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PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF *Escherichia coli*: NUCLEOTIDE SEQUENCE OF THE *pnt* GENE AND CHARACTERIZATION OF THE ENZYME COMPLEX

by

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ABSTRACT

Based on the rationale that Escherichia coli cells harboring plasmids containing the pnt gene would contain elevated levels of enzyme, three clones were isolated bearing the transhydrogenase gene from the Clarke and Carbon colony bank. The three plasmids were subjected to restriction endonuclease analysis. A 10.4-kilobase restriction fragment which overlapped all three plasmids was cloned into pUC13. Examination of several deletion derivatives of the resulting plasmids and subsequent treatment with exonuclease BAL31 revealed that enhanced transhydrogenase expression was localized within a 3.05-kilobase segment. This segment was located at 35.4 min in the E. coli genome. Plasmid pDC21 conferred on its host 70-fold overproduction of transhydrogenase. The protein products of plasmids carrying the pnt gene were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membranes from cells containing the plasmids and by in vitro transcription/translation of pDC21. Two polypeptides of molecular weights 52,000 and 48,000 were coded by the 3.05-kilobase fragment of pDC21. Both polypeptides were required for expression of transhydrogenase activity.

The transhydrogenase was purified from cytoplasmic membranes of E. coli by pre-extraction of the membranes with sodium cholate and Triton X-100, solubilization of the enzyme with sodium deoxycholate in the presence of 1 M potassium chloride, and centrifugation through a 1.1 M sucrose solution. The purified enzyme consists of two subunits, α and ß, of molecular weights 52,000 and 48,000.

During transhydrogenation between NADPH and 3-acetylpyridine adenine dinucleotide by both the purified enzyme reconstituted into liposomes and

the membrane-bound enzyme, a pH gradient is established across the membrane as indicated by the quenching of fluorescence of 9-aminoacridine. It was concluded that E. coli transhydrogenase acts as a proton pump which is regulated primarily by a pH gradient rather than a membrane potential.

Treatment of transhydrogenase with N,N'-dicyclohexylcarbodiimide results in an inhibition of proton pump activity and transhydrogenation, suggesting that proton translocation and catalytic activities are obligatorily linked. [^{14}C]Dicyclohexylcarbodiimide preferentially labelled the α subunit.

The transhydrogenase-catalyzed reduction of 3-acetylpyridine adenine dinucleotide by NADPH was stimulated over three-fold by NADH. It was concluded that NADH binds to an allosteric binding site on the enzyme.

The nucleotide sequences of the pntA and pntB genes, coding for the transhydrogenase α and ß subunits respectively, were established. The molecular masses of 53,906 (α) and 48,667 (ß) and the N-terminal sequences of the predicted polypeptides agree well with the data obtained by analysis of the purified subunits. Several hydrophobic regions large enough to span the cytoplasmic membrane were observed for each subunit.

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ABBREVIATIONS

AcNAD	3-Acetylpyridine adenine dinucleotide
AG-NAD	NAD coupled to agarose resin.
Brij 35	Polyoxyethylene(23) lauryl ether
DCCD	N,N'-dicyclohexylcarbodiimide
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	(Ethylenedinitrilo)-tetraacetic acid
EDC	1-Ethyl-3(3-dimethyl-amino-propyl)carbodiimide
EGTA	[Ethylenebis(oxyethylenenitrilo]-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid
MES	2-(N-morpholino)ethanesulphonic acid
MOPS	3-(N-morpholino)propanesulphonic acid
PEG	Polyethyleneglycol
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
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
U	Unit
$\Delta\psi$	Membrane potential
ΔpH	Difference in pH across the membrane

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INTRODUCTION

Pyridine nucleotide transhydrogenases (EC 1.6.1.1) catalyze the direct and reversible transfer of a hydride ion equivalent between NAD and NADP according to the equation:

$$NADH + NADP \rightleftharpoons NAD + NADPH$$

An enzyme possessing transhydrogenase activity was first discovered by Colowick et al. (1) in extracts from Pseudomonas fluorescens. Soon after the discovery of the Pseudomonas enzyme, Kaplan and co-workers (2) reported that transhydrogenase activity was found in bovine heart preparations. It became apparent that there were significant differences between the Pseudomonas and bovine heart transhydrogenases. Today these enzymes are known to be representatives of two distinct classes of transhydrogenases. Both classes of transhydrogenases have been recently reviewed (3,4,5).

The first class, termed BB-specific transhydrogenases, and represented by the Pseudomonas enzyme, catalyze the transfer of a hydride ion equivalent between the 4B locus of both NADH and NADPH. The BB-specific transhydrogenases are soluble, FAD-containing enzymes which are under allosteric regulation by nucleotides such as 2'-AMP. They are found in some heterotrophic bacteria such as Pseudomonas fluorescens (1), Pseudomonas aeruginosa (6), Azotobacter vinelandii (7), Azotobacter chroococcum, and Azotobacter agile (8). The transhydrogenase enzymes of Pseudomonas aeruginosa (9) and Azotobacter vinelandii (10,11) have been purified to homogeneity. The purified enzymes were isolated as large filamentous aggregates with molecular weights of several million. In the presence of 2'-AMP or NADP the Pseudomonas enzyme dissociated into smaller

fragments of molecular weight 900,000, composed of 20 polypeptides of molecular weight 40,000 to 45,000 (12). The Azotobacter transhydrogenase also disaggregated in the presence of NADP into fragments of molecular weight 58,000 (10). FAD was found as a prosthetic group in both enzymes (9,10).

The second class, termed AB-specific transhydrogenases, and represented by the bovine heart mitochondrial enzyme, catalyze the transfer of a hydride ion equivalent between the 4B locus of NADPH and the 4A locus of NADH. These enzymes are found in the cytoplasmic membrane of certain bacteria and in the inner membrane of mitochondria. They are not allosterically regulated by 2'-AMP nor do they require FAD insofar as is known. The AB-specific transhydrogenases are an interesting class of enzymes because upon membrane energization by respiration or ATP hydrolysis the rate of reduction of NADP by NADH is increased up to ten-fold (3). This energy-linked transhydrogenase is widespread; it is found in the mitochondria of heart, kidney, liver, arterial and muscle tissue, in heterotrophic bacteria such as Escherichia coli (13), Micrococcus denitrificans (14), Bacillus megaterium (15), Salmonella typhimurium (16) and Benekea natriegens (17), and in photosynthesizing bacteria including Rhodospirillum rubrum (18), Rhodopseudomonas spheroides (19), Rhodopseudomonas palustris and Rhodospirillum molischianum (18).

This thesis focused on the study of the energy-linked transhydrogenase of E. coli. The E. coli enzyme, along with the transhydrogenases of bovine heart mitochondria and R. rubrum are the most studied AB-specific transhydrogenases. The following will focus on advancements that have contributed to an understanding of the structure, function and properties of the aforementioned transhydrogenases.

Linkage with the Oxidative Phosphorylation System

AB-specific transhydrogenases are integral membrane proteins found in the inner mitochondrial membrane (20-22), the cytoplasmic membrane of E. coli (23), and R. rubrum chromatophore membranes (18,24). Energy-linked transhydrogenases are functionally linked to the energy-transfer system of the membrane in which they are located (4). The energy required may be generated through any of the coupling sites of the respiratory chain in mitochondria or respiring bacteria. In E. coli, the transhydrogenase may be driven by ATP hydrolysis or respiration (25-27). Energy to drive the mitochondrial enzyme may be furnished by hydrolysis of ATP or by oxidation of NADH, succinate or reduced cytochrome c (21,22). With photosynthetic bacteria such as R. rubrum, energy can be generated either by light-induced electron transport or by the hydrolysis of pyrophosphate, ATP or GTP (18,24). The presence of an energy source results in an energy dependent increase in both the rate (5) and the extent (28,29) of reduction of NADP by NADH. In mitochondria, the apparent equilibrium constant for the reaction is increased from unity to about 500 and the rate of reduction of NADP by NADH is stimulated about 10-fold in the presence of an energy source such as ATP. The effect of ATP is mediated by the energy-transducing ATPase (3). In mutants of E. coli lacking ATPase, ATP does not drive the transhydrogenase reaction (30), although respiration is still effective (31). An antibody to purified ATPase inhibits the stimulation by ATP of transhydrogenase activity as well as inhibiting ATPase activity (32). In addition, ATPase can be extracted from the membrane with loss of the ATP stimulation and reconstituted to restore the effect (27,33). Energy-linked transhydrogenation driven by any of the energy sources is inhibited by oxidative phosphorylation

uncouplers (28,34). The ATP-dependent reaction is specifically inhibited by the phosphorylation inhibitors, oligomycin and dicyclocarbodiimide (3,18,35). The available information suggests that the energy-linked transhydrogenase reaction and electron transport-linked phosphorylation utilize a common energy pool. A consequence of this assumption would be that a reversal of the transhydrogenase reaction (i.e., NADPH→NAD transhydrogenation) should result in the conservation of free energy. Dontsov and co-workers (36) showed that energy-linked transhydrogenation is reversible in studies on the distribution of the lipophilic anion phenyl dicarbaundecarborane (PCB^-), across submitochondrial particle and R. rubrum chromatophore membranes. NADPH→NAD transhydrogenation was linked to PCB^- uptake which is indicative of the formation of a membrane potential, positive on the inside of the vesicles (3). On the other hand, NADH→NADP transhydrogenation caused an efflux of PCB^-. Similar results were obtained using E. coli vesicles (37). By using tightly-coupled submitochondrial particles, Van de Stadt et al. (38) were able to couple ATP synthesis to NADPH→NAD transhydrogenation. Based on these observations it was proposed that the transhydrogenase functions as a reversible proton pump (39-42), consistent with the chemiosmotic hypothesis (43,44). One of the major goals of research on AB-specific transhydrogenases is to determine the mechanism of the transhydrogenase reaction and how the reaction is coupled to the energy conservation system. These studies require a purified enzyme which can be reconstituted into artificial membranes.

Purification of Transhydrogenase

Transhydrogenase is an integral membrane protein which must be released from the membranes for purification purposes. However, release of transhydrogenase from the membrane is complicated by the fact that delipidation inactivates the enzyme. Lipid-removing agents such as acetone and bile salts inactivate mitochondrial transhydrogenase (2). Rydstrom has demonstrated that bovine heart mitochondrial transhydrogenase is inactivated by ammonium sulfate precipitation in the presence of sodium cholate (45). Addition of phospholipids such as phosphatidylcholine, phosphatidylethanolamine or lysophosphatidylcholine to this preparation restored activity. Treatment of E. coli transhydrogenase with cholate and ammonium sulphate also caused inactivation of the enzyme (46). The preparation was reactivated by various phospholipids, particularly bacterial cardiolipin and phosphatidylglycerol. Therefore, solubilization of transhydrogenase requires a detergent that either solubilizes without stripping away essential lipids or can effectively substitute for native lipids.

Transhydrogenase was first purified from beef heart mitochondria in the laboratories of Rydstrom (47) and Fisher (48). Rydstrom's group extracted transhydrogenase from beef heart submitochondrial particles using sodium cholate in the presence of ammonium sulphate. Purification of such an extract by chromatography on DEAE-Sepharose and hydroxyapatite yielded a homogeneous preparation of transhydrogenase having a molecular weight of 97,000. No prosthetic group was detected. Fisher's group purified transhydrogenase from beef heart submitochondrial particles using a six-step procedure. Submitochondrial particles were first extracted with sodium perchlorate to remove peripheral proteins and then

transhydrogenase was solubilized using lysolecithin. Purification was then achieved by fractionation of the solubilized enzyme on alumina gel, calcium phosphate gel, and by chromatography on NAD affinity columns. The purified transhydrogenase was reported to have a molecular weight of 110,000. The enzyme was free of flavin, cytochromes, NADPH-dichlorophenolindophenol reductase, NADPH-ferricyanide reductase and NADH→NAD transhydrogenase activities. The stereochemistry of hydrogen transfer by purified transhydrogenase was shown to be identical to the submitochondrial enzyme with no exchange of protons with medium water (3). Both of the above procedures were laborious and yielded small amounts of transhydrogenase. A much simpler purification procedure for mitochondrial transhydrogenase was developed by Wu et al. (49). Bovine heart submitochondrial particles were washed with 2 M NaCl to remove peripheral proteins. This was followed by extraction of the membranes with 1.5% Triton X-100. The extract was then applied to an affinity column of NAD immobilized on agarose and the enzyme eluted with NADH. The enzyme preparation was judged to be homogeneous by analysis using sodium dodecyl sulphate polyacrylamide gel electrophoresis. This procedure resulted in a high yield of enzyme (47.4%). However, this purification procedure is not reproducible. Persson et al. (50), despite repeated attempts, could not obtain yields of transhydrogenase greater than 5% when using methods employing immobilized NAD. In addition, the specific activity of their preparations was only about 15-20 μmol/min/mg of protein, which was much less than the specific activity of 62.3 μmol/min/mg of protein reported by Wu et al. Persson et al. (50) purified the mitochondrial transhydrogenase by cholate-ammonium sulphate fractionation followed by DEAE-Sepharose chromatography and fast protein

liquid chromatography. The advantages of this preparation, as compared to other preparations, is its superior purity, the reproducibility of the method, and the ability to obtain large amounts of purified transhydrogenase.

Preparations of the purified mitochondrial transhydrogenase have shown that the minimal molecular weight of the enzyme is 97,000 to 115,000. The amino acid composition of the mitochondrial transhydrogenase has been determined (49,50). The polarity index (percentage of Asx, Glx, Ser, Thr, His, Lys and Arg residues) is about 40% (49). This is somewhat more nonpolar than the typical water-soluble protein (51). The subunit structure of purified bovine heart transhydrogenase was investigated using cross-linking reagents (52). Reaction of purified bovine heart transhydrogenase with the bifunctional cross-linking reagents dimethyl adipimidate, dimethyl pimelimidate, dimethylsuberimidate and dithiobis (succinimidyl propionate) results in the appearance of a dimer band on sodium dodecyl sulphate polyacrylamide gels with no higher oligomers being formed. Treatment of the enzyme with 6 M urea led to inactivation of transhydrogenase and prevented cross-linking. The subunit structure of membrane-bound mitochondrial transhydrogenase was also investigated using cross-linking reagents (53). It was concluded that transhydrogenase exists in the native membrane primarily as a dimeric species.

Attempts have been made by several laboratories to isolate or identify the polypeptide composition of the E. coli enzyme (46,54,55,56). Liang and Houghton (55) partially purified the E. coli transhydrogenase by deoxycholate extraction of membranes followed by ion-exchange and gel filtration chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the preparation showed two major protein bands of

molecular weights 94,000 and 50,000, and several minor bands. When E. coli cells are grown on complex media containing high levels of amino acids, the synthesis of transhydrogenase is repressed (57). Using this observation, Liang and Houghton (55) attempted to determine which polypeptides were components of transhydrogenase by incorporating ^{3}H-labelled Casamino acids in the initial repressive growth phase and nonrepressive levels of [^{14}C]leucine in the induction phase. Sodium dodecyl sulphate polyacrylamide gels of the preparation indicated that the protein bands with the highest $^{14}C/^{3}H$ ratio corresponded to polypeptides of molecular weights 94,000 and 50,000. The relationship of the two polypeptides is not known. The largest polypeptide may represent an unusually stable dimer of the 50,000-molecular weight polypeptide. Alternatively, the smaller component may represent a proteolytic fragment of the larger component. This is consistent with the sensitivity of the enzyme to proteolytic degradation and with the fact that the mitochondrial transhydrogenase is a single polypeptide of molecular weight 97,000 to 120,000. A third possibility is that the enzyme is composed of two components.

Chromatophores prepared from R. rubrum contain a transhydrogenase complex which is readily separable by dilution and centrifugation into a soluble protein factor having a molecular weight of about 70,000 and a membrane-bound component (58,59). Neither the soluble factor nor the membrane-bound component alone exhibits transhydrogenase activity (60). A 2000-fold purification of the soluble factor was obtained using ammonium sulphate precipitation followed by chromatography on DEAE-Sephadex (61). The membrane component was successfully extracted from both R. rubrum chromatophores and soluble factor-depleted membranes using

lysophosphatidylcholine (62). Transhydrogenase activity can be reconstituted by mixing the membrane component with partially purified soluble factor (62,63) in the presence of low concentrations of NADP or NADPH.

Reconstitution of Transhydrogenase

In order to test the hypothesis that transhydrogenase couples the transfer of protons across membranes to the transfer of a hydride ion equivalent between the substrates, the purified enzyme must be reconstituted into liposomes. Purified bovine heart transhydrogenase was first reconstituted into phosphatidylcholine vesicles by Hojeberg and Rydstrom (47). Reduction of NAD by NADPH catalyzed by reconstituted transhydrogenase generated an uncoupler-sensitive uptake of lipophilic anion indicative of the formation of a membrane potential, positive inside the vesicle. The rate of reduction of NAD by NADPH was enhanced over 10-fold by uncouplers. Other work showed that purified mitochondrial transhydrogenase could be reconstituted by dialysis of mixtures of transhydrogenase, sodium cholate and phosphatidylcholine to form small unilamellar proteoliposomes (64-66). Experiments with reconstituted transhydrogenase demonstrated that NADPH→NAD transhydrogenase is coupled to the acidification of the vesicle internal space and that transhydrogenation is stimulated several-fold in both directions upon addition of uncoupler (64-66). The inhibition of transhydrogenation in both directions upon reconstitution results from the rapid establishment of a pH gradient across the membrane (3). Uncouplers allow the cycling of protons across the membrane and relieve the inhibition of transhydrogenation.

The respiratory control ratio is the ratio of transhydrogenase activity in the presence to that in the absence of uncoupler (65). The respiratory control ratio serves as an indicator of functional reconstitution of transhydrogenase. Respiratory control was abolished by detergents such as Triton X-100 or lysophosphatidylcholine (65,66). No latent transhydrogenase activity was observed when reconstituted transhydrogenase was treated with detergents. Since pyridine nucleotides cannot cross the membranes, the enzyme molecules in the reconstituted vesicles must be oriented asymmetrically with their active sites exposed to the external medium.

Pennington and Fisher (67) demonstrated that reconstituted mitochondrial transhydrogenase is a transmembrane protein. Purified bovine heart mitochondrial transhydrogenase was asymmetrically inserted into phosphatidylcholine liposomes by cholate-dialysis procedure. N-(4-Azido-2-nitrophenyl)-2-aminoethylsulfonate, a membrane-impermeant photoprobe, when encapsulated in the vesicles, covalently modified the enzyme and inhibited transhydrogenation. External AcNAD (3-acetylpyridine analog of NAD) increased the rate of inactivation several-fold, whereas NADPH, NADP and NADH were without effect. Labelling of the enzyme by the isotopically labelled photoprobe was enhanced by AcNAD and NADP, decreased by NADH, and not significantly affected by NADPH. These results indicate that reconstituted mitochondrial transhydrogenase spans the membrane and that substrate binding alters the conformation of the enzyme. Neither the *E. coli* nor *R. rubrum* enzyme have been purified and reconstituted into membranes.

Transhydrogenase as a Proton Pump

Several lines of evidence indicate that reconstituted transhydrogenase functions as a proton pump. Direct evidence for proton translocation coupled to transhydrogenation has been provided by the use of pH probes such as 9-aminoacridine (50,64,65) and 9-amino-6-chloro-2-methoxyacridine (66). A decrease in intravesicular pH causes the uptake of the probes with a resultant quenching of fluorescence (68). During NADPH→NAD transhydrogenation by the reconstituted enzyme, the fluorescence of these probes was substantially quenched, indicating uptake of the probe in response to a decrease in intravesicular pH. Similar results were obtained using the nonpermeant pH indicator, fluorescein isothiocyanate-dextran trapped within the vesicle (65). A direct demonstration of proton translocation coupled to transhydrogenation in reconstituted vesicles was carried out by Earle and Fisher (3,69). Transhydrogenase was reconstituted into potassium loaded phosphatidylcholine vesicles and the vesicles were suspended in potassium-free buffer. The rate of NADPH→NAD transhydrogenation was measured using the NAD analogue AcNAD. Proton translocation was measured using a pH electrode. When NADPH→AcNAD transhydrogenation was carried out in the presence of valinomycin a concomitant uptake of protons from the medium was demonstrated by electrode measurements. About one proton was translocated for each hydride ion equivalent transferred between the substrates. Addition of valinomycin in the absence of substrates did not result in the uptake of protons indicating that transhydrogenase does not act as a proton pore.

Effect of an Electrochemical Potential on Reconstituted Transhydrogenase

Rydstrom (66) found that both pH gradients and membrane potentials influence the transhydrogenase reaction. When the reduction of AcNAD by NADPH was catalyzed by transhydrogenase vesicles with an internal pH of 8 in a medium of pH 6, a transient phase of high initial activity was observed which rapidly declined to a lower activity. This implied that the artificially imposed pH gradient promoted NADPH→AcNAD transhydrogenation. Interactions between transhydrogenase and imposed membrane potentials were investigated with potassium gradients in the presence of valinomycin. A high external concentration of potassium chloride (150 mM), generating a minimal membrane potential of 100 mV, positive inside the vesicles, caused an inhibition of the reduction of AcNAD by NADPH. The reduction of AcNAD by NADPH was stimulated when a membrane potential of the same size but negative inside the vesicles was generated by the presence of valinomycin and the same concentration of potassium chloride inside the vesicles. Thus, these results indicate that both a pH gradient and a membrane potential regulate the transhydrogenase reaction in vesicles. Earle and Fisher (65) studied the influence of pH gradients and membrane potentials on reconstituted transhydrogenase using valinomycin and nigericin. They found that creation of pH gradients, either acidic inside the vesicles, by addition of nigericin to proteoliposomes prepared with appropriate potassium gradients, had little effect on the transhydrogenase rate in either direction. However, valinomycin-dependent movement of potassium ions, in a direction opposite to proposed transhydrogenase-coupled proton movements, stimulated the rate of transhydrogenation markedly. These results indicate that reconstituted transhydrogenase is influenced primarily by membrane potentials with only a limited contribution by the pH gradient.

Chemical Modification with Dicyclohexylcarbodiimide

N,N'-Dicyclohexylcarbodiimide (DCCD) has been known as a potent covalently interacting inhibitor of a number of enzymes involved in proton translocation across biological membranes. DCCD inhibits proton-linked ATP synthase (70), ubiquinol-cytochrome c reductase from mammalian and yeast mitochondria (71,72), and cytochrome oxidase (73). In these systems DCCD inhibits proton translocation primarily rather than the hydrolytic or redox reactions catalyzed by these enzymes. Since transhydrogenase acts as a proton pump, it is anticipated that DCCD may modify the proton-binding domain in an analogous way.

Treatment of bovine heart submitochondrial particles with DCCD does cause inactivation of transhydrogenase (74-76). The kinetics of inactivation suggest that the reaction of 1 mol of DCCD per active enzyme complex results in complete inactivation. Pennington and Fisher (74) found that NADPH and NADP stimulated inactivation of transhydrogenase by DCCD, whereas AcNAD and NADH afforded no protection. They concluded that DCCD-modified the transhydrogenase outside the active site, possibly in a proton-binding domain that functions to translocate protons across the membrane. However, Phelps and Hatefi (75,76) reported that AcNAD and NADH protected the enzyme from inactivation and came to the conclusion that DCCD binds at, or near, the NAD(H)-binding site on transhydrogenase. Both purified and submitochondrial transhydrogenases were labeleled with [^{14}C]DCCD in a manner which paralleled the extent of inhibition (74). By contrast Persson et al. (50) found that treatment of reconstituted transhydrogenase with DCCD resulted in an inhibition of proton pump activity without an effect on uncoupled catalytic activity, suggesting that proton translocation and catalytic activities are not obligatorily

linked or that this agent separates proton pumping from the catalytic activity. Similar results had been observed by Pennington and Fisher (74). In experiments with transhydrogenase reconstituted potassium-loaded phosphatidylcholine vesicles, DCCD inhibited the rate of proton uptake into the liposomes to a significantly greater extent than transhydrogenation. These results support the hypothesis that DCCD may modify the proton-binding domain of transhydrogenase.

Reaction Mechanism of Transhydrogenase

The mechanism by which transhydrogenation is coupled to the energized state of the membrane is unknown. Mitchell has proposed a loop mechanism for the mitochondrial transhydrogenase (44). In this mechanism, the enzyme is reduced by a hydride ion equivalent donated by NADPH and a proton from the matrix side of the membrane to form a reduced-enzyme intermediate. This is followed by the transfer of a hydride ion to NAD and the release of the proton to the cytosolic side of the membrane.

Skulachev (85) suggested an alternative to Mitchell's scheme based on ligand-induced conformational changes. In the Skulachev model, the transhydrogenase was proposed to have catalytic and proton translocating subunits. A positive charge on the proton translocating subunit was envisioned to be near the NADPH binding site of the catalytic subunit. The proton binding site would reorient from one side of the membrane to the other when NADP, formed by the oxidation of bound NADPH by NAD, occupied the NADPH binding site.

Ligand-induced conformational changes in the transhydrogenase have been detected in proteolytic, thermostability and chemical modification studies. Both the bovine heart (86) and rat liver (87) mitochondrial

transhydrogenases were protected from thermal inactivation by NADPH, and became more thermally labile in the presence of NADP. Neither NADH nor NAD affected thermostability. The rate of inactivation of mitochondrial transhydrogenase by trypsin is affected by the presence of ligands. Bovine heart transhydrogenase was protected by low concentrations of NAD or NADH (86), whereas these substrates did not affect trypsin inactivation of the liver enzyme. The trypsin inactivation of both enzymes was stimulated in the presence of NADPH. NADP had little effect on the rate of inactivation. Hence, at least three different conformations of the mitochondrial transhydrogenase have been detected: unliganded enzyme, the NADPH-enzyme complex and the NADP-enzyme complex. Phenylglyoxal and 2,3-butanedione in borate buffer inhibit E. coli transhydrogenase activity (88). NADP, NAD and high concentrations of NADPH and NADH protected the enzyme against inhibition by 2,3-butanedione. Low concentratins of NADPH and NADH increased the rate of inhibition by 2,3-butanedione. Similar effects were observed for the inactivation of E. coli transhydrogenase by tryptic digestion in the presence of these coenzymes. It was concluded that there were at least two conformations of the active site of E. coli transhydrogenase.

Separate binding sites for the NAD(H) and NADP(H) substrates at the active site are indicated by kinetic studies (54,88,89,90), the existence of inhibitors specifically competitive for binding with either NAD or NADP (91,92), and by direct hydride ion transfer between the 4A locus of NADH and the 4B locus of NADPH. Kozlov et al. (96) presented evidence that there is a short distance between the NADP(H) and NAD(H) binding sites of mitochondrial transhydrogenase. They found that the 7-nitrobenzofurazan-4-yl derivative of dephospho-CoA is a competitive

inhibitor with regard to both binding sites. The kinetics of the inhibition indicated that one molecule of the inhibitor binds simultaneously to both the NADP(H) and NAD(H) binding sites of the enzyme.

The presence of separate binding sites indicated that partial transhydrogenase reactions could take place at the NAD(H) and NADP(H) binding sites. Bovine heart mitochondrial transhydrogenase was shown to catalyze an exchange reaction between NADH and NAD, but only in the presence of NADPH (93). The stereochemistry of the NADH→NAD reaction was the transfer of hydride ion equivalent directly from the 4A locus of NADH to the 4A locus of the NADH product. The stereochemistry of hydride ion transfer between NADH and NAD provided evidence against NADH binding to the NADP site. Because transhydrogenation involves only the 4B locus of NADPH, binding of NADH to the NADP site would result in the specific removal of the 4B hydrogen of NADH. It was proposed that NADH→NAD transhydrogenation represents a partial reaction of NADPH→NAD transhydrogenation which involves the participation of a reduced enzyme intermediate.

Bovine heart transhydrogenase was also shown to catalyze NADPH→NADP transhydrogenation (94,95). Wu and Fisher (95) demonstrated that during NADPH→NADP transhydrogenation the NADP was reduced exclusively at the 4B locus and that oxidation of NADPH was predominantly at the 4B locus. Wu and Fisher (95) proposed that NADPH→NADP transhydrogenation represents a partial reaction of NADH→NADP transhydrogenation which also involves the participation of a reduced enzyme intermediate.

More recent results obtained by Enander and Rydstrom (97) do not support these conclusions. They reported that the reduction of NADP by NADPH by bovine heart mitochondrial transhydrogenase requires catalytic

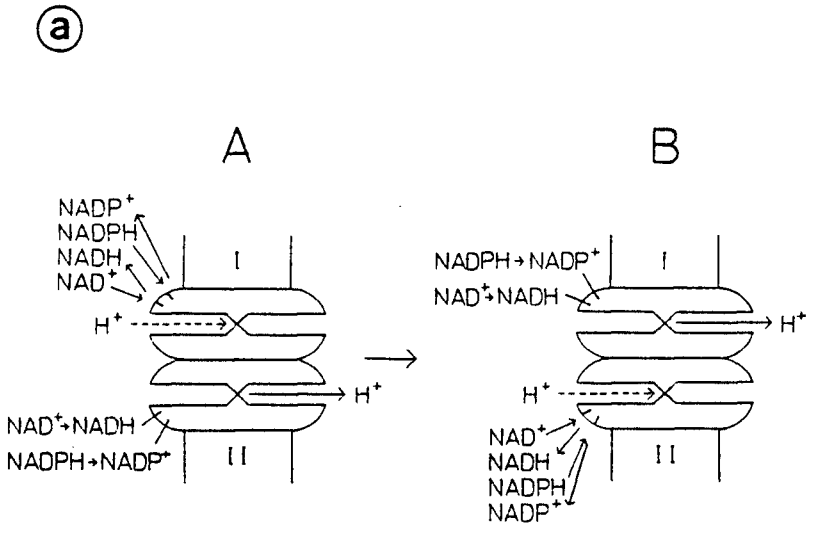
amounts of NADH. This argues against the involvement of a reduced enzyme intermediate. Other evidence against the participation of a reduced enzyme intermediate is the lack of exchange between substrate hydrogen and water hydrogen (93,97,98) and the lack of reducible groups (47,48).

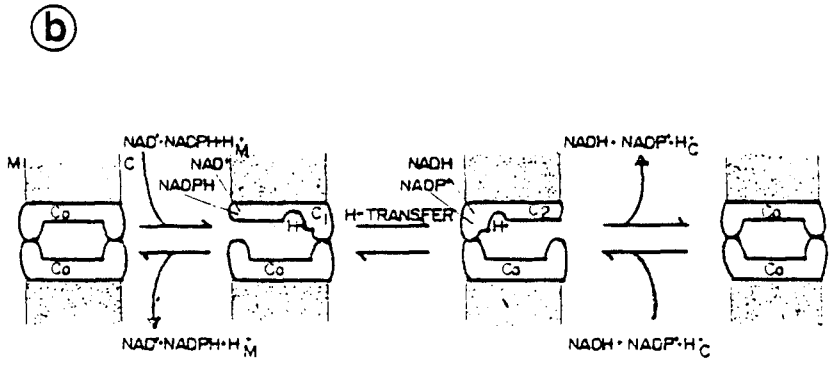
The kinetic mechanism of the transhydrogenase from E. coli (54,88) and bovine heart mitochondria (97) have been determined. In both cases double reciprocal plots of initial velocities for the reduction of NAD by NADPH versus substrate concentrations were convergent and intersecting indicating a ternary complex mechanism. The effect of site-specific inhibitors indicated that the order of addition of the substrates to the enzyme was random.

Enander and Rydstrom (97) used the knowledge that mitochondrial transhydrogenase was a dimer and a proton pump to propose a model for transhydrogenation shown in Fig. 1a. The model is based on the proposal that oligomeric proteins in general may exert the so-called half of the sites reactivity, i.e., only half of the subunits are catalytically active at a given time (99,100). In A (Fig. 1a), subunit I is involved in exchange of the products NADH and NADP for the substrates NAD and NADPH, and binding of a proton from the side of the enzyme facing the exterior of the vesicles. Simultaneously, subunit II is active in pumping one proton from the proton-binding site to the interior of the vesicles, driven by the reduction of NAD by NADPH.

A similar mechanism had been proposed by Pennington and Fisher (74). As shown in Fig. 1b, they proposed that the transhydrogenase dimer forms a proton channel which spans the inner mitochondrial membrane. In the unliganded native transhydrogenase conformation (C_0), the proton binding domain on each subunit is inaccessible to protons on either side of the

Fig. 1. Proposed proton pump mechanisms for mitochondrial transhydrogenase as outlined by Enander and Rydstrom (97)(a) and Pennington and Fisher (74)(b).





membrane. The binding of NADPH and NAD to either active site induces the formation of conformation C_1, forming and exposing the proton hydrophobic binding domain to the matrix side of the membrane. Subsequent to protonation, hydride ion transfer generates a second ternary complex having conformation C_2, in which the proton binding domain is exposed to the cytosolic side of the membrane. Products and protons are released and the enzyme returns to conformation C_0. This mechanism reflects the stoichiometry of protons translocated for each turnover of the enzyme ($H^+/H^- = 1$) as determined with homogenous transhydrogenase reconstituted into phospholipid vesicles (69).

Physiological Role of Transhydrogenase

The physiological function of transhydrogenase is unclear. Several physiological roles for the enzyme in mitochondria have been suggested such as supplying NADPH for biosynthesis and hydroxylation reactions (5) or participating in a pathway which inactivates hydroperoxides (77,78).

In *E. coli*, energy-linked transhydrogenase has been associated with the supply of NADPH for the biosynthesis of amino acids since the presence of the latter in the growth medium repressed the level of the enzyme in the cells (57). If the cells are initially cultured in a medium containing high levels of amino acids and then washed and placed in a medium containing low levels of amino acids, the level of transhydrogenase activity will increase. Incorporation of chloramphenicol in the induction medium inhibits the increase in transhydrogenase activity indicating that *de novo* protein synthesis is required for the induction of transhydrogenase (79). Gerolimatos and Hanson (80) presented evidence that the transhydrogenase of *E. coli* may play a role in branched-chain

amino acid transport as they found that leucyl-tRNA functions as a regulator of the enzyme. However, their hypothesis could not explain the repressive effects of the other amino acids on its formation.

The E. coli transhydrogenase may function as a component of the ammonia assimilation pathway. When E. coli was grown on glucose and various concentrations of NH_4Cl, a similarity in the regulation of transhydrogenase and glutamate dehydrogenase was observed (81). In the range of 0.5 to 20 mM NH_4Cl both transhydrogenase and glutamate denydrogenase activities increased two- to threefold. NH_4Cl concentrations of 20 to 60 mM resulted in relatively constant specific activities for both enzymes. Higher exogenous NH_4Cl, however, led to a decline in both activities. The coregulation of transhydrogenase and glutamate dehydrogenase activities may indicate that transhydrogenase may act as a direct source of NADPH in the ammonia assimilation system.

Mutants of E. coli lacking transhydrogenase activity have been isolated (82-84). Such mutants grow normally under growth conditions so far tested leading to the conclusion that under normal growth conditions an active transhydrogenase is not essential to cell viability.

Objective of this Study

Comparatively little work has been done on the transhydrogenase from E. coli despite advantages that genetic manipulation of this system can offer. One of the major goals of the work described in this thesis was to purify this enzyme, determine its subunit composition, and examine its possible role as a proton pump.

MATERIALS AND METHODS

Chemicals and Isotopes

The chemicals and column materials used in this work were the highest grade obtainable from commercial suppliers. Radioactive materials were purchased from Amersham International Corp. Restriction endonucleases were from Amersham International Corp., Boehringer Mannheim Biochemicals or Pharmacia P-L Biochemicals. Exonuclease BAL31, T4-DNA ligase, calf intestinal phosphatase and DNA polymerase I (Klenow fragment) were obtained from Boehringer Mannheim Biochemicals.

Strains and Bacterial Growth

Table 1 lists the strains of bacteria used in this study. These strains were stored at -50° to -70°C in 25% glycerol. Strains were prepared for storage by adding an equal amount of 50% glycerol to exponentially growing bacterial cultures.

Cells used in this study were grown on one of three types of medium. LB medium: 1% Bacto-tryptone, 1% NaCl and 0.5% yeast extract; YT medium: 0.8% Bacto-tryptone, 0.5% NaCl and 0.5% yeast extract; M9 medium: 0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 0.4% glucose, 100 µg/ml thiamine and supplemented with 40 µg/ml of the appropriate amino acids.

Cells were grown with shaking (at 250 rpm) at 37°C to an absorbance of 1.2 at 600 nm. Larger batches of 4.5 l were grown at 37°C with vigorous aeration (at 25 l/min) in a Lab-Line/S.M.S. Hi-Density Fermentor. The cells were harvested, washed with TED buffer (50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA) or 0.9% NaCl and either used immediately or stored at -70°C.

Table 1. Bacterial Strains

STRAIN	CHARACTERISTICS
RH-5	F⁻ pnt::Tn5 argE3 lacY1 galK2 mtl-1 rpsL700 λ⁻ supE44
AB1450	F⁻ thi-1 ilvD-16 argH1 metB1 hisG1 lacY1 or lacZ4 malA1 mtl-2 xyl-7 ara-13 gal-6 strA8 A9 or A17 tonA2 tsx-7 λ⁻ supE44 gltB13
W6	pro
MV-12	F⁺ trpΔ thr, leu recA
JM83	ara Δlac pro strA thi φ80d lacZ ΔM15
JM103	F' Δlac pro supE thi strA endA sbcB15 hsdR4 traD36 proAB $lacI^q$ ZΔM15
X1197	F⁻ thr leu arg lacY gal minA minB thi T6 recA1 str
A19	Hfr rna-19 his-95 relA1 metB1 spoT1
W1485	F⁺ supE lip
GMS343	F⁻ argE3 lacY1 galK2 mtl-1 rpsL700 λ⁻ supE44

Strains were supplied by B. Bachmann (E. coli Genetic Stock Centre).

Preparation of Membranes

All steps were performed at 0-4°C. The cells were suspended in TED buffer (50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA) and $MgSO_4$ and DNaseI were added to 5 mM and 1 μM, respectively. The cells were lysed by passage through an ice-cold French pressure cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12,000 x g for 10 min. The supernatant was centrifuged at 180,000 x g for 2 h. The supernatant obtained is referred to as the cytoplasmic fraction. The membranes were suspended in TED buffer.

Solubilization of Membrane Vesicles with Detergents

The solubilization characteristics of various detergents were determined as follows. Membranes of E. coli ML308-225 were prepared as described previously and suspended in TED buffer at a protein concentration of 5 mg/ml. The suspension was divided into several aliquots and to each was added dropwise, different amounts of detergents from either a 10% (w/v) or 20% (w/v) stock solution in TED buffer, or in some instances added without dilution. After incubation for 30 min at 0°C, they were centrifuged at 200,000 x g for 2 h and the transhydrogenase and protein levels determined in the supernatant and pellet fractions.

Purification of Transhydrogenase from Strain W6

All steps were performed at 0-4°C with 10-15 g of W6 cells as starting material. The cells were suspended in 40 ml of TED buffer and lysed by passage through a French pressure cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12,000 x g for 10 min. The supernatant was adjusted to final volume of 200 ml with TED buffer and

was centrifuged at 180,000 x g for 2 h. The resulting pellet was suspended in 20 ml of TED buffer and 5 ml of 8 M urea was added to the membrane suspension. After incubation at 0°C for 10 min, the membrane suspension was diluted to 100 ml with TED buffer and centrifuged at 180,000 x g for 2 h. The resulting pellet was washed by resuspension and recentrifugation at 18,000 x g for 1 h twice with 1 mM Tris-HCl, pH 7.8, 0.2 mM dithiothreitol, 0.2 mM EDTA. The washed membranes were suspended in 20 ml of TED buffer and KCl and sodium deoxycholate (0.25 M stock) were added to final concentrations of 1 M and 15 mM, respectively. The membrane suspension was stirred for 10 min at 0°C and then centrifuged at 180,000 x g for 1 h. The amber supernatant was adsorbed batchwise onto a minimal amount of phenyl-Sepharose (Pharmacia). The resin was washed with TED buffer containing (1 mg/ml) Brij 35 to remove KCl. The resin was transferred to a column and the protein eluted with 20 mg/ml Triton X-100 in the same buffer. The eluted material was adsorbed onto a 32 ml (1.5 x 18 cm) DEAE-Bio-Gel A (Bio-Rad) column equilibrated with TED buffer containing 10% (v/v) glycerol and 1 mg/ml Brij 35. Proteins were eluted with a 0-200 mM NaCl gradient (200 ml) in the same buffer used to equilibrate the column. Fractions containing transhydrogenase activity were pooled and concentrated by ultrafiltration under N_2 to a volume of 1 ml using an Amicon XM-50 membrane. The buffer in which the enzyme was dissolved was exchanged to 10 mM sodium phosphate pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml Brij 35 by gel filtration chromatography on Sephadex G-50. The enzyme was then adsorbed onto a 1 x 3 cm agarose/hexane/nicotinamide adenine dinucleotide column equilibrated with the same buffer. The column was washed sequentially with 10 ml of equilibration buffer, 10 ml of buffer containing 20 mM NaCl, 10 ml of

buffer, 5 ml of buffer containing 5 mM NADH, 5 ml of buffer, and the enzyme eluted by addition of 4 ml of buffer containing 10 mM NADH. This was followed by more buffer until all of the enzyme had been eluted. NADH was removed by desalting on a column of Sephadex G-50 equilibrated with sodium phosphate, pH 7.0, 1 mM dithiothreitol and 0.5 mg/ml Brij 35.

Screening of the Clarke and Carbon Collection for Plasmids Carrying the pnt Gene

Each of the 2,112 clones from the Clarke and Carbon collection was grown at 37°C for 48 h in 12.5 ml of M9 medium supplemented with 20 μg each of leucine, threonine, and tryptophan, 1 μg of thiamine, and 1 U of colicin E1 per ml. Cells were harvested by centrifugation at 12,000 x g for 5 min at 4°C. The supernatant was discarded and the cells suspended in 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol and 0.2 mM EDTA. The cells were broken by passage through an ice-cold French pressure cell at 1,400 kg/cm^2. Samples of 450 μl were used to measure transhydrogenase activity.

Preparation of Colicin E1

Colicin E1 was prepared from strain W3110 pColE1 (101). An overnight culture of W3110 pColE1 was streaked onto an LB plate and grown overnight at 37°C. Several of the colonies were then replica plated onto two LB plates. One plate was used as a master plate and grown overnight at 37°C. The remaining plate was incubated at 37°C for 2 h and then the colonies irradiated for 10 sec with a Mineralight short-wave lamp held 1 cm above the agar surface. The plate was incubated at 37°C for 1 h, then overlayed with a mixture of 0.5 ml of X1197 cells in 2.5 ml of top agar

(LB media with 0.8% agar), and incubated at 37°C overnight. Zones of clearing indicated that the colony contained the ColE1 plasmid.

A 50 ml culture of W3110 pColE1 (LB media) was grown at 37°C with vigorous shaking to an absorbance of 0.5 at 600 nm. To each of eight 2 l flasks containing 500 ml of LB media was added 5 ml of the W3110 pColE1 culture and the flasks were incubated at 37° with shaking (250 rpm) until the absorbance had reached 0.5 at 600 nm. Mitomycin C was then added to give a final concentration of 1 μg/ml and the flasks were shaken for 14 h at 37°C. The cells were harvested by centrifugation at 4,500 x g for 15 min and resuspended in 60 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 M NaCl. Cells were broken by passage three times through an ice-cold French pressure cell at 1,400 kg/cm^2. The broken cells were centrifuged at 18,000 x g for 90 min and the pellet discarded. Saturated ammonium sulfate (40 ml) was added dropwise to the supernatant which was then stirred for 30 min in an ice bath. The precipitate was removed by centrifugation at 12,000 x g for 10 min and the pellet was discarded. Saturated ammonium sulfate (35 ml) was added to the supernatant and the supernatant was stirred for 30 min in an ice bath. The precipitate was collected by centrifugation at 12,000 x g for 10 min and the supernatant was discarded. The pellet was suspended in 5 ml of 0.1 M potassium phosphate buffer, pH 7.5, and dialyzed for 4 h against 6 l of the same buffer. The volume was now 10 ml. 10 ml of 20% (v/v) glycerol was added and the crude colicin E1 was stored at -20°C.

Titration of the colicin was performed using strain X1197. Overnight cultures of X1197 grown in LB media were added to top agar (0.5 ml of cells to 2.5 ml of LB containing 0.8% (w/v) agar) and layered on LB plates. A drop of serially diluted colicin E1 was placed on the plate

which was then incubated overnight at 37°C. The number of units of colicin was calculated as the reciprical of the most dilute colicin solution which caused lysis of the cells on the plate. The preparations normally gave titrations of 1 x 10^8 to 1 x 10^{11} units of colicin per ml.

Preparation of Plasmid DNA

For large-scale preparations, plasmid DNA was amplified by treating the cells with chloramphenicol as described by Maniatis et al. (102). Cells were grown in LB media to an absorbance of 0.6 and chloramphenicol was added to a final concentration of 50 µg/ml. Cells were shaken a further 12-18 h. Plasmid DNA was extracted from lysozyme-Triton X-100 lysates (103,104) or by alkaline lysis (102). Normally, the alkaline lysis method was used. This was performed as described below. The bacterial pellet from a 500 ml culture was resuspended in 10 ml of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA containing 5 mg/ml lysozyme. After 5 min at room temperature, 20 ml of 0.2 N NaOH/1% SDS was added and the tubes were left on ice for 10 min. 15 ml of an ice-cold solution of 5 M potassium acetate, pH 4.8, was added. This solution was prepared from 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H_2O. The tubes were inverted several times and left standing in ice for 10 min. The cell DNA and bacterial debris were removed by centrifugation at 40,000 x g for 30 min at 4°C. The supernatant was transferred to a fresh tube and 0.6 volumes of isopropanol was added to each tube. The contents were mixed and left to stand at room temperature for 15 min. The plasmid DNA was recovered by centrifugation at 12,000 x g for 30 min at room temperature. The supernatant was

discarded and the pellet of nucleic acid dried briefly in a vacuum desiccator. The pellets were dissolved in 9 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 9 g of cesium chloride was added. The cesium chloride solution was transferred to a 16 x 76 mm sealable tube and the tube was filled with a solution of ethidium bromide (10 mg/ml in H_2O). The tube was sealed and centrifuged at 250,000 x g for 36 h at 15°C. Two bands of DNA were normally visible in ordinary light. The upper band consisted of linear bacterial DNA and nicked circular plasmid DNA; the lower band consisted of closed circular plasmid DNA. The lower band of DNA was collected using an 18G needle and the DNA transferred to a tube. Ethidium bromide was removed by adding an equal volume of isoamyl alcohol saturated with water and mixing the two phases. The phases were separated by centrifugation at 1,500 x g for 3 min at room temperature. The upper phase was discarded. The extraction was repeated several times until the pink color had disappeared. The aqueous phase was dialyzed against several changes of TE buffer.

For small-scale isolations, plasmid DNA was prepared from unamplified overnight cultures, using the alkaline lysis method (102). Typically, 1.5 ml of the culture was poured into a 1.5 ml tube and centrifuged for 1 min in a Micro-Centrifuge. The medium was removed by aspiration and the pellet resuspended in 100 µl of lysis buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA containing 4 mg/ml of lysozyme). After 5 min at room temperature, 200 µl of an ice-cold solution of 0.2 N NaOH in 1% SDS was added and the contents mixed by inverting the tube two or three times. The tube was kept on ice for 5 min. Then, 150 µl of an ice-cold solution of potassium acetate pH 4.8, made up as described for the large-scale plasmid isolation, was added. After 5 min at 0°C the solution

was centrifuged in a Micro-Centrifuge for 7 min. The supernatant was added to a fresh tube and extracted with an equal volume of phenol/chloroform. After centrifuging for 2 min in a Micro-Centrifuge, the supernatant was transferred to a fresh tube. Two volumes of ethanol were added. After standing at room temperature for 2 min, the tubes were centrifuged at room temperature for 5 min in a Micro-Centrifuge. The supernatant was removed and the pellet dried briefly in a vacuum desiccator. 50 µl of TE buffer containing 50 µg/ml DNase-free pancreatic RNase was added and the mixture incubated at 37°C for 5 min. The preparation of plasmid DNA was stored at -20° centrifugation.

Purification of Nucleic Acids

DNA was purified by extraction with phenol/chloroform and precipitation with ethanol. Liquified phenol was prepared by extracting several times with buffer (usually 1.0 M Tris-HCl pH 8.0, followed by 0.1 M Tris-HCl pH 8.0 and 0.2% ß-mercaptoethanol), until the pH of the aqueous phase was 7.6 (102). 8-Hydroxyquinoline was then added to a final concentration of 0.1%. This preparation is referred to as phenol. Chloroform as used in these experiments is a 24:1 mixture of chloroform and iso-amyl alcohol.

The DNA was mixed with an equal volume of phenol or phenol/chloroform in a polypropylene tube. The contents were mixed until an emulsion formed. The two phases were separated by centrifugation in a Micro-Centrifuge for 1 min. The upper aqueous phase was transferred to a fresh tube. The steps were repeated using chloroform alone. One-tenth volume of 0.3 M sodium acetate, pH 5.5, was added along with two volumes of ethanol. The tubes were stored at -20°C for at least 1 h. The DNA was

recovered by centrifugation for 10 min in a Micro-Centrifuge. The supernatant was discarded and the pellet dried in a vacuum desiccator. The DNA pellet was suspended in TE buffer.

Digestion with Restriction Enzymes

Restriction endonuclease digestions were done according to the manufacturer's instructions. Conditions for partial digestion of plasmid DNA were established by adding serial dilutions of enzymes to the plasmid DNA and incubating at 37°C for 1 h. Reactions were terminated by the addition of one-sixth volume of concentrated "loading" buffer (0.25% bromophenol blue, 40% sucrose, 75 mM EDTA) and a sample was loaded onto an agarose gel for electrophoresis. Once the correct dilution of enzyme had been determined, partial digestion of plasmid DNA was carried out accordingly and the reactions were stopped by the addition of 0.5 M EDTA to a final concentration of 12 mM.

Ligations with T4-DNA Ligase

200 ng of linearized plasmid DNA and a threefold molar excess of the fragment to be subcloned were mixed in a total volume of 8 µl. 1 µl of 10-fold concentrated ligation buffer (0.66 M Tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM dithiothreitol and 10 mM ATP) and 1 µl of 0.9 U/µl T4-DNA ligase were added. The contents were mixed with a vortex mixer and left at room temperature for 1 to 12 h.

Transformations

Bacteria were transformed with plasmid DNA by the calcium chloride procedure (102). About 40 ml of LB medium in a 250 flask was incubated

with 0.1 ml of an overnight culture of the cells to be transformed. The cells were grown at 37°C with vigorous shaking to an absorbance of 0.2 to 0.6 at 600 nm. The flask was placed on ice for 10 min and the cells collected by centrifugation at 3000 x g for 5 min at 2°C. The cell pellet was suspended in 20 ml of 10 mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$. The tube was left on ice for 25 min and then centrifuged as before. The supernatant was discarded and the cells were gently suspended in 4 ml of 10 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$ and kept on ice. For each transformation, 200 µl of the competent cells were placed in ice-cold tubes and about 50 ng of plasmid DNA was added. The tubes were left on ice for 40 min and then placed in a 42°C bath for 2 min. The cells were revived by adding 800 µl of LB media to each tube and incubating the cells at 37° for 1 h. Up to 100 µl of the transformed cells were streaked onto plates containing the desired medium and 1.5% agar. In the case of pUC plasmids 50 µl of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (2% in N,N'-dimethylformamide) was also present. Clones carrying an insert within the plasmid were identified as white colonies, whereas clones without inserts gave blue colonies.

Electrophoresis of DNA

Agarose slab gels (0.4 to 1.2%) were prepared (Bio-Rad, Mini-Gel) and run in TBE buffer (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA) at 60 V for 2 h. Gels were stained in ethidium bromide (0.5 µg/ml of H_2O) and DNA-containing bands were observed under UV light. Photographs were taken using a Polaroid MP-4 camera fitted with a red filter and using Type 667 film.

Extraction of individual DNA bands for ligation from low-melting point agarose was performed as described by Burns and Beacham (105). The piece of agarose containing the DNA was put in a volume of TE buffer so that the final concentration of agarose would be 0.1%. The agarose was melted by incubation at 65°C for 5 min and then cooled. The DNA in the solution was then ligated as described previously.

Dephosphorylation of DNA

The terminal 5' phosphates were removed from DNA by treatment with calf intestinal alkaline phosphatase (CIP)(102). The DNA was digested with the restriction enzyme of choice, extracted once with phenol/chloroform, and the DNA precipitated with ethanol. The DNA was dissolved in a minimum volume of 10 mM Tris-HCl, pH 8.0. Water and 5 µl of ten-fold concentrated buffer (0.5 M Tris-HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine) were added to bring the total volume to 50 µl. 0.01 units of CIP was added to remove the terminal phosphates from 1 pmole of DNA.

To dephosphorylate protruding 5' termini, the preparation was incubated at 37°C for 30 min, then a second aliquot of CIP was added and the incubation was continued for a further 30 min.

To dephosphorylate DNA with blunt ends or recessed 5' termini, the preparation was incubated for 15 min at 37°C followed by 15 min at 56°C. A second aliquot of CIP was added and the incubations were repeated at both temperatures. The CIP was then inactivated by adding 40 µl of H_2O, 10 µl of STE (100 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM EDTA), 5 µl of 10% SDS, and heating the sample at 68°C for 15 min. The DNA was extracted twice with phenol/chloroform and precipitated with ethanol.

Nuclease BAL31 Digestion

An equal volume of 2-fold concentrated buffer (24 mM $CaCl_2$, 24 mM $MgCl_2$, 0.4 M NaCl, 40 mM Tris-HCl pH 8.0, 2 mM EDTA) was added to the sample of DNA. The samples were incubated at 37°C for 3 min and then a pre-determined amount of BAL31 was added. At appropriate times EGTA (0.2 M pH 8.0) was added to a final concentration of 20 mM and the samples were placed on ice. The samples of BAL31-digested DNA were extracted with phenol/chloroform and precipitated with ethanol.

Purification of Transhydrogenase from JM83 pDC21

All steps were performed at 0-4°C with 3-5 g of JM83 pDC21 cells as starting material. The cells, suspended in 40 ml of TED buffer containing 5 mM $MgSO_4$ and a small amount of DNase I, were lysed by passage through an ice-cold French pressure cell at 1,400 kg/cm^2. Unbroken cells were removed by centrifugation of the lysate at 12,000 x g for 10 min. The supernatant was adjusted to a final volume of 100 ml with TED buffer and centrifuged at 210,000 x g for 2 h. The membrane pellets were suspended in 50 ml of TED buffer and Triton X-100 was added to a final concentration of 1% (v/v). After stirring at 0°C for 5 min, the membrane suspension was centrifuged at 210,000 x g for 90 min. The pellets were suspended in 50 ml of TED buffer, and sodium cholate (0.5 M) was added to a final concentration of 50 mM. After stirring at 0°C for 5 min, the membrane suspension was again centrifuged at 210,000 x g for 90 min. The pellet was suspended in 15 ml of TED buffer and KCl was added to a final concentration of 1 M. Sodium deoxycholate (0.25 M) and sodium cholate (0.5 M) were then added to final concentrations of 15 mM each. After stirring for 10 min at 0°C, the insoluble material was removed by

centrifugation at 210,000 x g for 45 min. The resulting supernatant was layered onto a cushion of 1.1 M sucrose in TED buffer containing 2.3 mM sodium cholate, and centrifuged at 260,000 x g for 16 h. The tube was punctured and ten 1.4 ml fractions were collected. Fractions containing transhydrogenase activity were pooled and stored at 0-4°C.

Peptide Mapping

Proteolytic digestion was carried out using the method described by Cleveland et al. (106). The first slab gel (15 x 14 x 0.075 cm) was composed of 10% polyacrylamide with a 4% stacking gel as described by Laemmli (7). Each well received 60 µg of purified transhydrogenase in Laemmli sample buffer. Electrophoresis was carried out at 100 mA/slab. The gels were stained in 0.1% (w/v) Coomassie Blue, 25% (v/v) isopropanol, 10% (v/v) acetic acid for 30 min, and then destained for 60 min in 10% (v/v) acetic acid. The desired polypeptides were excised from the gel, soaked for 30 min in buffer B (0.125 M Tris-HCl pH 6.8, 1 mM EDTA, 0.1% (w/v) SDS), and then loaded into the wells of a slab gel (15 x 14 x 0.15 cm) composed of 15% acrylamide with a 4% stacking gel. To each well was added 10 µl of chymotrypsin (0.25 mg/ml) in buffer B containing 10% (v/v) glycerol. Electrophoresis was performed at 100 mA/slab until the proteins had entered the stacking gel. The power was then shut off. After 30 min, electrophoresis was continued as before. The gels were stained overnight in 0.1% (w/v) Coomassie Blue, 25% (v/v) isopropanol, 10% (v/v) acetic acid and destained in 10% (v/v) acetic acid.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as described by Laemmli (107). The separating gel contained 0.375 M Tris-HCl, pH 8.8, 0.1% SDS and 7-15% acrylamide (prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide in water). For each 15 ml of gel, polyacrylamide was effected by the addition of 75 µl of freshly prepared 10% (w/v) ammonium persulfate and 15 µl of TEMED. A 15 x 14 x 0.075 or 0.15 cm gel was poured and approximately 0.5 ml of tertiary butanol was layered on the gel surface. After 1 h, the tertiary butanol was removed. A stacking gel of 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS and 4% acrylamide was prepared. Polymerization was initiated by the addition of 10 µl of TEMED and 15 µl of freshly prepared 10% (w/v) ammonium persulfate. The gel mixture was layered over the separating gel and allowed to polymerize for 1 h. Samples were prepared by adding an equal volume of SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% ß-mercaptoethanol). The samples were loaded onto the gel which was immersed in running buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS). Electrophoresis was carried out at 100 mA/slab. The gels were stained with Fairbank's stain (108) of 0.1% (w/v) Coomassie brilliant blue R, 25% (v/v) isopropanol and 10% (v/v) acetic acid. The gels were destained with 10% (v/v) acetic acid.

Reconstitution of Transhydrogenase

Purified transhydrogenase was reconstituted with egg yolk phosphatidylcholine by the cholate dilution method (65). Purified transhydrogenase (0.5 mg/ml, 26.4 U/mg of protein) was passed through a Sephadex G-50 column to exchange the buffer to 10 mM Hepes-KOH, pH 7.5,

300 mM KCl, 0.1 mM dithiothreitol, 1.5% (w/v) sodium cholate. One ml of egg phosphatidylcholine (10 mg/ml) was dried under nitrogen and the residue was suspended in 5 ml of the same buffer. The lipid suspension was clarified by passage through a French pressure cell at 1,400 kg/cm^2 twice. Ten µg of purified transhydrogenase was then added to 0.2 ml of lipid suspension and diluted into the appropriate assay medium.

In vitro Protein Synthesis

In vitro protein synthesis was carried out with a procaryotic DNA-directed translation kit obtained from Amersham International Corp. Proteins were translated in the presence of [^{35}S]methionine according to the instructions of the supplier. A premix for each reaction was prepared by mixing together 7.5 µl of supplement solution, 3 µl of amino acid mixture and 2 µl of [^{35}S]methionine (1460 Ci/nmole, 15.8 Ci/ml). Reaction mixtures were prepared adding 12.5 µl of DNA (3-5 µg) in dilution buffer to 12.5 µl of the premix. The reaction mixture was placed in a 37°C water bath and the reaction was started by adding 5 µl of the S-30 cell extract. After incubating the reaction at 37°C for 60 min, 5 µl of methionine chase solution was added and incubation was continued for a further 5 min. An equal amount of SDS sample buffer was added and the proteins were separated by SDS/polyacrylamide gel electrophoresis. The gel was stained with Fairbank's stain for 30 min, destained with 10% (v/v) acetic acid, treated with Amplify (Amersham) for 15 min, and then dried. Autoradiography was performed overnight using Kodak XAR-5 film.

In some cases an S-30 extract was used that was prepared from strain A19 as follows (109). The growth medium was composed of 2.6% (w/v)

K_2HPO_4, 0.5% (w/v) KH_2PO_4, 0.9% (w/v) yeast extract, 1% (w/v) glucose, 13.5 µl/ml thiamine and 50 µg/ml of methionine. Strain A19 was grown overnight in 200 ml of medium in a 1 l flask at 37°C with shaking. One ml of the overnight culture was used to inoculate each of twelve 2 l flasks containing 400 ml of medium. The cells were grown to an absorbance of 0.3 at 600 nm with shaking (250 rpm) at 30°C. The cells were harvested by centrifugation at 4,500 x g for 15 min. They were suspended in ice-cold buffer S (0.01 M Tris-acetate, pH 8.2, 0.014 M MgOAc, 0.06 M KOAc, 1 mM dithiothreitol) and centrifuged at 8,000 x g for 5 min. The cells were resuspended in 40 ml of buffer S and recentrifuged. The yield of cells was 2.5 g. The washed cells were suspended in 10 ml of buffer S and broken in an ice-cold French pressure cell at 560 kg/cm^2. Dithiothreitol was then added to give a final concentration of 1 mM. The extract was centrifuged twice at 30,000 x g for 30 min, keeping the supernatant each time. Equal parts of mix (1 M Tris-acetate pH 7.8, 5 ml; 0.14 MgOAc, 1.0 ml; 1 M dithiothreitol, 15 µl; 20 mM amino acids mixture lacking methionine, 25 µl) and mix 2 (0.2 M ATP, pH 7.0, 0.2 ml; 75 mM sodium phosphoenolphosphate, 6 ml; pyruvate kinase, 0.5 mg) were mixed and 2 ml was added to 8 ml of the cell extract. After incubation at 37°C for 90 min in the dark, the extract was dialyzed against 200 volumes of buffer S for 14 h at 4°, changing the buffer once. The extract was divided into aliquots and stored at -70°C.

DNA Sequence Determination

DNA sequence determination was performed using the chain termination procedure (110,111). The procedure consists of two parts; preparation of single-stranded phage and DNA sequence determination. Phages M13mp18 and M13mp19 were used.

Isolation of M13 Phage

0.1 ml of an overnight culture of JM103 cells grown in M9 media was added to 5 ml of YT medium and the culture incubated at 37°C for 2 h with shaking. This culture was added to 45 ml of YT medium and 2 ml was distributed to separate culture tubes. Each phage plaque was cored from a plate with a 50 µl disposable micropipet and the agar plug was blown into the medium in a culture tube. The tubes were incubated at 37°C for 5 h with vigorous shaking. The contents of each tube were poured into a 1.5 ml centrifuge tube and the cells pelleted by centrifugation in a Micro-Centrifuge for 1 min. The supernatant (0.8 ml) was added to 200 µl of 20% (w/v) PEG-6000/2.5 M NaCl, mixed, and let stand at room temperature for 15 min. The phage were collected by centrifugation in a Micro-Centrifuge for 5 min. The supernatant was removed and the phage pellet was suspended in 100 µl of TE buffer. The phage pellet was extracted with 50 µl of phenol by vortexing for 10 sec followed by centrifuging in the Micro-Cenrifuge for 1 min. The aqueous layer was then extracted three times with 500 µl of water-saturated diethyl ether. The single-stranded DNA was precipitated by adding 10 µl of 3 M sodium acetate, pH 5.5, and 300 µl of ethanol and chilling the tube at -20°C overnight. The DNA was collected by centrifugation in the Micro-Centrifuge for 5 min at 0-4°C. The pellet was dried in a vacuum desiccator and the DNA resuspended in 50 µl of TE buffer.

Sequence Reaction

The first step of the sequencing reaction was to anneal the primer to the template. To 5 µl of single-stranded M13 DNA was added 1 µl of 17 base deoxynucleotide primer (2.4 µg/ml) and 1 µl of 10-fold

concentrated annealing buffer (0.1 M Tris-HCl, pH 8.5, 0.1 M $MgCl_2$) and 1 µl of H_2O. The tube was placed in 70°C water in a heating block. The heating block was allowed to cool to about 40°C and 1 µl of 12 µM dATP and 1 µl of deoxyadenosine 5'-[α-^{32}P] triphosphate (3000 Ci/mmole, 10 mCi/ml) was added to the DNA. The contents of the tube were mixed and 2 µl was distributed to each of the four nucleotide reaction tubes each of which contained 2 µl of the appropriate terminator mix. The terminator mixes of the following compositions in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA: A, 0.5 mM ddTTP, 5.4 µM dTTP, 109 µM dCTP, 109 µM dGTP; B, 50 µM ddCTP, 109 µM dTTP, 5.4 µM dCTP, 109, µM dGTP; C, 0.5 mM ddGTP, 109 µM dTTP, 109 µM dCTP, 5.4 µM dGTP; D, 50 µM ddATP, 77 µM dTTP, 77 µM dCTP, 77 µM dGTP. The reaction was started by adding 0.5 units of the Klenow fragment of DNA polymerase in 2 µl of Klenow dilution buffer (10 mM Tris-HCl pH 8.0, 1 mM dithiothreitol, 50 µg/ml of nuclease-free BSA, 10% (v/v) glycerol) to each of the reaction tubes. The tubes were incubated at 37°C for 15 min and then 2 µl of 0.5 mM dATP was added to each tube and incubation was continued for a further 15 min. The reactions were stopped by adding 4 µl of formamide stop mix (90% (v/v) formamide, 20 mM EDTA, 0.03% (w/v) xylene cyanol, 0.03% bromophenol blue) to each reaction mixture. The reaction tubes were placed in a boiling water bath for 3 min and then cooled on ice. Polyacrylamide gels were prepared by first mixing together 25 g of urea, 7.5 ml of 38% (w/v) acrylamide/2% (w/v) N,N'-methylenebisacrylamide, 5 ml of 10-fold concentrated TBE buffer (1.78 M Tris, 1.78 M boric acid, 20 mM EDTA), 0.5 ml of 10% (w/v) ammonium persulfate in a total volume of 50 ml. The gel was degased and polymerization was initiated by adding 20 µl of TEMED. The gel was poured to form a 17 x 30 x 0.05 cm gel. After the gel

had polymerized, it was attached to the electrophoresis apparatus and pre-run at 28 amps/gel for 10 min using TBE buffer. About 2 µl of each sequencing reaction was loaded in each well and the gels were run at 28 mA/gel for either 2 or 5 h. The gels were then placed on 0.3 mm Whatman paper, covered with Saran Wrap and dried for one hour on a gel-dryer at 80°C. Autoradiography was performed overnight using Kodak XRP-1 film. The film was treated for 5 min with Kodak GBX developer and replenisher and then treated for 5 min with Kodak fixer. The developed film was rinsed with water for 30 min and dried.

Isolation of Transhydrogenase α and ß Subunits

A 15 x 14 x 0.15 cm 10% polyacrylamide gel with a 4% stacking gel was prepared as described for Laemmli gels except that the TEMED and ammonium persulfate concentrations were reduced by one-half. The slot in the stocking gel was 12 cm wide. Purified transhydrogenase (450 µg) in SDS-sample buffer was layered onto the gel. Electrophoresis was carried out at 100 mA per slab until the bromophenol blue dye front reached the end of the gel. The gel was placed in a glass dish, washed three times with water and cooled on ice. Ice-cold 0.2 M KCl was added to the gel (112). After 5 min the white protein bands could easily be seen against a black background. The protein bands were excised from the gel, cut into very small pieces and then placed in a screw-cap tube with 4-5 volumes of 0.1% (w/v) SDS. The tube was incubated with shaking for four hours at room temperature. The contents of the tube were placed in a 10 ml syringe and filtered through a 45 µm Acrodisc filter. Cold 100% trichloroacetic acid was added to the filtrate to give a final concentration of 12%. After incubation on ice for 2 h, the precipitate was collected by

centrifugation for 15 min in a Micro-Centrifuge at 0-4°C. The protein pellet was washed three times with ice-cold 10% trichloroacetic acid and three times with ice-cold acetone. The protein pellet was dried in a vacuum desiccator. The sample was then sent to the University of Victoria for protein sequencing. The amount of each subunit recovered was estimated to be between 50 and 100 µg on the basis of the intensity of staining when a sample was run on a Laemmli gel.

Isolation of Transhydrogenase Subunits using the Prep-Gel Apparatus

The BRL Prep-Gel apparatus was used according to the manufacturer's instructions. A 6 cm 10% Laemmli gel with a 1 cm 4% stocking gel was cast in the apparatus. Laemmli running buffer containing 0.1 mM sodium mercaptoacetate was used. A sample containing 50-200 µg of purified transhydrogenase in SDS-sample buffer was layered on the gel. Electrophoresis was carried out at 150 V, collecting 10 min fractions at a flow rate of 10 ml/h at room temperature. The transhydrogenase subunits eluted after 5-6 h.

Protein Assay

Protein concentration was determined according to the method of Lowry et al. (114) with the exception that 1% (w/v) SDS was incubated in the assays.

Assay of Energy-Independent Transhydrogenase Activity

The assay of transhydrogenase activity was based on the method of Kaplan (113). The reaction was carried out at 25°C in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM KCN, 1 mM dithiothreitol, 0.5 mM

EDTA, 1 mM AcNAD and 0.5 mM NADPH in a final volume of 1 ml. The reduction of AcNAD by NADPH was measured as an increase in the absorbance at 375 nm using a Coleman 124 spectrophotometer attached to a chart recorder. The extinction coefficient was taken as 5.1 l/mmol/cm (113). One unit of enzyme activity represents the conversion of 1 µmol of AcNAD to AcNADH per min. The assay was modified when assaying soluble transhydrogenase by including 0.025% (w/v) Brij 35 in the assay medium.

Assay of Energy-Dependent Transhydrogenase Activity

This was measured by a modification of the method of Fisher and Sanadi (25). Membrane-bound transhydrogenase was incubated in an assay medium of 50 mM Tris-HCl, pH 7.8, 10 mM $MgSO_4$, 1 mM dithiothreitol and 0.16 M sucrose. The cuvette was then transferred to a Perkin-Elmer model 124 spectrophotometer, maintained at 37°C by means of a circulating water bath. After 5 min, 10 µl of ethanol, 50 µl of yeast alcohol dehydrogenase (4 mg/ml) and 25 µl of 2.7 mM NAD were added. The absorbance of the reaction mixture at 340 nm was monitored. After 1 min, 50 µl of 15.7 mM NADP was added and the reduction NADP measured (aerobic-driven transhydrogenase). When the oxygen in the cuvette was exhausted, the formation of NADPH was now due to the energy-independent transhydrogenase. 10 µl of 65 mM ATP, pH 7.8, was added and the new rate of NADP reduction was measured (ATP-driven transhydrogenase). The rate of the energy driven transhydrogenase was corrected by subtracting the rate of energy-independent transhydrogenation. The extinction coefficient was 6.22 l/mmol/cm. One unit of enzyme activity represents the conversion of 1 µmole of NADP to NADPH.

Fluorescence Assays

Intravesicular pH changes were followed fluorometrically with 9-aminoacridine with a Turner Spectrofluorometer (model 420) using the indicated wavelength pair (excitation, 420 nm; emission, 500 nm). Assays were conducted in 10 mM Hepes-KOH buffer, pH 7.5, containing 300 mM KCl, 5 mM $MgCl_2$, 0.5-5 μM 9-aminoacridine, 250 μM NADPH and reactions were initiated by addition of 500 μM AcNAD.

Preparation of RNase that is Free of DNase Activity

Pancreatic RNase (RNase A) was dissolved in 10 mM Tris-HCl pH 7.5/15 mM NaCl at a concentration of 10 mg/ml. The enzyme preparation was boiled for 15 min and then allowed to cool slowly to room temperature. The RNase was dispensed into aliquots and stored at -20°C.

Glutamate Dehydrogenase Assay

Glutamate dehydrogenase was assayed using the method described by Sakamoto et al. (115). The following solutions were added to a 1 ml quartz cuvette: 0.5 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, 0.05 ml of 0.2 M 2-oxoglutarate, 0.05 ml of 0.4 M ammonium chloride, 0.01 ml of 10 mM NADPH, 0.39 ml of water and sample. The absorbance of the reaction mixture was followed at 340 nm using a Perkin-Elmer model 124 spectrophotometer maintained at 30°C by means of a circulating water bath. The reaction was started by addition of the sample. The same assay was used for glutamate synthase except that 40 mM glutamine was included in the assay instead of NH_4Cl.

P1 Transduction

Bacteriophage P1 vir was obtained from Dr. P. Dennis of this department. The preparation of lysates and transductions were carried out as described by Miller (116). The lysate was prepared as follows. An overnight bacterial culture from which the lysate was to be prepared was used to inoculate (100 µl) 10 ml of LB media containing 0.1% (w/v) glucose and 5 mM $CaCl_2$ in a 50 ml flask. This culture was incubated with shaking until an absorbance reading of 0.2 at 600 nm was obtained. One ml of this culture was placed in a test tube containing about 1 x 10^6 phage and left for 20 min at 37°C. To each tube was added 2.5 ml of R-top agar (1% (w/v) Bacto-tryptone, 0.1% (w/v) yeast extract, 0.8% (w/v) NaCl, 0.1% (w/v) glucose, 2 mM $CaCl_2$, 0.8% (w/v) agar) and the entire contents were poured onto plates containing R-medium (1% (w/v) Bacto-tryptone, 0.1% (w/v) yeast extract, 0.8% (w/v) NaCl, 0.1% (w/v) glucose, 2 mM $CaCl_2$, 1.2% (w/v) agar). The plates were incubated at 37°C for 5-6 h. The top agar was scrapped off and placed in a screw-top tube with 4 ml of LB media, 50 µl of 1 M sodium citrate and 10 drops of chloroform. The contents of the tube were mixed vigorously on a vortex mixer for 1 min and the tube was left at 0-4°C overnight. The agar was removed by centrifugation at the maximum speed in an International desk-top centrifuge for 30 min. The supernatant was removed and stored in screw-top tubes in the presence of a few drops of chloroform. The bacteriophage were titrated using strain W1485. The above procedures were repeated for the titration using different dilutions of the phage. The plates were incubated overnight at 37°C and the number of clear plaques were counted. Titers ranged from 1 x 10^8-1 x 10^{11} phage/ml.

Transductions were carried out as follows. Overnight cultures of the desired strain of bacteria in LB medium containing 10 mM $CaCl_2$ were added to different dilutions of the phage stock in a total volume of 400 µl. The tubes were incubated at 37°C for 20 min. An equal volume of 1M sodium citrate was added to the cells and 100 µl of samples of the transduction mix were streaked out onto the selection media. The plates were incubated overnight at 37°C.

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

[^{14}C]DCCD (50 mCi/mMole) was purchased in ether in a sealed vial. The ether was evaporated at room temperature under nitrogen and the dried contents were taken up in absolute ethanol (5 mM [^{14}C]DCCD).

Membranes were prepared and suspended in 10 mM Tris-HCl, pH 7.8, containing 0.25 M sucrose, 5 mM $MgSO_4$ and 0.2 mM dithiothreitol at a protein concentration of 5 mg/ml. To 0.5 ml of the membrane suspension was added 10 µl of 5 mM [^{14}C]DCCD in absolute ethanol and the suspension stirred at 4°C for 12 h. The labelled membranes were washed five times in buffer by resuspension and centrifugation at 250,000 x g for 1 h.

Crossed Immunoelectrophoresis

This was based on the methods of Nowotny (117), Bjerrum and Lundhal (118) and Mayer and Walker (119). 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 apart, was cut out with a Bio-Rad 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, Bio-Rad) 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.

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 onto 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. The proteins were stained with Fairbank's stain (108).

Reaction of E. coli Transhydrogenase with Mitochondrial Anti-Transhydrogenase

Membranes were prepared from 3 g each of JM83 pUC13 and JM83 pDC11. The proteins (70 µg) were separated on a 0.15 cm 10% SDS/polyacrylamide gel with a 4% stacking gel. The gel was equilibrated in 500 ml of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol for 30 min. The gel was

placed in a transfer apparatus (Bio-Rad) next to wetted nitrocellulose in equilibration buffer and the proteins were transferred to the nitrocellulose at 100 V for 12 h. The apparatus was cooled by circulating tap water. Staining of the gel indicated that the two transhydrogenase subunits had been transferred to the nitrocellulose. The membrane was washed twice for 30 min in TBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 3% (w/v) BSA) and placed in a sealed bag with 1:25 diluted antibody (gift from R.R. Fisher) and incubated overnight at room temperature. Excess antibody was removed by washing the membrane twice with 100 ml of TBS containing 0.05% (w/v) Tween 20. [^{125}I]Protein A was added and the membrane incubated for 1 h in a sealed plastic bag. The excess protein A was washed three times with TBS containing 0.05% (w/v) Tween 20. The nitrocellulose was air dried and developed by autoradiography. No bands were observed.

RESULTS

I. Physiological Role of Transhydrogenase

The physiological functions of the pyridine nucleotide transhydrogenase of E. coli have not been established. To gain insight into the role of this enzyme, mutants which lack transhydrogenase activity were isolated by Hanson's group (82,84). As shown in Table 2, one of these mutants, RH-5, has normal growth rates when grown aerobically on LB, or on a synthetic medium with glucose, glycerol or fructose as carbon source when compared to the growth rates of the parental strain. No difference was observed between anaerobic growth rates of the mutant and parent on synthetic media with glucose or glycerol plus fumarate as carbon source. These results confirm the observations of Hanson's group (82,84) and support his conclusion that under normal growth conditions an active transhydrogenase is not essential to cell viability.

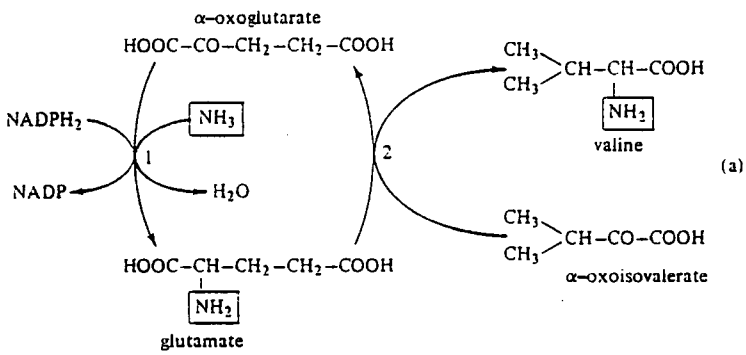
In E. coli, the energy-linked transhydrogenase has often been postulated to act as a source of NADPH for the biosynthesis of amino acids since the presence of the latter in the growth medium represses the level of the enzyme in the cells (57,120). An interrelationship between amino acid biosynthesis and transhydrogenase activity may occur during the assimilation of nitrogen. Recent work on the E. coli transhydrogenase has implicated this enzyme as a source of NADPH for glutamate dehydrogenase (81). As shown in Fig. 2, glutamate dehydrogenase is one of the pathways for assimilation of ammonia in E. coli. Liang and Houghton (81) reported that glutamate dehydrogenase and transhydrogenase are coregulated during nitrogen limitation. Similar results were obtained when E. coli strain W6 was grown on glucose at various concentrations of NH_4Cl (Fig. 3). In

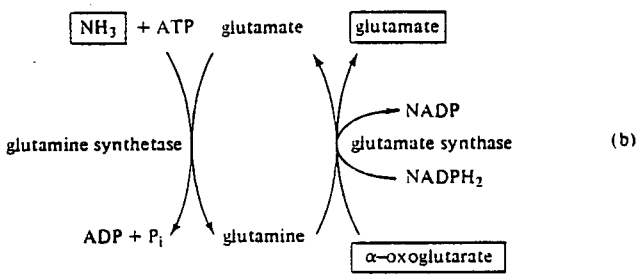
Table 2. Effect of transhydrogenase activity on aerobic growth rates.

Strain	Doubling Time (min)			
	Glucose	Glycerol	Fructose	LB
GMS 343 (*pnt*$^+$)	58	139	97	36
RH5 (*pnt*::Tn*5*)	64	131	95	38

Cells were grown in 200 ml of M9 medium containing 0.2% (w/v) of the indicated carbon sources or in LB medium in 2 l flasks at 37°C with shaking (250 rpm). RH-5 was grown in the presence of 25 µg/ml kanamycin. Cell growth was measured from the absorbance of the culture at 600 nm.

Fig. 2 Pathways of nitrogen assimilation in E. coli.





the range of 0.5 to 20 mM NH_4Cl the activities of both glutamate dehydrogenase and transhydrogenase increased two- to threefold. Higher exogenous NH_4Cl concentrations led to a decline in the specific activities of both enzymes. Coordinate changes in the levels of transhydrogenase and glutamate dehydrogenase could indicate that the enzyme is involved in the supply of NADPH, specifically for glutamate dehydrogenase when it functions in the synthesis of glutamate. This hypothesis was tested by inactivating the other pathway for nitrogen assimilation. This pathway includes glutamate synthase. The strain AB1450 (gltB13), lacking glutamate synthase activity, is dependent on glutamate dehydrogenase for assimilation of ammonia. The transhydrogenase activity of AB1450 was inactivated by P1 transduction from the pnt$^-$ strain RH-5. The gltB13 pnt mutant obtained did not require glutamate for growth on a minimal salts medium. Its growth rate was identical to that of the parent (Table 3,4). These results indicate that transhydrogenase is not the sole source of NADPH for nitrogen assimilation by glutamate dehydrogenase in E. coli.

II. Purification of transhydrogenase from Strain W6

Growth of Cells

E. coli strain W6 was used as a source of enzyme. The transhydrogenase activity of this strain is consistently higher than that of strain K-12 (81). Transhydrogenase activity is repressed when E. coli is grown on complex media containing high levels of amino acids (81,120). Transferring of such cells into a glucose minimal medium results in a 5- to 10-fold increase in transhydrogenase activity (55). Therefore, in

Fig. 3. Effect of exogenous NH_4Cl on glutamate dehydrogenase and transhydrogenase activities in E. coli W6. Cells were grown on M9 medium containing various concentrations of NH_4Cl. The cells were harvested in late-exponential phase (as determined by the absorbance of cultures at 600 nm) and washed twice with TED buffer (50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol). Membrane and cytoplasmic fractions were prepared from the cells, and the glutamate dehydrogenase (GDH) and transhydrogenase (PNT) activities measured as described in Materials and Methods. Measurements were obtained at least from two separate experiments.

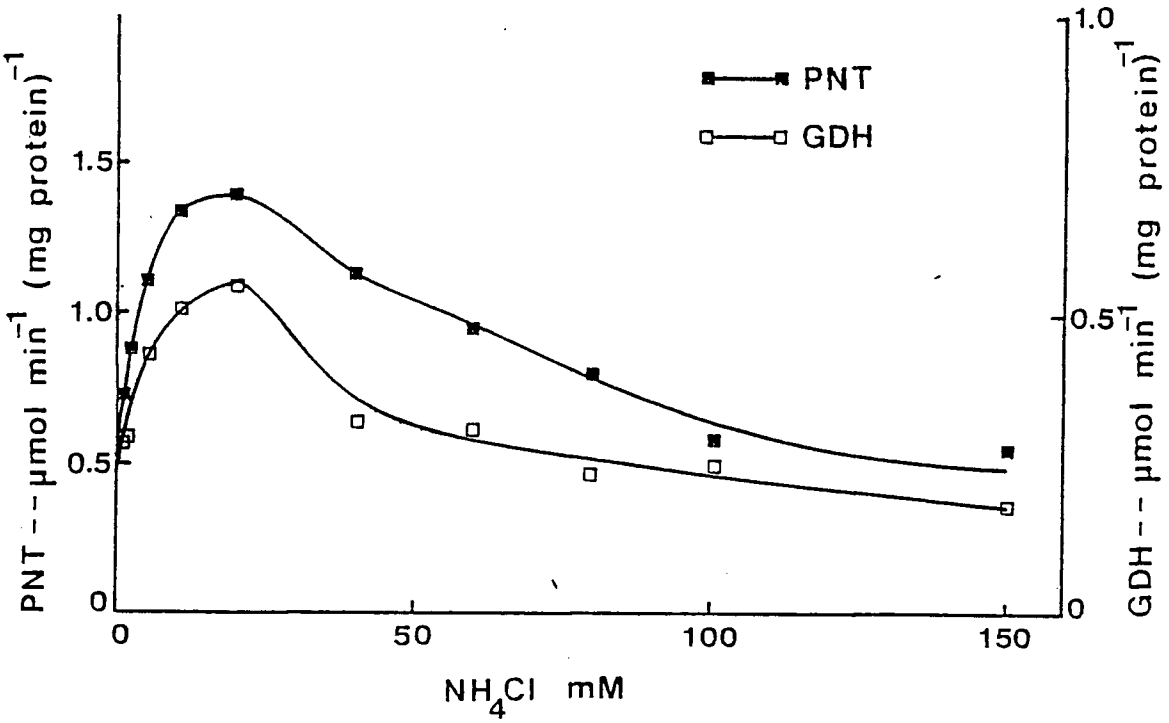


Table 3. Role of transhydrogenase in the assimilation of ammonia.

Strain	Doubling time (min)	Enzyme Activities [μmoles min^{-1} $(mg\ protein)^{-1}$] Trans-hydrogenase	Glutamate dehydrogenase	Glutamate synthase
GMS 343	68	0.36	0.28	0.38
RH-5(pnt::Tn5)	65	0	0.24	0.38
AB1450(gltB13)	78	0.18	0.42	0
AB1450(pnt::Tn5,gltB13)	80	0	0.37	0

Cells were grown in 200 ml of M9 media (containing 25 μg/ml of kanamycin for transhydrogenase mutants) in 2 l flasks at 37°C with shaking. The growth of the cells was measured using the absorbance at 600 nm. The cells were harvested in late-exponential phase and washed with TED buffer. Enzyme assays and the preparation of membrane and cytoplasmic fractions are described in Materials and Methods.

Table 4. Effect of transhydrogenase mutation on the growth of glutamate synthase mutants.

Strain	Doubling Time (min)			
	Glucose	Fructose	Glycerol	LB
AB 1450 (gltB13)	78	106	146	31
AB 1450 (gltB13, pnt::Tn5)	80	98	144	35

Cells were grown in 200 ml of M9 medium containing 0.2% (w/v) of the indicated carbon source or in LB medium in 2 l flasks at 37°C with shaking (250 rpm). AB 1450 (gltB13, pnt::Tn5) was grown in the presence of 25 µl/ml kanamycin. Cell growth was measured from the absorbance at 600 nm.

order to maximize the expression of transhydrogenase, cells were grown in a glucose minimal medium. The specific activity of transhydrogenase in membranes prepared from cells grown under these conditions ranged from 0.9 to 1.3 μmoles/min per mg of protein.

Selection of Detergent

Transhydrogenase is an integral component of the cytoplasmic membrane. In contrast to water-soluble proteins, membrane-bound proteins must be released from the membranes prior to purification. An effective detergent should satisfy certain conditions. First, the detergent should be able to release a significant percentage of the enzyme from the membrane. Second, it should not inactivate the enzyme under study. Third, it would greatly aid the purification scheme if the detergent could selectively solubilize a significant amount of the enzyme without solubilizing other membrane-bound proteins. On the basis of these criteria a number of detergents were tested to select a suitable detergent for the extraction of the transhydrogenase from the cytoplasmic membrane. Strain ML 308-225 was used as starting material for these preliminary experiments.

Membrane vesicles were prepared and treated with various levels of detergents. Transhydrogenase activity was considered as being solubilized if it was not sedimented following centrifugation at 200,000 x g for 2 h. The following detergents solubilized less than 25% of the membrane-bound transhydrogenase and were considered ineffective: cholate, taurodeoxycholate, deoxycholate, Triton X-100, Brij 35, Brij 96, Lubrol WX, Aminoxide WS 35 and n-octyl-ß-D-glucoside when used at concentrations up to 2%.

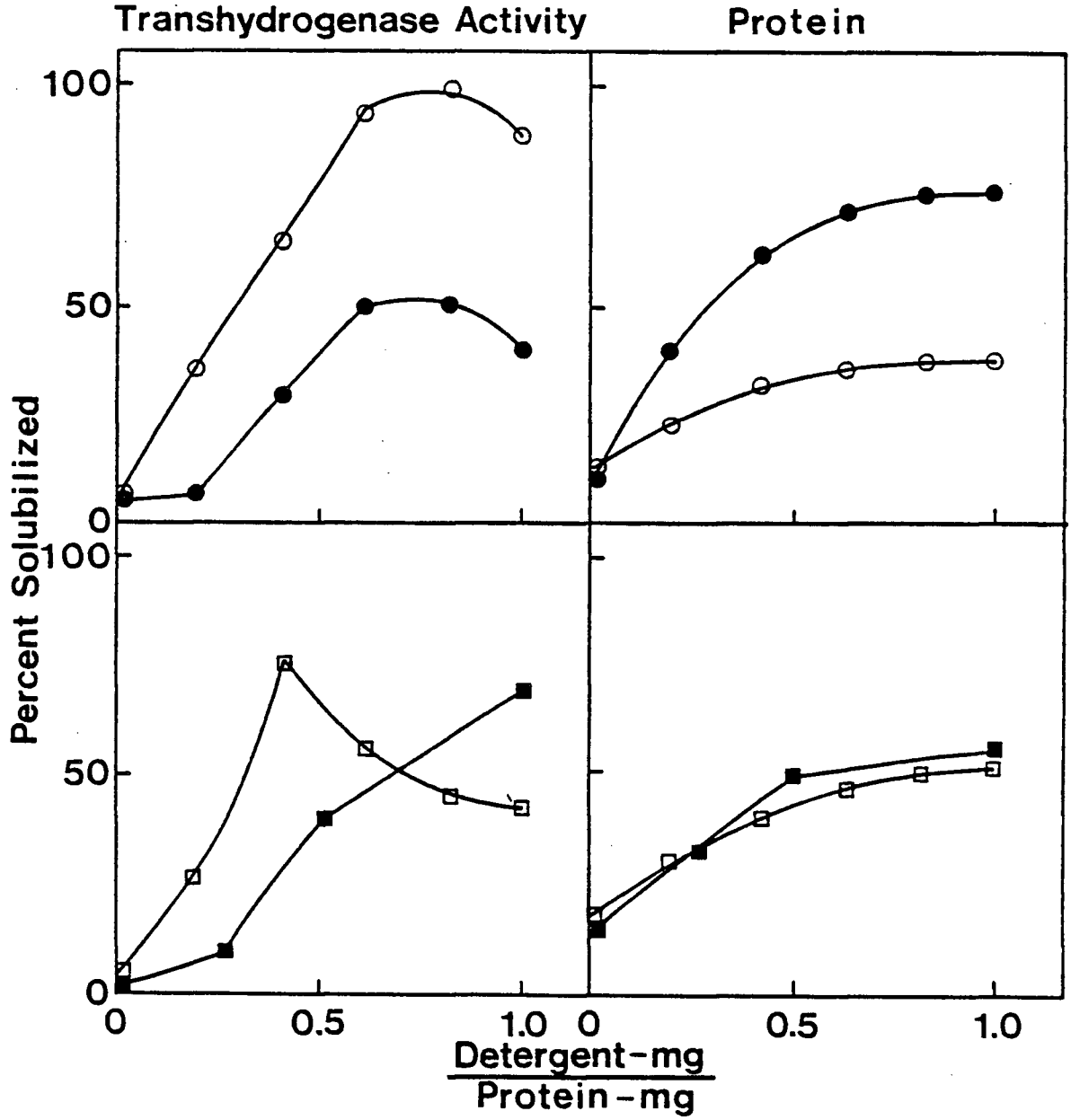
The results of the treatment with various detergents which solubilized more than 25% of the activity are shown in Fig. 4. Optimal solubilization of the transhydrogenase activity was obtained with the detergent sodium deoxycholate in the presence of 1 M KCl. At a detergent to protein ratio of 0.8, 100% of the activity was solubilized. Some stimulation of transhydrogenase occurs in the presence of deoxycholate as 12% of the original activity was detected in the pellet fraction. Deoxycholate exhibited some selectivity, in that only 34% of the membrane protein was solubilized at a detergent to protein ratio of 0.8.

Taurodexoycholate in the presence of 1 M KCl also solubilized significant amounts of transhydrogenase activity. At a detergent to protein ratio of 0.4, taurodeoxycholate solubilized 73% of the activity but only 37% of the membrane protein.

Dodecyl-ß-D-maltoside and N-lauroyl sarcosine solubilized over 50% of the transhydrogenase activity but with less selectivity when compared to the results obtained with deoxycholate and taurodeoxycholate. At a detergent to protein ratio of 1, dodecyl-ß-D-maltoside solubilized 64% of the transhydrogenase activity and 49% of the membrane protein. N-lauroyl sarcosine exhibited the least selectivity of the detergents tested. At a ratio of 0.6, 65% of the membrane protein was solubilized, but only 50% of the transhydrogenase was detected in the supernatant. Since only 5% of the activity was found in the pellet, 45% of the activity was inactivated.

It is clear from these studies that the most suitable detergent for the solubilization of transhydrogenase is deoxycholate in the presence of KCl.

Fig. 4. Solubilization of membrane-bound transhydrogenase with various detergents. Membrane vesicles in TED buffer at a protein concentration of 5 mg/ml were treated with various levels of detergent as described in Materials and Methods. N-lauroyl sarcosine (●-●); sodium deoxycholate in the presence of 1 M KCl (o-o); taurodeoxycholate in the presence of 1M KCl (⊔-⊔); dodecyl-ß-D-maltoside (■-■).



Purification of Transhydrogenase

The purification procedure outlined under 'Methods and Materials' is represented in Table 5. The purity of the transhydrogenase at various stages was assessed by SDS-polyacrylamide gel electrophoresis (Fig. 5).

To remove peripheral proteins likely to contaminate the solubilized transhydrogenase, membrane vesicles were extracted sequentially with 2 M urea and low salt buffer. Over 90% of the transhydrogenase activity was retained in the membrane vesicles after these washing procedures.

The extracted membrane vesicles were incubated with 0.6% deoxycholate in the presence of 1 M KCl. Cholate was added to prevent the deoxycholate from forming a gel. After clarification by centrifugation, 24% of the total membrane protein and 75% of the transhydrogenase activity remained in solution.

The high concentration of salt had to be removed prior to ion-exchange chromatography. A rapid desalting procedure was developed using hydrophobic chromatography. The solubilized transhydrogenase was adsorbed onto a minimal amount of phenyl-Sepharose. KCl was removed by washing the resin with a column volume of buffer and the transhydrogenase then eluted with Triton X-100. Although ion-exchange chromatography was performed in the presence of the detergent Brij 35, this detergent was found to be ineffective in eluting the bound transhydrogenase from phenyl-Sepharose. Recoveries of about 90% were obtained during this step when Triton X-100 was used.

The material from phenyl-Sepharose was adsorbed onto a DEAE-Bio Gel A column and then eluted using a 0-200 mM NaCl linear gradient containing 0.05% (w/v) Brij 35. As shown in Fig. 6, considerable amount of the applied protein did not bind to the ion-exchange resin. Transhydrogenase

Table 5. Partial purification of transhydrogenase from E. coli strain W6.

Fraction	Protein	Transhydrogenase Activity	
		Total	Specific
	mg	%	U/mg protein
Membranes	311	100	1.3
Extracted Enzyme	74	75	4.1
After phenyl-Sepharose chromatography	52	63	4.9
Combined fractions after chromatography on DEAE-Bio-Gel A	7.8	25	13
Combined fractions after chromatography on NAD-Agarose	1.4	3.9	22

Membranes were prepared from 12.1 g of cells. After treating the membranes with 2 M urea, the transhydrogenase was solubilized by the detergent deoxycholate in the presence of KCl. After desalting the extract using phenyl-Sepharose, the enzyme was partially purified using ion-exchange and affinity chromatography. Experimental details of the purification are described in Materials and Methods.

Fig. 5. SDS-polyacrylamide gel electrophoresis of fractions at various stages of the transhydrogenase purification from E. coli strain W6. A sample was removed at each of the various stages of the purification scheme outlined in Table 5 and analyzed by SDS/polyacrylamide gel electrophoresis as described in Materials and Methods. AG-NAD^+; NAD^+-agarose.

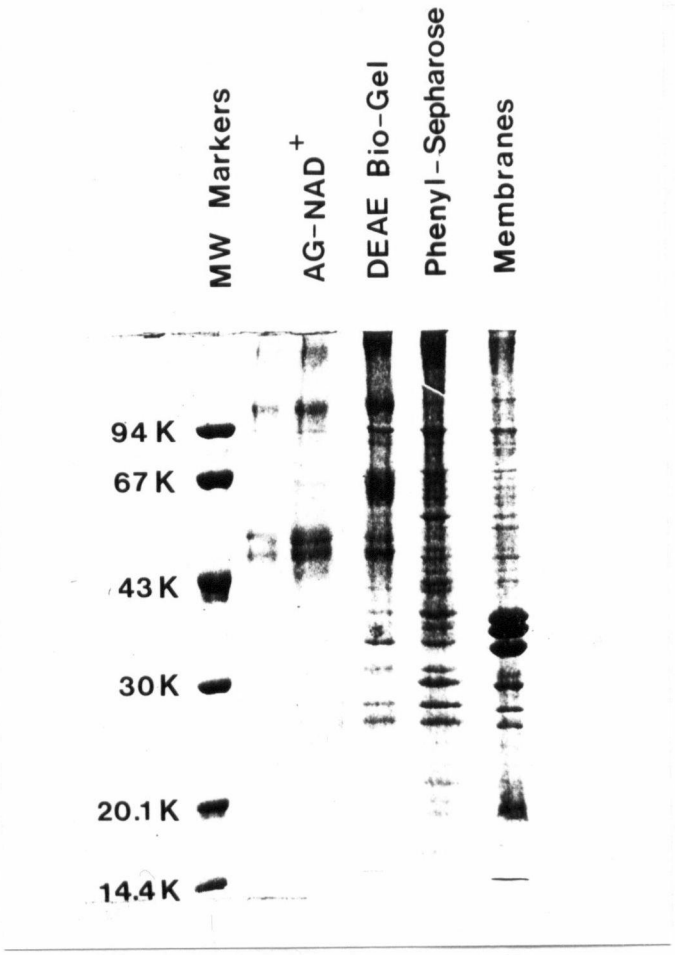
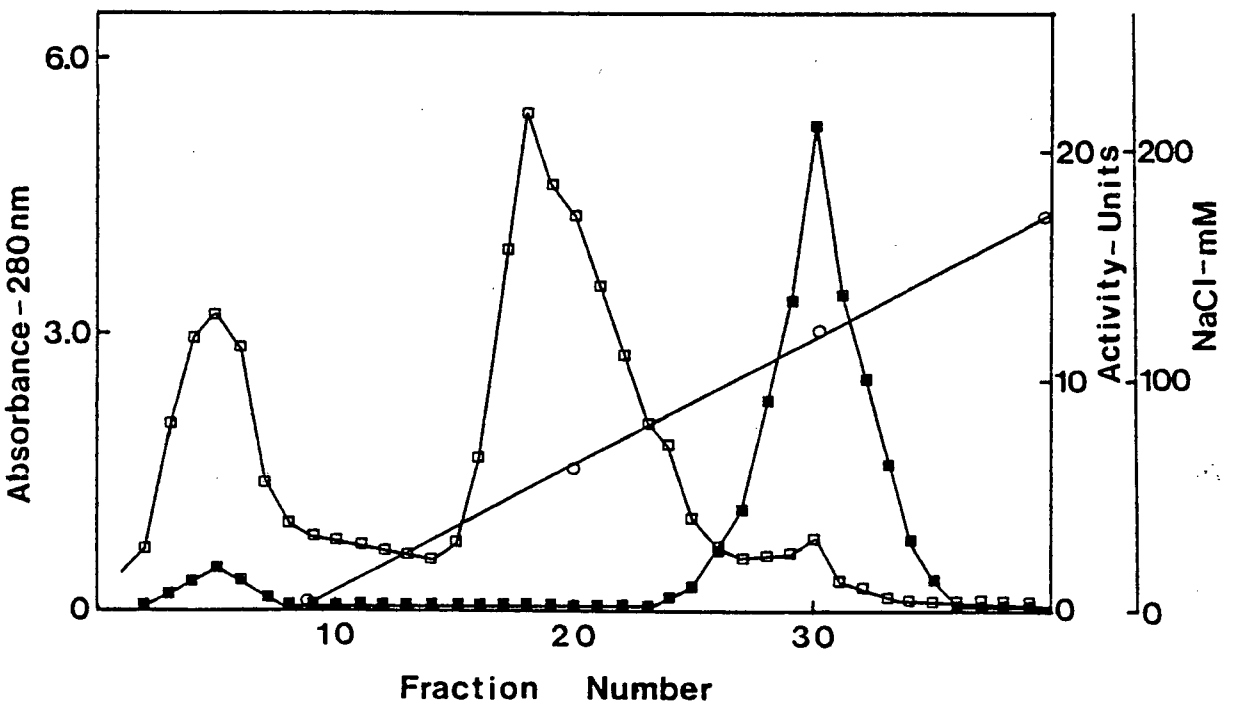


Fig. 6. Separation of transhydrogenase by ion-exchange chromatography. Solubilized transhydrogenase (50 mg of protein), which had been desalted by hydrophobic chromatography, was subjected to chromatography on a 1.5 x 18 cm DEAE Bio Gel A column as described in Materials and Methods. Fractions of 4.2 ml were collected and assayed for transhydrogenase activity (■-■). Protein was detected from the absorbance at 280 nm (□-□) and NaCl concentration by conductivity (o-o). Fractions 28 to 32 were saved.

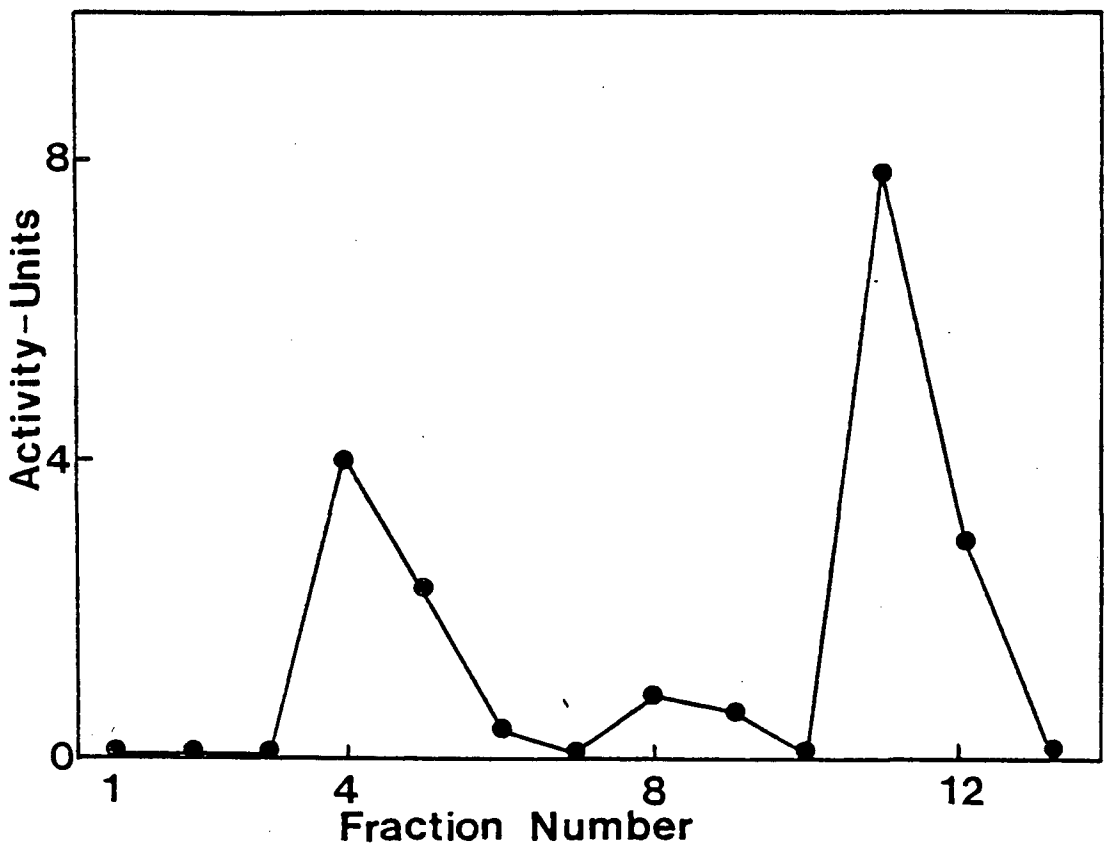


activity was detected in fractions 24-35 with the peak fractions eluting at 100 mM NaCl. Most of the applied protein was eluted off the column prior to the appearance of transhydrogenase activity in the eluant. Fractions 28-32 were pooled and the buffer was exchanged to 10 mM sodium phosphate pH 7.4, 1 mM EDTA, 1 mM DTT and 0.05 mg/ml Brij 35 using a Sephadex G-25 column.

The desalted transhydrogenase from the ion exchange column was applied to a column of NAD agarose. The column was washed successively with equilibration buffer, buffer containing 20 mM NaCl, buffer containing 5 mM NADH, and buffer with 10 mM NADH. As shown in Fig. 7, most of the transhydrogenase activity was eluted with 10 mM NADH, although significant amounts were eluted by the 20 mM NaCl wash. The total number of units of transhydrogenase recovered after this step represented 1-4% of the starting material. Analysis of the purified transhydrogenase using SDS-PAGE showed the presence of three major protein bands of molecular weights 100,000, 52,000 and 47,000, as well as several minor protein bands.

The purification procedure discussed above was not entirely satisfactory. Yields of transhydrogenase were low and the purity of the material varied greatly from purification to purification. The greatest variability occurred during the ion-exchange or affinity chromatography steps. During ion-exchange chromatography, the best purification occurred when the transhydrogenase eluted later than the bulk of the applied protein. Unfortunately, in many cases the two peaks would overlap slightly resulting in a less pure transhydrogenase preparation. The yield and purity of the transhydrogenase following affinity chromatography varied from run to run. Another problem with the purification procedure was that the transhydrogenase preparation was contaminated with several other proteins.

Fig. 7. Purification of transhydrogenase by affinity chromatography. Partially purified transhydrogenase (7.8 mg) was subjected to chromatography on a 1 x 3 cm AG-NAD Type I column (P-L Biochemicals) as described in Materials and Methods. The column was washed successively with buffer (fractions 1-3), buffer containing 20 mM NaCl (fraction 4), buffer containing 5 mM NADH (fraction 8), and transhydrogenase eluted with 10 mM NADH (fraction 11). Each fraction was assayed for transhydrogenase activity (●-●).



Transhydrogenase is found in relatively minor amounts in the E. coli cell membrane. This increases the difficulty of purification. This difficulty was overcome by increasing the expression of transhydrogenase by cloning the pnt gene onto a multi-copy plasmid.

III. Cloning of the pnt Gene

Identification of the pnt plasmids

Mutants defective in the expression of transhydrogenase do not exhibit a readily detectable phenotype. Hanson and Rose (84) reported that E. coli strains defective in both glucose-6-phosphate dehydrogenase and transhydrogenase produced much smaller colonies when grown anaerobically on minimal media plates when compared to the parent which was defective in glucose-6-phosphate dehydrogenase alone. However, I found the difference in colony size between the two strains to be negligible.

This property ruled out any screening of an E. coli plasmid bank based on a recognizable phenotypic change when seeking the pnt locus. Therefore, the Clarke and Carbon colony bank was screened based on the rationale that E. coli cells harboring multicopy plasmids containing the pnt gene would contain elevated levels of the enzyme. Among the approximately 2,200 clones of the Clarke and Carbon collection, clones pLC 10-19, pLC 26-24, and pLC 27-35 contained 8- to 10-fold more transhydrogenase activity when compared with the other clones of the bank (Table 6). Over 90% of the enzyme activity was associated with the membranes of these cells. Expression of E. coli transhydrogenase activity is maximal when cells are grown in minimal media free of high levels of

Table 6. Transhydrogenase activity in membranes of selected strains from the Clarke-Carbon colony bank

Strain	Transhydrogenase Activity (μmoles/min/mg protein)	
	LB medium	M9 medium
MV12(pLC 14-12)[a]	0.04	0.18
MV12(pLC 10-19)	0.32	1.33
MV12(pLC 26-24)	0.38	1.52
MV12(pLC 27-35)	0.42	1.80

[a]The level of transhydrogenase activity in this strain is typical of those strains not carrying the *pnt* gene on a Col EI plasmid.

amino acids (57). Increased levels of transhydrogenase were observed when each of the pnt bearing clones was grown in minimal M9 medium. Membranes prepared from cells grown in minimal medium exhibited a fourfold increase in transhydrogenase activity as compared with the transhydrogenase activity of membranes prepared from cells grown in LB medium (Table 6).

Restriction Endonuclease Analysis of the pnt Plasmids

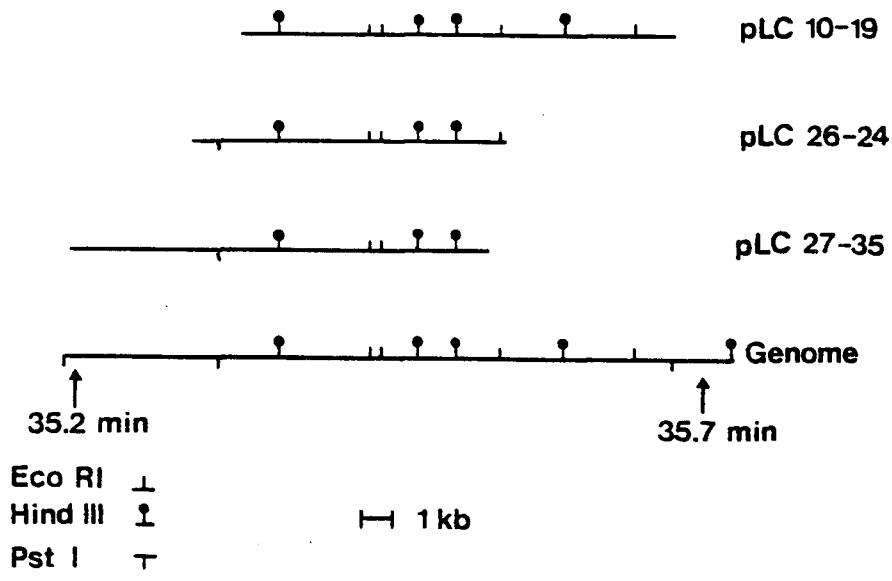
Restriction endonuclease analysis was performed on the three recombinant plasmids from the Clarke and Carbon colony bank to identify the regions of DNA containing the pnt gene. An 8.7-kilobase region was found to be common to the inserts of all three plasmids (Fig. 8).

The region of the E. coli genome bearing the transhydrogenase gene has been physically mapped by Bouché (121). Comparison of the restriction endonuclease maps of the plasmids with that of the genome revealed that the plasmid inserts overlap a region of the genome between 35.2 and 35.7 min (Fig. 8). The overlap region common to all three plasmids included the 35.4 min region which is the position mapped by Hanson and Rose (84) for the pnt gene.

Subcloning of the pnt Gene into pUC13

Plasmid pLC 26-24 was digested by restriction nuclease PstI to give four fragments (10.4, 5.9, 1.2 and 0.1 kilobases). The fragments were separated by gel electrophoresis in low-melting-point agarose, and the 10.4 kilobase fragment was excised from the gel. PstI-digested pUC13, which had been dephosphorylated with calf intestinal phosphatase, was ligated with the 10.4-kilobase fragment, and the ligated DNA was used to transform strain JM83. White ampicillin-resistant transformants were

Fig. 8. Comparison of restriction endonuclease maps of Col EI plasmid inserts with a region of the E. coli genome. The restriction map of the E. coli genome was determined by Bouché (121).



selected and screened for overproduction of transhydrogenase. Plasmid pDC1, containing the 10.4-kilobase PstI fragment inserted into pUC13, amplified transhydrogenase activity 20-fold in JM83.

A 4.8-kilobase HindIII fragment of pDC1 was subcloned into the HindIII site of pUC13 as shown in Fig. 9 to yield plasmid pDC3. Transhydrogenase activity of JM83 carrying pDC3 was 50-fold greater when compared with JM83 harboring pUC13.

Localization of the pnt Gene in pDC3

Plasmid pDC3 was subjected to restriction endonuclease analysis, using the restriction endonucleases HpaI, BstEII, XhoI, SmaI, and SalI. After the locations of the restriction endonuclease sites had been established, various segments of the 4.8-kilobase HindIII inserts of pDC3 or pDC4 were removed. Plasmids pDC3 and pDC4 differ only in the orientation of the insert in the pUC13 vector. Plasmid pUC13 contains a single EcoRI site. Plasmids in Fig. 10 are drawn so that this site is closest to one end of the insert. The constructed plasmids were used to transform JM83. The cells were grown and their transhydrogenase levels were determined (Fig. 10). Deletion of a 0.55-kilobase HpaI-SmaI fragment from pDC3 or a 1.6-kilobase HpaI-SmaI fragment from pDC4 to give plasmids pDC8 and pDC9, respectively, resulted in loss of enhanced expression of transhydrogenase activity. These results demonstrated that at least the 2.65-kilobase fragment bounded by the HpaI restriction sites was essential for the expression of enzyme activity. During the construction of pDC9 from pDC4, one clone was isolated which contained plasmid pDC11. This plasmid was missing 0.75-kilobases of DNA between the HpaI and HindIII sites (Fig. 10). There was no HpaI site at this point in pDC4 so the

Fig. 9. Subcloning of DNA carrying the pnt gene. The plasmids and relative positions of the restriction sites are drawn approximately to scale.

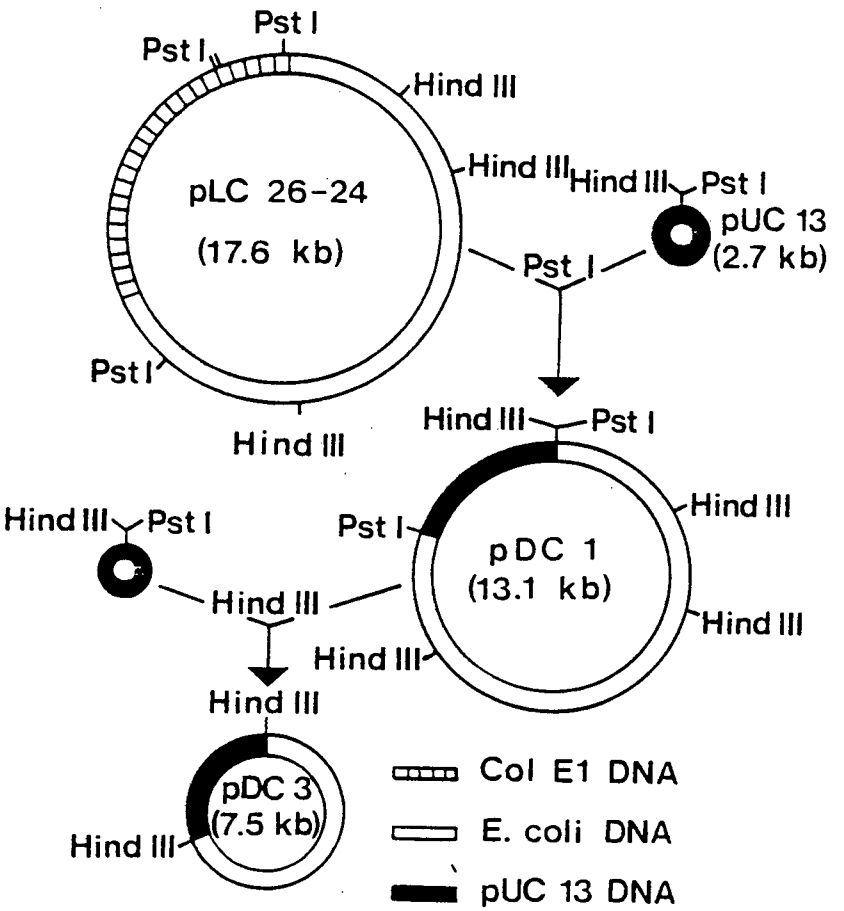
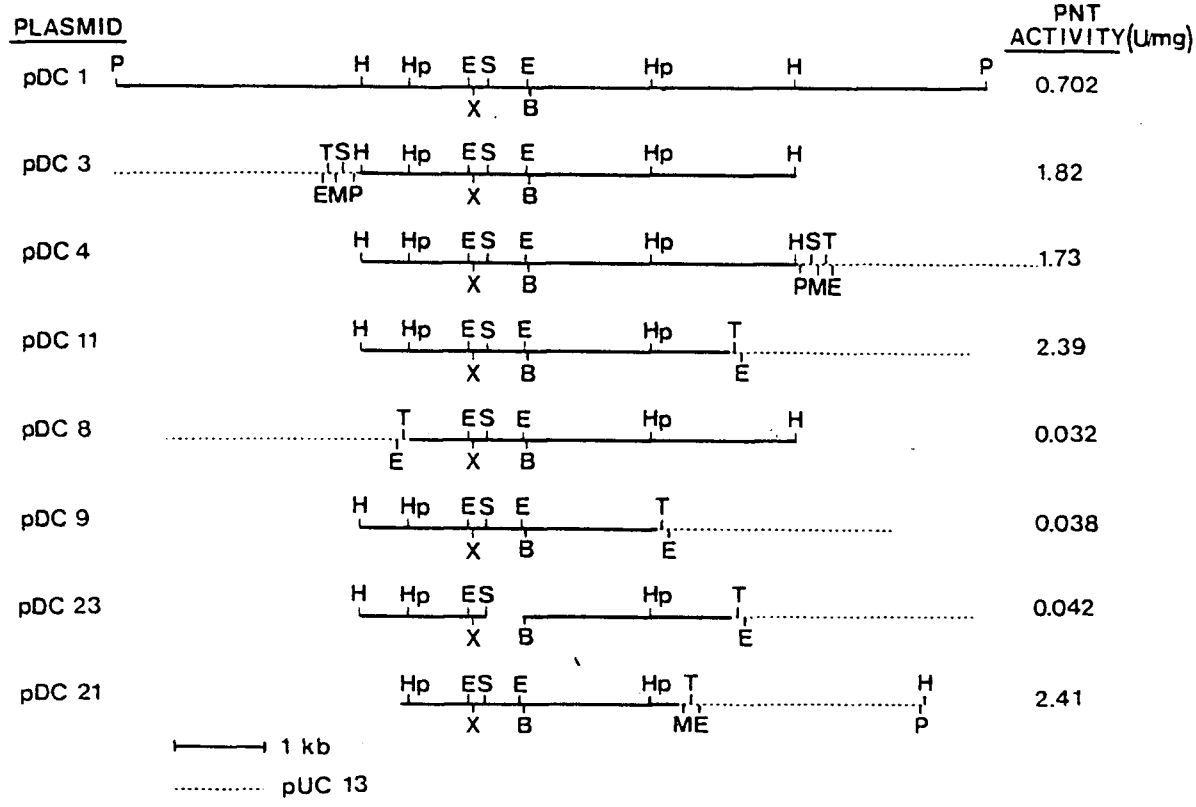


Fig. 10. Restriction endonuclease maps of plasmids containing the pnt gene and transhydrogenase activities of membranes prepared from cells barboring each of the plasmids. Plasmids were constructed and transformed into JM83. Membranes were prepared from transformants grown in LB medium and assayed for transhydrogenase (PNT) activity. The transhydrogenase activity of membranes prepared from JM83 pUC 13 was 0.035 U/mg protein. Symbols: P, Pst I; H, Hind III; B, Bst EII; Hp, Hpa I; E, Eco RI; S, Sal I; X, Xho I; T, Sst I; M, Sma I; solid lines are inserted DNA.



cleavage may have been made by another enzyme contained in the HpaI preparation. This plasmid conferred to 70-fold amplification of transhydrogenase activity in the membranes of JM83 (Fig. 10). The gene was further localized by treatment with the exonuclease BAL31. Plasmid pDC11 contains a single HindIII site at one junction of the pUC13 and insert DNA (Fig. 10) and a single SstI site within vector pUC13 at the other junction. The plasmid was first cleaved with SstI and then treated with BAL31 for different lengths of time. The BAL31-treated inserts were cleaved with HindIII to release the fragments and then ligated into the HindIII and HincII sites of pUC13. The resulting plasmids were used to transform JM83. The smallest plasmid pDC15 still retaining the transhydrogenase gene was then isolated, and the process described above was repeated for the other end of the insert in this plasmid by first cleaving with HindIII followed by digestion for various lengths of time with BAL31. The digested inserts ware released from the plasmid by cleavage with SstI and then ligated into the SstI and HincII sites of pUC13. One of the resulting plasmids, pDC21, contained a 3.05-kilobase insert. Inserts 50 to 100 base pairs smaller at either end of the insert of pDC21 did not exhibit transhydrogenase activity. Plasmid pDC21 conferred a 70-fold amplification of transhydrogenase activity in the membranes of JM83 (Fig. 10).

Identification of the pnt Gene Products

During the course of this study, we found that the pnt gene products irreversibly aggregated when solubilized in SDS gel electrophoresis sample buffer at 100°C and did not enter SDS-polyacrylamide gels during electrophoresis. However, two protein products of molecular weight 52,000

and 48,000 were observed in the gels of membranes from JM83 (pDC11), but not in the gels of membranes from JM83 (pUC13), when solubilization in SDS sample buffer was carried out at 37°C (Fig. 11).

To establish that the two protein products of molecular weights 52,000 and 48,000 were plasmid encoded, an in vitro transcription/translation system was used with plasmid pDC11 as template. The proteins, labelled with [^{35}S]methionine, were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography of the dried gels. Two radioactive polypeptides of molecular weights 52,000 and 48,000 were observed to be products of the in vitro translation of pDC11 but not of pUC13 (Fig. 12). These two products correspond to the two polypeptides amplified in membranes of JM83 (pDC11). The lower-molecular-weight radioactive polypeptides seen on the gel are products of the pUC13 vector DNA.

Two polypeptides of a combined molecular weight of 100,000 would require the coding capacity of about 2.7 kilobases of DNA. This agrees with the observation that at least a 3.05-kilobase insert is required for the expression of enzyme activity. To establish whether or not both polypeptides were needed for transhydrogenase activity, the products of the various plasmids (Fig. 10) were examined by SDS-polyacrylamide gel electrophoresis (Fig. 13). The expression of both polypeptides was observed in JM83 membranes containing either pDC11 or pDC21, and amplification of transhydrogenase activity occurred in both cases. Similar results were obtained with plasmids pDC1, pDC3, and pDC4 (data not shown). Neither of the two polypeptides was observed in the membranes of cells containing pUC13 or pDC8. Only one membrane-bound protein was observed as a product of plasmid pDC9. No increase in transhydrogenase

Fig. 11. SDS polyacrylamide gel electrophoresis of membranes of JM83 containing either pUC 13 or pDC 11. Membranes were solubilized in SDS sample buffer at either 37°C for 10 min or at 100°C for 3 min prior to electrophoresis. Lane 1, molecular weight markers; lane 2, JM83 (pUC 13) membranes solubilized at 100°C; lane 3, JM83 (pDC 11) membranes solubilized at 100°C; lane 4, JM83 (pUC 13) membranes solubilized at 37°C; lane 5, JM83 (pDC 11) membranes solubilized at 37°C.

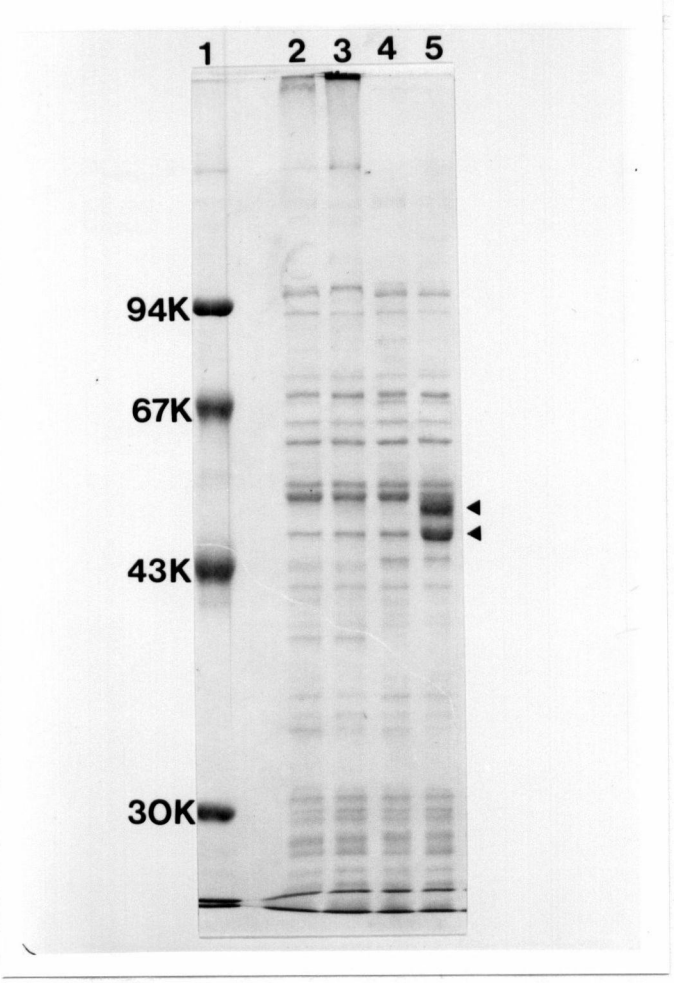


Fig. 12. Autoradiograph of SDS polyacrylamide electrophoresis gel of [^{35}S]methionine-labeled products using plasmids pUC 13 (lane 3) and pDC 11 (lane 4) as templates in an <u>in vitro</u> transcription/translation system. Plasmid DNA was added to final concentration of 100 µg DNA per ml. The reaction mixture was incubated at 37°C for 40 min. The reaction was terminated by cooling to 0°C. Samples of the reaction mixture were mixed with an equal volume of electrophoresis sample buffer and 10 µl was used for electrophoresis. The polypeptides of lane 1 (molecular weight markers) and lane 2 (membrane polypeptides of JM83 pDC 11) were stained with Coomassie blue.

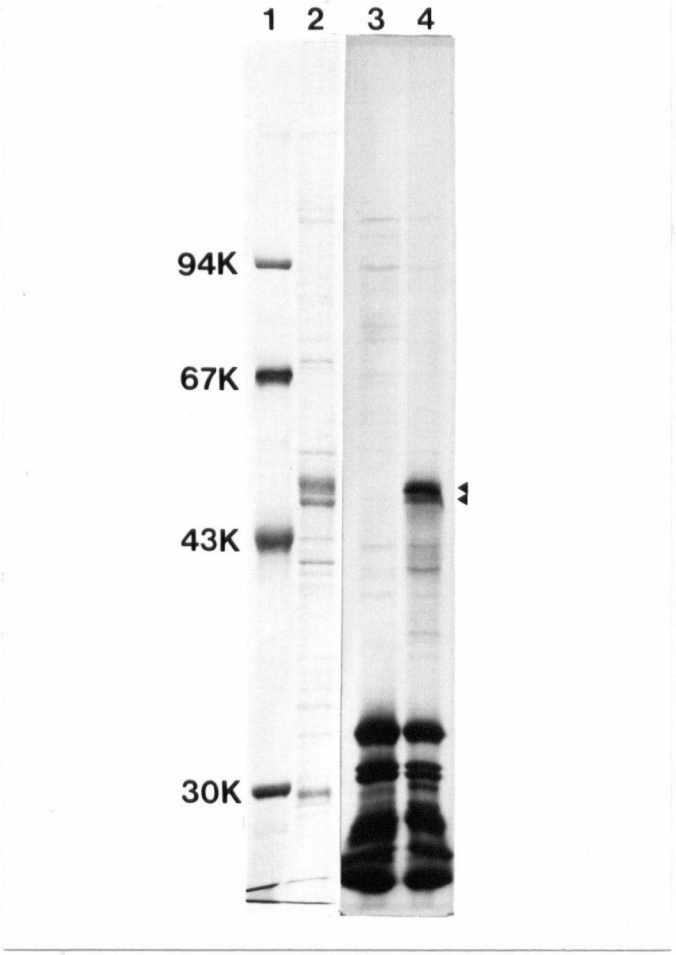
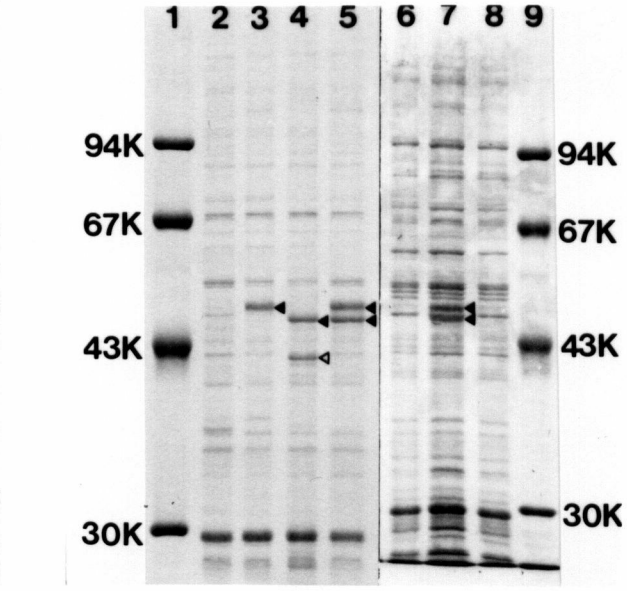


Fig 13. SDS polyacrylamide gel electrophoresis of membrane fractions of JM83 carrying hybrid plasmids. Lanes 1,9, molecular weight markers; lanes 2,8, JM83 (pUC 13); lane 3, JM83 (pDC 9); lane 4, JM83 (pDC 23); lane 5, JM83 (pDC 21); lane 6, JM83 (pDC 8); lane 7, JM83 (pDC11).



activity was detected in these membranes, indicating that the 52,000-molecular-weight polypeptide alone is not capable of transhydrogenation.

The possibility that the 48,000-molecular-weight polypeptide alone is responsible for transhydrogenase activity was examined by constructing plasmid pDC23 from pDC11. Plasmid pDC11 was digested with SalI and BstEII, and the recessed 3' ends of the DNA were filled in before ligation, using the Klenow fragment of E. coli DNA polymerase. The resulting plasmid, pDC23, was used to transform strain JM83. Membranes prepared from the transformed strain contained the 48,000-molecular-weight polypeptide and a polypeptide of molecular weight 42,000 (Fig. 13). The latter may be a translation product of the residual DNA resulting from the 0.45-kilobase deletion. No increase in transhydrogenase activity was detected in these membranes (Fig. 13), indicating that the 48,000-molecular-weight polypeptide alone is not capable of transhydrogenation.

Complementation of Transhydrogenase Activity

Further evidence that the 52,000- and 48,000-molecular-weight polypeptides compose the transhydrogenase enzyme and were not merely stimulating expression of the chromosomally encoded transhydrogenase gene comes from complementation tests with strain RH-5. In this strain, transposon Tn5 is inserted in the pnt locus with the result that there is complete loss of transhydrogenase activity (84). Since RH-5 transformed poorly, the defective pnt locus was transduced into AB1450, using bacteriophage P1 and selecting for kanamycin resistance. This strain was then transformed with plasmids pUC13, pDC9, pDC23, and pDC21.

Transhydrogenase activity was restored to the recipient by pDC21 only (Table 7), indicating that both polypeptides are part of the enzyme.

Transhydrogenase activity could also be restored by having replicons containing the pDC9 and pDC23 inserts transformed into the same cells. Plasmid pDC23 was cleaved with HindIII and BamHI and ligated into pACYC184 which had been cleaved with HindIII and BamHI to give plasmid pDC50. Plasmids pDC9 and pDC50 were transformed into AB1450 (pnt::Tn5). Transformants resistant to both ampicillin and chloramphenicol were selected. As shown in Fig. 14, these transformants contained both pDC9 and pDC50. Plasmid pDC50 is a low-copy-number plasmid and was found in lesser amounts than the high-copy number plasmid pDC9. Membranes prepared from clones containing both plasmids had an enhanced level of transhydrogenase activity (0.33 U/mg protein). No transhydrogenase activity was found in the supernatant prepared from AB1450 (pnt::Tn5). Membranes prepared from AB1450 had a specific transhydrogenase activity of 0.04 U/mg protein. Both the 52,000 and 48,000 proteins were found in membranes of the cells containing both plasmids in amounts corresponding to the plasmid copy number from which they were encoded (Fig. 15). These results confirm that the transhydrogenase consist of two subunits of molecular weights 52,000 and 48,000. These will be referred to as the α and ß subunits, respectively.

Morphological Effects of pnt Overproduction

The three plasmids, pDC9, pDC23 and pDC21, carrying intact α, ß and α/ß subunits of transhydrogenase, and pUC13 were transferred into the transhydrogenase inactivated strain AB1450 pnt::Tn5. High levels of the transhydrogenase subunits were found in the membranes (Fig. 13) from cells

Table 7. Complementation of chromosomal pnt::Tn5 by various pnt alleles on plasmids.

Strain	transhydrogenase activity in membrane (μmoles/min/mg protein)
AB 1450	0.044
AB 1450 pnt::Tn5	0
AB 1450 pnt::Tn5 pUC13	0
AB 1450 pnt::Tn5 pDC9	0
AB 1450 pnt::Tn5 pDC23	0
AB 1450 pnt::Tn5 pDC21	0.94

E. coli strain AB 1450 pnt::Tn5 was transformed with plasmids pUC13, pDC9, pDC23 and pDC21 and then grown in LB media supplemented with 25 μg/ml kanamycin and 50 μg/ml ampicillin. Membranes were prepared and assayed for transhydrogenase activity as described in Materials and Methods.

Fig. 14. Agarose gel electrophoresis of plasmids prepared from E. coli AB 1450 containing transhydrogenase subunits on separate replicons. Plasmids were prepared from AB 1450 pDC50 (lane 2), AB 1450 pDC50 and pDC9 (lane 3) and AB 1450 pDC9 (lane 4) cells grown in LB supplemented with 30 μg/ml chloramphenicol (pDC50) and/or 50 μg/ml ampicillin as described in Materials and Methods. The plasmids were digested with endonucleases XhoI and BamHI and the fragments separated by electrophoresis in an 0.8% agarose gel. Lane 1: λDNA cleaved with HindIII and EcoRI.

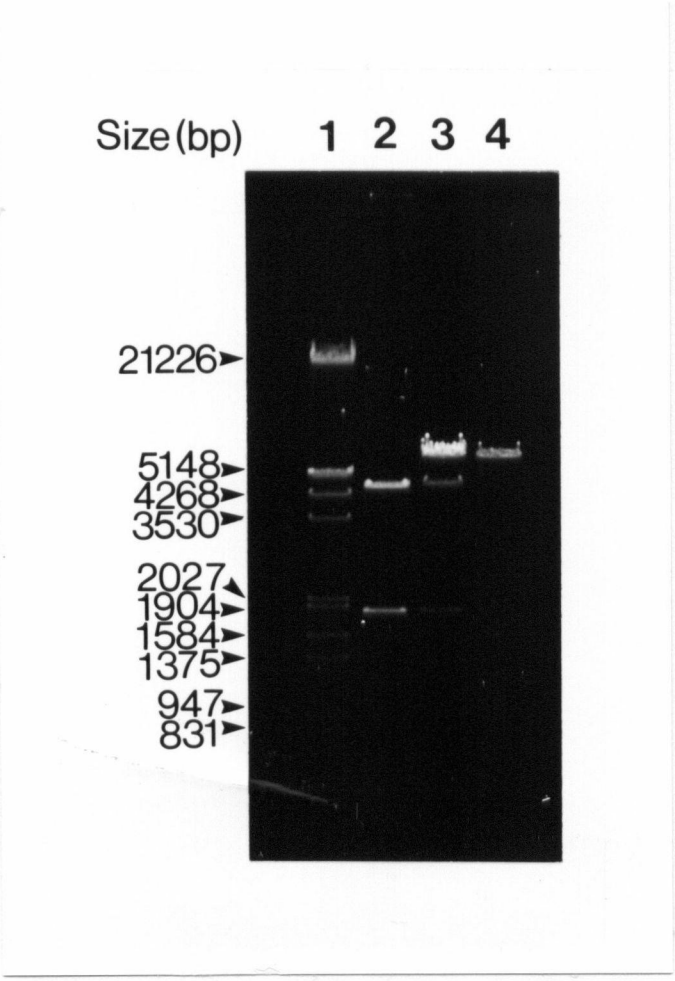
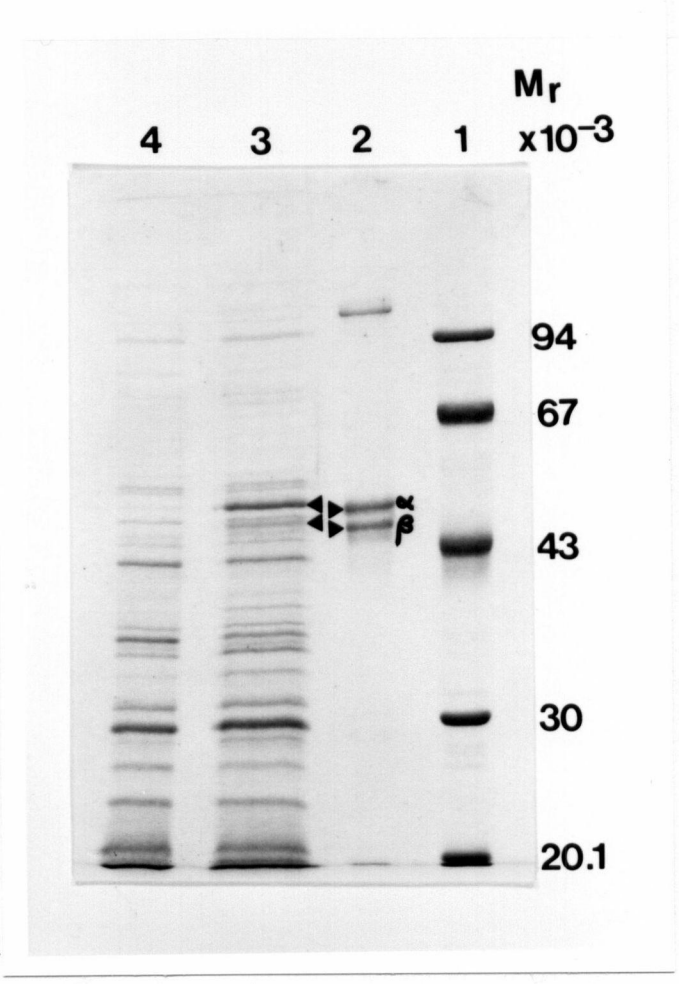


Fig. 15. SDS-polyacrylamide gel electrophoresis of membranes prepared from E. coli AB 1450 pnt::Tn5 and AB 1450 pnt::Tn5 pDC9, pDC50. Cells were grown in LB media supplemented with 30 μg/ml of kanamycin. Ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml) were included in the medium for the plasmid-containing strain. Membranes were prepared and electrophoresed in a 10% SDS/polyacrylamide gel as described in Materials and Methods. Lane 1: molecular weight markers; lane 2, purified transhydrogenase; lane 3, AB 1450 pnt::Tn5 pDC9 and pDC50 membranes; lane 4, AB 1450 pnt::Tn5 membranes.



carrying pDC21. There was an almost 80-fold greater expression of transhydrogenase activity when compared to the transhydrogenase levels of AB1450. No growth was observed when cells containing the plasmids pDC9, pDC23 or pDC21 were inoculated into M9 medium and shaken at 37°C for 18 h. Cells containing pUC13 grew normally. The presence of the plasmids containing transhydrogenase subunits also inhibited growth in LB medium and decreased the yield of cells (Table 8). The inhibition was most pronounced in cells containing plasmid pDC21. The yield of cells was reduced by almost 50% when compared to the yield of cells which contained the plasmid pUC13.

Observation of the cells using phase contrast microscopy revealed that the strains with a high level of transhydrogenase subunits had a heterogeneous size distribution (Fig. 16).

The cells in thin section preparation revealed the presence of tubular-like structures that were sometimes observed in cells overproducing the transhydrogenase subunits but not observed in cells containing the plasmid pUC13 (Fig. 17). These tubular-like structures were normally observed near the poles of the cells.

Another interesting effect of overproduction of transhydrogenase was the loss of aerobic-driven transhydrogenation. In membranes prepared from JM83, energy-independent, aerobic-dependent and ATP-dependent activities are 0.009, 0.036 and 0.064 U/mg protein respectively. The corresponding values were 0.14, 0, and 0.44 U/mg protein for JM83 pDC21.

IV. Purification of Transhydrogenase from Strain JM83 pDC21

Cloning of the *pnt* gene to form the multicopy plasmid pDC21 resulted

Table 8. Growth characteristic of JM83 carrying various plasmids.

Plasmid	Specific Activity of transhydrogenase U/mg protein	Relative Growth of Single Colonies	Growth Rate (%)	Growth Yield (%)
pUC13	0	1.0	100	100
pDC21	3.4	0.6	78	52
pDC9	0	0.6	80	64
pDC22	0	0.7	86	81
none	0	1.0	100	100

Cells were grown in LB medium supplemented with 100 μg/ml of ampicillin when the cells contained plasmids. The sizes of colonies were measured after growth on plates containing LB medium containing 100 μg/ml of ampicillin at 37°C overnight. Growth rate was measured from the absorbance at 600 nm. The growth yield was measured by adding an equal amount of cells to 200 ml of LB media and growing the cells for 14 h at 37° with shaking (250 rpm).

Fig. 16. Microphotographs (phase contrast, 40X objective) of E. coli JM83 cells containing the plasmids pUC13 (A) or pDC21 (B). Magnification - 1000X.

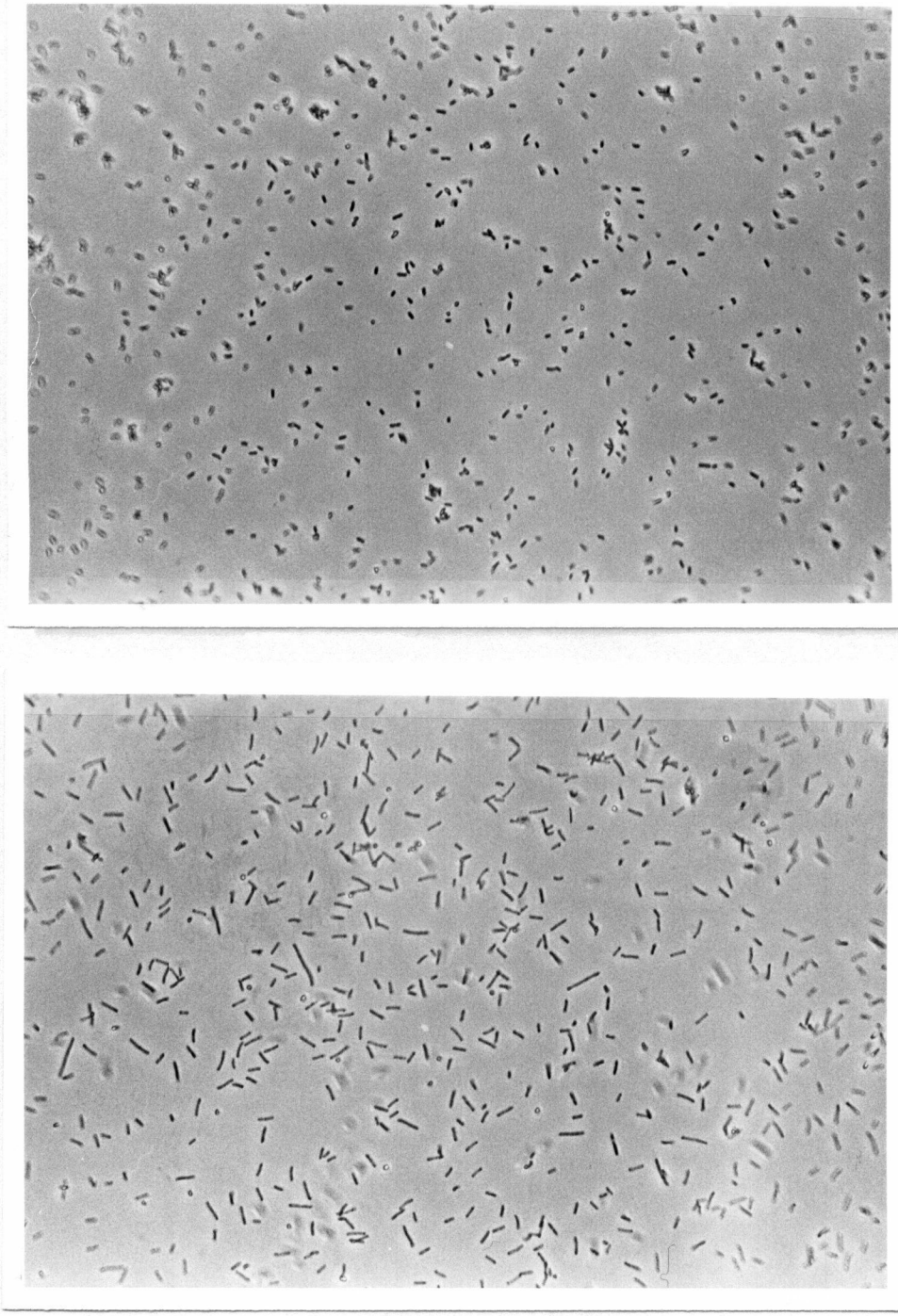
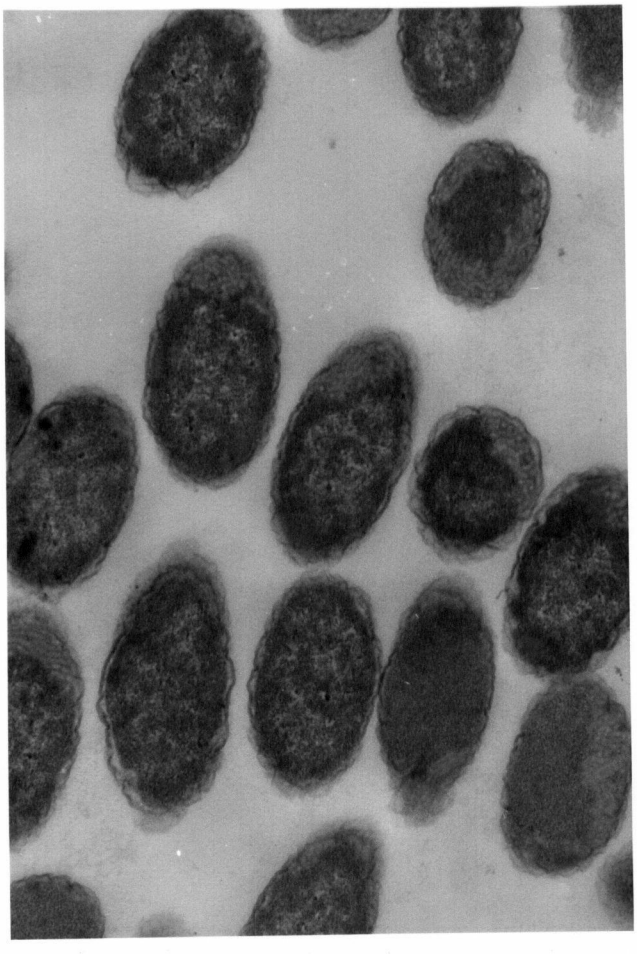


Fig. 17. Thin section micrographs of JM83 pDC21 cells. Cells were grown to stationary phase in LB medium. The cells were collected by centrifugation, washed with 0.2 M sodium phosphate (pH 7.0), and fixed with 2% glutaraldehyde. The cells were washed with phosphate-buffered sucrose, postfixed in 2% OsO_4, dehydrated in ethanol and embedded in Epon 812. Sections were stained with lead citrate and saturated uranyl acetate, and mounted on grids for electron microscopy.



in greater than 70-fold overproduction of transhydrogenase in cells harboring the plasmid. These cells served as an excellent starting material for the purification of transhydrogenase as the two subunits, α (M_r 52,000) and ß (M_r 48,000), were the two major proteins in the cytoplasmic membrane.

Extraction of membrane vesicles of E. coli strain JM83 pDC21 sequentially with 1% Triton X-100 and 2% sodium cholate resulted in solubilization of 80% of the membrane protein, while approximately 55% of the transhydrogenase remained in the particulate material after centrifugation (Table 9). Some selectivity was observed in the proteins extracted by the detergents. Several of the proteins remaining after Triton X-100 extraction were efficiently solubilized by subsequent treatment with cholate. However, only Triton X-100 was effective in solubilizing some flavoproteins and b-type cytochromes, as well as a number of other proteins.

The transhydrogenase could be solubilized from the extracted vesicles by 0.5% sodium deoxycholate in the presence of 1 M KCl. Although cholate (0.5%) alone was ineffective in solubilizing the transhydrogenase, it was necessary to include this detergent in order to prevent the solubilized material from forming a gel when stored for any length of time.

The deoxycholate extract was loaded over a 20% sucrose solution and centrifuged overnight at 260,000 x g. The transhydrogenase activity was found in the sucrose solution.

Pooled fractions containing transhydrogenase had a specific activity of 29.9 $\mu mol.min^{-1}.mg^{-1}$ of protein and represented 16% of the original activity. Recoveries of activity ranged from 10-18%. The purified enzyme retained over 90% of its activity when stored for a week

Table 9. Purification of transhydrogenase from E. coli strain JM83 pDC21.

Fraction	Protein	Transhydrogenase Activity	
		Total	Specific
	mg	%	U/mg protein
Membranes	188	100	5.2
After Triton X-100 treatment	61	77	12.1
After cholate treatment	33	55	16.2
Extracted enzyme	15	38	24.4
Combined fractions after centrifugation	5	16	29.9

Membranes were prepared from 4.7 g of cells; after treating the membranes sequentially with the detergents Triton X-100 and sodium cholate, the transhydrogenase was solubilized by the detergent sodium deoxycholate in the presence of KCl. The enzyme was purified from the extract by centrifugation through a sucrose solution. Experimental details of the purification are described in Materials and Methods.

at 4°C. A high degree of purification was achieved as shown by SDS-polyacrylamide gel electrophoresis of the purified material (Fig. 18). Two bands, α and ß, of apparent molecular weights 52,000 and 48,000 were observed. The absorption spectrum of the purified enzyme indicated that the preparation was devoid of flavin.

Both Liang and Houghton (55) and Voordouw et al. (122) have suggested that the 100,000-molecular-weight protein is a component of the E. coli transhydrogenase. A 100,000-molecular-weight protein was observed when transhydrogenase was purified from W6 (Fig. 18). A minor 100,000-molecular-weight protein was observed as a diffuse band in SDS-polyacrylamide gels of the enzyme preparation purified from JM83 pDC21. This polypeptide became more prominent in the gels when the samples were stored for several days at 4°C or when thiol reducing agents were omitted from the buffers. In order to determine if the 100,000-molecular weight protein represented an aggregate of the transhydrogenase α and ß subunits, the stained bands of the polypeptides were excised from the gels and partially digested with chymotrypsin using the technique described by Cleveland et al. (106). The patterns of chymotryptic fragments are shown in Fig. 19. The pattern of chymotryptic fragments for the 100,000 molecular-weight protein closely resembled the pattern of fragments obtained from the α and ß subunits of the transhydrogenase. This indicates that the 100,000-molecular-weight protein was an aggregate of the α and ß subunits.

Fig. 18. SDS-polyacrylamide gel electrophoresis of various fractions obtained during the purification of transhydrogenase from E. coli strains JM83 pDC 21 (lanes 1-5) and W6 (lane 6). Purification of the enzyme, gel electrophoresis, and staining were carried out as described in Materials and Methods. Lane 1, membranes, 36 µg; lane 2, Triton X-100 extracted membranes, 16 µg; lane 3, cholate-extracted membranes, 12 µg; lane 4, deoxycholate extract, 8 µg; lane 5, pooled sucrose gradient fractions, 6 µg; lane 6, affinity column eluent, 10 µg. The positions of migration of the molecular weight standards are indicated.

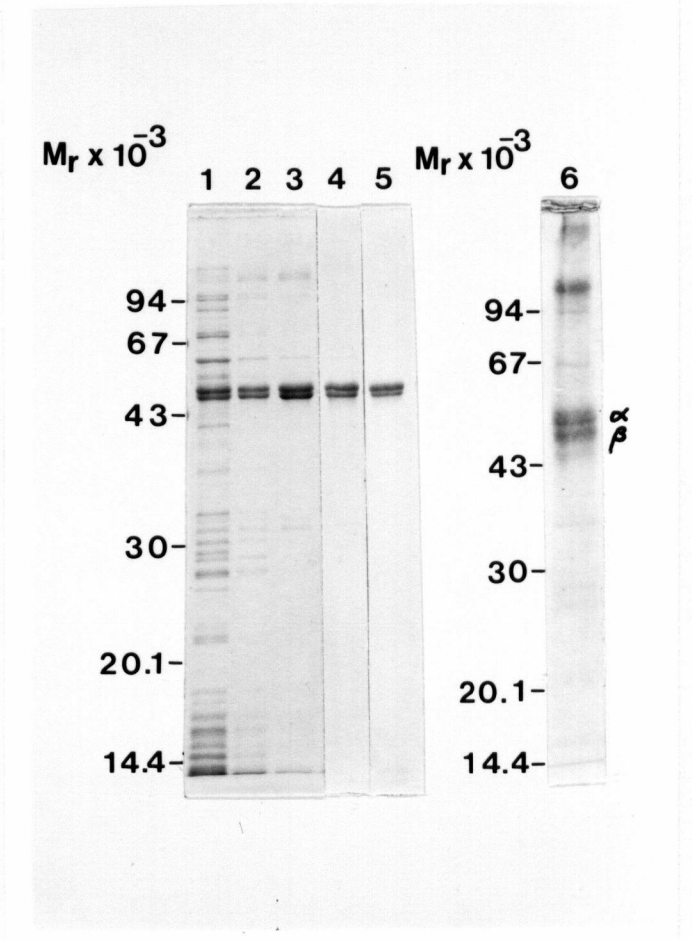
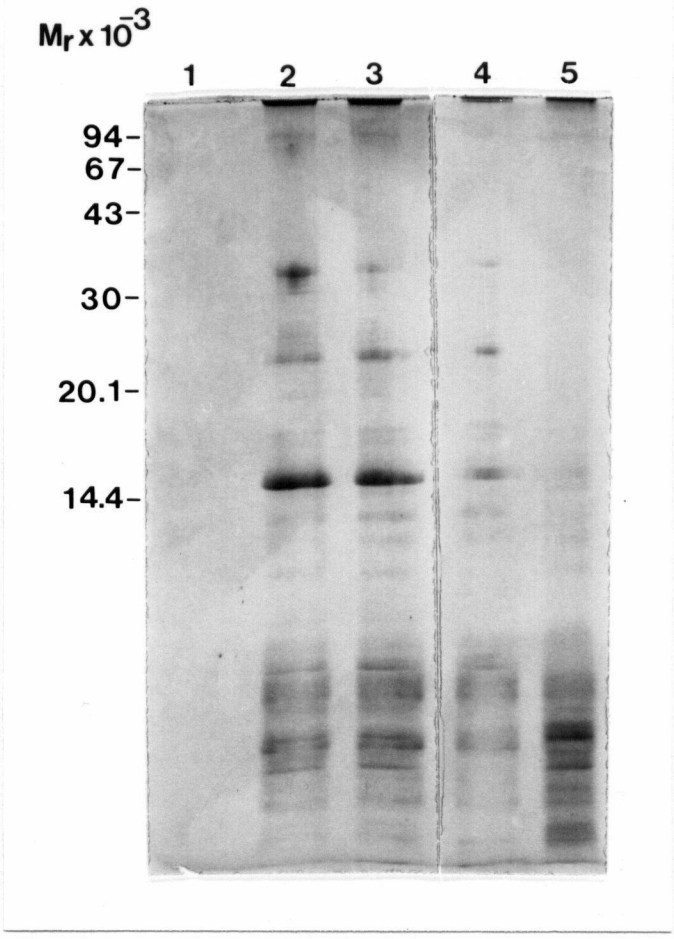


Fig. 19. Partial proteolysis of the 97,000-molecular-weight protein, and the α and ß subunits of the transhydrogenase. The 100,000-molecular-weight protein and the α and ß subunits of the transhydrogenase were subjected to Cleveland digestion using chymotrypsin as described in Materials and Methods. Lane 1, chymotrypsin alone; lane 2, 100,000-molecular-weight protein; lane 3, mixed α and ß transhydrogenase subunits; lane 4, transhydrogenase ß subunit; lane 5, transhydrogenase α subunit. The positions of migration of the molecular mass standards are indicated.



V. Properties of Transhydrogenase

Kinetic Parameters

The steady state kinetics for reduction of AcNAD by NADPH were determined (Fig. 20). The apparent Michaelis constants, Km, for NADPH and AcNAD were 23 μM and 33 μM, respectively, for the purified enzyme. The corresponding values for the membrane-bound enzyme were 29 μM and 41 μM, respectively.

Inactivation by Trypsin

Studies of the proteolytic inactivation of mitochondrial transhydrogenase by trypsin provided evidence that conformational changes in the transhydrogenase molecule are induced by the binding of NADPH (123). As shown in Table 10, E. coli transhydrogenase was also inactivated by trypsin. Proteolytic cleavage took place within the α subunit (Fig. 21). NADPH increased the degree of inactivation by trypsin whereas NADP, NADH and NAD did not have any significant effect on proteolysis (Fig. 22). These findings are similar to those reported for the mitochondrial transhydrogenase.

VI. Transhydrogenase as a Proton Pump

Proteoliposome Energization

During the early stage of purification when the transhydrogenase was still membrane-bound, maximum activity was observed in the presence of an uncoupler. Addition of the uncoupler FCCP to nonenergized, everted membrane vesicles enhanced the reduction of AcNAD by NADPH and the reduction of NADP by NADH by 3.4-fold and 3.6-fold, respectively

Fig. 20. Kinetic parameters of transhydrogenase. The activities of purified (A and B) and membrane-bound transhydrogenases (C and D) were assayed in the presence of a fixed concentration of 1.98 mM AcNAD (B and D) or 0.99 mM NADPH (A and C) and varying the concentration of the other nucleotide as described in Materials and Methods. The assays were carried out in the presence of 1 μM FCCP. V is expressed in μmoles min^{-1} mg^{-1}.

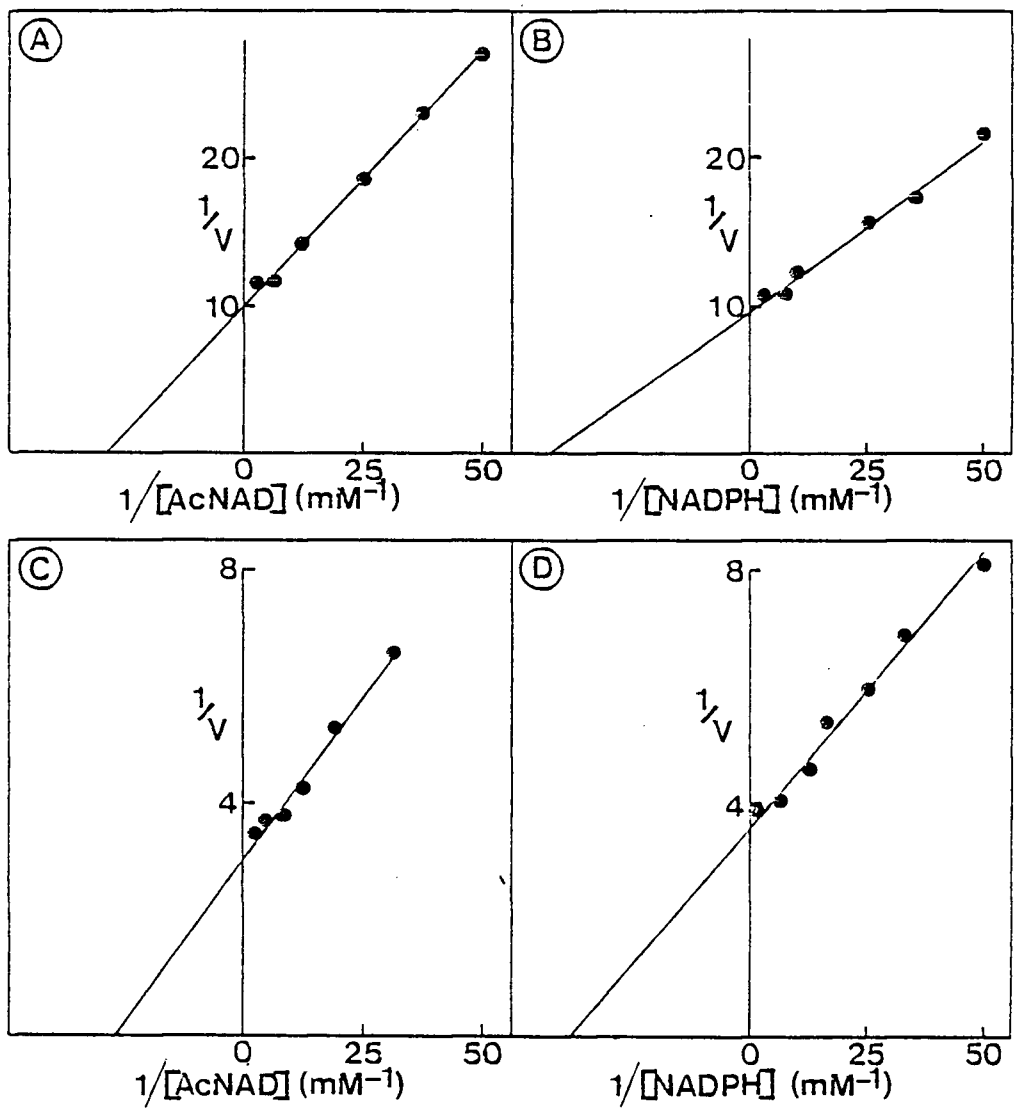


Table 10. Treatment of membrane vesicles prepared from E. coli JM83 pDC21 with various levels of TPCK-trypsin.

Trypsin added (ng)	% Control Activity
0	100
50	86.3
100	65.3
200	25.6
400	1.4

Membranes in TED at a protein concentration of 2.3 mg/ml were treated with various amounts of trypsin for 5 min at 37°C. Reactions were stopped by the addition of 10 µg of trypsin inhibitor. Transhydrogenase activity was then measured as described in Materials and Methods.

Fig. 21. SDS-polyacrylamide gel electrophoresis of membrane vesicles prepared from E. coli JM83 pDC21 and treated with 0 ng (lane 3), 50 ng (lane 4), 100 ng (lane 5), 200 ng (lane 6) or 400 ng (lane 7) of TPCK-trypsin as described in Table 10. The proteins were separated on a 10% SDS-polyacrylamide gel as described in Materials and Methods. Lane 1: molecular weight markers; lane 2: purified transhydrogenase.

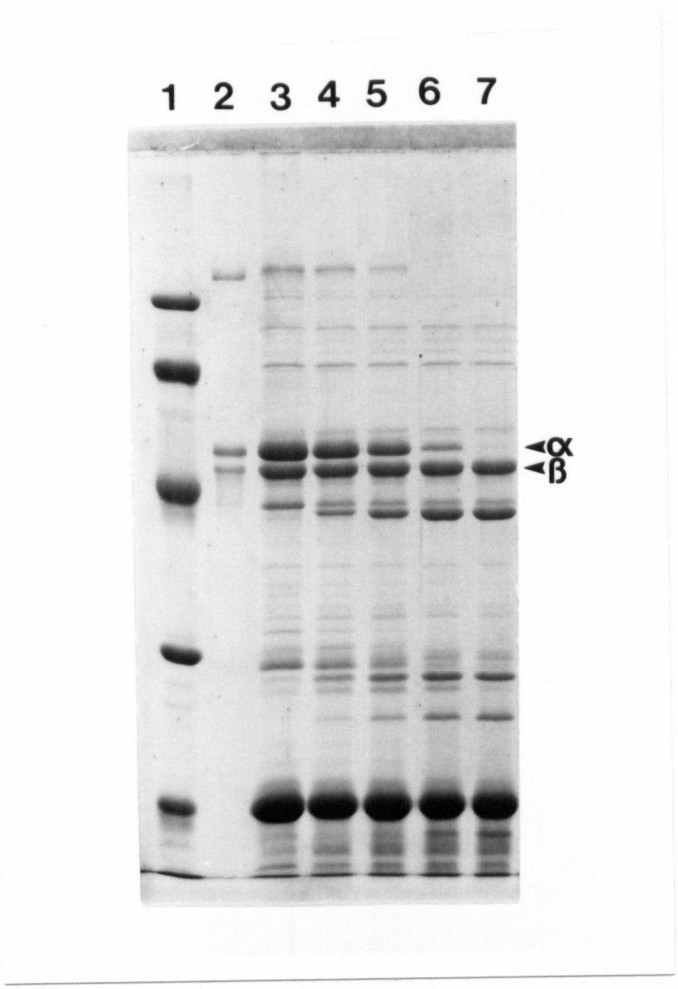
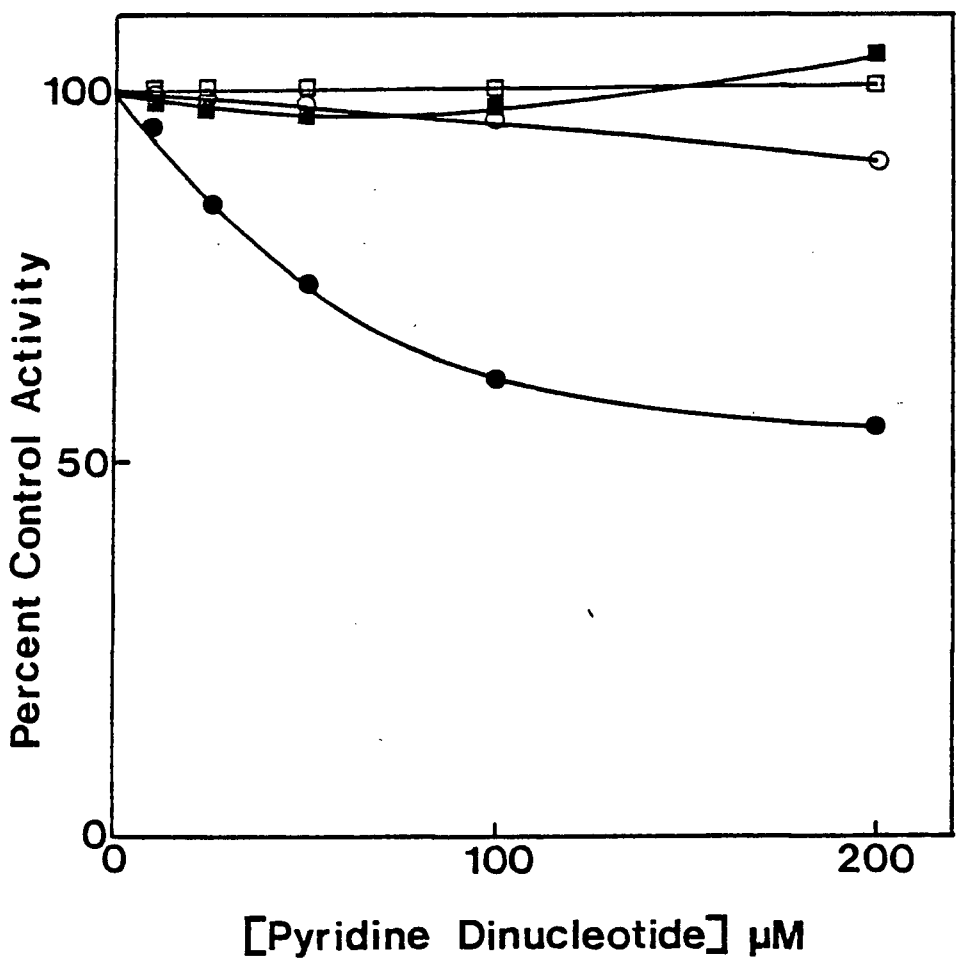


Fig. 22. Inactivation of membrane-bound transhydrogenase by TPCK-trypsin in the presence of various levels of nucleotides. Membrane vesicles were prepared and suspended in TED buffer at a protein concentration of 2.3 mg/ml. The membrane vesicles (0.5 ml) were treated with 75 mg of TPCK-trypsin for 5 min at 37°C in the presence of various amounts of NADPH (●-●), NADP (o-o), NADH (■-■) or NAD (□-□). The reactions were stopped by the addition of 10 µg of soybean trypsin inhibitor and the tubes placed on ice. The transhydrogenase activity was measured as described in Materials and Methods. It is expressed as a percentage of the transhydrogenase activity remaining in a control with no added nucleotide. Treatment of the control with 75 ng of TPCK-trypsin for 5 min at 37°C resulted in a 34% decrease in transhydrogenase activity.



(Fig. 23). Uncoupler did not affect the activities of solubilized or purified transhydrogenase. These data suggest that the inhibition of transhydrogenation in both directions derives from the establishment of a pH gradient across the membrane. Uncouplers would collapse the pH gradient either by providing a continual supply of protons to the vesicle interior to be pumped out during the reduction of NADP by NADH or by relieving the build up of protons in the vesicle interior during the reduction of AcNAD by NADPH.

Various pH probes, such as 9-aminoacridine, pyramine or neutral red may be used to monitor the internal pH of the vesicles (124-126). Proton translocation during the reduction of AcNAD by NADPH was followed by measuring the fluorescence of 9-aminoacridine (Fig. 24). Following the addition of both substrates, the fluorescence was substantially quenched indicating an uptake of protons into the vesicles during the reaction. Quenching was subsequently relieved by the addition of FCCP.

Measurement of the electrogenic activity of purified transhydrogenase may be carried out by reconstituting the enzyme into synthetic liposomes. When purified transhydrogenase was reconstituted into egg phosphatidylcholine vesicles, the rate of reduction of AcNAD by NADPH was increased threefold by the addition of the uncoupler FCCP. Proton pumping activity was also observed during the reduction of AcNAD by NADPH catalyzed by the reconstituted transhydrogenase as indicated by the quenching of the fluorescence of 9-aminoacridine (Fig. 24).

Interaction of Transhydrogenase with a pH Gradient or Membrane Potential

Uncouplers stimulate transhydrogenase in both directions (Fig. 23) suggesting that respiratory control seen in transhydrogenase containing

Fig. 23. Effect of FCCP on reverse and forward transhydrogenase activities. The influence of uncoupler on the activities of the soluble and membrane-bound transhydrogenase activities were assayed as described in Materials and Methods. Experiment A compares the rates of AcNAD reduction catalyzed by either (a) solubilized transhydrogenase or (b) membrane-bound transhydrogenase. Experiment B compares the rate of NADP reduction catalyzed by either (a) solubilized transhydrogenase or (b) membrane-bound transhydrogenase. FCCP (1 μM) was added where indicated.

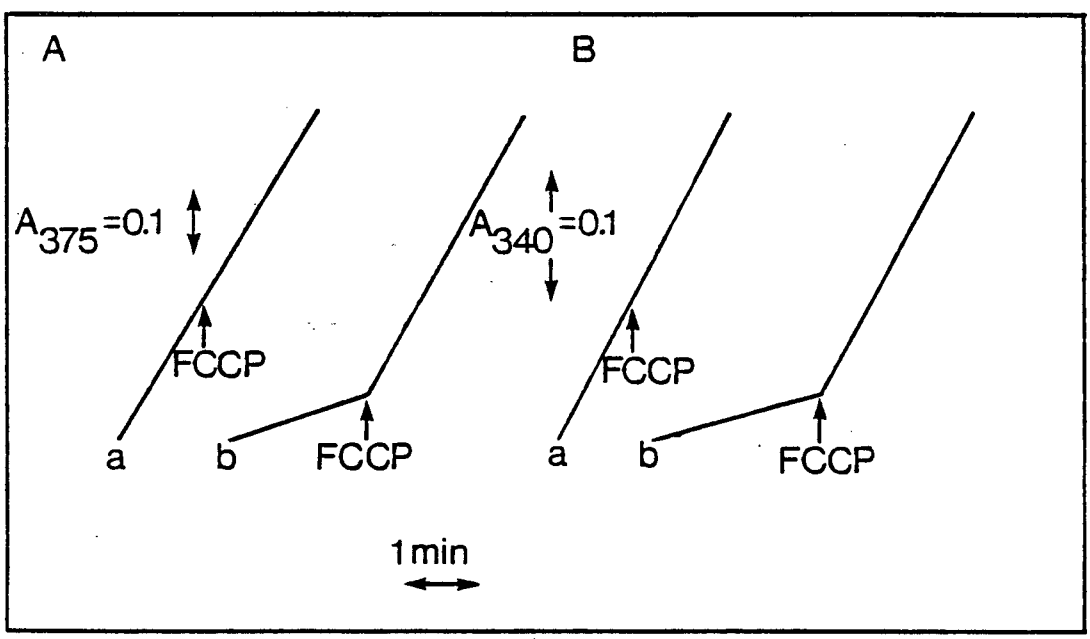
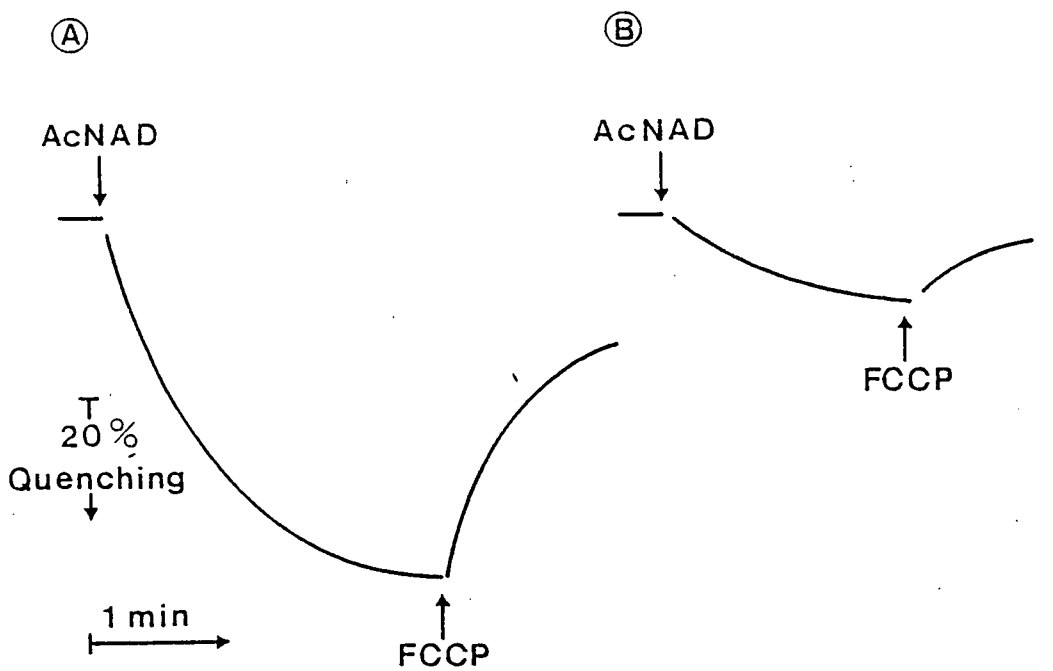


Fig. 24. Quenching of the fluorescence of 9-aminoacridine during the reduction of AcNAD by NADPH catalyzed by either membrane-bound or reconstituted transhydrogenase. The fluorescence of 9-aminoacridine was monitored during the reduction of AcNAD by NADPH. The excitation wavelength was 420 nm and emission was determined at 500 nm. Membranes were prepared from E. coli strain JM83 pDC21 as described in Materials and Methods with the exception that 5 mm $MgSO_4$ was included in all steps. The reaction mixtures (2.1 ml) at pH 7.5 contained 10 mM Hepes, 300 mM KCl, 5 mM $MgCl_2$ and 250 µM NADPH. In addition, experiment A contained 15.8 µg of washed membranes and 8.7 µM 9-aminoacridine. Experiment B contained 2.4 µg of purified transhydrogenase reconstituted into vesicles with egg yolk phosphatidylcholine, and 1.1 µM 9-aminoacridine. Reactions were started by the addition of 500 µM AcNAD. Where indicated 1.3 µM FCCP was added in experiment A and 0.3 µM FCCP was added in experiment B.



vesicles could result from a balancing of the substrate oxidation-reduction potential against the electrochemical hydrogen ion gradient formed across the membrane. The rate of reduction of NADP by NADH would decrease because of a depletion of internal protons whereas the rate of reduction of NAD by NADPH would be inhibited because of an increase in internal protons in everted membrane vesicles. Alternatively, transhydrogenation might be controlled primarily by the membrane potential ($\Delta\psi$).

An artificially imposed pH gradient was generated in membrane vesicles by carrying out the reduction of AcNAD by NADPH in a buffer at pH 6.0 using vesicles which had been equilibrated with buffer to give an internal pH of 8.0. As shown in Fig. 25A, there was a transient phase of high initial activity which gradually declined. Addition of uncoupler caused the expected enhancement of activity. No decrease in activity was observed when the reaction was carried out at an external pH of 7.4 (Fig. 25B).

Interactions between transhydrogenase and imposed membrane potentials were investigated with potassium gradients in the presence of valinomycin. A high external concentration of KCl (150 mM) and a low internal concentration of KCl (5 mM) would generate a minimal membrane potential of 100 mV positive inside the vesicles. A membrane potential of approximately the same size but negative inside the vesicles was generated by the presence of valinomycin and the same concentration of KCl inside the vesicles. No significant effect was observed on the rate of transhydrogenation under either of these conditions (Fig. 26). The same results were observed when everted vesicles were prepared in 0.1 M potassium phosphate or sodium phosphate and assayed in 0.1 M sodium phosphate or potassium phosphate, respectively.

Fig. 25. Influence of a transmembrane pH gradient on the rate of reduction of AcNAD by NADPH catalyzed by membrane vesicles. Membrane vesicles were prepared from E. coli JM83 pDC21 in 0.1 M Tricine buffer, pH 8.0 containing 5 mM $MgCl_2$, 0.2 mM dithiothreitol. The reduction of AcNAD by NADPH at 375 nm was measured in 0.1 M MES buffer, pH 6.0, containing 5 mM $MgCl_2$ and 0.2 mM transhydrogenase (A) or in 0.1 M Tricine, pH 7.4 containing 5 mM, $MgCl_2$ and 0.2 mM dithiothreitol. 35 µg of protein was added to each assay mixture. FCCP was added to a final concentration of 1.3 µM.

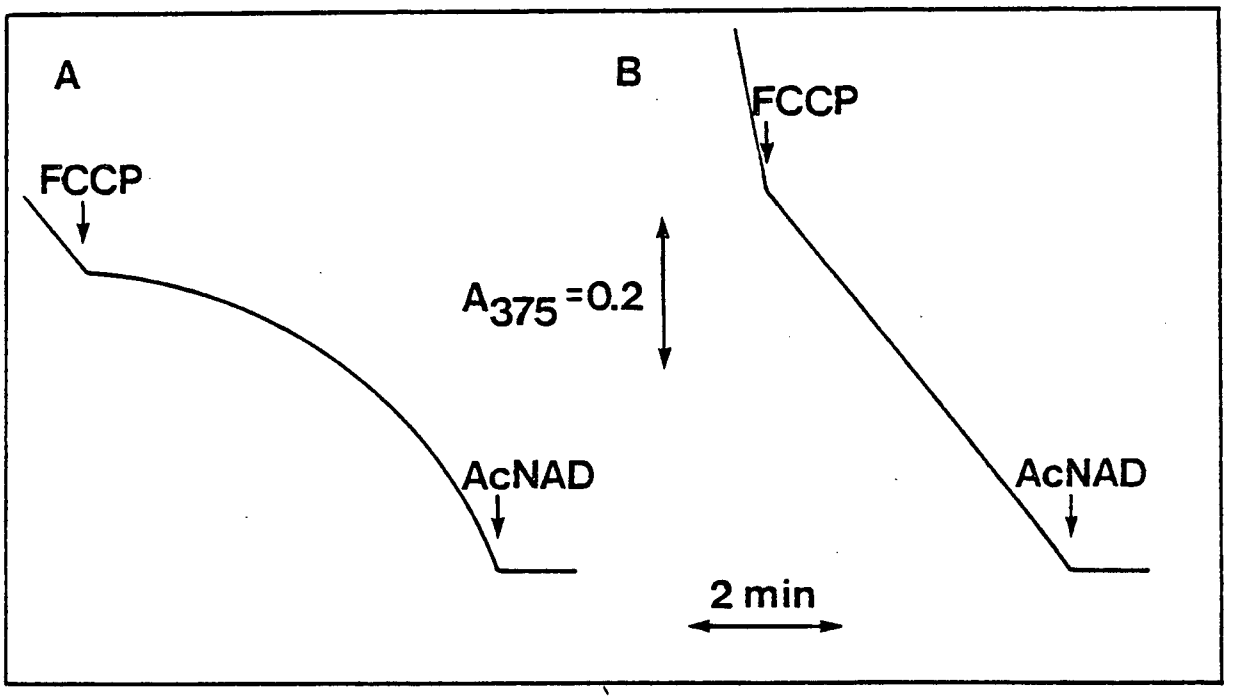
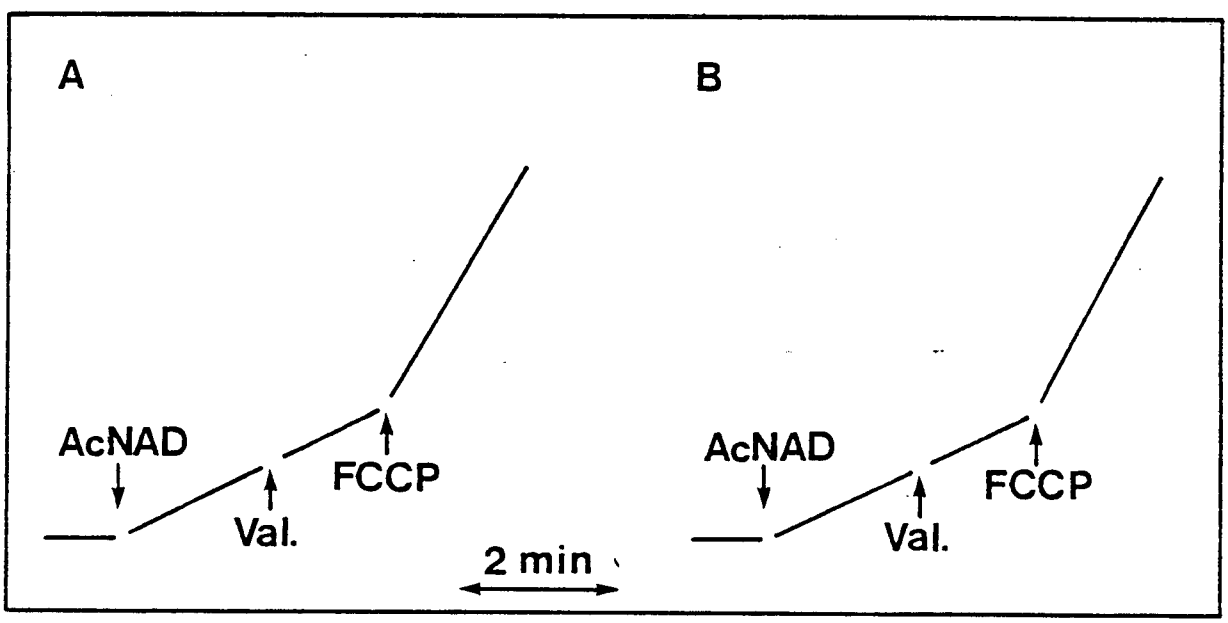


Fig. 26. Influence of a membrane potential positive inside (A) or outside (B) on the rate of reduction of AcNAD by NADPH catalyzed by membrane vesicles. Membrane vesicles of JM83 pDC21 were prepared in 0.1 M Tricine pH 7.4 containing 5 mM $MgCl_2$ and 0.2 mM dithiothreitol and assayed for transhydrogenase activity in the same buffer containing 150 mM KCl (A) or membrane vesicles were prepared in buffer with KCl and assayed in the absence of external KCl (B). A volume of 2 µl membrane vesicles containing 12 µg of protein was used in the assay. Valinomycin was added to a final concentration of 1 µM and FCCP to 1.5 µM. The transhydrogenase assay procedure is described in Materials and Methods.



Nigericin promotes the electroneutral exchange of K^+ for protons across membranes. Thus, it will change the intravesicular pH without altering the $\Delta\psi$ (127). As shown in Fig. 27, nigericin stimulated the reduction of AcNAD regardless of the orientation of the membrane potential in the everted membrane vesicles. When the external level of KCl is high, nigericin, which couples the efflux of protons to the uptake of potassium, would be expected to stimulate the reduction of AcNAD if the reaction were influenced by ΔpH. In the experiment with potassium loaded vesicles, nigericin again stimulated the reaction by coupling the efflux of protons to the uptake of potassium even though this required the movement of potassium ions against a potassium ion gradient. Thus, these results indicate that the reduction of AcNAD by NADPH is associated with an inward transport of protons and the reaction is controlled primarily by a pH gradient in everted membrane vesicles.

Inhibition by DCCD

Previous studies have shown that the activities of the mitochondrial transhydrogenase are affected by DCCD (74,128). This compound inhibited proton pump activity without affecting transhydrogenation (50). In addition to the mitochondrial transhydrogenase, DCCD also inhibits proton-linked ATP synthase (129), ubiquinol-cytochrome c reductase from mammalian and yeast mitochondria (130,131) and cytochrome oxidase (132). In these systems DCCD inhibits proton translocation primarily rather than hydrolytic or redox reactions catalyzed by these enzymes.

Treatment of both purified and membrane-bound transhydrogenases of E. coli with DCCD inactivated the enzyme. Fig. 28 shows the kinetics of inactivation of both purified and membrane-bound transhydrogenases by

Fig. 27. Effect of ionophores on the reduction of AcNAD by NADPH catalyzed by membrane-bound transhydrogenase in the presence of a transmembrane potential positive inside (A) or positive outside (B). Experimental details are outlined in Fig. 26 except that 10 mM KCl was included in the external assay medium in B. Nigericin, valinomycin and FCCP were added to give final concentrations of 0.1 μM, 1 μM and 1.5 mM, respectively.

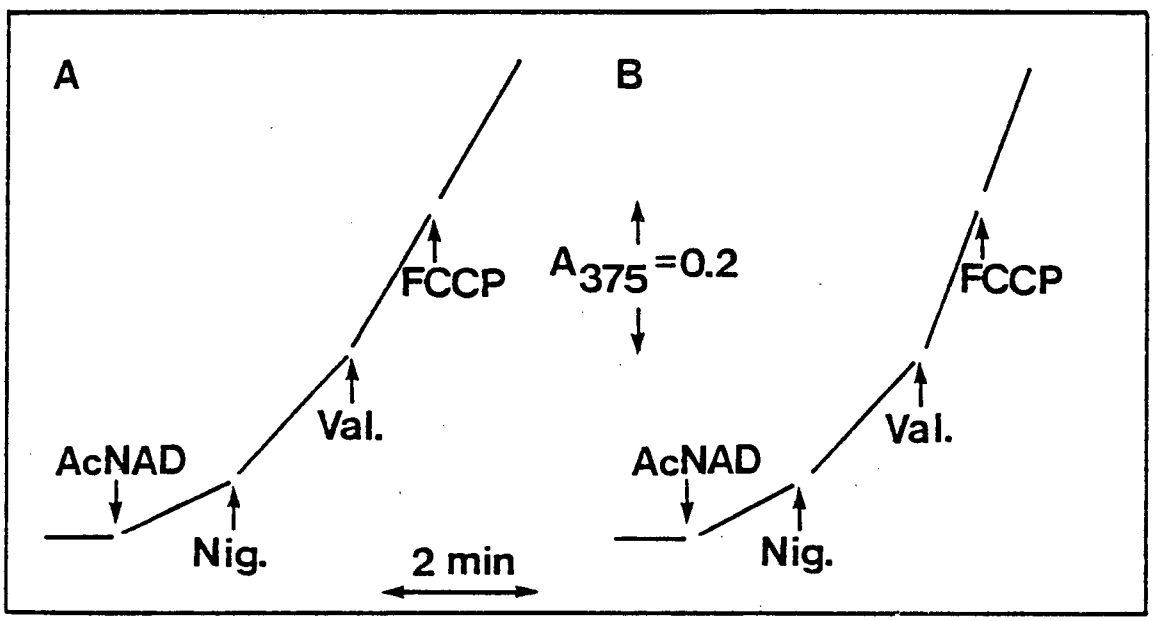
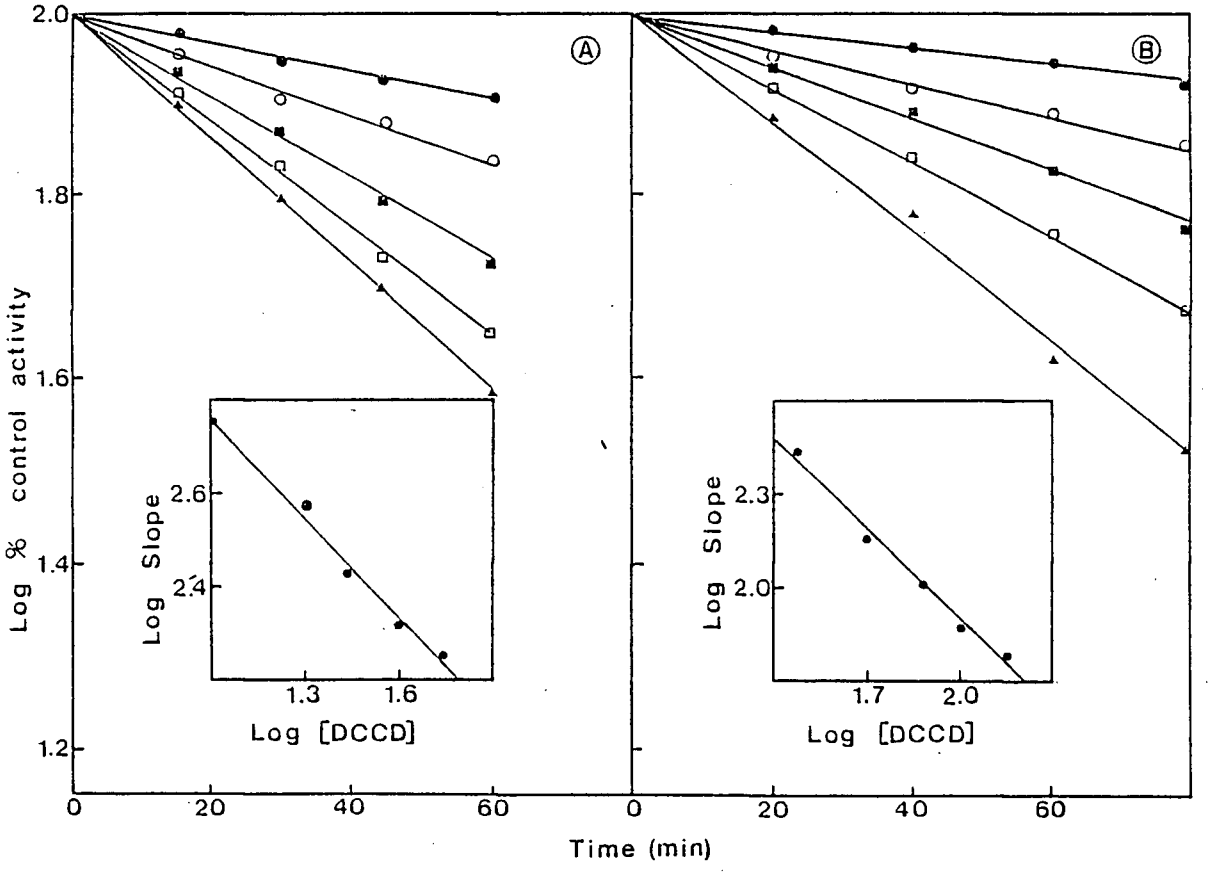


Fig. 28. Kinetics of inhibition of membrane-bound and purified transhydrogenase by DCCD. Washed membranes (380 µg) were incubated at 22°C with constant stirring in a 1 ml reaction mixture containing 40 µM Tris-HCl, pH 7.8, 0.8 mM dithiothreitol, 2% (v/v) ethanol, and 25 µM (●), 50 µM (o), 75 µM (■), 100 µM (□), or 150 µM (▲) DCCD (experiment B). Purified transhydrogenase (76 µg) was incubated at 22°C in a 1 ml reaction mixture containing 40 mM Tris-HCl, pH 7.8, 0.8 mM dithiothreitol, 2% (v/v) ethanol, 0.02% (w/v) Brij 35 detergent and 10 µM (●), 20 µM (o), 30 µM (■), 40 µM (□), or 50 µM (▲) DCCD (experiment A). Samples were removed at the times indicated and assayed for the reduction of AcNAD by NADPH. In both cases the transhydrogenase activity of the control remained constant. In the insets the log of the slope of the line obtained by plotting log control activity (%) against time is plotted against the log of the concentration of DCCD during preincubation.



DCCD. Plots of the logarithm of the activity, expressed as a percentage of the control value versus time, were linear, indicating that the modification of the enzyme was pseudo-first order. The insets show plots of the logarithm of the pseudo-first-order rate constants versus the logarithm of the corresponding concentrations of DCCD. The slopes of the lines, 1.16 for membrane-bound transhydrogenase, 1.03 for purified transhydrogenase, indicate that inhibition results from the interaction of approximately one molecule of DCCD per reactive enzyme complex. As shown in Fig. 29, both the catalytic and proton-pumping activities of transhydrogenase were inhibited at the same rate with 250 μM DCCD. The modification of transhydrogenase by DCCD exhibited some specificity as transhydrogenation was not affected after treatment with the water-soluble carbodiimide EDC (Table 11).

Since the enzyme consists of two subunits it was of interest to see whether inhibition of transhydrogenase activity by DCCD involved covalent modification by the inhibitor of only one of the subunits. Membranes from JM83 pDC21 were incubated with [^{14}C]DCCD, washed several times to remove excess reagent, and then the polypeptides separated by electrophoresis on an SDS-polyacrylamide gel. As can be seen in Fig. 30, the α subunit was preferentially modified. No labelling of the ß subunit was observed even when the gel was autoradiographed for a much longer period of time.

As shown in Table 12, NADH protected the enzyme against inhibition by DCCD, while NADP, and to a lesser extent NADPH, increased the rate of inhibition. NAD, AcNAD and AcNADH had little effect on the rate of inhibition by DCCD. Although both NADH and AcNADH can act as substrates for the enzyme, only NADH affected the rate of inhibition by DCCD. A further difference between NADH and AcNADH is shown in Fig. 31. NADH

Fig. 29. Effect of DCCD on proton translocation and catalytic activities of membrane-bound transhydrogenase. Washed membranes were prepared from E. coli strain JM83 pDC21 as described in Materials and Methods with the exception that 5 mM $MgSO_4$ was included in all steps. The membranes (1.26 mg) were incubated at 22°C with constant stirring in a 1 ml reaction mixture containing 7.5 mM Hepes/KOH (pH 7.5), 1 mM KCN, 0.15 mM dithiothreitol, 4 mM $MgCl_3$, 225 mM KCl and 2% (v/v) ethanol. Treatment was carried out with 250 µM (▲, △) or zero (■, □) DCCD and samples were removed at the times indicated to assay the reaction of AcNAD by NADPH (■, ▲) in the presence of 1 µM FCCP or to measure the quenching of fluorescence of 9-aminoacridine (□, △) during the reduction of AcNAD by NADPH. Control values at zero time were 30% quenching of 9-aminoacridine and 6 µmol of AcNAD reduced x min^{-1} x mg of $protein^{-1}$.

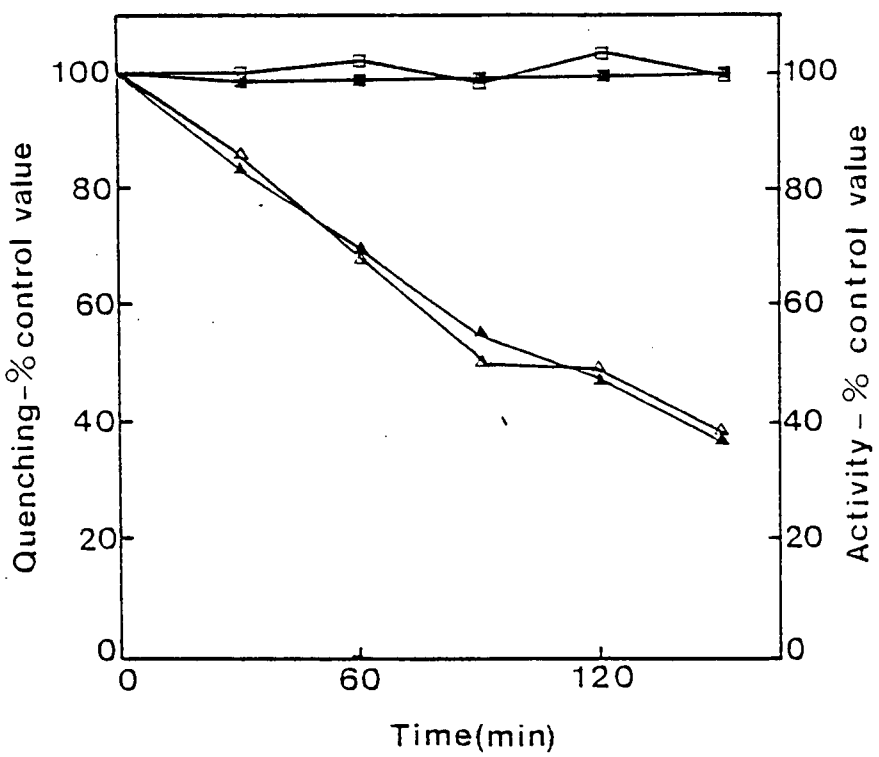


Table 11. Treatment of E. coli W6 pDC21 membrane with EDC [1-ethyl-3(3-dimethyl-amino-propyl)carbodiimide].

Concentration of EDC (μM)	Transhydrogenase Activity (U/mg of protein)
0	4.32
10	4.26
50	4.26
100	4.34
200	4.27
500	4.23
1000	4.26

Membranes (5 mg/ml of protein) prepared from E. coli W6 pDC21 were treated with various levels of EDC for 60 min. Transhydrogenase activity was measured as described in Materials and Methods.

Fig. 30. [^{14}C]DCCD labelling of membrane-bound transhydrogenase. Washed membranes from E. coli strain JM83 pDC21 (5 mg protein/ml) were incubated for 10 h at 4°C in the presence of 40 mM Tris-HCl (pH 7.8), 0.2 mM dithiothreitol and 160 µM DCCD (5 µCi). The membranes were then sedimented by centrifugation at 175,000 x g for 1 h, and resuspended in buffer without DCCD. This step was repeated until all of the free label was removed. Samples were submitted to electrophoresis, stained and fluorographed as described in Materials and Methods. Lane 1, molecular mass standards; lane 2, [^{14}C]DCCD labelled membranes stained with Coomassie blue; lane 3, fluorograph of [^{14}C]DCCD labelled membranes.

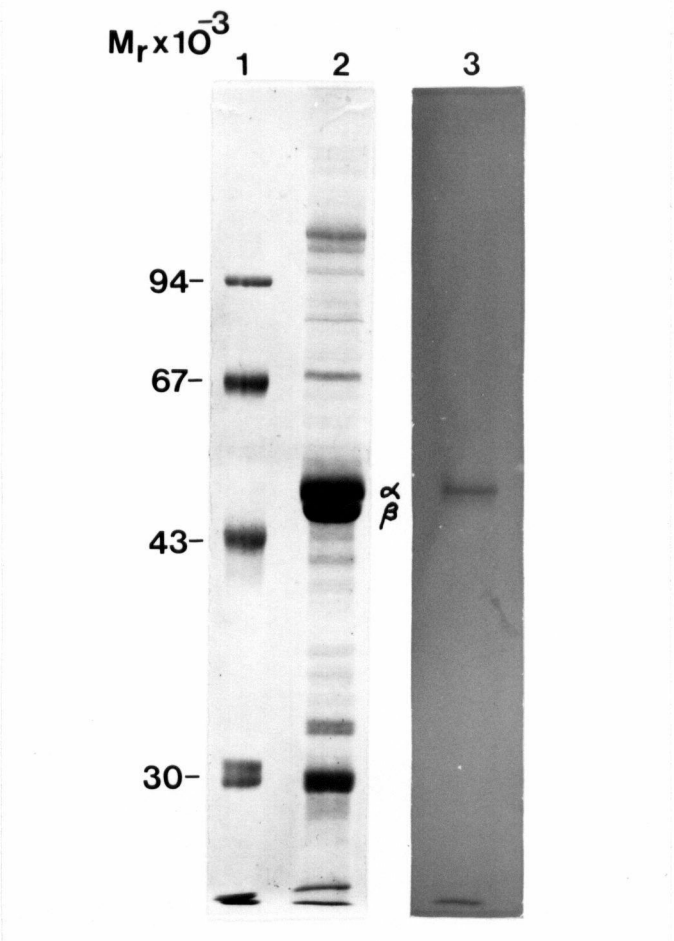
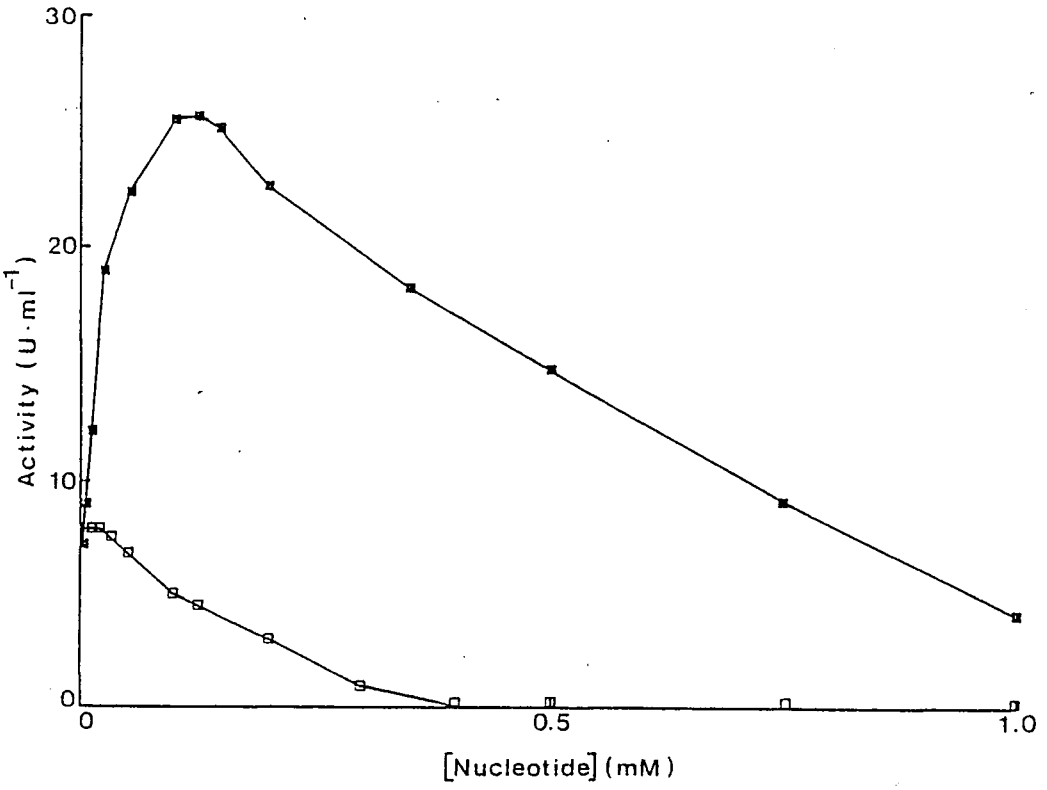


Table 12. Effect of substrates on the inhibition of transhydrogenase activity by DCCD.

Nucleotide added	$\underline{k}$
	min^{-1}
None	0.071
NADH	0.019
AcNADH	0.068
AcNAD	0.064
NAD	0.066
NADP	0.26
NADPH	0.15

Preincubation and assay conditions were the same as in Fig. 26, experiment B, except that the purified transhydrogenase was incubated in the presence of 75 μM DCCD. The nucleotides indicated were added to the preincubation mixture immediately before the addition of DCCD. All data were plotted as in Fig. 26 and inhibition rate constants were calculated from the slopes of the pseudo-first-order plots. $\underline{k}$ is the pseudo-first-order inhibition rate constant.

Fig. 31. Effect of NADH and AcNADH on the reduction of AcNAD by NADPH catalyzed by purified transhydrogenase. The reduction of AcNAD by NADPH catalyzed by purified transhydrogenase was assayed in the presence of different concentrations of NADH (■) or AcNADH (□).



stimulated the enzyme-catalyzed reduction of AcNAD by NADPH at concentrations up to 0.75 mM. Maximum stimulation occurred at 0.1-0.2 mM NADH. At concentrations higher than 0.75 mM, NADH inhibited this reaction by acting as a competitive inhibitor at the NAD site. In contrast, low concentrations of AcNADH did not affect the reaction rate, but at concentrations higher than 25 μM, inhibited the reaction.

VII. Isolation of Transhydrogenase Subunits for Amino Acid Sequence Analysis

Purification of Subunits Using Polyacrylamide Slab Gels

An important body of information that is needed to understand the mechanism and structure of transhydrogenase is the amino acid sequence of the transhydrogenase subunits. The amino acid sequence of the transhydrogenase subunits was elucidated by sequencing the DNA of the *pnt* gene (discussed later). The amino acid sequence of the amino-terminal ends of the transhydrogenase α and ß subunits was determined to aid in the identification of their respective DNA coding regions.

The α and ß subunits of transhydrogenase must be individually isolated for amino acid sequence analysis. This was achieved by SDS-polyacrylamide gel electrophoresis. Two methods of gel electrophoresis were used.

Isolation of the Transhydrogenase Subunits Using a Commercial Preparative Gel Electrophoresis System

The BRL preparative gel electrophoresis system was used to separate the transhydrogenase subunits. In this system, the proteins are separated

by electrophoresis in a tube gel and the components collected by continuous sampling from the base of the gel.

The system was used according to the manufacturer's instructions. A 6 cm 10% polyacrylamide gel with a 1 cm 4% stacking gel was poured and allowed to stand overnight at room temperature. A sample of 200 µl containing 50 to 400 µg of purified transhydrogenase in sample buffer was layered on top of the gel and electrophoresis was carried out at 150 V. Fractions were collected every 10 min at a flow rate of 10 ml/h. Sodium mercaptoacetate (0.1 mM) was included in the cathode buffer reservoir to minimize the destruction of tryptophan, histidine and methionine side chains by free radicals or oxidants trapped in the gel matrix (133). The mercaptoacetate travels at the dye front during electrophoresis and scavenges the destructive species in the gel before the proteins reach them. All of the buffers and the apparatus were thoroughly de-gassed to prevent accumulation of bubbles under the elution frit which would cause a poor separation. A portion of each fraction was lyophilized, suspended in SDS sample buffer and applied to a polyacrylamide slab gel. As shown in Fig. 32, some separation of the transhydrogenase subunits was obtained but there was still some overlap. The separation of the subunits was much worse when larger amounts of protein were applied to the system (Fig. 33). Therefore only small amounts of the transhydrogenase subunits (<10 µg of each subunit) could be recovered in a purified form using the apparatus. Attempts were made to isolate larger amounts of each subunit by pooling the partially purified subunits and re-running them through the electrophoretic system. Again the yields of recovered purified subunits were low and degradation was observed. Therefore it was concluded that the system could not be used to resolve the two subunits in quantities sufficient for sequencing.

Fig. 32. SDS-polyacrylamide gel electrophoresis of samples from fractions obtained during the separation of transhydrogenase subunits using the preparative gel electrophoresis system (BRL). 160 µg of purified transhydrogenase was applied to a 6 cm 10% polyacrylamide gel with a 5% stacking gel and electrophoresis was carried out at 150 V as described in Materials and Methods. 200 µl of each 1.6 ml fraction was lyophilized and the residue suspended in sample buffer. The lyophilized portions of fractions 30-45 were applied to a 10% polyacrylamide gel (lanes 2-17, respectively) along with intact transhydrogenase (lane 1).

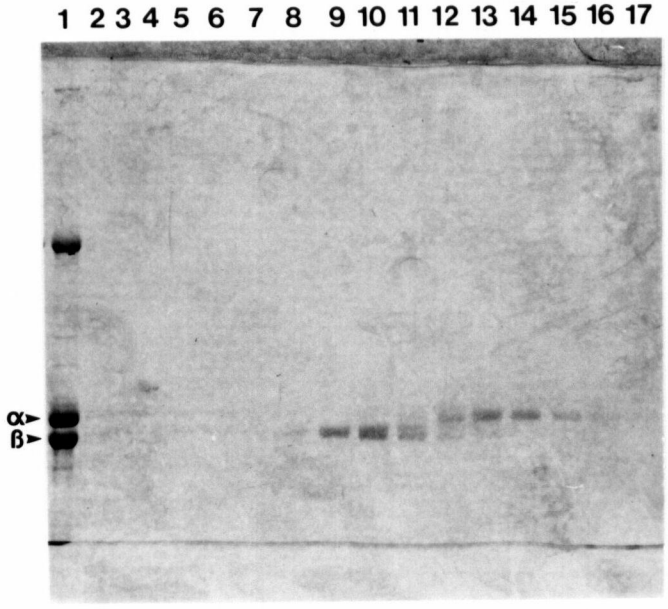
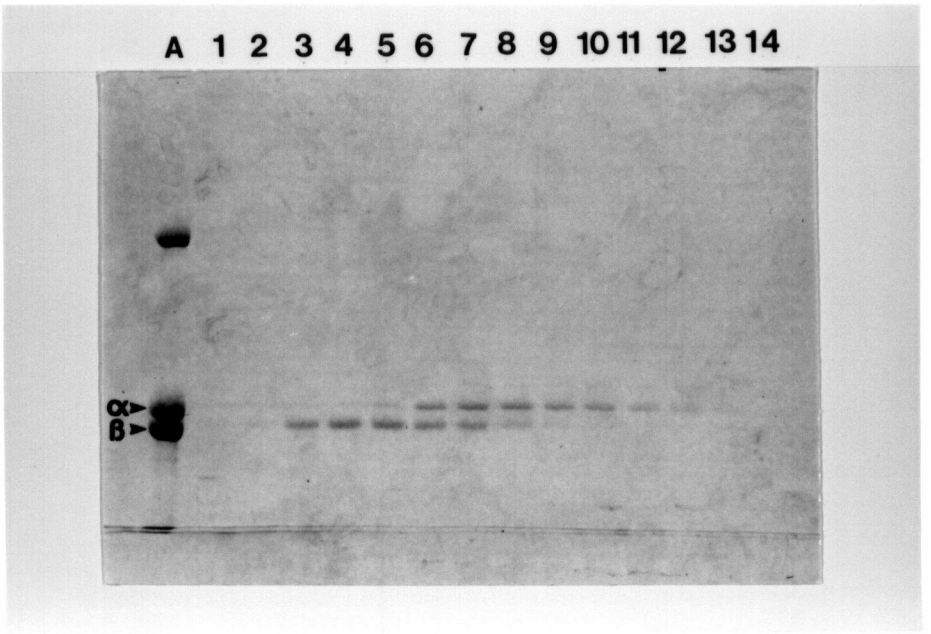


Fig. 33. SDS-polyacrylamide gel electrophoresis of samples of fractions obtained during the separation of transhydrogenase subunits using the preparative gel electrophoresis system (BRL). The conditions are the same as outlined in Fig. 32 except that 400 μg of purified transhydrogenase was applied to the gel. Lanes 1-18 contain samples from fractions 30-49.

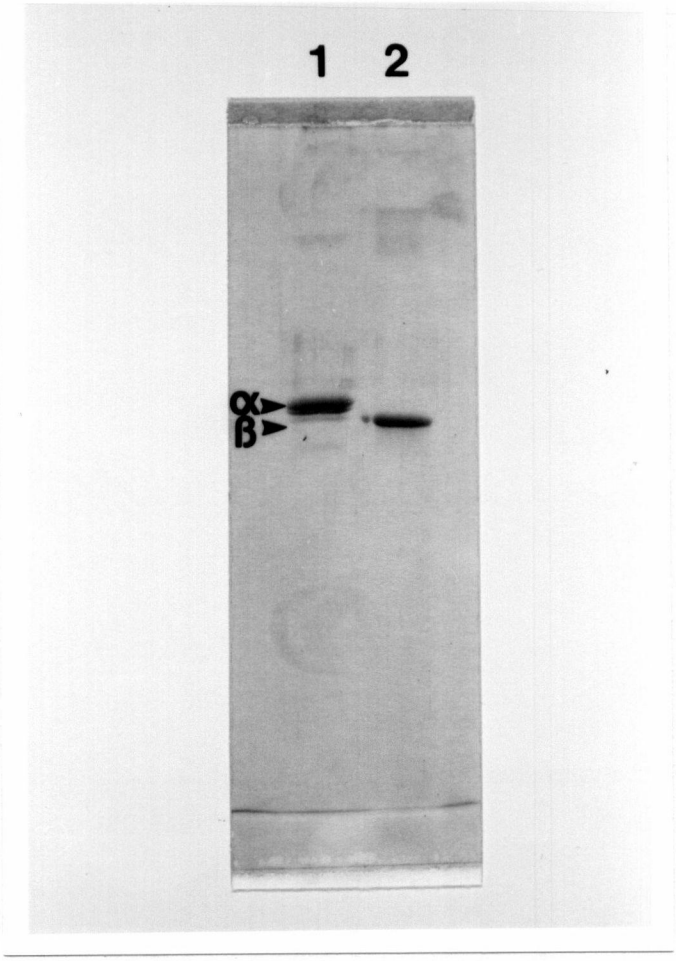


Isolation of Transhydrogenase Subunits by Excision from Polyacrylamide Gels

A better method of separating the two subunits was the excision of the protein bands from the gel. The two transhydrogenase subunits had first to be located in the gel. The protein bands were too closely spaced to use stained strips of gel as a guide. Furthermore the use of Coomassie Brilliant Blue to visualize the protein is time-consuming and some proteins, particularly those containing Asp-Pro bands, can be partially fragmented by brief exposure to low pH. The protein bands can be visualized without staining using the formation of protein-SDS complexes with potassium (134,135) or by precipitation of non-protein-bound SDS by 4M sodium acetate (136). A strip of gel containing the protein is cut out and the protein extracted from it.

Approximately 450 μg of purified transhydrogenase was separated on a 15 x 14 x 0.15 cm 10% polyacrylamide gel. The protein bands were visualized by treating the gel with cold 0.1 M KCl. The opaque protein bands were visible after 5 min when viewed against a black background. The strips of gel containing the proteins were cut out, diced into small pieces with a sharp razor blade and placed in a sealable tube with four volumes of 0.1% SDS/1 mM dithiothreitol. The tube containing the gel was incubated at room temperature with gentle shaking for 4 h. The gel pieces were removed by filtration through a 0.45 μM pore filter. The proteins were precipitated by adding trichloroacetic acid to a final concentration of 12% and allowing the samples to stand on ice for 1 h. The precipitate was collected by centrifugation and the protein pellet was washed three times with cold 10% trichloroacetic acid and three times with cold acetone. The samples were lyophilized for sequencing. As shown in Fig. 34 the two subunits were isolated in a highly purified form using

Fig. 34. SDS-polyacrylamide gel electrophoresis of transhydrogenase subunits purified by excision of the protein band from a gel. 450 μg of purified transhydrogenase was applied to a 10% polyacrylamide gel to purify the subunits as described in Materials and Methods. Samples representing 5% or 2.5% of the isolated α and ß subunits, respectively, were subjected to electrophoresis in a 10% polyacrylamide gel. Lane A, α subunit; lane B, ß subunit.



this technique. The α subunit did undergo some degradation but the multiple bands probably represent different oxidized forms of the enzyme.

The results of the protein sequencing of each of the transhydrogenase subunits are shown in Fig. 35. The identity of 26 of the first 28 amino acid residues was obtained for the α subunit and 8 residues were identified for the ß subunit. It was reported that the ß subunit was susceptible to the acidic conditions used to suspend the protein for sequencing. This caused the protein to fragment.

VIII. Nucleotide Sequencing of the pnt Gene

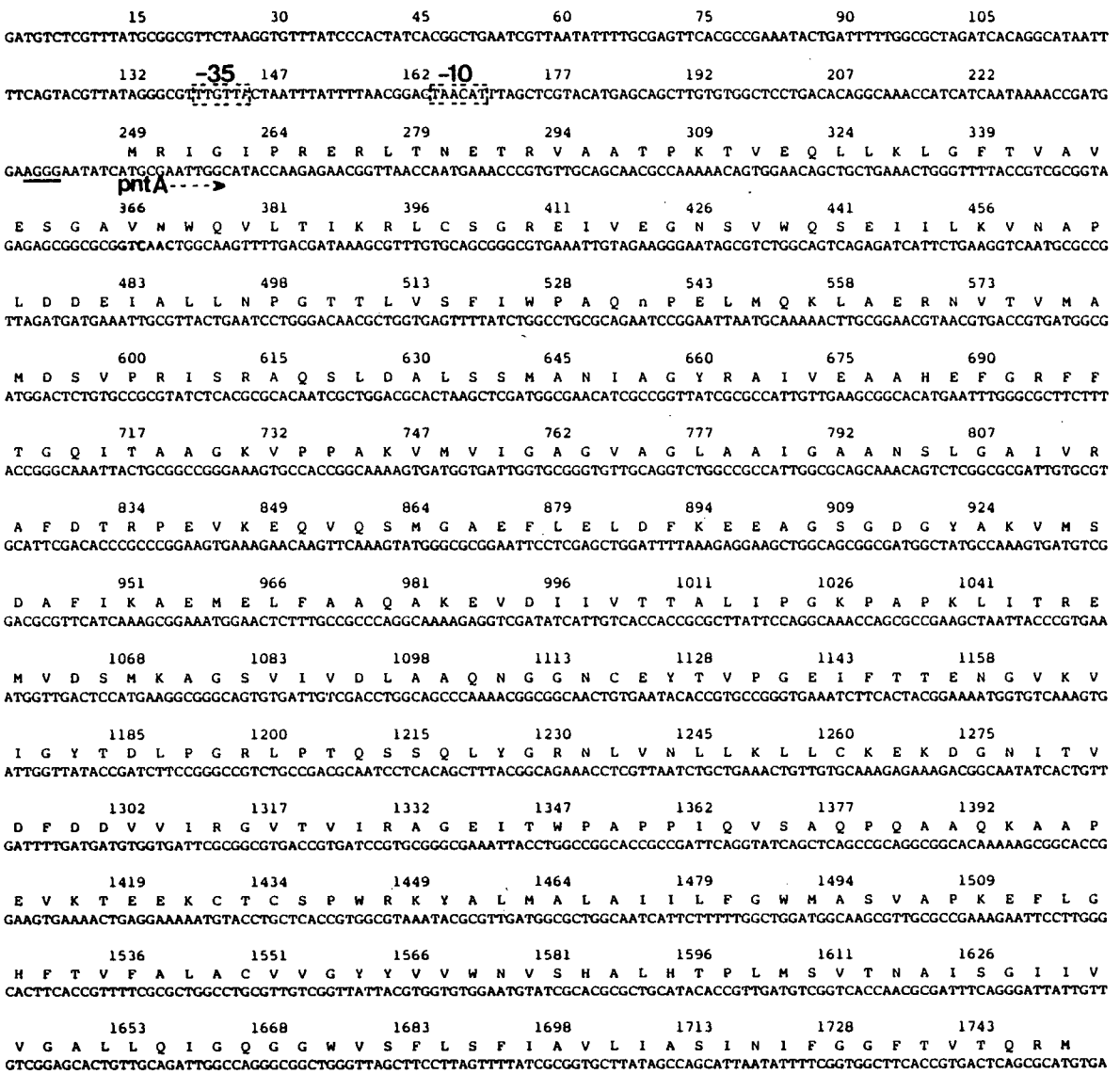
The DNA sequence described in Fig. 36 were determined by the dideoxy chain termination procedure (110) coupled with cloning into bacteriophage M13 (137). The phage M13 has been genetically engineered to contain part of the E. coli gene for ß-galactosidase and expresses this gene when grown on lac⁻ E. coli. This lac region serves as a marker system for distinguishing vector phage (blue plaque-formers) and recombinant phage (white plaque formers) on plates containing X-Gal. The phages used for sequencing the transhydrogenase gene were M13mp18 and M13mp19. These contain an insert of 57 bases with an array of restriction sites within the lac gene as shown in Fig. 37. Fragments can be inserted into this region and recombinant phages distinguished as white plaques. The plaques were grown in small cultures (2 ml), the phage isolated, and the single-stranded DNA uncoated using phenol. The fragments were then sequenced using a synthetic primer.

Two different strategies were applied at various stages of this work to sequence the transhydrogenase gene. Initially, various restriction

Subunit	Residue Number																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
α	MET	ARG	ILE	GLY	ILE	PRO	ARG	GLU	ARG	LEU	THR	ASN	GLU	THR	ARG	VAL	ALA	ALA or VAL	THR	PRO	LYS	THR	GLY	GLU	GLN	-	-	LYS
ß	MET	SER	GLY	THR	LEU	VAL	THR	ALA																				

Fig. 35. Amino acid sequence of the transhydrogenase α and ß subunits. The transhydrogenase subunits were isolated as described in Materials and Methods and sequenced on a gas-phase sequenator at the University of Victoria.

Fig. 36. Nucleotide sequence of the pnt gene region. Each gene is marked above the proposed points for initiation of translation. Proposed ribosome-binding sites are underlined. Promoter sequences are boxed and labelled -10 and -35.



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          1770          1785          1800          1815          1830          1845          1860
                                  M  S  G  G  L  V  T  A  A  Y  I  V  A  A  I  L  F  I  F  S  L  A  G  L  S  K  H  E  T
AAATGTTCCGCAAAAATTAAGGGGTAACATATGTCTGGAGGATTAGTTACAGCTGCATACATTGTTGCCGCGATCCTGTTTATCTTCAGTCTGGCCGGTCTTTCGAAACATGAAACG
                    pntB---->

          1887          1902          1917          1932          1947          1962          1977
 S  R  Q  G  N  N  F  G  I  A  G  M  A  I  A  L  I  A  T  I  F  G  P  D  T  G  N  V  G  W  I  L  L  A  M  V  I  G  G
TCTCGCCAGGGTAACAACTTCGGTATCGCCGGGATGGCGATTGCGTTAATCGCAACCATTTTTGGACCGGATACGGGTAATGTTGGCTGGATCTTGCTGGCGATGGTCATTGGTGGG

          2004          2019          2034          2049          2064          2079          2094
 A  I  G  I  R  L  A  K  K  V  E  M  T  E  M  P  E  L  V  A  I  L  H  S  F  V  G  L  A  A  V  L  V  G  F  N  S  Y  L
GCAATTGGTATCCGTCTGGCGAAGAAAGTTGAAATGACCGAAATGCCAGAACTGGTGGCGATCCTGCATAGCTTCGTGGGTCTGGCGGCAGTGCTGGTTGGCTTTAACAGCTATCTG

          2121          2136          2151          2166          2181          2196          2211
 H  H  D  A  G  M  A  P  I  L  V  N  I  H  L  T  E  V  F  L  G  I  F  I  G  A  V  T  F  T  G  S  V  V  A  F  G  K  L
CATCATGACGCGGGAATGGCACCGATTCTGGTCAATATTCACCTGACGGAAGTGTTCCTCGGTATCTTCATCGGGGCGGTAACGTTCACGGGTTCGGTGGTGGCGTTCGGCAAACTG

          2238          2253          2268          2283          2298          2313          2328
 C  G  K  I  S  S  K  P  L  M  L  P  N  R  H  K  M  N  L  A  A  L  V  V  S  F  L  L  L  I  V  F  V  R  T  D  S  V  G
TGTGGCAAGATTTCGTCTAAACCATTGATGCTGCCAAACCGTCACAAAATGAACCTGGCGGCTCTGGTCGTTTCCTTCCTGCTGCTGATTGTATTTGTTCGCACGGACAGCGTCGGC

          2355          2370          2385          2400          2415          2430          2445
 L  Q  V  L  A  L  L  I  M  T  A  I  A  L  V  F  G  W  H  L  V  A  S  I  G  G  A  D  M  P  V  V  V  S  M  A  E  L  V
CTGCAAGTGCTGGCATTGCTGATAATGACCGCAATTGCGCTGGTATTCGGCTGGCATTTAGTCGCCTCCATCGGTGGTGCAGATATGCCAGTGGTGGTGTCGATGGCTGAACTCGTA

          2472          2487          2502          2517          2532          2547          2562
 L  R  L  G  G  C  G  C  G  L  Y  A  Q  Q  R  P  V  I  V  T  G  A  L  V  G  S  S  G  A  I  L  S  Y  I  M  C  K  A  M
CTCCGGCTGGGCGGCTGCGGCTGCGGGCTTTATGCTCAGCAACGACCTGTGATTGTGACCGGTGCGCTGGTCGGTTCTTCGGGGGCTATCCTTTCTTACATTATGTGTAAGGCGATG

          2589          2604          2619          2634          2649          2664          2679
 N  R  S  F  I  S  V  I  A  G  G  F  G  T  D  G  S  S  T  G  D  D  Q  E  V  G  E  H  R  E  I  T  A  E  E  T  A  E  L
AACCGTTCCTTTATCAGCGTTATTGCGGGTGGTTTCGGCACCGACGGCTCTTCTACTGGCGATGATCAGGAAGTGGGTGAGCACCGCGAAATCACCGCAGAAGAGACAGCGGAACTG

          2706          2721          2736          2751          2766          2781          2796
 L  K  N  S  H  S  V  I  I  T  P  G  Y  G  M  A  V  A  Q  A  Q  Y  P  V  A  E  I  T  E  K  L  R  A  R  G  I  N  V  R
CTGAAAAACTCCCATTCAGTGATCATTACTCCGGGGTACGGCATGGCAGTCGCGCAGGCGCAATATCCTGTCGCTGAAATTACTGAGAAATTGCGCGCTCGTGGTATTAATGTGCGT

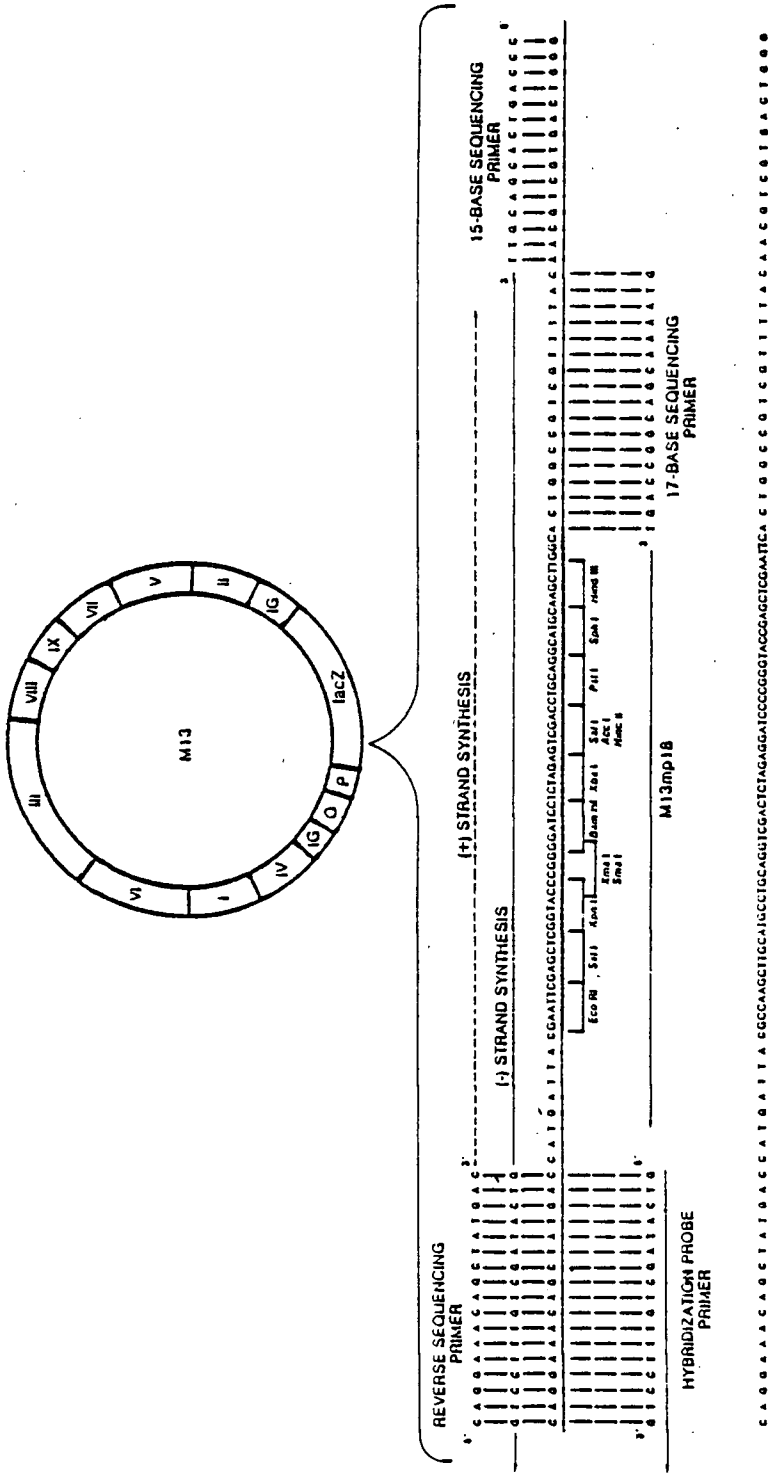
          2823          2838          2853          2868          2883          2898          2913
 F  G  I  H  P  V  A  G  R  L  P  G  H  M  N  V  L  L  A  E  A  K  V  P  Y  D  I  V  L  E  M  D  E  I  N  D  D  F  A
TTCGGTATCCACCCGGTCGCGGGGCGTTTGCCTGGACATATGAACGTATTGCTGGCTGAAGCAAAAGTACCGTATGACATCGTGCTGGAAATGGACGAGATCAATGATGACTTTGCT

          2940          2955          2970          2985          3000          3015          3030
 D  T  D  T  V  L  V  I  G  A  N  D  T  V  N  P  A  A  Q  D  D  P  K  S  P  I  A  G  M  P  V  L  E  V  W  K  A  Q  N
GATACCGATACCGTACTGGTGATTGGTGCTAACGATACGGTTAACCCGGCGGCGCAGGATGATCCGAAGAGTCCGATTGCTGGTATGCCTGTGCTGGAAGTGTGGAAAGCGCAGAAC

          3057          3072          3087          3102          3117          3132          3147
 V  I  V  F  K  R  S  M  N  T  G  Y  A  G  V  Q  N  P  L  F  F  K  E  N  T  H  M  L  F  G  D  A  K  A  S  V  D  A  I
GTGATTGTCTTTAAACGTTCGATGAACACTGGCTATGCTGGTGTGCAAAACCCGCTGTTCTTCAAGGAAAACACCCACATGCTGTTTGGTGACGCCAAAGCCAGCGTGGATGCAATC

     3169      3179      3189      3199      3209      3219      3229      3239
 L  K  A  L
CTGAAAGCTCTGTAACCCTCGACTCTGCTGAGGCCGTCACTCTTTATTGAGATCGCTTAACAGAACGGCGATGCGACTCTA
```

Fig. 37. Structure of the M13mp18 and M13mp19 cloning regions.



fragments (mostly EcoRI/HincII fragments) of pDC21 were cloned into M13 for sequencing. However, it proved difficult to isolate sufficient clones to cover the whole gene. Therefore, the majority of the clones for sequencing were generated using exonuclease BAL31. Three sets of fragments were generated using BAL31. The first set of fragments were generated by cleaving the unique SmaI site of pDC21, treating the linearized plasmid DNA with BAL31 for various lengths of time and then releasing the shortened pnt fragment with PstI and HindIII. The BAL31 fragments were cloned into PstI/SmaI cleaved M13mp19. The second set of fragments were generated by cleaving the unique PstI site of pDC21, treating the linearized plasmid with BAL31 for various lengths of time and then releasing the shortened fragments by cleavage with BamHI. The fragments were cloned into the BamHI/SmaI site of M13mp19. Clones containing fragments of the opposite strand of pDC21 were generated by cleaving pDC21 with BstEII, treating with BAL31 for various lengths of time and then releasing two fragments containing opposite ends of the pnt gene by cleaving with HindIII and BamHI. These fragments were cloned into either BamHI/HincII-treated M13mp18 or HindIII/HincII-treated M13mp19. The M13 phage containing the fragments were transformed into JM103 and the sequencing protocols carried out as described in Materials and Methods. Thus, the sequence was built up in an orderly and rapid manner in both orientations, as summarized in Fig. 38.

The predicted amino acid sequences for the α and ß transhydrogenase subunits are shown in Fig. 36. The amino acid composition of the two subunits is shown in Table 13. The predicted molecular weight for the α and ß subunits are 53,906 and 48,667 respectively.

Fig. 38. Summary of clones used to establish the nucleotide sequence. Horizontal arrows represent extent of sequences determined and their orientations. Symbols: Hi, HindIII; Ps, PstI; H, HpaI; P, PvuII; Hc, HincII; E, EcoRI; X, XhoI; Sa, SalI; Bs, BstEII; B, BamHI; Sm, SmaI; S, SstI. Boxed lines are inserted DNA and dotted lines are pUC13 vector DNA.

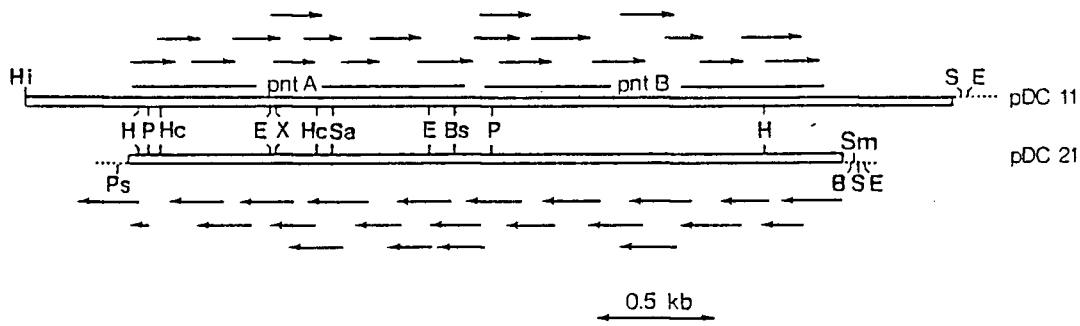


Table 13. Amino acid composition of the transhydrogenase subunits.

	No. of residues/subunit		Content (mole %)	
	α	ß	α	ß
GLY	41	48	8.17	10.39
ALA	59	55	11.75	11.90
LEU	42	47	8.37	10.17
ILE	38	38	7.57	8.22
VAL	51	48	10.16	10.39
PRO	24	17	4.78	3.68
GLU	31	19	6.18	4.11
GLN	20	10	3.98	2.16
ASP	16	18	3.19	3.90
ASN	18	18	3.59	3.90
THR	30	22	5.98	4.76
SER	27	25	5.38	5.41
MET	15	19	2.99	4.11
CYS	6	4	1.20	0.87
ARG	20	13	3.98	2.81
LYS	24	17	4.78	3.68
HIS	4	12	0.80	2.60
TYR	8	8	1.59	1.73
PHE	20	21	3.98	4.55
TRP	8	3	1.59	0.65
	502	462		

DISCUSSION

Physiological Role

Mutants have been isolated which lack transhydrogenase activity (82,84). One of the mutants, RH-5, had normal growth rates when grown aerobically on a synthetic medium with glucose, glycerol or fructose as carbon source, when compared to the growth rates of the parental strain. The mutant also grew normally under anaerobic conditions on synthetic media with glucose or glycerol and fumarate as carbon source (Table 2). These results agree with those of Hanson (82,84). Thus, transhydrogenase is not an essential source of NADPH for the cell.

In agreement with the findings of Liang and Houghton (85) glutamate dehydrogenase and transhydrogenase activities were found to be coregulated during nitrogen limitation. Coordinate changes in the levels of transhydrogenase and glutamate dehydrogenase suggest that transhydrogenase may be a direct source of NADPH for the glutamate dehydrogenase reaction. This hypothesis was tested by inactivating transhydrogenase in a strain of E. coli dependent solely on the glutamate dehydrogenase pathway for assimilation of ammonia. The mutant did not require glutamate for growth. This rules out the hypothesis that transhydrogenase is the sole source of NADPH for ammonia assimilation by glutamate dehydrogenase in E. coli.

These results suggest that the transhydrogenase of E. coli may not function as a component in a specific pathway. Since the catabolism of E. coli is almost entirely NAD-linked, the function of the energy-linked transhydrogenase may be to channel reduction equivalents from the NADH pool to the NADPH pool under thermodynamically unfavourable conditions. A

number of other enzymes including glucose 6-phosphate dehydrogenase and NADP-specific malic enzyme also contribute to the NADPH pool. Therefore, it is not surprising that transhydrogenase mutants grow normally. It is interesting to note that mutants defective in both glucose 6-phosphate dehydrogenase and transhydrogenase grow much slower than mutants defective in only one of the enzymes (84).

Cloning and Expression of Transhydrogenase

A great deal of time and effort was devoted to the purification of transhydrogenase from E. coli strain W6 during the initial stage of this project. The transhydrogenase was purified to near homogeneity by a combination of hydrophobic, ion-exchange and affinity chromatography. Analysis of the purified transhydrogenase using SDS-PAGE showed the presence of three major protein bands of molecular weights 100,000, 52,000 and 47,000 as well as several minor protein bands. The SDS-PAGE profile closely resembled the results reported by Liang and Houghton (55) although their gels showed only one protein band in the molecular weight 50,000 region.

The purification procedure from strain W6 was not entirely satisfactory. The reproducibility of the procedure was poor. Much of the variability occurred during the affinity chromatography step. Persson et al. (50) also reported that yields and purity of mitochondrial transhydrogenase varied greatly when purified using affinity chromatography with AG-NAD resins. Another problem with the purification procedure was that the transhydrogenase preparation was contaminated with several other proteins. Therefore it was decided to amplify the levels of transhydrogenase in the cells by cloning the pnt gene onto a multi-copy plasmid.

Based on the rationale that E. coli cells harbouring plasmids containing the pnt gene would contain elevated levels of enzyme, three clones were isolated from the Clarke and Carbon colony bank which contained the transhydrogenase gene. That the pnt gene had been cloned was shown by the following.

(i) An 8.7-kilobase fragment common to all three plasmids was included in the 35.5-min region of the E. coli genome previously mapped as the locus for the pnt gene (Fig. 8).

(ii) Transhydrogenase activity was repressed when the plasmid-bearing cells were grown in a rich medium (Table 6).

(iii) Analysis of membranes of the plasmid-bearing strains showed the amplified expression of the two polypeptides of molecular weights 52,000 and 48,000 (Fig. 11) which were observed in the partially purified preparation of the transhydrogenase of E. coli strain W6 (Fig. 5). Similar polypeptides were formed during in vitro transcription/translation of pDC11 DNA (Fig. 12).

(iv) Transhydrogenase activity was restored to a transhydrogenase-defective mutant when transformed with plasmid pDC21 (Table 7).

Subcloning of the pnt gene into pUC13 resulted in up to 70-fold amplification of transhydrogenase activity. Initial attempts to identify the protein products by SDS-PAGE failed because heating at 100°C in the presence of SDS gel electrophoresis sample buffer caused aggregation of the proteins so that they did not enter the gel (Fig. 11). Liang and Houghton demonstrated that the ratio of 100K to 50K protein could be altered upon alkylation. Therefore, the 100K protein probably represents an unusually stable dimer of one or both of the lower molecular weight

components. The rodA gene product (138), lactose permease (139), and glycerol phosphate permease (140) are other proteins which aggregate when boiled in SDS. All four proteins which behave in this manner are cytoplasmic membrane proteins.

Both polypeptides are needed for the expression of transhydrogenase activity. Deletion of a 1.6-kilobase HpaI-HindIII fragment from pDC4 to give pDC9 resulted in the loss of the 48,000-molecular weight polypeptide from the membranes of plasmid-bearing cells (Fig. 12). Deletion of the 0.55-kilobase HpaI-HindIII fragment from the 4.8-kilobase HindIII insert of pDC3 to give pDC8 resulted in the loss of both polypeptides. The 52,000-molecular weight polypeptide was lost when a 0.45-kilobase SalI-BstEII fragment was deleted from pDC11 (Fig. 12). No transhydrogenase activity was associated with any of these plasmids. These results suggest that all or part of the promoter region is found in the 0.60-kilobase HpaI-HindIII region followed by the regions coding for the 52,000 and 48,000-molecular weight polypeptides, respectively. Compatible plasmids which would express either the 50,000 or 48,000-molecular-weight polypeptides were constructed and transformed together into E. coli AB1450 (pnt::Tn5) lacking transhydrogenase activity. In such cells, active transhydrogenase was formed indicating that the transhydrogenase of E. coli is composed of two different subunits of molecular weights 52,000 and 48,000 (Fig. 15). The two subunits of molecular weights 52,000 and 48,000 were designated as the α and ß subunits respectively of transhydrogenase.

Expression of the pnt gene of the multicopy plasmid pDC21 resulted in greater than 70-fold overproduction of transhydrogenase in cells harbouring the plasmids. The α and ß subunits were the two most

abundant polypeptides in the cytoplasmic membranes of these cells. Biosynthesis of transhydrogenase to amounts greater than those of wild-type strains had effects on morphology, growth rate, growth yield and cell division of these cells (Table 8, Fig. 16). Deletion of portions of the genes coding for the α or ß subunits so that only one intact subunit was synthesized in the cells in large amounts also caused the abnormal physiological and morphological effects (Table 8). Therefore, the changes are not the result of a pyridine nucleotide imbalance which may be produced by excess levels of transhydrogenase. The physiological and morphological phenomena observed in the E. coli strains, which synthesize high levels of transhydrogenase, are thus to be regarded as a sole consequence of the abnormally high amount of protein being inserted in the membrane. Tubular-like structures were observed in the cells containing excess levels of the transhydrogenase subunits (Fig. 17). Lemire et al. (141) found unusual tubular vesicles in their membrane preparations of an E. coli strain overproducing the membrane-bound fumarate reductase. The same group did a thorough study on the morphological changes caused by overproduction of fumarate reductase under growth conditions where the enzyme accounted for more than 50% of the inner-membrane protein (142). They found that the membrane accommodated this excess fumarate reductase without reducing the levels of other membrane-associated enzymes. At the same time, the amount of membrane lipid increased such that the lipid/protein ratio remained constant, indicating that the total amount of membrane had doubled. The excess membrane was localized in tubular structures which branched from the cytoplasmic membrane and were composed of an aggregate of fumarate reductase and lipid. The tubules only appeared after the cytoplasmic membrane became highly enriched in fumarate

reductase. Changes in lipid composition were also observed. The major change in phospholipid composition upon amplification of fumarate reductase was the disappearance of the acyl phosphatidylglycerol and the appearance of cardiolipin. Similar tubular-like structures could be observed in some of the cells containing excess transhydrogenase. However, no transhydrogenase activity was detected in the supernatant fraction after centrifugation at 50,000 x g for 90 min of envelopes prepared by French press lysis. Under these conditions, Weiner et al. (141) reported that small fragments of the fumarate reductase-enriched tubules remained in the supernatant fraction.

Abnormal physiological and morphological effects were also reported by von Meyenburg et al. (143) on overproduction of membrane-bound ATP synthase in E. coli. They observed that 10- to 12-fold overproduction of ATP synthase resulted in pronounced inhibition of cell division and growth and in formation of membrane cistern(s) and vesicles within the cells. Inclusion bodies, probably representing deposits of excess ATP synthase, were also observed in these cells.

Purification and Characterization of Transhydrogenase

Cloning of the pnt gene to form the multicopy plasmid pDC21 resulted in greater than 70-fold overproduction of transhydrogenase in cells harbouring the plasmid. These cells served as excellent starting material for the purification of transhydrogenase as the α and ß subunits were the two major proteins in the cytoplasmic membrane (Fig. 11). The transhydrogenase was purified from the amplified membranes by a simple procedure employing differential solubilization of proteins by detergents followed by centrifugation through a 1.1 M sucrose solution (Fig. 18).

The presence of the two subunits (α and ß) in the purified enzyme confirmed that both gene products of the pnt gene are needed for a functional transhydrogenase. The two subunits are present in equimolar amounts. In solubilized preparations these two subunits irreversibly aggregate over a period of time to form a species of molecular weight 95,000-100,000. The extent of aggregation is increased when the subunits are solubilized in the absence of disulphide-reducing agents.

The structure of the E. coli transhydrogenase differs markedly from the well-studied transhydrogenase of the bovine heart mitochondrion and of R. rubrum. The bovine heart mitochondrial transhydrogenase has been purified to homogeneity and consists of a single polypeptide chain of molecular weight 97,000-120,000 (47-50). In contrast, the transhydrogenase of R. rubrum consists of a soluble peripheral protein factor having a molecular weight of 70,000 and an integral membrane-bound component of unknown molecular weight (59,60). Neither component alone exhibits transhydrogenase activity. The E. coli transhydrogenase differs from the R. rubrum enzyme in that both subunits are tightly bound to the cytoplasmic membrane and are not released even in the presence of M 6 urea. The two components can be released only by detergents such as deoxycholate in the presence of high concentrations of salts.

Reconstitution of purified transhydrogenase into egg phosphatidylcholine veiscles resulted in a 70-80% decrease in enzymatic activity. Addition of the uncoupler, FCCP, enhanced enzymatic activity. Similar results were obtained with membrane-bound transhydrogenase, catalyzing the reaction in either direction (Fig. 23). These data suggest that the inhibition of transhydrogenase in both directions derives from a rapid estalishment of a pH gradient across the membrane. Translocation of

protons can also be measured directly using pH-sensitive fluorescent probes such as 9-aminoacridine (124-126). During the reduction of AcNAD by NADPH by transhydrogenase reconstituted into vesicles, 9-aminoacridine fluorescence was substantially quenched, indicating that protons were taken into the vesicles (Fig. 24). Additional support for a proton-translocating function of transhydrogenase was provided by the ATP-dependent stimulation of the reduction of NADP by NADH, catalyzed by membrane vesicles containing both proton-translocating ATPase and transhydrogenase.

The available information thus seems to favour a chemiosmotic type of coupling mechanisms for energy-linked transhydrogenation. However, caution is warranted in interpreting the present data with regard to a coupling mechanism. Transmembrane proton translocation catalyzed by transhydrogenase remains to be shown directly, although the fluorescent probe used here clearly indicates an NAD plus NADPH-dependent acidification of the vesicles. Fluorescent probes may not probe the internal pH of the vesicles exclusively, but also interact with the surface or the interior of the membrane (144). Nevertheless, 9-aminoacridine has been reported to behave as an ideal monoamine which distributes across the liposomal membrane in response to a transmembrane pH gradient (145,146).

Experiments with nigericin and valinomycin suggest a preferential regulation of the enzyme by a proton gradient rather than a membrane potential (Fig. 25-27). Persson et al. (50) reported that the mitochondrial transhydrogenase from bovine heart was preferentially regulated by a proton gradient rather than a membrane potential.

Both transhydrogenation and proton pump activity of the enzyme were inhibited by covalent modification of one active enzyme unit by one molecule of DCCD (Fig. 29). Labelling of the transhydrogenase with [^{14}C]DCCD indicated that the α subunit was preferentially modified (Fig. 30). Mitochondrial transhydrogenase is also inhibited by DCCD (74, 128). Pennington and Fisher (74) postulated that DCCD may modify the mitochondrial transhydrogenase in a putative proton binding domain outside the active site. By contrast Phelps and Hatefi (128) have suggested that DCCD reacts near the NAD(H) binding site of beef heart mitochondrial transhydrogenase. Persson et al. (50) observed an inhibition of proton pump activity without an effect on hydride transfer suggesting that proton translocation and hydride transfer are not obligatorily linked. However, my results with E. coli transhydrogenase suggest that proton translocation and catalytic activities are tightly coupled (Fig. 29). This does not imply that DCCD reacts with the proton pump. It has become evident that this reagent is not a specific inhibitor of proton pumps (147).

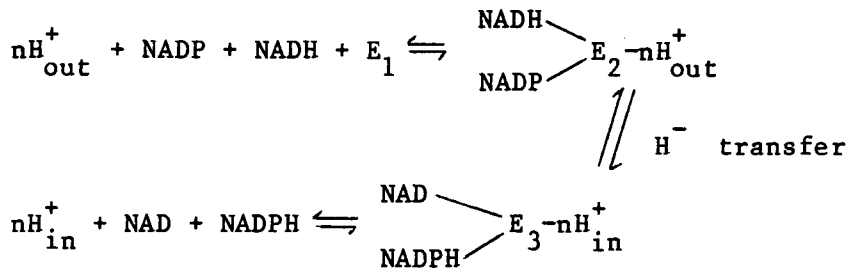
Homyk and Bragg (88) concluded that NADH may also bind to an allosteric site by studying the kinetics of modification by 2,3-butanedione of arginyl residues of the membrane-bound E. coli transhydrogenase. The present results are consistent with this conclusion (Fig. 31). Furthermore, the NADH analogue AcNADH appears not to bind to this site since it does not stimulate the enzyme-catalyzed reduction of AcNAD by NADPH or protect the enzyme from modification by DCCD (Table 12). The protection by NADH might mean that DCCD binds to the enzyme at or near the allosteric NADH-binding site that stimulates the reduction of AcNAD by NADPH. An alternative explanation is that the binding of NADH to

the enzyme induces a conformational change that makes the DCCD-binding residues less accessible. By contrast, the binding of NADP appears to induce a conformational change which causes the DCCD-binding residue to become more accessible to this reagent (Table 12).

Substrate-induced conformers of transhydrogenase are likely to play an important role in the function of the enzyme. Further evidence that binding of low concentrations of substrates cause a conformational change is seen from the inactivation of the enzyme by trypsin (Table 10). NADPH increased the degree of inactivation by trypsin whereas NADP, NADH and NAD did not have any significant effect on proteolysis (Fig. 20). It is interesting to note that similar results were obtained for the rat liver mitochondrial transhydrogenase (87). These results suggest that the NADP-enzyme complex has a different conformation from the NADPH-enzyme complex. This hypothesis is supported by the finding that sulfhydryl group modification by N-ethyl maleimide with either the mitochondrial enzyme or the enzyme from E. coli was enhanced by NADPH, whereas NADP afforded protection against modification (46,86).

A working hypothesis for a mechanism of the E. coli transhydrogenase can be based on the findings that transhydrogenase acts as a proton pump and can exist in three different conformations as shown in Fig. 39. The resting conformation (E_1) is transformed to E_2 by binding NADP. The conformation of the enzyme changes to E_3 upon hydride ion transfer. At the same time a proton is translocated from the outer surface of the membrane to the inner surface. The direction of transhydrogenation would be determined by the ratio of substrates and products, and also by the pH gradient.

Fig. 39. Proposed mechanism of transhydrogenase in intact E. coli cells. E_1, E_2 and E_3 are different conformations of the enzyme. Details of the model are found in the Discussion.



Nucleotide Sequence of the pnt Gene

In order to predict the amino acid sequence of the α and ß subunits of E. coli transhydrogenase, the nucleotide sequence of the insert of plasmid pDC21 was determined (Fig. 36). Two open reading frames of 1406 and 1386 nucleotides were found in the nucleotide sequence. The predicted amino acid sequences were compared with the N-terminal amino acid sequences of the α and ß subunits (Fig. 35). It was found that the 1406 and 1386 nucleotide open reading frames corresponded to the α and ß subunits respectively. However, only 45 nucleotides remained upstream of the α subunit and 69 nucleotides downstream of the ß subunit in the insert of plasmid pDC21. Therefore, promoter and transcription termination signals for pnt gene transcription in plasmid pDC21 were likely to be found in the vector DNA as expression of transhydrogenase was very high in cells containing plasmid pDC21. The missing nucleotide sequences of the pnt gene promoter region were derived from plasmid pDC11.

Promoter sequence elements, determining the position of transcriptional initiation in E. coli, contain two regions of conserved DNA sequence located at about 10 and 35 nucleotides upstream from the transcription start site (the '-10' and '-35' sequences [148]). The 246-base pair nucleotide sequence 5' to the α ATG codon was searched for a '-10' (TATAAT) and a '-35'(TTGACA) consensus sequence (149). A promoter-like sequence was found at positions 138-143 (TTGTTA) and positions 163-168 (TAACAT)(Fig. 36). The nucleotide sequence between the termination codon of the α subunit and the initiation codon of the ß subunit is too short (30 nucleotides) to contain a promoter. Therefore, the α and ß subunits are probably transcribed together.

Four-nucleotide sequences at positions 238-241 (AGGG) and positions 1775-1778 (AGGG) show some homology to the ribosome binding site consensus sequence (AGGAGGT)(150) and are located at the correct distance from the α and ß initiation points of translation respectively (Fig. 36). No obvious termination-like structure was observed in the sequence of the inserted DNA following the coding region.

The non-random usage of codons in the pnt gene coding regions further supports the assignment of the open reading frames. The codons CTA, ATA, AGA and AGG are rarely used in E. coli (151). The codons are used sparingly in the pntA and B genes (Fig. 36). Grojean and Fiers (151) have analyzed codon usage in E. coli genes. They found that an efficient in-phase translation is facilitated by proper choice of degenerate codewords ending with a T or C promoting a codon-anticodon interaction with intermediate strength (optimal energy) over those with very strong or very weak interaction energy. Generally, efficiently expressed genes show a clear preference for a C in the third base position of codons having A and/or T and a preference for a T in the third base position of codons having C and/or G. Conversely, codon usage in weakly expressed genes such as repressor genes follows exactly the opposite rules. Codon usage in both the α and ß subunit genes does not clearly resemble codon usage in either weakly or strongly expressed genes (Table 14). The codon usage reflects a moderately efficient translation of transhydrogenase mRNA. In normal E. coli cells, transhydrogenase only represents between 0.1% to 0.5% of the cytoplasmic membrane protein. This is much more expression than weakly expressed genes such as repressor proteins but much less than efficiently expressed genes such as RNA polymerase and ribosomal proteins.

Table 14. Codon usage in the *E. coli pnt* genes.

	α	ß		α	ß		α	ß		α	ß
TTT F	9	8	TCT S	1	7	TAT Y	4	5	TGT C	2	2
TTC F	11	13	TCC S	2	4	TAC Y	4	3	TGC C	4	2
TTA L	4	3	TCA S	6	1	TAA	0	0	TGA	0	0
TTG L	6	6	TCG S	5	6	TAG	0	0	TGG W	8	3
CTT L	8	3	CCT P	2	4	CAT H	2	7	CGT R	10	7
CTC L	4	3	CCC P	0	0	CAC H	2	5	CGC R	6	4
CTA L	2	0	CCA P	5	4	CAA Q	9	4	CGA R	1	1
CTG L	18	34	CCG P	17	9	CAG Q	11	6	CGG R	1	1
ATT I	24	19	ACT T	5	4	ATT N	10	4	AGT S	5	2
ATC I	11	18	ACC T	17	9	AAC N	8	14	AGC S	8	5
ATA I	3	1	ACA T	3	2	AAA K	20	12	AGA R	2	0
ATG M	15	19	ACG T	5	7	AAG K	4	5	AGG R	0	0
GTT V	13	9	GCT A	2	13	GAT D	9	11	GGT G	10	22
GTC V	11	10	GCC A	11	6	GAC D	7	7	GGC G	22	14
GTA V	4	7	GCA A	18	12	GAA E	24	15	GGA G	1	5
GTG V	23	22	GCG A	28	22	GAG E	7	4	GGG G	8	7

The amino acid sequences of the transhydrogenase α and ß subunits were predicted from the nucleotide sequences. Both subunits are rather hydrophobic. Analysis of the amino acid sequence of both subunits for local hydropathy and predicted secondary structure suggests that presence of at least five transmembrane segments in the α subunit and seven transmembrane segments in the ß subunit (Fig. 40,41). The reliability of such predictions are unknown because of an absence of a database of membrane proteins of known structure.

The α subunit of E. coli transhydrogenase reacts quite specifically and covalently with DCCD. Other protein-translocating enzymes react with DCCD and the site of interaction with DCCD has been identified in some cases (152). They exhibit striking similarity: in all cases DCCD reacts with a carboxyl residue located in an otherwise hydrophobic and highly conserved region of these polypeptides. This residue may be important to proton-translocation. However, no homologous sequence was found in the amino acid sequence of either subunit of E. coli transhydrogenase. This observation supports the supposition that DCCD is not a specific inhibitor of proton pumps (147).

Fig. 40. Hydropathy plot of the transhydrogenase α subunit based on the procedure of Kyte and Doolittle (153). The positions of basic residues (▲), acidic residues (Δ) and regions of predicted alpha helix (-) and beta sheet (ıtıtıtı) are indicated. Possible transmembrane segments are shown (I-V). Secondary structure was determined by the method of Garnier et al. (154).

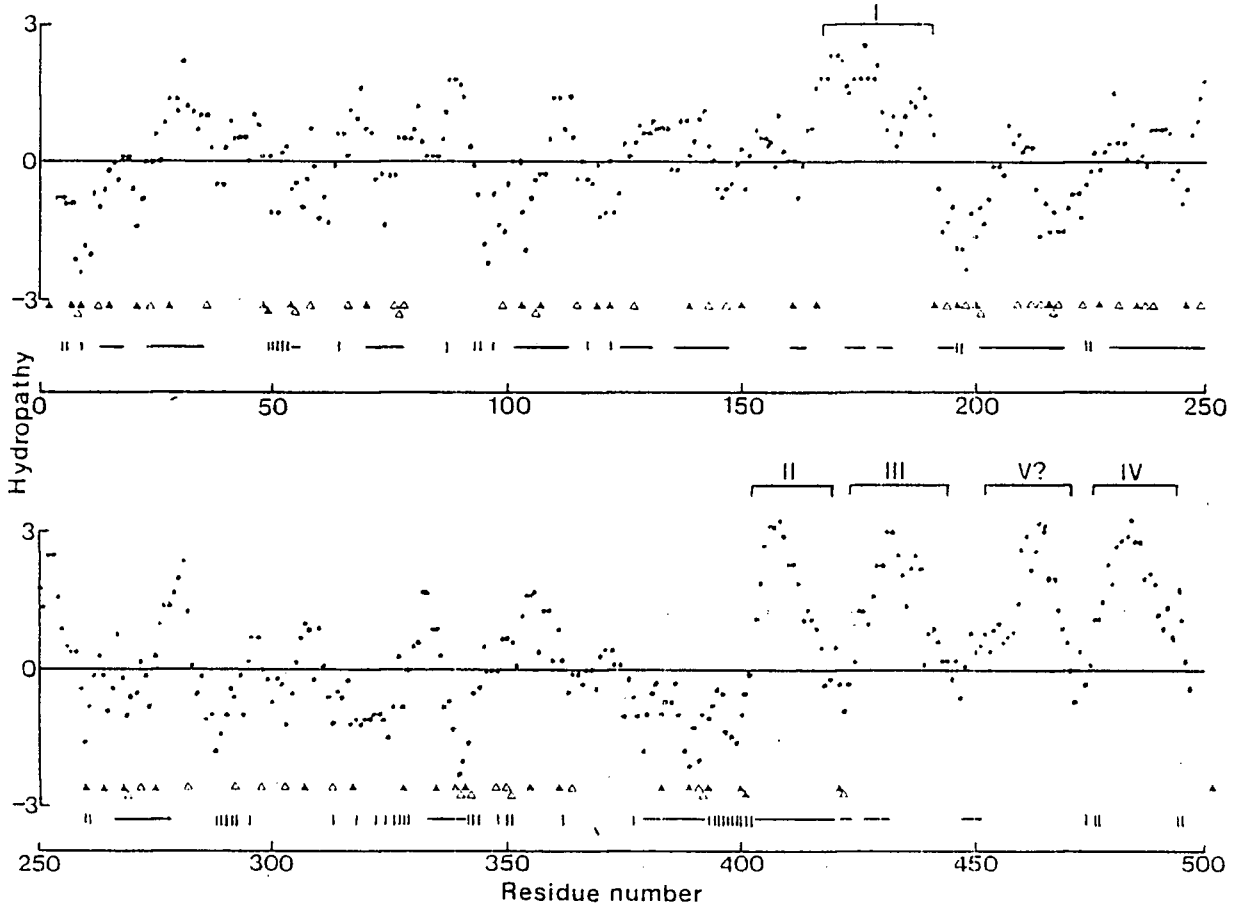
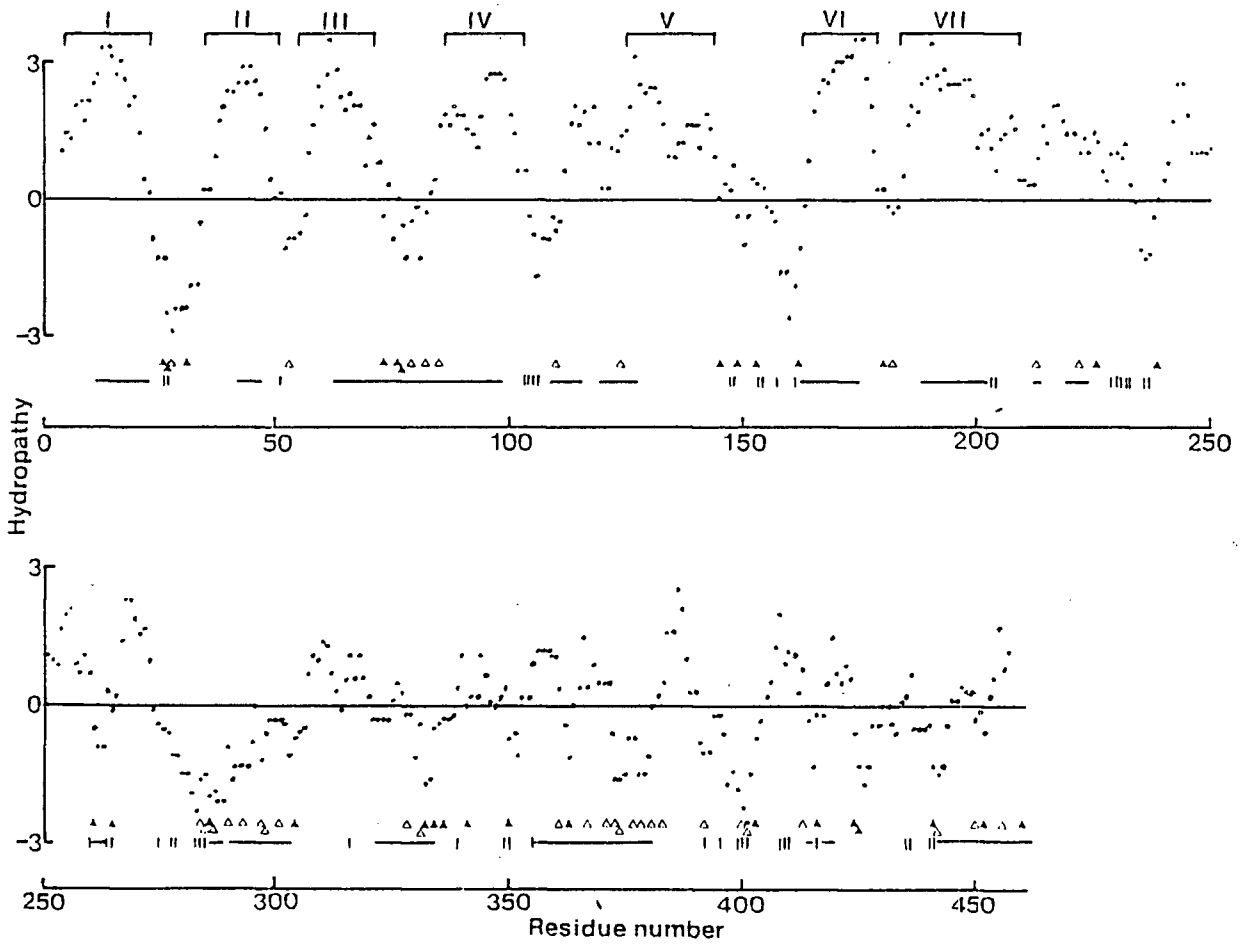


Fig. 41. Hydropathy plot of the transhydrogenase ß subunit based on the procedure of Kyte and Doolittle (153). The positions of basic residues (▲), acidic residues (Δ) and regions of predicted alpha helix (-) and beta sheet (ıı) are indicated. Possible transmembrane segments are shown (I-VII). Secondary structure was determined by the method of Garnier et al. (154).



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