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ARTICLE *in* BIOCHEMISTRY · JULY 1994

Impact Factor: 3.02 · Source: PubMed

Prediction and Site-Specific Mutagenesis of Residues in Transmembrane α-Helices of Proton-Pumping Nicotinamide Nucleotide Transhydrogenases from *Escherichia coli* and Bovine Heart Mitochondria†

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Received November 8, 1993; Revised Manuscript Received March 23, 1994®

ABSTRACT: Nicotinamide nucleotide transhydrogenase from bovine heart consists of a single polypeptide of 109 kD. The complete gene for this transhydrogenase was constructed, and the protein primary structure was determined from the cDNA. As compared to the previously published sequences of partially overlapping clones, three residues differed: Ala591 (previously Phe), Val777 (previously Glu), and Ala782 (previously Arg). The *Escherichia coli* transhydrogenase consists of an α subunit of 52 kD and a β subunit of 48 kD. Alignment of the protein primary structure of the bovine trashydrogenase with that of the transhydrogenase from *E. coli* showed an identity of 52%, indicating similarly folded structures. Prediction of transmembrane-spanning α-helices, obtained by applying several prediction algorithms to the primary structures of the revised bovine heart and *E. coli* transhydrogenases, yielded a model containing 10 transmembrane α-helices in both transhydrogenases. In *E. coli* transhydrogenase, four predicted α-helices were located in the α subunit and six α-helices were located in the β subunit. Various conserved amino acid residues of the *E. coli* transhydrogenase located in or close to predicted transmembrane α-helixes were replaced by site-specific mutagenesis. Conserved negatively charged residues in predicted transmembrane α-helices possibly participating in proton translocation were identified as βGlu82 (Asp655 in the bovine enzyme) and βAsp213 (asp787 in the bovine enzyme) located close to the predicted α-helices 7 and 9 of the β subunit. βGlu82 was replaced by Lys or Gln and βAsp213 by Asn or His. However, the catalytic as well as the proton pumping activity was retained. In constrast, mutagenesis of the conserved βHis91 residue (His664 in the bovine enzyme) to Ser, Thr, and Cys gave an essentially inactive enzyme. Mutation of αHis450 (corresponding to His481 in the bovine enzyme) to Thr greatly lowered catalytic activity without abolishing proton pumping. Since no other conserved acidic or basic residues were predicted in transmembrane α-helices regardless of the prediction algorithm used, proton translocation by transhydrogenase was concluded to involve a basic rather than an acidic residue. The only conserved cysteine residue, βCys260 (Cys834 in the bovine enzyme), located in the predicted α-helix 10 of the *E. coli* transhydrogenase, previously suggested to function as a redox-active dithiol, proved not to be essential, suggesting that redox-active dithiols do not play a role in the mechanism of transhydrogenase.

Nicotinamide nucleotide transhydrogenase (TH[1]) (EC 1.6.1.1) is a proton pump which catalyzes the reversible reduction of $NADP^+$ by NADH according to the reaction

$$n{H^+}_{out} + NADP^+ + NADH \rightleftharpoons n{H^+}_{in} + NAD^+ + NADPH$$

where n denotes the number of protons pumped from the cytosol or periplasmic space (out) to the matrix or the cytosol (in) in mitochondria and certain bacteria, respectively. Depending on the source of transhydrogenase and assay system used, the value of n is 0.5–1.0. Transhydrogenase from bovine mitochrondria, *Escherichia coli*, and the photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodospirillum rubrum* have been purified and characterized extensively with regard to molecular and catalytic properties in the detergent-dispersed state or in reconstituted liposomes with or without other proton pumps [for reviews, see Rydström (1977), Fisher and Earle (1982), Rydström et al. (1987), and Jackson (1991)].

The primary structure of transhydrogenase from *E. coli* (PNTX) has been determined (Clarke & Bragg, 1985; Clarke et al., 1986). *E. coli* transhydrogenase is composed of two different subunits, α with 510 residues and β with 462 residues, which are assembled into a tetramer $\alpha_2\beta_2$ (Hou et al., 1990) in a sequential manner (Ahmad et al., 1992a) in the inner membrane. Based on the algorithms of Kyte and Doolittle (1982), it was recently suggested that PNTX comprises 12 membrane-spanning segments (Tong et al., 1991).

The primary structure of the transhydrogenase from bovine heart mitochondria (NNTM) was subsequently also deduced

† This work was supported by the Swedish Natural Science Research Council and the Medical Research Council of Canada. Natalie Glavas acknowledges the award of a MRC Studentship.

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® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: TH, nicotinamide nucleotide transhydrogenase; NEM, *N*-ethylmaleimide; FSBA, (*p*-(fluorosulfonyl)benzoyl)-5′-adenosine; DCCD, *N,N′*-dicyclohexylcarbodiimide.

0006-2960/94/0433-7691$04.50/0

from the partial and overlapping sequences of cDNA clones (Yamaguchi et al., 1988). Bovine transhydrogenase was found to be a homodimer (Anderson & Fisher, 1981; Wu & Fisher, 1983; Persson et al., 1987) with 1043 amino acids in each monomer (Yamaguchi et al., 1988). The monomer consists of three domains: one extramembraneous N-terminal domain (1–420), one membrane-spanning domain (420–850), and one extramembraneous C-terminal domain (850–1043) (Weis et al., 1987; Yamaguchi et al., 1990; Yamaguchi & Hatefi, 1991). NAD(H) and NADP(H) have been shown to bind stoichiometrically (1 mol/monomer) to the N- and C-terminal extramembraneous domains, respectively (Yamaguchi & Hatefi, 1993). The central region, assigned to residues 420–850, was suggested to comprise about 14 membrane-spanning segments (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991) when studied by the method of Kyte and Doolittle (1982). However, it has recently been argued that algorithms and scales other than the ones from Kyte and Doolittle should be used when searching for membrane-spanning segments (Degli Esposti et al., 1990; Fasman & Gilbert, 1990). It has therefore been suggested that several methods should be utilized in order to obtain a clear model of the topology of a transmembrane protein (Turner & Weiner, 1993).

A number of coupling mechanisms have been proposed for transhydrogenase, all based on the fact that it is a proton pump most likely acting through some kind of chemiosmotic mechanism, whereby protons are translocated through the membrane by a transmembraneous proton-conducting structure (Rydström, 1977; Fisher & Earle, 1982; Rydström et al., 1987; Jackson, 1991). According to the only two structurally resolved cases of proton pumps, bacteriorhodopsin (Khorana, 1993) and H^+-ATPase (Fillingame, 1992), one or more acidic residues are required as proton-binding mediators during transport. Theoretically, the proton pumping mechanism may also involve conserved redox-active vicinal dithiols, either within a subunit or between subunits (Jackson, 1991; Persson & Rydström, 1987). However, the only conserved cysteine is βCys260 in the *E. coli* transhydrogenase, corresponding to Cys834 in the bovine enzyme, which means that such a dithiol mechanism would have to involve redox interactions between subunits.

In the present study, the complete gene for the bovine transhydrogenase was constructed and resequenced. Based on the revised sequences for the bovine and *E. coli* transhydrogenases, as well as the available biochemical information regarding the topology of the two enzymes, membrane-spanning α-helices were predicted from several algorithms. Possible proton-binding charged amino acid residues of the predicted transmembrane α-helices were identified and their roles tested by subjecting them to site-specific mutagenesis.

MATERIALS AND METHODS

Construction of the Bovine Transhydrogenase Gene. The complete gene for the bovine heart enzyme was constructed together with a suitable promoter in *E. coli*. The two partially overlapping cDNA clones λTH32 and λTH36 (Yamaguchi et al., 1988; obtained as gifts from Prof. Y. Hatefi, Division of Biochemistry, Research Institute of Scripps Clinic, La Jolla, CA), were used as starting material. Both clones lack the eight N-terminal amino acids, and this sequence was therefore synthetically made together with a consensus ribosome binding site as a 37-mer oligonucleotide (linker). The linker was put downstream of the T7 promoter in pTWa1 (*Xho*I–*Eco*RI). From this construction, the *Sca*I–*Eco*RI fragment was excised and inserted into pUC19 (*Ssp*I–*Eco*RI, see (Figure 1). The

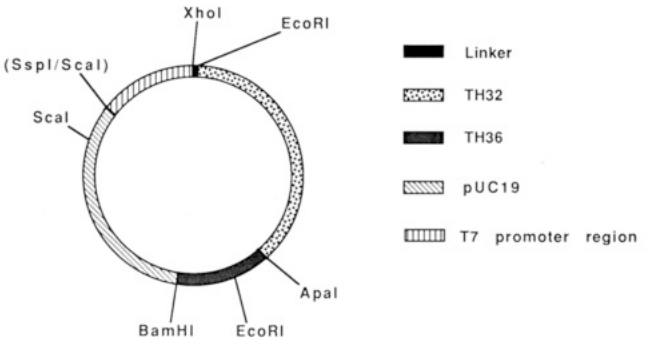

FIGURE 1: Construction of the complete gene for the bovine heart transhydrogenase. The construction of the gene is described in Materials and Methods.

*Eco*RI–*Eco*RI fragment of λTH32 was then inserted downstream of the linker. In the next step, the *Apa*I–*Sma*I fragment of this construction was replaced with the *Apa*I–*Cla*I fragment of λTH36 (*Cla*I filled in, no restriction site is formed). This construction was called pUGO4 and thus contained the complete structural gene for bovine transhydrogenase cloned between *Xho*I and *Bam*HI (cf. Figure 1). The structural gene was also transferred to pUC18 under control of the *lac* promoter. The data base accession number of the new bovine sequence in Genbank is L02543.

Automated Solid-Phase DNA Sequencing of the Bovine Transhydrogenase cDNA. A single bacterial colony containing the plasmid to be sequenced was transferred to 10 μL of PCR buffer (20 mM Tris-HCl, pH 8.3, at 20 °C, 50 mM KCl, and 0.1% Tween 20) and lysed at 99 °C for 5 min. One microliter of this lysis solution was used as the DNA source for the subsequent reactions. Polymerase chain reaction (PCR) amplification was carried out in 50 μL of PCR buffer including 200 μM of each dNTP, 1 unit of ampliTaq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA), and 5 pmol each of primers RIT 28 (5′-AAAGGGGGATGTGCTGCAAGG-3′) and RIT 29 (5′-biotin-GCTTCCGGCTCGTATGTTGTGTG-3′), complementary to regions downstream and upstream of the multilinker region of pUC-derived vectors, respectively. The temperature cycle consisted of denaturation at 95 °C for 30 s and annealing of primers and chain extension at 70 °C for 2 min. The reaction was carried through 30 cycles on a GenAmp PCR system 9600 (Perkin-Elmer).

Amplified biotin-labeled clones were subjected to automated solid-phase sequencing, using a robot workstation (Biomek-1000, Beckman Instrument, Fullerton, CA) as described by Hultman et al. (1989, 1991). In brief, the PCR mixture was immobilized on streptavidin-coupled magnetic beads (Dynabeads, Dynal, Oslo, Norway). After being washed, the immobilized double-stranded DNA was converted to single-stranded templates by treatment with NaOH, resulting in a single-stranded template immobilized in the 5′-end to the beads and the complementary strand in the NaOH supernatant. Each clone was thus sequenced from both directions: the immobilized strand using a fluorescent labeled M13 forward primer and the neutralized eluted strand using a fluorescent labeled reverse primer. The sequencing reactions were analyzed on an Automated Laser Fluorescent (ALF) sequencer (KABI-Pharmacia AB, Stockholm, Sweden).

Sequences and Structures. The primary structure of the bovine transhydrogenase was retrieved from the EMBL data bank "Swissprot" as NNTM$BOVIN. The 43 amino acid N-terminal signal sequence was excluded from the sequence. The primary structures of the *E. coli* transhydrogenase sequences (α and β subunit) were taken from the paper of Ahmad et al. (1992b).

Alignment. The alignment of the two transhydrogenases was performed with the Needleman and Wunsch algorithm (Needleman & Wunsch, 1970) included in the Biopolymer align subroutine in the SYBYL 1991 program (SYBYL, 1991, molecular modeling software, 5.41 Tripos Associates, Inc.). Both the pmutation and the physprop homology matrices were used. The gap penalty was set at both 8 and 35. PNTA and PNTB were aligned to different regions of NNTM. PNTA was aligned to the N-terminal region and PNTB to the C-terminal region of NNTM. Therefore, NNTM was cut into two pieces, NNTMA (1–560) and NNTMB 570–1043), which correspond to PNTA and PNTB. The sizes of the truncated bovine TH, NNTMA, and NNTMB were determined by running several alignments using truncations at different positions. This resulted in the truncated forms 1–560 for NNTMA and 570–1043 for NNTMB. The N-terminal start of PNTB was, however, ambiguous. By comparing predicted α-helices for NNTM and PNTB within this region, the start of PNTB was determined to be 571 in the bovine TH sequence. Different alignment matrices were tested. The results from the pmutation matrix with a penalty gap of 8 was chosen since this matrix yielded the highest score of 52% identity for both the α and the β subunits.

Algorithms for Predicting Membrane-Spanning Segments. The Jähnig algorithm developed by Fritz Jähnig (Jähnig, 1989) was obtained from the Vax Software utility in the EMBL data bank. The algorithm was retrieved as the program AMPHI and was run on a VAX-3000 workstation. The following scanning windows were used: amphipathic α-helices, 20; amphipathic β-sheets, 10; hydropathy index, 7 and 19. The RAO (Rao & Agros, 1986), MPH (Degli Esposti et al., 1990), NKD, (Kyte & Doolittle, 1982; Degli Esposti et al., 1990), and AMP (Degli Esposti et al., 1990) algorithms included in the basic program MAGINT were obtained as a gift from the group of Dr. Degli Esposti. The program was run on an IBM-compatible 486 computer. The scanning window for these four algorithms (RAO, MPH, NDK, and AMP) was set at 7. The base line for RAO was set at 1.05, while the base lines for the other three algorithms were set at 1.1. All these settings were recommended by the authors (Degli Esposti et al., 1990). The KKD algorithm uses the algorithm described by Klein et al. (1985). The SKD algorithm uses the algorithm described by Bangham (1988). KKD and SKD are available via anonymous FTP as the programs ALOM and SIEVE, respectively, from the work of Fasman and Gilbert (1990). These two programs, however, needed several changes before it was possible to compile and link them into runable versions. SKD was used with a mesh size of 9 (hydropathy index), and KKD was used with settings fixed by the authors (Fasman & Gilbert, 1990).

Prediction of Turns. The INFORMATION THEORY (INFORM) (Garnier et al., 1978) was included in the Biopolymer predict subroutine in the SYBYL (1991) program and was run on a VAX-3000 workstation. This algorithm was also included in the program MacMolly within the routine translate and was run on a Macintosh II.

Bacterial Strains and Plasmids. The *pnt* gene was introduced in the pGEM-7Zf(+) plasmid, giving the construct pSA2, and subsequently transformed into *E. coli* K12 strain JM109 (Ahmad et al., 1992a,b). Normally, a 40–80-fold overexpression was obtained.

Site-Specific Mutagenesis. Plasmid pSA2 carrying wild-type *pnt* genes was used to isolate single-stranded phagemid DNA. Site-directed mutagenesis to convert selected residues was performed by the method of Taylor et al. (1985) using degenerate primers. The reagents and protocols outlined in the Amersham mutagenesis kit were followed except that competent *E. coli* JM109 cells were used for transformation. Plasmid DNA was prepared from individual colonies, and the mutants were identified by double-stranded DNA sequencing.

Table 1: Revised Amino Acid Sequence of Nicotinamide Nucleotide Transhydrogenase from Bovine Mitochondria

reference	sequence residues		
Yamaguchi et al., 1988	Phe591	Glu777	Arg782
this work	Ala591	Val777	Ala782

Sequencing of E. coli Transhydrogenase Mutant DNA. In order to eliminate the possibility of unwanted changes in the DNA sequence, the entire coding region of the *pnt* genes from each mutant was completely sequenced by the dideoxy chain-termination method using Sequenase (Sanger et al., 1977).

Preparation of Membrane Vesicles. E. coli cells carrying the wild-type or mutant plasmid pSA2 were grown in LB medium supplemented with 0.1 g/L ampicillin to an OD_{550} of 1.0 before harvest, and membrane vesicles were prepared by a French press, essentially as described (Tong et al., 1991).

Catalytic Activity. The catalytic activity of transhydrogenase was measured spectrophotometrically at 375 nm as reduction of $AcPyAD^+$ by NADPH at pH 7.0 according to Clarke and Bragg (1985) in the absence or in the presence of 2.5 μmol of the uncoupler TCS. The reaction was started by the addition of membrane vesicles or NADPH, and the initial rate was calculated. The activity of membrane vesicles derived from nontransformed JM109 cells was 0.29 μmol/min/mg protein.

Proton Pump Activity. Initial relative rates of proton pumping catalyzed by freshly prepared membrane vesicles obtained from transformed JM109 cells were assayed fluorimetrically as quenching of quinacrine fluorescence (using excitation at 430 nm and emission at 505 nm) (Clarke & Bragg, 1985). All activity-dependent quenching was sensitive to uncouplers. Rates were expressed in terms of a decrease in arbitrary fluorescence units per minute per milligram of membrane protein relative to the rate of pumping obtained by membrane vesicles from pSA2/JM109 cells.

Polyacrylamide Gel Electrophoresis. SDS–PAGE analysis was performed on 12% (w/v) gels by the method of Laemmli (1970), and the gels were stained with Coomassie Blue.

Chemicals. Unless otherwise specified, biochemicals were of analytical grade and purchased from Sigma Chemical Co. or Boehringer Mannheim.

RESULTS AND DISCUSSION

Sequence of the Complete Bovine Transhydrogenase Gene. The bovine transhydrogenase gene was constructed as described in Materials and Methods. As shown in Table 1, sequencing of the cDNA resulted in changes of three amino acids as compared to the result previously reported (Yamaguchi et al., 1988). Phe591 was changed into an Ala, Glu777 was changed into a Val, and Arg782 was changed into an Ala, i.e., two charged residues were changed to hydrophobic residues. These changes in the charged residues are important since these residues proved to be located in the predicted transmembrane α-helix 9 (see below), which constitutes one of several possible proton-conducting pathways through the membrane. As compared to the sequence of the *E. coli* transhydrogenase, the corrected residues are not conserved.

The alignment (not shown) of the revised bovine transhydrogenase sequence with that recently determined (Ahmad et al., 1992b) for the *E. coli* transhydrogenase indicates a

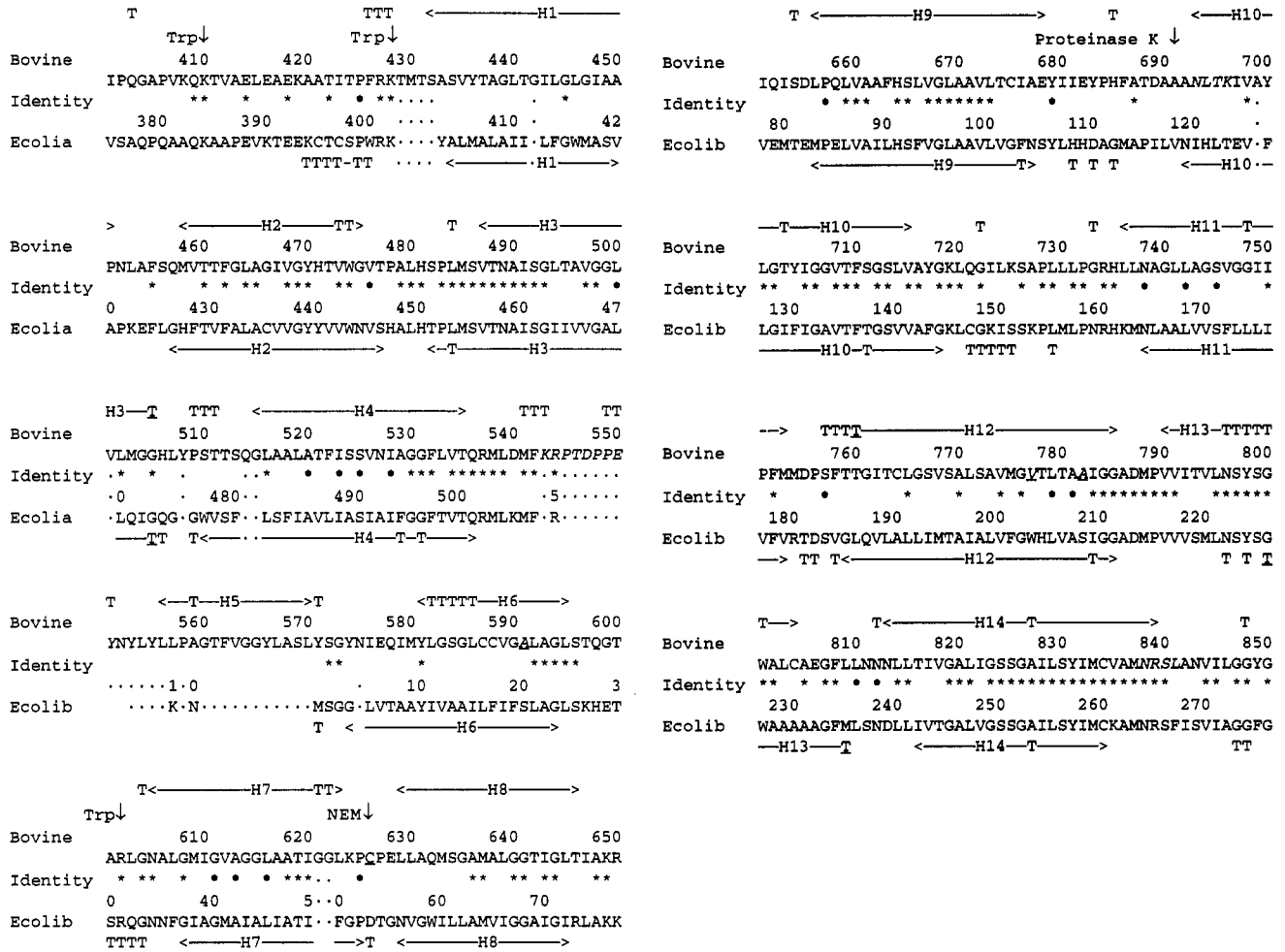

FIGURE 2: Alignment of the sequences of the membrane regions of the α and β subunits of *E. coli* transhydrogenase with those of the transhydrogenase from bovine heart. Stars indicate identity. The predictions of transmembrane α-helices are indicated within arrows. The maximal number of predicted transmembrane α-helices are denoted by an H-prefix (H1–H14), and the lengths of these are taken from the mean numbers in Table 4. Amino acids predicted as participating within a turn are indicated by a T. If such an amino acid is predicted at any end of a predicted transmembrane α-helix, the T is underlined. Experimentally obtained sites for tryptic and proteinase K cleavages, as well as for NEM binding, are indicated by ↓. The amino acids, obtained after sequencing of the cDNA, differing from the ones obtained previously (Yamaguchi et al., 1988), are shown in underlined italics.

52% identity between the two protein sequences (NNTM and PNTX) and identical seqeunces in certain regions, suggesting a common or at least a similarly folded structure. Figure 2 shows the regions of the bovine (residues 401–850) and *E. coli* (residues α375–α510 and β1–β276) sequences that form part of predicted transmembrane α-helices, turns, and connecting loops (see below), as well as other biochemical information (the α-helices indicated correspond to the mean lengths of the predicted α-helices 1–14 described in Table 4).

Prediction of Membrane-Spanning α-Helices and β-Sheets. Secondary structure elements within membranes can either be α-helices or β-sheets. However, β-sheets form only stable transmembrane-spanning elements as components of a β-barrel (Jähnig, 1989). Therefore, at least six or eight amphipathic β-sheets have to be predicted for a β-barrel. The algorithms SKD, KKD, Jähnig, RAO, MPH, NKD, and AMP were used to predict hydrophobic regions which can be either β-sheets or α-helices. In addition, the Jähnig algorithm could predict amphipathic α-helices and β-sheets. For bovine transhydrogenase, three amphipathic β-sheets (and two more possible) were predicted, while five β-sheets (and two more possible) were predicted for PNTX. In view of the large number of β-sheets required for a β-barrel, the predictions for amphipathic β-sheets were considered not to be strong enough (data not shown). No amphipathic α-helices were predicted.

The results from the predictions of α-helices and β-sheets in the transmembrane-spanning regions of bovine and *E. coli* TH, using the different algorithms, are shown in Tables 2 and 3, respectively. Note that the numbering of all predicted α-helices in tables and figures (except in the abstract and Figure 5), regardless of the total number of α-helices in a particular model, is based on the original 14 α-helix model of Yamaguchi et al. (1988).

There are several experimentally determined data to consider when assigning a folded structure of possible α-helices, some of which are described in Figure 2. It has previously been suggested that essentially the entire N- and C-terminal domains of bovine transhydrogenase protrude from the inner membrane into the mitochondrial matrix (Yamaguchi & Hatefi, 1991). It has also been reported that *E. coli* transhydrogenase has the N-terminus of its α subunit and the C-terminus of its β subunit exposed to the cytoplasmic surface of the cell membrane of *E. coli* (Tong et al., 1991). Proteinase K and several other enzymes split the enzyme in mitoplasts between residues 690 and 691 (Yamaguchi & Hatefi, 1991), suggesting that this protease-sensitive loop is exposed to the cytosolic side of the inner mitochondrial membrane. Antibodies raised to a synthetic, hydrophilic pentadecapeptide, corresponding to position 540–554 within the central hydrophobic domain of bovine transhydrogenase, bound to the

Table 2: Predictions of α-Helices in the Bovine Transhydrogenase[a]

helix no.	Hatefi	SKD	KKD	Jähnig	RAO	MPH	NKD	AMP
0			**187–203**	**184–204**	**187–201**	**188–210**		
		405–424						
1	**432–450**		*439–455*	*435–455*	**432–453**	**431–448**	**432–453**	
2	**458–478**	**455–470**	*458–474*	**470–489**	**455–471**	**455–474**		
3	**484–503**		**487–503**		**487–505**	**482–506**	**481–505**	**491–505**
4	**515–535**	**517–545**	**516–535**	**513–534**	**514–533**	**515–533**	**515–536**	
5	**553–573**		*554–570*	*555–570*	**556–571**			
6	**579–599**		**579–595**	*581–601*	**581–596**	**581–595**		**582–594**
7	**603–623**	**603–- - -**	**607–623**	*616–637*	**606–621**	**606–620**	**604–623**	**606–620**
8	**629–648**	- - - - - - -	*629–645*		**632–646**	**631–646**	**628–648**	
9	**659–679**	- - - -**702**	**656–678**	**657–682**	**659–678**	**659–678**	**656–680**	**661–678**
10	**697–717**		*694–710*	*692–710*	**697–715**		**691–718**	
11	**735–754**	**716–757**	**736–752**	**744–766**	**738–752**			
12	**758–776**		**767–784**		**759–785**	**760–785**	**759–797**	**760–774**
13	**790–810**	790–- - -	*788–804*	**770–804**				
14	**814–836**	- - - - - - -	**813–837**	**808–843**	**815–835**	**815–839**	**811–839**	**816–836**
15		- - - -**848**	*832–848*					

[a] The α-helix numbers correspond to the helix numbers of bovine TH in the prediction made by Yamaguchi et al. (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991). See Materials and Methods for details of prediction algorithms. Boldface, strong prediction; italics, weak prediction.

Table 3: Prediction of α-Helices in *E. coli* Transhydrogenase[a]

helix no.	Ahmed et al.	SKD	KKD	Jähnig	RAO	MPH	NKD	AMP
0			**165–185**	**165–190**	**168–192**	**168–193**	**166–193**	**168–184**
1	**403–421**	**404–- - -**	**402–422**	**409–419**	**405–418**	**404–419**		**405–418**
2	**425–446**	- - - -**446**	**424–446**	**426–451**	**426–440**	**427–448**	**426–446**	**427–440**
3	**451–472**		**452–473**	**455–- - -**	**456–471**	**451–470**	**450–473**	
4	**479–500**	**463–496**	**475–502**	- - - -**498**	**477–501**	**478–506**	**477–502**	**478–498**
5								
6		**7–27**	**1–27**	*5–20*	**4–23**	**4–22**	**3–24**	**4–22**
7	**32–51**	**39–52**	**34–52**	**40–- - -**	**38–49**	**38–49**		**38–49**
8	**56–75**	**60–- - -**	**54–76**	- - - -**72**	**57–72**	**58–72**		**58–72**
9	**86–104**	- - - -**104**	**83–105**	*84–99*	**86–104**	**88–104**	**84–104**	**88–102**
10	**123–143**	**112–150**	**124–144**	**119–149**	**123–145**	**123–145**	**113–149**	**123–145**
11	**161–180**	**160–- - -**	**161–186**	**167–- - -**	**165–178**	**164–179**		**166–178**
12	**184–202**	- - - - - - -	**181–210**	- - - -**215**	**186–211**	**186–209**	**185–221**	**186–209**
			205–221					
13	**216–236**	- - - - - - -				**226–235**	**227–- - -**	
14	**240–262**	- - - -**274**	**239–256**	**235–257**	**242–260**	**242–262**	- - - -**261**	**242–261**
15		**319–336**			**309–320**			

[a] The α-helix numbers correspond to the homologous helix numbers in the bovine transhydrogenase predicted by Yamaguchi et al. (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991) and Ahmad et al. (Tong et al., 1991; Ahmad et al., 1992a). See Materials and Methods for details of prediction algorithms. Boldface, strong prediction; italics, weak prediction.

enzyme in submitochondrial particles but not to that in mitoplasts (Yamaguchi & Hatefi, 1991). These results indicate that this sequence protrudes from the inner membrane into the mitochondrial matrix (Yamaguchi & Hatefi, 1991).

All known tryptic cleavage sites are located on the matrix side of the bovine transhydrogenase. Trypsin cleaves the amide bond Arg602-Leu603 in bovine transhydrogenase faster in the presence of NADPH (Yamaguchi et al., 1990). The β-subunit of *E. coli* transhydrogenase is susceptible to digestion by trypsin only if NADPH is present (Tong et al., 1991). *N*-Ethylmaleimide (NEM) modifies Cys893 and Cys626 in bovine transhydrogenase, and it was found that Cys893 is modified slower in the presence of NADH/NADP but faster in the presence of NADPH (Yamaguchi et al., 1990). Cys626 has been proposed to be located on the cytosolic side in bovine transhydrogenase (Yamaguchi & Hatefi, 1991); however, the support for this location does not appear to be strong.

For a model having both the N- and C-termini on the matrix side in bovine transhydrogenase, an even number of α-helices has to be predicted. In *E. coli* transhydrogenase, the N-terminus of the α subunit and the C-terminus of the β subunit are on the cytosolic side. It is not experimentally clarified if the C-terminus of the α subunit and the N-terminus of the β subunit are on the same side of the periplasmic membrane. Thus, both an even and an odd number of α-helices are possible in *E. coli* transhydrogenase.

A comparison of the predicted α-helices in the two enzymes is presented in Table 4 (see also Figure 2). Some of the predicted α-helices were adjusted in the N- and/or the C-terminal region in order to fit with knowledge about amino acids commonly occurring as N-caps and C-caps, etc. (e.g., Pro, Gly, and Asn) (Richardson & Richardson, 1989). The prediction of a transmembrane α-helix in the extramembranous N-terminal region of both the bovine and the *E. coli* transhydrogenases (Tables 2 and 3) was not included, since the assignment of this region as a NADH-binding region with a general βαβ-fold makes this prediction rather unlikely (Hu et al., 1992; Yamaguchi & Hatefi, 1993).

Complex membrane proteins from the bacterial membranes as well as from the endoplasmic reticulum, Golgi, plasma, inner mitochondrial, and thylakoid membranes of eukaryotic cells and chloroplasts have a charge bias for nontranslocated polar segments shorter than 70–80 residues (von Heijne & Gavel, 1988; Gavel et al., 1991). The basic residues Arg and Lys are four times more prevalent in cytosolic as compared to periplasmic connecting loops in bacterial inner membranes, whereas no comparable distribution has been observed for the acidic residues aspartate and glutamate (von Heijne, 1986). The bovine transhydrogenase is expressed from chromosomal DNA (Rydström et al., 1982; Wu et al., 1985). In analogy

Table 4: Comparison Between the Predicted α-Helices in Bovine and *E. coli* Transhydrogenase[a]

	bovine				*E. coli*				
helix no.	mean[b]	score[c]	adj[d]	length[e]	mean[b]	score[c]	adj[d]	corr[f]	length[e]
1	432–451	3		20	**404–419**	**6**		**434–450**	**16**
2	455–474	4 + 1	*458–475*	18	**426–446**	7		**457–477**	**21**
3	**487–505**	**5**		**19**	**452–473**	**5**	**451–473**	**482–505**	**23**
4	**515–535**	**6**		**21**	**477–501**	7		**510–536**	**25**
5	*556–570*	1 + 2		*15*					
6	581–595	4 + 1*		15	**4–23**	6 + 1		**574–594**	**20**
7	**606–623**	6 + 4	**605–623**	**19**	**38–52**	**6**	**37–52**	**608–625**	**16**
8	629–646	4 + 1		18	**57–72**	**6**	**56–72**	**629–645**	**17**
9	**657–678**	7	**656–678**	**23**	**84–104**	6 + 1	**83–104**	**656–677**	**22**
10	*694–715*	2 + 2	*693–715*	*23*	**123–145**	7	**119–144**	**692–718**	**26**
11	736–757	4	*736–753*	18	**164–179**	**6**		**738–753**	**16**
12	**760–785**	**5**		**26**	**185–211**	7		**759–785**	**27**
13	*790–804*	2 + 1*		*15*	*226–235*	**3**		*800–809*	*10*
14	**813–839**	7		**27**	**242–261**	7	**242–260**	**816–834**	**20**

[a] The α-helix numbers correspond to the helix numbers of bovine transhydrogenase in the prediction made by Yamaguchi et al. (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991). [b] A "mean" number of amino acids as judged from comparison of the results from the different algorithms. The ends of the helices were set at the position where ≥50% of the strong predictions coincided. The predictions made by Yamaguchi & Hatefi (bovine transhydrogenase) and Ahmad et al. (*E. coli* transhydrogenase) were not accounted for. Boldface, strong prediction (≥5 strong predictions); regular type, medium prediction (3–4 strong predictions); italics, weak prediction (≤2 strong predictions). [c] Number of strong (bold) and weak predictions; star indicates predictions of many amino acids as participating within a turn. [d] Adjusted length due to the type of amino acids occurring in the ends of the helices. [e] Based on the mean/adjusted number of amino acids. [f] The amino acid sequence number corresponding to the residue number in the bovine transhydrogenase.

with this "positive-inside rule" for bacteria by von Heijne (1986), positively charged loops in bovine TH should not be able to traverse the mitochondrial inner membrane from the matrix to the cytosol once pre-transhydrogenase has entered the mitochondrion. Thus, according to this model, positively charged loops on the matrix side should be expected for bovine transhydrogenase.

Based on the "score" of algorithms predicting α-helices (Table 4, score) and the number of participating amino acids, different folds of bovine transhydrogenase were predicted. The previously predicted fold (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991) is shown in Figure 3a. Using the algorithms from Table 2, a corresponding fold originating from the prediction of α-helices of the revised sequence was obtained (not shown). Such a fold also has the positively charged loops predominantly on the matrix side. By omitting the weakly predicted α-helices 5 and 13, considering the score and the number of participating amino acids for bovine transhydrogenase (Table 4), the reverse situation may be obtained (not shown). In this model, however, the sites for proteolysis and NEM binding are not supported by the experimental data.

Further omitting α-helices 6 and 10 yields a fold that is possible when considering the experimental data mentioned above, except for the positive-inside rule (von Heijne, 1986). Further omitting α-helices 8 and 11 yields a fold having the proteinase K site erroneously on the matrix side instead of the cytosolic side. By considering α-helix 10 to be more likely than α-helix 11, due to the length of the α-helix rather than the score, a model is obtained which agrees with known experimental data but has a high positive charge bias for the matrix (Figure 3b). Although α-helix 1 has a score of 3 (Table 4), it was not excluded as a membrane-spanning segment due to the homology with the α-subunit of *E. coli*. Since all models comprise α-helices 1–4 in the N-terminus, they have the antibody-binding loop 540–554 correctly on the matrix side.

For *E. coli* transhydrogenase, the different algorithms agree quite well (Table 3). Considering the score (Table 4), the only weak prediction is obtained for α-helix 13. All other α-helices have rather high scores, except for helix 5, which was predicted very weakly in bovine transhydrogenase and is impossible in *E. coli* transhydrogenase, since this α-helix

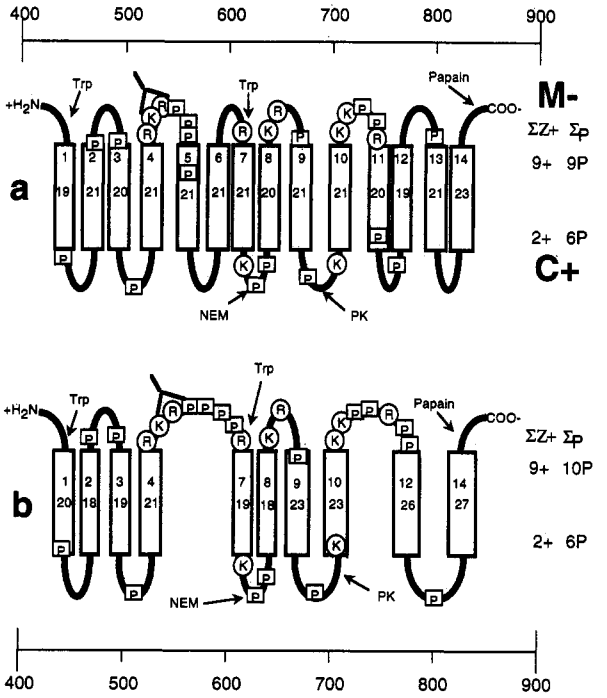

FIGURE 3: Possible folds of the transmembrane α-helices predicted in bovine TH. (a) The model predicted by Yamaguchi et al. (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991). (b) The fold proposed as most promising by the authors. The amino acids arginine (R) and lysine (K) are indicated. The sites for trypsin (Trp), proteinase K (PK), and papain cleavage and *N*-ethylmaleimide (NEM) and antibody binding (λ) are also shown. M– denotes matrix side (positively charged relative to the cytosolic side) and C+ denotes cytosolic side (negatively charged relative to the cytosolic side). The sum of positively charged (ΣZ+) amino acids (K and R) and the sum of prolines (ΣP) within the loops are given in the margin. The α-helices are positioned relative to a scale indicating the amino acid sequence numbers. The width of each α-helix is proportional to its relative length.

corresponds to the gap between the C-terminus of the α-subunit and the N-terminus of the β-subunit.

The model in Figure 4a was deduced from recently obtained data for the *E. coli* transhydrogenase (Tong et al., 1991; Ahmad et al., 1992a) and contains 12 α-helices. α-Helix 5 (impossible, see above) and α-helix 6 are missing in this

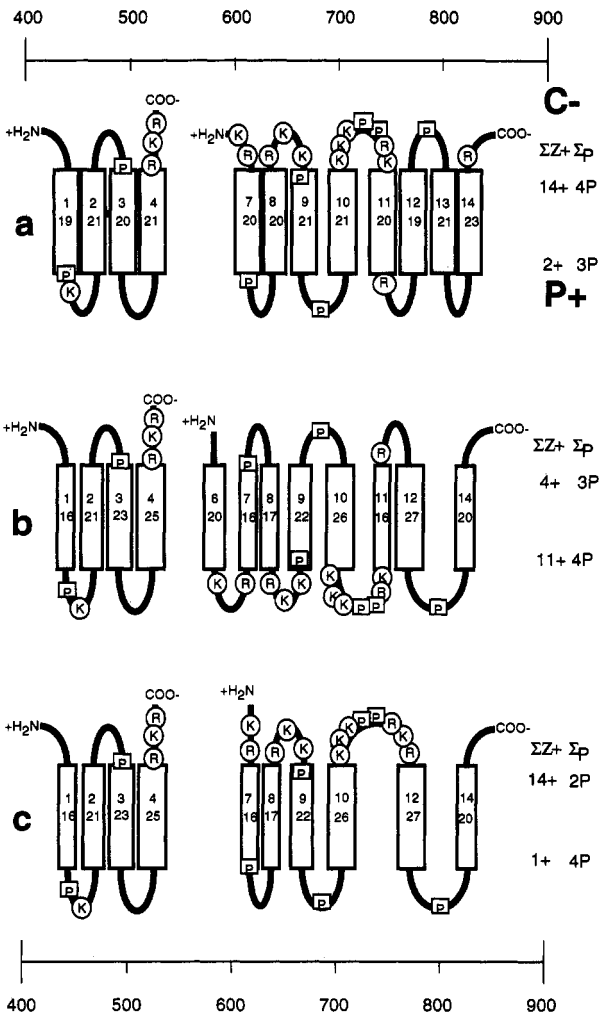

FIGURE 4: Possible folds of the transmembrane α-helices predicted in *E. coli* transhydrogenase. (a) The model predicted by Ahmad et al. (Tong et al., 1991; Ahmad et al., 1992a). (b) The fold predicted on the basis of the revised sequence (Ahmad et al., 1992b) of the *E. coli* transhydrogenase. (c) The fold proposed as most promising by the authors. The amino acids arginine (R) and lysine (K) are indicated. The sum of positively charged (ΣZ+) amino acids (K and R) and the sum of prolines (ΣP) within the loops are given in the margin. The α-helices are positioned relative to a scale indicating the amino acid sequence numbers. The width of each α-helix is proportional to its relative length.

model, but the remaining α-helices agree with the bovine 14-helix model of Figure 3a. Based on the prediction of α-helices in the revised sequence (Ahmad et al., 1992b) and using the algorithms in Table 3, a fold inconsistent with the positive-inside rule (von Heijne, 1986) was obtained (Figure 4b). Omitting the weakly predicted α-helix 13, omitting α-helix 6 as being less likely than α-helices 7 and 8 and weakly predicted in the bovine transhydrogenase, and omitting α-helix 11 gives a 10-helix model (Figure 4c) which is fully consistent with the positive-inside rule (von Heijne, 1986), as well as with the model in Figure 3b for the bovine transhydrogenase. The main uncertainty with this model is α-helix 11, which is weakly predicted in the case of the bovine transhydrogenase but more strongly predicted in the case of the *E. coli* transhydrogenase (Table 4).

Due to the dipolar nature of biological membranes, it is possible that loops with excess of positively charged amino acids are always found on the matrix side of mitochondria and on the cytosolic side of *E. coli*. The respiratory chain generates an electrochemical proton gradient across the inner mitochondrial membrane by electron transfer and proton transport. The difference in potential between the outside and the inside of the membrane is approximately 0.14 V, the outside being positively charged (Cramer & Knaff, 1991). This has recently been concluded to explain why positively charged loops are predominantly found on the matrix side of mitochondria and on the cytosolic side of bacteria (Andersson & von Heijne, 1994).

Prediction of Proline and Glycine Residues in Turns and/or Loops. The membrane-spanning segments have to be connected to turns and/or loops on both sides of the membrane. Taking into account the prediction of turns within predicted transmembrane α-helices in the bovine transhydrogenase (Table 4), the weakness of the prediction of α-helix 6 and α-helix 13 is further emphasized.

The amino acids most commonly occurring as α-helix breakers and in turns are prolines and glycines (Richardson & Richardson, 1989). Prolines are enriched in periplasmic loops of the *E. coli* membrane (cytosolic side in mitochondria), and they may even be present within membrane-spanning α-helices which have their N-termini toward the cytosolic side (matrix side in mitochondria) (von Heijne, 1986, 1991).

None of the bovine and *E. coli* transhydrogenases have a proline in the middle of any α-helix, but rather at the ends. Nevertheless, the model in Figure 3b of the bovine transhydrogenase agrees with the observation made by von Heijne (1991), by having prolines in those α-helices which have their N-termini toward the matrix side. The prolines in loops/turns are not enriched in the cytosolic loops of bovine transhydrogenase. The model in Figure 3b has, however, rather evenly distributed prolines. The loop binding to antibodies is on the matrix side in all models.

Glycine is a very commonly occurring residue in proteins, and it is also present within α-helices. An interesting observation is that the glycine content in bovine TH is 11%, and in the transmembrane region (432–848) it is as high as 19%, i.e., nearly every fifth amino acid is a glycine. In contrast, the glycine content in the corresponding transmembrane region (α403–α510 and β1–β276) for *E. coli* transhydrogenase is 11%, whereas it is 9% in the α subunit and 10% in the β subunit. Glycines are therefore not enriched in the transmembrane region of *E. coli* TH.

It should be emphasized that the models in Figures 3b and 4c do not provide any information as to how the α-helices are organized relative to each other. As mentioned previously, the prediction does not support a barrel type of organization of amphipathic β-sheets, and it appears unlikely that any of the predicted α-helices are amphipathic. However, a model where the hydrophobic 10 α-helices are organized around a more hydrophilic core of the remaining transhydrogenase protruding through the membrane is a possibility that presently cannot be excluded.

Localization and Mutagenesis of Charged Amino Acids of a Putative Proton Channel. Charged amino acids, especially Asp and/or Glu residues, have been implicated as protonatable residues in, for example, bacteriorhodopsin and subunit c of F_1F_0-type ATPases (Cain & Simoni, 1988; Lightowlers et al., 1987). Histidine residues may also have a role in proton conduction across membranes. Thus, an essential His residue has been found in subunit a of the *E. coli* F_1F_0-ATPase (Cain & Simoni, 1988). Since these residues have to be located in or close to transmembrane α-helices, it was considered of interest to locate charged residues with a similar function in bovine and *E. coli* transhydrogenases and to replace these by site-specific mutagenesis. Altered conserved charged residues, as well as the conserved βCys260, in or close to predicted

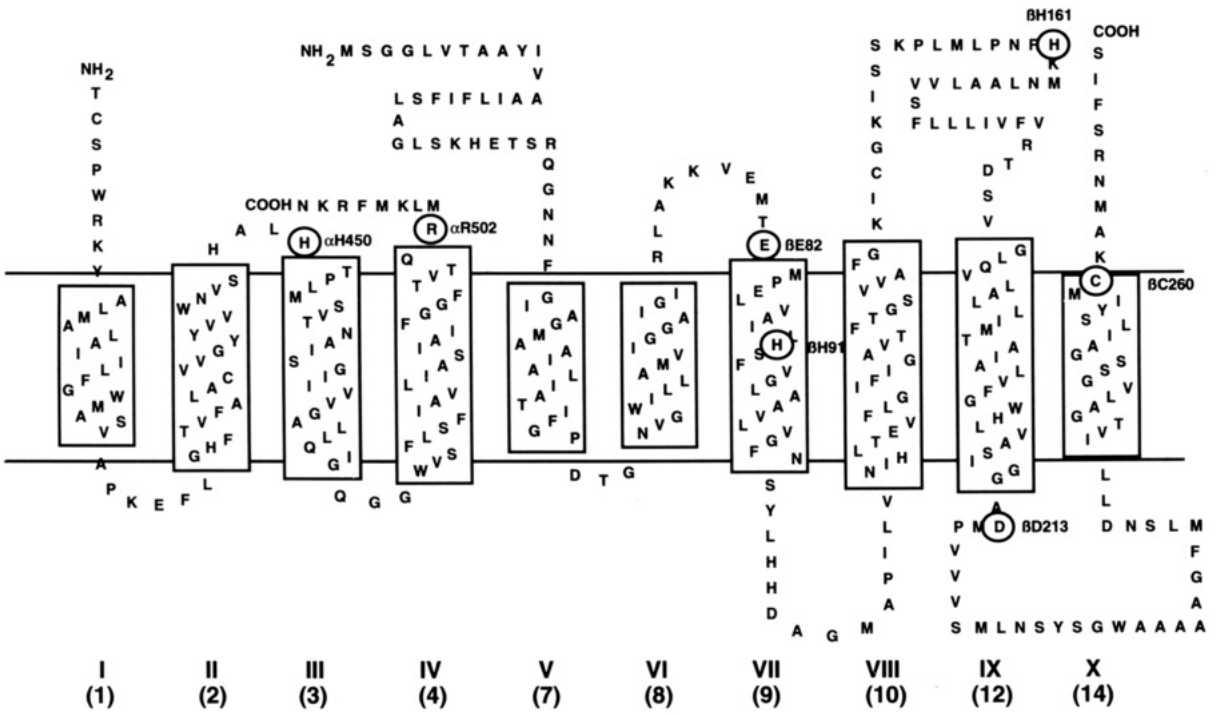

FIGURE 5: Proposed model of the distribution of amino acid residues of transmembrane α-helices for *E. coli* transhydrogenase. The model is based on Figures 2 and 4c and also shows the residues which have been changed by site-directed mutagenesis (circles). Note that the α-helices are numbered as I–X and as 1–14 (in parentheses), the latter being the conventional labeling used throughout this paper (except in the abstract) based on that used by Hatefi and co-workers (Yamaguchi et al., 1988; Yamaguchi & Hatefi, 1991).

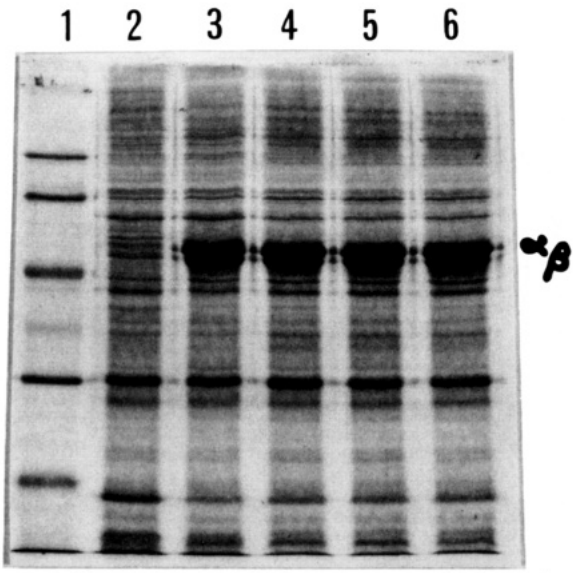

FIGURE 6: SDS–PAGE analysis of membrane proteins of transformed *E. coli* JM109 cells. SDS–PAGE analysis of total membrane proteins of *E. coli* JM109 cells containing the parent plasmid pGEM-7Zf (lane 2), recombinant plasmid containing wild-type transhydrogenase genes (plasmid pSA2) (lane 3), and recombinant plasmids containing the mutant transhydrogenase genes βH91S (lane 4), βH91T (lane 5), and βH91C (lane 6). Ten micrograms of protein was loaded in each lane. Lane 1: molecular mass standards 94 000, 67 000, 43 000, 30 000, and 20 000.

membrane α-helices of Figure 4c are shown in Figure 5. All mutant transhydrogenases were correctly folded and expressed at levels which were similar to that of wild-type, as indicated by PAGE analysis of the membrane vesicle preparations (not shown, but see Figure 6 above regarding βHis91 mutants).

The only conserved acidic residues, βGlu82 (Asp655 in the bovine enzyme) and βAsp213 (Asp787 in the bovine enzyme) of the *E. coli* transhydrogenase, are located close to α-helices 9 and 12 (see Figure 5) and could constitute essential components of a proton channel. These were therefore replaced by other residues in the *E. coli* transhydrogenase to determine their possible involvement in the transmembrane proton pathway. Replacement of βGlu82 by Lys or Gln and of βAsp213 by Asn or His resulted in an active enzyme that also retained proton pumping activity (Table 5). These data suggest that βGlu82 and βAsp213 are not involved in proton pumping by transhydrogenase. It may also be noted that the residues flanking βGlu82 and βAsp213 do not show any homology with those of the transmembrane acidic residues in the subunit c of the H^+-ATPases (Fillingame, 1992). Although there are several conserved acidic residues in the bovine and *E. coli* transhydrogenases, which like subunit c of the H^+-ATPases can be covalently modified by DCCD, notably αAsp232, αGlu238 (corresponding to Glu257 in the bovine transhydrogenase), and αGlu240, these are located in the substrate (NAD(H))-binding region. We have already shown

Table 5: Effects of Site-Specific Mutation on Catalytic Activity and Proton Pumping of *E. coli* Transhydrogenase[a]

mutant	catalytic activity (%)	proton pumping activity (%)
control	100	100
αH450T	17	51
αR502S	71	93
βE82Q	63	85
βE82K	79	66
βH91S	6	8
βH91T	2	8
βH91C	3	0
βH161S	90	100
βH161T	88	108
βH161C	59	94
βD213N	92	44
βD213H	82	34
βC260S	78	57

[a] Catalytic and proton pumping activities are specific activities expressed as percentages of the activity of control membranes. Conditions were as described under Materials and Methods. 100% catalytic activity corresponds to 14.5 μol/min/mg protein.

by site-directed mutagenesis that they are not essential for proton pumping (Glavas et al., 1993).

Only a single conserved histidine residue, βHis91 in the *E. coli* transhydrogenase (His664 in the bovine enzyme), is found in the predicted transmembrane α-helix 9 of the β subunit (see Figure 5). Replacement of βHis91 by Cys gave 3% residual catalytic activity but no proton pumping activity (Table 5). The corresponding Ser and Thr mutants showed 2% and 6% residual catalytic activity, respectively, and 8% residual proton pumping activity (Table 5). In this context, it should be pointed out that quenching of quinacrine (as well as other 9-amino-acridine derivatives) is logarithmic rather than linear. Thus, although 8% residual quinacrine fluorescence is significant, the activity is much lower on a linear scale. In addition, about 2% catalytic activity is accounted for by genomic transhydrogenase, which also contributes to proton pumping. As can be seen from Figure 6, the expression levels for all βHis91 mutants were indistinguishable from that of the overexpressed wild-type transhydrogenase.

An interesting question is whether mutagenesis of a residue involved in proton pumping should give a complete inhibition of catalytic and/or proton pumping activity. In the case of bacteriorhodopsin, both Asp96 and Asp85 are believed to be directly involved in proton translocation (Henderson et al., 1990; Khorana, 1993). Replacement of these residues with Asn inhibits activity completely in the case of Asp96 but only about 90% in the case of Asp96 (Mogi et al., 1988). Also, the complete inhibition of proton translocation in Asp61 mutants of the subunit c of *E. coli* F_1F_0-ATPase was relieved partially by a substitution of the nearby Ala24 with Asp. One interpretation of these results, which may be relevant for the present investigation, is that mutagenesis of an "essential" proton-conducting residue may give rise to alternative pathways by which a limited proton translocation can take place.

The conserved αHis450 (His481 of the bovine transhydrogenase), located in the loop connecting the predicted α-helices 2 and 3, was replaced by Thr in the *E. coli* enzyme. There was greater than 80% reduction in enzyme catalytic activity, but proton pumping was largely retained (Table 5). Similar results were obtained with Ser mutant of the conserved αArg502 (Table 5). Mutagenesis of the conserved βHis161 close to α-helix 11, earlier assumed to be membraneous but not predicted as such in this study (cf. Figures 4c and 5), also had little effect (Table 5).

Thus, the data from site-directed mutagenesis of conserved charged residues in or close to transmembrane α-helices implicates only βHis91 as a candidate residue involved in proton pumping by transhydrogenase. That a residue with a pK_a of about 6.0–7.0, which may correspond to a histidine, is involved in proton translocation was recently proposed on the basis of kinetic analysis of the solubilized *E. coli* transhydrogenase (Hutton et al., 1994). It should be stressed that the conclusion based on the present results holds also for the 14-helix model of Figure 3a for the bovine transhydrogenase and the 12-helix model of Figure 4a for the *E. coli* transhydrogenase.

Role of Conserved Cysteine Residues. Only a single cysteine residue is conserved in both the *E. coli* and bovine transhydrogenase sequences, i.e., βCys260 (Cys834 in the bovine sequence), which in *E. coli* is located in the predicted α-helix 14 (Figure 5). Thus, if redox-active dithiols are involved in the transhydrogenase reaction, including proton pumping, then the two conserved βCys260 residues of the β-subunits of the active $\alpha_2\beta_2$ enzyme would be involved in catalysis. However, replacement of βCys260 by Ser yielded an active enzyme that retained proton pumping activity (Table 5). These data therefore suggest that dithiols are not involved in the catalytic mechanism of transhydrogenase.

In conclusion, the secondary membrane structure of proton-pumping nicotinamide nucleotide transhydrogenase was predicted using several prediction algorithms. The prediction included experimental data, the positive-inside rule, and observation of the positions of prolines in α-helices and loops. A model having 10 α-helices was predicted both for the bovine and for the *E. coli* transhydrogenases. The role of predicted acidic and basic residues and thiols in these transmembrane α-helices was tested by site-directed mutagenesis. Of these residues, only mutants of βHis91 were essentially inactive, suggesting that this residue, but not acidic residues, may mediate proton translocation and that thiols are not involved.

ACKNOWLEDGMENT

We thank Liang Liem for converting the programs ALOM and SIEVE into versions runnable on our VAX system, system manager Martin Norin for invaluable computational help, and Gunnar von Heijne for discussions.

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