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Review

Molecular biology of nicotinamide nucleotide transhydrogenase –a unique proton pump

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1. Introduction

In 1953 Kaplan and co-workers [1] discovered that extracts of *Pseudomonas fluorescens* catalyzed the reduction of NAD^+ by NADPH according to the reversal of the reaction

$$NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ \quad (1)$$

Somewhat later the same year this group demonstrated that a similar activity was present in animal tissue [2]. It then took 10 years before Danielsson and Ernster showed that transhydrogenase was linked to the energy-transfer system of the respiratory chain, i.e., utilizing the same intermediate as the ATP synthase [3]. The existence of a so-called energy-linked transhydrogenase, which drives the reaction from left to right at an enhanced rate and a concomitantly shifted apparent equilibrium towards NADPH formation, had indeed been proposed earlier on the basis of the difference in redox levels of NAD and NADP in intact

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mitochondria [4]. This discovery by Danielsson and Ernster [3] triggered an intense research on the mode of interaction of transhydrogenase with the energy-transfer system. Major recent advances in transhydrogenase research have been the purification [5,6] and reconstitution of beef heart transhydrogenase [5,7], the demonstration that beef heart transhydrogenase is a proton pump [7], reconstitution of ATP and bacteriorhodopsin-driven transhydrogenase [8,9], and the cloning and sequencing of the DNA/cDNA of *E. coli* [10,11] and beef heart transhydrogenases [12]. Several reviews have covered these aspects as well as the kinetics, regulation and structure–function relations of different transhydrogenases up to about 1990/91 [13–19].

A separate previous review has been devoted to possible physiological roles of transhydrogenase in mammals [20]. In a more recent review an additional role of transhydrogenase was proposed in which the enzyme is suggested to regulate the flux of substrates through the NADP and NAD-linked isocitrate dehydrogenases [21]. However, the physiological role(s) of transhydrogenases remains an open question.

The transhydrogenase reaction catalyzed by proton-pumping transhydrogenases of the so-called AB-type (denoted H^+-TH in the following text), in contrast to the BB-transhydrogenases which are soluble and different with regard to a number of properties, may be described as

$$nH^+_{out} + NADP^+ + NADH \rightleftharpoons nH^+_{in} + NADPH + NAD^+ \quad (2)$$

where 'n' denotes the number of protons pumped across the membrane, and 'in' and 'out' the matrix and intermembrane space, respectively, of mitochondria, or cytoplasm and periplasmic space, respectively, of bacteria. The reaction from left to right is the predominant direction of the reaction in the intact mitochondrion/bacterial cell, driven by the existing electrochemical proton potential, Δp. It may be safely concluded that no other protein(s) is required for the Δp-driven H^+-TH to function, which has been demonstrated by the co-reconstitution of H^+-TH with either ATPase or bacteriorhodopsin as Δp-generators [8,9]. H^+-TH's offer a number of advantages as model system for studies of the mechanism of proton pumps, e.g., they are composed of only 1–3 polypeptides and therefore represent a relatively simple structure, they are relatively hydrophilic for a membrane protein, and are easily purified and reconstituted in liposomes with or without other proton pumps. In addition, the reaction can be followed directly spectrophotometrically by using various substrate analogues, and there are no scalar protons consumed or generated in the reaction which complicate measurements of vectorial protons.

Site-directed mutagenesis is an additional and attractive possibility to study structure–function relations of transhydrogenase, so far using primarily the *E. coli* transhydrogenase. An overexpression of this enzyme of about 70-fold allows essentially unlimited amounts of pure enzyme to be produced. To date, no other transhydrogenase(s) has been expressed in any host system. However, the genes of six H^+-TH's have now been cloned and sequenced, which allow a reliable determination of conserved regions and domains of importance for NAD(P)-binding and proton pumping. Not all of these genes have as yet been shown to code for a H^+-TH, although this is very likely. Nevertheless, based on amino acid sequences and similarities with other enzymes, three-dimensional structures of functional domains can now be predicted and tested by, for example, mutagenesis. Such functional domains are also being produced by expression in *E. coli* host cells and their structures are being examined by NMR.

It is increasingly evident that the H^+-TH's characterized so far represent a unique set of enzymes, in which two redox sites interact directly to generate a redox potential difference sufficient to drive proton pumping. The finding that especially the hydrophilic N-terminal domain of H^+-TH (the α-subunit in *E. coli* transhydrogenase) shows a high degree of amino acid sequence identity with alanine dehydrogenase [22,23] as well as other dehydrogenases [24], suggests that H^+-TH's have evolved from dehydrogenases/reductases rather than from, for instance, ATPases or other proton pumps driven by electron transport. This evolutionary pathway is supported by the proposed proton-pumping/conducting structure of H^+-TH involving a single histidine which has not been demonstrated previously in other pumps (cf. Sections 8 and 9). Obviously, this raises the question whether H^+-TH's will remain a unique class of proton pumps or whether they will turn out to be representatives of pumps of hitherto unknown structures. A very interesting development in this context is that cDNA-derived amino acid sequences homologous to those of the major H^+-TH's have been found in the protozoan parasites *Eimeria tenella, Eimeria acervulina* and *Entamoeba histolytica* [25–27], which also show sequence homology to proteins involved in sugar transport [25].

The present review will emphasize structure–function relations of H^+-TH at the molecular level and also includes aspects related to properties and expression of H^+-TH genes. These areas of H^+-TH research have been essentially lacking in previous reviews and the special emphasis of this review is therefore justified by the recent developments in molecular biology-related approaches used in investigations of H^+-TH's and the mechanism of proton pumping catalyzed by these enzymes.

2. Proton-pumping transhydrogenases

Like most proton pumps, H^+-TH can either use or generate a Δp depending on the direction of the reaction catalyzed. The direction from left to right in reaction 2 (the

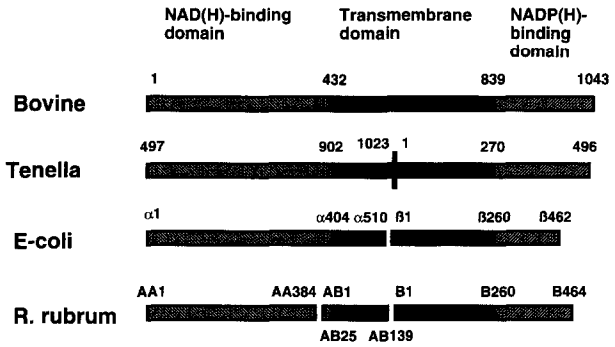

Fig. 1. A comparison of the NAD(H)-binding, transmembrane and NADP(H)-binding domains of bovine, *Tenella*, *E. coli* and *R. rubrum* H^+-TH.

'forward' reaction) is slow in the absence of Δp but is nevertheless coupled to proton pumping. In the presence of a Δp generated by any other proton pump(s), the rate of this reaction is stimulated 5–10-times and the product/substrate ratio, i.e., [NADPH][NAD^+]/[NADH][$NADP^+$], is increased by a factor of up to 500 (the so-called 'energy-linked' reaction). However, this latter ratio is normally not reached in the intact mitochondrion/bacterial cell because of a number of competing reactions which will reoxidize NADPH and rereduce NAD^+. The right to left direction of reaction 2 (the 'reverse' reaction) is faster and its maximal velocity approaches that of the Δp-driven forward reaction.

Despite the fact that H^+-TH is an integral and ion-translocating membrane protein, it is surprisingly hydrophilic, with an approximate molecular mass of 100–110 kDa for the monomeric form or minimal assembly of subunits, of which only approximately 35% is located in a hydrophobic membrane-bound domain. As judged from the bovine H^+-TH which has two separate binding sites for NAD(H) and NADP(H) [28], respectively, all H^+-TH's have their substrate-binding sites located in two hydrophilic domains of the enzyme, separated by a hydrophobic domain. These binding sites are exposed to the matrix in mitochondria and cytoplasm in bacteria. Presumably, the hydrophobic domain contains the proton-conducting structure, which is assumed to consist of a helical bundle containing 10–12 predicted transmembrane α-helices (cf. Section 6). Only a minor part of the hydrophobic domain, approximately 5%, is exposed on the cytosolic side in mitochondria (periplasmic space in bacteria).

The three domains of all H^+-TH's the amino acid sequences of which have been deduced from their cDNA/DNA, i.e., the bovine [12], *E. coli* [11], *Rhodospirillum rubrum* [29,30], *Eimeria acervulina* [25], *Eimeria tenella* [26] and *Entamoeba histolytica* [27] enzymes, are shown in Fig. 1, in which the parasite H^+-TH's are represented by the *Tenella* enzyme. The $\alpha 1 + \alpha 2$-subunits of the *Rhodospirillum rubrum* enzyme correspond essentially to the α-subunit of the *E. coli* enzyme [29,30] (in the text below the gene products PntAA, PntAB and PntB of the *R. rubrum* enzyme will be referred to as $\alpha 1$, $\alpha 2$ and β). Human brain H^+-TH has been identified and shown to be homologous to the bovine enzyme [31] and its gene was shown to be located to chromosome 5 [32], but the whole gene has not yet been sequenced. Interestingly, the parasite enzymes differ with regard to the intramolecular distribution of the three domains. Whereas the three non-parasite H^+-TH's are transcribed from the N-terminal to the C-terminal of the monomer, or the N-terminal to the C-terminal of the α-subunit followed by the N-terminal to the C-terminal of the β-subunit, etc., the three parasite H^+-TH's start with the N-terminal of a sequence which is

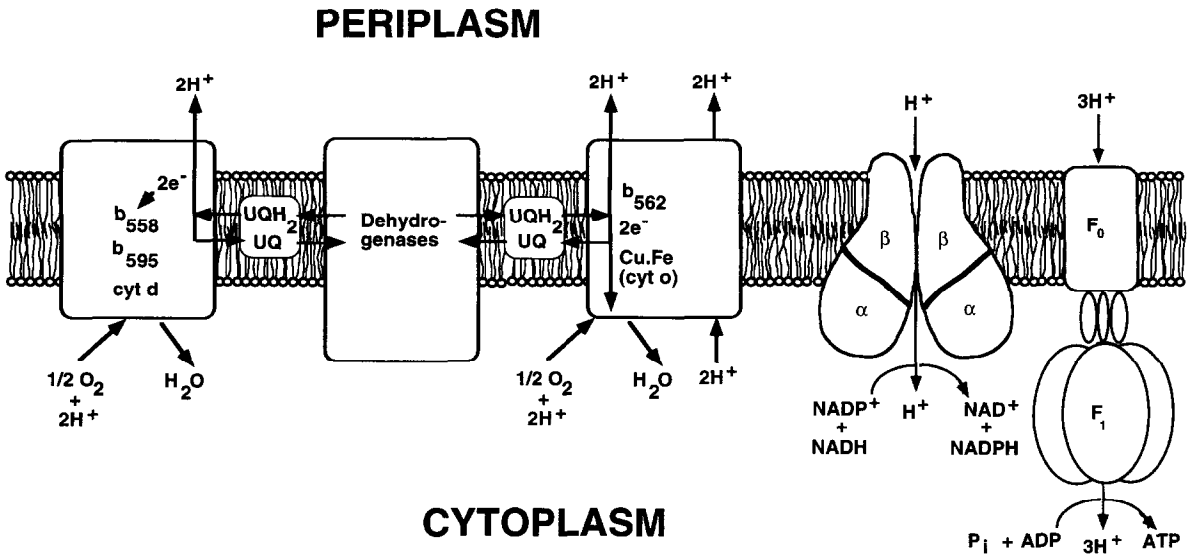

Fig. 2. The major proton pumps of the plasma membrane of *E. coli* . H^+-TH is shown as the active dimer form. However, the remaining pumps/complexes contributing to a Δp are not necessarily shown as their active forms.

homologous with the β-subunit of the non-parasite H^+-TH's. This has implications for the assembly of H^+-TH discussed in Section 7.

As exemplified with the *E. coli* H^+-TH, Fig. 2 shows the topology of this enzyme in relation to the electron transport system and ATPase. Presumably, the topology of the bovine [12,19], *Rhodospirillum rubrum* [29,30] and *Rhodobacter capsulatus* [22] enzymes are similar to that of the *E. coli* enzyme. Little is known about the topology of the *Eimeria* transhydrogenases, but apart from the reversed order of transcription there is nothing that indicates that their topology would differ from the majority of the H^+-TH's. Since the parasite of the *Entamoeba histolytica* type does not have mitochondria [27] the localization of H^+-TH in this organism is presently unclear.

Table 1 summarizes some key properties of H^+-TH's from different sources. The cloning and sequencing of the *E. coli* and bovine H^+-TH genes, and recently also the gene for the *Rhodospirillum rubrum* enzyme, have led to a considerable advancement in the characterization of these enzymes. Thus, the subunit compositions of the active enzymes have been elucidated in all three cases except for the *R. rubrum* enzyme which, however, may be assumed to be $\alpha 1_2 \alpha 2_2 \beta_2$, where $\alpha 1$ is loosely and reversibly bound to the remainder of the complex. Both the bovine and *E. coli* enzymes have a k_{cat} value of about 50–100 s^{-1}, a value range which probably is typical for most H^+-TH's. The parasitic enzymes are not included in Table 1 mainly because their biochemical properties have not yet been sufficiently characterized.

As shown in Table 1, the value of H^+/H^-,or '*n*' in Eq. (2), has been determined to be 1.0 in the case of the bovine H^+-TH and 0.5 in the case of the *Rhodobacter capsulatus* enzyme. It is unlikely that the true '*n*' values for the two enzymes are different, which means that either of the two determinations, or both, are erroneous. Indeed, the methods used to arrive at the values were different. The bovine value was determined independently by Earl and Fisher [7] and by Rydström and co-workers [8,33], in both cases employing pure enzyme reconstituted in phospholipid vesicles. Jackson and co-workers [40] used intact membranes from *Rhodobacter capsulatus* and an indirect method based on absorbance changes of endogenous carotenoid pigments. Recently, Jackson and co-workers (T. Bizouarn, L.A. Sazanov and J.B. Jackson, unpublished data), using a pH-indicator dye (Cresol red) to monitor proton translocation on a rapid time-scale, measured values of the H^+/H^- in the region of 1.0 with chromatophore membranes isolated from an over-expressing strain of *Rhodospirillum rubrum* and with liposome membranes inlaid with mitochondrial H^+-TH. Even though it is generally easier for obvious reasons to obtain too low a ratio, a recent demonstration that movements of protons along the membrane surface are considerably faster than the equilibration between membrane surface protons and the bulk solute [41] may indicate a source of underestimation of the H^+/H^- ratio in complex systems. In fact, this is the first independent support for a localized interaction between proton pumps originally suggested by Ernster and coworkers on the basis of the energetics of the ATP synthase and H^+-TH systems [42,43]. However, there is a large and increasing body of evidence in favour of a delocalized interaction of H^+-TH with other proton pumps (cf. [9,16–19,44,45]).

As is evident from Fig. 2, H^+-TH competes with, for example, the ATP synthase for the available Δp generated by electron transport when the direction of the transhydrogenase reaction is that of NADPH generation. The transhydrogenase reaction may also be reversed so as to generate, for instance, ATP, but only at a very high $[NADPH][NAD^+]/[NADH][NADP^+]$ ratio [46]. Despite repeated attempts, reconstituted transhydrogenase-ATPase vesicles have not been found to generate ATP under similar conditions, possibly because of the presence of uncoupled/excess ATPase in these preparations (G. Eytan and J. Rydström, unpublished data). In this context, the number of protons being pumped/translocated in the transhydrogenase reaction is of course essential [20,21]. If the value is 0.5, H^+-TH is a relatively poor pump which is locked into a state of NADPH generation. However, experimentally it has been shown repeatedly that H^+-TH nevertheless is an efficient consumer of Δp, much more efficient than, for instance, ATP synthase when the two systems compete for the same Δp [14,47,48]. If '*n*' equals 1.0, a more potent pump is obtained which may contribute

Table 1
Properties of H^+-TH's from different sources

Source	Subunits			Active form	k_{cat} (s^{-1})	H^+/H^-	Refs
	Mol. mass (kDa)						
Bovine heart	109			dimer	60	1.0	[7,8,12,33–36]
E. coli	α		β	$\alpha_2 \beta_2$	100	n.d.	[10,37,38]
	54		48				
Rhodosp. rubrum	α1	α2	β	$\alpha 1_2 \alpha 2_2 \beta_2$?	n.d.	n.d.	[29,30]
	40	15	48				
Rhodob. caps.	53		48	n.d.	n.d.	0.5	[39,40]

substantially to the redox level of NADP, as well as generate a Δp under conditions when the activities of other proton pumps are decreased. This is of course provided that the [NADPH][NAD^+]/[NADH][$NADP^+$] ratio is sufficiently high, since this ratio is the only input of free energy. However, under normal cellular conditions it is assumed that H^+-TH is a device that basically converts NADH into NADPH at the expense of a Δp. The recent interesting suggestion by Sazanov and Jackson [21] that H^+-TH regulates the flow of substrates mainly through the NAD^+ and $NADP^+$-dependent isocitrate dehydrogenases, has provided another theoretical function which may be subjected to an experimental test. One observation that apparently does not fit the latter suggestion is that the Δp-driven H^+-TH is rapidly saturated at increasing Δp and, unless NADPH is rapidly oxidized by other systems, probably relatively inactive at, for example, State 4 due to product inhibition [20]. Indeed, this may be the reason why it has been difficult in the past to estimate transhydrogenase activity in intact mitochondria using redox couples [43], or bacteria [49]. However, as indeed pointed out by Hoek and Rydström [20] and by Sazanov and Jackson [21], the enzyme may well have different functions depending on the type of cell/organism in which it is located. An interesting method to investigate the role of H^+-TH in mammals is that of gene knock-out, which is presently being pursued in our laboratory. The role of H^+-TH in parasites, especially the mitochondria-deficient *Entamoeba histolytica*, also poses a challenge and may indeed involve a separate function.

3. Cloning, expression and mutagenesis

The first gene, encoding *E. coli* H^+-TH, was isolated by Clarke and Bragg [10]. They grew each of the 2112 clones in the Clarke and Carbon colony bank and screened for elevated levels of H^+-TH activity. Three such clones were identified, subcloned into pUC13 and cut down to the smallest possible fragment expressing H^+-TH by use of restriction enzymes and exonuclease *BAL*31. The final construct, pDC21, contains the EcTH gene inserted into the *Sst*I-*Hin*cII sites of pUC13 and overexpresses, under control of the *lac*Z promoter, H^+-TH activity 70-fold in the inner membrane of *E. coli*. The EcTH gene was shown to be located in the 35.4 min region of the *E. coli* genome previously mapped as the locus for the EcTH gene [50]. Sequencing of the gene and its products, i.e., the α-and β-subunits which probably are co-transcribed, proved the gene to be coding for *E. coli* H^+-TH [11]. The EcTH gene was also expressed from a pGEM-7Zf(+) vector (also *lac*Z promoter) at similar levels [51]. This construct, pSA2, and the pDC21 construct have been used extensively for site-directed mutagenesis and expression studies as described below.

Yamaguchi et al. [12] isolated two partially overlapping cDNA clones (λTH36-1 and λTH32-1) encoding bovine mitochondrial H^+-TH by screening a bovine λgt10 [genomic] cDNA library with two synthetic oligonucleotides and a cDNA restriction fragment as probes. Synthetic oligonucleotides were designed from the sequences of two tryptic fragments of purified bovine mitochondrial H^+-TH. Both the N-terminal sequence and the presequence were determined from sequenced mRNA after reverse transcription [52]. The complete gene encoding bovine mitochondrial H^+-TH was constructed by Holmberg et al. [53] from the partially overlapping cDNA clones mentioned above. The eight missing N-terminal amino acids were added together with a consensus ribosome binding site and this construct was put under control of the T7 or the *lac*Z promoter. However, these constructs, pUGO4 and pUGO5, respectively, failed to express detectable levels of H^+-TH. Resequencing of the bovine gene showed that the previously reported sequence [12] contained three errors; Phe-591, Glu-777 and Arg-782, which were identified as Ala-591, Val-777 and Ala-782, respectively [53]. These changes are important, since they eliminate two charged amino-acid residues in the predicted transmembrane region [53]. Adams et al. [31] reported that the bovine enzyme shows 93% identity to an 'expressed sequence tag' (86 amino acids) from human brain. The method used by Adams et al. [31] for rapid characterization of vast numbers of expressed genes by partial DNA sequencing will most likely be very useful also for cloning of new H^+-TH genes.

Three H^+-TH genes were recently isolated from different protozoan parasites: *Eimeria acervulina*, *Eimeria tenella* and *Entamoeba histolytica*. The *Eimeria* genes were isolated through immunoscreening of λgt10 and λgt11 cDNA libraries constructed from mRNA isolated from *Eimeria* oocysts [25,26]. The *Entamoeba histolytica* H^+-TH gene was isolated from genomic and cDNA λZAP II libraries through hybridization with a radioactive PCR-fragment by using primers in the PCR derived from amino acid sequences in two highly conserved regions of H^+-TH [27]. These three H^+-TH genes share the somewhat surprising feature that they are assembled in a $\beta \Rightarrow \alpha$ order, in contrast to the $\alpha \Rightarrow \beta$ order of the *E. coli*, bovine and *R. rubrum* genes (cf. Fig. 1 and Section 7). The *Eimeria* enzymes show 91% mutual identity, 40% identity to the bovine enzyme and 45% identity to the *E. coli* enzyme. The *Entamoeba histolytica* enzyme shows 37% overall identity with *E. tenella*, 46% with *E. coli* and 42% identity with the bovine enzyme. No expression studies on the three protozoan H^+-TH genes have been reported.

Cloning and sequencing of H^+-TH from *Rhodospirillum rubrum* was recently reported by Williams et al. [29] and by Yamaguchi and Hatefi [30]. They constructed degenerate oligonucleotides from the N-terminal amino acid sequences of the soluble part (TH_s) and proteolytic fragments thereof which were used as PCR primers. This PCR-fragment was checked by sequencing and was then used for screening a pBR322 library of *R. rubrum* DNA.

Positive clones were restriction enzyme mapped and compared to a map of the PCR-fragment before they were subcloned into pUC18 and sequenced. Alignment of the *R. rubrum* sequence with the *E. coli* and bovine sequences indicated 50% and 43% identity, respectively. The gene corresponding to the α-subunit of *E. coli* H^+-TH proved to be split into two parts, the former (*PntAA*) coding for the soluble domain α1 and the latter (*PntAB*) coding for the membrane bound domain α2 (cf. Fig. 1). The product of the third gene (*PntB*), the β-subunit, corresponds to the β-subunit of *E. coli*.

So far, only *E. coli* transhydrogenase has been reported to be overexpressed. However, the *Rhodospirillum rubrum* pntAA H^+-TH gene has recently been overexpressed as a dimer, is reconstitutively active and has a single unique tryptophan (Trp-72) whose very short wavelength emission

Table 2
Mutants of *E. coli* H^+-TH and the effects of the mutation on catalytic and proton-pumping activity

Mutant	Cat. act. (%)	H^+ act. (%)	Ref.	Comment
Wt	100	100		wild type
αY226H	33/51	n.d.	57	NAD(H)-binding region
αY226L	38	n.d.	57	NAD(H)-binding region
αY226F	45	n.d.	57	NAD(H)-binding region
αY226N	42	n.d.	57	NAD(H)-binding region
βY315H	28	n.d.	57	NADP(H)-binding region
βY315I	4	n.d.	57	NADP(H)-binding region
βY315F	62	n.d.	57	NADP(H)-binding region
βY315D	6	n.d.	57	NADP(H)-binding region
βY315N	25	n.d.	57	NADP(H)-binding region
βY315V	4	n.d.	57	NADP(H)-binding region
βY315L	13	n.d.	57	NADP(H)-binding region
βY431H	2/5	n.d.	57	NADP(H)-binding region
βY431L	6	n.d.	57	NADP(H)-binding region
βY431F	60/80	n.d.	57	NADP(H)-binding region
βY431N	4	n.d.	57	NADP(H)-binding region
βY431I	4	n.d.	57	NADP(H)-binding region
βG314E	0	0	57,	NADP(H)-binding region
βY315F,βY431F	62	n.d.	57	NADP(H)-binding region
αY226F,βY315F,βY431F	50	n.d.	57	NADP(H)-binding region
αD232N	45	67	58	DCCD-reactive
αD232E	89	110	58	DCCD-reactive
αD232K	75	65	58	DCCD-reactive
αD232H	84	98	58	DCCD-reactive
αE238Q	71	18	58	DCCD-reactive
αE238Q	44	27	58	DCCD-reactive
αD232N,αE238Q,αE240Q	55	59	58	DCCD-reactive
αD232H,αE238Q,αE240Q	44	51	58	DCCD-reactive
αH450T	17	51	58	possible H^+ pathway
αR502S	71	93	58	possible H^+ pathway
βE82Q	63	85	58	possible H^+ pathway
βE82K	79	66	58	possible H^+ pathway
βH91S	6	8	58	possible H^+ pathway
βH91T	2	8	58	possible H^+ pathway
βH91C	3	0	58	possible H^+ pathway
βH161S	90	100	58	possible H^+ pathway
βH161T	88	108	58	possible H^+ pathway
βH161C	59	94	58	possible H^+ pathway
βD213N	92	44	58	possible H^+ pathway
βD213H	82	34	58	possible H^+ pathway
βC260S	78	57	58	possible H^+ pathway
αG172E	2	2	59	NAD(H)-binding region
αG174A	90	58	59	NAD(H)-binding region
αG174S	28	24	59	NAD(H)-binding region
αG174C	1.5	4	59	NAD(H)-binding region
αE210V	87	97	59	NAD(H)-binding region
αE213K	140	105	59	NAD(H)-binding region
αE210Q,αE213Q	97	99	59	NAD(H)-binding region
αE210V,αE213D	93	100	59	NAD(H)-binding region

Catalytic activity was assayed as reduction of $AcPyAD^+$ by NADPH [57–59] and proton pumping as initial rates of quenching of quinacrine or ACMA [57–59].

is sensitive to binding of NADH, allowing on and off rate constants to be determined (Diggle, C., Hutton, M., Jones, G.R., Thomas, C.M. and Jackson, J.B., unpublished data). Several other attempts to clone H^+-TH genes are in progress, e.g., the mouse and *Klebsiella pneumoniae* genes. The latter bacterium was recently shown to contain a transhydrogenase, most likely an H^+-TH but possibly Na^+-pumping, which cross-reacts with polyclonal antibodies raised against the bovine H^+-TH [54].

The effects of substituting various amino acids in *E. coli* H^+-TH on the catalytic and proton pumping activities are summarized in Table 2. Mutants were produced based on PCR methodology or single-stranded phagemid DNA [55], all of which were sequenced using the dideoxy chain termination method [56]. Most of the mutations in or close to the active NAD(H) and NADP(H) sites affected the affinity for the substrates [57–59]. Some mutations affected proton pumping. The properties of these mutants are described in the following sections.

4. Sequence homologies with other proteins

The first H^+-TH amino acid sequence published by Clarke and Bragg [11] revealed a putative NAD(P)-binding sequence but no residues possible involved in proton translocation. Naturally, the predictive strength increased dramatically when the bovine sequence was subsequently published by Hatefi and coworkers [12], since conserved residues now could be identified with some certainty. The most striking similarities concerned those sequences that conceivably are involved in substrate binding, i.e., $\beta\alpha\beta$ or Rossman folds containing the consensus sequence GXGXXG [60–62]. Four such folds were identified in the sequence regions 182–232, 230–280, 880–937 and 994–1043 of the bovine enzyme [12,24] and the corresponding regions of the *E. coli* enzyme [11,24]. These sequences were denoted sequences 1–4 (Table 3). Even though the substrate-binding sites of the bovine or *E. coli* H^+-TH at this time had not been definitely identified, sequence homologies between these regions and those of various dehydrogenases and reductases (Table 3) indicated that the two N-terminal (in the α-subunit of the *E. coli* H^+-TH) and the two C-terminal (in the β-subunit of the *E. coli* H^+-TH) sequences of the bovine enzyme corresponded to possible NAD(H) and NADP(H)-binding sites, respectively [24]. By using FSBA, an unspecific label for nucleotide-binding sites, Hatefi and co-workers [12] attempted to identify the substrate-binding sites by exposing purified bovine H^+-TH to the label in the absence and in the presence of either of the substrates NADPH or NADH. The result was that sequences 2 (Tyr-245) and 4 (Tyr-1006) were labelled [12], and these were therefore suggested to form part of the NAD(H) and NADP(H)-binding sites, respectively [12]. Unexpectedly, site-specific labelling of the bovine enzyme with 8-azido-AMP, constituting part of the substrate NAD(H), proved to label sequence 4 assumed to be an NADP(H)-site [24]. Because of the lack of reducible (pros-

Table 3
Amino acid homologies between the beef heart and *E. coli* H^+-TH and various NAD(P)/flavine-dependent enzymes (from Ref. [24])

ENZYME	A. NAD(H)-binding sites
TH-BOV 1 (aa182-232)	VPPAKILIV-**GGG**V**A**G**L**ASAGAAKSMG-A-IVRGF**D**TRAAALEQFKSLG
TH-ECOLI 1 (A,aa163-213)	VPPAKVMVI-**GAG**V**A**G**L**AAIGAANSLG-A-IVRAF**D**TRPEVKEQVQSMG
GSH HUMAN (FAD)	VASYDYLVI-**GGG**S**G**G**L**ASARRAAELG-A-RAAVV**E**SHKLGGTCVNVGC
GLYCERALDP DH PIG	---VKVGVN-**GFG**R**I**G**R**LVTRAAFNSGKVDIVAIN**D**PFIDLHYMVYMFQ
LIPOAMID DH HUMAN	KVPEKMVVI-**GAG**V**I**G**V**ELGSVWQRLG-AD-VTAV**E**FLGHVGGVGIDME
LIPOAMID DH PIG	KVPEKMVVI-**GAG**V**I**G**V**ELGSVWQRLG-AD-VTAV**E**LLGHVGGIGIDME
LDH PIG M	VPHNKITVV-**GVG**A**V**G**M**ACAISILGKELADEIALV**D**VMEDKLKGEMMDL
TH-BOV 2 (aa230-280)	PLEVDVKES-**GEG**Q**G**G**Y**AKEMSKEFIEAEMKLFALQCKEVDILISTALI
TH-ECOLI 2 (A,aa211-221)	FLELDFKEEA**G**S**G**D-**G**YAKVMSDAFIKAEMELFAAQAKEVDIIVTTALI

ENZYME	B. NADP(H)-binding sites
TH-BOV 3 (aa880-937)	EANSIIITP**G**Y**G**L**C**AAKAQYPI**A**DLVKMLSEQ**G**KKVRFGI**H**PVAG-**R**MPGQLNVLLAEA
TH-ECOLI 3 (B, aa305-362)	NSHSVIITP**G**Y**G**M**A**VAQAQYPV**A**EITEKLRAR**G**INVRFGI**H**PVAG-**R**LGGHMNVLLAEA
GSH HUMAN	ELPGRSVIV**GAG**Y**I**AVEM----**A**GILSAL---GSKTSLMI**R**HDKVL**R**SFDSMISTNCTE
GSH ECOLI	ALPERVAVV**GAG**Y**I**AVEL----**A**GVINGL---**G**AKTHLFV**R**KHAPL**R**SFDPMISETLVE
THIORED RED ECOLI	YRNQKVAVI**G**G**G**NT**A**VEE----**A**LYLSN----IASEVHLI**H**RRDGF**R**AEKILIKRLMDK
TRYPT RED	EPPRRVLTV**GGG**F**I**SVEF----**A**GIFNAYKPV**G**GKVTLCY**R**NNPIL**R**GFDYTLRQELTK
MERC RED SHIG	TIPKRLAVI**G**S**S**VV**A**LEL----**A**QAFARL---G**A**KVTILA**R**STLFF**R**E-DPAIGEAVTA
MICR MONO RABBIT	FKDKRVLVV**G**MGNS**G**TDI----**A**VEASHV---**A**KKVFLST
TH-BOV 4 (aa994-1043)	QVIVMKRSL**G**V**G**YA**A**VDVPIFY**K**PNTAMLLGD**A**KKTCDALQA-KV-RESYQHCOOH
TH-ECOLI 4 (B,aa419-462)	NVIVFKRSMNT**G**YA**G**VQNPLFF**K**ENTHMLFGD**A**K---ASVDA--IL**K**ALCOOH

thetic) groups in H^+-TH, the two substrates NAD(H) and NADP(H) have to interact directly at least during catalysis. Consequently, the NAD(H) and NADP(H) binding sites have to be located very closely. Presumably, the latter labelling was therefore a result of binding of 8-azido-AMP to the NAD(H) site, but because of the close location of the NADP(H) site and the nearby reactive Tyr-1006, it reacted with this residue. It was later shown by site-specific mutagenesis with *E. coli* H^+-TH that the corresponding residues αTyr-226 and βTyr-431 are not essential [57]. However, the β-subunit of *E. coli* H^+-TH binds immobilized NAD^+ ([63]; Glavas, N.A. and Bragg, P.D., unpublished data) and it is therefore also possible that, at least in *E. coli* H^+-TH, the β-subunit indeed contains an NAD(H)-binding site which may be catalytically inactive.

By using equilibrium binding of radiolabelled substrates, Hatefi and coworkers showed that sequence(s) 1 and/or 2 binds NADH and sequence(s) 2 and/or 4 binds NADPH at a ratio of 1 mol substrate/dimer [28]. Thus, there is now little doubt that the N-terminal half of the bovine enzyme (α-subunit of the *E. coli* H^+-TH) harbours the NAD(H)-binding site and that the C-terminal half (β-subunit of the *E. coli* H^+-TH) harbours the NADP(H)-binding site. However, recent multiple alignment analyses of H^+-TH indicate that the earlier conclusions based on covalent labelling of tyrosine residues regarding the locations of the active NAD(H) and NADP(H) binding sequences in sequences 2 and 4 probably are erroneous [24,59]. Table 4 shows an alignment of sequence 1 with those of several other nucleotide-dependent enzymes. As will be discussed in depth in Section 5, sequence 1, rather than 2, gives the correctly 3D-predicted

Table 4
Sequence comparison of ADP binding of $\beta a \beta$ folds of category I dinucleotide binding protein

Source	Sequence	Reference	
	<-βA-> <-----αB-----> <-βB->		
	^¤ ¤ * * * ¤ ¤ ¤ ¤ ●		
	194		
ADH (NAD)	TCAVFGLGGVGLSVIMGCKAAGAA-RIIGVDI	Jornvall, 1970	[64]
	22		
LDH (NAD)	KITVVGVGAVGMACAISILMKDLADEVALVDV	Taylor, 1977	[65]
	2		
GPD (NAD)	KIGIDGFGRIGRLVLRAALSCGAQ-VVAVNDP	Davidson et al., 1967	[66]
	4		
PHBH (FAD)	QVAIIGAGPSGLLLGQLLHKAGI--DNVILER	Weijer et al., 1982	[67]
	22		
GRS (FAD)	DYLVIGGGSGGLASARRAAELGA--RAAVVES	Krauth-Sieghel et al., 1982	[68]
	167		
EcTH (NAD)	KVMVIGAGVAGLAAIGAANSLGA--IVRAFDT	Ahmad et al., 1992	[51]
	186		
BTH (NAD)	KILIVGGGVAGLASAGAAKSMGA--IVRGFDT	Holmberg et al., 1994	[53]
	174		
RrTH (NAD)	RVLVFGVGVAGLQAIATAKRLGA--VVMATDV	Williams et al. 1994	[29]
	665		
EtTH (NAD)	KVFVIGAGVAGLQAISTAHGLGA--QVFGHDV	Kramer et al., 1993	[26]
	205		
EaTH (NAD)	KVFVIGAGVAGLQAISTAHGLGA--QVFGHDV	Vermeulen et al., 1993	[25]
	^¤ ¤ * * * ¤ ¤ ¤ ¤ ●		
	<-βA-> <-----αB-----> <-βB->		

The fingerprint amino acids in the fold are indicated at the bottom. Underlined residues have deviating properties. "□" indicates hydrophobic residues, "*" the dinucleotide GXGXXG consensus sequence, ^ polar residues, and ● acidic residues. ADH represents alcohol dehydrogenase; LDH, lactate dehydrogenase; GPD, glyceraldehyde-3-phosphate dehydrogenase; PHBH, *p*-hydroxybenzoate hydroxylase; GRS, glutathione reductase; *EcTH*, *E. coli* transhydrogenase; *BTH*, bovine transhydrogenase; *RrTH*, *Rh. rubrum*; *EtTH*, *E.tenella* transhydrogenase; *EaTH*, *E. acervulina* transhydrogenase. Numbers within brackets indicate reference numbers (Refs. [25,26,29,51,53,64–68]).

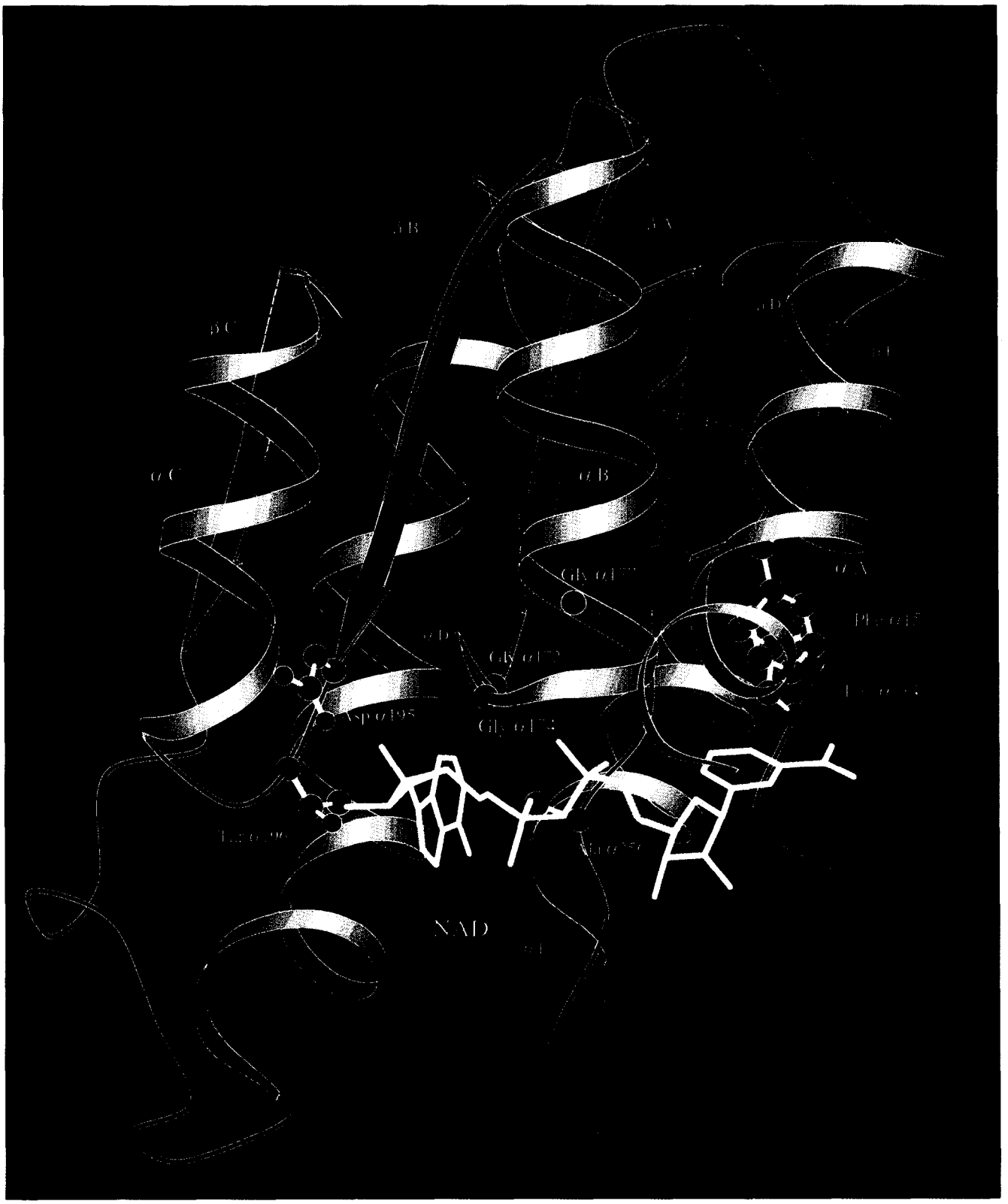

Fig. 3. A representation of the NAD binding domain of *E. coli* H^+-TH. The picture was prepared using the MOLSCRIPT package [74], and is a computer-derived model based on comparisons with fingerprint sequences of homologues NAD/FAD-dependent enzymes with known 3D structures. The nomenclature is that used by Eklund and Bränden [80]. The β-strands are shown as blue shaded arrows and the α-helices as yellow shaded ribbons. The loops extending from βA and βD which create the switchpoint are shown in red. The NAD substrate molecule is displayed as sticks, and residues involved in interactions with NAD(H) are shown with ball and sticks. From Ref. [59].

$\beta\alpha\beta$-fold of the NAD(H) site. In this context it must be emphasized, however, that the $\beta\alpha\beta$ fold only binds the pyrophosphate moiety of the dinucleotide and that a dinucleotide domain covers approximately 150–200 residues [69], which means that e.g both of the sequences 3 and 4 theoretically may form part of the NADP(H)-binding domain. Nevertheless, judging from other dehydrogenases/FAD enzymes, there can only be one $\beta\alpha\beta$-fold per dinucleotide site [69], which raises the very interesting question; what are the roles of the $\beta\alpha\beta$-like folds in sequences 2 and 4? Since the β-subunit of *E. coli* H^+-TH binds to immobilized NAD^+ ([63]; Glavas, N.A. and Bragg, P.D., unpublished data) one possibility is that they represent inactive remnants of gene-duplicated nucleotide-binding sites in the fusion precursor of this domain. It is intriguing that the counterparts of the two reactive tyrosines (Tyr-245 and Tyr-1006) in bovine H^+-TH are also found in the β-subunit of F_1-ATPase as part of a 2-azido-ATP and FSBI-reactive site [24].

The resolution of the three-dimensional structure of F_1-ATPase from bovine heart [70] has established the coordinates for the βTyr-345 reacting with 2-azido-ATP or FSBI bound at the catalytic site, and for the βTyr-368 reacting with these agents when they are bound at the non-catalytic site [71]. Interestingly, the sequences containing the 2-azido-ATP/FSBI-reactive βTyr-345 in F_1-ATPase and the 8-azido-AMP-reactive βTyr-431 in *E. coli* H^+-TH are similar. However, H^+-TH's do not contain the so-called P-loop motif GXXXXGT/S.

A very interesting development was the recent demonstration that the sequence of alanine dehydrogenase from *Bacillus* is homologous with that of the N-terminal half of the bovine H^+-TH and the α-subunit of *E. coli* H^+-TH [22,23]. Alanine dehydrogenase catalyses the reversible reductive amination of pyruvate to alanine. However, the soluble α1-subunit of *Rhodospirillum rubrum* does not function as an alanine dehydrogenase [22]. There is only one NAD(H)-binding site and therefore one $\beta\alpha\beta$-fold in alanine dehydrogenase, which is almost identical with that located in sequence 1. Delforge et al. [23] speculated that a putative H^+-TH of *Mycobacterium tuberculosis* may have a role in the virulence mechanism of this important bacterium.

The consensus sequence GXGXXG for an NAD(P)-bi-

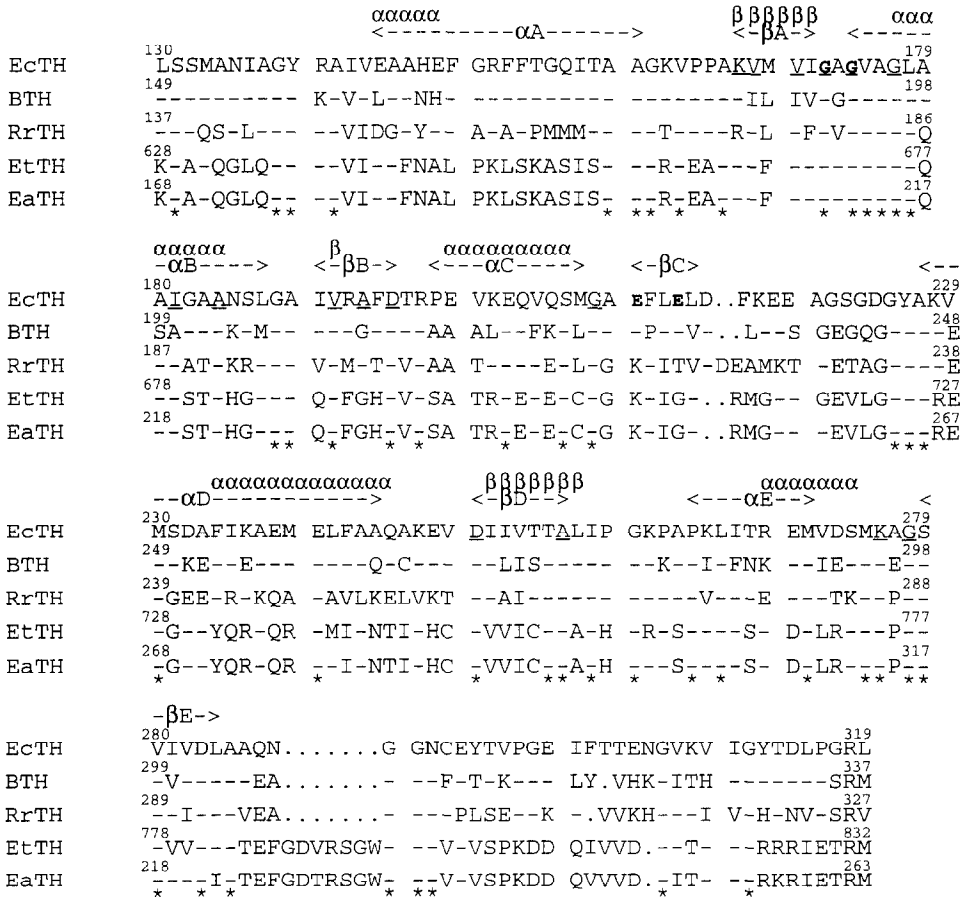

Fig. 4. Alignment of amino acid sequences of transhydrogenases from five different sources. Residues identical to the corresponding amino acids in the *E. coli* sequence are represented as dashes. Dots indicate gaps. Underlined residues are structurally important or involved in NAD(H) binding. Stars denote positions with totally conserved residues. The prediction of α-helices and β-sheets are shown within arrows.

nding site can vary and there are a number of different structural requirements for an NAD-site and an NADP-site [59]. Both the spacer 'X' can be extended to, for example, GXXGXXG or GXXXGXXG [72], and especially the first and the last glycine can be exchanged for, e.g., alanine, or asparagine in the case of the first glycine [62,73]. In contrast to the NAD(H)-binding domain, the putative NADP(H)-binding domain in sequence region 3 shows fewer conserved/identical residues with other NADP-dependent enzymes and reductases. This is simply because considerably fewer primary structures of this type have been solved. Despite this limitation sequence region 3 fulfils all requirements for an NADP(H)-site, i.e., the consensus sequence of the $\beta\alpha\beta$-fold is GXGXXA/V instead of GXGXXG, and it contains the two His-920 and Arg-925 residues downstream the GXGXXA/V sequence of the bovine enzyme stabilizing the acidic 2′-phosphate moiety [24]. Although sequence region 4 has NxGxxG, which is acceptable for an NAD(P)-binding site, it lacks at least two of the essential residues downstream from the NXGXXG [24].

Using the more flexible definition of an NAD(P)-binding site, e.g., GXXXGXXG, there is in fact a third possible binding sequence, sequence 3b, in the region 719–826 (bovine H^+-TH), with the sequence GALIGSSG. However, again, this sequence lacks the essential basic residues downstream stabilizing the 2′-phosphate moiety. Just as in the case with sequences 1 and 2, the possible role(s), if any, of the sequences 3b and 4 remains to be clarified.

As will be discussed in Section 6, the predicted transmembrane sequences are apparently not homologues with those of other known membrane protein sequences.

5. The extended $\beta\alpha\beta$-folds of the catalytic NAD(H) and NADP(H) sites

5.1. The NAD(H) binding site

To elucidate the mechanism for the coupling between NADPH production and proton gradient utilization at the molecular level, structural knowledge is essential. Also, the more structural information available the greater is the opportunity to fine tune further experimental work, i.e., mutagenesis studies. Therefore, a model of the NAD binding site with *E. coli* H^+-TH as an example has been constructed [59]. The result of the three-dimensional prediction is presented in Fig. 3 prepared with the MOLSCRIPT [74] package. Structurally important residues and amino acids involved in substrate binding that are described in this section can be localized in Fig. 4. The following methods have been used for prediction: the primary structures of the H^+-TH's from *E. coli* [11], bovine [12,53], *Rh. rubrum* [29,30], and the putative transhydrogenases from *E. tenella* [26] and *E. acervulina* [25] were aligned, and conserved features were compared to sequences of structurally solved enzymes that use NAD as coenzyme or substrate; hydrophilicity profiles according to Kyte and Doolittle [75] and secondary structure predictions according to Chou and Fasman [76], Garnier et al. [77] and mainly PHD [78] aided in the assignment of the different secondary structure elements in the open twisted α/β structure.

This study supports previous suggestions that the monomeric transhydrogenase contains one NAD binding site which is located in the soluble part of the α-subunit in *E. coli* transhydrogenase [24]. Each of the five transhydrogenases includes one stretch that exactly correlates to the Wierenga fingerprint [62,79] for ADP-binding $\beta\alpha\beta$ folds of category I dinucleotide binding proteins. In the *E. coli* enzyme, residues α167 to α196 correspond to this particular fold. The region contains the GXGXXG consensus sequence (α172 to α177) located in the N-terminal end of the dinucleotide binding helix αB. The first glycine provides for a tight turn between βA and αB, the second glycine is sterically important for the close binding of the pyrophosphate bridge in NAD to αB, and the third glycine determines the packing of αB to the β core [62]. The first two glycines in *E. coli* transhydrogenase have been exchanged by the method of site directed mutagenesis. The αG172E mutant yielded a catalytically inactive enzyme as did the αG174C mutant [59]. However, the αG174S was found to retain 30% catalytic activity and the αG174A mutant was almost as active as the wild type enzyme [59]. The observation that five out of seven 6-phosphogluconate dehydrogenases in the Swissprot protein database contain an alanine in the equivalent second glycine position is supportive of the partial activities found in the latter two mutants.

An acidic residue located in the end of βB is almost invariably present in enzymes binding NAD. The role of this residue is to, via its carboxyl group, form one or two hydrogen bonds to one or both hydroxyl groups in the adenosine ribose moiety. In the *E. coli* enzyme this amino acid corresponds to Asp-195. A hydrophobic residue commonly follows the conserved acidic amino acid, and its function is to create half of a hydrophobic sandwich [80] in which the adenine part of the dinucleotide is embedded. αThr-196 is the only candidate for this function. The second half of the adenine sandwich is provided for by a residue located in the C- terminal end of βD.

The positions of helix αC and strand βC were deduced from the finding of a striking similarity between transhydrogenases and 29 alcohol dehydrogenases, three out of four formate dehydrogenases, and six sorbitol dehydrogenases extracted from the Swissprot database [59]. Namely, the thirteenth residue from the conserved acidic residue in the end of βB happens to be a glycine in all these sequences. The crystal structure of alcohol dehydrogenase from horse liver reveals that the function of this glycine is to terminate αC and initiate the turn to βC. Presumably, the situation is the same for transhydro-

genases. The assignment of residues αl98 to α207 to αC is also strongly supported by PHD secondary structure predictions for equivalent regions in all transhydrogenases.

αD, including residues α226 to α244, was mainly assigned based on secondary structure predictions, since this part varies quite extensively amongst the different NAD binding enzymes [59]. On the other hand, βD, the strand which together with βA create the switchpoint into which the dinucleotide is fitted, contains certain characteristic features: a charged residue at the N-terminal end of βD is prevalent; at least five of the six following residues are mainly hydrophobic and and are suggested to adapt almost invariably β-strand structure, and the sixth of these residues forms the second half of the adenine sandwich. The stretch α250 to α256 and corresponding regions in the four other transhydrogenases contain all the appropriate features (Fig. 4). Accordingly, αAla-256 is situated such as to complete the sandwich around the adenine moiety. The role of the highly conserved charged residue in the beginning of βD seems to be to establish an interaction, presumably often as a salt bridge, with a residue in the neighbouring βE for proper alignment of the two β-strands. Indeed, the transhydrogenases contain a totally conserved lysine just after a predicted α-helix and a couple of positions before a hydrophobic stretch predicted as a β-strand. This pattern is also observed in many dehydrogenases, such as formate, malate, and glyceraldehyde-3-phosphate dehydrogenase. It is noteworthy that an additional conserved feature is the presence of a glycine immediately preceding or initiating βE. The pocket provided for the nicotinamide part of the dinucleotide is partly created by hydrophobic residues extending from αA and βE. Here, αPhe-152 and αLeu-284 are tentatively suggested to form this pocket.

5.2. The NADP(H) binding site

According to experimental evidence, the soluble C-terminal domain of the β-subunit in *E. coli* H^+-TH accommodates the NADP(H) binding site [14–19,28] This part of the enzyme is particularly intriguing for two reasons. First of all, multiple sequence analysis of transhydrogenases from *E. coli*, bovine, *R. rubrum* and *E. tenella* reveals a 35% identity in this region compared to 26% in the full length protein. A spontaneous reflection about this fact is that the structure and function of this domain is especially important for the mechanism of the enzyme. Second, binding of the NADP(H) substrate is accompanied by a conformational change, as concluded from the variation in tryptic digestion patterns observed in the presence or absence of NADP(H) [63]. The mechanistic implication of this structural change has not been fully determined, but it has been suggested that the movement is coupled to proton translocation. However, the elucidation of the molecular details by which this occurs requires knowledge about the three dimensional structure.

Sequence comparisons with some NADP(H)-dependent enzymes have been presented by Hu et al. [24]. In addition to the proposal of a catalytic NADP(H) binding site centered around the dinucleotide binding consensus-like residues β314–β319, they suggested the presence of a noncatalytic dinucleotide site at β428–β433. The latter site is unlikely to bind NADP(H) for the following reasons: a little over 200 residues in the soluble C-terminal domain can hardly compose two dinucleotide binding sites; no conserved positive residues, that are commonly found to stabilize the 2′ phosphate group, are present downstream of the ill-defined and poorly conserved consensus sequence. The proposed catalytic site in transhydrogenase, however, show considerable similarities with those of other NADP-binding enzymes [24]. This indicates a resemblance in the tertiary folding arrangement. A nomenclature analogous to the one presented for the NAD(H) domain will be used in the following discussion. The alanine in the GXGXXA consensus sequence for NADP(H)-dependent enzymes that adopt a compact dinucleotide βαβ fold [73], such as glutathione, thioredoxin and trypanothione reductases, corresponds to valine in all transhydrogenases except for the bovine enzyme, where this residue is an alanine. The former valine, βVal-319 in *E. coli* H^+-TH, can be replaced with glycine without any change in catalytic or proton pumping activity (Ahmad, S., Glavas, N.A. and Bragg, P.D., unpublished data). Further differences include a longer αB helix which presumably comprises a bend at residues β323–β325. There are three major reasons for believing that the NADP(H) domain in transhydrogenases adopts a conformation that can be compared to the reductases mentioned above. First, the similarity in the consensus sequence is considerable and includes the two conserved glycine residues. Second, the PHD method yields strong β-strand secondary predictions at residues β309–β311 and β340–β344, immediately preceding and succeeding αB, respectively, and corresponding regions in all transhydrogenases (except for two residues in the *E. tenella* enzyme). Third, the positions downstream of βB include two conserved positively charged residues separated by four amino acids, which could well be responsible for the interactions with the 2′ phosphate group in the NADP(H) substrate. In addition, the mutations of βGly-314 to Glu, Ala, Val and Cys all yielded inactive enzymes incapable of performing the NADP(H)-induced conformational change [51].

6. Predicted secondary structures of the transmembrane domain

Following the cloning and sequencing of the DNA for *E. coli* H^+-TH [10,11], the transmembrane domain of this enzyme was proposed to involve a 14 α-helix structure [11]. This model was later refined to include 12 α-helices [51,63] and a similar model was recently suggested for the

Rhodospirillum enzyme [29,30]. The transmembrane domain of the bovine enzyme was proposed to contain 14 helices [12,81]. In the discussion below, possible transmembrane helices are numbered according to the 14-helix model of Hatefi and co-workers [12,81], which represents the maximal number of transmembrane helices regardless of the prediction algorithm applied. Thus, for example, helix 14 is denoted 14 even though there are only 12 helices in a particular model. It is also assumed that all proton-translocating transhydrogenases have a common membrane structure of the helix bundle type, which is the dominating structure for membrane proteins with known 3D structures (e.g., bacteriorhodopsin).

Because of the number of restraints the prediction of transmembrane regions of membrane proteins is considerably easier than that of hydrophilic domains. Several prediction algorithms exist today which partly are based on the same assumptions. However, a combination of several algorithms has recently proven more efficient in predicting transmembrane secondary structures, mainly α helices, as applied to H^+-TH [53]. In the case of the bovine H^+-TH and *E. coli* H^+-TH application of the various algorithms led to an elimination of 4 and 2 helices from the original 14 and 12 helix models, respectively, leading to two 10 helix models [53]. Recently, a novel prediction method was published based on weighted propensity values for multiply aligned sequences of hydrophobic core and terminal regions of potential transmembrane helices [85]. The latter method gave 12 helices, similar to a 12-helix model discussed previously [53] except that helix 14 also was extended to contain both helix 13 + 14 (Persson, B., unpublished data).

All of the above models are based mainly on the following biochemical information: (i) antibodies towards the N-terminal and the C-terminal hydrophilic domains as well as the peptide corresponding to the sequence positions 540–554 in bovine H^+-TH were all found to bind to the enzyme in submitochondrial particles but not in mitoplasts, indicating that these peptides are located on the matrix side of the membrane [81]; (ii) proteinase K hydrolyzes the Ala690-Ala691 bond of bovine H^+-TH in mitoblasts but not in submitochondrial particles, suggesting that this bond is located on the cytosolic side of the membrane [81]; (iii) considering the 'positive-inside rule' of Von Heijne [82–84] the strongly charged loop β150–166 (5 positive charges) of the *E. coli* H^+-TH (as well as bovine H^+-TH) is assumed to be located on the cytosolic side. In addition to prediction of α helices, predicted information regarding β-sheets, connecting loops and turns have been included in the modelling procedure.

The preferred predicted *E. coli* H^+-TH 10-helix model is shown in Fig. 5. In this model the helix numbering used in this text follows the original 14-helix bovine model (helix numbers in parentheses). The four helices in the

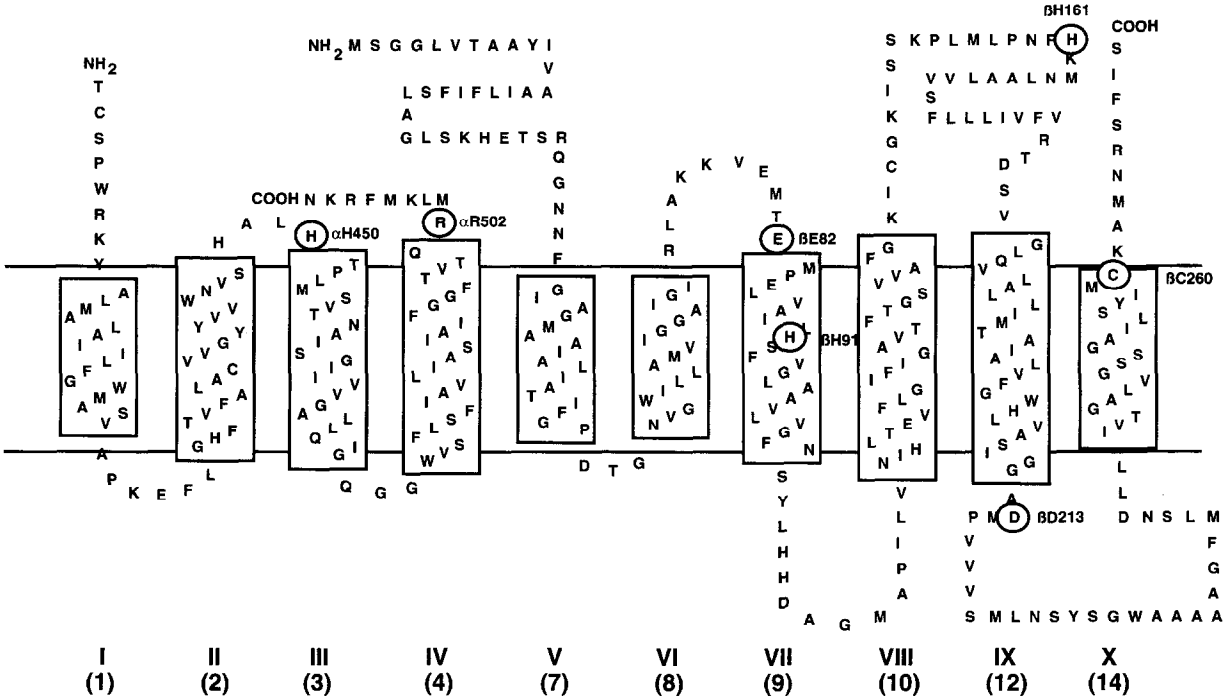

Fig. 5. Predicted membrane α helices of the *E. coli* H^+-TH. Circled residues have been subjected to mutagenesis (cf. Table 2). Upper side faces the cytoplasm and the lower side faces the periplasmic space. From Ref. [53].

α-subunit of the *E. coli* H^+-TH and in the corresponding regions of other transhydrogenases, except *Rhodospirillum rubrum* H^+-TH, are predicted with a high degree of probability. A single hydrophobic helix was predicted in the soluble α1-subunit of *Rhodospirillum rubrum* H^+-TH, but this helix is probably not a membrane helix because of the homology with the soluble alanine dehydrogenase [29]. In the α2-subunit of *Rhodospirillum rubrum* H^+-TH three helices are strongly predicted and one weakly [29]. In the β-subunit of *E. coli* H^+-TH, helix 10 with its C-terminal on the cytosolic side is very likely because of the proteinase K cleavage site in the bovine enzyme and the assumed distribution of charged residues on the cytosolic side. Because of the short loop connecting helix 9 and 10, this also means that the periplasmic location of the C-terminal of helix 9 is quite certain. The C-terminal location of the *E. coli* sequence corresponding to the antibody-binding site of helix 4 in the bovine enzyme, the location of the C-terminal of this helix on the cytosolic side, and the lack of helix 5 in *E. coli* H^+-TH (this sequence is missing in the *E. coli* enzyme) are certain. Helix 13 is likely to be included in an extended helix 12 or 14. This leaves helices 6 and 11, i.e., the ends of the domain corresponding to the β-subunit of *E. coli* H^+-TH as weakly predicted and they have therefore been removed. Another uncertainty is the sidedness of the N-and C-terminal residues. In analogy with the majority of other membrane proteins with reasonably well known topologies, it has generally been assumed that these residues are on the cytosolic side [53], despite the fact that examples with reverse topologies indeed do exist (e.g., Ref. [86]). Comparing the bovine and *E. coli* enzymes, it is in fact likely that the C-terminus of the α-subunit and the N-terminal of the β-subunit correspond to the sequence in the bovine H^+-TH that links these domains, and that both termini therefore would be located on the same side as the bovine sequence.

In addition to what already has been deduced from the bovine and *E. coli* H^+-TH's structures, the high extent of sequence identity in the predicted membrane helices of all H^+-TH's so far sequenced has not provided much new information derived from, for example, multiple alignments. From the above information it is therefore obvious that, although the structure of transhydrogenases may be modelled, the establishment of the true membrane structure requires a considerable effort. This is a major future task which of course will be carried out in parallel with attempts to crystallize transhydrogenases.

7. Assembly of transhydrogenases

The active forms of both the *E. coli* H^+-TH and bovine H^+-TH have been shown to be composed of $\alpha_2\beta_2$ [37] and a homodimer [12,35,87], respectively. As suggested by Jackson and co-workers [29] and others [25,26] transhydrogenases are transcribed in different orders and thus probably assembled differently. The *E. coli* and *Rhodospirillum rubrum* enzymes are transcribed in the order α, β and α1, α2 and β, respectively, from the N-to the C-terminal (cf. Fig. 1). However, using the nomenclature of the *E. coli* enzyme, the *Tenella* and *Entamoeba* enzymes are transcribed in the order β, α [25–27]. The various domains of transhydrogenases may be derived from different genes, i.e., using the *Rhodospirillum* enzyme as the most primitive enzyme and an ancestor of other transhydrogenases, domain α1 would be derived from an alanine dehydrogenase or similar protein, the α2 domain from a second protein, and the β domain from a third protein possibly involved in carbohydrate transport. The latter is indicated by the presence in the *Tenella* sequence [25] of a consensus sequence typical of proton-coupled carbohydrate (hexose) symporters with a 12-transmembrane α-helix structure [88,89]. At least part of the latter protein type may represent the proton-translocating function of transhydrogenases. The more advanced bovine H^+-TH gene would have been formed by fusion of the corresponding genes of the *Rhodospirillum* enzyme as they are ordered in this organism, whereas in *Tenella* and *Eimeria* the order has been reversed.

A systematic study of the assembly of the α-and β-subunits of *E. coli* H^+-TH has been carried out by Bragg and coworkers [90,91]. Deletion of the first two α-helices of the α-subunit from the N-terminal end abolished activity but not assembly although both subunits were loosely attached, whereas deletion of the two last helices proved that they are essential for both activity and assembly [90]. Deletion of one or several of the helices of the β-subunit resulted in a correct assembly but with loosely attached subunits, and loss of activity [90]. These and other results suggest that the α-subunit is first incorporated in the membrane and that subsequent the assembly of the β-subunit requires a correct or nearly correct assembly of the α-subunit. In contrast, the assembly of the α-subunit is independent of the β-subunit. The last 10 hydrophilic amino acids in the C-terminal of the α-subunit proved to be important for obtaining an active enzyme but not assembly. Presumably, a stretch of positively charged residues in the C-terminal of the α-subunit are important, i.e., at least one positive charge is required for assembly of the enzyme in a functional form [91]. However, a very interesting finding was that mutant enzymes that lacked activity but showed a relatively proper assembly, also showed a trypsin sensitivity of the β-subunit which is only seen in the wild-type enzyme in the presence of NADPH [63]. These results suggest that improper assembly of the α-subunit leads to a trypsin-sensitive conformational change of the β-subunit similar to that normally induced by NADPH. As will be discussed in the following sections, this also indicates that a particular conformation of the α-subunit may trigger a catalytically active conformation of the β-subunit, and vice versa, during the catalytic/proton pumping cycle. Because of the different

order of transcription of the *Tenella* H^+-TH, this type of transhydrogenase is probably assembled differently.

8. Charged residues potentially involved in proton pumping

Acidic and or basic residues located in the membrane domain are believed to constitute essential residues in proton pumps like F_1F_0-ATPases [92], bacteriorhodopsin [93,94] and pyrophosphatases [95]; membrane acidic residues in these proteins are usually reactive to DCCD. Charged residues are probably also essential for transporters like *lac*-permease [96]. The only conserved acidic residues in transhydrogenases are βGlu-82 and βAsp-213 (in *E. coli* H^+-TH) located close to the predicted helices 9 and 12, respectively (cf. Fig. 5). However, these residues were recently shown by site-specific mutagenesis not to be essential [53], and they are apparently not modified by DCCD. In contrast to F_1F_0-ATPases [92] and bacteriorhodopsin [93,94], DCCD-reactive residues in *E. coli* H^+-TH are located in the hydrophilic NAD(H)-binding domain and have been identified as αAsp-232, αGlu-238 and αGlu-240. These residues have also been replaced by site-specific mutagenesis and found not to be essential [58]. There are also DCCD-reactive residues in the β-subunit of *E. coli* H^+-TH [58], but these have not yet been identified. Indeed, in *E. coli* H^+-TH, DCCD apparently labels the β-subunit more than the corresponding region of the bovine H^+-TH (N.A. Glavas and P.D. Bragg, unpublished data), and in *R. rubrum* H^+-TH the main DCCD-reactive residues are located in the β-subunit [97].

Regardless of prediction algorithms used, the only positively charged residues located in a predicted membrane α-helix are βHis-91 and βHis-161. In fact, a more refined prediction [53] suggests that only βHis-91 is located in a transmembrane helix, namely helix 9 (cf. Fig. 5). Mutagenesis of these residues showed that βHis-161 is not essential, whereas βHis-91 is essential for both catalytic and proton-pumping activity [53]. Thus, the membrane domain of transhydrogenases only contains a single essential positive residue but no acidic residues, which makes transhydrogenases unique among proton pumps. Fig. 6 shows a model of helix 9 where βHis-91 is located close to the middle of the helix. Interestingly, the transverse surface of helix where βHis-91 is located is indeed conserved in all transhydrogenases and contains mostly smaller hydrophobic residues, which suggests that this surface has an important function. One of the relevant questions that can be asked in this context is what other residues the βHis-91 would interact with as part of a potential proton wire. Thus, again assuming a helix bundle structure, the only conceivable but hypothetical proton-carrying structure may be bound water molecules.

In addition to other residues, histidine(s) has previously been proposed to be involved in proton transport catalyzed

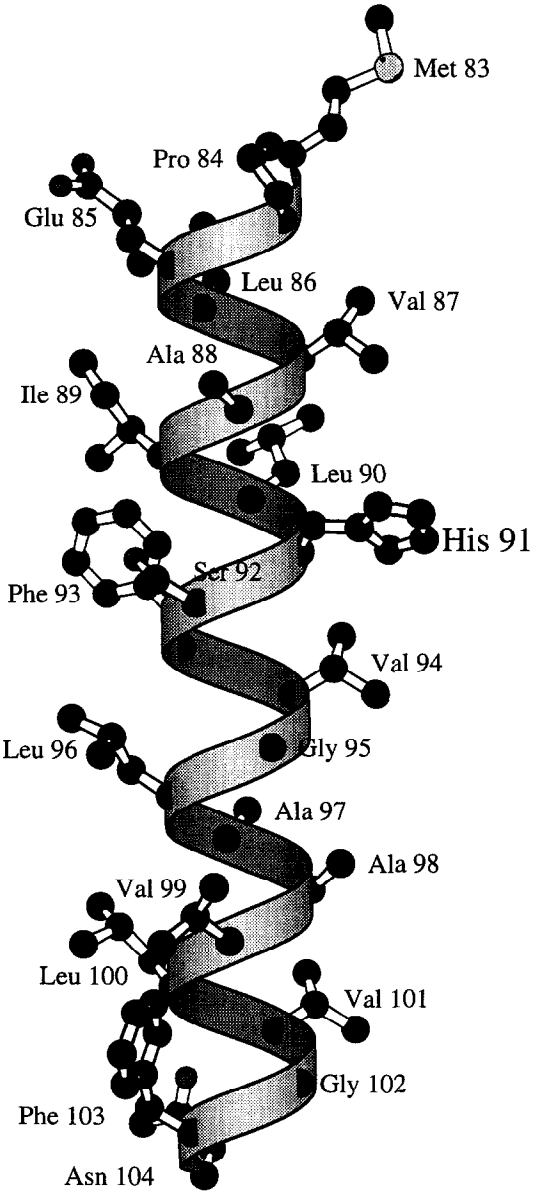

Fig. 6. A predicted model of helix 9 of the *E. coli* H^+-TH

by *lac* permease [96,98,99], in the photosynthetic reaction centre [100], and in the response of a Na^+/H^+ antiporter of *E. coli* to pH [101]. It was recently found that His-326 of the glutamate transporter from rat brain is the only charged membrane residue and this residue was shown by site-specific mutagenesis to be essential for transport [102]. His-326 is also conserved in the sodium-dependent transporter of certain neutral amino acids like serine, threonine and alanine, which also belongs to the glutamate transporter family [103,104]. In addition to the key role of histidines in these transport systems, the fact that an alanine transporter is included in this family is of obvious interest because of the similarity between the α-subunit of *E. coli* H^+-TH (and the corresponding domains of other transhydrogenases) and alanine dehydrogenase from *Bacillus* (cf. Sections 4 and 7).

9. Mechanism of redox-driven proton pumping

Several hypothetical mechanisms for proton-pumping catalyzed by transhydrogenases have been proposed in the past which have been amply reviewed [14–19]. Because of the fact that the difference in free energy between the substrates and products is negligible, all of these mechanisms involve substrate-induced conformational changes which are converted into a vectorial proton translocation. The extent of this translocation is a function of the substrate/product ratio and the number of protons translocated/product formed (cf. Section 2).

A role of a redox-active dithiol involving cysteine residues suggested previously to be involved in transhydrogenation/proton pumping [18,33] has been eliminated since the only conserved cysteine in transhydrogenases (βCys-260 in *E. coli* H^+-TH) is not essential as demonstrated by site-specific mutagenesis [53]. Also, the 2′-OH group of the ribose moiety of NAD(H) is probably not involved, since 2′-deoxy-NADH indeed does function as a substrate, although with a considerably lower affinity [59].

An interesting aspect of the coupling mechanism is the interaction between the NAD(H) and NADP(H)-binding domains of transhydrogenases. As represented by the *E. coli* H^+-TH these domains correspond to the α- and β-subunits. It was shown in the preceding section that a modification of the C-terminal of the α-subunit influenced the conformation of the β-subunit. In contrast, the availability of specific DCCD-reactive acidic residues in the NAD(H)-binding domain is influenced markedly by NADP(H) binding to the β-subunit [58]. Thus, these interactions between the substrate-binding domains are reversible and represent long-range conformational changes which may extend over at least 500 amino acid residues.

A substantial contribution to the understanding of the coupling mechanism of transhydrogenases was provided by the demonstration that NADH may reduce $AcPyAD^+$ (an analogue of NAD^+) in the presence of NADPH, catalyzed by either coupled bovine H^+-TH vesicles [105,106], DCCD-treated purified *Rhodobacter capsulatus* H^+-TH [97], or detergent-dispersed partially purified *E. coli* H^+-TH at a low pH [107,108]. On the basis of affinity changes of the enzyme for its substrates in the presence of an electrochemical proton gradient [14,16,17] reduction of $AcPyAD^+$ by NADH in the presence of NADPH was originally suggested to involve bound NADH and/or $NADP^+$ [106]. The corresponding reactions catalyzed by the *Rhodobacter capsulatus* H^+-TH [97] and the detergent-dispersed partially purified *E. coli* H^+-TH [107,108] were also suggested to involve bound NADP(H) (but not NADH) and Jackson and co-workers stressed that the reactions catalyzed by the bovine enzyme reconstituted in liposomes and the detergent-dispersed *Rhodobacter capsulatus* and *E. coli* enzymes were different [97,107,108]. DCCD was proposed to lead to an inhibition of the rate of release of $NADP^+$ and NADPH [97,107,108]. However, DCCD did not increase binding of NADPH to the bovine enzyme under equilibrium conditions [28]. Moreover, the affinity of the bovine H^+-TH for $AcPyAD^+$ was markedly decreased by the presence of an electrochemical proton gradient in reconstituted vesicles, whereas that for NADPH was unchanged [106]. Using purified *E. coli* H^+-TH reconstituted together with bacteriorhodopsin, it was recently shown that a Δp generated by light led to a strongly increased affinity and binding of NADH, whereas the corresponding properties for thio-NADP (an analogue of $NADP^+$) were unchanged [109]. Thus, there is evidence for an alteration of the NAD(H)-binding site of H^+-TH induced by Δp.

Recently, Hutton et al. [107] described a mechanism for transhydrogenization which was based on the pronounced pH-dependency of the reduction of $AcPyAD^+$ by NADH in the presence of NADP(H) and the effect of DCCD on this reaction. It was proposed that an acidic group 'X' is protonated when either $NADP^+$ or NADPH binds to the enzyme. In the NADH-enzyme-$NADP^+$ complex 'X' is exposed on the periplasmic space (outside) with a pK_a of < 5, whereas in the NAD^+-enzyme-NADPH complex 'X' is exposed on the cytosolic side (inside) with a high pK_a of 6–7. There are minor differences between this model and earlier models proposed by others workers in the field [14–19]. However, a new feature is that protonation of 'X' is dependent on bound NADP(H) (not NAD(H)) and that release of the proton is linked to the dissociation of

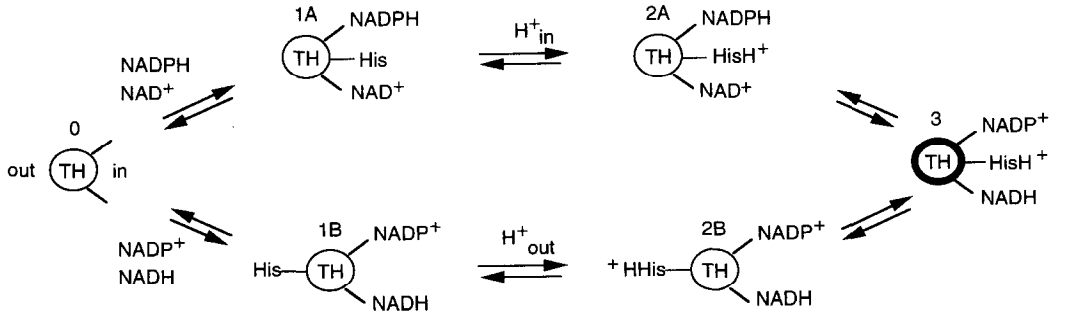

Fig. 7. A proposed mechanism of proton pumping catalyzed by H^+-TH. 'In' and 'out' denote the cytosolic and periplasmic space in bacteria, respectively. 'His' denotes βHis-91. The hydride ion transfer occurs between states 2A and 3, and state 3 (bold) represents a metastable state. Modified from Ref. [109].

NADP(H) (not NAD(H)). It is also stressed that the mechanism is fully reversible and that, therefore, the stimulation by Δp of the reduction of $NADP^+$ by NADH can be explained by the same mechanism.

The finding that βHis-91 constitutes the only charged and essential residue in the predicted membrane domain of transhydrogenases suggests a coupling mechanism where this residue undergoes an alternating cycle of rate-limiting protonation/deprotonation steps described by Hu et al. [109] in Fig. 7. Indeed, βHis-91 may be identical to residue 'X' in the model of Hutton et al. [107] described above. In the model of Hu et al. [109], NADH and $NADP^+$, or NAD^+ and NADPH, determine on which side of the membrane βHis-91 is exposed and also the pK_a of βHis-91. When NAD^+ and NADPH are bound it is proposed that βHis91 is directed to the cytosolic side and buried in the hydrophobic phase of the membrane thus increasing its pK_a. When NADH and $NADP^+$ are bound, it is proposed the βHis-91 is directed to the periplasmic space side and freely accessibly to the bulk solute giving a relatively normal pK_a. The interaction between substrate/product binding and βHis-91 is reversible, i.e., an extensive protonation of βHis-91 on the periplasmic side leads to an increased binding of the substrates NADH and $NADP^+$ and a decreased binding of the products NAD^+ and NADPH, in agreement with the kinetics of the enzyme in submitochondrial particles exposed to a Δp [110]. Indeed, it is proposed that it is the extent of protonation of βHis-91 on the periplasmic space side which regulates the activity of the enzyme by Δp in the presence of NADH and $NADP^+$. DCCD is suggested to block the protonation/deprotonation cycle due to the modification of a residue in its vicinity or along the remaining proton-conducting pathway, in a manner that favours the reduction of $AcPyAD^+$ by NADH in the presence of NADPH. Possibly, this modification by DCCD leads to a state where NADP(H) remains bound to the enzyme. In agreement with this proposal, the pH dependencies for the reduction of $AcPyAD^+$ by NADH supported by NADPH is a mirror image of that for proton pumping driven by NAD^+ plus NADPH [109], the former being active at low pH and the latter at high pH, with an intersection of the two curves at about pH 5.8. This pH is indeed close to the pK_a of free histidine. A consequence of the assumed lack of a deprotonation at low pH as part of the normal cycle of βHis-91 is that NADP(H) will remain bound to the enzyme, and the kinetics of the reduction of $AcPyAD^+$ by NADH supported by NADPH will therefore be a ping-pong mechanism typical of reducible enzymes [108]; the kinetics of the normal transhydrogenase reaction at neutral pH is that of a random bi-bi mechanism [14,15,17,108,111,112].

10. Future perspectives

This review has attempted to convey the large amount of information that now is available about structure–function relationships of proton-pumping transhydrogenases, and the rate at which this information is increasing. In fact, we believe that this class of membrane proteins presently constitutes one of the best systems for studying long-range conformational changes in ion-transporting membrane proteins in general, and mechanisms of redox-driven proton pumping in particular. With the increase in the number of transhydrogenase genes cloned, expressed and characterized, our knowledge of the intriguing physiological roles of these proteins will also increase rapidly. Basic knowledge about structure–function relationships in proteins is ultimately a source of improved human health. Transhydrogenases may well have a key role in regulating essential redox-linked functions in the cell. This remains perhaps the most challenging future problem of this interesting group of proton-pumps.

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