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PURIFICATION AND CHARACTERIZATION OF CHLOROPLAST COUPLING FACTOR FROM WHEAT

by

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ABSTRACT

Chloroplast coupling factor (CF_1) from thylakoids of wheat (*Triticum aestivum*, var Thatcher) was purified by chloroform extraction and subsequent sucrose density gradient centrifugation. The wheat enzyme, in common with most coupling factors, contains five subunits, designated α, β, γ, δ and ε, of molecular weights 57, 55, 39, 25 and 13 kd respectively. Inclusion of proteolysis inhibitors in all steps of the purification prevented the loss of the two smallest subunits during the sucrose gradient step.

Although proteolysis inhibitors did not interfere with the extraction of CF_1 by chloroform, they completely prevented CF_1 release from the thylakoid by the hypotonic sucrose method of Strotmann et al. (1973). However, CF_1 release by hypotonic sucrose did not appear to require a proteolytic event in any CF_1 subunit, as no difference in apparent molecular weight of any CF_1 subunit was observed when CF_1 was released by this method.

A double band or apparent mw's 37 and 39 kd for the γ subunit in SDS-PAGE was identified as reduced and unreduced forms of the same subunit, suggesting an internal disulfide bridge in the γ subunit of wheat CF_1.

The latent CaATPase of wheat CF_1 is activated by trypsin digestion but not by heat, unlike spinach CF_1. Wheat CF_1 hydrolyzes GTP at 32% the rate of ATP, but no other nucleotide triphosphate is effective as a substrate. A 1:1 ratio of Ca:ATP gave optimal ATPase activity. K_M and V_{max} were determined in the presence of a large excess of calcium (10 mM) and with an equimolar amount of calcium and ATP. In the presence of excess calcium, a K_M of 0.125 mM CaATP and a V_{max} of 18.9 units/mg

protein were obtained. Under conditions of equimolar calcium and ATP, the K_M was 0.018 mM CaATP and the V_{max} was 6.77 units/mg protein. The order of magnitude difference in K_M's is thought to be due to an alteration of affinity of the enzyme for substrate by the presence or absence of excess calcium.

Electron microscopy of negatively-stained wheat CF_1 particles showed a solid hexagonal particle. *Escherichia coli* F_1 gave a similar structure. Markham rotation supported the conclusion that wheat CF_1 exhibits a six-fold symmetry.

Antibodies to wheat CF_1 were generated by injecting the purified enzyme into a rabbit. The resulting antiserum both precipitated wheat CF_1 and inhibited its latent, trypsin-activated CaATPase. The serum cross-reacted with spinach CF_1, showing that there is considerable antigenic similarity between the two enzymes. Crossed immunoelectrophoresis showed that this antiserum reacts specifically with the α and β subunits of wheat CF_1.

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INTRODUCTION

The "common currency" of energy in living cells is the molecule adenosine triphosphate (ATP). The phosphodiester bond between the β and γ phosphates of this molecule is of sufficient energy that its cleavage can provide energy to drive the many processes of the living cell (Lipmann, 1946). The mechanism of ATP synthesis and the organization of enzymes involved in this process now appear to be remarkably similar among the many living systems studied.

By 1960 it was known that chloroplasts (Arnon et al., 1954) and mitochondria (Kennedy and Lehninger, 1948) are the sites of photosynthetic and oxidative phosphorylation of ATP in plants and animals. These organelles each contain membrane-bound compartments and an electron transport chain. At certain points in the electron transport chains, the passage of electrons is tied to ATP synthesis. It was postulated that a high energy chemical intermediate produced at these sites donated its energy for the synthesis of ATP (Slater, 1953).

In 1961, the English biochemist Peter Mitchell proposed a radically different scheme for the synthesis of ATP. In the chemiosmotic hypothesis (Mitchell, 1961), he postulated that the flow of electrons through the electron transport chain carried with it protons, delivering them to one side of a membrane. This results in the accumulation of protons on one side of the membrane, and the formation of an electrochemical potential gradient across the membrane. The dissipation of this gradient by the leakage of protons back across the membrane was proposed to be the driving force for ATP synthesis. Critical to this hypothesis is the membrane across which the electrochemical potential gradient is generated, and the assym-

metrical arrangement of components of the electron transport chain, resulting in the accumulation of protons on one side of the membrane.

In chloroplasts, the first evidence for a proton gradient driving ATP synthesis came in 1966 from the work of Jaegendorf and Uribe. Chloroplasts, transferred from high pH to low pH solutions in the dark, synthesized ATP when ADP and inorganic phosphate were provided. A pH gradient across the thylakoid was sufficient driving force for ATP generation.

Over the past twenty years, much evidence has been accumulated to support the chemiosmotic hypothesis of ATP synthesis, although the mechanism by which ATP is made in conjuction with the dissipation of the electrochemical potential gradient is still incompletely understood. For six views on this subject by six scientists active in this field, see the multiauthored review by Boyer et al.(1977).

The coupling of ATP synthesis to the electron transport-generated electrochemical potential gradient is through an enzyme complex which is dubbed "coupling factor" or "F_1". In both chloroplasts and mitochondria, it has been shown that extraction with an EDTA solution removes a protein component, resulting in uncoupling (Jaegendorf and Smith, 1961; Penefsky et al.,1960). When this coupling factor is added back to the deficient membranes in the presence of an appropriate divalent cation, electron transport-dependent ATP synthesis is resumed (Avron, 1963; Penefsky et al., 1960) under appropriate conditions.

The solubilized coupling factor will catalyze the reverse reaction to ATP synthesis, that is, ATP hydrolysis. For the mitochondrial F_1, ATPase activity is manifest in the soluble enzyme (Pullman et al., 1960), but the activity of the chloroplast enzyme is exhibited only after a short digestion

with trypsin, or by heating to 65°C for two minutes (Vambutas and Racker, 1965).

A number of different ATPase activities are associated with chloroplast coupling factor (CF_1), as demonstrated by the fact that separation of the activity from the thylakoid results in a loss of coupling, and reassociation of this protein with the thylakoid restores it. Baltscheffsky (1959) and Wessels and Baltscheffsky (1960) first showed that a Mg-dependent ATPase activity was associated with thylakoids. Avron (1962, 1963) demonstrated a MgATPase extracted from thylakoids by EDTA solution. Vambutus and Racker (1965) prepared a stable protein fraction which showed a CaATPase activity after either a short trypsin digestion, or two minutes heating at 65°C. Farron (1970) and Farron and Racker (1970) characterized the properties of the heat-activated CaATPase. By heat activiation at pH 6.0 in maleate buffer and 8 mM $MgCl_2$, Nelson et al. (1972a) obtained a MgATPase activity from soluble CF_1.

In vivo, CF_1 is attached to the outer surface of the thylakoid membrane. It has been identified as the knob-like projections seen on the surface of thylakoids that have been negatively stained (Lein and Racker, 1971a). These knobs can be removed by a number of treatments known to uncouple chloroplasts, such as EDTA wash (Howell and Moudrianakis, 1967) or hypotonic sucrose wash (Miller and Staehelin, 1976). Miller and Staehelin (1976) demonstrated, by freeze-fracture techniques, that CF_1 projections on the thylakoid surface are limited to those regions which are not stacked. In the mitochondrion, F_1 has also been identified with knob-like projections that can be removed by procedures known to remove F_1. In the native conformation of mitochondrial membranes, F_1 is attached to the inner surface of the inner mitochondrial membrane, projecting into the mitochondrial matrix. Protons

are pumped by the electron transport chain into the intrathylakoid space of chloroplasts, while in mitochondria, protons are pumped out of the matrix. The difference in direction of proton pumping explains the different orientation of these two coupling factor complexes.

The coupling factor complex binds to an intrinsic membrane complex, denoted F_0. Together F_0-F_1 comprises a complex which contains both the proton channel by which the cross-membrane proton gradient is dissipated in a controlled manner, and the enzyme which utilizes the electrochemical potential gradient to synthesize ATP. CF_0 has been purified and reconstituted into phospholipid vesicles. When CF_1 was added to these vesicles, ATP was synthesized in response to an acid-to-base transition (Pick and Racker, 1979). When the proton-translocating chromatophore bacteriorhodopsin was incorporated into CF_0-containing vesicles, CF_1 binding gave vesicles which catalyzed light-dependent ATP formation (Younis and Winget, 1977). The number of polypeptides in CF_0 is unclear. Three or four CF_0 polypeptides have been reported (Younis and Winget, 1977; Pick and Racker, 1979; Ellenson et al., 1978), although one polypeptide, of mw 8300, when reconstituted into phospholipid vesicles, functioned as a proton pore that could be blocked by the uncoupler dicyclohexylcarbodiimide (DCCD) (Nelson et al., 1977). The other polypeptides in the CF_0 complex may act as regulatory or CF_1-attachment components.

The CF_1 complex is released from the thylakoid by a number of different treatments, including washing in EDTA (Lien and Racker, 1971b), hypotonic sucrose (Strotmann et al., 1973), or silicotungstic acid (Lien and Racker, 1971a), and emulsifying a thylakoid suspension in chloroform (Younis et al., 1977). Once released from the thylakoid, CF_1 is cold labile unless cryoprotected in a number of ways. Lability is characterized by a loss of

CaATPase activity, due to dissociation of the complex. CF_1 can be stored at 4°C only in a saturated solution of ammonium sulfate. Upon freezing, CF_1 is cold-labile unless stored in the presence of 2 mM ATP and sucrose or glycerol (Lien et al., 1972). Reassociation of CF_1 with the thylakoid to give restoration of photophosphorylation requires divalent cations, and it appears that both EDTA and hypotonic sucrose wash methods for CF_1 extraction act by removing those critical cations required for the binding of CF_1 to the thylakoid. Silicotungstic acid is a chaotrope, and it probably acts by altering the hydrophobic interactions between CF_1 and its membrane-associated link, permitting the solubilization of the complex. A new method for the solubilization of F_1 by chloroform treatment has proven useful. Beechey et al. (1975) found that F_1 could be extracted from bovine heart mitochondria by emulsifying them in chloroform. Presumably this acts as an organic solvent, neutralizing forces involved in attachment of F_1 to the membrane. F_1 prepared by chloroform extraction appears to have much the same properties as F_1 prepared by other methods except that it is no longer cold labile (Spitsberg and Blair, 1977; Penin et al., 1979). Younis et al. (1977) modified the chloroform method to extract spinach CF_1.

The properties of CF_1 noted here are characteristic of the enzyme complex from spinach. Since spinach is an easy plant with which to work, it has been the plant of choice for the study of CF_1, and indeed has served as a model organism for the study of coupling factors in general.

The polypeptide composition of CF_1, and many other coupling factor complexes, has been well studied. CF_1 is a large complex, with a molecular weight in the range of 350,000. It is composed of five subunits, denoted α, β, γ, δ and ε in order of decreasing molecular weight (Racker

et al., 1971; Lien et al., 1972). The observed pattern of polypeptides obtained in SDS-PAGE has proven to be characteristic of coupling factors, having been found for the coupling factors of mitochondria (Brooks and Senior, 1971; Catterall and Pederson, 1971), *Escherichia coli* (Wilson and Smith, 1977), *Streptococcus faecalis* (Abrams et al., 1976), *Micrococcus lysodeikticus* (Huberman and Salton, 1979), the thermophilic bacterium PS3 (Yoshida et al., 1975), and the thermophilic blue-green alga *Mastigocladus laminosus* (Binder and Bachofen, 1979). Although the number of different polypeptides comprising coupling factor complexes is generally accepted as five, the stoichiometry of these subunits is by no means clear. Stoichiometries of 3α: 3β: 1γ: 1δ: 1ε (Senior and Brooks, 1971; Yoshida et al., 1979), 2α: 2β: 2γ: ?δ: 2ε (Senior, 1975) and 2α: 2β: 1γ: 1δ: 2ε (Binder et al., 1978) have been reported for CF_1 and other coupling factors.

Exceptions exist in the literature. There are several reports of proton-translocating ATPases that have a smaller number of polypeptides, such as the two polypeptide F_1's of *Bacillus magaterium* (Mirsky and Barlow, 1973) and *Bacillus subtilis* (Serrahima-Zeiger and Monteil, 1978), and the three polypeptide F_1 of *Clostridium pasteurianum* (Clarke et al., 1979).

Roles in photophosphorylation have been assigned to some of the subunits of CF_1. Removal of the δ subunit prevents binding of the complex to the thylakoid (Younis et al., 1977), but though coupling is prevented, this deficient complex still has ATPase activity. Coupling activity can be restored by addition of a minor subunit fraction containing the δ and ε subunits. Recently Cox et al., (1978) demonstrated that extraction of *E. coli* F_1 by a low ionic strength wash was prevented by an inhibitor of proteolysis, *p*-aminobenzamidine. Bragg and Hou (1979) found that the δ

subunit of ECF_1 undergoes a proteolytic cleavage during release from the bacterial membrane. Inhibition of this proteolysis prevents release of ECF_1. This evidence indicates that the δ subunit of coupling factors is involved in the attachment of the complex to the membrane.

The ε subunit acts as an inhibitor of the latent, heat-activated ATPase of spinach CF_1 (Nelson et al., 1972b). Its inhibitory activity is lost upon digestion by trypsin (Nelson et al., 1975). It has been proposed that the ε subunit prevents ATP hydrolysis by CF_1 when it is attached to the membrane, as well as being responsible for the latency of ATPase activity of the soluble CF_1 (Bakker-Grunwald and Van Dam, 1974; Nelson et al., 1975). Activation of CF_1 probably proceeds by removal or digestion of the ε subunit.

The actual catalytic site of CF_1 has been difficult to locate, as CF_1 deficient in one or more subunits does not give coupling of phosphorylation to electron transport. In studying the location of the active site of ATPase activity in the soluble, activated enzyme, one makes the assumption that ATP synthesis and ATP hydrolysis occur at the same site.

Farron and Racker (1970) observed an increase in the number of titrable sulfhydryl groups on the native CF_1 after heat activation of the latent ATPase. These newly-exposed sulfhydryl groups could be oxidized with *o*-iodosobenzoate to form two new disulfide bridges, one each in the β and γ subunits (Andreo et al., 1979). Similar experiments with the membrane-bound CF_1 showed an increase in N-ethylmaleimide binding to the γ subunit upon illumination (McCarty and Fagen, 1973). These observations suggest a conformational change in the γ subunit of CF_1 upon stimulation of photophosphorylation by illumination or upon heat activation of the latent CaATPase.

Nelson et al. (1973) attempted to determine which subunits of CF_1 were involved in ATP synthesis and hydrolysis by reacting CF_1 with anti-

bodies generated against each of the subunits. No single antiserum was able to inhibit the ATPase activity of isolated CF_1. Either anti-α or anti-γ serum inhibited photophosphorylation and the light-triggered MgATPase activity in isolated chloroplasts. Although the authors concluded that the α and γ subunits are involved in photophosphorylation, Koenig and co-workers (1978) obtained inhibition of ATPase activity with antisera against any of the subunits of CF_1. The difference in results is most likely due to different antigenic sites between antibody preparations. This demonstrated that one should be extremely cautious about making conclusions about active sites solely upon the evidence of monospecific antibody inhibition of enzyme activity.

Photochemical labelling of heat-activated CF_1 demonstrated ATP binding sites on the β subunit, and ADP sites on both the α and β subunits (Carlier et al., 1979). However, it is difficult to say whether these sites are all catalytic, or all regulatory in character, or a mixture of both. Nucleotide binding analyses tend to give more information on the mechanism of phosphorylation than on the function of the component polypeptides. For an overview of information on nucleotide binding sites on coupling factors, see the review by Harris (1978).

The reconstruction experiments of Yoshida et al. (1977) have provided some very suggestive information on the role of individual subunits in ATPase activity. Individual subunits of F_1 from the thermophilic bacterium PS3 were added back together and the reconstituted complex was assayed for the ability to hydrolyze ATP. No ATPase activity was obtained unles the β subunit was included in the reconstituted complex, and all combinations of subunits containing the β and γ subunits exhibited identical catalytic properties and amount of ATPase activity to those of the native PS3 F_1 .

However, reconstitution of the coupling factors from organelles and mesophilic bacteria seems to be more difficult, probably because the amino acid sequences of their polypeptides make them less resistant to the dissociating agents and the manipulation necessary to reconstruct the complex (Kagawa, 1978).

Another approach to determining the subunit or subunits necessary for ATPase activity has been to remove subunits from F_1 by trypsin digestion, as was done with the enzyme from *Micrococcus lysodeikticus* (Mollinedo et al., 1980). Trypsin removes considerable amounts of the α and γ subunits, but no β subunit. A form of the ATPase with a subunit ratio of 1.67α: 3β: 0.17γ exhibited more than twice the activity of the native enzyme, but removal of more α and γ gave an inactive ATPase. Thus it appears that the β subunit in *M. lysodeikticus* F_1 is absolutely required for ATPase activity, but α and γ may be necessary in lesser amounts as regulators or effectors.

The evidence from studies of other coupling factor complexes is that the β subunit is probably the active site of ADP phosphorylation and ATPase activity. It is tempting to compare catalytic functions of the subunits of the above mentioned coupling factors with those of CF_1, but only further research will prove whether this is a valid comparison.

The object of this research was to characterize some of the properties of the CF_1 from the wheat chloroplast. To this end, a method was devised for the purification of wheat CF_1 containing all its subunits. Electrophoretic and electron microscopic analyses gave information about the structure of wheat CF_1. Functional data was obtained from enzymatic analysis of the latent ATPase. The value of such a study is two-fold. The structural and enzymatic properties of CF_1 from a monocotyledon have

never been characterized. One cannot assume that an enzyme from a monocotyledon has identical properties to that from dicotyledons such as spinach, lettuce or snapdragon. In addition, any information on the structure and function of a coupling factor from an unstudied species will add to the bulk of information from which to draw when formulating a unifying model about structure and function of energy-transducing coupling factors.

This research project originally included a study of the biosynthesis of wheat chloroplast coupling factor. It was decided to approach this problem by isolating wheat RNA, translating it in an *in vitro* protein synthesizing system, then isolating components of CF_1 translated in such a system by immunoprecipitation, using monospecific antibodies to the subunits of wheat CF_1. Of special interest are the subunits of CF_1 synthesized outside the chloroplast and transported post-translationally into the organelle. Mendiola-Morgenthaler et al. (1976) showed that the isolated intact chloroplasts from spinach synthesize only the α, β and ε subunits of CF_1. From this they concluded that the γ and δ subunits are synthesized in the cytoplasm. These findings were confirmed by two other groups (Ellis, 1977; Bouthyette and Jaegendorf, 1978). Recently, Nelson et al. (1980) found evidence that the γ subunit, as well as α, β and ε are synthesized in isolated intact spinach chloroplasts. They reported that the δ subunit is synthesized as a slightly larger precursor polypeptide in the cytoplasm. Studies on the cytoplasmically-synthesized subunits of yeast mitochondrial F_1 (Maeccecchini et al., 1979) and the small subunit of chloroplast ribulose-1,5-bisphosphate carboxylase (Highfield and Ellis, 1978; Cashmore et al., 1979; Chua and Schmidt, 1978) show that these polypeptides are originally made as longer polypeptides. At some point during their transport into the organelle, the polypeptides are trimmed down to their final size,

probably by specific enzymes. Time did not permit the completion of such a study of the wheat CF_1 subunits. Presented here are the results of the immunization of one rabbit against wheat CF_1, and the characterization of the antigenic properties of the wheat antibodies produced.

MATERIALS AND METHODS

The following abbreviations are used throughout this text: SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl) aminomethane; Tricine, N-tris (hydroxymethyl) methylglycine; EDTA, ethylenediamine-tetraacetate acid disodium salt; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DTT, dithiothreitol; F_1, coupling factor; CF_1, chloroplast coupling factor; ECF_1, *Escherichia coli* coupling factor; F_0, an intrinsic membrane protein complex, the site of attachment of F_1 to the membrane; ATPase, adenosine triphosphatase; CaATPase, calcium-dependent ATPase; TEMED, N,N,N',N'-tetramethylethylene-diamine; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonylfluoride; kd, kilodalton; mw, molecular weight. Coupling factor and ATPase are used interchangeably in referring to the two activities of the same enzyme complex. A unit of ATPase activity is the amount of enzyme that will catalyze the release of one umole of inorganic phosphate per minute. "Proteolysis inhibitors" is used to signify the inclusion of 6 mM *p*-aminobenzamidine di-HCl, 40 mM ε-amino-*n*-caproic acid and 0.1 mM PMSF.

Materials

Miracloth (Chicopee Mills, Inc.) was supplied by Calbiochem. Sigma provided ε-amino-*n*-caproic acid, PMSF, agarose (low EEO), Lubrol PX, polyethylene glycol (PEG), trypsin (DPCC-treated) and soybean trypsin inhibitor. Molecular weight standards for SDS-PAGE were from Pharmacia (ovalbumin) or Sigma (pyruvate kinase, carbonic anhydrase and ribonuclease A). Aldrich supplied *p*-aminobenzamidine di-HCl. The colour reagent for the Bradford (1976) protein assay was purchased from Bio-Rad. Freund's Complete Adjuvant was supplied by Difco. Sodium deoxycholate came from Schwarz/Mann. All other chemicals were of reagent grade.

Plant Material

Young wheat seedlings (*Triticum aestivum*, var Thatcher) were grown from seed supplied by Buckerfield's Ltd., Vancouver, B.C. or by Alberta Wheat Pool, Calgary, Alta. Seeds were vacuum infiltrated with 3% hypochlorite solution for five minutes, soaked in the same for 30 minutes, washed thoroughly and germinated by bubbling air through the seeds suspended in distilled water overnight. Germinated seed was sown in vermiculite and grown in a greenhouse for 8 to 15 days, with 16 L: 8 D light supplement as necessary. Spinach leaves were cut from four-week-old seedlings (*Spinacea oleracea*, var Longstanding Bloomsdale) grown in soil in a greenhouse.

CF_1 Extraction and Purification

All steps up to the extraction of CF_1 from thylakoids were done at 4°C. The blades of young wheat seedlings were removed, washed in tap water, then distilled water, chopped with scissors, and blended in 10 mg/g fresh weight in cold Isolation Medium (0.3 M mannitol, 50 mM Tricine, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.0) in a chilled blendor for ten seconds. The brei was filtered through four layers of Miracloth and the chloroplasts pelleted by centrifuging at 6,000 x g for ten seconds. The crude chloroplast pellet was resuspended in 1.5 volumes of Isolation Medium and pelleted as before. Stromal ribulose-1,5-bisphosphate carboxlyase was removed by stirring for 30 minutes in about 1 ml/g fresh weight cold 10 mM sodium pyrophosphate pH 7.4 (Strotmann et al., 1973) to which was added proteolysis inhibitors (6 mM *p*-aminobenzamidine di-HCl, 40 mM ε-amino-*n*-caproic acid, 0.1 mM PMSF). This wash was repeated for a total of four times at 4°C, the thylakoids being pelleted at 10,000 x g between washes. During the final wash, the chlorophyll concentration was determined by the method of Arnon (1949).

Subsequent work was performed at room temperature. Chloroform extraction was performed via a modification of the method of Younis et al. (1977). The thylakoids were suspended at 2.0 to 2.5 mg chlorophyll/ml in 0.25 M sucrose, 10 mM Tris/SO_4 pH 7.6, 1 mM EDTA, 2 mM ATP, 5 mM DTT plus proteolysis inhibitors. To this suspension, ½ volume of chloroform was added and the mixture was emulsified by stirring with a magnetic stir bar for 15 seconds. The emulsion was broken by low speed centrifugation and the aqueous phase was recentrifuged at 48,000 x g 30 min. The supernatant was concentrated to 1 to 3 ml with a Millipore Immersible CX Molecular Separator. This sample is designated "crude CF_1".

To further purify CF_1, the crude sample was loaded onto a 37 ml sucrose gradient (8 to 25% sucrose in 10 mM Tris/SO_4 pH 7.6, 1 mM EDTA, 2 mM ATP, 5 mM DTT, plus proteolysis inhibitors) and centrifuged at 26,000 rpm in a Beckman SW27 rotor for 28 hours. The gradient was fractionated by pumping denser sucrose into the bottom and collecting 30-drop fractions off the top. Sucrose content of gradient fractions was determined using a refractometer. Protein was determined by the dye-binding method of Bradford (1976). The CF_1 thus prepared was greater than 98% pure, as determined by SDS-PAGE.

Spinach CF_1 was prepared in a similar way except that the ratio of Isolation Medium to fresh weight of young leaves was 3:1.

In various experiments, proteolysis inhibitors were omitted at certain stages of the procedure outlined above. These changes are noted in the text and figures as they occur.

A second method for the preparation of a crude CF_1 extract, according to the procedure of Strotmann et al. (1973) was used for comparative purposes. Thylakoids were washed four times in cold 10 mM sodium

pyrophosphate pH 7.4 without proteolysis inhibitors. CF_1 was then released by washing in a hypotonic sucrose solution (0.3 M sucrose, 2 mM Tricine-KOH pH 7.8) containing no proteolysis inhibitors. This crude CF_1 extract was purified to homogeneity by sucrose density gradient centrifugation as above.

Electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed according to the method of Kirchanski and Park (1976). The resolving gel contained 10% (w/v) acrylamide, 0.27% (w/v) bisacrylamide, 1.1347 M Tris-HCl, pH 9.8, 0.1% (w/v) SDS, 0.47 (v/v) TEMED, and 0.1% (w/v) ammonium persulfate. This last component was added after de-aeration, to initiate polymerization. The stacking gel contained 5% (w/v) acrylamide, 0.14% (w/v) bisacrylamide, 0.1 M Tris-HCl pH 6.1, 0.1% (w/v) SDS, 0.16% (v/v) TEMED, and 0.025% (w/v) ammonium persulfate. The running buffer consisted of 0.192 M glycine, 0.025 M Tris and 0.1 (w/v) SDS. Protein samples were dissociated in 65mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, and either 0.2% (v/v) β-mercaptoethanol or 10 mM DTT. Slab gels (2 mM thick) were used throughout. Electrophoresis was routinely run at 15 to 20 mA per slab for four to six hours at room temperature.

At the end of electrophoresis, polyacrylamide slabs were stained for 30 minutes in a solution of 0.2% (w/v) Coomassie Blue R, 50% (v/v) methanol, 7% (v/v) acetic acid, and destained in several changes of 20% (v/v) methanol, 7% (v/v) acetic acid. A tuft of unbleached wool was added during destaining to speed up the process.

Molecular weight analysis of polypeptide bands in SDS-PAGE was done by the method of Webber and Osborne (1969), using as standards pyruvate kinase (mw 57 kd), ovalbumin (mw 45 kd), carbonic anhydrase (mw 30 kd) and ribonuclease A (mw 13.7 kd).

In-gel limited proteolysis was performed according to the method of Cleveland et al. (1977). Protein bands separated by SDS-PAGE were visualized by staining for five minutes, then destaining to the point where bands were visible. These were excised with a razor blade and soaked for 30 minutes in 10 ml of soaking buffer (0.125 M Tris-HCl pH 6.8, 0.1% SDS, 1mM EDTA). A 15% SDS-PAGE slab gel (as described above, but containing 1 mM EDTA in addition) with a four cm long stacking gel containing 3% acrylamide (no EDTA) was made. Into each slot on the slab was placed the excised soaked band and 10 ul of 20% glycerol in soaking buffer. Over this, 10 ul of 50 ug/ml solution of *Staphylococcus aureus* V8 protease in 10% glycerol, 0.1% Bromphenol blue and soaking buffer was overlaid. A current of 15 mA was run until the sample and tracking dye had stacked in the middle of the 3% gel. The current was turned off for 40 minutes to allow digestion to occur. The current was turned back on at the end of this period and electrophoresis proceeded at 20 mA until the tracking dye had run off the end of the 15% gel. Staining and destaining were done the same as for other SDS gels.

ASSAYS

The latent CaATPase of CF_1 was activated by trypsin digestion and assayed according to the method of Lien and Racker (1971b). Briefly, to a sample containing 50 ug protein was added 0.01 ml ATP (0.1 M pH 7 stock), 0.02 ml EDTA (0.2 M pH 7.6 stock), 0.03 ml Tricine-KOH (1 M pH 8 stock), and the volume was brought to 0.93 ml with distilled water. Trypsin (0.02 ml of a 5 mg/ml stock prepared in 1 mM H_2SO_4) was added and digestion proceeded for 6 minutes at room temperature. Digestion was halted by the addition of 0.05 ml soybean trypsin inhibitor (5 mg/ml in 5 mM Tricine-KOH pH 7).

Aliquots of the activated enzyme containing 2.5 ug CF_1 were brought to 0.2 ml with 20 mM Tricine-KOH pH 8. To this was added 0.8 ml of substrate mix containing 40 mM Tricine-KOH pH 8, 1 mM ATP, and 10 mM $CaCl_2$. After a 10 minute incubation at 37°C, the reaction was halted by the addition of 1 ml 0.5 M cold TCA. Samples were analyzed for inorganic phosphate (Chen et al., 1956). In all assays for ATPase activity, zero time inorganic phosphate was determined, and a correction was made for ATPase activity of the unactivated enzyme.

ATP was omitted from the trypsin digestion step in experiments for determination of K_M and V_{max}, in order to assay ATPase activity at very low concentrations of substrate.

Prior to enzyme activation with trypsin, proteolysis inhibitors were removed by dialysis against two changes of 10 mM Tris-SO_4 pH 7.4, 1 mM EDTA, 1 mM ATP, 5 mM DDT.

The latent, heat activated CaATPase of CF_1 was activated and assayed according to the method of Lien and Racker (1971b). To a 0.2 ml aliquot of protein containing 150 to 200 ug CF_1, 0.05 ml ATP (0.2 M pH7), 0.015 ml DTT (0.1 M) and 0.03 ml Tricine-KOH (1 M pH 8) was added. The mixture was heated in a 60°C water bath for four minutes, and then 1.7 ml Tricine-KOH-EDTA buffer (40 mM Tricine KOH pH 8.0, 2 mM EDTA) at room temperature was added. ATPase activity was assayed as described above.

Nucleotide specificity of the trypsin-activated ATPase was assayed by substituting 1 mM of the various substrates for ATP, and activating in the absence of ATP.

For determination of K_M and V_{max} graphically, the velocity of the enzyme reaction was determined by sampling a reaction mixture at one minute intervals over a three minute reaction. Velocity was plotted against the

concentration of $Ca^{2+}ATP$ complex, rather than the concentration of ATP. The concentration of $Ca^{2+}ATP$ was determined using the following equation of Ahlers et al. (1975):

$$[Ca^{2+}ATP] = \frac{[Ca]_t + [ATP]_t + K_{CaATP}\left(1 + \frac{[H^+]}{K_1}\right)}{2} - \sqrt{\frac{[Ca]_t + [ATP]_t + K_{CaATP}\left(1 + \frac{[H^+]}{K_1}\right)}{2} - [Ca]_t\,[ATP]_t} \quad (1)$$

with K_{CaATP}= 0.725 mM (Nanninga, 1957; obtained from Sillen and Martell, 1964) and $K_1 = 3 \times 10^{-7}$ (Sillen and Martell, 1964). $[Ca]_t$, $[ATP]_t$ and $[H^+]_t$ refer to the total concentration of each of these components.

Electron Microscopy

A light coating of carbon was evaporated onto collodion-coated 200 mesh electron microscopy grids, and the plastic backing was removed by gently dipping the grid into acetone for five seconds. CF_1 samples were dialyzed overnight against 2 mM Tris-HCl pH 7.5 to remove salts prior to negative staining. Samples of wheat CF_1 (greater than 98% purity as determined by SDS-PAGE) or *Escherichia coli* F_1 (kindly provided by Dr. P.D. Bragg, UBC Department of Biochemistry) with about 0.1 to 0.2 mg protein/ml were mixed with an equal volume of negative stain for 30 seconds, and sprayed onto the carbon film with the spray gun apparatus described by Backus and Williams (1950). Negative stains used were 2% ammonium molybdate in 12 mM sodium oxalate pH 7.2 or 2% phosphotungstic acid-KOH pH 7.0. Uranyl acetate (2% solution, pH 4.3) was not a satisfactory

stain.

Negative stained CF_1 and ECF_1 were examined using a Zeiss EM-10 electron microscope, at magnifications of 40,000 X or greater, with a voltage setting of 80 kV. CF_1 diameters were measured on photographic prints and the actual size of the particle was calculated from the magnification of the print and the negative. For the magnifications used, calibration of the EM-10 gave magnifications that were within 0.8 to 2.2% of the instrument settings.

Image enhancement of electron micrographs was done by the method of Markham et al. (1963).

Preparation and Characterization of Monospecific Antibodies to Wheat CF_1

200 ug of CF_1 (greater than 98% pure) was emulsified in an equal volume of Freund's Complete Adjuvant and injected intramuscularly into the hind leg of a New Zealand white rabbit. At ten day intervals, second and third injections were given and two weeks later the rabbit was bled from the ear vein. After clotting, serum was separated from the blood cells by centrifugation. Preservative (0.1% sodium azide) was added to all sera and they were stored at 4°C.

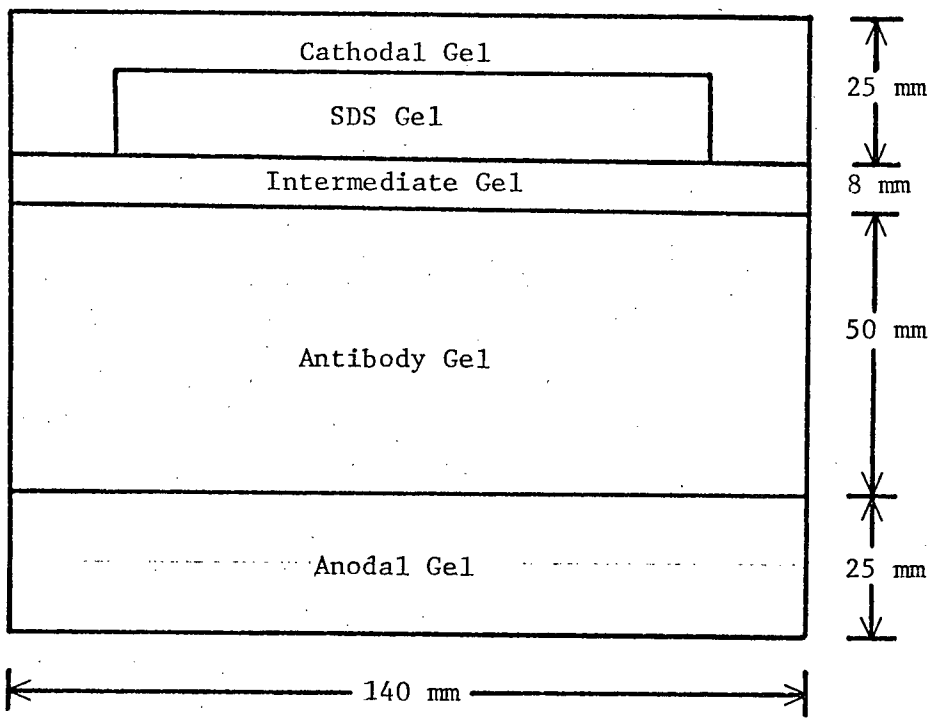
Antibody titer was determined by the double diffusion method of Ouchterlony (1968). Crossed immunoelectrophoresis was performed according to the method of Chua and Blomberg (1979) except that whole serum, rather than purified IgG, was used. The following stock solutions were prepared: (a) buffer A: 90 mM Tris pH 8.6 with acetic acid, 40 mM sodium acetate, 1 mM EDTA; (b) 20% (v/v) Lubrol PX in distilled water; (c) 10% (w/v) sodium deoxycholate in distilled water; (d) 50% PEG (w/v) in buffer A; (e) 1.2% (w/v) agarose in buffer A. The gel was cast between two glass plates with

3 mm thick spacer bars on the sides and bottom. The apparatus was clamped together in a vertical position during casting, and individual layers were cast from bottom to top as diagrammed in fig. 1. Solution (e) was dissolved by heating in a boiling water bath, and all solutions were kept in a 65 C water bath prior to casting. The anodal gel was prepared by mixing 2.9 ml of solution (b) with 35.1 ml of solution (e). Mixing was accomplished by gentle swirling in a pre-warmed flask. The gel was then cast by pouring the warm mixture between the plates, being careful not to make bubbles. After this layer had solidified, successive layers were poured on top in the same manner. To prepare the antibody gel, 1.3 ml of solution (d) and 18.7 ml of solution (e) were mixed, and added to 1 ml of antiserum that had been prewarmed. The short intermediate gel was cast from extra anodal gel mixture. An unstained, unfixed lane from a SDS-PAGE gel was excised, rinsed in distilled water, and placed above the intermediate gel as diagrammed. The cathodal gel, composed of 1 ml of solution (c) and 9 ml of solution (e), was cast around the SDS gel. The bottom spacer bar was removed and J-Cloth (Johnson and Johnson) wicks were fused to the cathodal and anodal ends of the gel using warm solution (e) as a binder.

The composite gel was then placed horizontally across the supports of a Gelman flatbed electrophoresis chamber. Each reservior was filled with about 400 ml of buffer A. Electrophoresis was run at a constant voltage of 100 V for 18 to 20 h. The starting current was about 20 mA.

Immunoprecipitates in both double diffusion plates and crossed immunoelectrophoresis gels were visualized by staining with Coomassie Blue after soaking the gel in 0.15 M NaCl for one day and in distilled water for two days. Due to the fragility of agarose gels, staining and destaining were done without shaking.

Figure 1: Diagram of crossed immunoelectrophoresis gel, as described in Materials and Methods.


Cathodal Gel
SDS Gel
Intermediate Gel
Antibody Gel
Anodal Gel
25 mm
8 mm
50 mm
25 mm
140 mm

RESULTS

Purification of Wheat CF_1

A satisfactory method was developed for obtaining homogeneous wheat CF_1, using a modification of the chloroform extraction method of Younis et al. (1977). CF_1, from thylakoids stripped of the chloroplast envelope and stromal proteins, was released into the aqueous medium by emulsifying the thylakoid suspension in chloroform. The extract was purified to greater than 98% homogeneity on a linear 8 to 25% sucrose gradient. Proteolysis inhibitors were included in all solutions except the initial grinding medium. Fractionation of a typical sucrose gradient gave the profile illustrated in fig. 2. CF_1 bands at 20 to 21% sucrose.

Analysis of the major protein peak in the sucrose gradient by SDS-PAGE revealed that all five subunits of CF_1 were present (fig. 3). The smaller protein peak at the bottom of the sucrose gradient contains polypeptides corresponding in apparent molecular weight to the large and small subunits of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39), the major protein in the chloroplast stroma (lane 6, fig. 3). As can be seen in lane 1 of fig. 3, the "crude CF_1" is very nearly homogeneous, so that further purification by sucrose gradient centrifugation resulted in only a 1.5 to 2-fold increase in CaATPase activity at best. Results of a typical experiment are given in Table I.

Figure 2: Profile of sucrose density gradient used to purify wheat CF_1. A crude chloroform extract of wheat thylakoids containing 4.9 mg protein was loaded onto a 34 ml gradient (8 to 25% sucrose in buffer containing proteolysis inhibitors) with a 50% sucrose cushion, and the gradient was centrifuged 28 h at 26,000 rpm in a Beckman SW 27 rotor. The gradient was fractionated into 30 drop aliquots. Sucrose content, protein concentration and trypsin-activated ATPase activity were assayed as described in Materials and Methods.

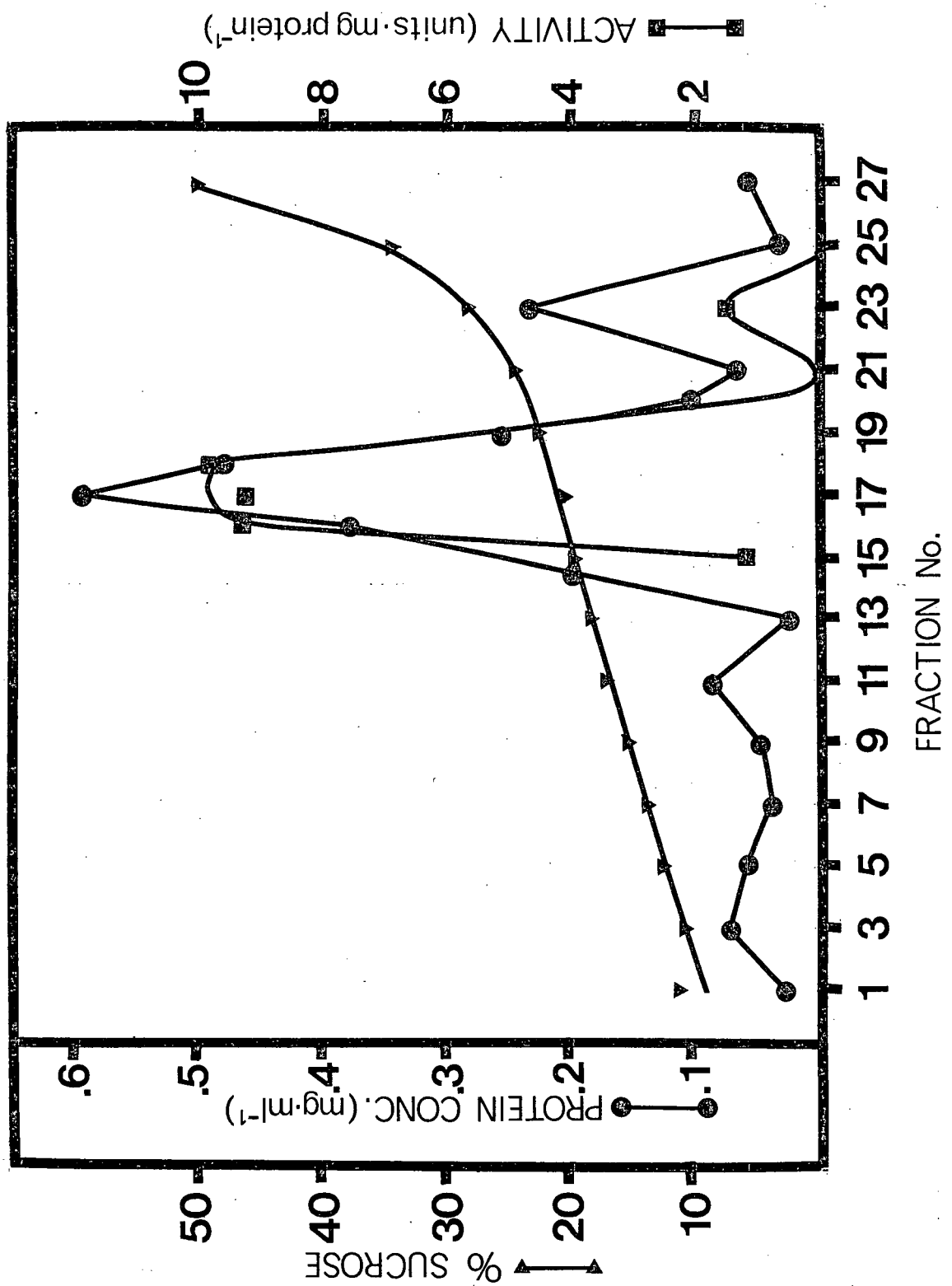

% SUCROSE
PROTEIN CONC. (mg·ml⁻¹)
ACTIVITY (units·mg protein⁻¹)
FRACTION No.
10
20
30
40
50
.1
.2
.3
.4
.5
.6
2
4
6
8
10
1 3 5 7 9 11 13 15 17 19 21 23 25 27

Figure 3: SDS-PAGE of crude CF_1 sample and protein fractions from a sucrose density gradient similar to the one shown in Figure 2. Lane 1, 20 ug crude chloroform extract; lane 2, 19 ug of gradient fraction 16; lane 3, 35 ug of gradient fraction 17; lane 4, 33 ug of gradient fraction 18; lane 5, 18 ug of gradient fraction 19; lane 6, 9.6 ug of gradient fraction 23. LS and SS refer to the large and small subunits of ribulose-1,5-bisphosphate carboxylase.

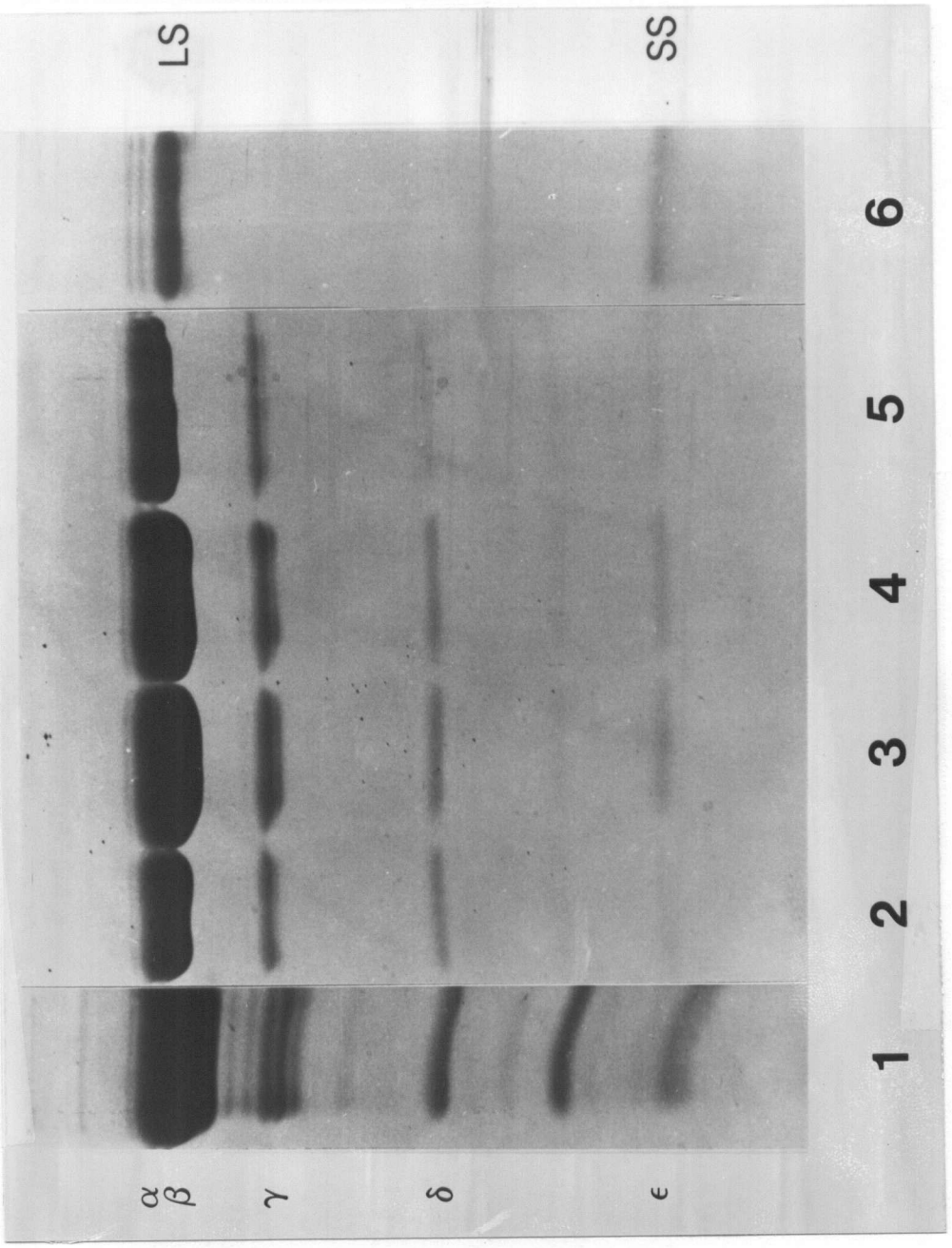

LS
SS
α
β
γ
δ
ϵ
1
2
3
4
5
6

Table I. Purification of wheat CaATPase

Sample	Conc. Protein, mg/ml	Total Volume, ml	Total Protein, mg	Specific Activity*	Total Activity, units	Degree of Purification
Crude CF_1	2.96	4.6	13.62	5.42	71.4	1
Sucrose gradient peak	0.69	1.3	0.90	10.42	9.4	1.99

*units/mg protein

Spinach CF_1 was prepared by the same procedure for comparative purposes. It was found to have the same sedimentation velocity as wheat CF_1 in sucrose gradients, and the same polypeptide composition, although the apparent molecular weights of the subunits differ somewhat (Table II).

Table II. Apparent molecular weights of subunits of wheat and spinach CF_1, as determined by the method of Webber and Osborne (1969).

Subunit	Apparent molecular weight (kd)	
	Wheat CF_1	Spinach CF_1
α	57	58
β	55	54
γ	37-39	36-37
δ	25	19
ε	13	13

The Effect of Proteolysis Inhibitors on Extraction of Wheat CF_1

In initial CF_1 extractions, proteolysis inhibitors were not included in solutions used for CF_1 release and purification. CF_1 extracted in this manner contained only the three largest subunits. When proteolysis inhibitors were added to all solutions used in the extraction and purification of wheat CF_1, all five subunits co-purified. To test at what stage the presence of proteolysis inhibitors is critical for the preservation of the intact enzyme complex, wheat CF_1 was prepared by chloroform extraction with and without 6 mM *p*-aminobenzamidine, 40 mM ε-amino-*n*-caproic acid and 0.1 mM phenylmethylsulfonylfluoride. The crude chloroform extracts were identical in polypeptide composition (fig. 4, lanes A and B), but when the crude CF_1 prepared without proteolysis inhibitors was purified on a sucrose gradient also without proteolysis inhibitors, the CF_1 peak in the gradient lacked the δ and ε subunits (fig. 5). It appears that proteolysis inhibitors prevent the loss of these minor subunits from the enzyme complex during the prolonged room temperature density gradient centrifugation.

In addition to chloroform extraction, there are several other methods for releasing CF_1 from the thylakoid, as mentioned in the Introduction. It was decided to compare CF_1 extracted by the hypotonic sucrose method (Strotmann et al., 1973) with the method described here. Addition of proteolysis inhibitors to the hypotonic sucrose extraction medium completely prevented CF_1 release (fig. 4, lanes C and D). Extraction of CF_1 with hypotonic sucrose minus proteolysis inhibitors released many more polypeptides than are released by chloroform treatment (compare in fig. 4, lane A with lane D). Chloroform thus seems to be a much more specific method for CF_1 extraction than the sucrose method.

Figure 4: Comparison of crude CF_1 prepared by the chloroform method with that prepared by the hypotonic sucrose method, and the effect of the inclusion of proteolysis inhibitors upon each. A. Crude chloroform extract, prepared in the presence of all proteolysis inhibitors. 25 ug protein. B. Crude chloroform extract, for which all proteolysis inhibitors were omitted from both the sodium pyrophosphate wash and the chloroform extraction medium. 25 ug protein. C. Hypotonic sucrose wash in which proteolysis inhibitors were included. The small amount of α and β seen in this lane was washed over from the adjacent lane. 6.25 ug protein. D. Hypotonic sucrose wash of thylakoids, prepared in the absence of proteolysis inhibitors. 73 ug protein.

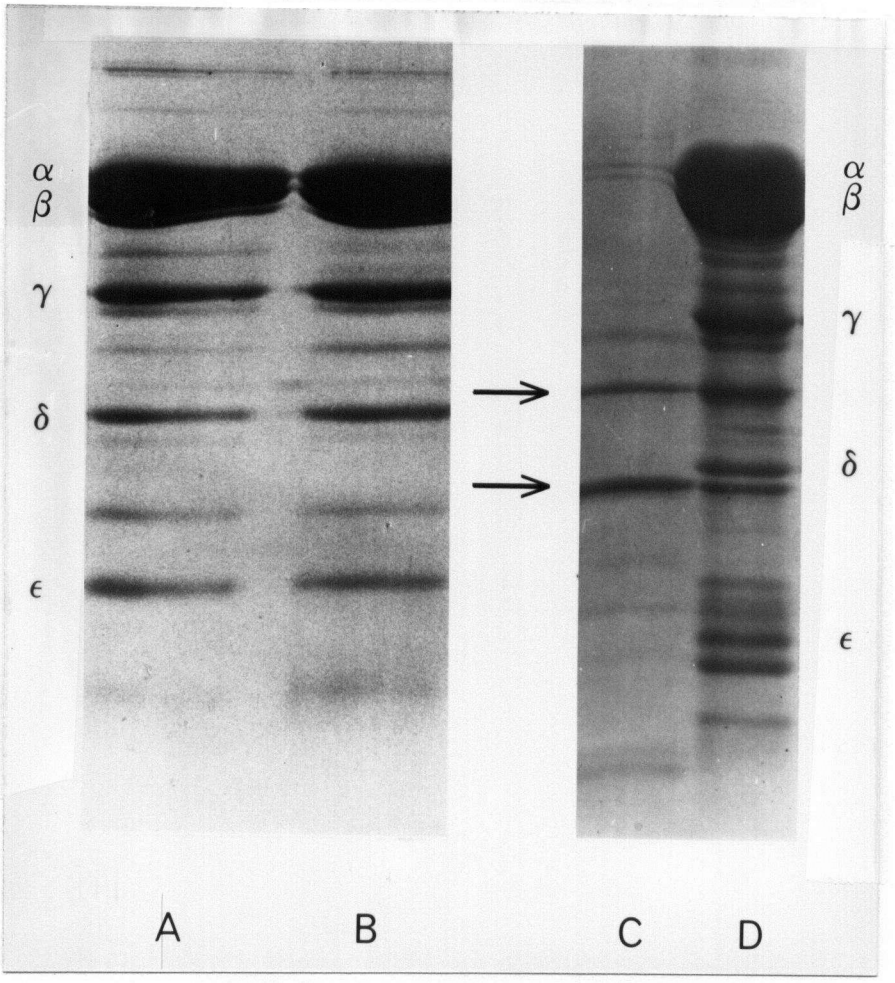
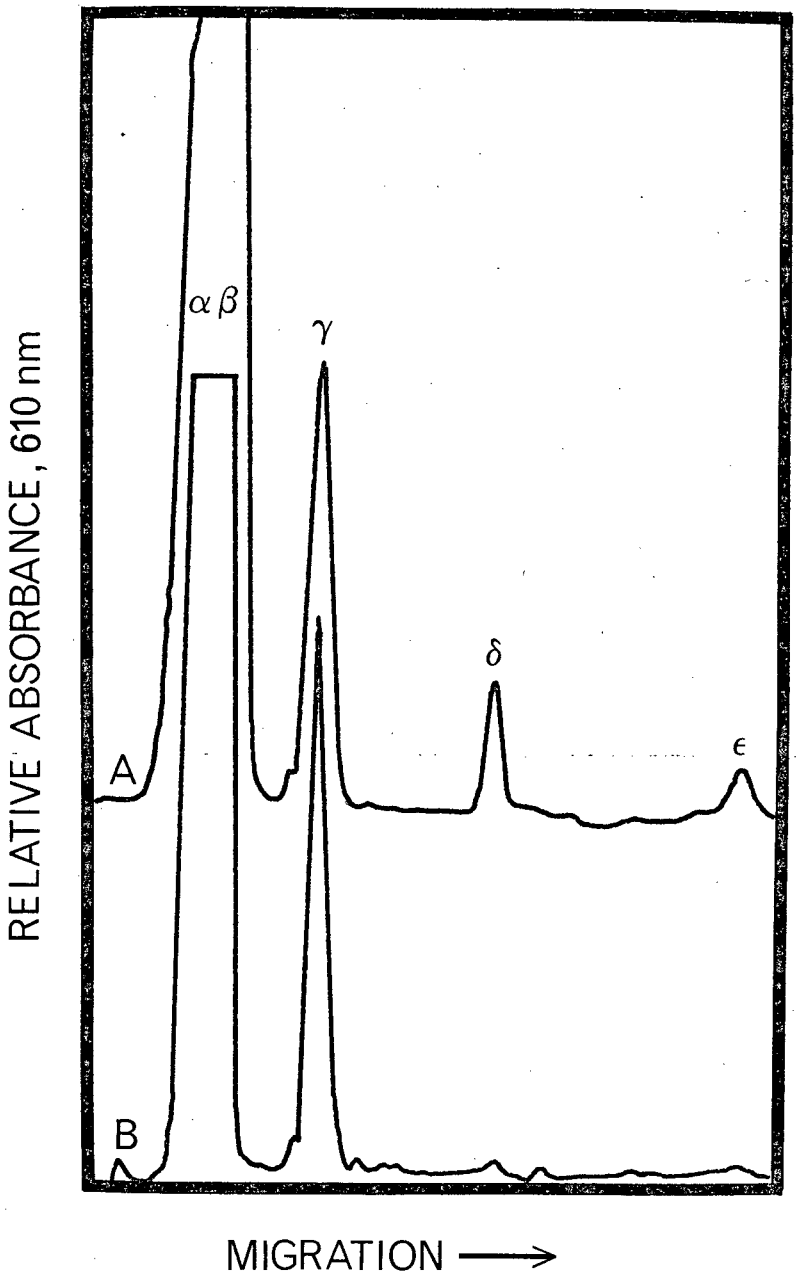

α
β
γ
δ
ϵ
α
β
γ
δ
ϵ
A
B
C
D

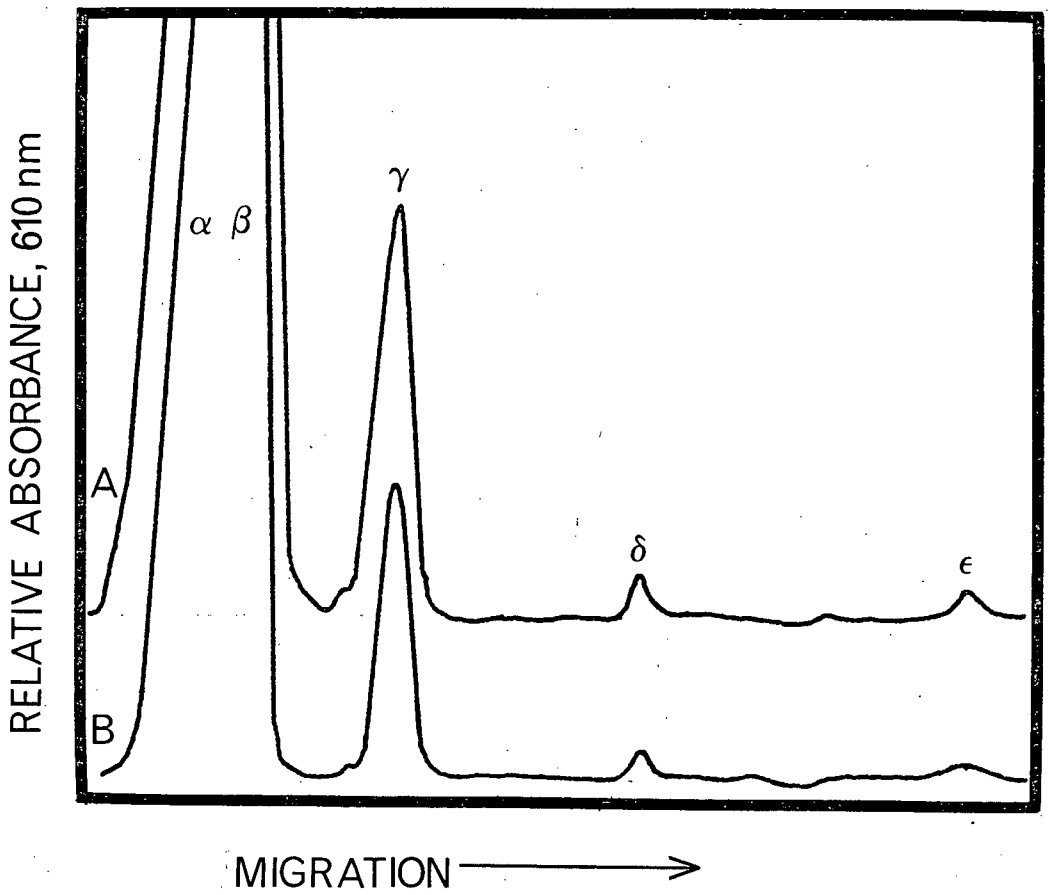
Figure 5: Effect of the inclusion of proteolysis inhibitors during CF_1 extraction and purification upon subunit composition of wheat CF_1. A. CF_1 extracted by the chloroform method and purified, all in the presence of proteolysis inhibitors. B. CF_1 extracted by the chloroform method and purified in the absence of proteolysis inhibitors.


RELATIVE ABSORBANCE, 610 nm
αβ
γ
δ
ε
A
B
MIGRATION ⟶

The prevention of CF_1 release by hypotonic sucrose when proteolysis inhibitors are included raises the question of whether a proteolytic event is required for CF_1 release by this method. Such a proteolytic event has been reported for ECF_1 (Cox et al., 1978, Bragg and Hou, 1979). Bragg and Hou (1979) compared ECF_1 released by chloroform in the presence of proteolysis inhibitors with that released by low ionic strength buffer in the absence of proteolysis inhibitors. They found a slightly larger δ subunit is released by chloroform, and showed that the chloroform-released ECF_1 rebinds to *E.coli* membranes to a lesser extent than ECF_1 released by a low ionic strength wash. They proposed that proteolysis of the δ subunit is required for the release of ATPase from *E.coli* membranes with low ionic strength buffer.

To test whether the inhibition of a proteolytic cleavage in one of the CF_1 subunits accounts for the inhibition of CF_1 release by hypotonic sucrose when proteolysis inhibitors are included, two methods of CF_1 release were compared. Thylakoids from the same batch of wheat plants were used for this comparison. In the first case, CF_1 was released from thylakoids by the chloroform method, in the presence of proteolysis inhibitors. In the second method, no proteolysis inhibitors were present, in either the sodium pyrophosphate washes, or in the extraction of CF_1 with hypotonic sucrose. Both crude CF_1 extracts thus obtained were purified on sucrose gradients containing all proteolysis inhibitors. When the two samples of purified CF_1 were compared (fig. 6), no difference in apparent molecular weight in any of the subunits was evident, and the δ and ε subunits were preserved in both. Thus is seems that the release of CF_1 from the thylakoid by hypotonic sucrose does not involve any detectable proteolysis of CF_1, although these results do not rule out a proteolytic event at the CF_1

Figure 6: Proteolysis inhibitors present during sucrose density gradient centrifugation prevent the loss of the δ and ε subunits of wheat CF_1. A. Thylakoids were prepared without proteolysis inhibitors, washed in sodium pyrophosphate without proteolysis inhibitors, and a crude CF_1 extract was prepared by washing in hypotonic sucrose containing no proteolysis inhibitors. CF_1 was purified by sucrose density gradient centrifugation in a gradient containing all proteolysis inhibitors. 62 ug protein. B. Wheat CF_1 extracted by the chloroform method in the presence of all proteolysis inhibitors and purified on a sucrose density gradient containing all proteolysis inhibitors. 44 ug protein.

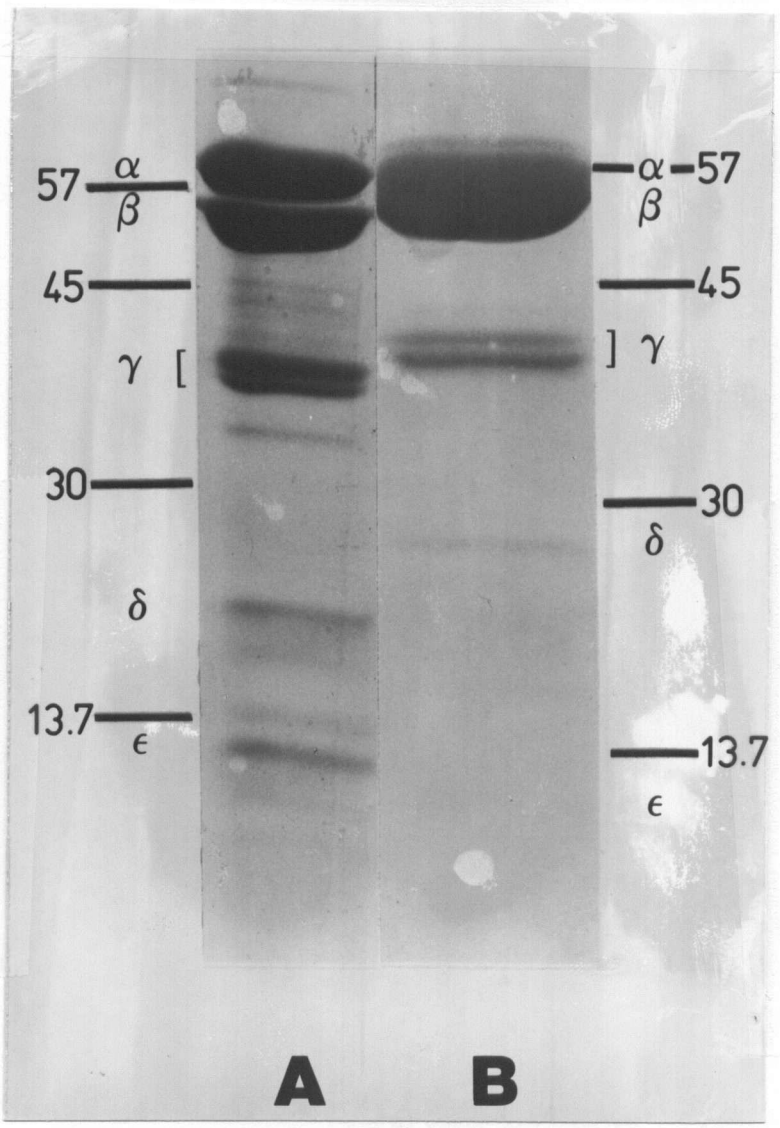

RELATIVE ABSORBANCE, 610 nm
α β
γ
δ
ϵ
A
B
MIGRATION

attachment site on CF_0. It may be that some other factor, such as osmotic strength, is the reason why CF_1 is not released in the presence of proteolysis inhibitors with the hypotonic sucrose method. However, the loss of δ and ε in the sucrose gradients run at 18 C for 28 hours without proteolysis inhibitors does suggest that enzyme prepared by both methods contains traces of protease activity.

The γ Subunit of Wheat CF_1 Contains an Internal Disulfide Bridge

In many, but not all CF_1 preparations, a pair of bands of about 2 kd difference in apparent mw was seen in the region of the γ subunit of both wheat and spinach CF_1 upon SDS-PAGE (fig.7) It was first thought that one of these two bands might be the result of proteolysis of the α or β subunit, as found by Ryrie and Gallagher (1979) in the yeast mitochondrial F_1. With that enzyme, the authors found that a 40 kd polypeptide purifying with F_1 is actually a proteolytic fragment of the α subunit. Proteolysis of the α subunit was due to a latent, SDS-activated protease contaminating their F_1 preparation in small quantities. To test whether proteolysis occurs during the preparation of wheat CF_1, proteolysis inhibitors were added to all solution used to prepare wheat CF_1. Both of the γ subunit polypeptides were still present on SDS-PAGE. In order to determine whether one of the bands was a breakdown product of α or β, peptide analysis was performed according to the method of Cleveland et al. (1977). With *Staphylococcus aureus* V8 protease, a comparison of the limited proteolysis fragments of the γ doublet with those of the α and β subunits showed no clear similarities between any of the two largest subunits and the dimer. However, when the two bands of the doublet were separately subjected to the same procedure, the resulting peptide fingerprint shows

Figure 7: SDS-PAGE of wheat and spinach CF_1, showing a double band in the γ subunit position. A. Spinach CF_1, dissociated in 65 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 0.2% β-mercaptoethanol, 50 ug protein. B. Wheat CF_1, dissociated in 65 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 2.5 mM DTT, 38 ug protein.


57
α
β
45
γ [
30
δ
13.7
ϵ
α
β
57
45
] γ
30
δ
13.7
ϵ
A
B

that the upper and lower γ bands contain identical fragments (fig. 8). If there had been a 2 kd mw difference between the two bands, it should have shown up as at least one different band in the limited proteolysis. This strongly suggests the two polypeptides at the γ position are identical.

Electrophoresis up to this point had been performed upon samples dissociated in a buffer containing 65mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 0.2% β-mercaptoethanol. In order to determine if the difference in mobilities was due to incomplete disulfide bond reduction of the γ subunit with β-mercaptoethanol, samples were dissociated in increasing amounts of a stronger reducing agent, DTT. Figure 9 shows that with increasing amounts of DTT the lower band of the γ doublet is decreased while the upper band is increased. This suggests that the lower band of the doublet migrates faster than the upper one because one or more disulfide bonds are not reduced, making a shorter effective length polypeptide. It was concluded that complete reduction was obtained with 33 nmoles DTT/mg CF_1 since no further increase in apparent mw was observed when CF_1 was dissociated in a higher ratio of DTT to protein.

Andreo et al. (1979) showed that new disulfide bridges can be formed in the β and γ subunits of spinach CF_1 upon heat activation of ATPase activity in the presence of an oxidizing agent, *o*-iodosobenzoate. To test if the lower apparent molecular weight polypeptide of the doublet is an artefact of the heating step used to dissociate polypeptides for electrophoresis, CF_1 was dissociated without heating, in the presence or absence of reducing agents (fig. 10). CF_1 dissociated in 2% SDS without heat gives the same two bands for the γ subunit as that heated in SDS. Addition of DTT to CF_1 during dissociation yields only one band for the γ subunit (fig. 10, lanes b and c) independent of heating the sample. Thus it

Figure 8: Limited proteolysis of each of the two bands of the γ subunit dimer and of the α and β subunits. A sample of purified CF_1 containing 44 ug protein was dissociated in buffer containing 65 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS and 0.2% β-mercaptoethanol and the subunits were separated by SDS-PAGE. The gel was stained for five minutes, and destained to the point where bands were visible. These were excised and subjected to limited proteolysis according to the in-gel method of Cleveland et al. (1977) as described in Materials and Methods. A. Proteolytic pattern generated from the upper polypeptide of the γ dimer. B. Pattern from the lower polypeptide of the γ dimer. C. Pattern from a combined sample of the α and β subunits.

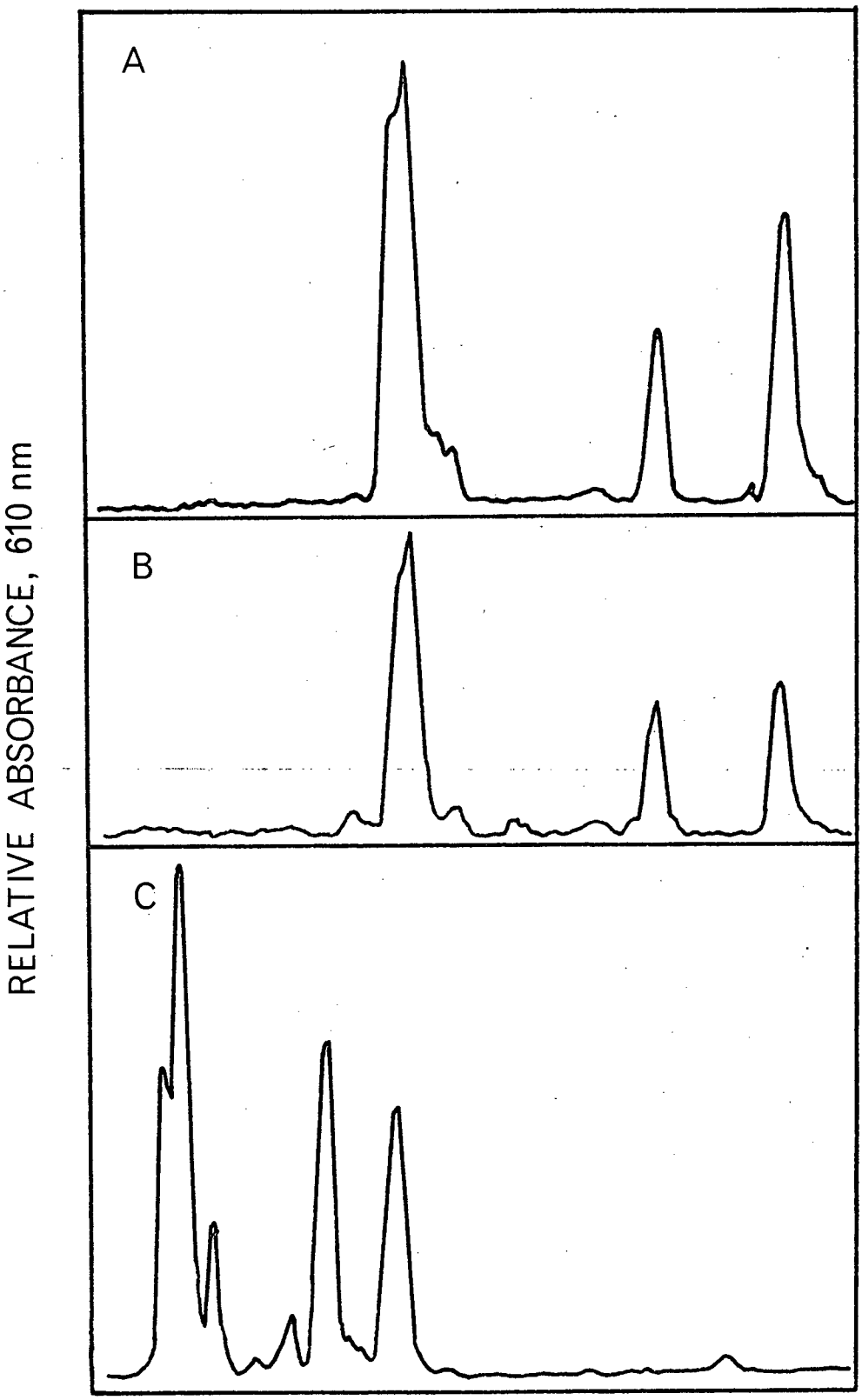

A
B
C
RELATIVE ABSORBANCE, 610 nm
MIGRATION ⟶

Figure 9: Effect of reducing agent upon the mobility of the γ subunit of wheat CF_1 in SDS-PAGE. All lanes contain 38 ug protein. A. CF_1 dissociated in a 2% SDS solution containing 11 nmoles DTT/mg CF_1. B. 15.4 nmoles DTT/mg CF_1. C. 33 nmoles DTT/mg CF_1.

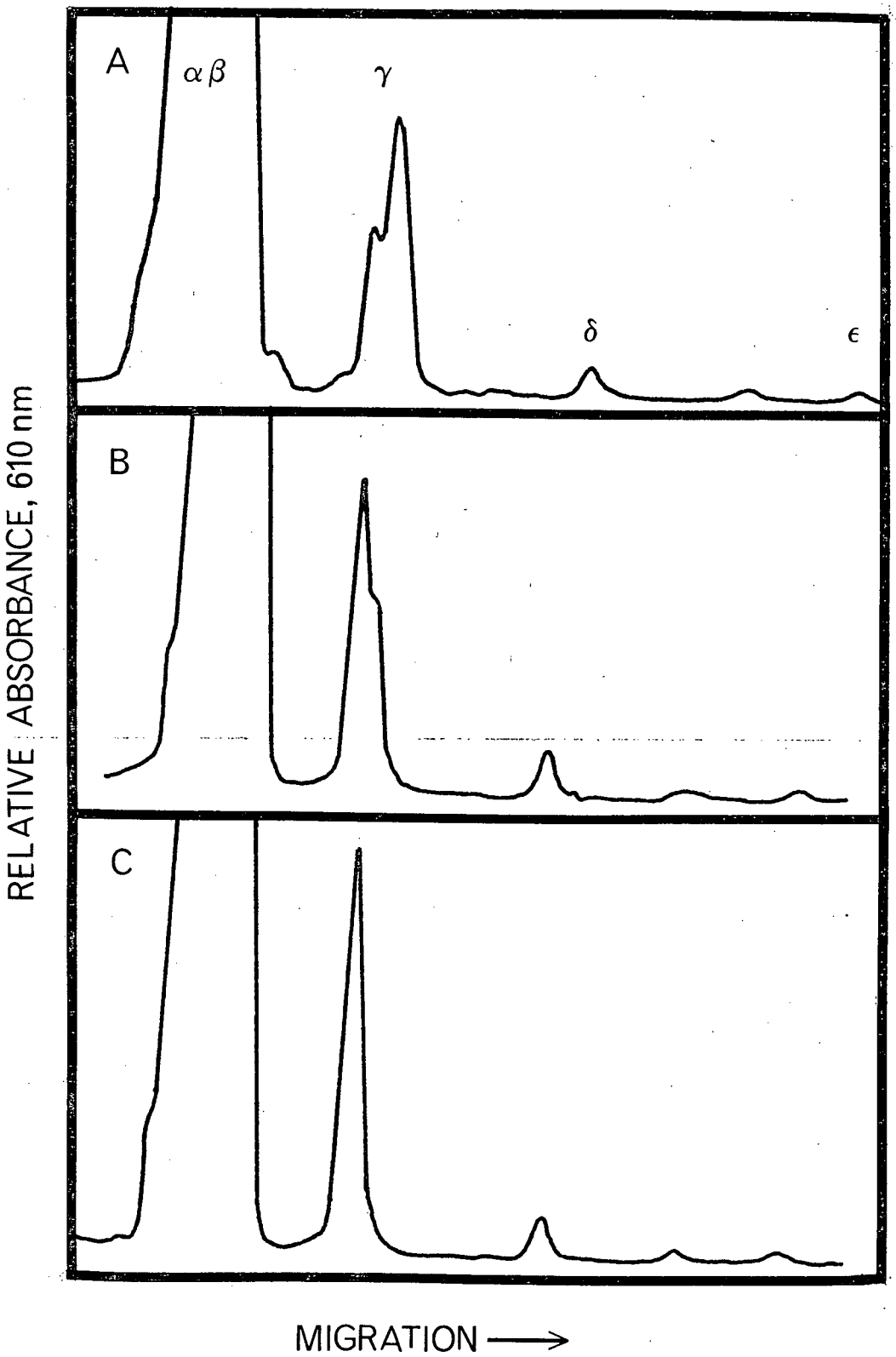
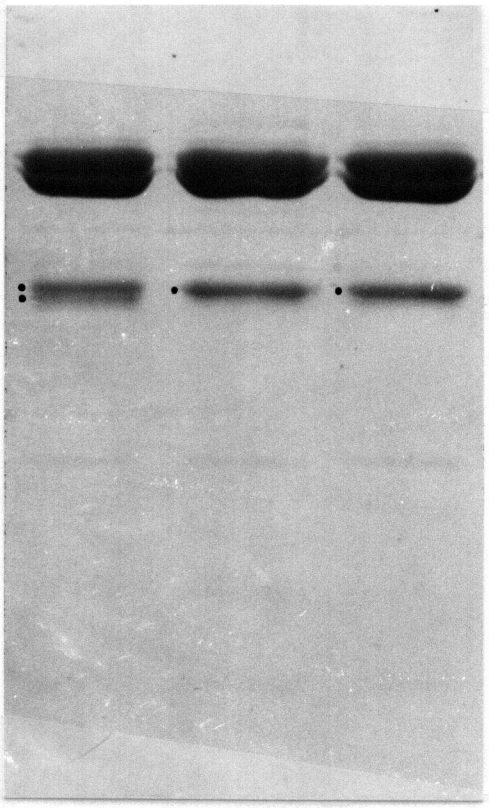

A
αβ
γ
δ
ϵ
B
C
RELATIVE ABSORBANCE, 610 nm
MIGRATION ⟶

Figure 10: Effect of heat upon the number of bands found in the γ subunit in SDS-PAGE. All lanes contain 37.5 ug protein.

a. CF_1 dissociated in 2% SDS, 2.5 mM DTT, no heating.

b. CF_1 dissociated in 2% SDS, 12.5 mM DTT, and heated 60 sec at 100 C.

c. CF_1 dissociated in 2% SDS, 12.5 mM DTT, no heating.



a b c

appears that heat does not artificially introduce a disulfide bridge into the γ subunit of wheat CF_1. Since two discrete bands are obtained, one can suggest that there is at least one internal disulfide bridge in this subunit. The two bands could be the completely reduced subunit and the subunit with its internal crosslink(s).

ATPase Activity of Wheat CF_1

Spinach chloroplast coupling factor, the best characterized of the plant CF_1's, exhibits a latent Ca^{2+}-dependent ATPase activity that can be activated by a short trypsin digestion (Vambutas and Racker, 1965) or by heat treatment in the presence of a low concentration of DTT (Farron, 1970). In the initial wheat CF_1 preparations for this study, CaATPase activity was routinely unmasked by trypsinization, but when it was discovered that the inclusion of proteolysis inhibitors improved the isolation of intact CF_1 complex, it was decided to determine whether the wheat CF_1 has a heat activated CaATPase activity also. Table III gives the result of an experiment to induce a CaATPase activity by heating at various temperatures.

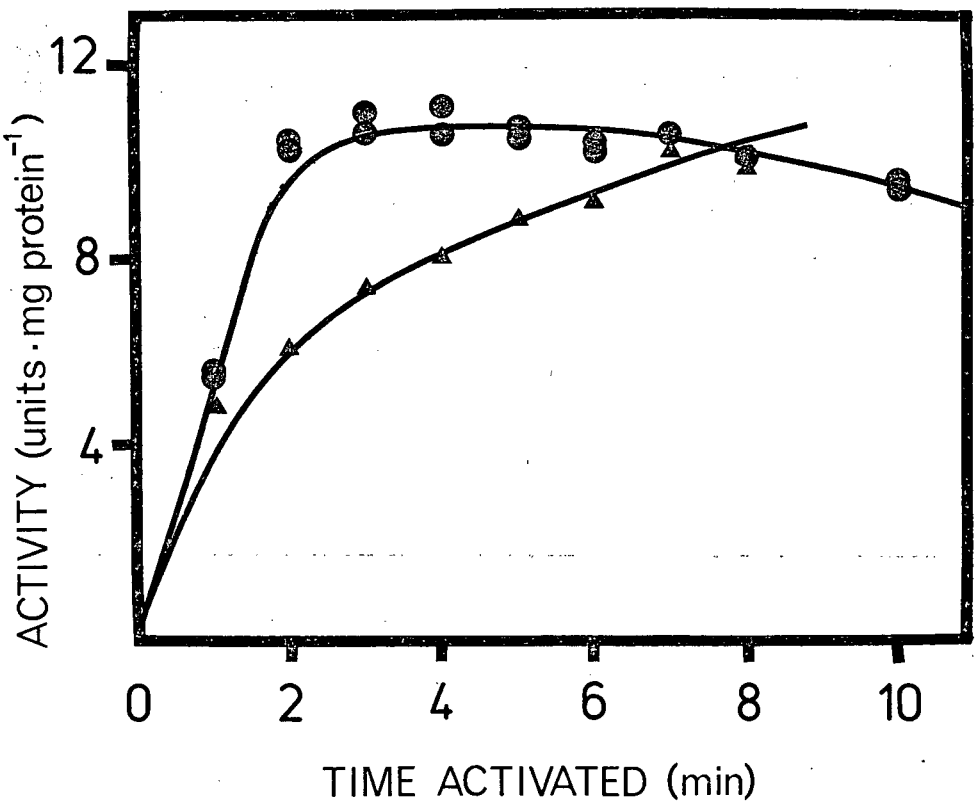
TABLE III. Heat activated CaATPase activity of wheat CF_1 treated at various temperatures and assayed according to the method of Lién and Racker (1971b).

Length of time heated (minutes)	Ca^{2+}-dependent ATPase activity of wheat CF_1 activated at the following temperatures (units/mg protein)		
	49°C	56°C	61°C
0	0	0	0
0.5	0.10	0	0
1	0.22	0	0
2	0.46	0	0
3	0.48	0	0
4	0.40	0	0
6	0.30	0	0

In comparison, activation by the trypsin method routinely gives 7 to 20 units/mg protein of CaATPase activity. Thus the maximal heat-activated activity obtained (0.48 units/mg protein) can be considered negligble. The inability to heat activate is a property of the wheat CF_1 complex, since spinach CF_1 activated by heating for 4 min at 60°C showed 10 units/mg protein CaATPase activity. On the basis of these results it was decided to remain with the trypsin method for activating the latent CaATPase of wheat CF_1.

Since proteolysis inhibitors included in the wheat CF_1 preparation solutions (*p*-aminobenzamidine, ε-amino-*n*-caproic acid and PMSF) are specific inhibitors of serine proteases, including trypsin, it was necessary to determine what effect the presence of these inhibitors might have upon the activation of CaATPase activity. Figure 11 shows that a longer trypsin

Figure 11: Time course of trypsin activation of wheat CaATPase in the presence and absence of proteolysis inhibitors. ● CF_1 sample dialyzed to remove proteolysis inhibitors before trypsin activation. ▲ Trypsin activation of CF_1 in the presence of proteolysis inhibitors. Trypsin activation was done in a sample of protein with a final concentration of 2.9 mM ε-amino-*n*-caproic acid, 0.43 mM *p*-aminobenzamidine and 0.0072 mM PMSF.


12
8
4
ACTIVITY (units · mg protein^{-1})
0
2
4
6
8
10
TIME ACTIVATED (min)

digestion is required to reach maximal activity in the presence of these proteolysis inhibitors than in their absence. In either the presence or absence of inhibitors, the six minutes trypsin digestion routine used was adequate to activate the latent CaATPase of wheat CF_1 in the experiment shown. However, depending upon the volume of enzyme added to the activation mix, the concentration of proteolysis inhibitors in any one enzyme activation will vary greatly. Thus it was decided to routinely dialyze out proteolysis inhibitors prior to critical enzyme assays, and remain with the six minutes digestion.

Substrate specificity of wheat CaATPase was determined, and the results are given in Table IV. About one-third of the nucleotide triphosphatase activity with ATP as substrate was obtained with GTP, the other purine nucleotide triphosphate. Negligible activity was obtained with CTP, UTP or TTP as substrates. Wheat CaATPase is thus very substrate specific, accepting only purine base nucleotide triphosphates into the enzyme active site(s), but cleaving GTP to a lesser extent than ATP.

Table IV. Substrate specificity of the CaATPase activity of wheat CF_1. Latent ATPase activity was activated by trypsin digestion in the absence of ATP. All substrates were 1mM nucleotide triphosphate in 40 mM Tricine-KOH pH 8.0, 10 mM $CaCl_2$.

Substrate	% of activity with ATP
ATP	100
GTP	32
UTP	0
CTP	1.4
TTP	0

For assaying the latent CaATPase activity of wheat CF_1, 1 mM ATP was used as substrate. To test whether the wheat ATPase is strictly calcium-dependent, the calcium concentration was varied, holding the ATP concentration constant at 1 mM. As shown in fig. 12, optimal activity is obtained with a 1:1 ratio of calcium to ATP, implying that the cation-ATP complex is the true substrate for the ATPase reaction. Excess calcium (10 Ca^{2+}: 1 ATP) does inhibit enzyme activity about 25%.

K_M and V_{max} for the CaATPase of wheat CF_1 were determined under two different sets of conditions. In the first, initial velocities of the reaction were determined at various concentrations of ATP with Ca^{2+} in excess, at a constant concentration of 10 mM. Substrate was plotted as the concentration of CaATP complex, as calculated using equation (1). The result of one such experiment is shown in fig. 13. Eadie-Hofstee analysis of this data gives a K_M of 0.125 mM CaATP and a V_{max} of 18.9 units/ mg protein. V_{max} was found to vary greatly from enzyme preparation to enzyme preparation, but under the same conditions of excess Ca^{2+}, K_M was found to be in the range of 0.1 to 0.3 mM CaATP.

In the second set of conditions, the calcium level was kept equimolar to that of ATP. Using equation (1) to calculate the concentration of CaATP complex, a K_M of 18 uM CaATP and a V_{max} of 6.77 units/mg protein were obtained. (fig. 14). It was observed that the maximal enzyme activity, but not the K_M, varies from enzyme preparation to enzyme preparation. The enzyme used in this determination was from a different preparation and a different batch of seed, than that used for the experiment shown in fig. 13, so V_{max} values are not comparable. However, the K_M in the case of equimolar calcium and ATP is an order of magnitude lower than that obtained with a large excess of calcium, and appears to be a direct effect of the different levels of calcium.

Figure 12: Effect of the ratio of calcium to ATP upon ATPase activity of the trypsin-activated CF_1 from wheat. The ATP concentration in the assay mix was held constant at 1 mM and the calcium concentration was varied.

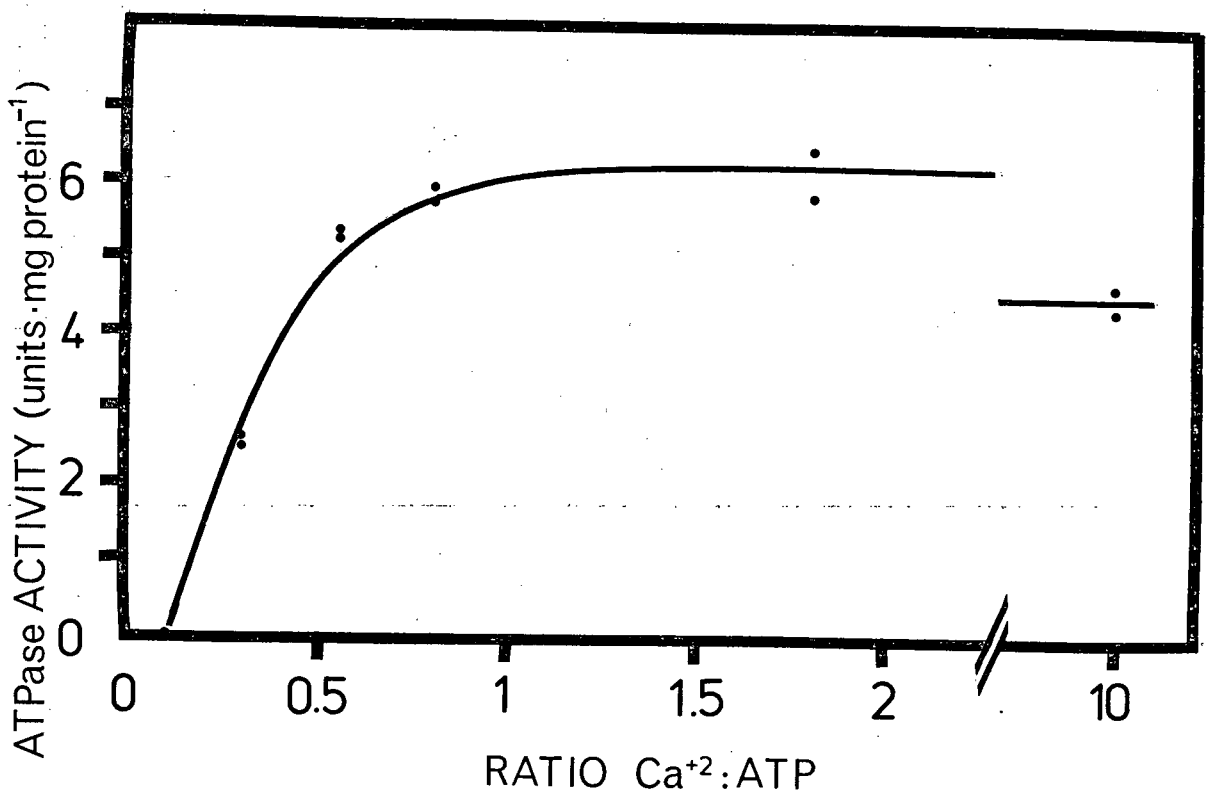

ATPase ACTIVITY (units·mg protein^{-1})
0
2
4
6
0
0.5
1
1.5
2
10
RATIO Ca^{+2}:ATP

Figure 13: Michaelis-Menten kinetics of the trypsin-activated CaATPase of wheat CF_1 in the presence of 10 mM $CaCl_2$. Substrate concentration (S) was determined by calculating CaATP according to equation (1). Inset is an Eadie-Hofstee plot of this data, used for the determination of K_M and V_{max}. This line has a correlation coefficient of 0.93.

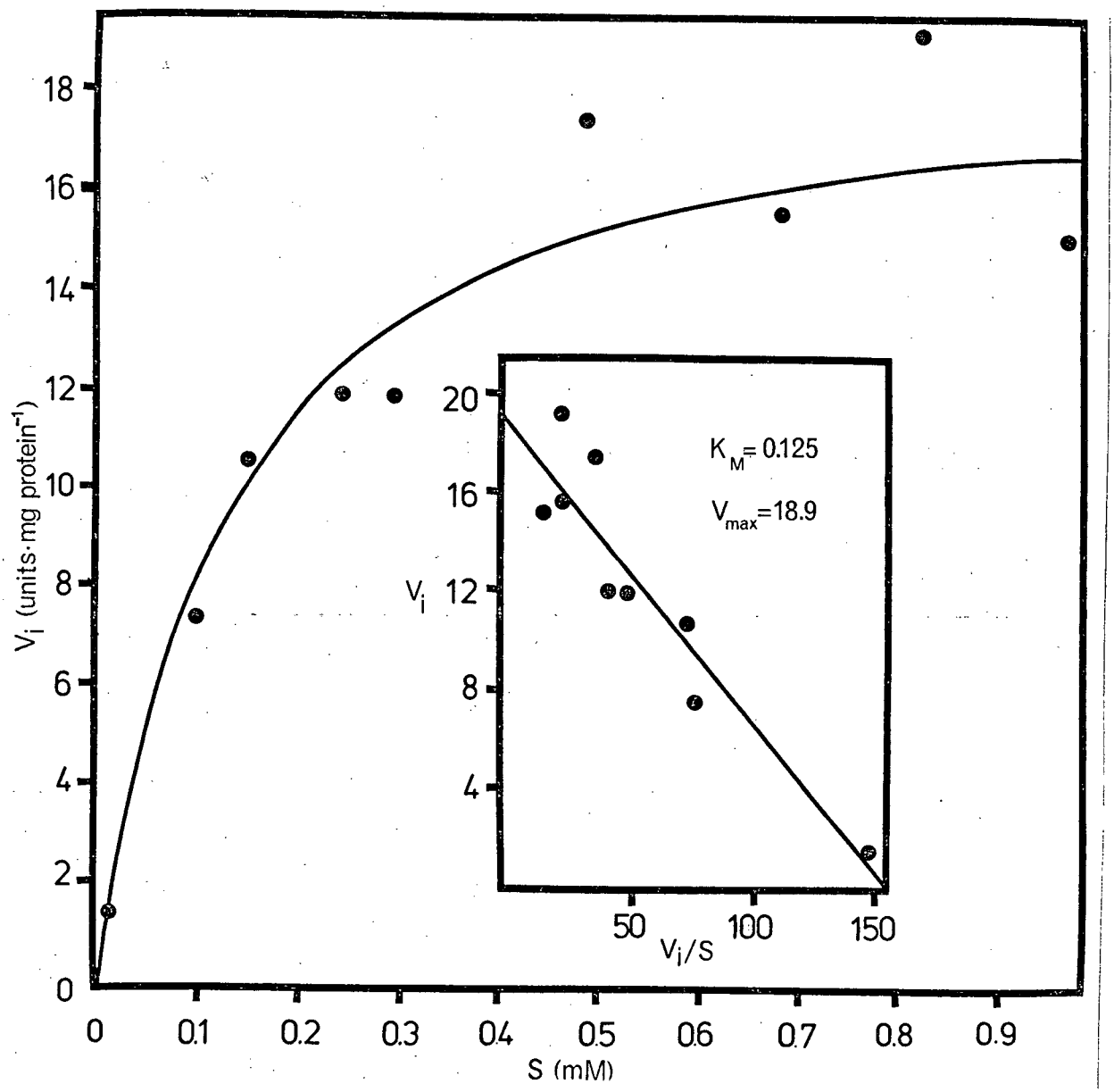
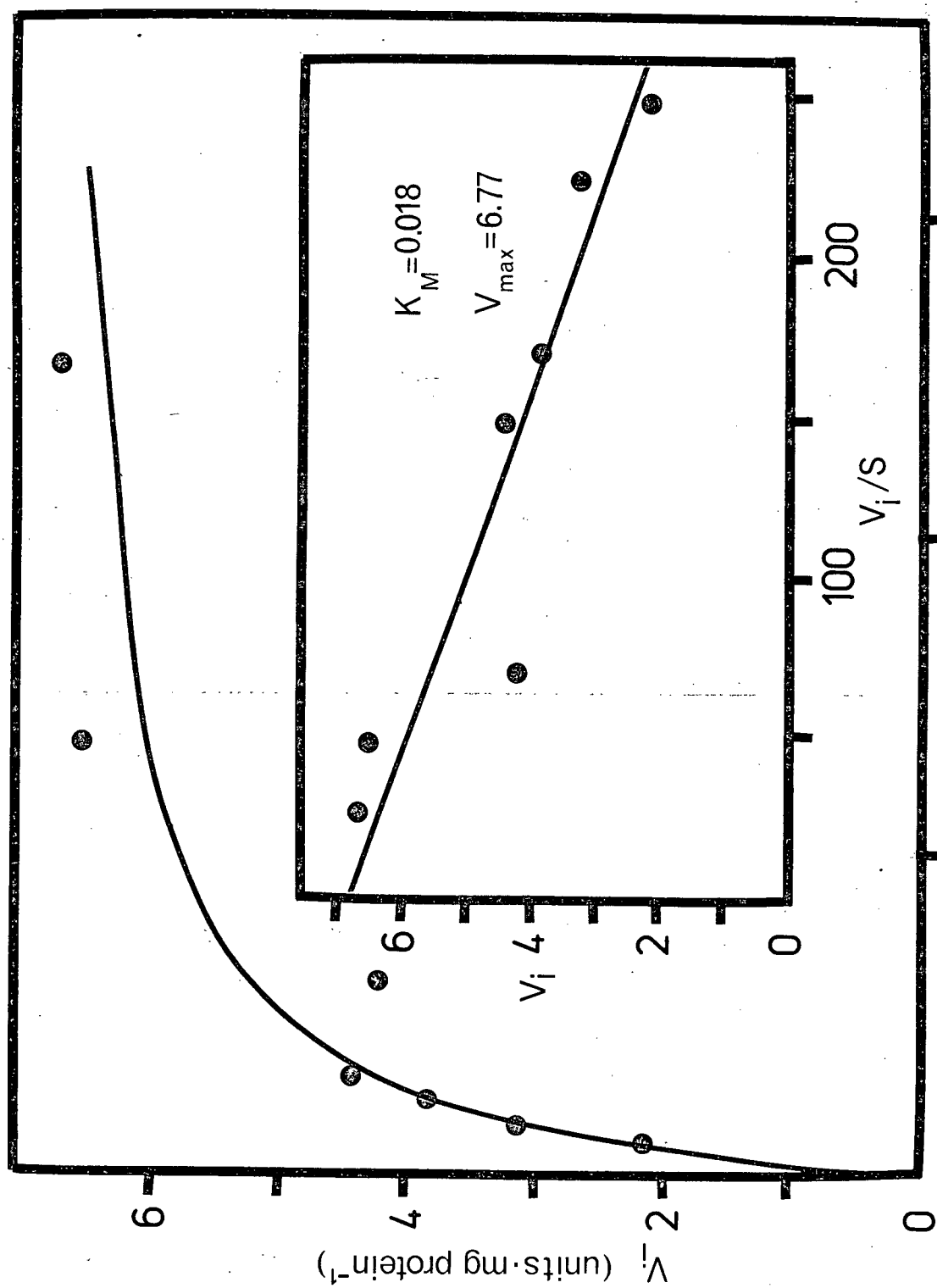

V_i (units·mg protein^{-1})
S (mM)
K_M= 0.125
V_{max}=18.9
V_i
V_i/S

Figure 14: Michaelis-Menten kinetics of the trypsin-activated CaATPase of wheat CF_1. Calcium and ATP were added in equimolar amounts to the assay mix, and substrate concentration was calculated according to equation (1). Inset is an Eadie-Hofstee plot of this data, used for the determination of K_M and V_{max}. This line has a correlation coefficient of 0.87.


V_i (units·mg protein^{-1})
0
2
4
6
0.1
0.2
0.3
S (mM)
K_M=0.018
V_{max}=6.77
V_i
0
2
4
6
100
200
V_i/S

Preliminary experiments to test the effect of ADP upon CaATPase activity showed that ADP was indeed inhibitory. However, these results do not give a clear picture as to whether ADP is a competitive inhibitor or acts by some allosteric mechanism. Furthermore, these experiments were done before the effect of high concentration of calcium was observed, and part of the inhibition seen may have been due to excess calcium rather than to the presence of ADP.

Electron Microscopy of Wheat CF_1

Negative staining of wheat CF_1, with either ammonium molybdate or phosphotungstic acid, gave identical particles when viewed in the electron microscope (fig. 15). Particle diameters were estimated to be 108 Å ± 9.5 Å with ammonium molybdate stain, and 110 Å ± 6.3 Å with phosphotungstic acid. Phosphotungstic acid gave slightly better resolution, and more consistent results than did ammonium molybdate. Uranyl acetate stain caused CF_1 to dissociate into its subunits.

E. coli F_1 shows a very similar structure to CF_1 when stained by the same procedure (fig. 16). ECF_1 has a particle diameter of 100 Å ± 5.1 Å when particles are negatively stained with ammonium molybdate.

Particles of CF_1 show many orientation when negatively stained, as they settle onto the electron microscopy grid at random. It is possible to pick out particles which have a hexagonal structure, with a central core and what may be six subunits at the verticies (fig. 17). Many times such an orientation seems to be tilted out of the plane, giving particles with a central depression.

However, at such high magnifications, it is difficult to obtain good resolution of enzyme particles because of the large background grain size.

Figure 15: Electron micrographs of wheat CF_1, negatively-stained with two different stains. A. 2% phosphotungstic acid KOH pH 7.0. B. 2% ammonium molybdate in 12 mM sodium oxalate pH 7.2. Magnification in both micrographs is 516,000 x.

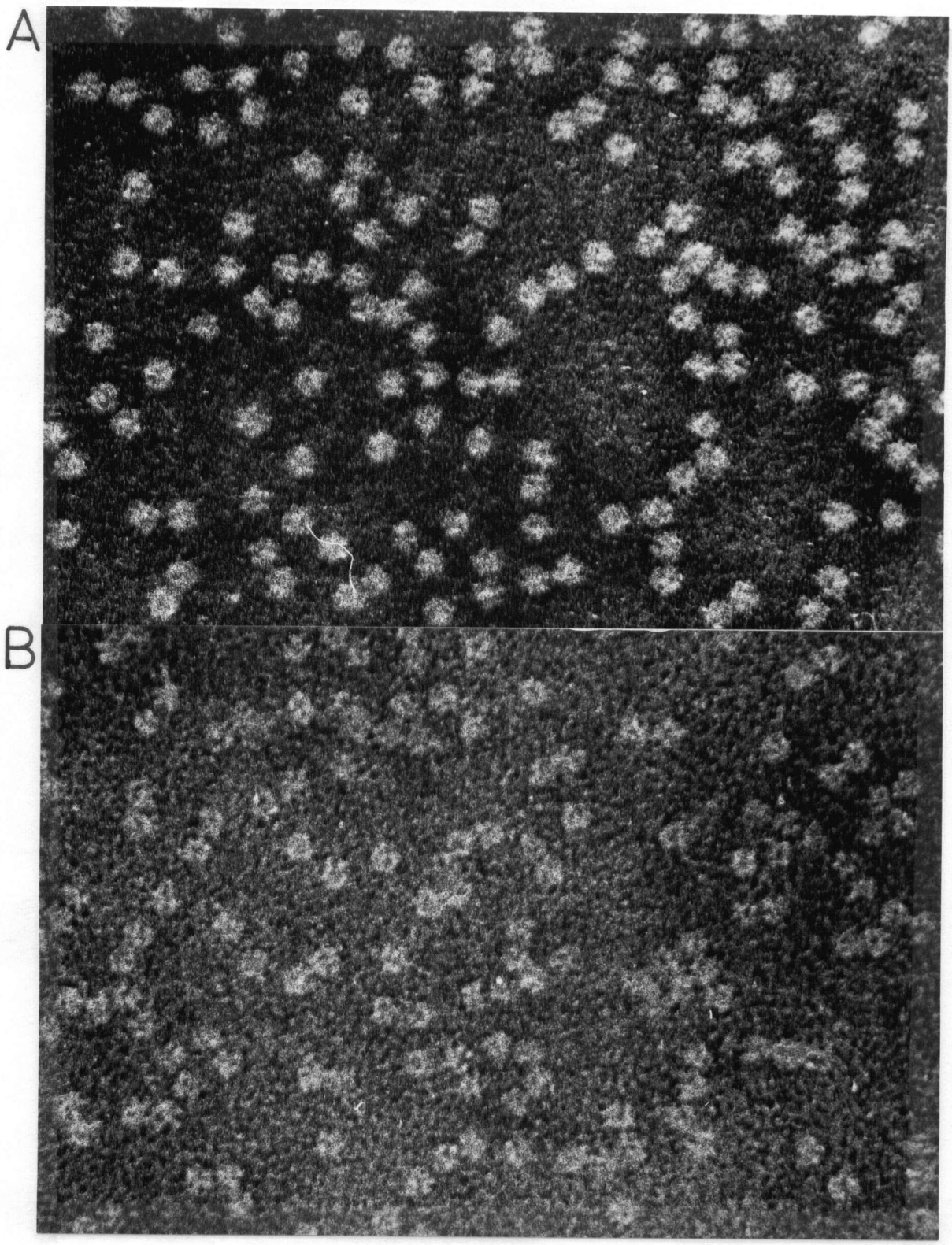

A
B

Figure 16: Electron micrographs of *E. coli* F_1, negatively-stained with 2% ammonium molybdate in 12 mM sodium oxalate pH 7.2. Magnification is 413,000 x.

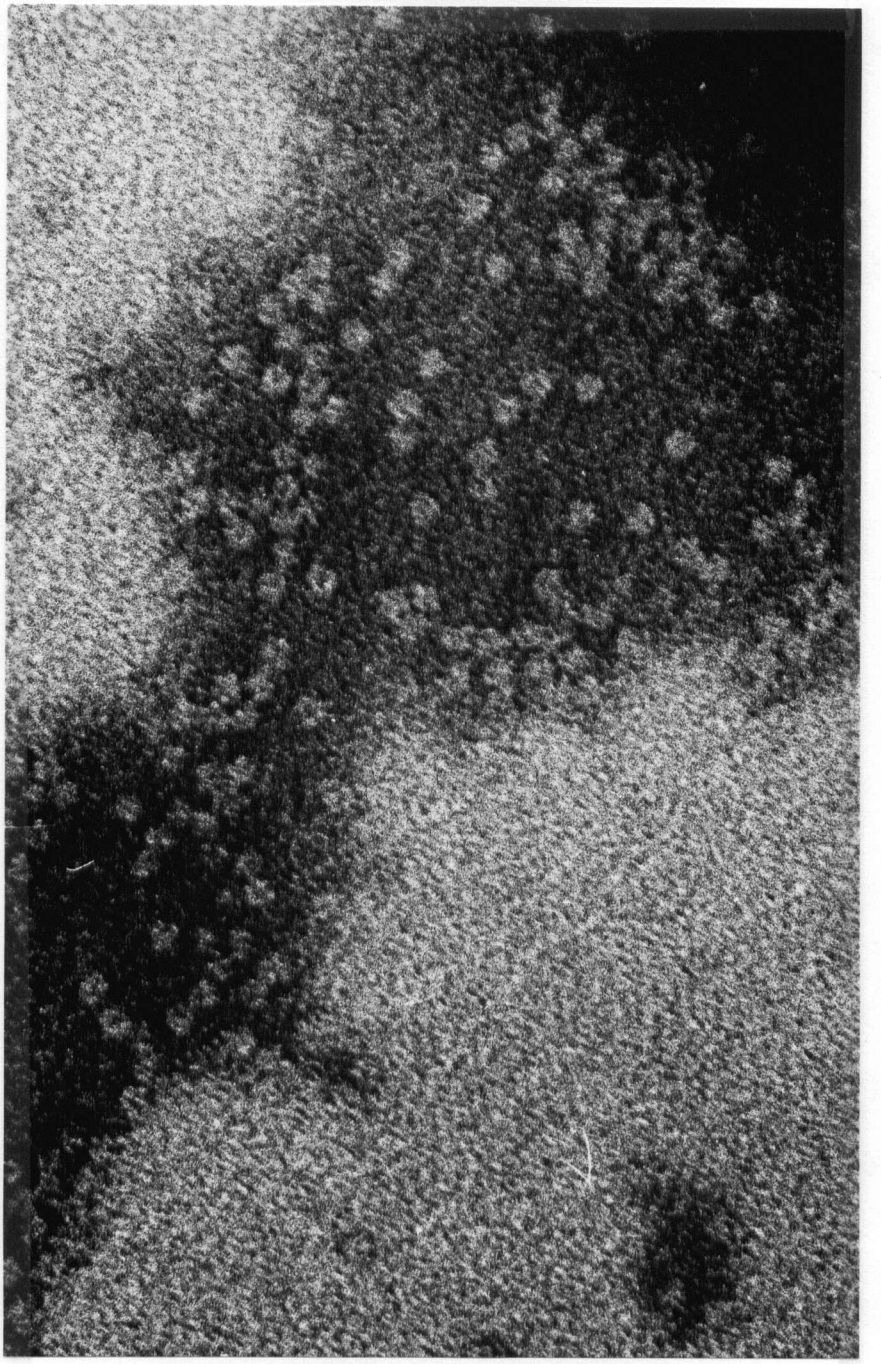
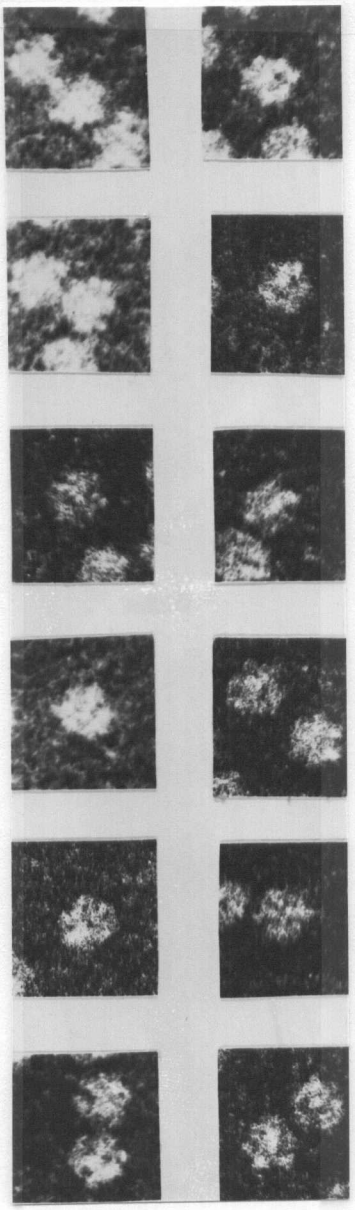


Figure 17: Selected CF_1 particles, negatively stained with 2% phosphotungstic acid-KOH pH 7.2, showing various degrees of hexagonal structure. Magnification is 739,000 x.



For this reason, it was decided to try image enhancement of the most symmetrical molecules printed to bring out any inherent structure. CF_1 particles exhibiting what appeared to be a six-fold symmetry were blown up photographically and image analysis was performed by Markham rotation (Markham et al., 1963). Figure 18 shows the result of rotation of three such particles. From the original image it is difficult to differentiate between five-, six-, and seven-fold symmetry, but for the most part fig. 18 shows the best resolution of apicies with a six-fold rotation, with two- and three-fold rotations also giving hexagonal particles.

Immunological Study of Wheat CF_1

Anti-wheat CF_1 serum, with an antibody titer of 1/80, was obtained from a rabbit after three injections of 0.2 mg purified CF_1 (fig 19). Serum taken from the rabbit prior to immunization did not bind to CF_1, since it gave no precipitin line against wheat CF_1 in double diffusion plates. Wheat CF_1 antiserum also precipitated purified spinach CF_1, indicating similar antigenic sites on the CF_1 complexes from the two plants.

Complete CF_1 was injected into the rabbit to generate antibodies. In order to determine against which CF_1 subunit or subunits the antibodies reacted, two dimensional immunoelectrophoresis was performed (fig. 20). A precipitin rocket developed against the α and β subunits, but not against the three smaller subunits. It is impossible to ascertain whether the anti-CF_1 serum reacts against both the α and β subunits, or only one subunit, because these two subunits are not well resolved in a 10% SDS-PAGE gel. However, the immunoprecipitation rocket is symmetrical, so it probably reacts with both of the largest subunits of wheat CF_1. Thus this antiserum would

Figure 18: Markham rotation of three wheat CF_1 particles. The numbers denote the divisions of a circle through which the photographic paper was rotated in making the multiple exposures.

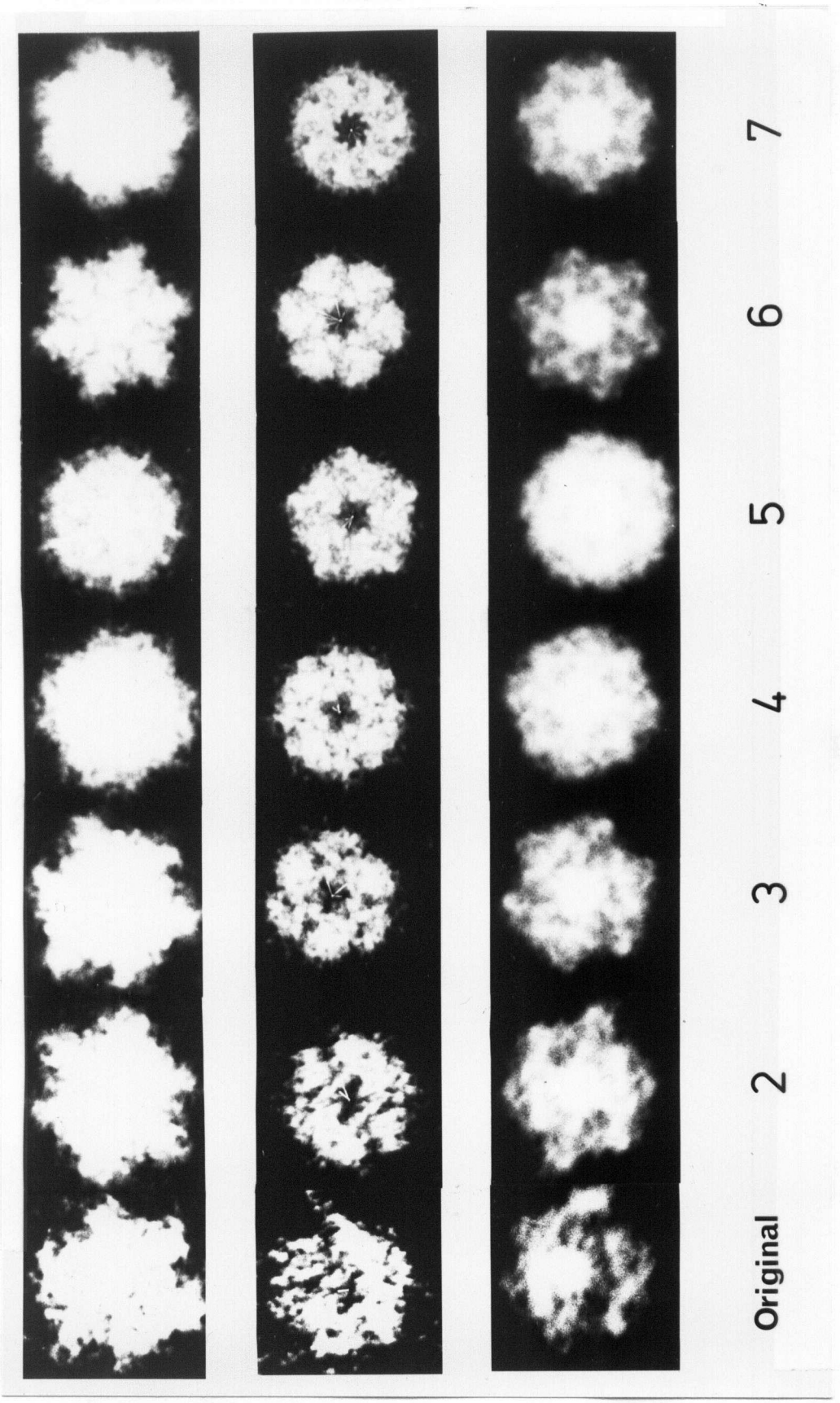
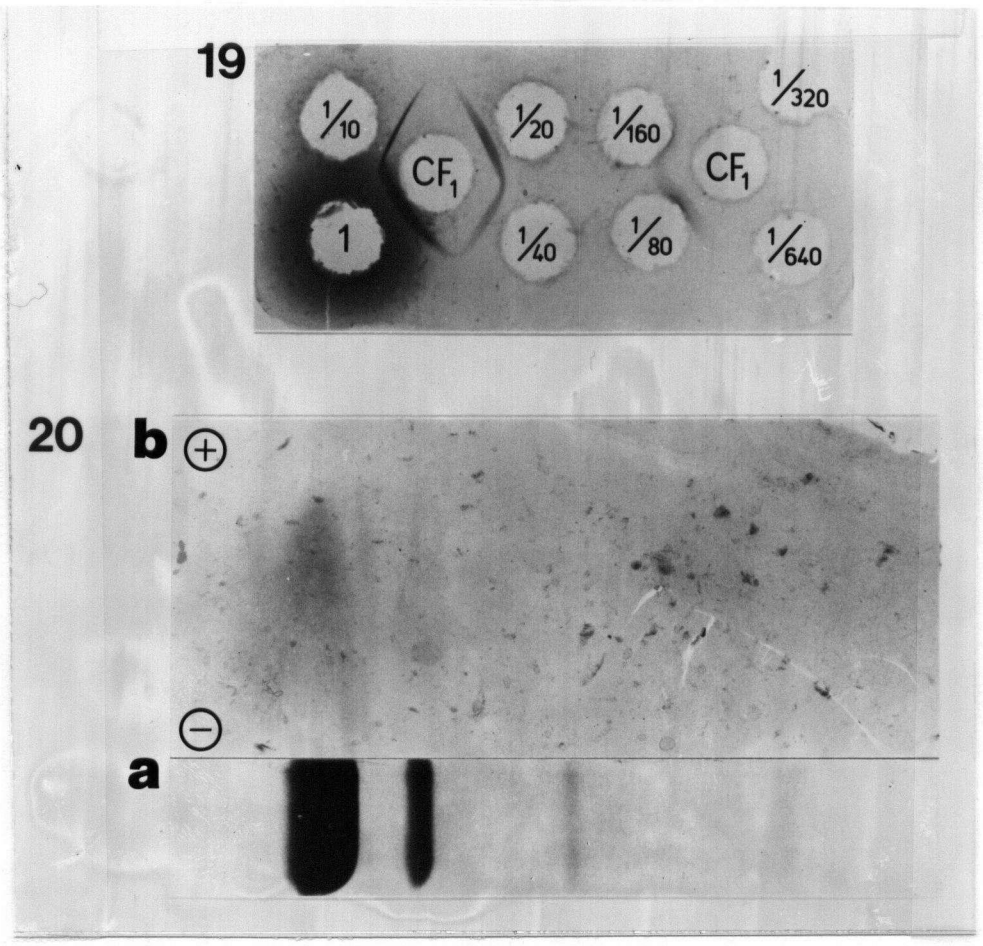

Original
2
3
4
5
6
7

Figure 19: Ouchterlony double diffusion plate estimation of antibody titer in serum from rabbit immunized against wheat CF_1. Whole serum was diluted as indicated in the peripheral wells, and precipitin lines were developed against CF_1 overnight. These precipitin lines were then visualized by staining with Coomassie blue as described in Materials and Methods.

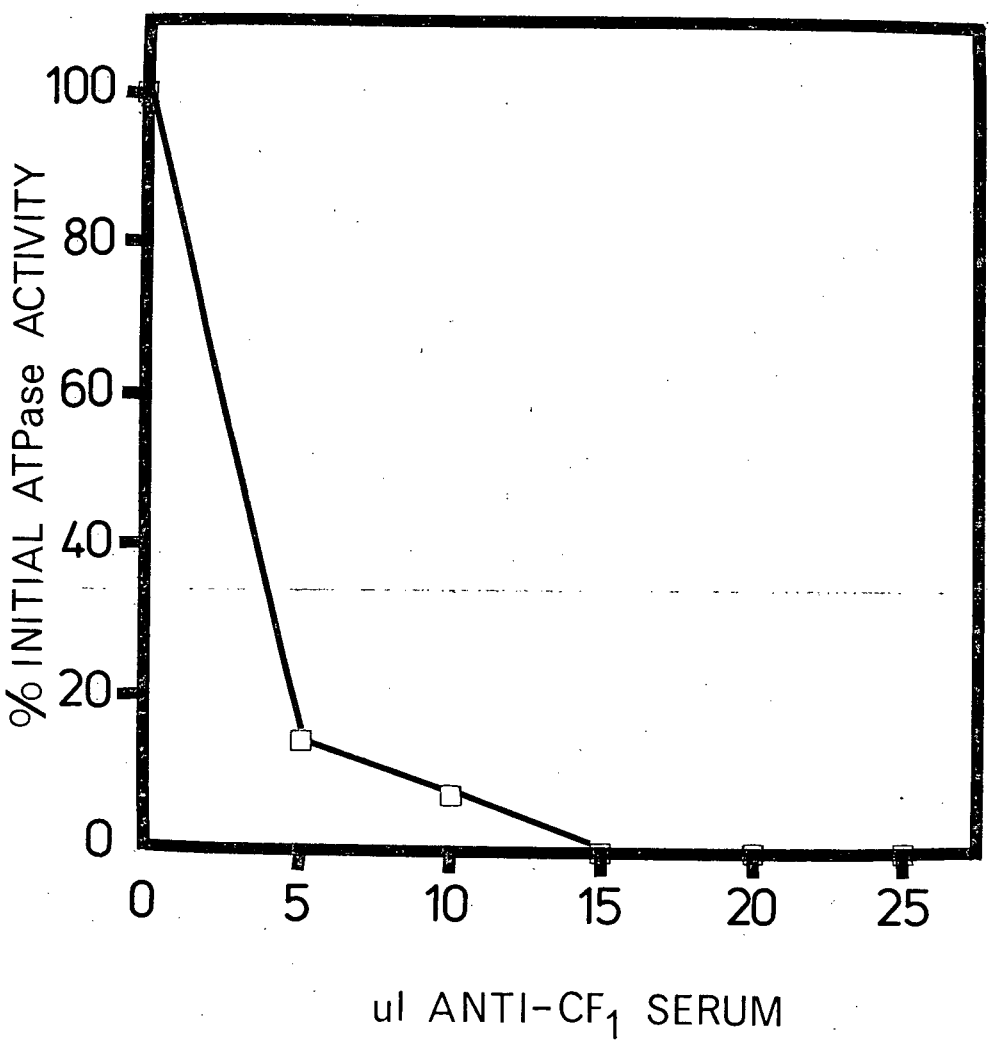
Figure 20: Crossed immunoelectrophoresis of the separated subunits of wheat CF_1 against anti-wheat CF_1 serum, according to the method of Chua and Blomberg (1979). a. Wheat CF_1 subunits, separated by SDS-PAGE on a 10% gel. The sample contained 47 ug protein. The lane shown here is a lane adjacent to the one electrophoresed into the antibody gel. b. Antibody gel, showing immunoprecipitin rocket which has developed against the α and β subunits of wheat CF_1. The antibody gel contained 1 ml of unfractionated rabbit antiserum. Precipitin lines were visualized by staining with Coomassie blue as described in Materials and Methods.


19
1/10
CF1
1/20
1/160
1/320
CF1
1
1/40
1/80
1/640
20
b
⊕
⊖
a

be useful in precipitating the α and β subunits in a cell-free translation of whole wheat RNA.

The antiserum obtained also inhibits the latent CaATPase of trypsin-activated CF_1 (fig. 21). Very small amounts (15 ul) of antiserum completely inhibit ATPase activity.

Figure 21: Inhibition of the trypsin-activated CaATPase activity of wheat CF_1 by anti-wheat CF_1 serum. The latent CaATPase was activated by trypsin digestion, and anti-serum was added to 2.5 ug aliquots of the activated enzyme. CaATPase was assayed immediately, according to the method of Lien and Racker (1971b).


100
80
60
40
20
0
% INITIAL ATPase ACTIVITY
0
5
10
15
20
25
ul ANTI-CF1 SERUM

DISCUSSION

In the procedure developed here, wheat chloroplast coupling factor was removed from the surface of the thylakoid and purified to homogeneity by a simple two-step procedure consisting of chloroform extraction and sucrose density gradient centrifugation. Better preservation of a CF_1 complex containing all subunits was obtained by the inclusion of proteolysis inhibitors during isolation and purification. In experiments in which wheat CF_1 was isolated in the absence of proteolysis inhibitors, both the δ and ε subunits were lost during the sucrose gradient step. Inclusion of proteolysis inhibitors yielded a CF_1 with all five subunits present. This could be due to a number of factors. Several authors have noted the tendency of this enzyme to lose subunits once it has been separated from the thylakoid. Binder et al. (1978) found that the δ subunit of spinach CF_1 was often lost in preparations where CF_1 was chloroform-released from large amounts of thylakoids. Passage of spinach CF_1 through a DEAE-Sephadex A50 column also caused this complex to lose the δ subunit (Younis et al., 1976). It seems that this complex is inherently fragile, above and beyond its cold lability properties. Whether the protection of CF_1 obtained with proteolysis inhibitors reported here is due to inhibition of proteolysis or to some osmotic or ionic effect of these compounds is unknown.

Characterization of the subunits of wheat CF_1 by SDS-PAGE gave several interesting results. Molecular weights of the γ and δ subunits are significantly higher than those of spinach CF_1, especially that of the δ subunit (25 kd versus 19 kd for spinach). In addition, a number of discrete forms of the γ subunit were found, dependant upon the degree of reduction prior to electrophoresis. It is not sufficient to dissociate wheat CF_1 in a stock solution of 2% SDS plus 0.2% β-mercaptoethanol

or 5 mM DTT. A critical amount of reducing agent is necessary to insure complete reduction of the γ subunit, at least 33 nmoles DTT/mg CF_1. Care should be taken to completely reduce and dissociate all polypeptides when molecular weight determinations are made on a basis of mobility in SDS-PAGE.

Because two discrete γ bands are obtained when CF_1 is electrophoresed in low amounts of reducing agent, it is tempting to conclude that there is at least one internal disulfide bridge in the γ subunit. Farron and Racker (1970) showed that there are two disulfide bonds in the spinach CF_1 molecule. These are not broken during ATPase activation, as trypsin or heat activation of the latent ATPase did not result in an increase in the number of titrable half cystines (also Farron and Racker, 1970). It is proposed here that there is at least one internal disulfide bridge in the γ subunit of wheat CF_1. A similar internal cystine which alters mobility in electrophoresis has been identified in the 33 kd polypeptide of Photosystem II particles in spinach (Kuwabara and Murata, 1979).

The effect of proteolysis inhibitors in preventing release of CF_1 from the thylakoid is striking, and specific for the CF_1 complex. In fig. 4 (lanes c and d) one can see that proteolysis inhibitors do not prevent the release of two other unidentified polypeptides normally extracted by hypotonic sucrose (arrows) but they completely inhibit release of any of the polypeptides of CF_1. One could thus argue that the inhibitors, by their specificity, prevent CF_1 release because proteolysis is prevented, rather than because of some general osmotic effect. In addition, the discovery that proteolysis inhibitors prevent the loss of the minor subunits of CF_1 during sucrose gradient centrifugation suggests that a protease extracts with CF_1.

All attempts to heat activate the latent CaATPase of wheat CF_1 were unsuccessful. Under identical conditions, the spinach CF_1 activated to a maximum of 10 units/mg protein. This leads one to conclude that there is a basic difference in the structure of these two enzymes. Farron (1970) and Farron and Racker (1970) studied the process of heat activation of spinach CF_1. In both the activated and unactivated CF_1 complexes, eight SH groups were titrable in urea or SDS. Heat activation did not result in the cleavage of either of the two disulfide bridges of spinach CF_1. However, most of the eight SH groups are not on the surface of the enzyme, since in the absence of denaturing solvents, only two SH groups per molecule of CF_1 could be titrated. Heat activation made accessible four additional SH groups. These newly-exposed SH groups can be induced to form intrapeptide disulfide bridges by the addition of the strong oxidizing agent *o*-iodosobenzoate during heat activation (Andreo et al., 1979). It has been shown that N-ethylmaleimide or iodoacetamide inhibit the activation of CF_1 by heat but not by trypsin (Farron and Racker, 1970), implying that the two types of activation of the latent CaATPase proceed by different mechanisms. Thus it is quite possible that wheat CF_1 should show trypsin activation of the latent CaATPase but not heat activation.

The optimal Ca:ATP ratio for ATPase activity of the activated CF_1 from spinach is 1:1 (Vambutas and Racker, 1965). Similar results are reported here for wheat CF_1. This suggests that the actual substrate for the ATPase reaction is the $Ca^{2+}ATP$ complex.

The formation of the cation-ATP complex is pH dependent as well as dependent upon the dissociation constant for the complex, according to the relationship given in equation (1). It is important to calculate

substrate concentration as complexed CaATP rather than free ATP, for at very low ATP concentrations there is a large difference between the two values. The complex kinetic curves published by Carreira and Munoz (1975) for the ATPase activity of *E. coli* F_1 were called into question by Ahlres (1976) for this very reason. In experiments where Carreira and Munoz used a Ca:ATP ratio of 0.5:1 in determining reaction rates with ATP as the substrate, non-linear Lineweaver-Burke plots were obtained. Ahlers has shown that these unusual kinetic curves are due to faulty choice of substrate and failure to calculate the effective concentration of the cation-ATP complex. In the study reported here, by plotting initial velocities against the calculated CaATP concentration, a Michaelis-Menten relationship is obtained (figs. 13 and 14).

A number of K_M values for the CaATPase have been reported in the literature. Nelson et al. (1972), assaying the spinach ATPase in Tricine-maleate buffer at pH 8 and in the presence of 8 mM $CaCl_2$, found a K_M of 0.8 mM ATP. In lower pH medium (pH 6) in the presence of 2 mM $MgCl_2$ a MgATPase with a K_M of 0.1 mM ATP was observed, though V_{max} was very low at this pH. In pH 8 Hepes-NaOH buffer, Hochman et al. (1976) obtained a K_M of 2.7 mM CaATP for the lettuce enzyme. In the results presented in this dissertation, the K_M obtained in the presence of excess calcium (0.125 mM CaATP) is comparable to values reported in the literature, but the K_M obtained in experiments with equimolar calcium and ATP (18 uM CaATP) is much lower than those reported for spinach or lettuce ATPases. This is probably due to a difference in the enzyme from wheat.

The kinetic data of Hochman et al. (1976) indicates that the cation-ATP complex is also the substrate of ATPase activity of lettuce CF_1. By substituting Mn^{2+} for Ca^{2+}, they measured direct binding of MnATP by

the amplitude of the Mn^{2+} electron paramagnetic resonance signal. Their results show two tight and three loose cation binding sites on CF_1. The tight cation binding sites have a K_M in the micromolar range, similar to the K_i values for the competitive inhibition of free cations. These authors propose that these high affinity cation sites are the catalytic sites, while the low affinity sites are regulatory. They conclude that the rate of catalysis is regulated by the low affinity site, which has a K_M in the millimolar range.

In experiments reported here, the K_M of the trypsin-activated ATPase was determined using CaATP as substrate in the presence of a vast excess of Ca and with a 1:1 ratio of Ca:ATP. With excess Ca, a K_M of 0.1 to 0.3 mM was obtained, while with equimolar amounts of Ca and ATP a K_M of 18 uM was found. One may suggest several reasons for the different affinity constants in these two types of determiniations. It is possible that excess calcium binds at other sites than the active sites on the ATPase, decreasing the affinity of the enzyme for substrate. Alternatively, the increase in K_M observed with excess calcium could be due to the excess calcium binding competitively for the same high affinity site as CaATP. The second postulate would call for a competitive inhibition of the ATPase reaction by calcium. Hochman et al. (1976) give a K_i of 7 mM for Ca, working with the heat-activated ATPase of lettuce CF_1. The preliminary nature of the results presented here prevents making any conclusions about the affinity of substrate binding in low and high Ca concentrations.

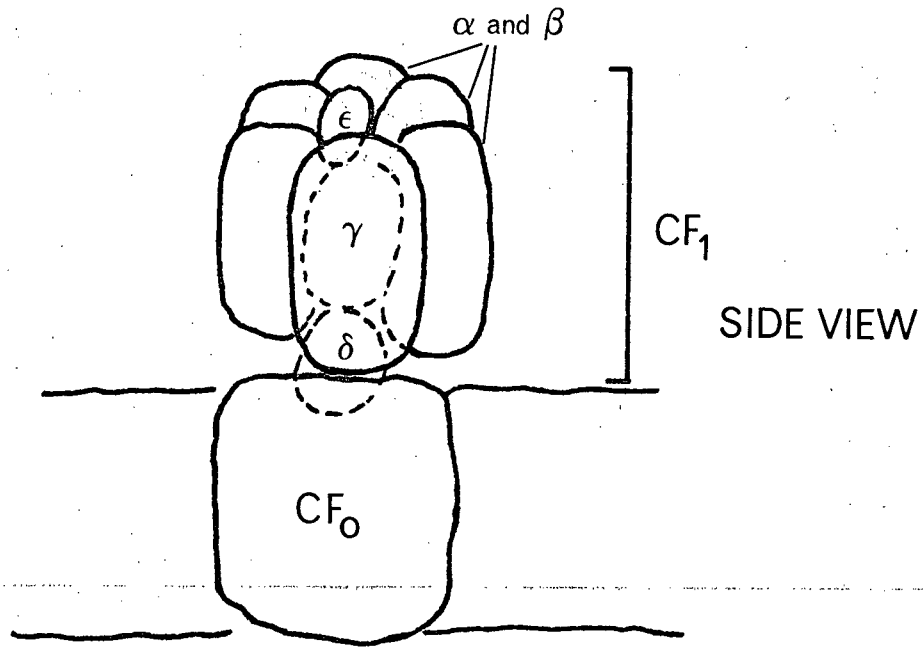
Negative-stained wheat CF_1 has a particle diameter of 108-110 Å when viewed in the electron microscope using two different negative stains. The micrographs show a hexagonal structure in particles of one particular orientation. The electron micrographs presented here are strikingly similar

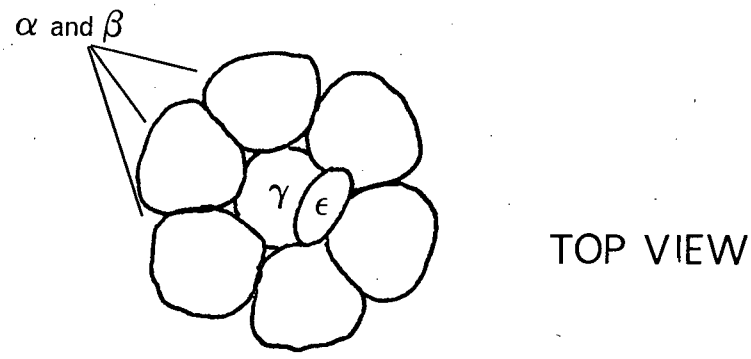
to published micrographs of yeast mitochondrial F_1 (Schatz et al., 1967) and beef heart mitochondria F_1 (Kagawa and Racker, 1966). These F_1 preparations gave 80-90 Å spheres which consist of about six discrete subunits. Markham rotation of wheat CF_1 particles enhances a six-fold symmetry. However, it must be remembered that one introduces bias when using this image enhancement technique, by selecting particles that suggest a particular symmetry. Particles will fall in many orientations upon the electron microscopy grid. In addition, one enhances artefacts of staining as well as the actual structures. From fig. 18 one can conclude only that one particular orientation of wheat CF_1 shows a six-fold symmetry. For the most part, these hexagonal particles appear solid, as if there is a central core of subunit or subunits (fig. 17).

On the basis of my observations and the work of others, I am proposing a model of wheat CF_1 (fig. 22). The δ and ε subunits are placed at exposed regions of the solubilized complex because of evidence presented here (fig. 5) and by other authors (Younis et al., 1977; Binder et al., 1978) that these polypeptides are easily lost during enzyme purification. The δ and ε subunits are drawn at opposite ends of the $\alpha_3\beta_3\gamma$ core on the basis of the cross-linking studies of Baird and Hammes (1976), in which it was found that these are the only subunits not in close enough proximity to be chemically cross-linked.

Stoichiometry and structure are closely linked. As noted in the Introduction, the question of stoichiometry of the subunits of the coupling factor complexes is still a controversial issue. Any $\alpha_2\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma$ stoichiometry would not be expected to give a hexagonal particle because of the inequality in size of these subunits. A core particle of $\alpha_3\beta_3\gamma$ can account for a regular hexagonal particle, since the small size

Figure 22: Proposed model for the arrangement of the subunits of wheat CF_1 within the enzyme complex.


α and β
ε
γ
δ
CF1
SIDE VIEW
CFo


α and β
γ
ε
TOP VIEW

of the δ and ε subunits probably means that they contribute little to the gross shape of the particle. Evidence for a near-perfect hexagon has been reported. Kagawa and his co-workers obtained optical diffraction patterns of two-dimensional crystals of F_1 from the thermophilic bacterium PS3 showing a hexagonal structure (Wakabayashi et al., 1977). They further used a Fourier transform programme to computer construct the optically filtered image of this F_1. The two-dimensional crystal showed a 90 Å spacing with considerable, but not perfect, six-fold and three-fold symmetry.

Much of the problem in determining the subunit stoichiometry for coupling factors may be due to unreliable molecular weight determinations. Yoshida et al. (1979) re-evaluated the molecular weight of F_1 from the bacterium PS3, chloroplasts and mitochondria by equilibrium cemtrifugation and gel filtration, and that of the subunits by equilibrium centrifugation and gel filtration by high speed liquid chromatography. They report an over-estimate of the molecular weights of F_1 subunits in all cases, and when the new molecular weights were used to calculate the stoichiometry of the enzyme complex, an $\alpha_3\beta_3\gamma\delta\varepsilon$, or "hexagonal" model could be accomodated.

In support of this stoichiometry, Esch and Allison (1979) found one specific site for arrachment of *p*-fluorosulfonylbenzoyl-5'-adenosine, an inhibitor of F_1-ATPase, on each of the β subunits of beef heart F_1. A total of three specific sites per F_1 complex were found, suggesting three β subunits per complex. In addition, 0.5 non-specific sites of attachment per α subunit were found, with a total of 1.5 per F_1, giving three α per complex also.

Conflicting data also exist. ^{3}H-N-ethylmaleimide binds to F_1 from beef heart with a stoichiometry of 2α:2β:2γ:?δ:2ε (Senior, 1975; the number of δ subunits is unknown because this reagent does not bind to this subunit)

but binding of this molecule requires free sulfhydryl groups, so any subunit low or lacking in SH moieties will not bind it stoichiometrically. Earlier work from the same laboratory (Senior and Brooks, 1971) gave an $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry on the basis of relative staining of subunits in SDS-PAGE bands. Accuracy in determining stoichiometry by this method depends upon a good value for the molecular weight of the F_1 complex. As noted earlier, several authors have shown that the complex is very fragile, and the loss of the smaller subunits will give lower values for the molecular weight of F_1.

One should not propose a model solely on the basis of electron microscopy, but taken in conjunction with the more recent literature, the electron micrographs presented here tend to support a model of $\alpha_3\beta_3$ for the major subunits, with the δ and ε subunits relatively exposed for easy removal.

Antiserum generated against wheat CF_1 precipitated this enzyme complex as well as inhibited the latent CaATPase activity of CF_1. Rocket immunoelectrophoresis has shown that this antiserum reacts only with the two largest subunits of wheat CF_1.

However, this does not conclusively prove that the enzyme active site is located on the α and/or β subunits of wheat CF_1. Antibody binding may occur at any site on an antigenic polypeptide, and depending upon whether this site is critical to enzyme activity, the antibody may or may not interfere with the enzyme function. This has been demonstrated with antiserum to the γ subunit of *Antirrhinum majus* (snapdragon) CF_1. In one case, the anti-γ serum inhibited the MgATPase (Koenig et al., 1978), while serum from another rabbit immunized against the γ subunit stimulated the same activity (Koenig et al., 1976). Antibody binding at one site may

cause a conformational change at a distant site on the enzyme, resulting in activation or inhibition, depending upon the location of the antibody. Koenig et al. (1978) warns that "...conclusions concerning the uninvolvement of a component can be drawn with certainty only, if a large number of antisera to the same component have been investigated." Since serum from only one rabbit has been characterized no conclusions can yet be made about the active site of wheat CF_1.

SUMMARY

Wheat CF_1 exhibits many characteristics in common with coupling factor complexes from other plant sources, as well as mitochondrial and microbial F_1 complexes. These similarities include the number of subunits, extraction properties, substrate specificity, and a similar structure when negative stained.

A number of properties unique to the wheat enzyme have been elucidated in this study. A requirement for proteolysis inhibitors during CF_1 extraction by chloroform and subsequent purification has been described here. This may be due to the concomitant extraction of a protease with CF_1. The demonstration that extraction of CF_1 by hypotonic sucrose wash is inhibited by the inclusion of proteolysis inhibitors, while described for ECF_1, has never before been shown with a plant coupling factor. However, unlike ECF_1, this is not due to an inhibition of proteolysis of a subunit of CF_1. In addition, I have shown that wheat CF_1 contains an internal disulfide bridge in its γ subunit.

Unlike spinach CF_1, the wheat enzyme does not exhibit a heat-activated CaATPase activity. The trypsin-activated ATPase activity is both calcium-dependent and inhibited by calcium. Two different K_M values for the CaATPase activity were obtained, a low K_M when calcium and ATP are in equimolar amounts, and a higher K_M when calcium is in excess. These K_M values differ by an order of magnitude. More research is necessary to determine whether this reflects a competitive inhibition or an allosteric control by calcium.

Electron microscopy of negatively stained wheat CF_1 particles shows a structure similar to other F_1's viewed in the same manner. CF_1 shows a six-fold symmetry in one orientation, a symmetry which is best shown with

the image enhancement technique of Markham rotation. A comparative analysis of ECF_1 by negative staining showed a very similar structure.

The beginnings of a study of the biosynthesis of wheat CF_1 are reported here. Rabbit antibodies to the enzyme were characterized. With antibodies against the smaller subunits of wheat CF_1, one would then be prepared to examine the size of CF_1 subunit polypeptides synthesized in an *in vitro* translation of wheat RNA. In view of the recent work of Nelson et al. (1980) showing the γ subunit made by the intact spinach chloroplast, a result in direct conflict with results from three other groups (Mendiola-Morgenthaler et al., 1976; Ellis, 1977; Bouthyette and Jaegendorf, 1978) an additional study of the site of synthesis of the γ and δ subunits of CF_1 would be most informative. The site of synthesis and method of transport of those polypeptides made outside the chloroplast is an intriguing example of cooperation between nuclear and chloroplast genomes.

The coupling factors are probably one of the most important groups of protein complexes yet studied. Their structure, mode of action, regulation and biosynthesis are all questions which fascinate scientists today. As techniques and insights are gradually improved, this is one enzyme which will receive much attention.

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