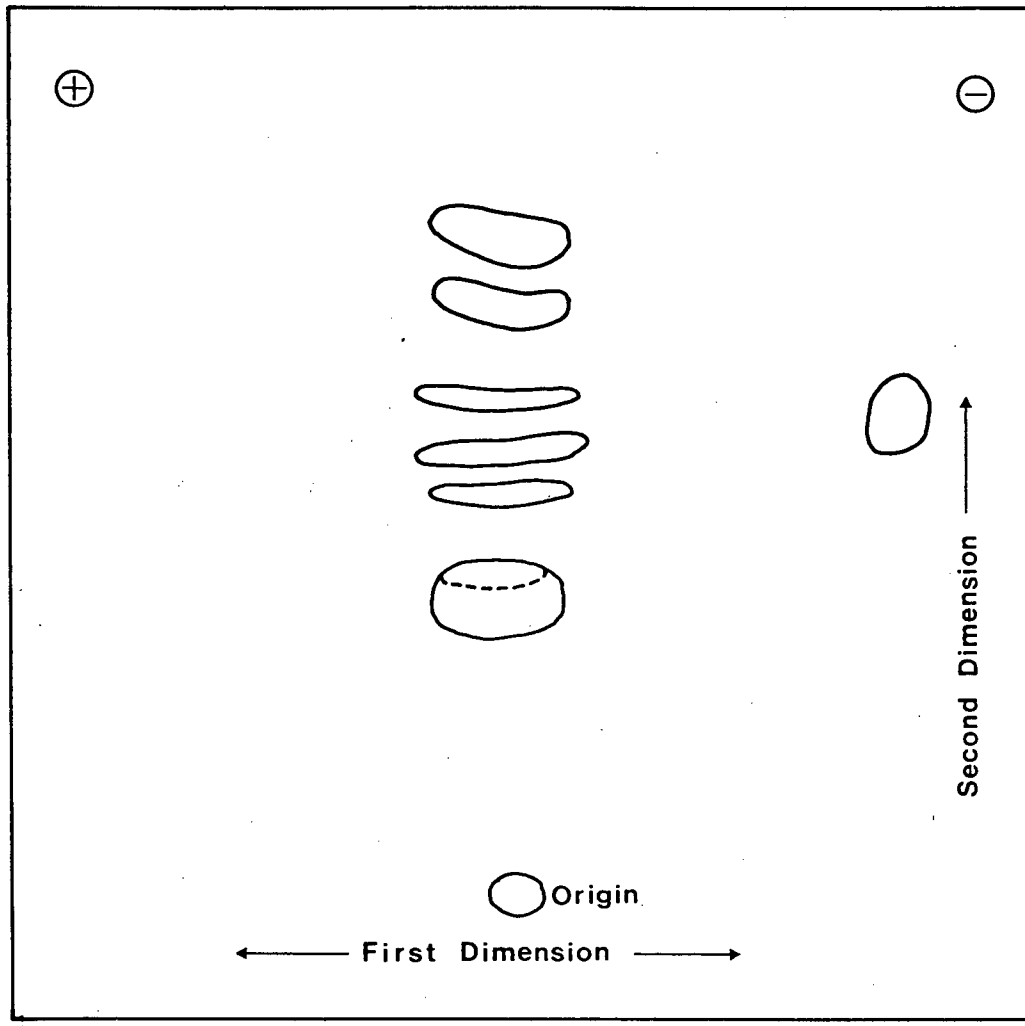


Fig. 26 Two-dimensional thin-layer chromatography of cyanogen bromide cleaved fragments of the DCCD-binding protein of E. coli CBT-302.

The DCCD-binding protein was treated with cyanogen bromide and the fragments (30-50 µg protein) separated by two-dimensional thin layer chromatography on cellulose. Separation of the cyanogen bromide fragments in the first dimension was by electrophoresis and in the second dimension by ascending chromatography, as described in MATERIALS AND METHODS. The position of migration of the peptides were visualized with ninhydrin.

for proton-impermeability was present on the other subunits of the F_0 complex, the mutant membranes were analyzed by two-dimensional isoelectric focusing gel electrophoresis.

Fig. 27 illustrates the pattern of polypeptides obtained with the membranes from the parent (WS1) and the mutant (N_{I44}) strains. None of the subunits of ECF_1 were found to be present in the membranes of E. coli N_{I44}. The γ subunit of ECF_1 is not seen on these gels since it migrates off because of its very basic nature (pI ≈ 8.9).

Careful examination of the gels shown in Fig. 27 revealed that a major polypeptide of molecular weight 18 000 (shown in the square) was absent in the mutant, E. coli N_{I44}. (An adjacent minor polypeptide of identical molecular weight was also absent in the mutant. This adjacent spot may have been caused by carbamylation of the major polypeptide by the cyanate formed from the urea present in the sample. Alternatively, this doublet could be electrophoretic variants of the same subunit. Fillingame et al. (204) have attempted to explain this occurrence as due to spontaneous deamination of the polypeptide since it occurred most frequently in aged samples). It is likely that this polypeptide is polypeptide b of F_0 since it migrates in this position on gels of this type (126). Unfortunately, polypeptide a of F_0 (24 000 daltons) does not enter these polyacrylamide gels (126). Thus, its presence in the membranes of the mutants could not be determined by using this gel system.

Comparison of the pattern of polypeptides obtained with the membranes of the mutants E. coli CBT-302 and its parent, E. coli CBT-1 revealed the absence of the subunits of ECF_1 in the mutant membranes. However, no differences were observed in the 18 000 molecular weight region.

By contrast, the subunits of ECF_1 were present in the membranes of

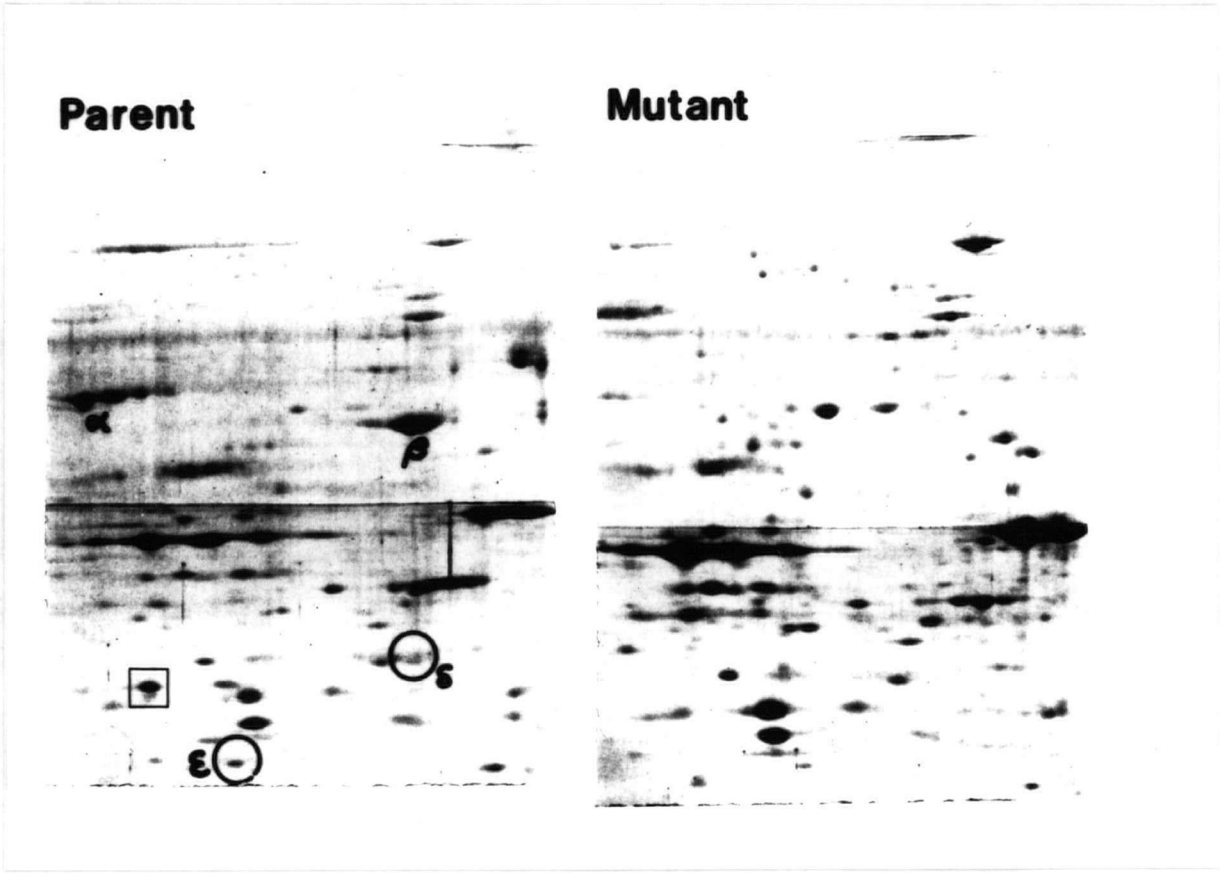


Fig. 27 Two-dimensional isoelectric focusing gel electrophoresis of membranes of parent (WS1) and mutant (N_{I44}) strains of E. coli.

The first-dimension gel (horizontal direction) contained 0.4% (w/v) pH 3.5-10 and 1.6% (w/v) pH 5-7 ampholytes. The second dimension (vertical direction) was a discontinuous polyacrylamide gel (Tris-buffered system) consisting of a layer of 15% (w/v) acrylamide below a layer of 11% (w/v) acrylamide. The samples for electrophoresis were prepared by the method of Merril et al. (175), as described in MATERIALS AND METHODS. The α, ß, δ and ε subunits of the ECF_1-ATPase, which are present only in the membranes of the parent strain, are indicated. The polypeptide outlined by the rectangle is absent in the mutant strain.

the mutant E. coli AN382. But again, no differences were observed in the 18 000 dalton region. However, minor changes which do not involve a change in the molecular weight or the net charge of the 18 000 dalton polypeptide of F_0 could not be excluded. Furthermore, since polypeptide a (24 000 daltons) does not enter these gels, any changes in this polypeptide could not be ascertained.

PART III STUDIES ON THE DCCD-BINDING PROTEIN OF THE F_0 COMPLEX OF E. coli

EFFECT OF ANTISERUM TO THE DCCD-BINDING PROTEIN ON THE ENERGIZATION OF THE MEMBRANE OF UREA-STRIPPED EVERTED MEMBRANE VESICLES

In my study, attempts to demonstrate proton-translocating activity by reconstituting the DCCD-binding protein into phospholipids, by various methods (206,207), were not successful. But evidence from reconstitution experiments (7) involving ECF_1 and ECF_1-depleted membranes or by the use of mutants (25,121) suggested that the DCCD-binding protein is directly involved in proton translocation in E. coli. This polypeptide should be transmembranous if it is the sole component of the proton translocation pathway through F_0. The orientation of the DCCD-binding protein in the membrane of E. coli was investigated by using antiserum to the DCCD-binding protein.

As was shown in the previous section, removal of ECF_1 from the membrane vesicles of normal strains of E. coli resulted in the leakage of protons through F_0. Consequently, reactions such as the quenching of the fluorescence of the dye 9-aminoacridine, which required the presence of a transmembrane pH gradient, could not occur. Fluorescence quenching could be observed if the leakage of protons through F_0 was blocked by the readdition of ECF_1 or by the reaction with DCCD. Fig. 28A shows the effect on fluorescence quenching of various additions to urea-stripped everted vesicles of E. coli. Bovine serum albumin at a concentration normally present in serum (approximately 37 mg per ml) had no effect on the residual fluorescence quenching of the ECF_1-depleted vesicles. Both antiserum to ECF_1 and preimmune serum had only a slight effect on the

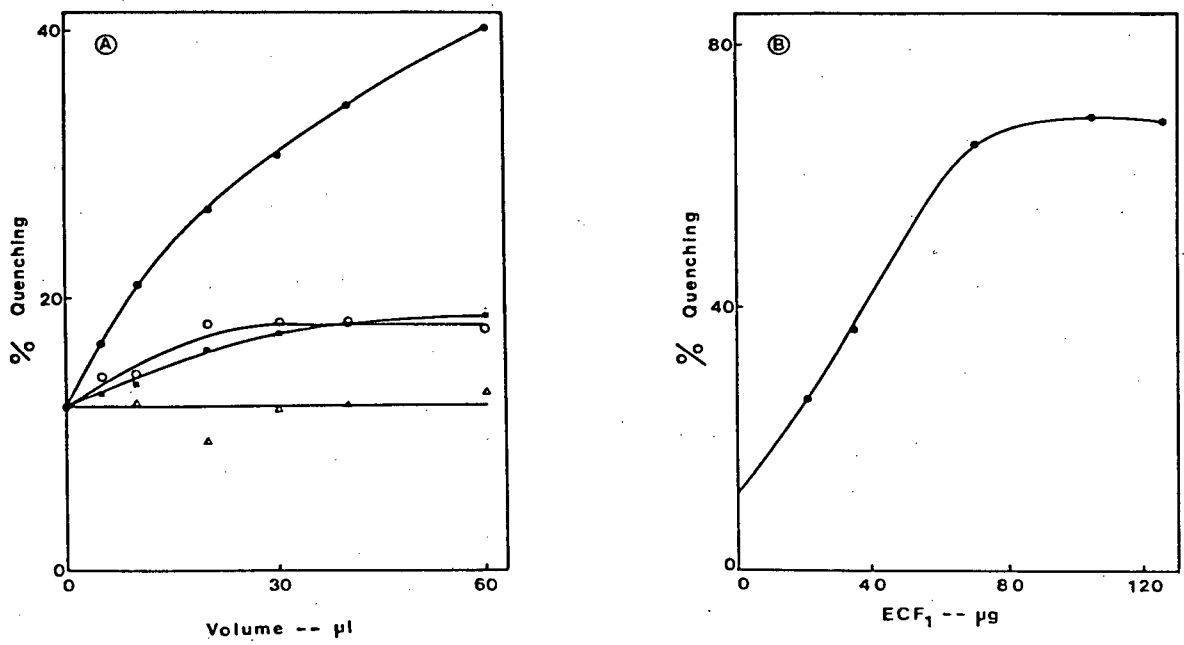


Fig. 28 Effect of the antiserum to the DCCD-binding protein and of ECF_1 on the ascorbate-oxidation-dependent quenching of fluorescence of 9-aminoacridine by urea-stripped everted membrane vesicles.

PANEL A: 2M urea-stripped everted vesicles of E. coli WS1 (10 mg protein) in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$ were incubated for 5 h at 4°C with different amounts of antiserum to the DCCD-binding protein (●-●), antiserum to ECF_1 (■-■), preimmune serum (O-O) or bovine serum albumin (37 mg/ml) (Δ-Δ), in a final volume of 1 ml. Samples (0.1 ml) were assayed for the ability to quench the fluroescence of 9-aminoacridine with ascorbate as substrate (in the presence of phenazine methosulphate) as described in MATERIALS AND METHODS. PANEL B: Urea-stripped everted vesicles of E. coli WS1 (1.0 mg protein) in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$ were incubated at 20°C for 5 min with various levels of ECF_1 in a final volume of 0.1 ml. Ascorbate-oxidation-dependent quenching of fluorescence of 9-aminoacridine was measured as described in MATERIALS AND METHODS.

quenching. This small effect was likely due to non-specific interaction of serum proteins with the subunits of F_0.

By contrast, antiserum to the DCCD-binding protein markedly stimulated fluorescence quenching indicating that the reaction of the antiserum with this polypeptide blocked the leakage of protons through F_0. If the data of Fig. 28A were examined as a Lineweaver-Burk plot, a value could be calculated for the maximum quenching to be expected at saturating levels of antiserum. This value (62% quenching) was in good agreement with that observed following the addition of saturating levels of ECF_1 (69% quenching, Fig. 28B).

EFFECT OF ANTISERUM TO THE DCCD-BINDING PROTEIN ON THE BINDING OF ECF_1 TO UREA-STRIPPED EVERTED MEMBRANE VESICLES

The addition of antiserum to the DCCD-binding protein or ECF_1 to ECF_1-depleted membrane vesicles resulted in the stimulation of fluorescence quenching during substrate oxidation. This suggested that both ECF_1, and antiserum to the DCCD-binding protein, were reacting at the same binding site(s) to prevent the leakage of protons through F_0. The effect of antiserum to the DCCD-binding protein on the binding of ECF_1 to ECF_1-depleted membranes, was examined as follows. Stripped everted vesicles of E. coli WS1 were incubated with different amounts of antiserum. The vesicles were sedimented by centrifugation and then washed to remove unbound antiserum. Various amounts of ECF_1 were added to the suspension of the treated vesicles and the extent of binding of ECF_1 measured by the increase in the ATPase activity of the vesicles. Stripped vesicles had no ATPase activity. The extent of binding of ECF_1 as a function of the amount of ECF_1 added to the treated and untreated vesicles is shown as a

Lineweaver-Burk plot in Fig. 29. The lines intersecting close to the ordinate suggested that antiserum to the DCCD-binding protein interferred with the binding of ECF_1 in a near competitive manner. Preimmune serum at a concentration of 110 µl serum per mg membrane protein also slightly inhibited the binding of ECF_1 to the stripped vesicles. However, the extent of inhibition was much lower than that seen with antiserum to the DCCD-binding protein.

Similar binding studies were carried out on the unc mutants E. coli N_{I44} and CBT-302. Stripped everted vesicles of E. coli N_{I44} and CBT-302 were treated with preimmune serum or antiserum to the DCCD-binding protein at a concentration of 120 µl serum per mg membrane protein. Reconstitution with ECF_1 was performed as described earlier and the results shown as Lineweaver-Burk plots in Fig. 29. In both mutants, the results were similar to those obtained with the wild-type strain, E. coli WS1, in that the antiserum to the DCCD-binding protein interfered with the binding of ECF_1 in a near competitive manner.

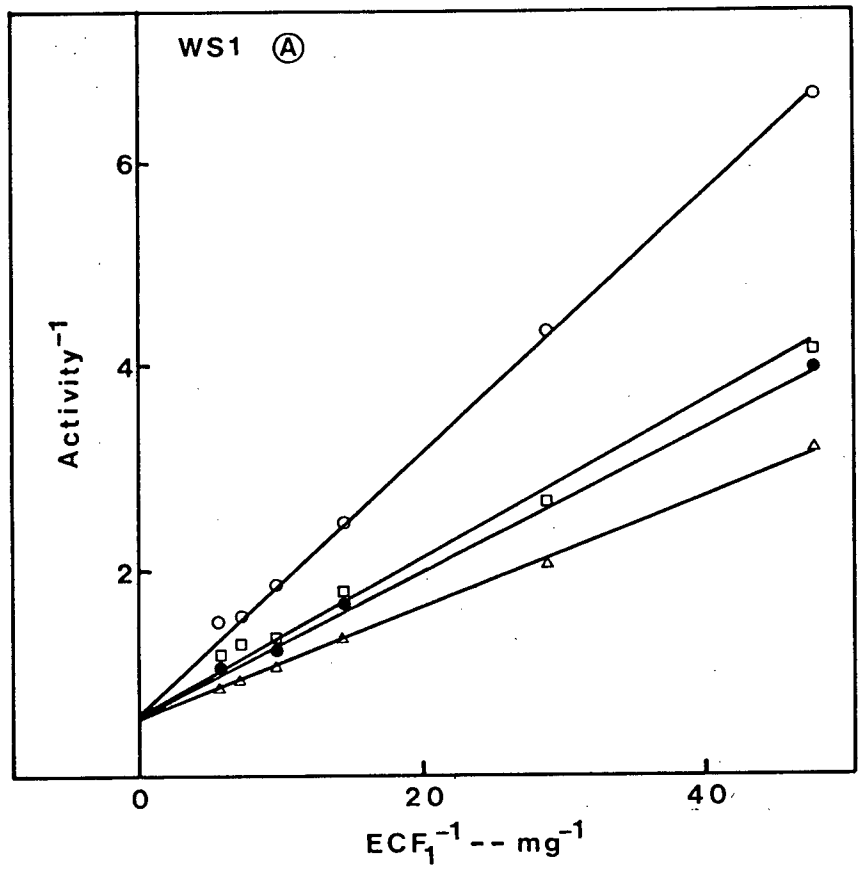
However, in contrast to the wild-type strain, preimmune serum did not inhibit the binding of ECF_1 to the mutant membranes. In addition, the extent of inhibition of the binding of ECF_1 by antiserum to the DCCD-binding protein was lower in the mutants than in the wild-type membranes. This was further evidence that the F_0 complex in E. coli N_{I44} and CBT-302 was different from that of the wild-type strain (E. coli WS1).

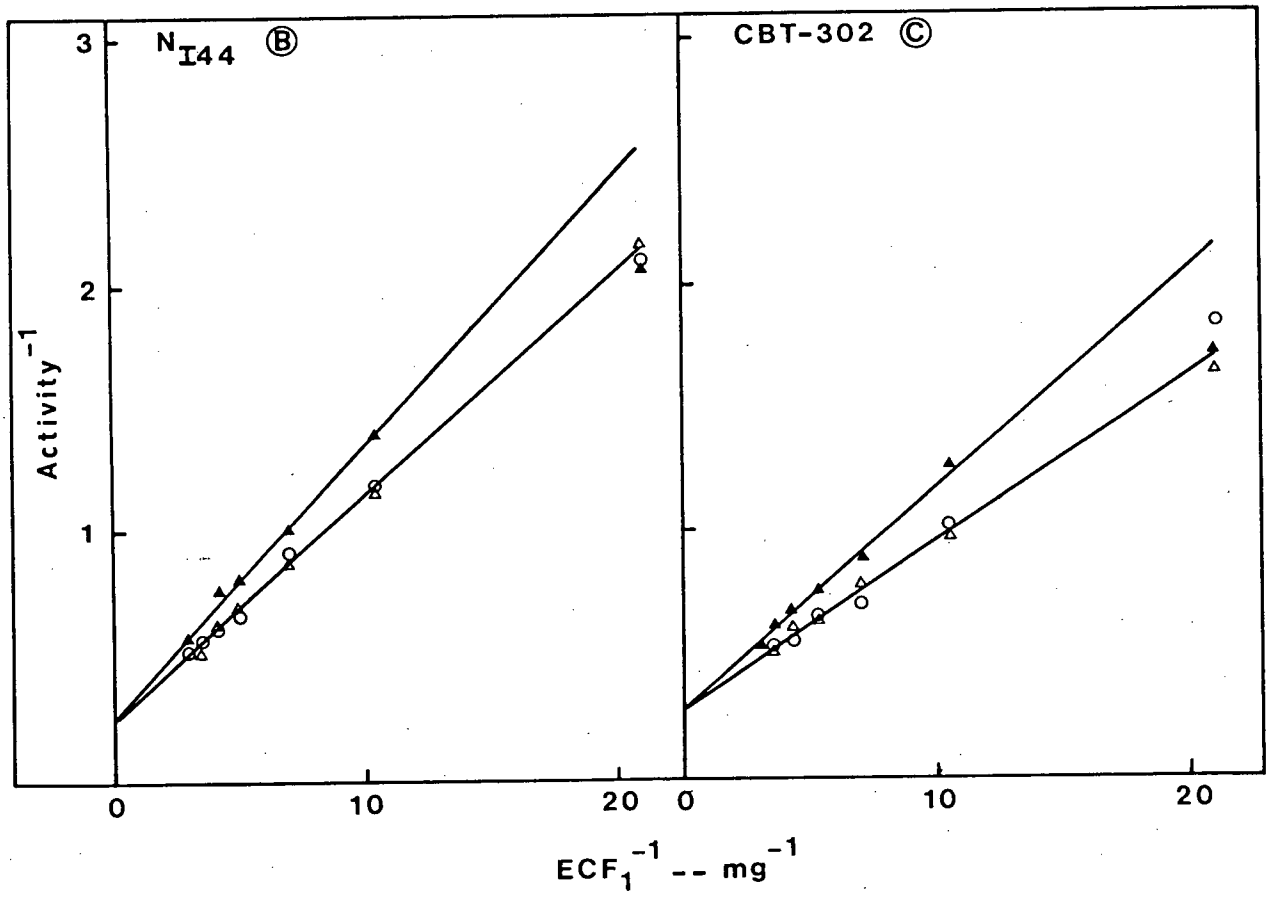
EFFECT OF ANTISERUM TO THE DCCD-BINDING PROTEIN ON THE PROTON PERMEABIITY OF THE RIGHT-SIDE OUT VESICLES OF E. coli

The experiments described earlier had detected the exposure of the DCCD-binding protein at the cytoplasmic surface of ECF_1-depleted membrane

Fig. 29 Effect of antiserum to the DCCD-binding protein on the binding of ECF_1 to urea-stripped everted membrane vesicles.

Urea-stripped everted vesicles (25 mg protein) from E. coli WS1, N_{I44} and CBT-302 in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol were incubated at 4°C for 20 h with different amounts of antiserum in a final volume of 4.4 - 4.5 ml ml. After a 4-fold dilution in buffer, the mixture was centrifuged at 250 000 xg for 2.5 h. The sedimented vesicles were washed once by suspension in buffer followed by resedimentation as before. The washed vesicles were resuspended in buffer at 6.5 mg protein/ml. Different amounts of ECF_1 were incubated at 4°C for 45 min with 2.5 mg of vesicles protein in buffer. The mixtures were diluted 8 to 10-fold in buffer and the vesicles sedimented as before. The ATPase activity of the vesicles was assayed as described in MATERIALS AND METHODS. Activity is expressed as units per mg protein. PANEL A: 0 µl (△-△), 50 µl (●-●), 75 µl (□-□) and 110 µl (○-○) of antiserum to the DCCD-binding protein per mg of membrane protein. PANELS B and C: 0 µl (△-△), and 120 µl (▲-▲) of antiserum to the DCCD-binding protein per mg membrane protein. 120 µl (○-○) of preimmune serum per mg membrane protein.


WS1 Ⓐ
Activity^{-1}
6
4
2
0
20
40
ECF_1^{-1} -- mg^{-1}


N_{I44} Ⓑ
CBT-302 Ⓒ
Activity^{-1}
3
2
1
0
10
20
0
10
20
ECF_1^{-1} -- mg^{-1}

vesicles. The extent of exposure of this polypeptide at the external (periplasmic) surface of the cell membrane was also examined by using antiserum to the DCCD-binding protein. In this experiment, the antiserum was partially purified by ammonium sulphate precipitation to reduce the buffering effect of the serum. The partially purified antiserum was determined by crossed immunoelectrophoresis to be still active (Fig. 30).

The ECF_1-ATPase-defective mutant, E. coli DL-54, readily loses ECF_1 from its binding sites on F_0. Consequently, vesicles of DL-54 leak protons through F_0 (26,205). Addition of valinomycin to K+-loaded "right-side out" vesicles results in an efflux of K+ concomitantly with a compensatory influx of protons, provided that a pathway for protons is available. This pathway is provided in DL-54 by the ECF_1-depleted F_0 proteins (26,205).

The basis of this assay is illustrated in Fig. 31. The influx of protons is detected as a rise in the pH of the medium external to the vesicles. A typical result is shown in Fig. 32. Addition of DCCD to the vesicles blocked the movement of protons through F_0 (Fig. 33B). A maximum of about 60-65% of the total protons entering the vesicles could be inhibited by DCCD (880 μM). The residual uptake of protons was likely due to passive movement of the protons across the membrane. In contrast to the effect of DCCD, the antiserum to the DCCD-binding protein had no effect on the total amount of protons taken up (Fig. 33A).

However, if the data were analyzed with respect to the rate, not extent, of proton uptake, an effect was observed with antiserum to the DCCD-binding protein. These results are shown in Fig. 33 (panels C and D). The rate of proton uptake was inhibited to a maximum of 65% at a DCCD concentration of 440-880 μM. Antiserum to the DCCD-binding protein also

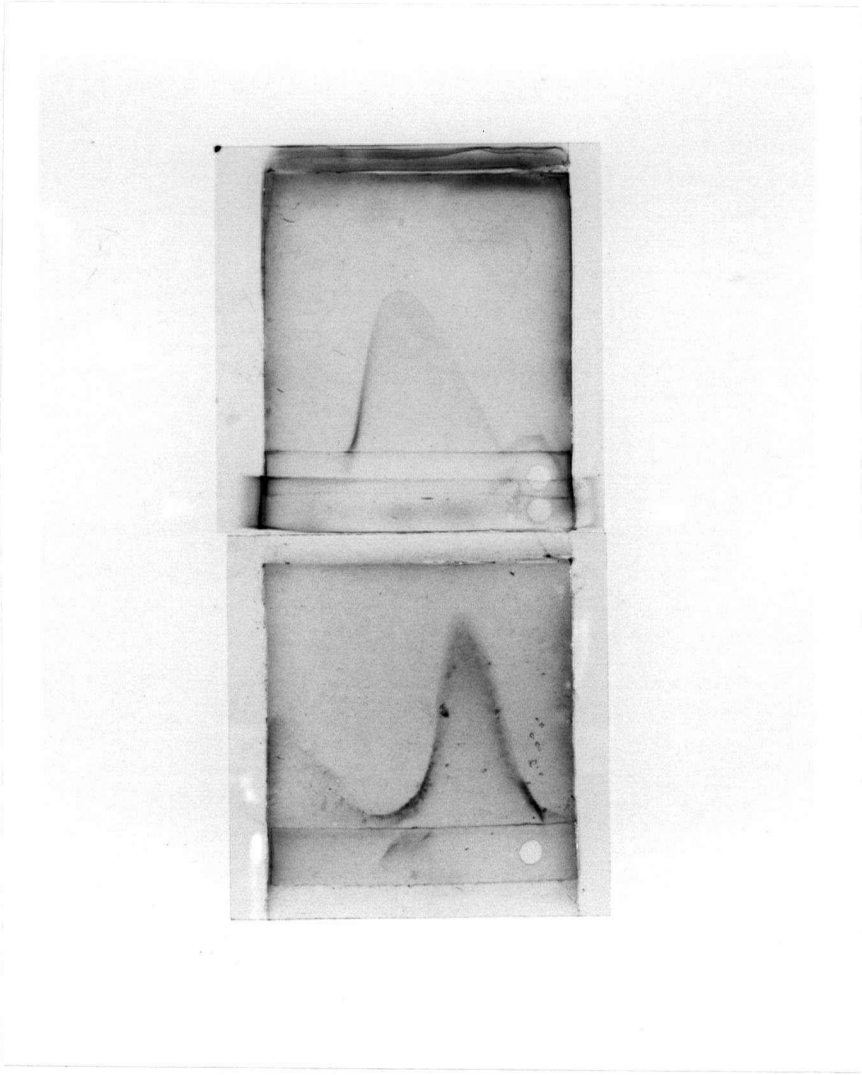


Fig. 30 Crossed immunoelectrophoresis of antiserum to the DCCD-binding protein.

Crossed immunoelectrophoresis of the DCCD-binding protein was carried out as described in MATERIALS AND METHODS. UPPER SLIDE: Antigen in the first-dimension was 4 µg DCCD-binding protein in Bjerrum buffer, pH 8.8, containing 100 mM glycine, 38 mM Tris base and 1% (w/v) Triton X-100. 150 µl of antiserum to the DCCD-binding protein was present in the second-dimension gel. LOWER SLIDE: Antigen in the first-dimension was 8 µg DCCD-binding protein in Bjerrum buffer, pH 8.8. 175 µl of ammonium sulphate-purified antiserum to the DCCD-binding protein was present in the second-dimension gel.

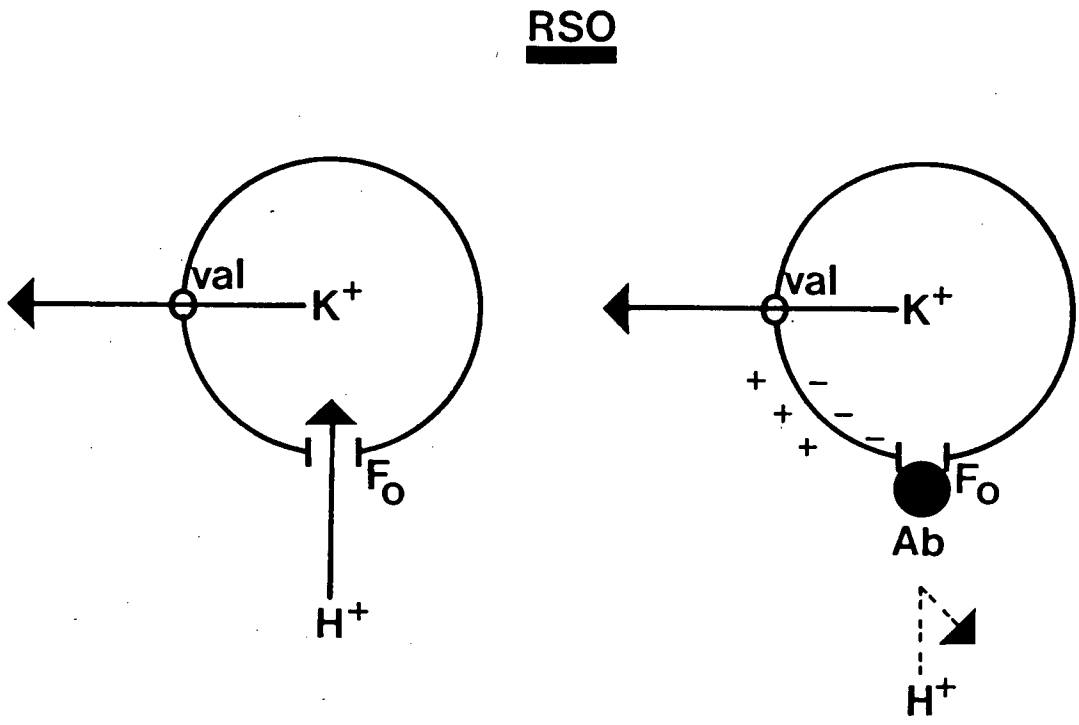


Fig. 31 Schematic representation of the proton-pathway provided by the "right-side out" vesicles of E. coli DL-54.

The figure on the left shows that the addition of valinomycin to K^+-loaded "right-side out" vesicles of E. coli results in the efflux of K^+ with a concomitant influx of protons through F_0. The influx of proton is blocked by addition of antibody to polypeptide(s) of F_0 (figure on the right). Details of this type of experiment are described in the legend to Fig. 32.

Val, valinomycin; Ab, antibody; RSO, "right-side out" vesicles.

(Drawings courtesy of Dr. P.D. Bragg.)

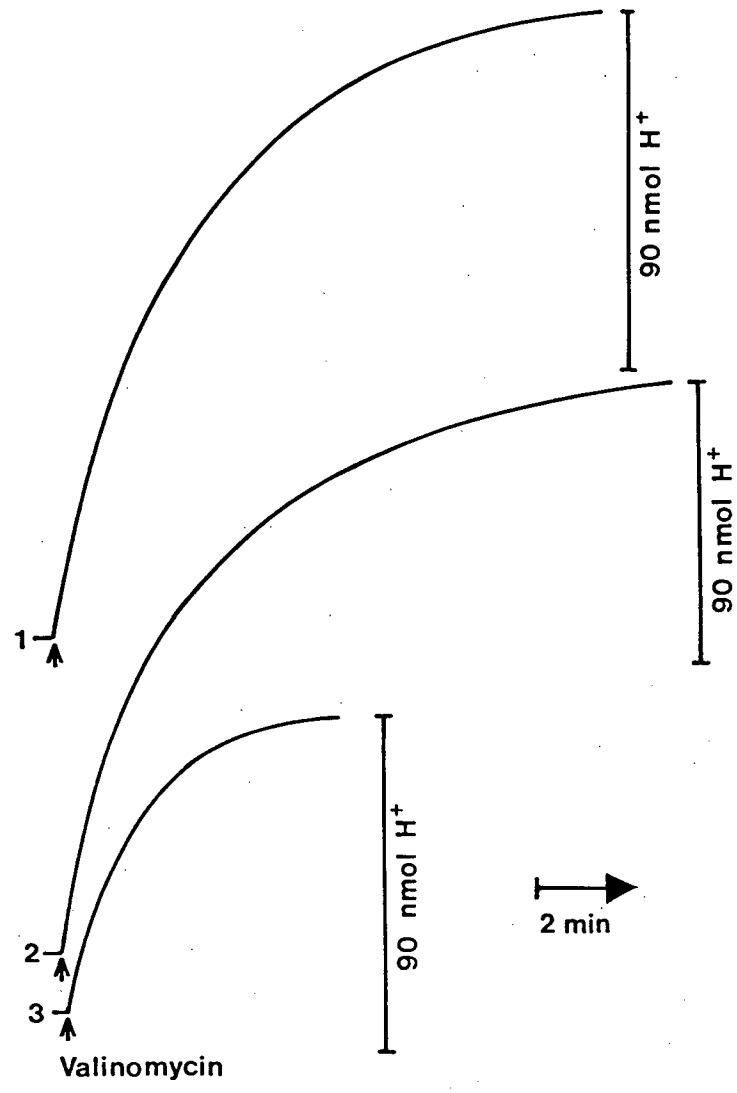


Fig. 32 Effect of DCCD and of antiserum to the DCCD-binding protein on the proton permeability of "right-side out" membrane vesicles.

E. coli DL-54 was grown to the late exponential phase and converted to spheroplasts by treatment with lysozyme in the presence of EDTA. The spheroplasts were then loaded with K^+ as described in MATERIALS AND METHODS. The pH changes occurring following the addition of 3 µl of valinomycin (5 mg/ml) to 0.85 ml K^+-loaded vesicles (1.5 mg protein) in 0.4 M sucrose - 10 mM $MgCl_2$ were measured with a glass pH electrode connected to a Fisher Accumet Model 325 expanded scale pH meter as described in the MATERIALS AND METHODS section. In some experiments, the vesicles were pre-incubated with DCCD (15 µl in ethanol) or with ammonium sulphate-purified antiserum to the DCCD-binding protein for 45 min at 20°C. Each assay was internally calibrated by addition of a known concentration of acid (HCl or H_2SO_4). 1, no addition; 2, 50 µl antiserum to the DCCD-binding protein; 3, 880 µM DCCD. The amounts and rates of proton influx are summarized in Fig. 33.

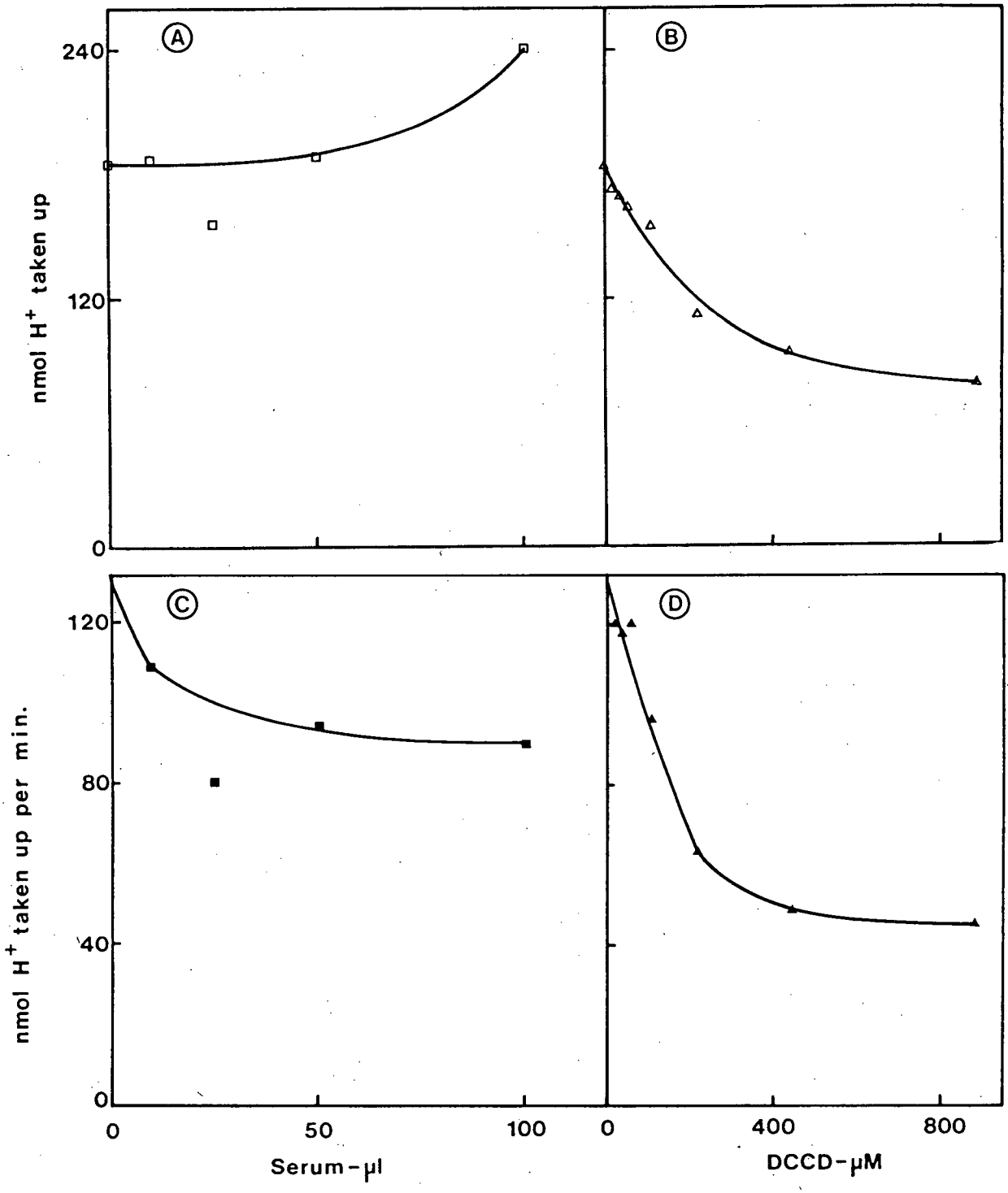


Fig. 33 Effect of DCCD and of antiserum to the DCCD-binding protein on the proton-permeability of "right-side out" membrane vesicles of E. coli DL-54.

The experiment was carried out as described in the legend to Fig. 32. PANELS A and B show the amounts of protons taken up by the "right-side out" vesicles, whereas PANELS C and D show the rates of proton-uptake, in the presence of DCCD or antiserum to the DCCD-binding protein.

decreased the rate of proton uptake, but only to a maximum of 30%.

Thus, there appeared to be some correlation between the effect of DCCD on the rate of proton uptake and on the total amount of protons taken up by these vesicles. This relationship was not shown when the antiserum to the DCCD-binding protein was used, suggesting that DCCD and antiserum to the DCCD-binding protein affected proton movement through F_0 by different mechanisms.

EFFECT OF ANTISERUM TO THE DCCD-BINDING PROTEIN ON THE ENERGIZATION OF THE MEMBRANE OF NATIVE EVERTED MEMBRANE VESICLES

The effect of antiserum on the quenching of fluorescence during substrate oxidation and ATP hydrolysis by native everted vesicles was also investigated.

Everted vesicles of *E. coli* WS1 were incubated in the presence of different amounts of antiserum and then assayed for fluorescence quenching as previously described. Bovine serum albumin and preimmune serum did not affect fluorescence quenching during oxidation of ascorbate (in the presence of PMS) (Fig. 34, panel A). By contrast, antiserum to the DCCD-binding protein increased the level of quenching from 50% to 60%. Addition of saturating levels of ECF_1 to these vesicles stimulated the quenching to 70%.

Similar results were obtained with quenching during ATP hydrolysis (Fig. 34, panel B). Bovine serum albumin and preimmune serum had, at the most, only a slight effect on the fluorescence quenching of the everted vesicles. Stimulation of fluorescence quenching was observed when antiserum to the DCCD-binding protein was present.

These results suggested that there was loss of some ECF_1 from the

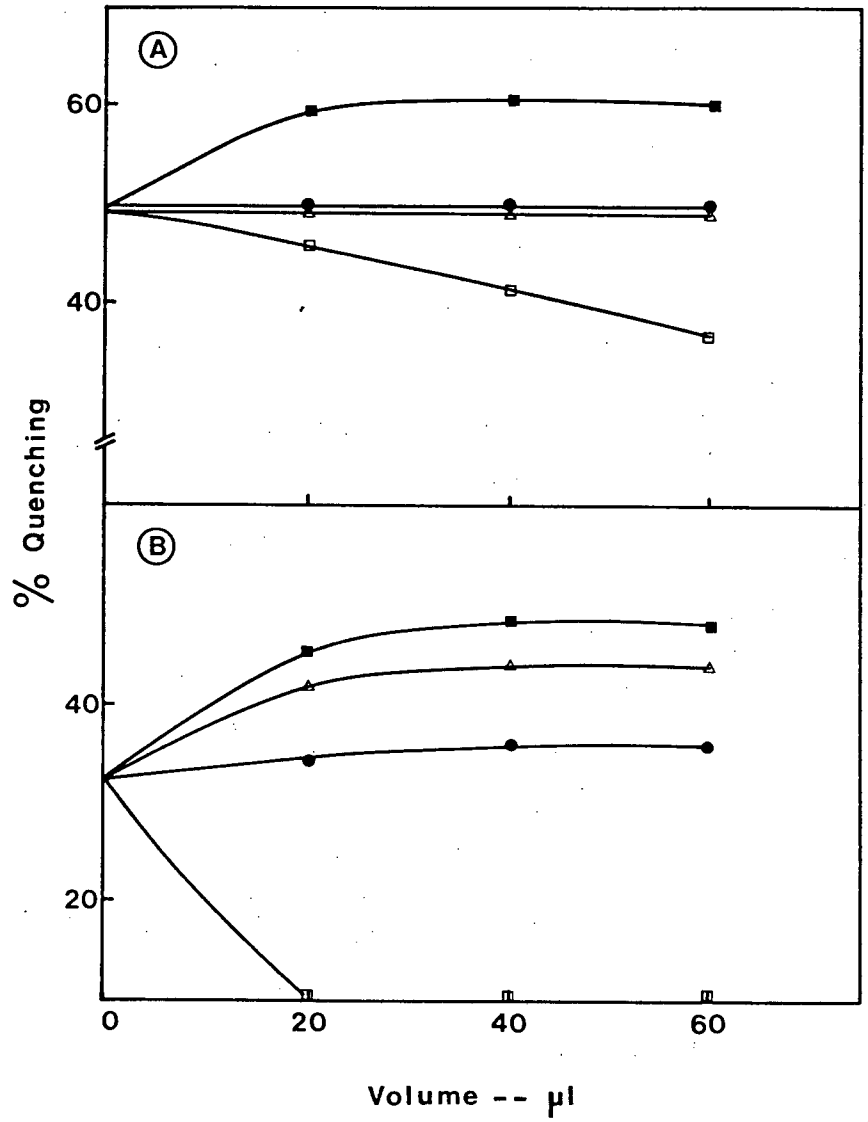


Fig. 34 Effect of antiserum to the DCCD-binding protein on the energization of untreated everted membrane vesicles.

Membrane vesicles of E. coli WS1 (10 mg protein) in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$ were incubated for 5 h at 4°C with various amounts of antiserum to the DCCD-binding protein (■-■), antiserum to ECF_1 (□-□), preimmune serum (△-△), or bovine serum albumin (37 mg/ml) (●-●), in a final volume of 1 ml. Samples (0.1 ml) were assayed for the ability to quench the fluorescence of a 9-aminoacridine during: PANEL A, oxidation of ascorbate in the presence of phenazine methosulphate; PANEL B, ATP hydrolysis.

membrane during preparation.

The most striking effect was observed when native everted vesicles were treated with antiserum to ECF_1. During substrate oxidation, antiserum to ECF_1 caused a decrease in the quenching of fluorescence (Fig. 34, panel A) indicating that the membranes became leaky to protons. Fluorescence quenching during ATP hydrolysis was completely abolished in vesicles treated with antiserum to ECF_1 (Fig. 34, panel B).

The cause(s) of the decrease in fluorescence quenching during substrate oxidation in the treated vesicles is not known. It has been suggested (208,209) that the membrane and/or membrane proteins undergo conformational changes during membrane energization. Therefore, it is possible that antiserum to ECF_1 prevented the conformational change of ECF_1 during substrate oxidation such that the F_0 was not completely blocked. Alternatively, the antiserum to ECF_1 could have caused the detachment of ECF_1 from the membrane (65).

Quenching during ATP hydrolysis was abolished in vesicles treated with antiserum to ECF_1 because of inhibition of the ATPase activity rather than because of membranes becoming leaky to protons. Addition of antiserum to ECF_1 to purified ECF_1 inhibited the ATPase activity.

ENERGIZATION OF THE MEMBRANE OF TRYPSIN-TREATED UREA-STRIPPED EVERTED VESICLES

ATP and respiration-dependent fluorescence quenching could be restored to urea-stripped everted vesicles reconstituted with ECF_1. By contrast, ECF_1 could not restore these activities in stripped vesicles which had been treated with trypsin (Table 14). Since the energized membrane was detected indirectly by the quenching of the fluorescence of 9-aminoacridine,

Table 14 Energization of the membrane of trypsin-treated everted membrane vesicles of *E. coli*

System	Percent Quenching		
	NADH	Ascorbate/PMS	ATP
Urea-treated vesicles	7.5	5.9	0-2
+ DCCD	35.1	7.3	0-2
+ ECF_1	40.5	28.3	37.5
Trypsin -treated stripped vesicles	5.0	5.2	3.5
+ DCCD	15.0	6.4	0-2
+ ECF_1	7.1	4.8	4.3

Urea-treated everted vesicles of *E. coli* WS1 were treated with TPCK-trypsin. The treated vesicles were suspended in 50mM HEPES-KOH buffer, pH 7.5, containing 5mM $MgCl_2$ and 10% (v/v) glycerol at a protein concentration of 10 mg/ml. Samples (0.1 ml.) were removed and assayed for the ability to quench the fluorescence of 9-aminoacridine during substrate (NADH; ascorbate, in the presence of PMS) oxidation or during hydrolysis of ATP, as described in MATERIALS AND METHODS. In experiments in which DCCD or ECF_1 was used, each was incubated with the membrane suspension for 5 min. at 20°C, prior to assay. The final concentration of DCCD in the assay mixture was 375 µM and the amount of ECF_1 used was 80 - 100 µg protein.

it was not known if the absence of quenching in trypsin-treated vesicles was due to the complete destruction of the respiratory chain or to the vesicles becoming leaky to protons as a result of protease treatment.

It was unlikely that the absence of fluorescence quenching activity was due to the destruction of the respiratory chain since NADH-oxidation-dependent fluorescence quenching in the presence of DCCD was observed (Table 14). Quenching was not observed during ascorbate oxidation because DCCD inhibited its activity. This was supported by the observation that trypsin-treatment of the membranes from the unc mutants, E. coli AN382 and CBT-302 did not destroy NADH- or ascorbate-oxidation-dependent fluorescence quenching (Fig. 20).

Since ECF_1 did not inhibit the proton-translocating properties of F_0 in trypsin-treated vesicles, it was of interest to determine if antiserum to DCCD-binding protein could restore respiration-dependent fluorescence quenching.

Urea-stripped everted vesicles of E. coli WS1 were treated with trypsin and then incubated with different amounts of antiserum. Fig. 35 shows the effect of various additions to trypsin-treated vesicles on the fluorescence quenching. These results appeared to be similar to those in Fig. 28, panel A, for urea-treated vesicles. Bovine serum albumin had no effect on the residual fluorescence quenching. Preimmune serum and antiserum to ECF_1 only slightly stimulated the quenching of fluorescence. By contrast, antiserum to the DCCD-binding protein markedly stimulated fluorescence quenching indicating that reaction of the antiserum with this polypeptide blocked the leakage of protons through F_0.

These results were different from those obtained with urea-treated everted vesicles (Fig. 28A) in two ways. Firstly, the maximum quenching

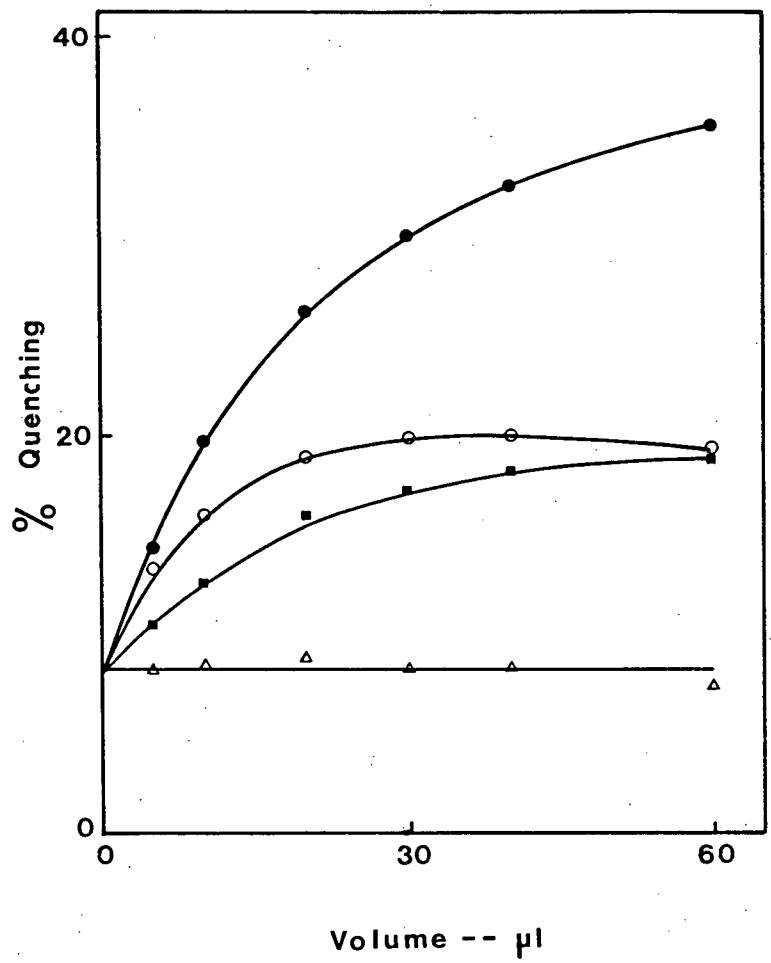


Fig. 35 Effect of antiserum to the DCCD-binding protein on the ascorbate-oxidation-dependent quenching of fluorescence of 9-aminoacridine by trypsin-treated everted membrane vesicles.

Urea-stripped everted vesicles of E. coli WS1 were treated with TPCK-trypsin at 20°C for 30 min as described in MATERIALS AND METHODS. The trypsin-treated vesicles (10 mg) in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$ were incubated for 5 h at 4°C with various levels of antiserum to the DCCD-binding protein (●-●), antiserum to ECF_1 (■-■), preimmune serum (○-○) or bovine serum albumin (37 mg/ml) (△-△), in a final volume of 1 ml. Samples (0.1 ml) were assayed for the ability to quench the fluorescence of 9-aminoacridine with ascorbate as substrate (in the presence of phenazine methosulphate) as described in the MATERIALS AND METHODS section.

expected at saturating levels of antiserum was lower (42% quenching) than with urea-treated everted vesicles (62% quenching). Secondly, the addition of ECF_1 to trypsin-treated vesicles (at levels which caused maximum fluorescence quenching in urea-treated vesicles), did not stimulate fluorescence quenching. The lower extent of quenching may be due to damage of the membrane by trypsin, causing an increase in proton leakage other than through F_0.

BINDING OF ECF_1 TO PROTEASE-TREATED MEMBRANE VESICLES

Treatment of stripped everted vesicles with trypsin did not completely destroy the respiratory chain. However, the absence of fluorescence quenching in trypsin-treated vesicles reconstituted with ECF_1 suggested that the coupling- and/or ECF_1-binding sites on the membrane were affected by protease treatment. This was examined as follows.

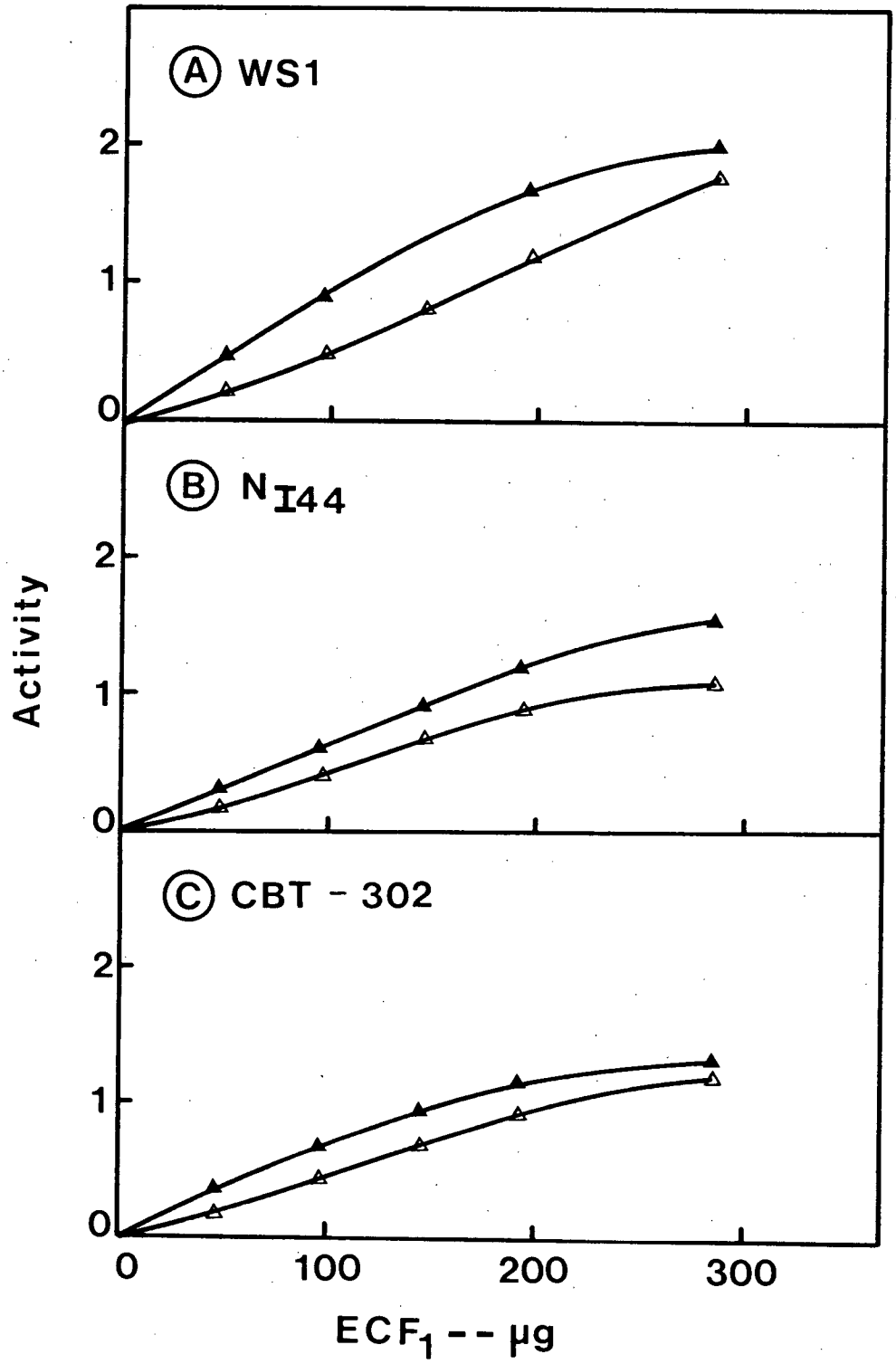
Urea-stripped everted vesicles of E. coli WS1 were treated with trypsin and reconstituted with ECF_1 as described earlier. The extent of binding of ECF_1 was measured by the increase in the ATPase activity of the vesicles since the trypsin- or urea-treated vesicles had no ATPase activity. ATP- and respiration-dependent fluorescence quenching was not observed in these trypsin-treated vesicles which had been reconstituted with ECF_1.

The results in Fig. 36 (panel A) shows that the digestion of the stripped vesicles with trypsin for up to 4 h at 37°C did not affect the ability of the membranes to bind ECF_1.

Trypsin-treatment of stripped membranes of the unc mutants, E. coli N_{I44} and CBT-302 also did not alter the capacity of these membranes to bind ECF_1 (Fig. 36, panels B and C).

Fig. 36 Binding of ECF_1 to trypsin-treated everted membrane vesicles.

Urea-stripped everted vesicles of *E. coli* WS1, N_{I44} and CBT-302 were treated with TPCK-trypsin and then reconstituted with various levels of ECF_1 as described in MATERIALS AND METHODS. Treatment with TPCK-trypsin was for 4 h at 37°C. The amount of membrane vesicle protein reconstituted with ECF_1 was 2 mg. The extent of binding of ECF_1 to urea-stripped (▲–▲) or trypsin-treated (△–△) vesicles was measured by the increase in ATPase activity. Activity is expressed in units per mg protein.


Ⓐ WS1
Ⓑ N_{I44}
Ⓒ CBT - 302
Activity
0
1
2
0
100
200
300
ECF_1 -- µg

Double-reciprocal plots of the data in Fig. 36 (not shown) revealed that the maximum ATPase activity to be expected in urea-treated membrane vesicles of E. coli WS1, N_{I44} and CBT-302, at saturating levels of ECF_1, was similar (8.33 units per mg protein). The half-saturation values for E. coli WS1, N_{I44} and CBT-302 were determined to be 0.55, 1.11 and 1.11 mg ECF_1, respectively. This suggested that the urea-treated vesicles of E. coli WS1 (wild-type) had a higher affinity for ECF_1, as would be expected. Similar analysis of the data on the binding of ECF_1 to trypsin-treated vesicles resulted in more complex kinetics (not shown).

Attempts to abolish the ability of urea-treated vesicles to bind ECF_1 were made also by treating the stripped vesicles with Staphylococcus aureus V_8 protease. Stripped vesicles which were treated with V_8 protease were still able to bind ECF_1 (Fig. 37). Analysis of the data on Lineweaver-Burk plots, revealed that the maximum activity expected at saturating levels of ECF_1 was 8.33 units per mg protein for E. coli WS1 only. Similar analysis of the data on the binding of ECF_1 to V_8 protease-treated vesicles of E. coli N_{I44} and CBT-302 were also very complex (not shown).

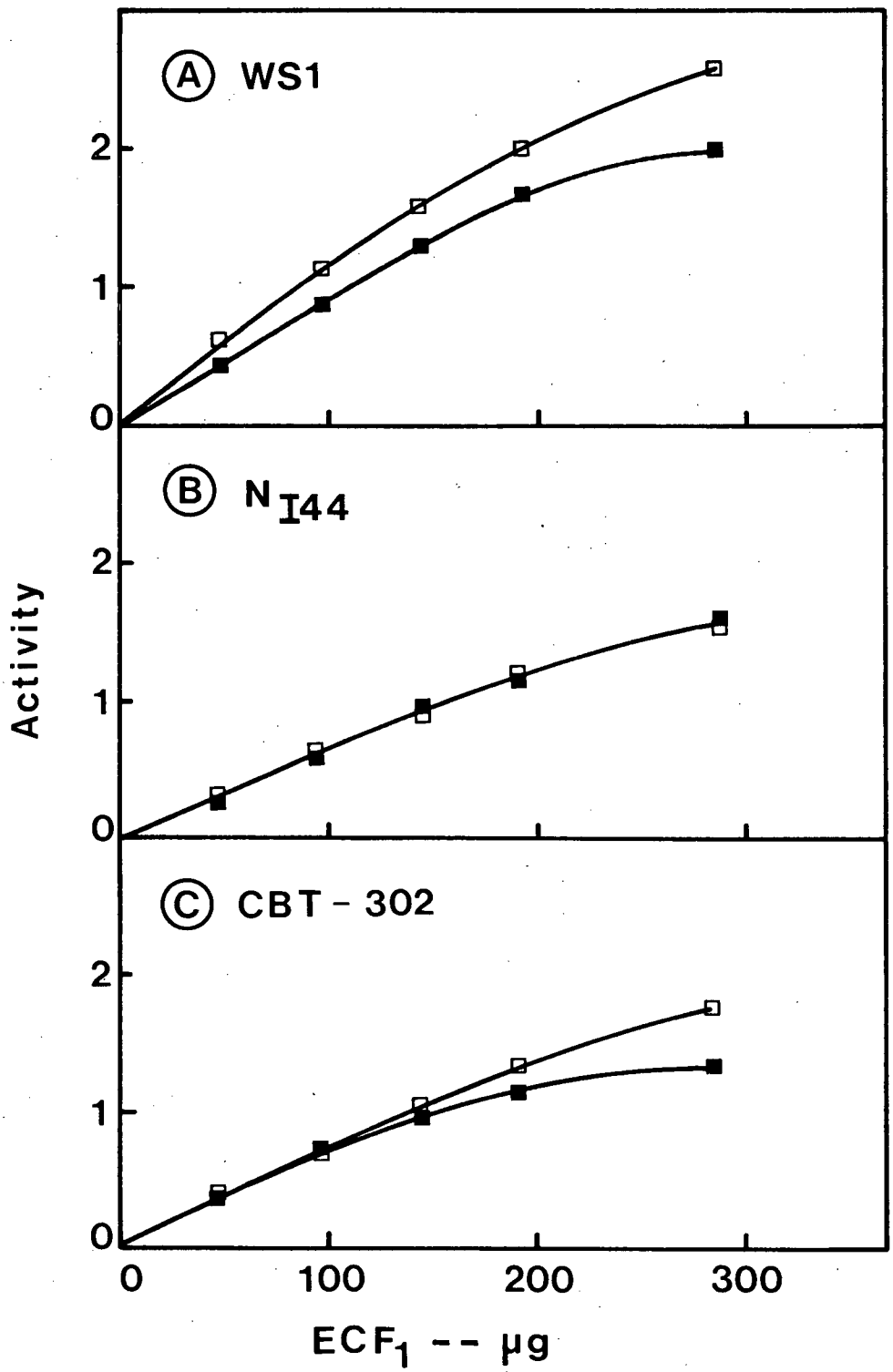
EFFECT OF DCCD ON THE ATPase ACTIVITY OF THE ECF_1 BOUND TO TRYPSIN-TREATED VESICLES

The activity of the intact membrane-bound ATPase enzyme can be inhibited by DCCD. Since trypsin-treated vesicles were still capable of binding ECF_1, it was of interest to determine if the activity of the rebound ATPase enzyme was still sensitive to DCCD.

Urea-stripped everted vesicles of E. coli WS1 were treated with trypsin and then reconstituted with ECF_1 as previously described. The

Fig. 37 Binding of ECF_1 to Staphylococcus aureus V_8 protease-treated everted membrane vesicles.

Urea-stripped vesicles of E. coli WS1, N_{I44} and CBT-302 were treated with Staphylococcus aureus V_8 protease and then reconstituted with various levels of ECF_1 as described in MATERIALS AND METHODS. Treatment with V_8 protease was for 4 h at 37°C. 2 mg of membrane vesicle protein was reconstituted with ECF_1. The extent of binding of ECF_1 to urea-stripped (■-■) or V_8 protease-treated (□-□) vesicles was measured by the increase in ATPase activity. Activity is expressed in units per mg protein.


A WS1
B N I44
C CBT - 302
Activity
0
1
2
0
100
200
300
ECF1 -- µg

reconstituted vesicles were incubated with different amounts of DCCD and the ATPase activity measured. The results are illustrated in Fig. 38. After a short period of incubation (45 min at 37°C), the enzyme activities present in the native membrane vesicles and in the trypsin-treated vesicles were inhibited by about 40% at a concentration of 120 nmol DCCD per mg protein. With a longer period of incubation (12 h at 4°C), the inhibition of the ATPase acitivty in both cases increased to 60%. Under identical conditions, the ATPase activity of the purified ECF_1 was inhibited by 5-10%.

Therefore, DCCD inhibited the ATPase activity of the rebound ECF_1 to an extent similar to that found in native everted membrane vesicles.

IMMUNOPRECIPITATION OF THE ECF_1F_0 COMPLEX WITH ANTISERUM

Since ECF_1 could still bind to trypsin-treated vesicles, it was conceivable that the reconstituted vesicles could be solubilized with detergents and the ECF_1F_0 complex immunoprecipitated with antiserum to determine which polypeptide of F_0 was likely to be affected by trypsin-treatment.

Initially, attempts were made to determine which polypeptides of the ECF_1F_0 complex could be immunoprecipitated. Native everted vesicles of *E. coli* WS1 were washed with a low ionic strength buffer in the presence of protease inhibitors and then solubilized with Aminoxid WS-35 as described in the legend to Fig. 39. The solubilized fraction was treated with the test antiserum.

The immunoprecipitate obtained with antiserum to the DCCD-binding protein contained many bands when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 39). The major protein-staining polypeptides had

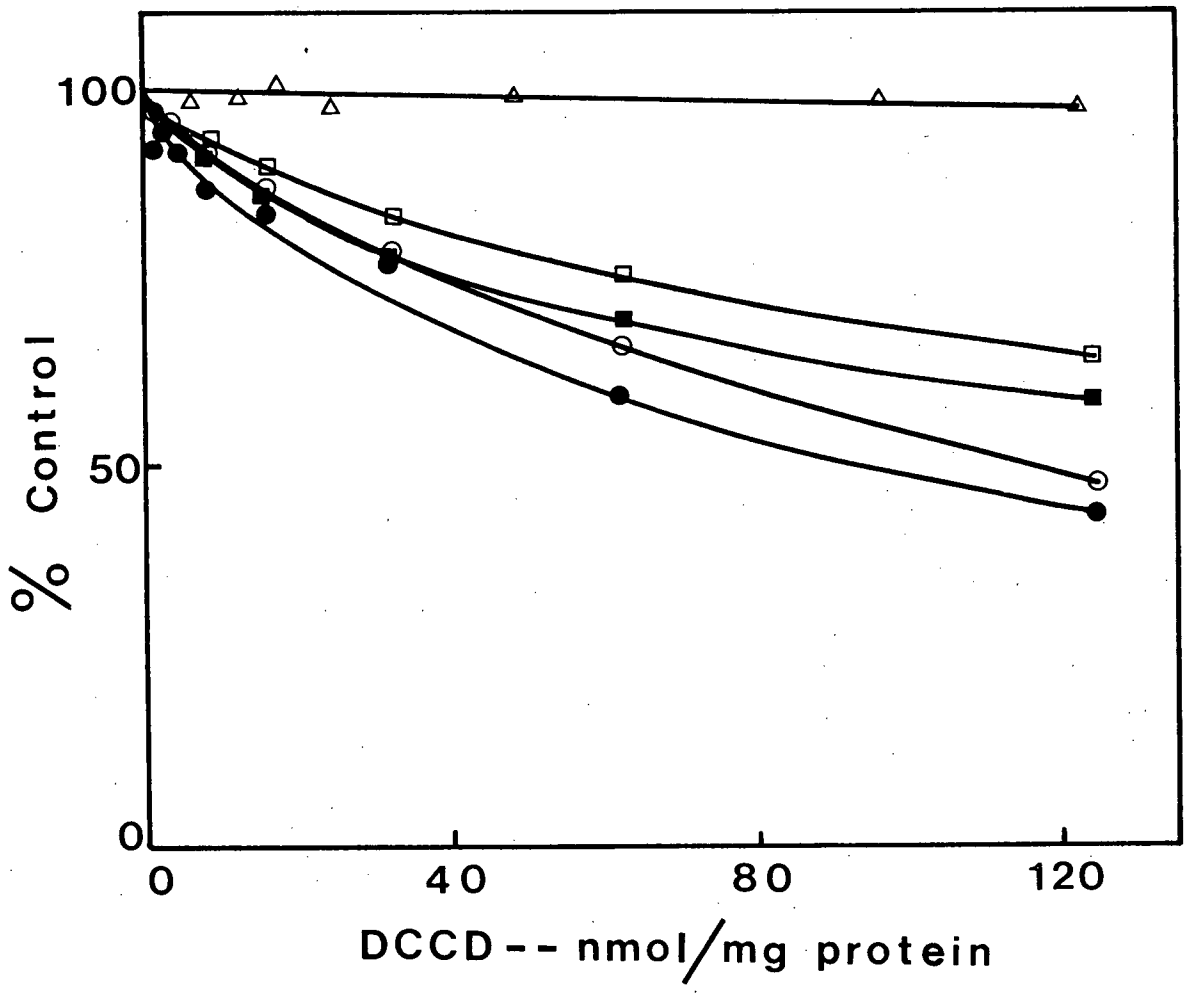


Fig. 38 Effect of DCCD on the ATPase activity of the ECF_1 bound to trypsin-treated everted membrane vesicles.

Urea-stripped everted vesicles of E. coli WS1 were treated with TPCK-trypsin for 45 min at 20°C as described in MATERIALS AND METHODS. Reconstitution of the vesicles with ECF_1 was carried out as follows: 40 mg of membrane vesicle protein in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol was reconstituted with 2.4 mg of ECF_1 in a final volume of 4 ml. The suspension was incubated at 4°C for 45 min, diluted 8-10 fold with buffer, and the vesicles sedimented by centrifugation at 250 000 xg for 2.5 h. The sedimented vesicles were resuspended in buffer and samples containing 4 mg membrane protein incubated with various levels of DCCD in a final volume of 1 ml either for 45 min at 37°C or for 45 min at 37°C followed by 12 h at 4°C before the ATPase activity was determined. Reconstituted urea-treated (■-■) or trypsin-treated (□-□) vesicles incubated for 45 min at 37°C; reconstituted urea-treated (●-●) or trypsin-treated (○-○) vesicles incubated for 45 min at 37°C followed by 12 h at 4°C. ECF_1 (△-△) treated with DCCD for 45 min at 37°C followed by 12 h at 4°C. The specific activities of ECF_1 and of the reconstituted urea-stripped and trypsin-treated vesicles were 18.5, 1.34 and 1.08 units/mg protein, respectively.

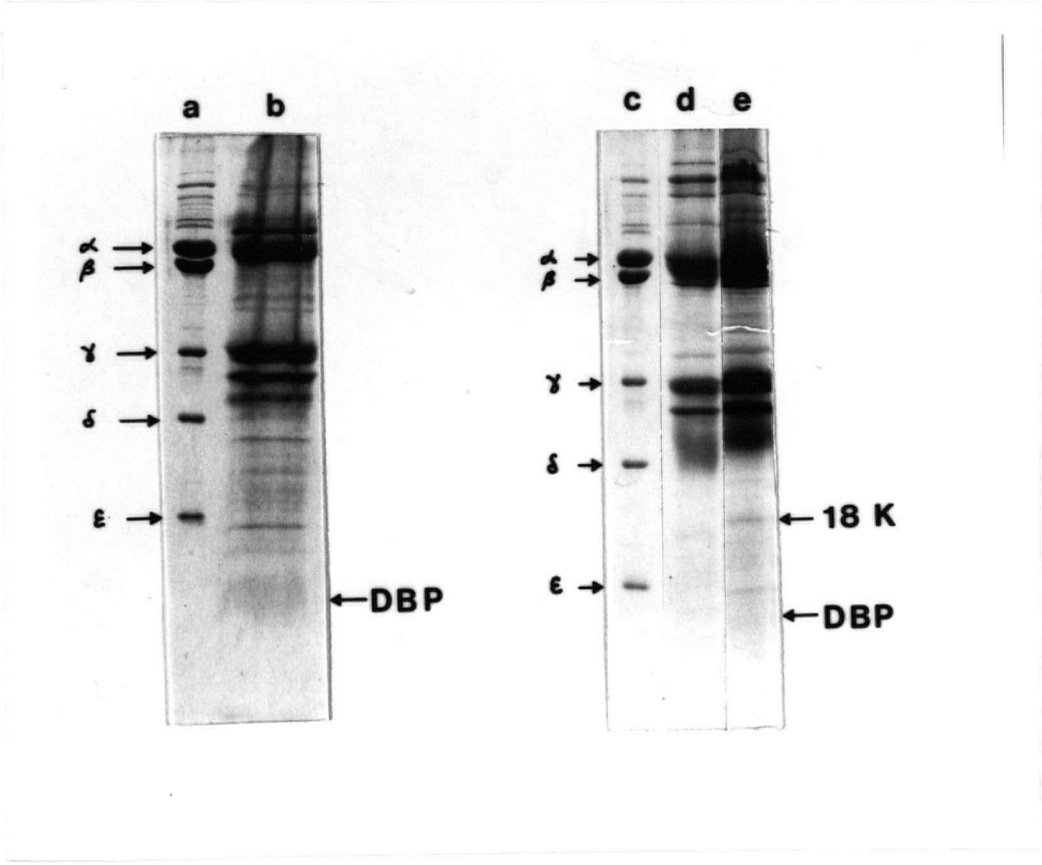


Fig. 39 SDS-polyacrylamide gel electrophoresis of the ECF_1F_0 complex immunoprecipitated with antiserum.

Everted membrane vesicles of E. coli ML 308-225 were suspended in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM KCl, 40 mM ε-aminocaproic acid, 24 mM p-aminobenzamidine, 0.8 mM PMSF, 0.4% (w/v) Merthiolate and 2% (v/v) methanol at a protein concentration of 10 mg/ml. The membrane vesicles were solubilized with Aminoxid WS-35 at a detergent to protein ratio of 6, as described in MATERIALS AND METHODS. To 10 ml of solubilized material was added 0.5 ml of antiserum to the DCCD-binding protein or antiserum to ECF_1 and the mixture incubated at 4°C for 24-30 h. The precipitate was collected by centrifugation at 12 000 xg for 30 min and washed once with 0.9% (w/v) NaCl containing 1% (w/v) Aminoxid WS-35, and twice with 0.9% (w/v) NaCl. The immunoprecipitate was subjected to SDS-electrophoresis, as described by Laemmli (171). The separating gel consisted of 13% (w/v) acrylamide and the stacking gel was 4% (w/v) acrylamide. The gel was stained with 0.1% (w/v) Coomassie Blue as described previously. Lanes a and c, purified ECF_1; lane b, immunoprecipitate obtained with antiserum to the DCCD-binding protein; lanes d and e, immunoprecipitate obtained with antiserum to ECF_1 with lane e containing twice the amount of material as that in lane d. The migration positions of the subunits of ECF_1, of the DCCD-binding protein (DBP), and of the 18 000 dalton polypeptide of F_0 are indicated.

molecular weights of 60 000, 55 000, 32 000, 30 000, 28 000, 22-25 000 (stained diffusely), 18 000, 11 000 and 9 000. The polypeptide of 9 000 daltons migrated as a diffuse Coomassie Blue staining region and was identified as the DCCD-binding protein since it co-migrated with purified DCCD-binding protein. However, the 56 000 (α), 52 000 (ß), 22 000 (δ) and 13 000 (ε) dalton subunits of ECF_1 were absent. Similarly, the subunits of ECF_1 could not be detected in the immunoprecipitate when the ATPase complex, purified by Phenyl-Sepharose CL-4B and centrifugation at 250 000 xg for 16-17 h, was treated with antiserum to the DCCD-binding protein. It is possible that the interaction of the antibody to the DCCD-binding protein with the ECF_1F_0 complex resulted in the displacement of ECF_1 since it has been shown that the antibody binds close to the ECF_1-binding site on F_0 (Fig. 29A). By contrast, treatment of the solubilized fraction with antiserum to ECF_1 precipitated polypeptides with molecular weights of 94 000, 90 000, 56 000 (α), 52 000 (ß), 48 000, 35 000, 32 000, 30 000, 28 000, 22-25 000 (stained diffusely), 18 000, 13 000 (ε), 11 000 and 9 000 (stained diffusely). The presence of the α, ß and ε subunits suggested that the 32 000 dalton polypeptide was likely to be the γ subunit of ECF_1. The 9 000 dalton polypeptide was identified as the DCCD-binding protein as before. These results indicated that the immunoprecipitate obtained with antiserum to ECF_1 contained a more "intact" ECF_1F_0 complex.

Urea-stripped vesicles of E. coli WS1 also were treated with trypsin and reconstituted with ECF_1. The reconstituted vesicles were solubilized with Aminoxid WS-35 and the solubilized fraction immunoprecipitated with antiserum to ECF_1. The immunoprecipitates were analyzed on the two-dimensional gel electrophoresis system of O'Farrell (176). This technique

was used to analyze the contents of the immunoprecipitates because of the characteristic migration patterns of the α, ß, δ and ε subunits of ECF_1 and the 18 000 dalton subunit (polypeptide b) of F_0 (Fig. 27). These preliminary results are shown in Fig. 40. Many polypeptides were present in the precipitate from the urea-stripped vesicles which had been reconstituted with ECF_1 (Fig. 40A). The ECF_1F_0 complex in this preparation was presumably intact. The α, ß, ε subunits of ECF_1 were easily distinguishable. The δ subunit of ECF_1 was missing in this preparation. This was probably due to the susceptibility of this subunit to proteases (Dr. Helga Stan-Lotter, personal communication) and the problem was further enhanced by the prolonged incubation period (30-36 h) involved in the experiment. In addition to these three subunits of ECF_1, a polypeptide of 18 000 daltons was present (shown in square) and was likely to be polypeptide b of F_0.

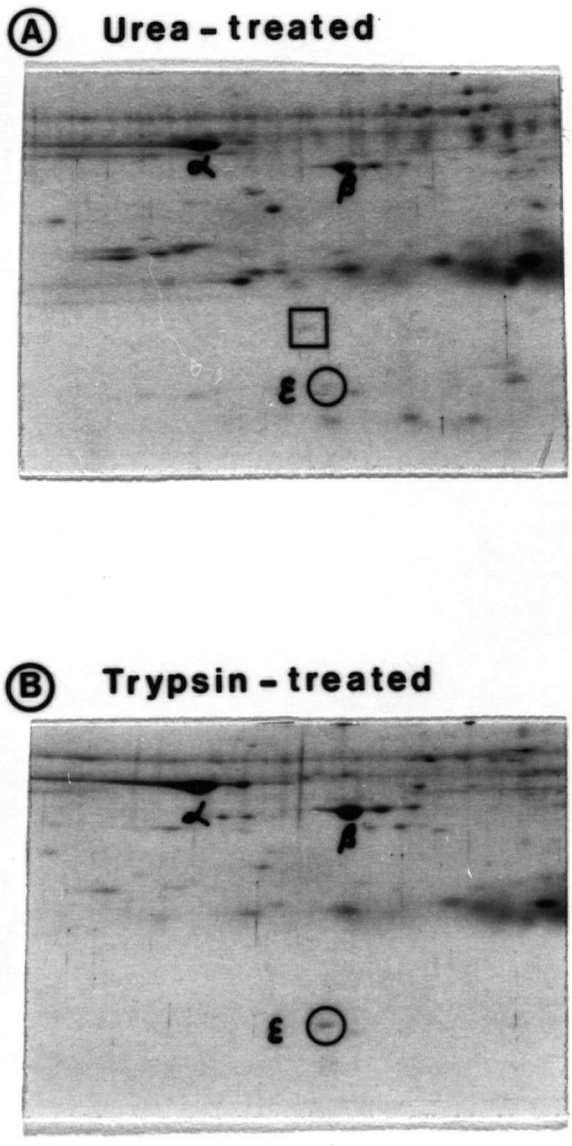
Similarly, the α, ß and ε subunits of ECF_1 were present in the precipitate obtained from the trypsin-treated vesicles which had been reconstituted with ECF_1 (Fig. 40 B). In contrast to the urea-treated vesicles, trypsin-treatment resulted in the cleavage of the 18 000 dalton subunit. This polypeptide was absent, even though the gel was loaded with more protein than in Fig. 40A. (Note the relative staining intensity of the α, ß and ε subunits of ECF_1.)

Analysis of the precipitates from the urea- and trypsin-treated vesicles on one dimensional SDS-polyacrylamide gels revealed that the 9 000 dalton polypeptide (DCCD-binding protein) did not appear to be affected by trypsin-treatment. However, the possibility that trypsin digested a fraction of the DCCD-binding protein as well as the 24 000 dalton subunit (polypeptide a) of F_0 could not be excluded.

Fig. 40 Two-dimensional gel electrophoresis of the ECF_1F_0 complex obtained by immunoprecipitation with antiserum to ECF_1.

Urea-stripped everted vesicles of E. coli ML308-225 were treated with TPCK-trypsin and suspended in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol at a protein concentration of 10 mg/ml. Twenty ml of the treated vesicles were reconstituted with 0.3 ml of ECF_1 (9.5 mg/ml) as described in MATERIALS AND METHODS. The reconstituted vesicles were suspended in 50 mM Tris-HCl buffer, pH 7.4 containing 100 mM KCl, 40 mM ε-aminocaproic acid, 24 mM p-aminobenzamidine, 0.8 mM PMSF, 0.4% (w/v) Merthiolate and 2%(v/v) methanol at a protein concentration of 10 mg/ml and solubilized with Aminoxid WS-35 at a detergent to protein ratio of 6, as described in the legend to Fig. 39. To 25 ml of the solubilized fraction was added 1.5 ml of antiserum to ECF_1 and the mixture incubated at 4°C for 30-36 h. The immunoprecipitate was collected by centrifugation at 12 000 xg for 30 min and washed three times with 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $MgCl_2$. The immunoprecipitate was subjected to two-dimensional gel electrophoresis. The first-dimension gel (horizontal direction) consisted of 0.8% (w/v) pH 3.5-10 and 1.6% (w/v) pH 5-7 ampholytes. The second-dimension gel (vertical direction) was a linear gradient of 7.5-16.5% (w/v) acrylamide in the Tris-buffered system. The gels were stained with 0.1% (w/v) Coomassie Blue, as described previously. PANEL A: Immunoprecipitate obtained from urea-stripped everted vesicles reconstituted with ECF_1. PANEL B: Immunoprecipitate obtained from trypsin-treated everted vesicles reconstituted with ECF_1.

The α, ß and ε subunits of ECF_1 are indicated. The polypeptide outlined by the rectangle is absent in the immunoprecipitate from trypsin-treated everted vesicles.


A
Urea - treated
α
β
ε
B
Trypsin - treated
α
β
ε

DETECTION BY SOLID PHASE RADIOIMMUNE ASSAY OF THE REACTION OF ANTIBODY WITH MEMBRANE VESICLES

The experiments described earlier, in which the decrease in proton leakage through F_0 in the presence of antiserum to the DCCD-binding protein was measured, were attempts to determine if the DCCD-binding protein was transmembranous. The ability of the antibody to the DCCD-binding protein to react with this polypeptide in "right-side out" and everted membrane vesicles was also examined by the competitive inhibition assay (195). In this assay, the DCCD-binding protein of the membrane vesicles (urea-stripped everted vesicles or right-side out vesicles) competed for the antibody with the DCCD-binding protein immobilized in microtitre wells.

Poly-L-lysine was required to immobilize the antigen. The titration curve in Fig. 41 could not be reproduced if the antigen was passively dried (55-60°C) onto the microtitre wells. The results of the competitive inhibition assay are shown in Fig. 42 (panel A). The right-side out vesicles of E. coli WS1 were approximtely one-third as effective in binding the antibody compared with urea-stripped everted vesicles. Almost identical results were obtained with the vesicles from E. coli ML380-225. In both of these strains, 50% inhibition of binding was obtained with a concentration of 10 µg everted vesicle protein per ml, while that by the right-side out vesicles of E. coli WS1 and ML308-225 was 26.6 µg protein per ml.

The ability of both types of vesicles to bind the antibody, but with different effectiveness, could be due to the presence of a mixed population of antibodies (i.e. antibodies to determinants exposed on the inside (cytoplasmic side) and outside of the cell). Alternatively, the binding of antibody to the right-side out vesicles was likely due to reaction with

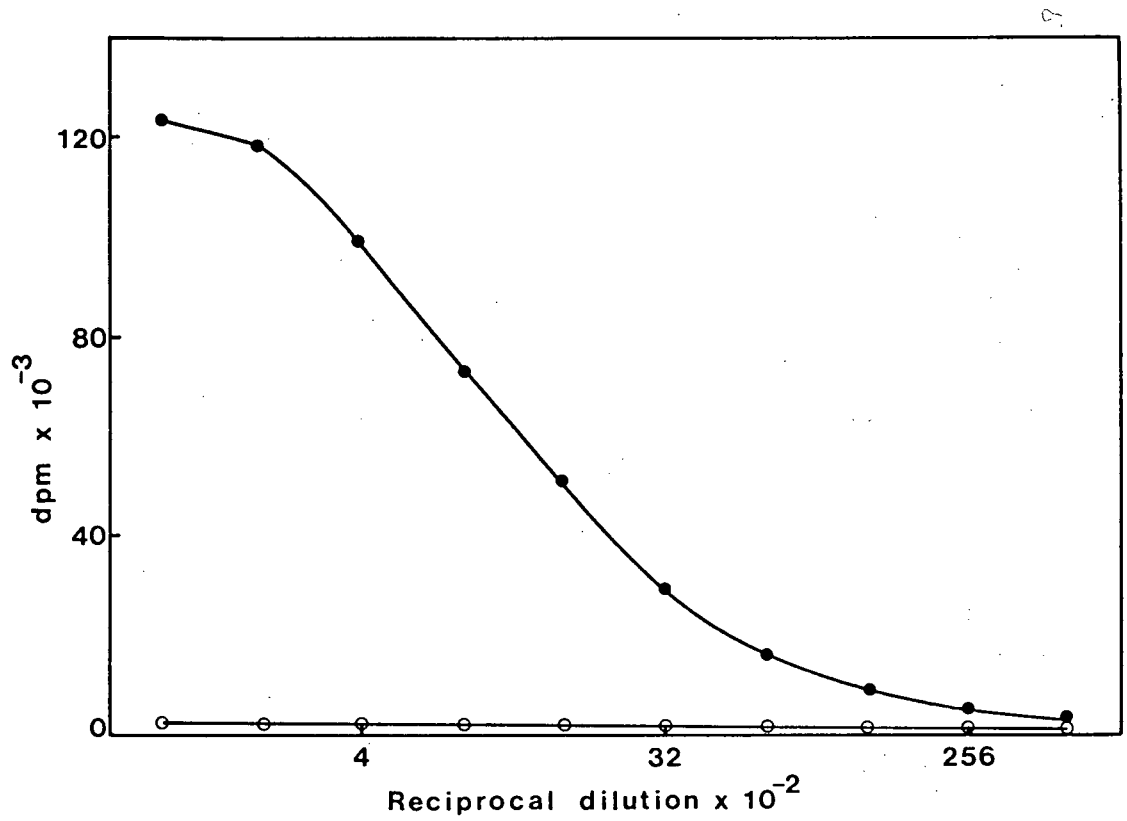
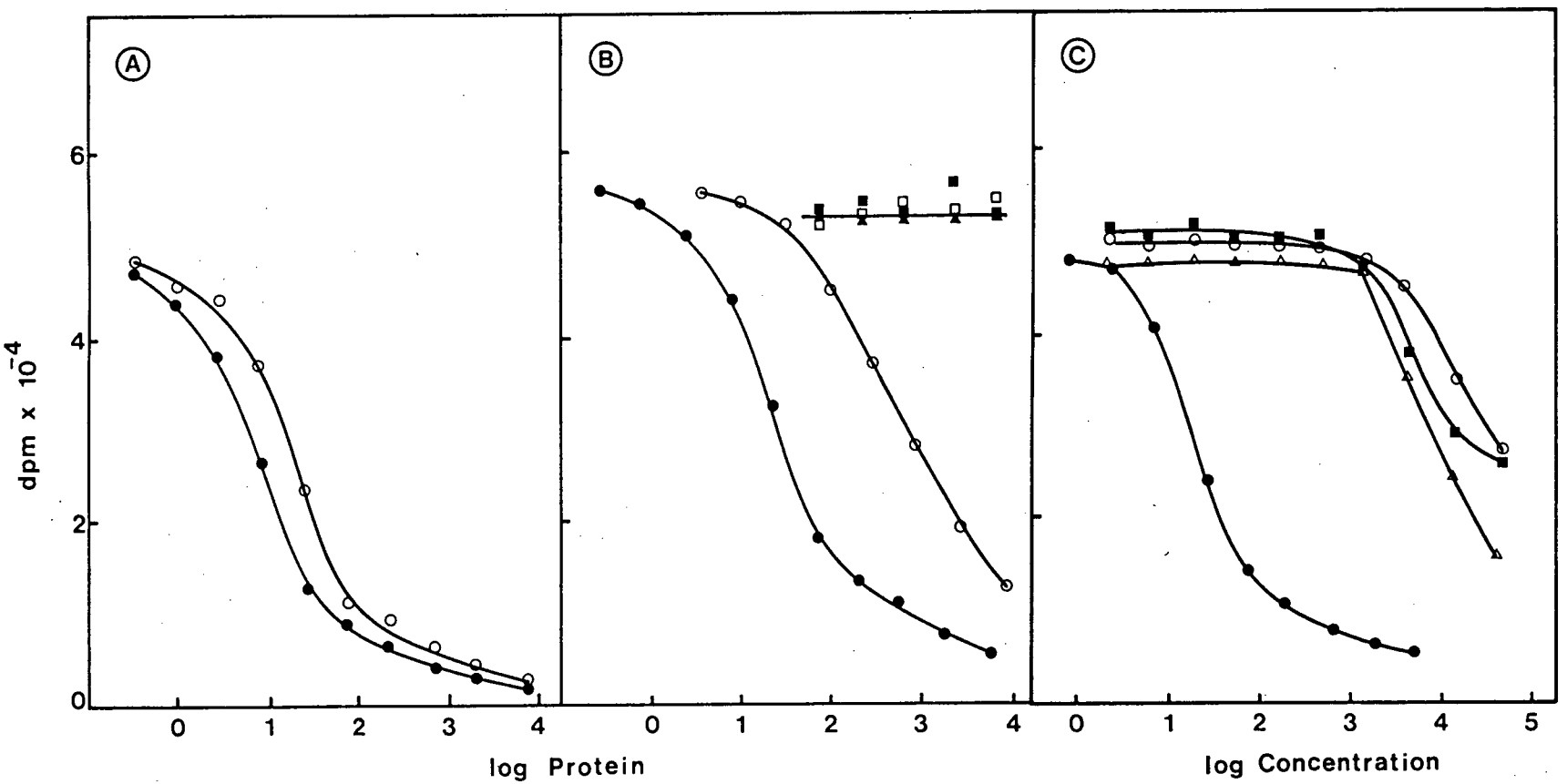


Fig. 41 Titration of the DCCD-binding protein with antiserum to this polypeptide.

DCCD-binding protein (3.3 µg) in 100 mM sodium borate buffer, pH 8.8, containing 2% (w/v) Triton X-100 was immobilized onto polylysine-coated microtitre plate wells. Non-specific binding sites were quenched with RIA buffer and the immobilized DCCD-binding protein was titrated with serial dilutions of antiserum to the DCCD-binding protein (●-●) or preimmune serum (○-○). The extent of binding of the rabbit antiserum was measured with ^{125}I-labelled goat anti-rabbit immunoglobulin as described in MATERIALS AND METHODS.

Fig. 42 Inhibition of antibody binding to immobilized DCCD-binding protein by membrane vesicles of E. coli, PS3 and rat liver mitochondria, and by phospholipid vesicles.

Urea-stripped everted vesicles were suspended in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol. "Right-side out" vesicles were suspended in 0.4 M sucrose containing 10 mM $MgCl_2$. The phospholipids were taken up in 100 mM sodium borate buffer, pH 8.8 containing 2% (w/v) Triton X-100. DCCD-binding protein (3.3 µg protein) was immobilized to polylysine-coated microtitre plate wells. Inhibition of antibody binding to the immobilized polypeptide by various amounts of membrane vesicles or phospholipid vesicles was measured by the "competitive inhibition assay" as described in MATERIALS AND METHODS. PANEL A: (●-●), urea-stripped everted vesicles of E. coli WS1; (○-○), right-side out vesicles of E. coli WS1. PANEL B: (●-●), urea-stripped everted vesicles of E. coli WS1; (○-○), urea-stripped everted vesicles of PS3; (□-□), phosphate-washed mitochondrial inner membranes; (▲-▲), sonicated phosphate-washed mitochondrial inner membranes; (■-■), urea-stripped phosphate-washed mitochondrial inner membanes. PANEL C: (●-●) urea-stripped everted vesicles of E. coli WS1; (△-△), soybean phosphatidylcholine vesicles; (○-○), egg yolk phosphatidylcholine; (■-■), synthetic phosphatidylcholine (1-palmityl-2-oleoyl phosphatidylcholine). Concentration of the everted membrane vesicles is expressed in µg protein/ml whereas the phospholipid concentration is expressed as µg/ml (w/v).


A
B
C
dpm x 10^{-4}
0
2
4
6
0
1
2
3
4
log Protein
0
1
2
3
4
5
log Concentration

re-oriented ATPase complexes (210-212) or to a contamination of the right-side out vesicles with a population of inside-out vesicles.

In contrast to the results obtained with E. coli WS1 and ML308-225, 50% inhibition of binding by the urea-stripped vesicles of the unc mutants E. coli DL-54, N_{I44} and AN382 occurred at a concentration of 19 µg everted vesicle protein per ml. In addition, 50% inhibition of binding by right-side out vesicles of E. coli DL-54 and N_{I44} were at 24 and 19 µg membrane protein per ml, respectively. The cause(s) of this difference in the value of the 50% inhibition of binding by everted and right-side out vesicles of the wild-type and mutant strains of E. coli is not known. However, Cox et al. (140) have proposed, on the basis of genetic studies, that the α and /or ß subunits of ECF_1 were needed for the proper assembly of a functional F_0. In its (their) absence, the (F_0 polypeptide) DCCD-binding protein was suggested to insert randomly into the membrane. Therefore, if the DCCD-binding protein does exist as a "loop" in the membrane, then in a random insertion, one would expect that 50% of the antigenic determinant normally exposed on the cytoplasmic surface to be also exposed on the external surface of the cell. In this event, differentiation between "right-side" out and "inside-out" vesicles would not be possible and the 50% inhibition of antibody-binding by both types of vesicles should be identical.

The ATPase complex of the thermophilic bacterium PS3 has been well characterized (4). In spite of considerable homology between the DCCD-binding proteins of E. coli and PS3 (146), the antibody reacted only weakly with everted membrane vesicles of PS3 (Fig. 42, panel B). Everted inner mitochondrial membrane vesicles (submitochondrial particles) from rat liver, or purified mitochondrial membranes from which MF_1 had been stripped, did

not react with the antibody to the E. coli DCCD-binding protein, at least at the concentration of antigen used (Fig. 42, panel B). It would appear that a very high concentration of membrane vesicles (E. coli, PS3 or mitochondrial membranes) could result in the trapping or non-specific interaction of antibody with the vesicles. As shown in Fig. 42 (panel C), at extremely high concentrations (greater than 1.8 mg per ml) of phospholipids, a significant amount of antibody was bound by the phospholipid vesicles.

REACTION SITE(S) FOR THE ANTIBODY ON THE DCCD-BINDING PROTEIN

The antiserum to the DCCD-binding protein could bind to the purified DCCD-binding protein. Therefore, attempts were made to determine the reaction site(s) for the antibody on this polypeptide. The DCCD-binding protein was modified with several group-specific reagents (213). The modified polypeptide was then used in the competitive inhibition assay. The immobilized antigen in the microtitre wells was unmodified DCCD-binding protein.

Cleavage of the polypeptide at methionyl residues with cyanogen bromide, or oxidation of methionyl residues with performic acid to greater than 95% (Table 13) (184,213), resulted in a reduction in the affinity of the modified polypeptide for the antibody by almost two orders of magnitude (Fig. 43). The polypeptides modified by these two reagents were soluble in borate-Triton buffer only up to a level of 90-100 µg protein per ml. Although the extent of cleavage of the DCCD-binding protein by TPCK-trypsin or Staphylococcus aureus V_8 protease was not known, the affinity of the protease-treated polypeptide was also reduced 3-fold. Modifiction of the arginyl residue(s) of the DCCD-binding protein with phenylglyoxal or 2,3-butanedione had no effect on its reaction with the antibody. Similarly,

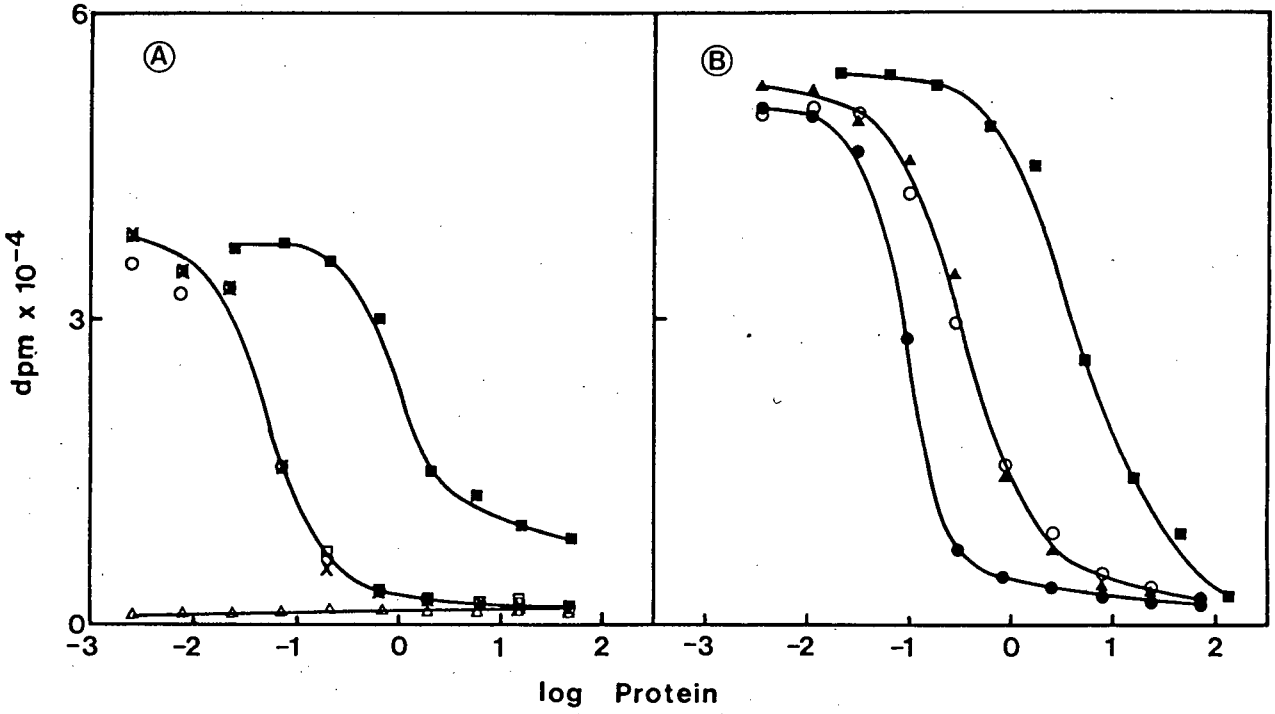


Fig. 43 Inhibition of antibody binding to immobilized DCCD-binding protein by protease-treated or chemically-modified DCCD-binding protein.

The protease-treated or chemically-modified DCCD-binding protein was suspended in 100 mM sodium borate buffer, pH 8.8, containing 2% (w/v) Triton X-100. DCCD-binding protein (3.3 µg) was immobilized to polylysine-coated microtitre plate wells. Inhibition of antibody binding to the immobilized polypeptide by various amounts of protease-treated or chemically-modified DCCD-binding protein was measured by the "competitive inhibition assay", as described in MATERIALS AND METHODS. PANEL A: (x-x), untreated DCCD-binding protein; (□-□), 2,3-butanedione-treated DCCD-binding protein; (○-○), phenylglyoxal-treated DCCD-binding protein; (■-■), performic acid-treated DCCD-binding protein; (△-△), antibody replaced in (control) experiments by preimmune serum. PANEL B: (●-●), untreated DCCD-binding protein; (○-○), TPCK-trypsin-treated DCCD-binding protein; (▲-▲), V_8-protease-treated DCCD-binding protein; (■-■), cyanogen bromide-treated DCCD-binding protein.

treatment of the DCCD-binding protein with chloramine T or hydrogen peroxide also did not affect its ability to bind the antibody (not shown). But the extent of modification of the DCCD-binding protein by these reagents was not known.

In contrast to the effect of trypsin and V_8 protease on the ability of the purified DCCD-binding protein to react with the antibody, urea-stripped vesicles treated with trypsin or V_8 protease did not cause a reduction in the affinity for the antibody (Fig. 44). Similar results were obtained with urea-stripped vesicles which had been treated with phenylglyoxal or Chloramine T (not shown).

BINDING OF ECF_1 BY PURIFIED DCCD-BINDING PROTEIN

The ability of urea-stripped vesicles of the unc mutant E. coli N_{I44}, which presumably contains only the DCCD-binding protein in the F_0 complex, suggested that this polypeptide may be involved in the binding of ECF_1. Indeed, ECF_1 could be bound by the purified DCCD-binding protein.

This was shown in two ways. In the first method, DCCD-binding protein immobilized in microtitre plate wells ("fixed antigen") was reacted with ECF_1 ("free antigen"). Bound ECF_1 was titrated with various dilutions of anti-ECF_1 serum. The extent of binding of the antibody was then measured with ^{125}I-labelled goat anti-rabbit immunoglobulin. (This assay is schematically represented in Fig. 45.) Nonspecific binding of antiserum to the fixed antigen (and to the wells) and nonspecific binding of ECF_1 to the wells were corrected for by omitting the ECF_1 and the DCCD-binding protein, respectively. As shown in Fig. 46, panel A, nonspecific binding of anti-ECF_1 serum was negligible. Significant nonspecific binding of ECF_1 could be detected but this was much less than the binding of ECF_1

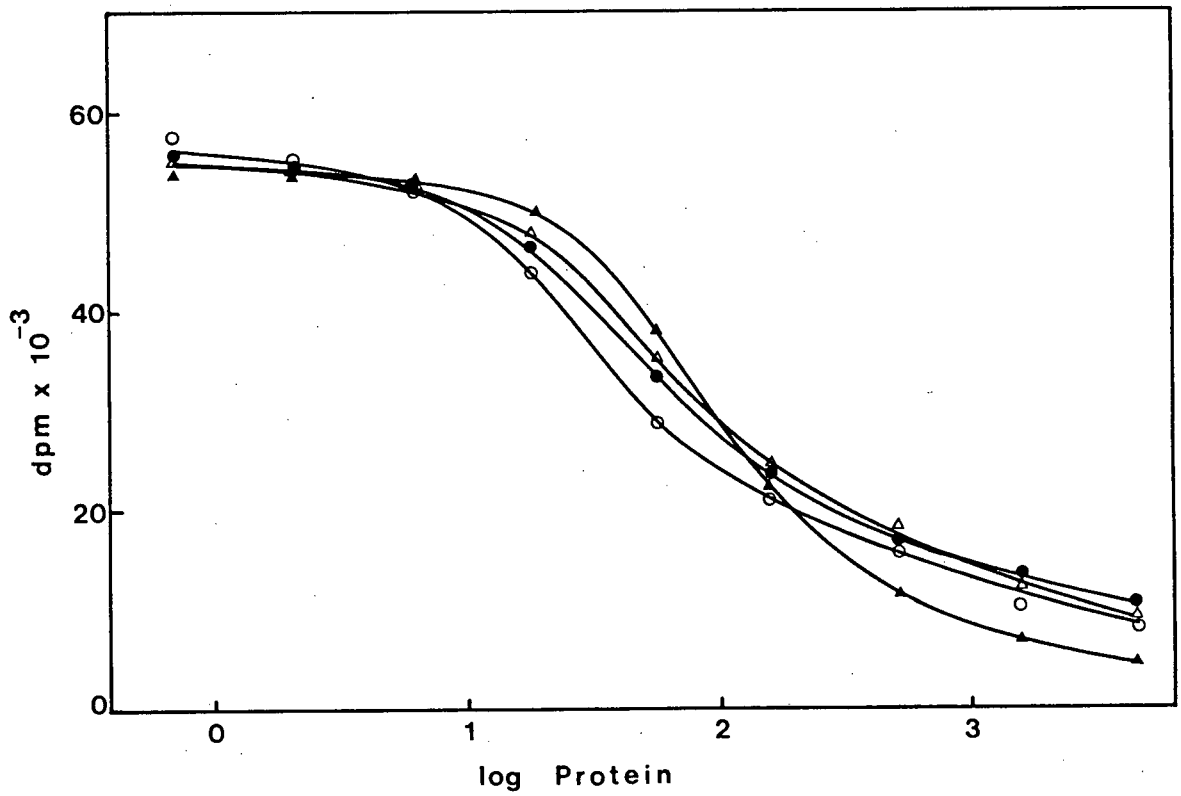


Fig. 44 Inhibition of antibody binding to immobilized DCCD-binding protein by protease-treated or chemically-modified everted membrane vesicles.

Urea-stripped everted membrane vesicles of E. coli WS1 were treated with phenylglyoxal, Staphylococcus aureus V_8 protease, or TPCK-trypsin as described in MATERIALS AND METHODS. The treated vesicles were then suspended in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol. DCCD-binding protein (3.3 µg) was immobilized to polylysine-coated microtitre plate wells. Inhibition of antibody binding to the immobilized polypeptide by various amounts of protease-treated or chemically-modified vesicles was measured by the "competitive inhibition assay" as described in the MATERIALS AND METHODS section. (●-●), urea-stripped everted vesicles; (▲-▲), phenylglyoxal-treated everted vesicles; (△-△), V_8 protease-treated everted vesicles; (○-○), TPCK-trypsin-treated everted vesicles.

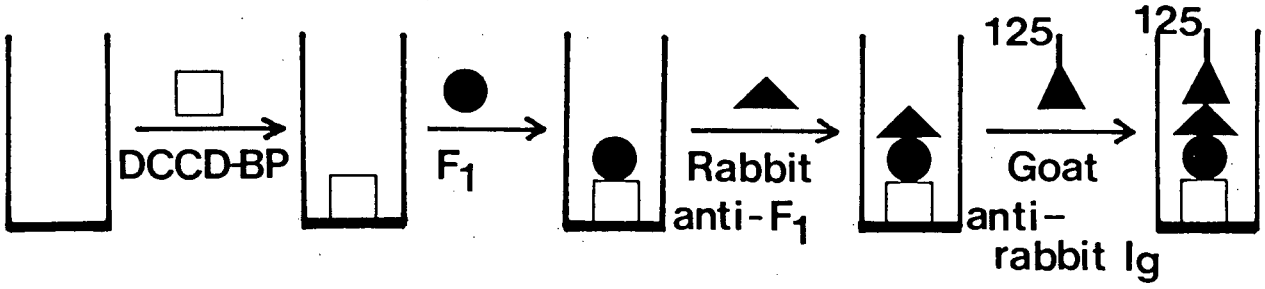


Fig. 45 Schematic representation of the radioimmune binding assay.

This figure shows the binding of the free antigen (F_1) to the fixed antigen (DCCD-binding protein). The amount of F_1 bound was measured by treating the contents of the wells with antiserum to F_1 and finally with ^{125}I-labelled goat anti-rabbit immunoglobulin. See MATERIALS AND METHODS for details.

(Drawings courtesy of Dr. P.D. Bragg.)

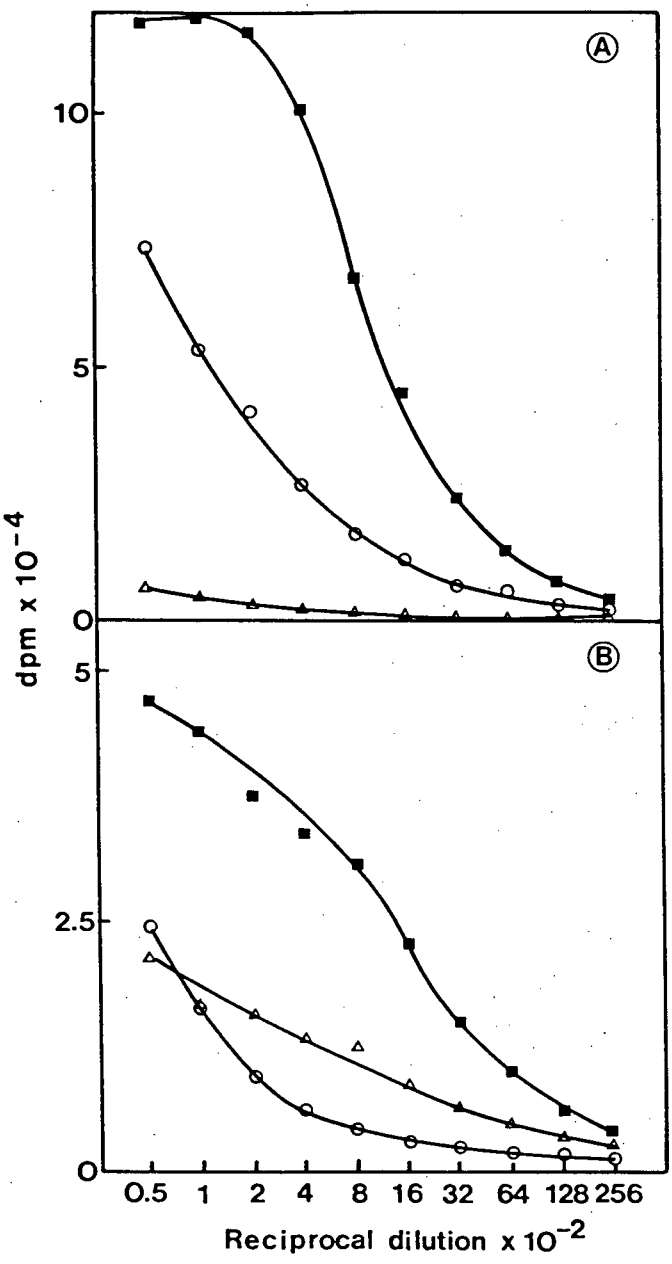


Fig. 46 Binding of ECF_1 to the DCCD-binding protein.

See MATERIALS AND METHODS for details of the binding assay (Solid Phase Radioimmune Assay) PANEL A: Immobilized DCCD-binding protein (3.3 µg protein) in the presence (■-■) or absence (△-△) of ECF_1 (19 µg protein) was titrated with various dilutions of anti-ECF_1 serum. (○-○), ECF_1 (19 µg protein) added but the immobilized DCCD-binding protein was omitted. PANEL B: Immobilized ECF_1 (19 µg protein) in the presence (■-■) or absence (○-○) of DCCD-binding protein (3.3 µg protein) was titrated with various dilutions of anti-DCCD-binding protein serum. (△-△), DCCD-binding protein was present but immobilized ECF_1 was omitted.

found in the presence of the DCCD-binding protein. The extent of nonspecific binding could be decreased by lowering the concentration of ECF_1 used in the experiment. In the second method (Fig. 46, panel B), the fixed antigen was purified ECF_1 and the binding of DCCD-binding protein to this was measured using antiserum to the polypeptide. Again, significantly more DCCD-binding protein was bound to ECF_1 than in the control.

Prior reaction of immobilized DCCD-binding protein with ECF_1 did not affect its subsequent reaction with antibody (Fig. 47, panel A). (A similar result was obtained for the reaction of immobilized ECF_1 with its antibody (Fig. 47, panel B).) There are two possible explanations of this. Either the reaction of the DCCD-binding protein with its antibody is sufficiently strong to displace pre-bound ECF_1 or the ECF_1-binding site on the polypeptide is separate from the binding site for the antibody. As described below, our results favour the latter explanation.

REACTION SITE(S) ON ECF_1 FOR THE DCCD-BINDING PROTEIN

The subunits of ECF_1 responsible for binding to the DCCD-binding protein were explored. Treatment of ECF_1 with TPCK-trypsin followed by reisolation of the enzyme on a sucrose gradient resulted in the complete removal of the δ and ϵ subunits (Fig. 48) and cleavage of the amino-terminal fifteen residues of the α subunits (68,214). The γ subunit also appeared to be cleaved in our preparation. The trypsin-treated ECF_1 was bound by the DCCD-binding protein almost as effectively as the native enzyme (Fig. 49, panel A). Pronase treatment of ECF_1 reduced the extent of binding dramatically due to extensive cleavage of all subunits of the ECF_1. Treatment of ECF_1 with other proteases also reduced the extent

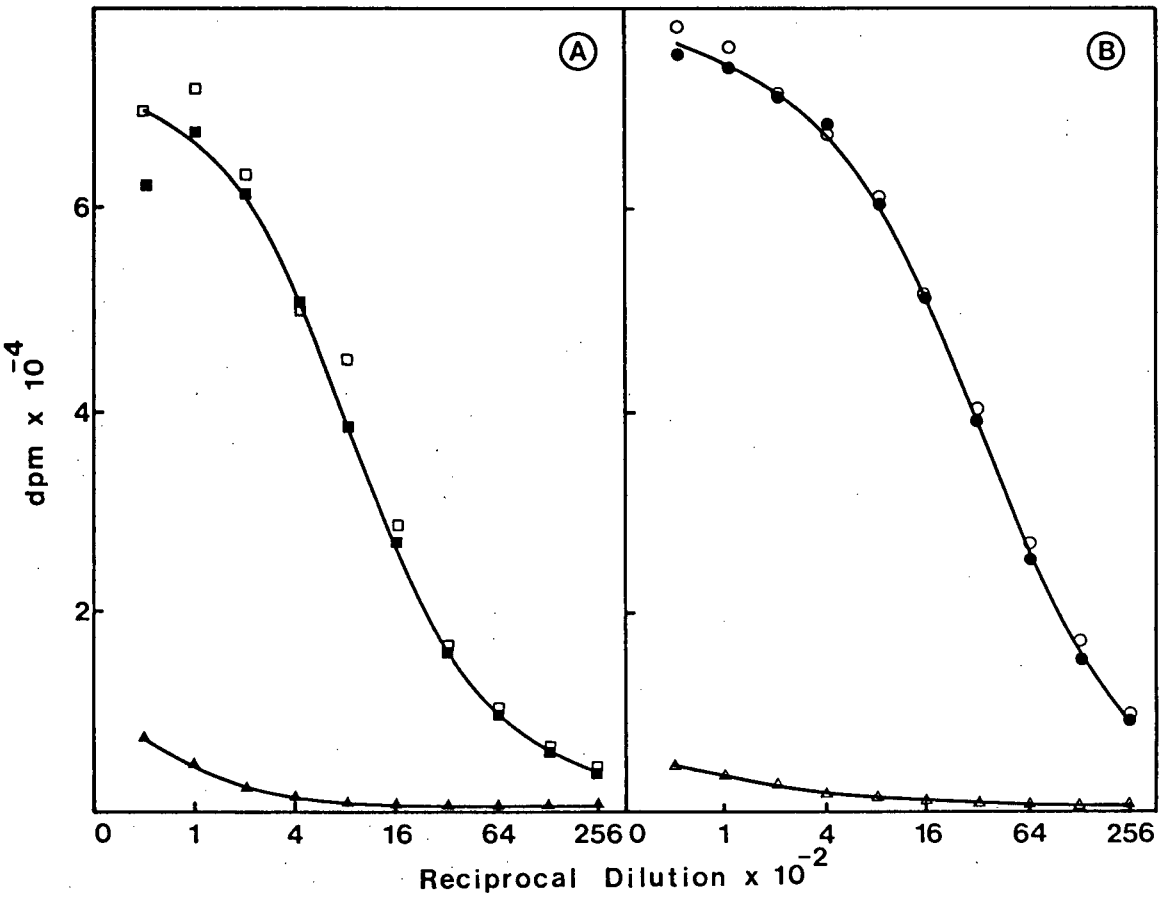


Fig. 47 Effect of ECF_1 on the binding of anti-DCCD-binding protein serum to the DCCD-binding protein and the effect of DCCD-binding protein on the binding of anti-ECF_1 serum to ECF_1.

See MATERIALS AND METHODS for details of the radioimmune binding assay. PANEL A: Immobilized DCCD-binding protein (3.3 µg protein) was titrated with serial dilutions of anti-DCCD-binding protein serum in the absence (■-■) and presence (□-□) of ECF_1 (19 µg protein). (▲-▲), ECF_1 (19 µg protein) was added but the immobilized DCCD-binding protein was omitted. PANEL B: Immobilized ECF_1 (19 µg protein) was titrated with serial dilutions of anti-ECF_1 serum in the absence (●-●) and presence (○-○) of DCCD-binding protein (3.3 µg). (△-△), DCCD-binding protein (3.3 µg) was added but the immobilized ECF_1 was omitted.

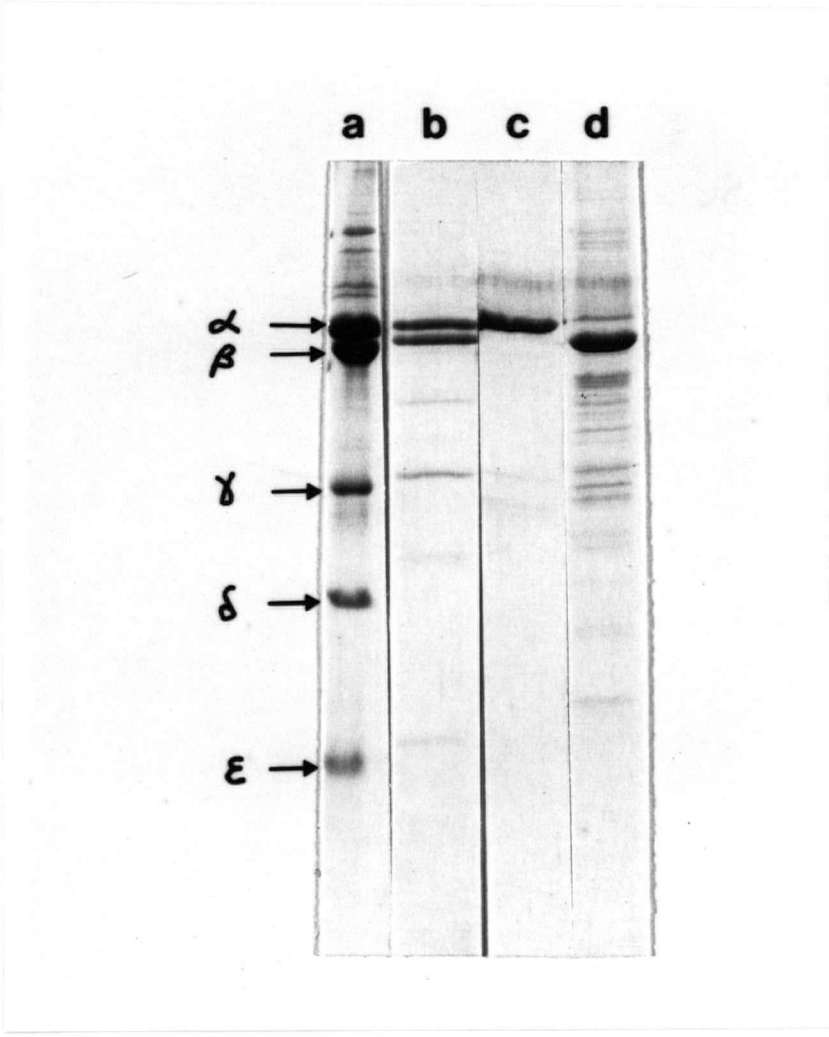
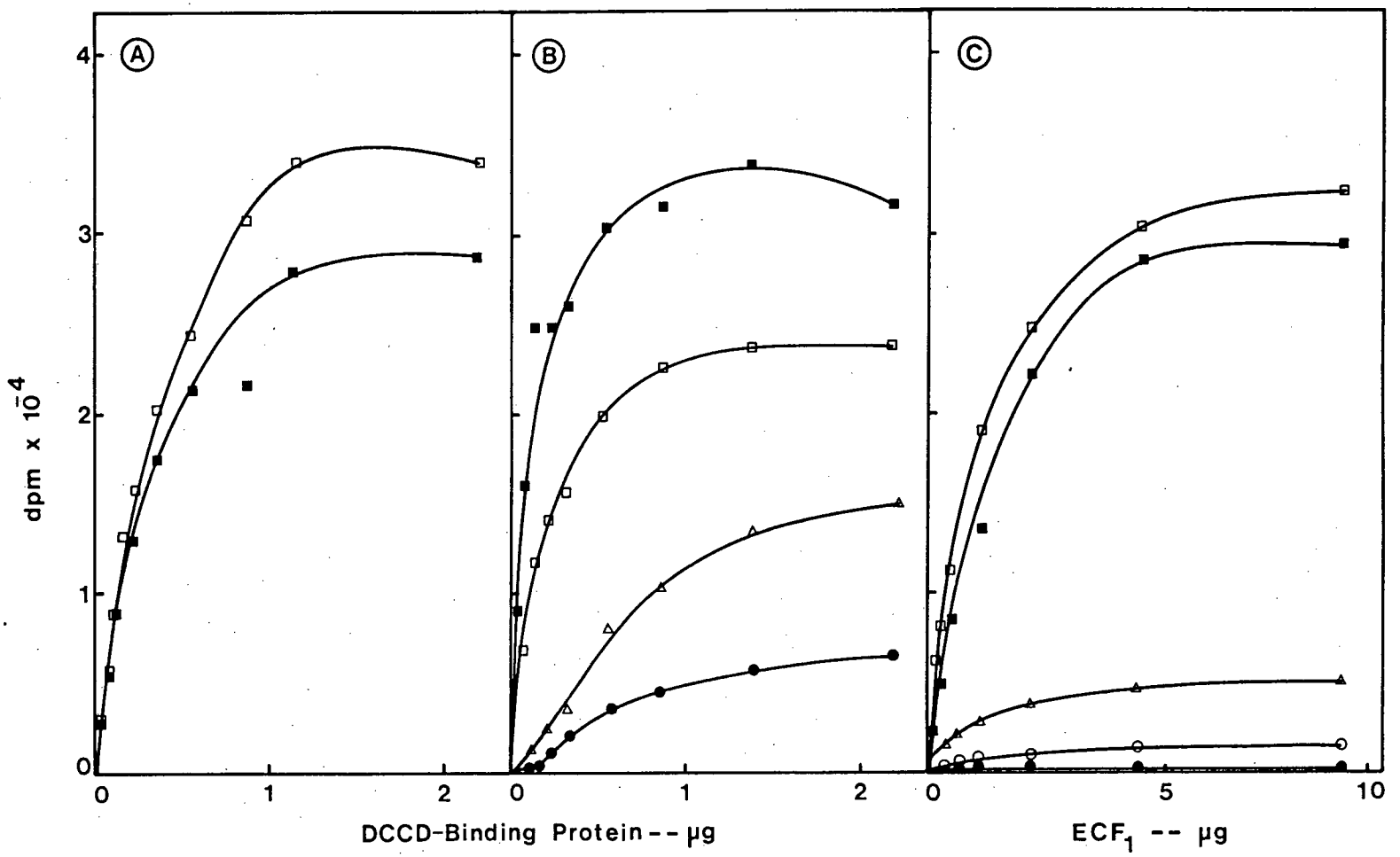


Fig. 48 SDS-polyacrylamide gel electrophoresis of subunits of ECF_1.

The subunits of ECF_1 were prepared from E. coli ML308-225 as described in MATERIALS AND METHODS. The SDS-gel consisted of 13% (w/v) acrylamide with a 4% (w/v) stacking gel, in the Tris-buffered system. The gel was stained with 0.1% Coomassie Blue as previously described. Lane a, purified ECF_1; lane b, TPCK-trypsin treated ECF_1; lane c, purified α subunit of ECF_1; lane d, purified ß subunit of ECF_1. The migration positions of the subunits of ECF_1 (α - ε) are indicated.

Fig. 49 Binding of ECF_1 to the DCCD-binding protein: Effect of protease treatment.

ECF_1 and DCCD-binding protein were treated with various proteases and the binding assay carried out as described in MATERIALS AND METHODS. PANEL A: Binding of DCCD-binding protein to TPCK-trypsin treated ECF_1. Immobilized ECF_1 (■-■) (19 µg protein) and TPCK-trypsin treated ECF_1 (□-□) (14 µg protein) were titrated with DCCD-binding protein. PANEL B: Binding of DCCD-binding protein to protease-treated ECF_1. ECF_1 (19 µg protein) was treated with V_8 protease (□-□), α-chymotrypsin (△-△) or pronase (●-●), or not treated (■-■), and immobilized on polylysine-coated microtitre plate wells and then titrated with DCCD-binding protein. PANEL C: Binding of ECF_1 to protease-treated DCCD-binding protein. Titration by ECF_1 of immobilized DCCD-binding protein (3.3 µg) which had been treated with V_8 protease (□-□), α-chymotrypsin (△-△), TPCK-trypsin (○-○), or pronase (●-●), or not treated (■-■).


A
B
C
dpm x 10^{-4}
0
1
2
3
4
0
1
2
0
1
2
0
5
10
DCCD-Binding Protein -- µg
ECF_1 -- µg

of its binding of the DCCD-binding protein but the effect was not as large as that seen with pronase (Fig. 49, panel B). Similarly, the binding of ECF_1 to the immobilized DCCD-binding protein was almost completely abolished when the polypeptide was treated with pronase or TPCK-trypsin prior to immobilization (Fig. 49, panel C).

The role of the major subunits of ECF_1 in binding the DCCD-binding protein was investigated following the purification of the α and ß subunits from the salt-dissociated enzyme by the procedure of Dunn and Futai (43). The purity of these subunits is shown in Fig. 48. While the isolated α subunit was about 100% pure, the isolated ß subunit was still contaminated (5-10%) with some low molecular weight polypeptides. These isolated subunits were immobilized in polylysine-treated microtitre plate wells and the extent of binding of the DCCD-binding protein measured using its antibody. Both subunits bound the DCCD-binding protein effectively (Fig. 50).

EFFECT OF CHEMICAL MODIFICATION OF THE DCCD-BINDING PROTEIN ON ITS REACTION WITH ECF_1

By contrast with its inhibitory effect on the binding of antibody, performic oxidation of methionyl residues of the DCCD-binding protein had little effect on the binding of ECF_1 (Fig. 51, panel A). However, modification of its arginyl residues with phenylglyoxal and 2,3-butanedione reduced the affinity of the DCCD-binding protein for ECF_1 with a relatively small effect on the total ECF_1-binding capacity of the polypeptide (Fig. 52, panel A). Double-reciprocal plots of binding versus concentration gave half-saturation values (under the conditions of this experiment) of 0.26, 0.59 and 0.95 µg for untreated, phenylglyoxal-, and

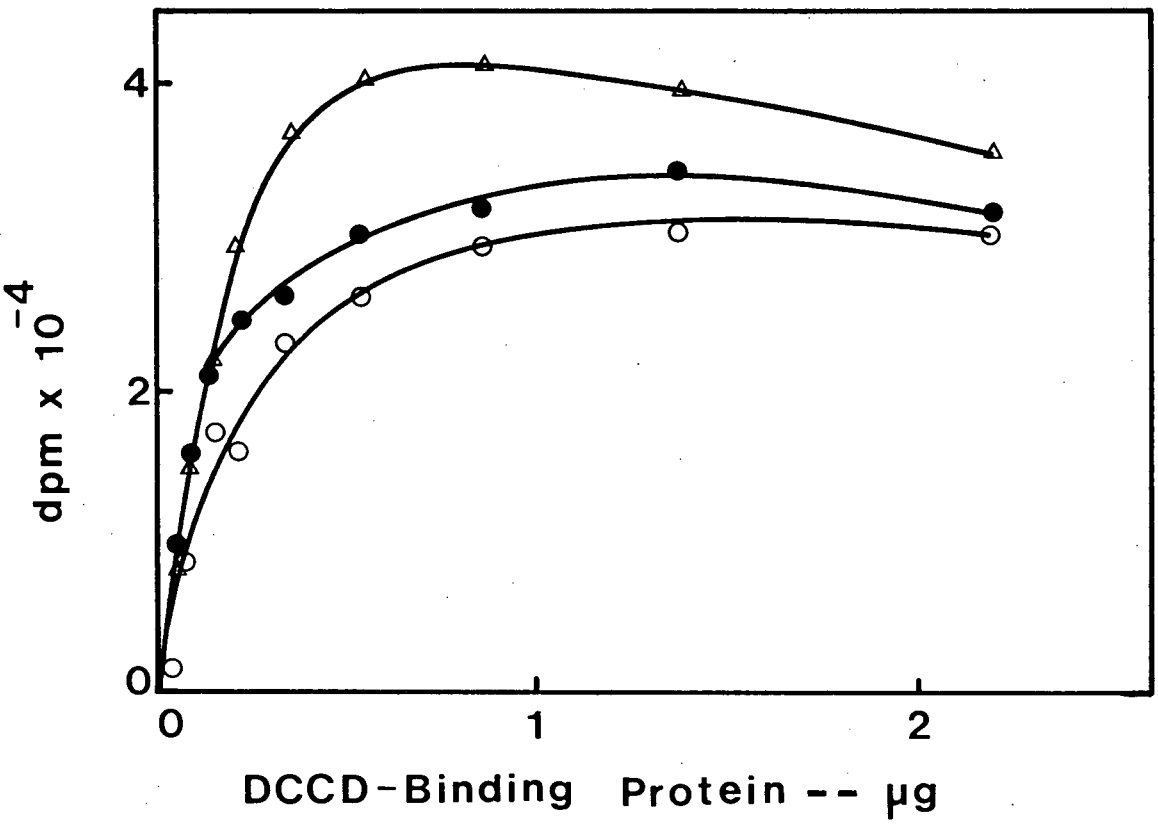


Fig. 50 Binding of DCCD-binding protein to subunits of ECF_1.

See MATERIALS AND METHODS for details of the radioimmune binding assay. Titration of the immobilized ECF_1 (●-●) (19 µg protein), α subunit (Δ-Δ) (6 µg protein) and ß subunit (○-○) (9̊ µg protein) by DCCD-binding protein.

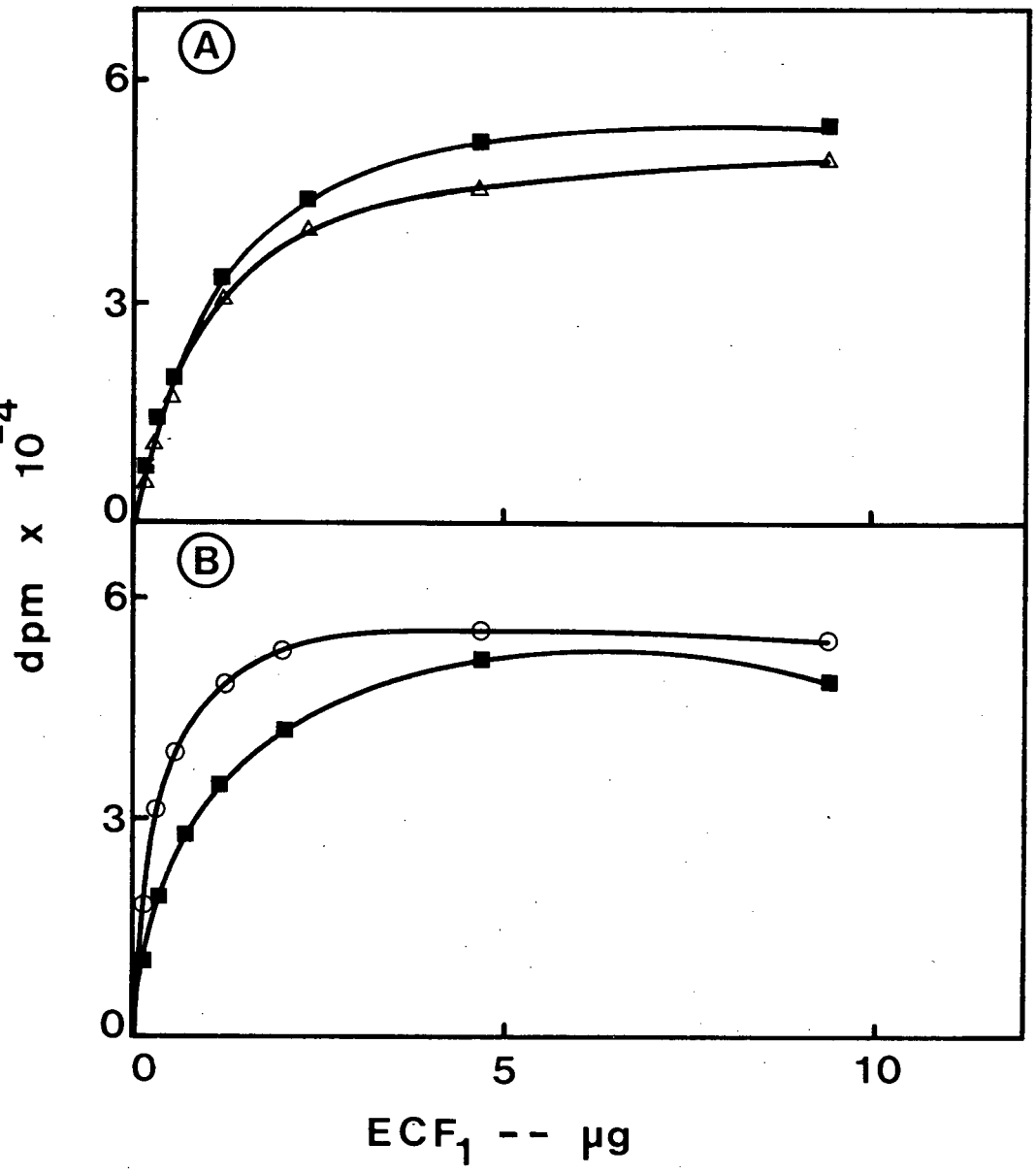
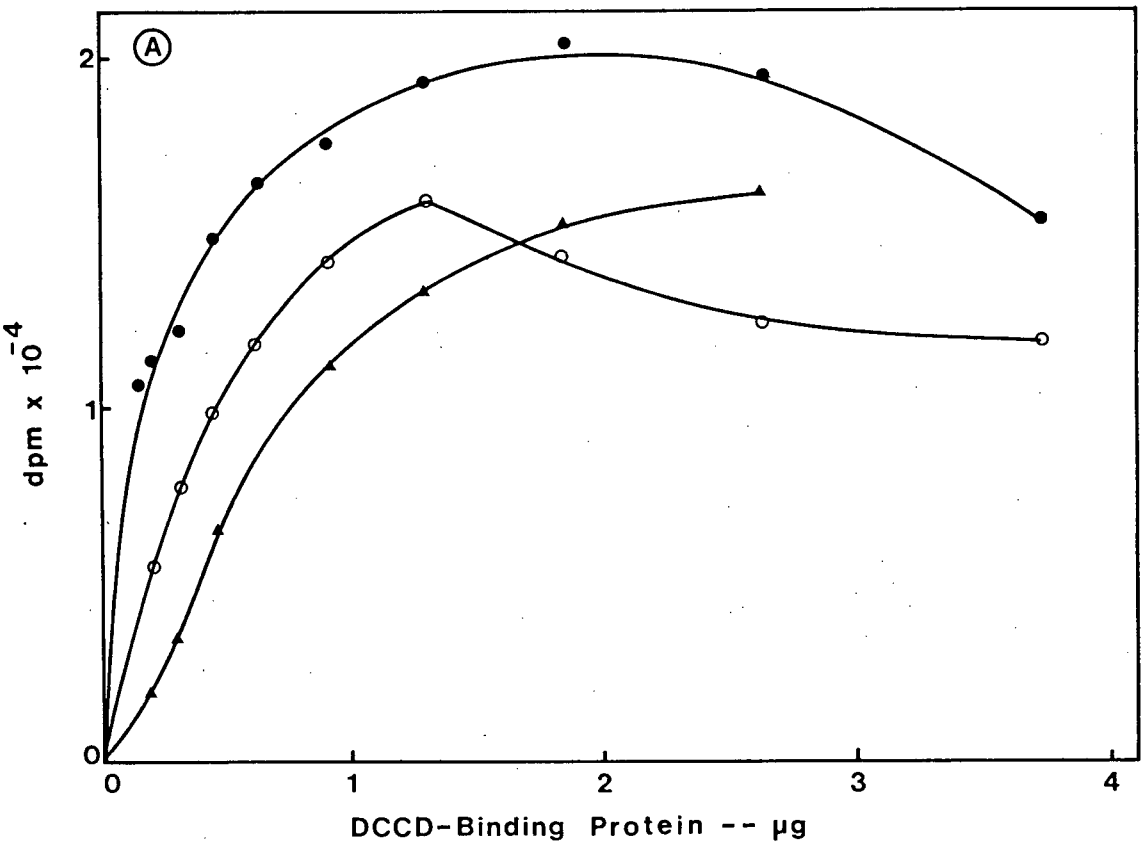


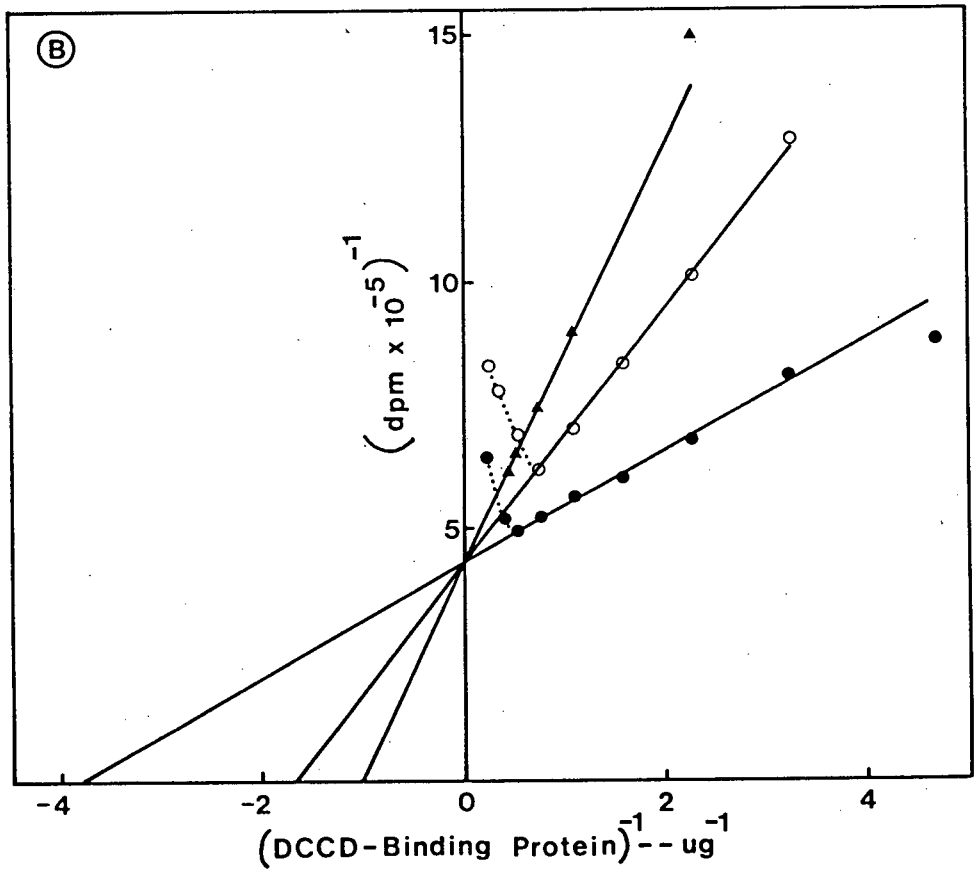
Fig. 51 Effect of chemical modification of the DCCD-binding protein on the binding of ECF_1.

The experimental details are described in the MATERIALS AND METHODS section. The following modification was made. The chemically-modified and the mock-treated DCCD-binding protein (controls) were suspended in 90% formic acid at a protein concentration of 0.13 mg/ml. 25 µl of this mixture (3.3 µg DCCD-binding protein) was immobilized on polylysine-coated microtitre plate wells and the binding experiment carried out as described previously. PANEL A: Titration of the immobilized mock-treated (■-■) or performic acid-treated (△-△) DCCD-binding protein (3.3. µg protein) with ECF_1. PANEL B: Titration of the immobilized mock-treated (■-■) or cyanogen bromide-treated (○-○) DCCD-binding protein (3.3 µg protein) with ECF_1.

Fig. 52 Effect of modification of the arginyl residue(s) of the DCCD-binding protein on the binding of ECF_1.

Details of the experiment are described in MATERIALS AND METHODS. PANEL A: Titration of immobilized ECF_1 (19 µg protein) with mock-treated (●-●), phenylglyoxal-treated (○-○) and 2,3-butanedione-treated (▲-▲) DCCD-binding protein. PANEL B: The above data analyzed as a Lineweaver-Burk plot.


A
dpm x 10^{-4}
DCCD-Binding Protein -- µg


B
$(dpm \times 10^{-5})^{-1}$
$(DCCD\text{-}Binding\ Protein)^{-1}$ -- ug^{-1}

2,3-butanedione-treated DCCD-binding protein, respectively (Fig. 52, panel B). This suggests that ECF_1 binds to the polypeptide in the region of its arginyl residues and is supported by the finding that the binding of ECF_1 to trypsin-treated DCCD-binding protein was significantly reduced (Fig. 49C). These arginyl residues are located in the central polar region (residues 41 to 50) (Fig. 55) of the DCCD-binding polypeptide molecule. This region should remain intact when the polypeptide is cleaved by cyanogen bromide at methionyl residues 17 and 57. As shown in Fig. 51 (panel B), the DCCD-binding protein had increased affinity for ECF_1 following cleavage by cyanogen bromide. Half-saturation of the binding sites on the untreated and cleaved polypeptide under the conditions of the experiments were given by 0.55 and 0.2 μg ECF_1, respectively. The ECF_1-binding capacities of the polypeptides were similar.

EFFECT OF PHENYLGLYOXAL ON THE BINDING OF ECF_1 TO UREA-STRIPPED EVERTED VESICLES

Since *in vitro* binding of ECF_1 to the DCCD-binding protein was reduced by treatment of the polypeptide with phenylglyoxal, it was of interest to determine the effect on intact vesicles. The effect of phenylglyoxal on the binding of ECF_1 to urea-stripped vesicles was examined as follows. Stripped everted vesicles of *E. coli* WS1 and N_{I44} were treated with phenylglyoxal and then washed to remove any unreacted phenylglyoxal. Various amounts of ECF_1 were added to the treated vesicles and the extent of binding of ECF_1 was measured by the increase in ATPase activity of the vesicles. Phenylglyoxal inhibited the binding of ECF_1 to the stripped membranes of the wild-type (*E. coli* WS1) and the mutant (*E. coli* N_{I44}) strains (Fig. 53).

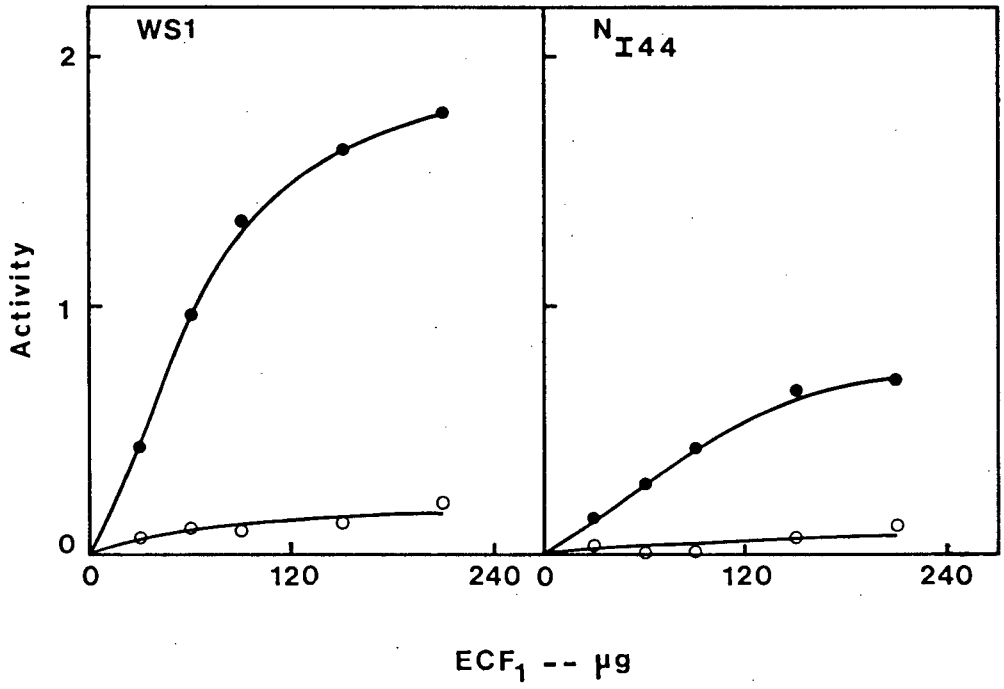


Fig. 53 Binding of ECF_1 to phenylglyoxal-treated vesicles.

Urea-stripped vesicles of E. coli WS1 and N_{I44} were treated with phenylglyoxal as described in MATERIALS AND METHODS. Reconstitution with ECF_1 was carried out as follows: 2 mg samples of the treated membrane vesicles at 10 mg protein per ml in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol were incubated with various levels of ECF_1 at 4°C for 45 min in a final volume of 0.5 ml. The mixture was then diluted 8-10 fold with buffer and the membranes sedimented at 250 000 xg for 2.5 h. The membranes were resuspended in buffer and the ATPase activity in the reconstituted urea-stripped (●-●) and phenylglyoxal-treated (O-O) vesicles determined as described previously. Enzyme activity is expressed as units per mg protein.

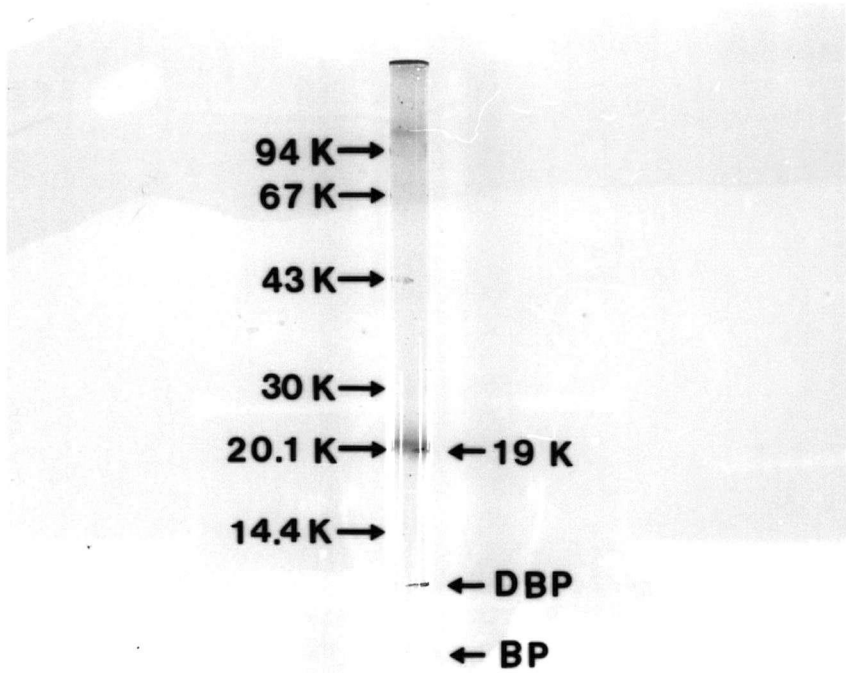
It was possible that the effect of phenylglyoxal on the binding of ECF_1 was not due to the modification of the arginyl residue(s) of the DCCD-binding protein. In order to determine whether the arginyl residues of the DCCD-binding protein were modified, the urea-stripped vesicles of E. coli WS1 were treated with [7-^{14}C]phenylglyoxal. The DCCD-binding protein was extracted from the treated vesicles with chloroform-methanol (2:1) and precipitated with ether. Gel electrophoresis of this material gave three polypeptide bands of molecular weights 96 000, 19 000 and 9 000. The 19 000 dalton polypeptide was stained very intensely with Coomassie Blue (Fig. 54, panel A). However, more than 95% of the total radioactivity recovered from the gel was associated with the protein-staining peak of 9 000 daltons (DCCD-binding protein). The remaining radioactivity was coincident with the peak of 96 000 daltons (Fig. 54, panel B). The product(s) of the reaction of phenylglyoxal with the arginyl residues has been reported to be quite unstable at alkaline pHs (213). This may likely be the cause of the decreased amount of radioactivity recovered from the gel following staining with Coomassie Blue (Fig. 54, panel A). These results suggested that the arginyl residue(s) of the DCCD-binding protein were modified in the stripped vesicles.

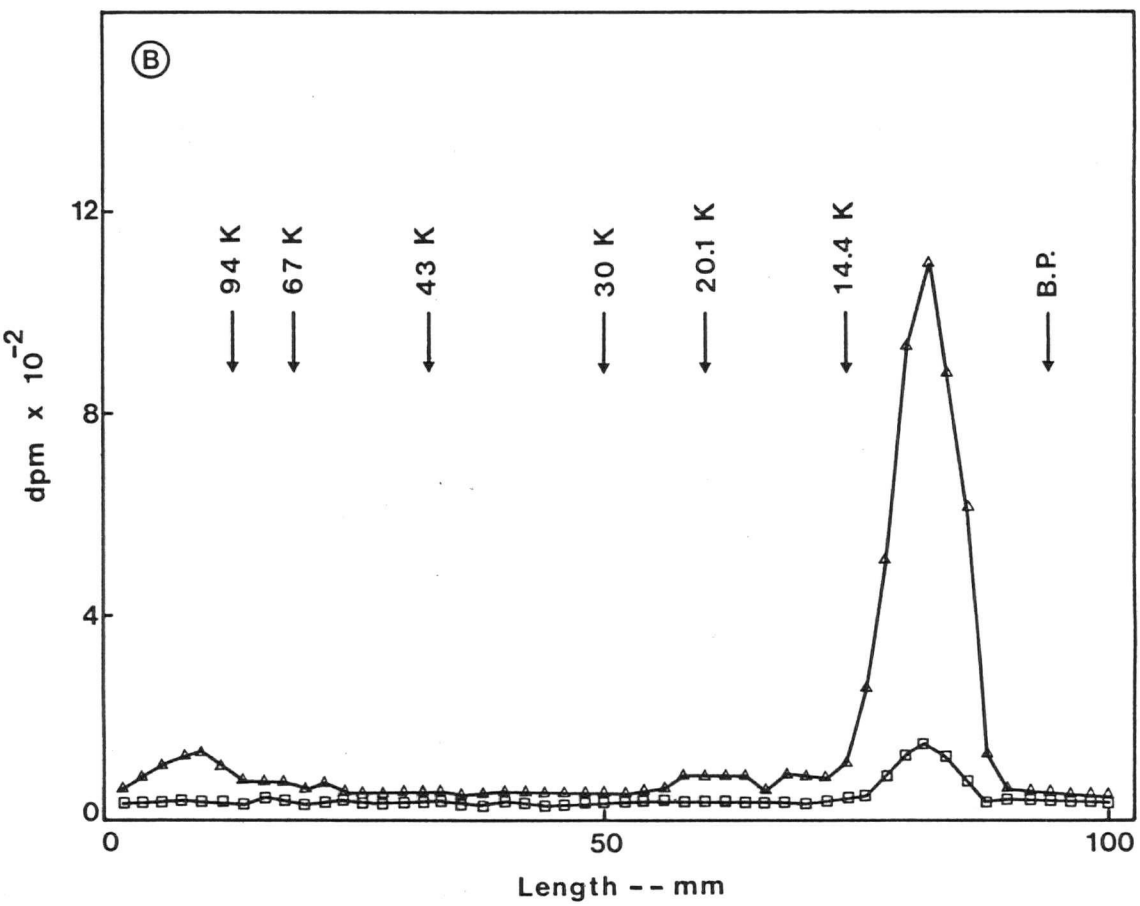
Treatment of the untreated vesicles with chloroform-methanol (2:1) does not normally result in the extraction of the 19 000 dalton polypeptide. Only the DCCD-binding protein is extracted from the membrane. Therefore, the extraction of a 19 000 dalton polypeptide from the phenylglyoxal-treated vesicles suggested that the reaction of the arginyl residue(s) of this polypeptide with phenylglyoxal caused the polypeptide to become quite non-polar. This resulted in its extraction from the membrane with chloroform-methanol (2:1) (Fig. 54B).

Fig. 54 SDS-polyacrylamide gel electrophoresis of the DCCD-binding protein of E. coli labelled with [7-^{14}C]phenylglyoxal.

1.25 ml of urea-stripped everted vesicles of E. coli WS1 (13.5 mg protein per ml) in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10% (v/v) glycerol was mixed with 15 µl of 16.45 mM [7-^{14}C]phenylglyoxal (specific activity, 15.1 mCi/mmol) (final concentration, 195 µM). The reaction mixture was incubated at 20°C for 2 h after which the phenylglyoxal concentration was increased to 34.1 mM with the non-radioactive reagent. The mixture was incubated for another 60 min. The reaction was stopped by addition of 1 volume of 100 mM arginine hydrochloride in buffer. After incubation for another 30 min at 20°C, the reaction mixture was diluted 10-15 fold with buffer and then centrifuged at 250 000 xg for 2.5 h. The sedimented vesicles were resuspended in distilled water (10 ml) and the DCCD-binding protein extracted with chloroform-methanol (2:1) as described in MATERIALS AND METHODS. The ether precipitate containing the DCCD-binding protein was subjected to electrophoresis on an SDS-urea (8M) gel. The gel was fixed with 50% (w/v) TCA for 4-6 h at 20°C, stained with 0.1% (w/v) Coomassie Blue, sliced into 1 mm segments and the radioactivity of each slice determined. PANEL A: Coomassie Blue-stained gel of the [7-^{14}C]phenylglyoxal labelled proteins. The migration positions of the molecular weight marker proteins (M_r, 94 000 - 14 400), the 19 000 dalton polypeptide, the DCCD-binding protein (DBP) and the bromophenol blue (BP) tracking dye are indicated. PANEL B: Distribution of the radioactivity of identical gels which had been cut into 1 mm slices immediately after fixing in 50% (w/v) TCA (▲-▲) or after staining with Coomassie Blue (□-□). BP indicates the position of the bromophenol blue tracking dye. The migration positions of the molecular weight marker proteins (M_r, 94 000 - 14 400) are also shown.

Ⓐ


94 K
67 K
43 K
30 K
20.1 K
19 K
14.4 K
DBP
BP


Ⓑ
94 K
67 K
43 K
30 K
20.1 K
14.4 K
B.P.
dpm x 10^-2
0
4
8
12
0
50
100
Length -- mm

Furthermore, the absence of radioactivity associated with the 19 000 dalton polypeptide indicated the reactive arginyl residue(s) on this polypeptide had a low affinity for phenylglyoxal such that reaction occurred only at a high concentration of phenylglyoxal (34 mM cold phenylglyoxal).

Labelling with phenylglyoxal was also suggested by the brownish-yellow polypeptide band (19 000 dalton) observed when the gel was fixed with 50% TCA prior to staining with Coomassie Blue. It has been reported (215) that the reaction of the arginyl residue(s) of a polypeptide with phenylglyoxal results in an orange-brown product. Therefore, the reactive arginyl residue(s) of the DCCD-binding protein must have a higher affinity for phenylglyoxal.

DISCUSSION

PURIFICATION OF THE ECF_1F_0 COMPLEX

Attempts to purify the ECF_1F_0 complex by gel filtration chromatography only were not successful. The majority of the proteins coeluted with the ATPase activity. In addition, the activity was eluted as a broad peak suggesting that the ATPase complexes were of different molecular weights (Table 11). The enzyme eluted as larger aggregates (M_r, 680 000 - 890 000) in the presence of non-ionic detergents than in the presence of ionic detergents (M_r, 450 000 - 580 000). This difference in the molecular weight of the solubilized enzyme was likely due to the association of the detergent molecules with the membrane proteins to form lipoprotein-detergent complexes as suggested by Tanford and Reynolds (216). Therefore, the size of the eluted enzyme and the broadness of the activity peak will depend on the extent of association of the detergent molecules with the ECF_1F_0 complex and on the CMC of the detergent.

In the present study, the best purification of the ECF_1F_0 complex was achieved by chromatography on Phenyl-Sepharose CL-4B followed by sedimentation of the enzyme at 250 000 xg for 16-17 h. The purified enzyme was judged to be an intact ECF_1F_0 complex. This was determined by the sensitivity of its activity to inhibition by DCCD (Fig. 14) and through labelling studies involving [^{14}C]-iodoacetic acid and 5-iodoacetamidofluorescein. The purified ECF_1F_0 complex consisted of eleven major polypeptides of molecular weight 56 000, 52 000, 32 000, 30 000, 28 000, 22 000, 18 000, 14 000, 12 000, 9 000 and 7 500. Minor bands of 85 000, 71 000 and 24 000 were also present. The polypeptides with molecular weights of 56 000 (α), 52 000 (β), 32 000 (γ), 22 000 (δ), and 12 000 (ε)

were subunits of ECF_1. Labelling studies with [^{14}C]DCCD revealed that the polypeptide of 9 000 daltons was the DCCD-binding protein. The polypeptides of molecular weights 28 000, 18 000 and 14 000 may also be subunits of F_0. The identification of some of these polypeptides as subunits of F_0 was made by comparison with the purification of others. The subunit composition of F_0 obtained in the present study and those obtained by other workers is summarized in Table 15. The DCCD-binding protein (8-9 000 daltons) and the 18-19 000 dalton polypeptides are clearly subunits of F_0. However, it was not clear whether the 28 000, 24 000 and 14 000 dalton subunits were polypeptides of F_0.

Genetic and DNA sequencing evidence (42,128-133) indicates that the ECF_1F_0 complex consists of eight subunits of molecular weights 55 264 (α), 50 157 (ß), 34 100 (γ), 30 258 (a), 19 310 (δ), 17 233 (b), 14 194 (ε) and 8 365 (c), and are coded for by the unc genes listed in Table 4. The polypeptides of molecular weight 30 258 (a), 17 233 (b) and 8 365 (c) are the subunits of F_0, although polypeptide a migrates with an apparent molecular weight of 24 000 on SDS-polyacrylamide gels (138). Foster and Fillingame (94) reported the identical subunit composition to the above in a preparation of the ECF_1F_0 complex. This subunit composition was subsequently confirmed by Friedl et al. (108).

Schneider and Altendorf (107) have also purified the ECF_1F_0 complex and showed that the F_0 also consisted of three subunits. But, in contrast to that found by Foster and Fillingme (94), the F_0 subunits had molecular weights of 28 000, 19 000 and 8 300. The 24 000 dalton polypeptide was present in only a minor amount. In this respect, the ECF_1F_0 complex appears to be similar to that purified in this thesis. However, from the DNA sequence of the unc operon and the known anomalous migration of poly-

Table 15 Subunit Composition of F_0 from Various ECF_1F_0 and F_0 Purifications.

Reference	Source	Subunit composition of F_0 ($M_r \times 10^{-3}$)
Foster and Fillingame (94)	F_1F_0	24; 19; 8.4[a]
Negrin et al (9)	F_0	24; 19; 8.4[a]
Friedl and Schairer (108)	F_1F_0	24; 19; 8.3
	F_0	24; 19; 8.3
Schneider & Altendorf (107)	F_1F_0	28; 19; 8.3[a]
	F_0	19; 14; 8.3
This Thesis	F_1F_0	28; 24; 18; 14; 9[a]

a. This subunit identified as the DCCD - binding protein using [^{14}C] DCCD

peptide a on SDS gels, it is unlikely that the 28 000 dalton polypeptide is a subunit of F_0. Therefore, the question remains as to the identity of the 28 000 dalton polypeptide.

Foster and Fillingame (94) also reported that a 14 000 dalton polypeptide copurified with the preparation of ECF_1F_0 complex only from cells grown in a medium containing succinate, acetate and malate. The 14 000 dalton polypeptide could be the Gene 1 product of the unc operon (128). The presence of a 28 000 and a 14 000 dalton subunit in the ECF_1F_0 complex purified in the present study suggest that the former might be a dimer of the latter. This possibility was first postulated by Schneider and Altendorf (107). They found that urea-treatment of their purified ECF_1F_0 complex consisting of the 28 000, 19 000 and 8 300 dalton polypeptides as the major subunits of F_0 resulted in the disappearance of the 28 000 dalton polypeptide, with a concomitant appearance of a 14 000 dalton polypeptide. The resulting F_0 preparation consisted of the 19 000, 14 000 and 8 300 dalton polypeptides.

The function of the 14 000 dalton subunit is not known, although it has been postulated (128) that it might function in regulating the assembly of the F_0 polypeptides. Schneider and Altendorf (107) also reported that the absence of the 14 000 dalton polypeptide in F_0 preparations resulted in a non-functional F_0 complex. However, recent genetic studies have shown that the 14 000 dalton subunit (Gene 1 product) is not essential for the biosynthesis or the activity of a functional ECF_1F_0 complex (217).

Critical examination of the densitometric scans of the gels of the ECF_1F_0 complex prepared by other workers (105-108) clearly revealed the presence of variable amounts of 28 000 and 14 000 dalton polypeptides. This contrasts with the apparent stoichiometric amounts of these polypep-

tides found in the preparation of ECF_1F_0 complex described in this thesis.

The causes of this inconsistency in the relative amounts and type(s) of subunits of F_0 in the various preparations are not known. It is possible that these differences are a function of the details of the purification procedures (Tables 2 and 3), of the cell strain, of the growth conditions (94), of the oligomeric nature of the F_0 polypeptides (149,155) and of proteolytic digestion (95).

Alternatively, these differences could also be due to the possibility that there are polypeptides which are closely associated with the ECF_1F_0 complex *in vivo*. For example, the polypeptides required by the localized proton hypothesis (218). According to this hypothesis, protons are not extruded into the external medium during membrane energization. Instead, the protons are localized within the bilayer and are transported through specific channels consisting of a series of connecting polypeptides between the respiratory chain and the ATPase complex. Such polypeptides could copurify with the ECF_1F_0 complex.

In conclusion, it is likely that the polypeptides of molecular weight 28 000, 24 000, 18 000, 14 000 and 9 000 are subunits of F_0 or are closely associated with it in the membrane.

SOME MUTANTS OF *E. coli* DEFECTIVE IN PROTON TRANSLOCATION

Removal of ECF_1 from membranes of normal strains of *E. coli* results in the leakage of protons through F_0. As a result, reactions such as the quenching of fluorescence of the dye 9-aminoacridine (Fig. 19), or the energy-dependent transhydrogenation of $NADP^+$ by NADH (23), which require the presence of a transmembrane proton gradient cannot occur. The *unc*

mutants of E. coli N_{I44} and CBT-302 were isolated as ATPase-deficient mutants (Table 7). Although the membranes of these mutants were found to be lacking ECF_1 (Table 12), they were still capable of maintaining a transmembrane proton gradient (Fig. 20). Several explanations for this are possible. (i) Although intact ECF_1 is absent in these mutants, a subunit of the ECF_1 could still be present and prevent the leakage of protons through F_0. Mutants in which only the ß or δ subunits of ECF_1 are retained by the membrane (following removal of ECF_1) without it becoming leaky have been described (219-221). (ii) F_0 or the DCCD-binding protein could be absent from the mutant membranes. (iii) The aspartyl residue of the DCCD-binding protein with which DCCD reacts specifically to block proton translocation through F_0 has been replaced (159-161). Mutants in which this aspartyl residue has been replaced by a glycyl or glutamyl residue are defective in proton translocation (Table 6). (iv) A polypeptide of F_0 other than the DCCD-binding protein and which is involved in the proton channel could be absent from or modified in the mutant membranes. These possibilities were investigated in the unc mutants, E. coli N_{I44}, CBT-302 and AN 382. Although the membranes of E. coli AN 382 contain an active ECF_1, the membrane cannot be energized through the hydrolysis of ATP, suggesting that there is a defect in the F_0 portion of the ATPase complex.

The extensive stripping procedures used to remove ECF_1 and subunits of ECF_1 resulted in the loss of the ability of the normal membranes (E. coli WS1) to set up and maintain a proton gradient. By contrast, none of these treatments destroyed the capacity of the mutant membranes to generate and maintain a proton gradient. Therefore, it was unlikely that retention of individual subunits of ECF_1 on the membranes of these mutants was responsible for the relative impermeability of these membranes to protons.

The absence of the α, ß, δ and ε subunits of ECF_1 on the membranes of E. coli N_{I44} and CBT-302 was confirmed by comparing their polypeptide composition with that of the parent strains, E. coli WS1 and CBT-1.

The presence of a [^{14}C]DCCD-binding protein of 8 000 daltons in the membranes of these mutants, and the similarity of the amino acid compositions to the parent strains (Table 13), indicated that the DCCD-binding protein occurred in these three mutants and that the DCCD-reacting aspartyl residue was present. Therefore, the defect(s) in F_0 responsible for the relative impermeability of these three mutant membranes to protons was likely to be on the other subunits(s) of F_0 (polypeptides a and b). Analysis of the membranes of these mutants by two-dimensional isoelectric focusing gel electrophoresis revealed that the 18 000 dalton polypeptide b of F_0 was missing in the mutant, E. coli N_{I44} (Fig. 27). This difference was not observed in the membranes of the unc mutants, E. coli, AN382 and CBT-302. However, changes in this polypeptide which did not affect its molecular weight or its net charge could not be excluded.

Recently, Friedl et al. (222) have reported the isolation of an unc mutant of E. coli which contained only the 24 000 dalton (polypeptide a) and the DCCD-binding protein (polypeptide c) of the ECF_1F_0 complex. The absence of the 18 000 dalton subunit (polyeptide b) of F_0 in this mutant drastically reduced the permeabiity of the membranes to protons. Thus, the mutant, E. coli N_{I44} appears to be similar to that isolated by Friedl et al. (222). From the arrangement of the genes of the unc operon, it would appear either that E. coli N_{I44} is a deletion mutant with only the proximal unc B and E genes (coding for polypeptide a and c of F_0) of the whole operon being retained or there is a polar effect of the mutation on the expression of the distal genes of the operon.

Therefore, these results suggest that the DCCD-binding protein and polypeptide a are insufficient to form a functional proton channel through the membrane. However, it is not known if polypeptide b forms part of the proton channel or acts indirectly by influencing the conformation of the DCCD-binding protein and/or of polypeptide a of F_0.

Fillingame et al. (204) have recently characterized the defect in the unc mutant E. coli AN382 by genetic techniques. It was shown that the lesion responsible for relative impermeabiity of the membranes to protons in this mutant was due to an amber-suppressible, chain-terminating mutation in the unc B gene that resulted in the loss of the 24 000 dalton subunit (polypeptide a) from the F_0 complex.

Although the membranes of E. coli CBT-302 did not contain any subunits of ECF_1, no change(s) in the composition of F_0 could be detected. Thus, the nature of the lesion responsible for proton impermeability in E. coli CBT-302 could not be characterized.

Therefore, the picture which emerges from the studies on the unc mutants, E. coli N_{I44} and AN 382 is that all three subunits of F_0 (polypeptides a, b and c) are required for a functional proton channel and this has recently been confirmed by the results of Friedl et al. (232). This is also supported by the finding that an unc mutant containing only the 24 000 dalton subunit (polypeptide a) of F_0 was relatively impermeable to protons (140). This picture is complicated by the observation that another unc mutant of E. coli containing all three subunits of F_0 (a, b and c) was also relatively impermeable to protons (140). Apparently, the presence of all three subunits of F_0 is not adequate to form a functional proton channel through the membrane. Cox et al. (140) have suggested that only the proper assembly of all three subunits in the membrane will result in

functional proton channel and that proper assembly of F_0 requires the involvement of the α and/or ß subunit of ECF_1. But the presence of the α and ß subunits in the membranes of E. coli AN382 suggests that mutations in the polypeptide(s) of F_0 can also affect the assembly of a functional proton channel.

The ability of the F_0 of E. coli AN382 to bind ECF_1, although more weakly than in the wild-type strain, in the absence of the 24 000 dalton subunit of F_0 suggests that this polypeptide is not necessary for the binding of ECF_1 to the membrane. This is supported by the finding that the membranes of another unc mutant containing only the 24 000 dalton subunit of F_0 was incapable of binding ECF_1 (140). Therefore, the ability of the isolated membranes of E. coli N_{I44}, containing only the 24 000 and 9 000 dalton subunits of F_0, to bind purified ECF_1 suggests that the DCCD-binding polypeptide is responsible for binding ECF_1 to the membrane. The absence of the 24 000 or the 18 000 dalton polypeptide in the mutant affected only the affinity, but not the capacity of the membrane for ECF_1. The decreased affinity of the mutant membranes (half saturation of binding for E. coli WS1, 0.55 mg ECF_1; E. coli N_{I44}, 1.10 mg ECF_1) could be explained on the basis that the other F_0 subunits most likely influence the conformation of the DCCD-binding protein. Alternatively, the DCCD-binding protein could affect the conformation of the missing polypeptide, thus causing it to interact with ECF_1.

Therefore, the membranes of the unc mutants, E. coli N_{I44}, AN382 and CBT-302 could still bind ECF_1 but not with as high an affinity as in the wild-type strain (E. coli WS1). Studies on the unc mutants, E. coli AN382 and N_{I44} suggest that all three subunits of F_0 may be needed for optimal ECF_1-binding affinity and are absolutely required for a functional proton

channel.

ORIENTATION OF THE DCCD-BINDING PROTEIN IN THE MEMBRANE

An understanding of the arrangement of the polypeptides of F_0 in the membrane is obviously an important prerequisite for determining the mechanism of proton translocation through F_0. The orientation of the DCCD-binding protein in the membrane of E. coli was studied by using antiserum against this polypeptide.

The antiserum to the DCCD-binding protein blocked the leakage of protons through F_0 in urea-stripped everted vesicles suggesting that the DCCD-binding protein was exposed on the cytoplasmic surface of the cell membrane (Fig. 28A). Competitive inhibition assays confirmed that the antibodies to the DCCD-binding protein reacted preferentially with the cytoplasmic surface of the cell membrane.

Although the antibody was raised against the purified DCCD-binding protein, it could still recognize and bind to this polypeptide when it was assembled as an oligomer in the membrane. The antibody is presumably recognizing a limited amino acid sequence in the molecule since it is unlikely that the isolated polypeptide would retain its native oligomeric structure following extraction by chloroform:methanol (2:1). The amino acid sequence of the DCCD-binding polypeptide exposed on the cytoplasmic surface of the cell membrane and which is recognized by the antibody is not known.

However, in vitro studies with purified DCCD-binding protein have shown that the oxidation of methionyl residues in the isolated polypeptide almost completely abolished its ability to react with the antibody. Cleavage of the polypeptide at the methionyl residues with cyanogen bromide

had a similar effect. These results are consistent with the antibody-binding site being close to one or more methionyl residues. If the binding-site on the isolated polypeptide is the same as that of the membrane-bound polypeptide, then the most likely methionyl residue is that at position 57 (Fig. 55). This assignment depends on the validity of the evidence that tyrosyl residues 10 and 73 are on the periplasmic surface of the membrane (223). Modification of arginyl residues of the DCCD-binding protein (in the isolated or membrane-bound form) with phenylglyoxal and 2,3-butanedione had little effect on the binding of the antibody (Fig. 43). Thus it is unlikely that the antibody reacts with residues 41 to 50 of the polar segment of the polypeptide molecule. On this basis, the DCCD-binding protein molecule must have a looped arrangment in the membrane and the other methionyl residues at position 6, 11, 16, 17, 65 and 75 could not be close to the cytoplasmic surface of the membrane.

The exposure of the DCCD-binding protein on the external (periplasmic) surface of the cell membrane could not be unambiguously determined. Addition of valinomycin to K^+-loaded "right-side out" vesicles of E. coli DL-54 resulted in an efflux of K^+ concomitantly with a compensatory influx of protons primarily through the ECF_1-depleted F_0 proteins. This influx of protons was inhibited by DCCD. The total amount of protons as well as the rate of proton uptake was inhibited by DCCD to a maximum of 65%. By contrast, antiserum to the DCCD-binding protein did not reduce the total amount of protons taken up by these vesicles, but did reduce the rate of proton uptake to a maximum of 30% (Fig. 33).

The absence of an effect on the movement of the total amount of protons could be due to several causes. (i) The DCCD-binding protein may not be exposed on the external surface of the cell membrane. However, the

results of Schneider et al. (223) indicate that the amino- and carboxy-terminals of this polypeptide are exposed on the external surface. (ii) The determinants of the DCCD-binding protein on the external surface are inaccessible to the antibodies. (iii) The portion(s) of the DCCD-binding protein exposed on the external surface may be so weakly antigenic that the antiserum does not contain antibodies to them. (iv) The antibodies did bind to the portion of the molecule exposed on the external surface without affecting proton translocation.

The effect of the antiserum to the DCCD-binding protein on the rate of proton movement could also be due to several causes. (i) Antibodies to the determinants on the DCCD-binding protein exposed on the external surface of the membrane are present. (ii) There may be a re-orientation of the ATPase complex to the external surface during preparation of the membranes. (iii) The "right-side out" vesicles may be contaminated with a population of "inside-out" vesicles. Energetically, this appears to be more feasible than (ii). However, Wickner (210), Adler and Rosen (211) and Owen and Kaback (212), have suggested that the re-orientation event occurs readily. The extent to which this occurs seems to vary between workers and perhaps is a function of the cell strain and of the details of the procedures used to prepare the vesicles. Thus one explanation of the above results is that the antibody is reacting preferentialy with the DCCD-binding protein exposed on the cytoplasmic surface of the cell membrane of E. coli and that the binding to right-side out vesicles is due to the reaction with re-orientated ATPase complexes. Recently, van der Plas et al. (224) reported that 31% of the ATPase enzyme re-orientated to the external surface in right-side out vesicles. This would account for the results that "right-side out" vesicles of E. coli WS1 and ML308-225 (Fig. 42 A) were only one-third as effective

in competing for the antibody to the DCCD-binding protein compared with everted vesicles.

It is likely that the problems associated with the determination of the antigenic sites of the DCCD-binding protein which are exposed and the possible re-orientation of the ATPase enzyme across the membrane may only be solved by using monoclonal antibodies.

INTERACTION OF THE DCCD-BINDING PROTEIN WITH ECF_1

The amino acid sequence of the DCCD-binding protein is suggestive of structure. Four regions of the molecule can be recognized (Fig. 55) (146,150). Seven amino-terminal, predominantly polar amino acids are followed by a non-polar region (residues 8 to 32). Amino acids 34 to 52 are predominantly polar. The fourth region (residues 53 to 79) is non-polar. It has been suggested by Altendorf et al. (150) that the two non-polar regions of the molecule are transmembranous, forming a looped structure in which the middle polar region is exposed at the cytoplasmic surface of the membrane. The looped arrangement is supported by the findings that (i) replacement of amino acyl residue 28 (with isoleucine or valine, Table 6) in the first non-polar segment influenced the reaction of DCCD with aspartyl-61 in the other non-polar segment (162), (ii) tyrosyl residues 10 and 73 were accessible on the periplasmic (outer) surface of the cell membrane (223), and (iii) antiserum against the DCCD-binding protein blocked the leakage of protons through F_0 in ECF_1-stripped everted membrane vesicles (Fig. 28A). The accessibility of the DCCD-binding protein at the cytoplasmic surface suggested that it could react with ECF_1.

In contrast to the effect of modification of amino acyl residues of the DCCD-binding protein on its reaction with antibodies, the effect on the

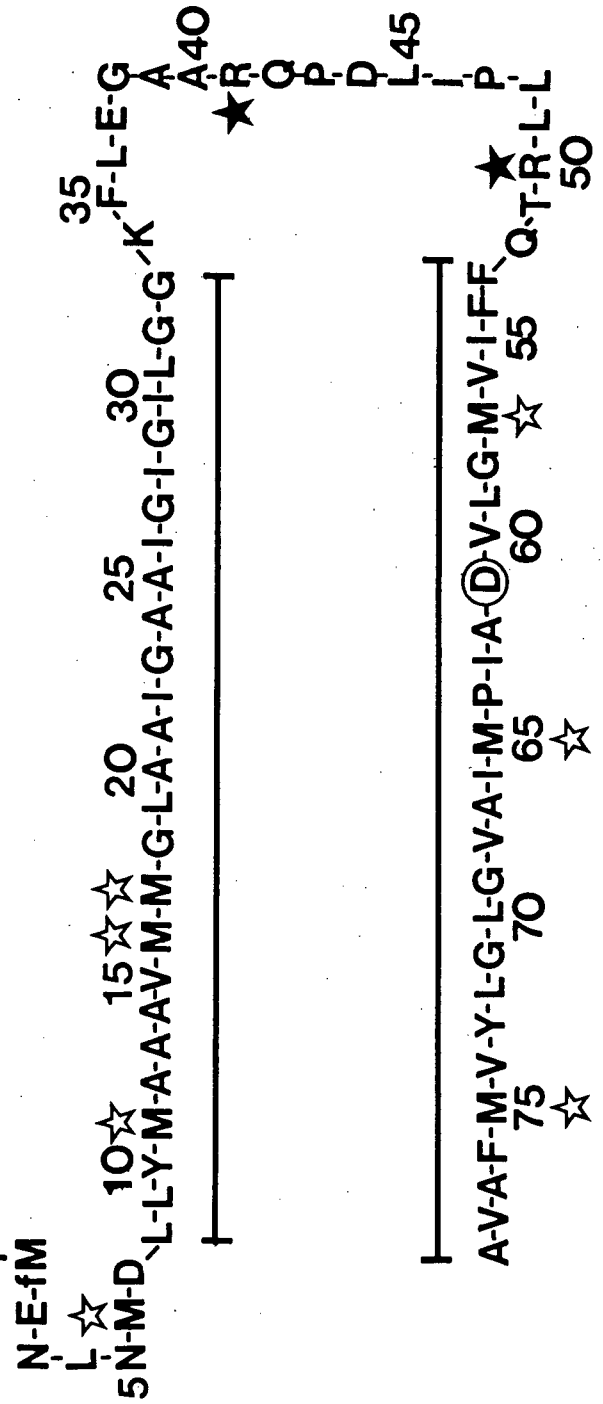


Fig. 55 Amino acid sequence of the DCCD-binding protein of E. coli (146)

The amino acid sequence of the DCCD-binding protein is drawn as a "hairpin" loop. The two nonpolar regions of the molecule are shown. DCCD is bound to the circled aspartyl residue. The methionyl (☆) and arginyl (★) residues of the polypeptide are also indicated.

binding of ECF_1 was different. Modification of the arginyl residues of the DCCD-binding protein with phenylglyoxal 2,3-butanedione (Fig. 52), or treatment of the DCCD-binding protein with TPCK-trypsin (Fig. 49C), reduced the binding of ECF_1 to the polypeptide. However, oxidation of the methionyl residues of the DCCD-binding protein, or cleavage of the chain at these residues with cyanogen bromide, did not affect the reaction of ECF_1 with the polypeptide (Fig. 51). Therefore, the ECF_1-binding site must be near residues 41 to 50 in the polar segment of the polypeptide. This site is consistent with the "looped model" in which this region is proposed to be at the cytoplasmic surface of the cell membrane (223). The independence of the ECF_1-binding and the antibody-binding sites was confirmed directly. Binding of ECF_1 to the DCCD-binding protein did not prevent the binding of the antibody. The reverse was also true (Fig. 47). However, it was shown (Fig. 29) with everted membrane vesicles that the binding of antibody interfered with the rebinding of ECF_1 to ECF_1-stripped membranes. The most likely reason is that the antibody-binding site is closer to the ECF_1-binding site in the oligomeric form of the DCCD-binding protein present in the membrane (5,146). Such conformational differences of the DCCD-binding protein *in vivo* and *in vitro* is also suggested by the lower level of inhibition by antiserum to the DCCD-binding protein in the binding of ECF_1 to the membranes of the *unc* mutants, *E. coli* N_{I44} and CBT-302 (Fig. 29). As discussed earlier, the polypeptides of F_0 may not be correctly assembled in these two mutants.

The significance of the binding of ECF_1 by the isolated DCCD-binding protein is unclear. Evidence for the *in vivo* involvement of the DCCD-binding protein in binding F_1-ATPase to the membrane comes from the purification of the F_1F_0 complex from *Clostridium pasteurianum* (16) and

from the chloroplast (225). In both cases, the DCCD-binding protein was the only subunit of the F_0 complex. It was mentioned previously that arginyl residues are involved in the interaction of ECF_1 with the isolated DCCD-binding protein, and they also appear to be involved in the interaction of ECF_1 with F_0 in everted vesicles. Treatment of ECF_1-stripped everted vesicles with phenylglyoxal, an arginyl-modifying reagent, almost completely abolished the rebinding of ECF_1 in both the wild-type (E. coli WS1) and the mutant strain (E. coli N_{I44}) (Fig. 53). Labelling of stripped everted vesicles with [7-^{14}C]phenylglyoxal modified the arginyl residue(s) of the DCCD-binding protein (Fig. 54). This suggests that the binding of ECF_1 to the isolated DCCD-binding protein may be physiologically significant. Binding to the polypeptide involved the α and/or ß subunits of ECF_1 since both of these subunits could bind independently. This is not surprising in view of their sequence homology (226). However, the δ and ε subunits of ECF_1 have also been implicated in the binding of ECF_1 to the membranes of E. coli (6,63). These subunits were not required for the interaction with the isolated DCCD-binding protein. Mutants have been isolated in which the ß (unc D) or δ (unc H) subunits are retained by the membranes in the absence of other subunits (219-221). The polypeptide(s) with which the ß or δ subunits interact has not been identified, but clearly ECF_1 is able to form linkages with F_0 not involving the δ and ε subunits. Recently, Andreo et al. (227) have concluded also that the δ subunit of chloroplast F_1 is not absolutely required for binding to the membrane, but is required to block the leakage of protons through F_0. Therefore, it is becoming clearer that ECF_1 may not be attached to the membrane solely by a "stalk" consisting of the δ and ε subunits (19,20). It has been suggested instead (231), that a large portion of the ECF_1

molecule is embedded in the membrane and that the extent to which it is embedded is regulated by the energy state of the cell.

Walker et al. (228) have proposed that ECF_1 is linked via its δ and ε subunits to polypeptide b of F_0. The amino acid sequence of polypeptide b suggests that the molecule is composed of two α-helical regions which are anchored by the amino-terminal sequence of non-polar amino acids. The helices are suggested to provide a pathway for conduction of protons to ECF_1 (132,228). Walker et al. (228) also quoted unpublished work of Hoppe et al. (229) which suggests that this polypeptide of F_0 is particularly sensitive to degradation by proteases. Similar results (Fig. 40) have been obtained in my studies. The immunoprecipitate obtained from the ECF_1-reconstituted trypsin-treated vesicles did not contain a 18 000 dalton polypeptide indicating that it had been removed by proteolysis. Hoppe et al. (233) have recently confirmed that trypsin-treatment of stripped-everted vesicles of E. coli results in the proteolytic degradation of the 18 000 dalton (polypeptide b) of F_0.

In the present study, trypsin-treatment of ECF_1-stripped everted vesicles of E. coli did not prevent the rebinding of ECF_1. The rebound ECF_1 was still sensitive to DCCD. However, even following rebinding of ECF_1, F_0 was still leaky to protons. (A somewhat similar behaviour was observed with trypsin-treated submitochondrial particles (230).) One possible explanation of these data is that in the absence of polypeptide b, destroyed by protease treatment, the proton pathway through F_0 and the δ and ε subunits of ECF_1 is disrupted. In this case, binding of ECF_1 to F_0 could be explained by the interaction of the α and/or ß subunits with the DCCD-binding protein. The involvement of the DCCD-binding protein in the proton pathway is supported by the fact that the antibody to this polypep-

tide will also block proton leakage even in the trypsin-treated vesicles (Fig. 35).

In summary, the results presented in this thesis are consistent with the looped arrangement of the DCCD-binding protein in the membrane, as proposed by Altendorf et al. (150), in which the polar central region of this molecule is at the cytoplasmic surface of the cell membrane. This region may interact with the α and/or ß subunits of ECF_1. Additional linkage(s) to the F_0 may involve the interaction of the δ and ε subunits of ECF_1 with polypeptide b of F_0 as proposed by Walker et al. (228).

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