J Bioenerg Biomembr (2008) 40:463–473
DOI 10.1007/s10863-008-9170-x

Proton-translocating transhydrogenase: an update of unsolved and controversial issues

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Received: 23 June 2008 / Accepted: 11 August 2008 / Published online: 30 October 2008

Abstract Proton-translocating transhydrogenases, reducing $NADP^+$ by NADH through hydride transfer, are membrane proteins utilizing the electrochemical proton gradient for NADPH generation. The enzymes have important physiological roles in the maintenance of e.g. reduced glutathione, relevant for essentially all cell types. Following X-ray crystallography and structural resolution of the soluble substrate-binding domains, mechanistic aspects of the hydride transfer are beginning to be resolved. However, the structure of the intact enzyme is unknown. Key questions regarding the coupling mechanism, i.e., the mechanism of proton translocation, are addressed using the separately expressed substrate-binding domains. Important aspects are therefore which functions and properties of mainly the soluble NADP(H)-binding domain, but also the NAD(H)-binding domain, are relevant for proton translocation, how the soluble domains communicate with the membrane domain, and the mechanism of proton translocation through the membrane domain.

Abbreviations

TH	transhydrogenase
dI	domain I of transhydrogenase
dII	domain II of transhydrogenase
dIII	domain III of transhydrogenase
ecIII	domain III of *E. coli* transhydrogenase
rrI	domain I of *R. rubrum* transhydrogenase

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Introduction

The recent demonstration that the proton-translocating transhydrogenase is involved in the defence against oxidative stress, e.g. the control of insulin release (Toye et al. 2005; Freeman et al. 2006; Arkblad et al. 2005; Huang et al. 2006) have caused a renewed interest in the physiological role of this enzyme (reviewed in refs Rydström 2006a and Rydström 2006b), as well as its structure-function relationships. Following the discovery in 1963 of the so called "energy-linked" transhydrogenase by Danielsson and Ernster (1963), driven by respiration or ATP-hydrolysis in submitochondrial particles, "energy" was of course subsequently identified as an electrochemical proton gradient (Δp), giving a transhydrogenase reaction described as

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

The direction of proton movement is the same as that for e.g. ATP synthase, where Δp strongly stimulates the forward reaction (left to right) and shifts the apparent equilibrium towards NADPH formation, through a conformationally driven mechanism. From 1963 to date the main goal in studying the enzyme has been to understand its physiological role (Rydström 2006a, b; Hoek and Rydström 1988; Sazanov and Jackson 1994) and the structure–function relationship that forms the basis for the coupling mechanism, i.e. mechanism of proton transport/utilization. Highlights in this area of transhydrogenase

research, especially structure–function studies in a broader context are shown in Table 1. The purpose with the present review is to identify major steps in the progress of transhydrogenase research, and to discuss problems and controversial issues in the area. Thus, this review does not claim to cover the entire field of transhydrogenase.

Structural properties

All known proton-translocating transhydrogenases share the same composition, i.e. an NAD(H)-binding domain (dI), an NADP(H)-binding domain (dIII) and a membrane domain (dII). These can be linked as one polypeptide as in the case of the mitochondrial enzyme, an α and a β subunit

Table 1 Structure–function studies of proton-translocating TH in a broader context

Year	Structure–function of proton-translocating TH—major findings/conclusions	Reference
1953	Membrane-associated mitochondrial TH is discovered	Kaplan et al. (1953)
1963/64	Demonstration of an energy-linked mitochondrial TH driven by ATP hydrolysis and respiration	Danielsson and Ernster (1963); Lee and Ernster (1964)
1965	Mitochondrial TH is stereospecific for the 4A-NADH and 4B-NADPH hydrogens and is concluded not to contain a reducible prosthetic group	Lee at al. (1965)
1966	Mitochondrial TH is proposed to be a proton pump	Mitchell (1966)
1967	Photosynthetic *R. rubrum*, contains an energy-linked TH	Keister and Yike (1967)
1969	A soluble domain of *R. rubrum*TH is isolated, which is required for activity	Fisher and Guillory (1969)
1971	*E. coli* contains an energy-linked TH	Sweetman and Griffiths (1971)
1971	Reversed energy-linked mitochondrial TH can make ATP	van de Stadt et al. (1971)
1971	Mitochondrial TH follows a ternary complex conformational mechanism	Rydström et al. (1971)
1975	Partially purified and reconstituted mitochondrial TH generates a membrane potential	Rydström et al. (1975)
1975	Co-reconstitution of Complex I (containing mitochondrial TH) and V gives an ATP-driven mitochondrial TH	Ragan and Widger (1975)
1977/78	Mitochondrial TH is composed of a single polypeptide with a mol mass of about 100–110 kD	Höjeberg and Rydström (1977); Anderson and Fisher (1978)
1979	*E. coli*TH follows a random ternary complex reaction mechanism	Hansson (1979)
1980	Purified and reconstituted mitochondrial TH is a proton pump	Earle and Fisher (1980)
1981/82	Purified and reconstituted mitochondrial TH catalyzes a rapid and uncoupler-sensitive NADH-AcPyAD$^+$ exchange mediated by bound NADP(H)	Wu et al. (1981); Enander and Rydström (1982)
1985	Cloning, expression of the *E. coli*TH gene(s), and purification of recombinant *E. coli*TH	Clarke and Bragg (1985a,b)
1985	Cell-free translation of mitochondrial TH	Carlenor et al. (1985)
1987	Active mitochondrial TH is a dimer	Persson et al. (1987)
1987	Co-reconstitution of purified mitochondrial TH and Complex V gives an ATP-driven TH with a H^-/NADPH stoichiometry of 1.	Eytan et al. (1987b)
1987	Co-reconstitution of purified mitochondrial TH and bacteriorhodopsin gives a lightdriven TH reaction and proof for a chemiosmotic mechanism	Eytan et al. (1987a)
1988	Cloning and primary structure of beef heart mitochondrial TH	Yamaguchi et al. (1988)
1991	Membrane domain of mitochondrial TH predicted to contain 14 helices	Yamaguchi and Hatefi (1991)
1994	Demonstration of a cyclic NADH-AcPyAD$^+$ reaction in *E. coli*TH, in the presence of bound NADP(H). Proposal that proton translocation is linked to dissociation of NADPH	Hutton et al. (1994)
1994	Cloning and structure prediction of *R. rubrum*TH	Williams et al. (1994)
1994	Identification of the conserved His91 as a potentially key residue in the proton channel of *E. coli* TH	Holmberg et al. (1994)
1997	Generation of an active cysteine-free *E. coli*TH	Meuller et al. (1997)
1999	Crystallography and 3D-resolution of the NADP(H)-binding domain III of bovine heart and *R. rubrum* TH	Prasad et al. (1999); White et al. (2000)
1999	Membrane topology proposed for *E. coli*TH based on cysteine labeling of predicted helices	Meuller and Rydström (1999)
2000	Crystallography and 3D-resolution of the NAD(H)-binding domain I from *R. rubrum*TH	Buckley et al. (2000)
2001	Intact *E.coli* TH has an aqueous cavity between His91 and the cytosolic side	Bragg and Hou (2000)
2001	Crystallography and 3D-resolution of the dI_2:dIII complex from *R. rubrum* TH	Cotton et al. (2001)
2001	H91R/K and N222R/K mutants of *E. coli* TH have occluded NADP(H)	Bragg and Hou (2001)
2002	An alternating site, binding change mechanism is proposed	Jackson et al. (2002)
2004	Helix packing model proposed for the membrane domain of *E. coli* TH based on crosslinking	Althage et al. (2004b)
2005	Crystal structures of the dI_2:dIII complexes from *R. rubrum* TH with saturating NAD(P)(H)	Sundaresan et al. (2005)
2005	Crystal structure of dI from *E. coli* TH	Johansson et al. (2005)

as in the case of *E. coli* transhydrogenase, or a three-subunit enzyme (α1, α2 and β) as in the case of the *Rhodospirillum rubrum* enzyme. Because structural information was essentially absent prior to 1999 (Table 1), most of the research at that time had to rely on resolution–reconstitution experiments and other biochemical approaches. This changed drastically with the solution of the dIII structures (Prasad et al. 1999; White et al. 2000), and subsequently dI (Buckley et al. 2000; Johansson et al. 2005) and dI_2–dIII structures (Cotton et al. 2001; Sundaresan et al. 2005), that initiated a large number of structurally based mutagenesis experiments. For example, the E115W mutant in dIII from *R. rubrum* made it possible to directly measure the extremely fast hydride transfer between bound substrates (Pinheiro et al. 2001). Subsequently, redox-dependent conformational changes in the NAD(H)-site induced by NADH/NAD^+ (Prasad et al. 2002; Mather et al. 2004) led to the suggestion that this site may be important in gating hydride transfer in the intact enzyme (Mather et al. 2004). All of these structures contained bound NAD^+ and/or $NADP^+$ (for an updated description of the structural and functional properties of dI, dIII and dI_2dIII proteins, see refs. Bhakta et al. 2007; Jackson et al. 2005). Based on knowledge derived from the crystal structure of the dI_2.dIII complex from *R. rubrum* (Cotton et al. 2001), and subsequently extended to the complete enzyme, a working hypothesis for hydride transfer and proton translocation was proposed by Jackson and coworkers (Jackson et al. 2002). The model, denoted the alternating site, binding change mechanism, is utilizing different catalytic activities and structural information as support for a proton-pumping mechanism (Bhakta et al. 2007; Jackson et al. 2005). However, although there was essentially no structural information at that time, an alternating site binding change model analogous to that for the ATP synthase system was indeed proposed already in 1981 by Rydström et al. (1981) and further discussed in 1982 by Enander & Rydström (1982) as applied to the mitochondrial enzyme.

The structure of the dI_2.dIII complex led to a proposed architecture of the complete $dI_2dII_2dIII_2$ enzyme (Pedersen 2006) which involves a direct contact of dIII with dI, and dIII sitting on top of dII (Fig. 1a). This agrees with the previous conclusion that the environment of the NADP(H)-site is more hydrophobic than that for NAD(H) (Rydström 1972). However, it does not take into account the fact that dI in the α subunit of *E. coli* transhydrogenase as well as in the mitochondrial enzyme is linked to 4 membrane helices in dII through a potentially important connecting peptide (see "Proton-pump mechanism section"), and that dI, in addition, probably is closer to the membrane. Figure 1b shows an alternative structure, based on a previous model (Bizouarn et al. 2002), with both dI and dIII functionally connected to the dII surface. This model will be discussed

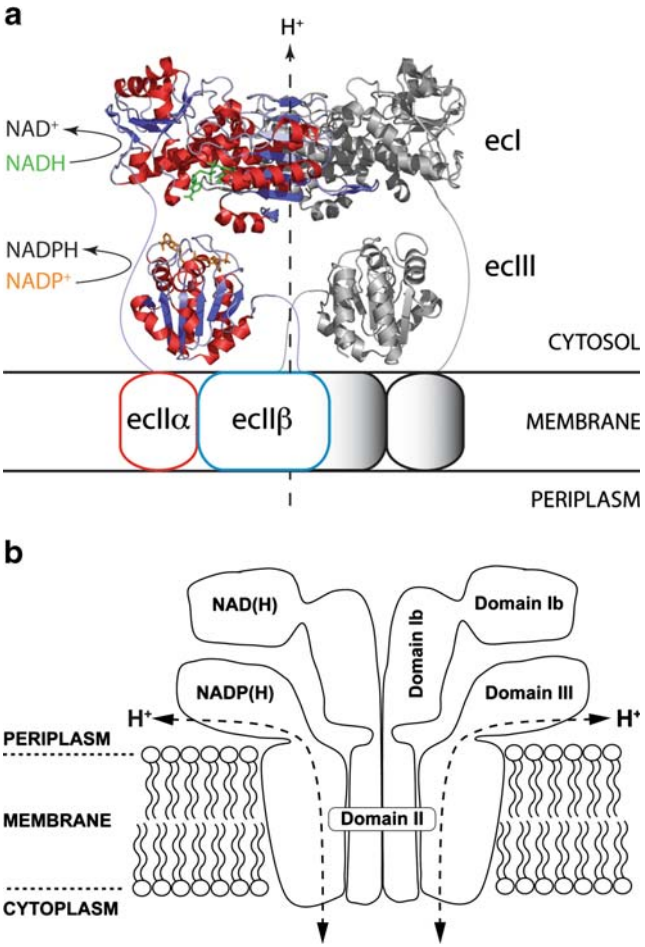

Fig. 1 2D-structural models of proton-translocating transhydrogenase without (**a**) and with (**b**) functional α and β-linkers connecting dI and dII, and dIII and dII, respectively. Note that, in a 3D model of the dimeric intact enzyme viewed from the top, the model may have the four dI and dIII domains arranged in an alternating fashion, i.e., dI(1)–dIII(1)–dI(2)–dIII(2), in agreement with the helix packing model for dII shown in Fig. 6

in more detail below in the Section on Proton pump mechanism. The solution of the domain organisation obviously has to await the structure of the intact enzyme.

It should be emphasized that essentially all structural information obtained so far is based on the structure of isolated domains (dI and dIII), or a crystallization product of dI+dIII (dI_2dIII), which may or may not constitute an intermediate in the overall reaction. The detailed hydride transfer mechanism has still not been resolved, mainly because of the missing structure of the complete enzyme, especially the missing second dIII domain in the dI_2dIII structure (Cotton et al. 2001) and, most importantly, the missing dII. The missing dIII can not be introduced in a structural model without steric clashes, suggesting that there are several required movements of both dIII domains, and therefore dI–dIII conformational states, to complete a catalytic cycle, and these are at the moment not defined

(Sundaresan et al. 2005). The conformations of both loop D and E determine whether the two dIIIs fit in the overall structure relative to the dI dimer (Sundaresan et al. 2005). Conserved residues and their roles in hydride transfer have been reviewed recently (Brondijk et al. 2006). However, unless they are relevant for the proton pumping mechanism, the details of the hydride transfer mechanism is outside the scope of this review.

Domain III structural features and remaining controversies

Despite all the structural information on domain III, both in complex with dI and as a separate entity, a number of key questions addressing known biophysical properties of the domain remain to be answered. The existing high-resolution X-ray structures of dIII with $NADP^+$ or NADPH bound have not provided the means to explain the marked 50-fold affinity difference between the two substrates, but rather the structures appear to be identical (Mather et al. 2004; Sundaresan et al. 2003). Whether the affinity difference can be attributed to altered binding site dynamics upon redox change or a structural difference not identified with X-ray crystallography, e.g. a crystallographic artefact, is unclear at present. However, a pH-titration study on *E. coli* dIII (ecIII) observed by amide proton 2D NMR clearly showed that conserved residues in the NADP(H)-binding site displayed redox-dependent changes of pK_as, indicative of a conformational change (Pedersen et al. 2005).

The solution structure of the *E. coli* domain in both redox forms (Pedersen et al., unpublished; Pedersen 2006) unfortunately lacks the resolution in the substrate binding site, mainly due to lacking assignments and signals to essential residues e.g. Arg350, Lys424, Arg425 and Tyr431, to be useful in elucidating any conformational change around the dinucleotide. Figure 2 shows the $NADP^+$-bound form determined by Pedersen et al. (unpublished) and Pedersen (2006). A previously determined solution structure of the *R. rubrum* domain (Jeeves et al. 2000) has $NADP^+$ modeled into the site, presumably using the 1.2 Å bovine structure (Prasad et al. 1999) as template. The resulting structure must be far from the true situation as the reported protein-$NADP^+$ NOE distances do not fit with the actual structure, and very strong NOE possibilities as judged form the structure are not even mentioned, or are not observed.

It may be concluded that none of the reported dIII structures, derived from X-ray crystallography or NMR, can explain the redox-dependent change in biophysical properties. Presumably, only the structure of the intact enzyme can reveal the different redox-dependent conformations of dIII.

Bound NAD(P)(H) and the cyclic reactions

The first observation of a cyclic reduction of $AcPyAD^+$ by NADH, mediated by bound NADP(H), was made by Fisher and coworkers (Wu et al. 1981), who showed that purified mitochondrial transhydrogenase reconstituted in liposomes catalyzed the reaction but only in coupled vesicles. However, the cyclic reaction itself was not linked to proton pumping. Indeed, this was the first indication that binding and release of NADP(H) in the mitochondrial enzyme is related to Δp. Subsequently, Enander and Rydström (1982) confirmed these findings, and showed that the cyclic reaction was related to a change in the affinity for NAD^+, but not for NADPH (cf. also ref. Rydström et al. 1971), and which was altered in an uncoupler-dependent manner. It was suggested that a conformationally different form of the enzyme, generated by Δp, was responsible for the tight binding of NADP(H) (Enander and Rydström 1982). A similar observation was later made with purified *E. coli* transhydrogenase co-reconstituted with bacteriorhodopsin

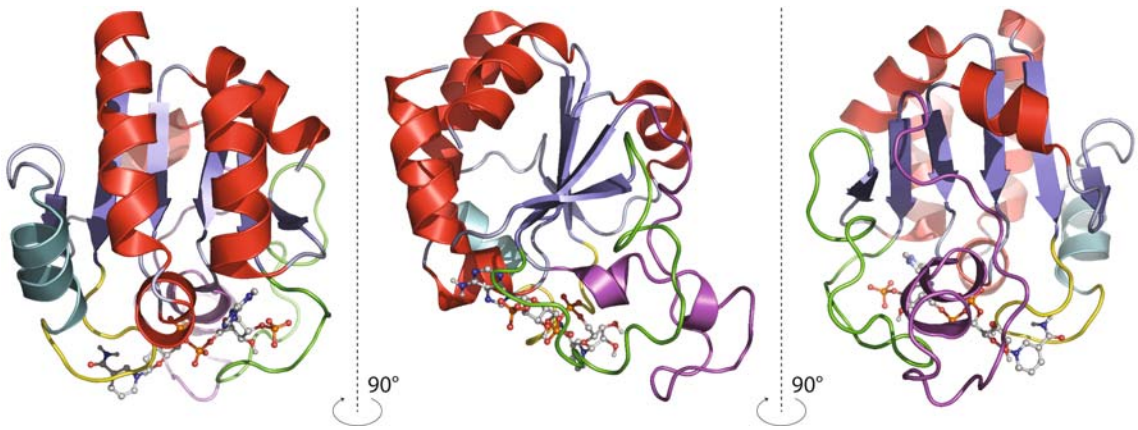

Fig. 2 NMR structure of ecIII with bound $NADP^+$. Cartoon representation of ecIII with bound $NADP^+$ (*black*) bound. Important structural elements are marked in colour; loop B in *yellow*, α-helix 3 in *cyan*, loop D in *purple*, and loop E in *green*. From Pedersen (2006)

(Hu et al. 1995). Here, the forward reaction was driven by light and the affinity for NADH, but not for thio-$NADP^+$, was strongly dependent on Δp. At low pH, the vesicles also catalyzed a cyclic reaction, but in contrast to the mitochondrial reaction, it was unrelated to proton pumping, i.e. the reaction did not pump protons and it did not require or was affected by the light-generated Δp. Most of the observations, but not all, made by Fisher and coworkers (Wu et al. 1981) and Rydström and coworkers (1982) using the purified and reconstituted beef heart enzyme, were later reproduced by Sazanov and Jackson (1995). However, the cyclic reactions were also observed at low pH using both detergent-dispersed purified beef heart and *E. coli* enzymes (Sazanov and Jackson 1995; Bizouarn et al. 1995; Stilwell et al. 1997). Despite these conflicting results, and despite the fact that scalar protons irrelevant for proton pumping may be involved in the latter observations at low pH, it was concluded (Sazanov and Jackson 1995; Bizouarn et al. 1995; Stilwel et al. 1997) that the results were compatible with the original proposal (Hutton et al. 1994), i.e. that the cyclic reaction at low pH was related to protonation/deprotonation steps involved in proton pumping.

A remarkable and unexpected finding was made with purified ecIII (which contains bound NADP(H) like all dIIIs) following the removal of the 2′-phosphate of the bound NADP(H) by treatment with alkaline phosphatase (Pedersen et al. 2003). The activity of ecIII was routinely assayed as reduction of $AcPyAD^+$ by NADH mediated by the bound NADP(H) (of which 87% is $NADP^+$, 5% NADPH and 8% the apo-form (Johansson et al. 2002)) in the presence of dI from *R. rubrum*. To our great surprise the NADP(H)-free ecIII, and without added NADP(H), essentially retained the catalytic capacity to reduce $AcPyAD^+$ by NADH (Pedersen et al. 2003). The activity did not last very long since the NADP(H)-free apo-form of ecIII is unstable and loses activity after approx. 10–15 min on ice, but it raises a very interesting possibility, namely that the NADP(H)-binding site can be unspecific. Reduction of $AcPyAD^+$ by NADH catalyzed by transhydrogenase has earlier routinely been rejected as due to contaminations by either NADP(H) or other proteins (Stilwell et al. 1997, but see Jackson et al. 2005). However, in addition to the above report, others show that this activity without doubt is real but variable and catalyzed by transhydrogenase at a high rate under various conditions (Glavas and Bragg 1995b; Bragg 1996). In addition, various mutants of residues in the so called "hinge" region, connecting dII and dIII, show dramatically increased and reproducible NADH-$AcPyAD^+$ activities approaching that of the normal reverse activity, e.g. up to 60% in the case of the cfR265C (cysteine-free intact *E. coli* transhydrogenase) mutant (Althage et al. 2001). In this context, it is interesting to note the ten-fold difference in NADH-$AcPyAD^+$ activity between the wild-type and cysteine-free *E. coli* enzyme (Johansson et al. 2002), and the lack of NADH-$AcPyAD^+$ activity of the βG314A mutant (Glavas and Bragg 1995b). In the case of the βR265C mutant, 85% of the NADH-$AcPyAD^+$ activity remained after phosphatase treatment (Pedersen 2006; Karlsson 2006).

Consequently, an interesting question is whether the NADH-$AcPyAD^+$ reaction is relevant for the catalytic mechanism. It is well known that, because of protonation of the 2′-phosphate, NAD(P)-dependent enzymes generally become nucleotide-unspecific at lower pH. At pH 6 and below, the NADH-$AcPyAD^+$ reaction can be visualized as an artificial reverse reaction where NADH is bound to the NADP(H) site, or a cyclic reaction where NADH is bound to the NADP(H) site long enough to cycle between the oxidized and reduced state, and interact with NADH and $AcPyAD^+$ in the NAD(H) site. The first alternative is unlikely since the stereospecificity is maintained (4A for NADH; it would be the 4B hydrogen if the NADP(H) site was used; Stilwell et al. 1997), which leaves the second alternative. The available evidence is consistent with a mechanism where NADH, at low pH and added in excess over bound NADP(H) in dIII, fully or partly replaces the bound NADP(H). As will be seen below, this unspecific interaction may be crucial in the overall catalytic mechanism.

Roles of loops D and E in NADP(H) binding

As compared to other NADP-dependent enzymes, NADP(H) in dIII is bound in an inverted manner by a web of bonds to the binding site (Prasad et al. 1999; White et al. 2000). In *E. coli* transhydrogenase, loops D (N391-V411) and E (M427-G434; *E. coli* enzyme numbering) connect the beta strands β4 and β5, and β5 and β6, respectively (Fig. 3). The important D392 interacts with the pyrophosphate bridge in NADP(H), and the K424-R425-S426 stretch binds the 2′-phosphate group of NADP(H). Loop E forms a "lid" covering the bound NADP(H) and contributes to maintaining it in the "occluded" state; loop D also contributes to the occluded state and its movements are somehow linked to those of loop E (Bhakta et al. 2007; Jackson et al. 2005). Both loops are involved in redox-sensitive dI–dIII surface interactions (Bergkvist et al. 2000). However, the role of loop D may be more complex and involve key movements and interactions with the RQD-loop in dI during hydride transfer (Cotton et al. 2001; Sundaresan et al. 2005). Loop D also seems to be involved in the two forms of NADP(H)-bound states, the "closed" and "open" states (Sundaresan et al. 2005; Sundaresan et al. 2003), where the "open" state corresponds to that denoted "occluded" by Jackson et al. (2002). However, the "closed" state has not been observed in NMR measurements on ecIII

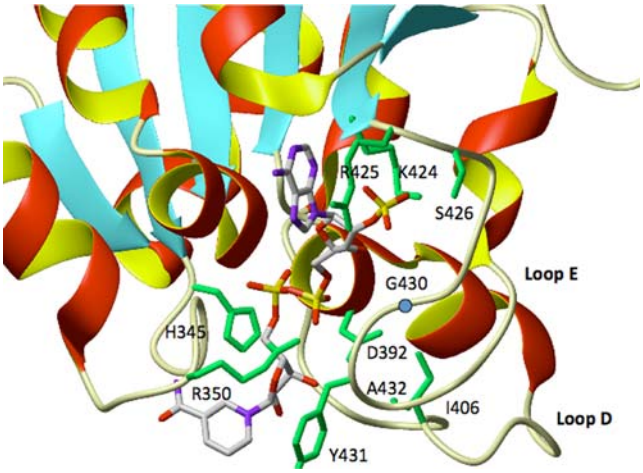

Fig. 3 Structure of the NADP(H)-binding site of *E. coli* TH. Key residues and loops D and E are indicated. From Prasad et al. (1999)

(Pedersen et al. 2005) or by Jackson and coworkers using X-ray crystallography on the *R. rubrum* dIII (Jackson, personal communication), leaving the question of whether this state is relevant for the intact enzyme mechanism or is an artifact of crystallization.

In an extensive effort to characterize conserved residues in loops E and D, these were mutated to cysteines in ecIII and in cfTH (Johansson et al. 2002; Pedersen 2006; Karlsson 2006). Remarkably, some mutants, e.g. ecIIID392C (Fjellström et al. 1999) in loop D and ecIIIR425C (Bergkvist et al. 2000) in loop E, have no bound NADP(H), whereas other mutants, e.g. ecIIIG430C and ecIIIA432C, show an inverse content of NADPH, i.e. no $NADP^+$ (Johansson et al. 2002), in contrast to wildtype ecIII that contains essentially only $NADP^+$ (Johansson et al. 2002). In addition, ecIIIG430C in the presence of rrI showed pronounced selective increases in the rates of both the forward and reverse reactions by almost 300-fold in the former and eight-fold of the latter (Johansson et al. 2002), associated with a 100-fold increased substrate release rate from the ecIIIG430 protein.

In order to further elucidate the role of loop D, the thiol-reactive fluorescent agent 2-(4-maleimidylanilino)naphthalene-6-sulphonic acid (MIANS) was used to label the mutant ecIII-I406C, or the corresponding mutant of the intact cysteine-free enzyme, cfTH-βI406C (Pedersen 2006). Both of these enzymes showed approximately the same fluorescence which, surprisingly enough, was drastically quenched upon the addition of low concentrations of $NADP^+$ ($NADPH$ could not be added because of fluorescence interference with MIANS; Fig. 4a), and an apparent "affinity" for this quenching could be calculated (Fig. 4b). In double mutants, these fluorescence changes were strongly affected especially by the residues in the 91 position in dII, e.g. in the cfTH-H91K-I406C mutant (Pedersen 2006; Karlsson 2006). The significance of these observations are not yet clear, but they show that it is possible to directly study the communication between dII and loop D in the intact enzyme.

Taken together, the structural information suggests that the difference in substrate-binding characteristics between the wildtype enzyme, which binds NADP(H) loosely, and isolated domain III, which binds NADP(H) tightly in the nanomolar range, depends mainly on the conformational states of loops D and E. Presumably, this reflects small but critical effects of dII (and dI) on dIII in the catalytic cycle of the intact enzyme.

The membrane domain

Following the prediction and experimental verification by accessibility labelling of both sides of all potential transmembrane helices in the *E. coli* transhydrogenase (Meuller and Rydström 1999), it is now generally accepted that this enzyme has a total of 13 transmembrane helices, of which four reside in the α subunit and nine in the β subunit (Fig. 5, mitochondrial helix numbering). The mitochondrial single polypeptide transhydrogenase was assumed to have an extra helix, helix 5, located between helix 4 and 6 in the *E. coli* enzyme, linking the end and beginning, respectively, of the α and β subunits. Indeed, the additional mitochondrial sequence of 27 amino acids was mimicked by the introduction of an extra homologous 32 amino acids long peptide that was used to fuse the α and β subunits, creating the extra helix 5 and a normally active enzyme (Meuller et al. 2001). Helix packing studies showed for the first time that the functional composition of dII indeed is a dimer, and the location of the assumed proton channel in helices 9, 10, 13 and 14 (Althage et al. 2004a; Fig. 6). Predictions of additional/missing transmembrane helices in e.g. dI of the *R. rubrum* enzyme (Jackson et al. 2005), as compared to those proposed for the *E. coli* and mammalian transhydrogenases, essentially lack experimental support.

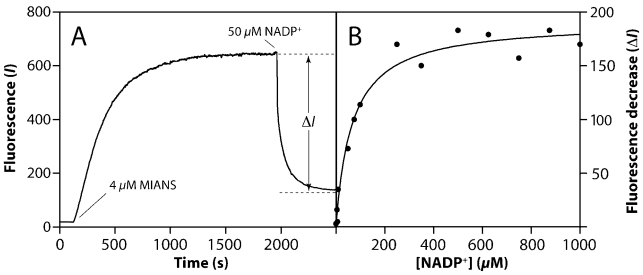

Fig. 4 MIANS modification of the cfTH-βIle406Cys mutant followed by fluorescent quenching by $NADP^+$ (**a**) and quantitation of fluorescence quenching by $NADP^+$ (**b**). Conditions were as described in Pedersen (2006)

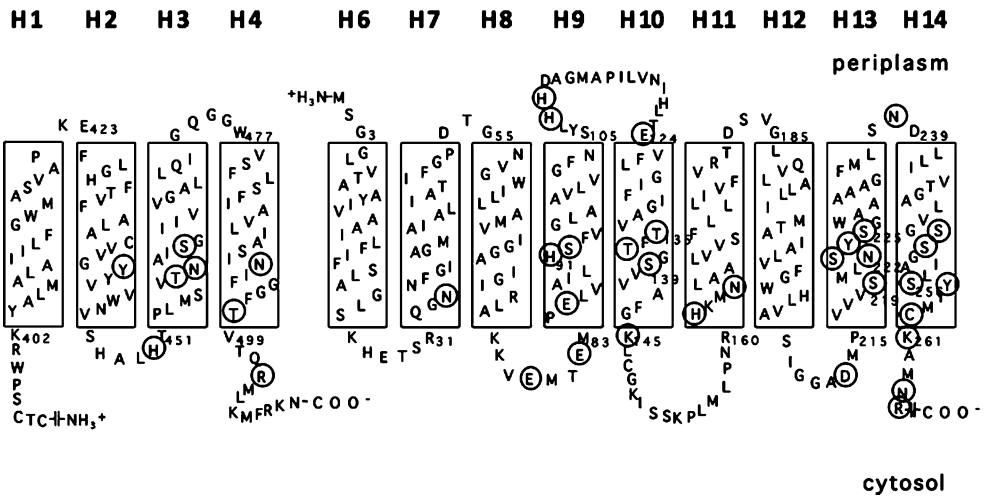

Fig. 5 Predicted helical content of the membrane domain (dII) of TH from *E. coli*. Semiconserved and conserved hydrophilic and hydrophobic residues are *circled*. From Meuller and Rydström (1999)

Figure 5 shows that the majority of conserved residues are located in helices 2–4, 9, 10 and 13 and 14. Several residues were concluded to be located in the intramembrane part of the proton channel, i.e. His91 in helix 9 (Holmberg et al. 1994; Bragg and Hou 2000; Glavas and Bragg 1995a), Ser139 in helix 10 (Yamaguchi et al. 2002), and Asn222 in helix 13 (Bragg and Hou 2001). In addition, Asp213 in the loop between helices 13 and 14 was found to be important (Yamaguchi and Hatefi 1995). Judged by the NADP(H)-induced trypsin sensitivity (Tong et al. 1991), Arg265 was early recognized as strongly mobile. This mobility was later demonstrated also by an NADP(H)-induced increased reactivity of the R265C mutant to fluorescent thiol reagents (Althage et al. 2001), and the NADP(H)-dependent formation of a D213C-R265C disulphide (Althage et al. 2001). Reduction of the latter disulphide led to a four-fold increased K_m^{NADPH} (Ålander et al. unpublished; Rydström 1972; Pedersen 2006). Based on these results, it was concluded that the mobile "hinge" region, and the potential salt bridge between D213 and R265 in the wildtype enzyme suggested by the D213C-R265C disulphide, play a crucial role in the communication between dII and dIII (Althage et al. 2001). Gly252 in helix 14 was identified as an important residue since the G252A mutation led to occlusion of NADP(H), blockage of the reverse reaction and a high cyclic reaction (Yamaguchi and Stout 2003), whereas the G252C mutant was essentially inactive regarding all activities (Karlsson et al. 2003).

In general, membrane helices are held together by interactions between amino acid side chains in a "groove-ridge" system, where glycines are grooves and mostly isoleucines and valines are ridges (Senes et al. 2000). As expected, mutations of conserved glycines were therefore strongly inhibitory due to a possible interference with this helix packing (Karlsson et al. 2003). That dII is held together by strong helix–helix interactions involving amino acid side chains functioning as grooves-ridges, is indicated by an essentially fully functional enzyme in which the short connecting peptide between helix 9 and 10 had been truncated (Althage et al. 2003).

All residues, one at a time, in both helix 13 (Karlsson and Rydström unpublished) and 14 (Karlsson et al. 2003) were mutated to cysteines as well as other residues. Both helices are almost fully conserved, but helix 14 was the first choice for these studies since it is linked to the "hinge" peptide, i.e. K261-I294, connecting dII to dIII. Mutation of the three conserved glycines (βG245, βG249 and βG252), which were all located on one side of the helix, led to severely inhibited activities, whereas mutation of the three conserved serines (βS250, βS251 and βS256) led to enhanced activities (Karlsson et al. 2003). The M258C

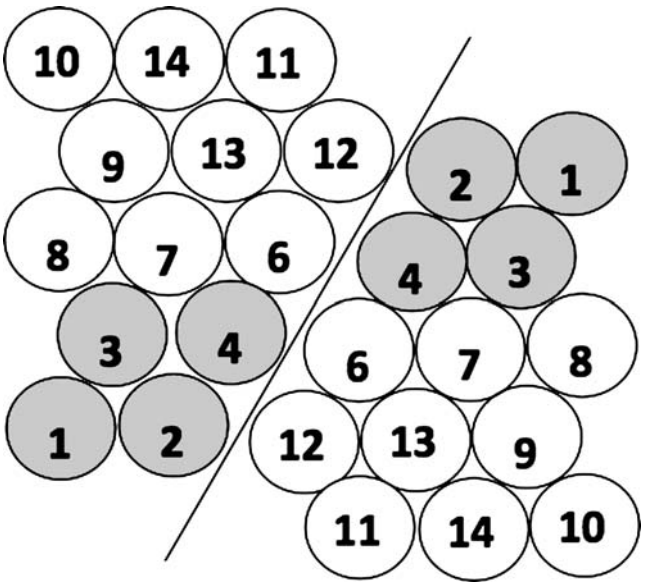

Fig. 6 Predicted helix packing of the membrane domain in *E.coli* TH based on crosslinking. *Grey* and *white* helices denote those of the α and β-subunit, respectively. Helices 9, 10, 13 and 14 form the proposed proton channel. From Althage et al. (2004a)

mutant located just before the "hinge" behaved very much like the R265C mutant (Althage et al. 2001), i.e. it showed a high cyclic NADH-AcPyAD$^+$ activity in the absence of NADP(H) (Karlsson et al. 2003).

Proton-pump mechanism

As mentioned in the Introduction it was concluded for the first time more than 30 years ago that proton translocation by transhydrogenases involves a conformational change mechanism (Rydström et al. 1971; Rydström 1974; Rydström 1977). It is comforting to learn that also Jackson and coworkers now recognise this general principle (Pinheiro et al. 2001). That Δp affects primarily the affinities for NAD(H) but also those for NADP(H) was first shown by Rydström et al. (1971) using beef heart submitochondrial particles. The affinity for NADH increased (K_m dropped) whereas that for NAD$^+$ decreased. Similar but much less extensive patterns were seen for NADP$^+$ and NADPH. These pronounced effects associated with the NAD(H)-binding site in domain I have never been satisfactorily explained, and are incompatible with the notion that coupling is exclusively linked to binding and release of NADP(H) in domain III (Jackson et al. 2002). The recent suggestion that gating in the intact enzyme is associated with the NAD(H) site (Mather et al. 2004) may provide an explanation for these affinity changes for NAD(H).

The fact that neither the NADH-AcPyAD$^+$ cyclic reaction in the presence of bound NADP(H), nor that in the absence of NADP(H), is linked to proton translocation strongly suggests that association and dissociation of the 2′-phosphate group of NADP(H) to/from the NADP(H) site governs proton uptake and release in dII. The K424-R425-S426 region in the NADP(H) site involved in binding the 2′-phosphate group is near loop E which is closing down on the larger part of NADP(H) thus facilitating occlusion. Loop D is involved in the interactions with dI and may regulate the accessibility of Asp392 through Ile406. The movement of loop E is somehow coordinated with that of loop D. Thus, it is reasonable to assume that the movements of these loops are involved in proton translocation. Asp392 in loop D is an attractive candidate for a potential proton-carrying residue. The importance of Asp392 was first discovered by Meuller et al. (1996) who showed that all mutants tested (Ala, Lys, Asn, Trp and Thr) were inactive with regard to the reverse reaction and proton pumping, but that some cyclic activity (+NADPH) was retained, especially in the D392A mutant.

Without doubt the most significant finding in the context of coupling and communication between dII and dIII is the demonstration by Bragg and coworkers (Bragg and Hou 2001) that the introduction of a positively charged residue in the 91 (His) or 222 (Asn) positions (in *E. coli* TH), presumably mimicking protonation, leads to occlusion of NADP(H) in dIII. This was explained as a consequence of a deprotonation of Asp392 (Bragg and Hou 2001). Conversely, it was implied that its protonation (by vectorial protons) could potentially release NADP(H) (Bragg and Hou 2001). However, such a protonation/deprotonation cycle of Asp392 is unlikely at least in isolated ecIII, since the pK_a of this residue is between 9.6 and 10,2 regardless of the redox state of the bound NADP(H) (Pedersen et al. 2005). The D and E loops have also been proposed to be involved in proton translocation by Rydström and coworkers (Bizouarn et al. 2002; Pedersen et al. 2003) and Jackson and coworkers (Jackson et al. 2002; Bhakta et al. 2007; Jackson et al. 2005). In a recent investigation of the intact *E. coli* transhydrogenase by ATR-FTIR spectroscopy, Jackson and coworkers found evidence that binding of NADP(H) opens up a deep cleft in the enzyme (Iwaki et al. 2006).

The scheme proposed by Rydström and coworkers (Ålander et al. unpublished; Pedersen 2006; Karlsson 2006; Fig. 7) is based on a similar mechanism proposed earlier (Bizouarn et al. 2002). It is assumed that dII is quite rigid, fixed largely by a "groove-ridge" interlocking system, and that the main mobile region, relevant for coupling, involves helices 9, 10, 13 and 14, with the residues H91, N222 and S139, and the superficially located residue D213 making up the proton channel (Bizouarn et al. 2002). Thus, apart from the movement of the "hinge" region, which may be substantial, other movements are presumably very small as indicated by e.g. the G252A mutant in helix 14 which locks the NADP(H)-site in the occluded state (Yamaguchi and Stout 2003). Other key components of the mechanism are D213 and R265 alternating between a salt bridge in the absence of NADP(H) and no salt bridge in the presence of NADP(H) (Althage et al. 2001). In addition, the model is consistent with the suggestion that there is an NADP(H)-regulated aqueous cavity connecting H91/N222 with the cytosol (Bragg and Hou 2000). In this model, the communication between dII and dI/dIII occurs through the α- and β (hinge)-linkers discussed previously and below, as well as possibly the conserved loop between helices 13 and 14.

Considering the possible roles of the hydrophilic C-terminus of the α subunit (Bragg 1996) and the hydrophilic connecting peptide between dII and dIII in the β subunit of the *E. coli* enzyme (the "hinge" region), forming a stalk-like structure (Fig. 1b), these are 20–30 residues long and potentially functional (Ålander et al. unpublished; Pedersen 2006; Karlsson 2006) rather than only structural (Jackson et al. 2005). Especially conserved residues in these peptides have been mutated (Ålander et al. unpublished; Pedersen 2006; Karlsson 2006). The former peptide, the so called α-linker, contains the PAPPIQVSAQPQ sequence. Reverse

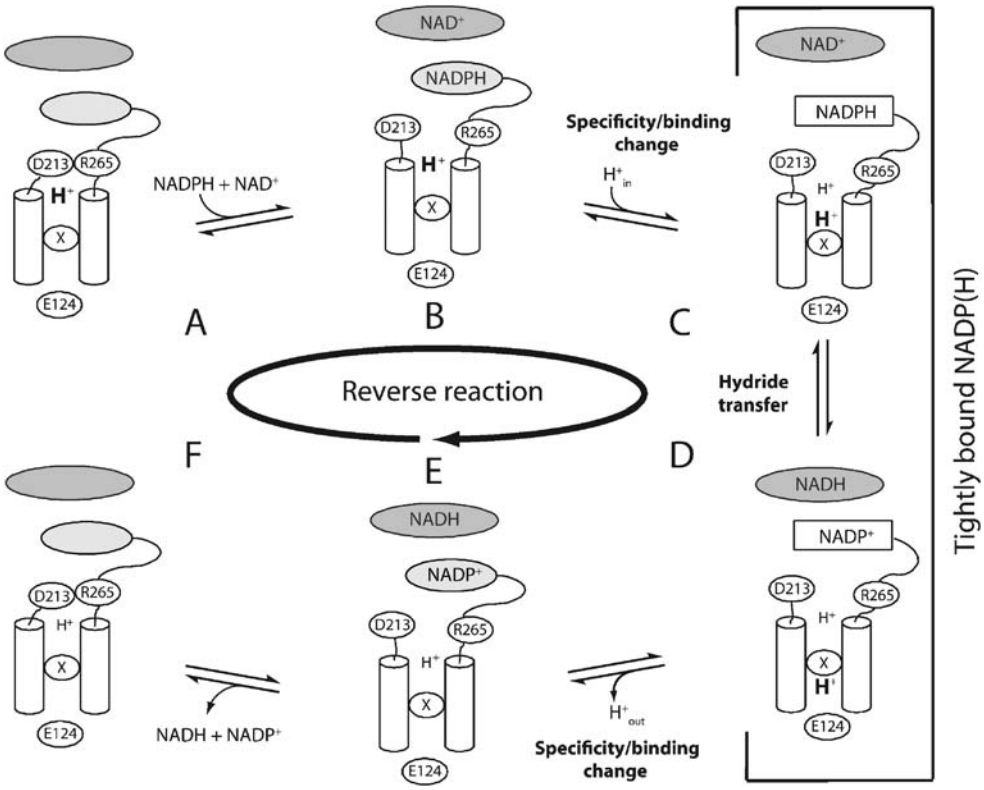

Fig. 7 Proposed mechanism of proton pumping by *E. coli* TH. A proton is prebound in the resting state. This proton is the one that is eventually translocated across the membrane. NADPH and $NADP^+$ bound to *rectangular sites* denote tight binding and different conformational states, essentially corresponding to the conformational state and properties of isolated dIII. *Grey binding sites* indicate loose binding. The indicated helices are not specific helices, but "X" is His91. From Pedersen (2006)

activities of the mutants P369C, A370C, P371C, P372C, Q374C, P379C and Q380C were between 16–49% of control, P371C being the most inhibited (Karlsson 2006). Cyclic activities were slightly less affected. Accessibility to NEM indicated that the beginning of the PAPP-region (P369 and A370) showed a lower accessibility than the remainder of the α-linker. A similar study of residues (I271, G273, F275, S280) in the β-linker showed that cysteine mutants were inhibited to various extents (17–105%, I271C being the more inhibited) but that none was essential (Ålander et al. unpublished; Pedersen 2006). Thus, the α- and β-linkers appear to be catalytically important but not essential, and are assumed to play a role in the dII–dI/dIII communication.

In a recently proposed proton pump mechanism by Jackson and coworkers, a key feature is a piston-like movement of an extended transmembrane helix (unknown which), containing a protonatable residue (unspecified) that works as a proton carrier (Jackson et al. 2005). However, such an extended helix is inconsistent with the careful analyses of helices in the *E. coli* transhydrogenase by prediction and mutagenesis (Mueller and Rydström 1999), and crosslinking of cysteines located in the ends of each helix (Althage et al. 2004a). In addition, the proposed movement of this helix is also approximately half of the width of the membrane, i.e. some 15Å (Jackson et al. 2005). In view of the argument above regarding the flexibility of dII, such movements are less likely.

Physiological role

Cellular functions of transhydrogenases have already been mentioned in the Introduction, and have recently been reviewed (Rydström 2006a,b). Briefly, it is now established that proton-pumping transhydrogenases constitute one of the main sources of cytosolic NADPH in some prokaryotes and mitochondrial NADPH in eukaryotes, and that this NADPH is used in the prevention against oxidative stress by maintaining a high GSH/GSSG ratio. This prevention may indirectly involve other defense systems linked to glutathione/NADPH, e.g. thioredoxin, glutaredoxin and peroxyredoxin (Rydström 2006a). In pancreatic β cells transhydrogenase contributes to the regulation of insulin release (Toye et al. 2005; Freeman et al. 2006), and in heart it has a cardioprotective role (Huang et al. 2006). In the mouse heart, a combination of a lack of transhydrogenase and a decrease in a second defence enzyme, e.g. mitochondrial superoxide dismutase, which individually are not lethal, is indeed lethal (Huang et al. 2006). A proposal that transhydrogenase, together with the NADP-isocitrate dehydrogenase in the Krebs cycle, regulates this cycle (Sazanov and Jackson 1994) is attractive but has no experimental support. That transhydrogenase and a reversal of the mitochondrial NADP-isocitrate reaction can form isocitrate and contribute to cytosolic NADPH (through transport of isocitrate to the cytosol, and the cytosolic NADP-isocitrate dehydrogenase) was demonstrated more than 30 years ago

(Hoek and Ernster 1974). In fact, co-existence of mitochondrial NADP-isocitrate dehydrogenase and proton-pumping transhydrogenase is common but not obligatory. A knockout of both transhydrogenase genes (proton-pumping transhydrogenase, PntAB and the soluble *E. coli* transhydrogenase, UdhA) in *E. coli* results in a markedly elevated concentration of citrate, *more than 60-fold* (Timishl et al. 2008). The latter is contrary to what would be expected. However, glutamate is decreased, which indeed is expected (Timishl et al. 2008).

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