

Proton translocating nicotinamide nucleotide transhydrogenase from *E. coli*. Mechanism of action deduced from its structural and catalytic properties¹

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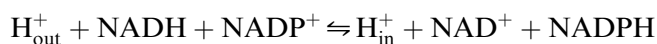
Abstract

Transhydrogenase couples the stereospecific and reversible transfer of hydride equivalents from NADH to NADP⁺ to the translocation of proton across the inner membrane in mitochondria and the cytoplasmic membrane in bacteria. Like all transhydrogenases, the *Escherichia coli* enzyme is composed of three domains. Domains I and III protrude from the membrane and contain the binding site for NAD(H) and NADP(H), respectively. Domain II spans the membrane and constitutes at least partly the proton translocating pathway. Three-dimensional models of the hydrophilic domains I and III deduced from crystallographic and NMR data and a new topology of domain II are presented. The new information obtained from the structures and the numerous mutation studies strengthen the proposition of a binding change mechanism, as a way to couple the reduction of NADP⁺ by NADH to proton translocation and occurring mainly at the level of the NADP(H) binding site. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Transhydrogenase; Proton pump; NADP; NAD; Membrane protein

1. Introduction

Energy-linked transhydrogenase (E.C. 1.6.1.1) is an integral membrane enzyme, which is situated in the cytoplasmic membrane of bacteria and in the inner membrane of mitochondria. The enzyme participates in the bioenergetic processes of the cell, and utilizes the electrochemical proton gradient across the membrane to drive NADPH formation from NADH (see reviews [1–3]) according to the reaction:



where ‘out’ and ‘in’ denote the cytosol and matrix, respectively, in mitochondria and periplasmic space

Abbreviations: dI, dII, dIII, domain I, domain II and domain III, respectively of transhydrogenases in general; dIA, dIB, sub-domains of dI; rrI and rrIII, domain I and domain III constructs, respectively, of *Rhodospirillum rubrum* transhydrogenase; ecI and ecIII, domain I and domain III constructs, respectively, of *Escherichia coli* transhydrogenase; ecβ, preparation of *E. coli* transhydrogenase in which the portion of the α-subunit has been degraded; AcPyAD⁺, 3-acetylpyridine adenine dinucleotide (oxidized form); AADP, 3-aminopyridine adenine dinucleotide phosphate

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¹ This review is dedicated to the memory of Professor Lars Ernster.

and cytosol, respectively, in bacteria. The enzyme is stereospecific for the 4A hydrogen of NADH and the 4B hydrogen of NADPH [4,5]. It has been estimated that one proton is translocated across the membrane per net transfer of one hydride equivalent, i.e. the H^+/H^- is 1 [6].

Transhydrogenase from *Escherichia coli* is composed of an α -subunit of about 54 kDa and a β -subunit of 48 kDa. The active form is $\alpha_2\beta_2$ [7,8]. Like all proton translocating transhydrogenases, the *E. coli* enzyme is composed of three domains, i.e. hydrophilic domain I ($\alpha 1$ – $\alpha 402$) containing the NAD(H) binding site, hydrophobic domain II ($\alpha 403$ – $\beta 260$) containing the membrane spanning α -helices and hydrophilic domain III ($\beta 261$ – $\beta 462$) containing the NADP(H) binding site (Fig. 1). Since 1995, the DNA encoding for domain I (dI) from *E. coli* [9,10], *Rhodospirillum rubrum* [9] and for domain III (dIII) from *E. coli* [10], *R. rubrum* [11,12], bovine [13] and human [14] transhydrogenases has been overexpressed and the proteins purified and characterized. Even in the absence of the transmembrane domain II (dII), a mixture of domain I and III catalyzes transhydrogenation reactions. This remarkable property shows that the whole apparatus necessary for hydride transfer is contained in the hydrophilic domains and therefore the reconstituted

dI+dIII system gives valuable information on the mechanism of the reaction, in particular on the hydride equivalent transfer step [15–17]. In parallel, important progress has been made in resolving the structures of the three domains from *E. coli* and *R. rubrum* transhydrogenases [18–21].

2. Activity assays

Three different transhydrogenase activities are routinely measured to characterize kinetic and thermodynamic properties of intact wild-type and mutant forms of transhydrogenases and also the isolated dI+dIII mixtures. These are the forward, reverse, and cyclic reactions. In the forward reaction, NADH reduces the NADP⁺ substrate analogue thio-NADP⁺, and in the reverse reaction, NADPH reduces the NAD⁺ analogue acetylpyridine adenine dinucleotide (AcPyAD⁺). The cyclic reaction is more complex; in the presence of NADP(H), hydride equivalents are transferred from NADH to AcPyAD⁺ via bound NADP(H) in domain III [22,23]. This reaction follows a ping-pong bi-bi mechanism [24].

3. Analysis of *E. coli* domain I, II and III structures

3.1. The nucleotide binding sites

The AB stereospecificity of the transhydrogenase hydride equivalent transfer reaction [4,5], and later thermostability experiments performed with *R. rubrum* transhydrogenase [25], led to the proposal that these membrane bound transhydrogenases have different binding sites for NAD(H) and NADP(H). The presence of multiple binding sites for NAD(H) and NADP(H) in the transhydrogenase monomer has also been suggested on the basis of its ability to bind on affinity column after proteolysis treatment [26,27], despite the lack of firm support from kinetics, e.g. no evidence of cooperative behavior. Binding studies on the soluble domains have ruled out this possibility and confirmed the proposal of one NAD(H) binding site on domain I [28] and one NADP(H) binding site on domain III [10,11].

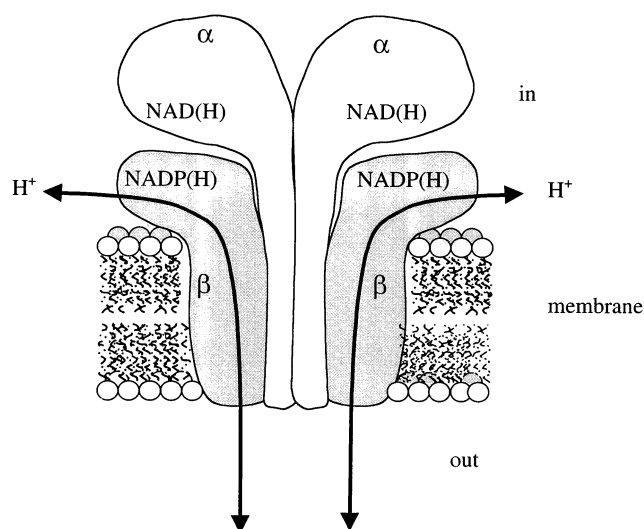


Fig. 1. Schematic representation of the *E. coli* transhydrogenase tetramer ($\alpha_2\beta_2$) where 'in' and 'out' represent the cytoplasm and periplasm, respectively.

3.2. The NAD(H) binding domain

Transhydrogenase was proposed to utilize a classical dinucleotide binding fold for NAD(H) recognition. A three-dimensional model was predicted based on PHD secondary structure predictions, hydrophobicity analyses and comparisons of conserved features of transhydrogenase with those of structurally defined NAD(H) binding proteins [29]. The prediction clearly supported the notion of one NAD(H) binding site.

In 1998, the crystal structure of alanine dehydrogenase (L-AlaDH) was solved [30]. The 31% sequence identity between *E. coli* transhydrogenase dI and alanine dehydrogenase from *Phormidium lapi-*

deum indicates homology and thus structural resemblance. Indeed, the L-AlaDH structure supports the main feature of the transhydrogenase NAD(H) binding domain model and gives an opportunity to extend the structural information to the entire dI. A Bestfit [31] alignment, manually modified to avoid insertions or gaps in α -helices and β -strands, is shown in Fig. 2A and provided the basis for homology modeling using SwissModel [32,33]. A model of transhydrogenase dI is depicted in Fig. 3.

The L-AlaDH monomer contains two similar domains. Each domain consists of mainly a parallel β -sheet surrounded by helices. The active site cleft is situated between domain A (residues M1-L128 and W306-A361) and domain B (residues L129-P305), the

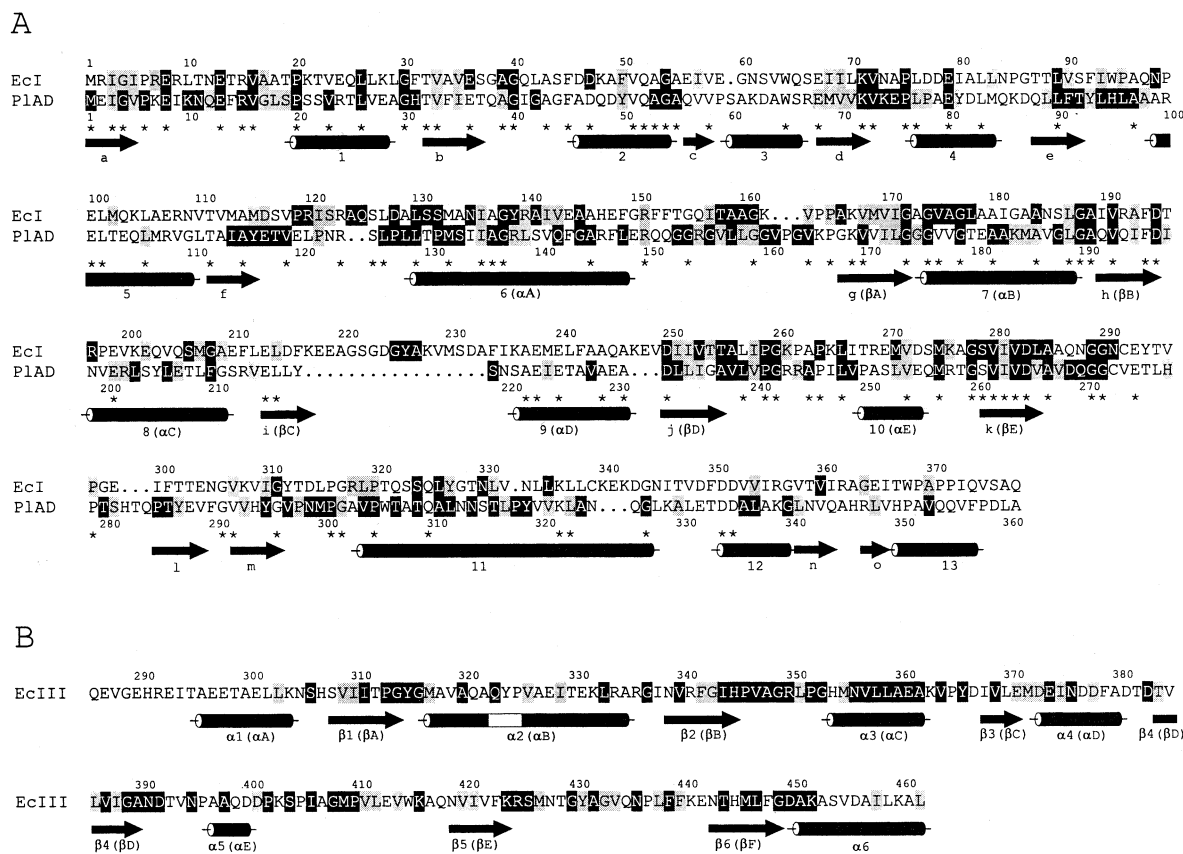


Fig. 2. (A) A sequence alignment of *E. coli* transhydrogenase dI (ecI) and *P. lapideum* alanine dehydrogenase (PIAD). The alignment is based on the Bestfit [31] program and manually modified to avoid insertions in regular secondary structures. Stars denote identities between ecI and PIAD. The numbering and position of secondary structures are adopted from [30]. The nomenclature used for a classical dinucleotide binding domain [34], and referred to in the text is shown within parentheses. (B) Sequence and structure information of *E. coli* domain III (ecIII). The secondary structures are based on NMR data (see text). Black shaded residues are conserved and gray shaded residues are similar (according to [35]) amongst L-AlaDHs from five species [30] or amongst H⁺-transhydrogenases from at least 11 of 12 species [36]. Helices and strands are depicted as cylinders and arrows, respectively.

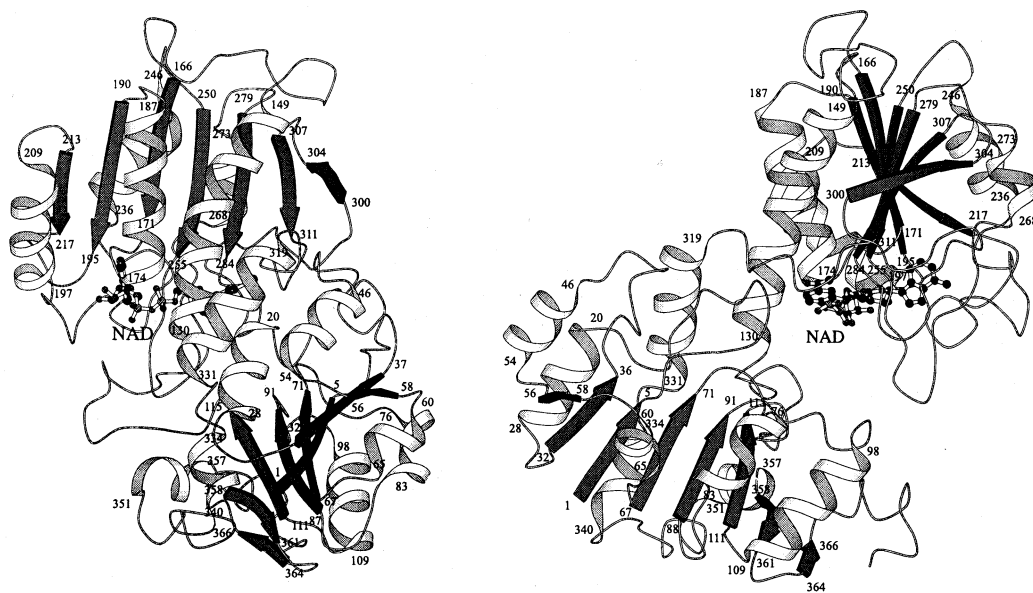


Fig. 3. MOLSCRIPT [37] illustrations of the predicted transhydrogenase dI structure. The two views relate to one another through a 90 degree rotation around the Y-axis. The homology model is based on the coordinates of alanine dehydrogenase (PDB entry: 1PJC). The sequence alignment in Fig. 2A was used as input to the automated comparative protein modeling server SwissModel. The resulting model was accepted with the exception that the loop region comprising residues α K217 to α F234 was manually modified. The coordinates of the model were subjected to the Verify3D algorithm [38] using the Verify3D Structure Evaluation Server, and the three-/one-dimensional scores were always positive and in the range of those for highly refined correct X-ray structures (data not shown).

latter binding NAD(H). In H^+ -transhydrogenase, the two corresponding domains will be referred to as dIA (α M1- α A129 and α T321- α Q378) and dIB (α L130- α P320), respectively. dIB binds NAD(H). In alanine dehydrogenase, the two domains, connected by a flexible hinge, work in synchrony to form the active site cleft. One can at most speculate on the role of dIA in H^+ -transhydrogenase. dIA may share an interface surface with dIII and could be important for guiding the nicotinamide ring of NADP(H) to its catalytically active orientation near the nicotinamide ring of NAD(H).

A segment of approximately 17 residues, which is present in transhydrogenase and absent in alanine dehydrogenase, is positioned between β C and α D in the model (α K217- α F234) (see Figs. 2A and 3). NMR studies revealed that this stretch constitutes a loop which interacts with the adenosine part of the NAD(H) dinucleotide [39].

3.3. The NADP(H) binding domain

3.3.1. Structure of domain III

In 1999, the secondary structure of the NADP(H)

binding domain from *E. coli* [18,20] and from *R. rubrum* [19] transhydrogenase was simultaneously proposed, both based on secondary structure prediction and NMR characterization. The two models showed that domain III essentially adopts a classical dinucleotide binding fold. However, the models differ in the position of the β -strands β C and β D. Because of the very similar behavior of the two domains in terms of activities, nucleotide binding properties, affinities to domain I, and their sequence similarity, it seems unlikely that these discrepancies are due to different origins of the domains.

The predicted structure of the *E. coli* domain III (ecIII) was primarily based on the conserved dinucleotide binding consensus-like sequence at residues β G314- β V319, the strong PHD secondary structure prediction of a $\beta\alpha\beta$ structure, and perhaps most importantly, on the conserved positively charged residues β H345 and β R350 downstream of β B with a presumed function to stabilize the 2'-phosphate group of NADP(H) [18]. The partial structure was confirmed and extended by NMR studies ([20], Fig. 2B). From the NOE pattern seen in the 3D ^{15}N NOESY-HSQC spectrum recorded at 800 MHz on a

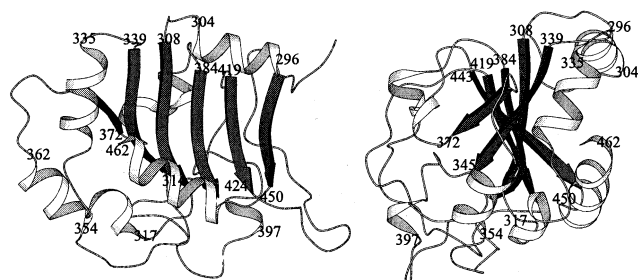


Fig. 4. Structure model of *E. coli* transhydrogenase domain III. The model was calculated with DYANA [45] using NMR data as reported in [20]. MOLMOL [44] was used to make the illustration. The two views relate to one another through a 90 degree rotation around the Y-axis. The protein sequence is preceded by a His-tag, visible in the illustration as the random coil at the N-terminus before α A. The NMR data unambiguously define a central six-stranded parallel β -sheet. The α -helices α B, α C and α D are positioned in a classical dinucleotide binding motif, the Rossmann fold, which is confirmed by NMR. The NMR data also suggest the current orientation of α -helices α A and α F on the same side of the β -sheet as α B and α C. The loops between β D and β E, and between β E and β F, as well as the position of α -helix E, are not defined in the model but are located on the same side of the β -sheet as α D for steric reasons. In agreement with the crystal structures for the mitochondrial dIII [40,41], NMR data also define the substrate binding site to be situated at the C-terminal end of the β -sheet, as expected in a classical dinucleotide binding motif, but no information is currently available on the substrate conformation and the substrate was thus not included in the structure calculation.

93% deuterated sample, a β -sheet containing six parallel β -strands with the strand order C, B, A, D, E, F was clearly identified (Fig. 4). This fact together with the topological arrangement of flanking helices and irregular structures revealed a three-layered structure of the type $\alpha/\beta/\alpha$. The recently published crystal structures of the bovine [40] and the human [41] mitochondrial transhydrogenase dIII essentially agree with the fold suggested from NMR data [20]. However, an important piece of new information obtained from these highly resolved structures was that NADP^+ is bound in an extended configuration, where the orientation of the molecule is turned 180 degrees as compared to the previously suggested orientation [18] and in e.g. NADP -dependent alcohol dehydrogenase [42] or quinone oxidoreductase [43]. Furthermore, the bound NADP^+ is well stabilized by a network of hydrogen bonds and other interactions [40,41].

All structures have been determined in the pres-

ence of substrate NADP(H) , since isolated dIII have been found to be unstable in the apoform.

3.3.2. Role of important residues in *ecIII*

As described in Section 3.3.1, the structure of the transhydrogenase domain III has characteristics of a classical $\beta\alpha\beta\alpha\beta$ binding fold (Fig. 4). Many residues of this domain have been mutated and the resulting proteins characterized. The effects of these mutations support fairly well the predicted structures and are consistent with the crystal structures of the mitochondrial domain III. The key residue β G314, located at the end of β A, and proposed to be the first glycine of the NADP(H) binding sequence GXGXXA/V , was effectively found to be essential [46]. The pronounced reactivity of β R350C towards diazotized AADP [18] indicated that, in agreement with the crystal structure, this residue is very close to the nicotinamide ring. Replacement of the positively charged residues β H345 and β R350 caused a dramatic loss of reverse activity but had less effect on the cyclic reaction and little effect on K_m^{NADPH} [18,27,47]. Furthermore, these mutants were found to be rather unstable and those at position β H345 were also highly sensitive to trypsin proteolysis [27]. Mutation of β A348, which is directly involved in the substrate binding, resulted in a substantial decrease of activity similar to the two previous residues, revealing the importance of the loop following β B in either the regulation of NADP(H) release or the conformational change triggered by the redox state of the molecule [18]. Residues proposed to be situated between α C and β D, and therefore on the outside of the binding site, such as β E371, β E374 and β D383, were found not to be important for activities or binding of nucleotides [48]. The stretches following β A and β D create a switchpoint, a cleft into which the dinucleotide is fitted. Residues β A390 and β D392, located between β D and α E and found to interact with the nicotinamide ribose [40,41], were shown to be important for the binding of nucleotides [18,48,49]. The β E strand is followed by a stretch of about 20 residues (β K424– β N443) which was seen by NMR spectroscopy as a loop [50]. Furthermore, most of β K424 and β R425 mutants displayed a drastic decrease of reverse activity compared to the wild-type enzyme and also elevated $K_m^{\text{NADP(H)}}$ [18,49]. By introducing a cysteine in a cysteine-free transhy-

drogenase and labelling it with sulfhydryl-specific probes, it was shown that β K424 and β R425 are located in or close to the binding site [18]. This was confirmed by the crystal structures where these two residues were found to stabilize the 2'-phosphate of the NADP molecule [40,41]. The pattern of medium and short range NOEs, in association with a chemical shift analysis, observed in NMR ^{15}N NO-ESY-HSQC spectrum, revealed the existence of an α -helix (β A451– β L462) following β F [20] which was affected by the redox state of the nucleotide [50]. β K452, which interacts with the adenine, was reported to be important for activity and the two or three last residues of this helix were found to be critical for the assembly and probably the folding of the β -subunit [51].

3.4. Structure and function of domain II

Domain II of transhydrogenases consists of approximately 370–400 residues which form the membrane spanning part of the enzyme responsible at least partly for the proton translocation. The topology of the transmembrane domain of transhydrogenases has been studied using both predictions and experimental approaches which resulted in different models ranging from 10 to 14 membrane spanning α -helices [52–58]. In order to clarify the topological ambiguities, the lengths of the transmembrane seg-

ments and their orientations were recently determined by an experimental approach in which unique cysteines were introduced into potential loops of a cysteine-free *E. coli* transhydrogenase [59]. These single cysteine mutants were then labelled with membrane impermeable thiol-specific reagents in two oppositely oriented membrane preparations. The number of helices and their topological orientations were unequivocally determined for the entire membrane spanning domain of the protein and a 13-helix model was presented (Fig. 5) [21].

The odd number of helices suggested for the *E. coli* enzyme seems, at a first glance, to be in disagreement with the mitochondrial transhydrogenases, which require an even number of helices since both the N- and C-termini of the single polypeptide are located on the matrix side [60]. However, the high sequence identities and the similar predictions obtained indicate a common topology for all transhydrogenases. Topology predictions of the mitochondrial enzymes suggested an additional helix constituted of the residues absent in the *E. coli* enzyme and located in the gap between the α - and β -subunits [21,58]. Consequently, the fusion of the *E. coli* α - and β -subunits, in which an additional stretch of residues similar to the bovine sequence were inserted, gave a more active enzyme than if the two subunits were fused directly to each other (J. Meuller, P.D. Bragg, J. Rydström, unpublished). It is likely that the model in

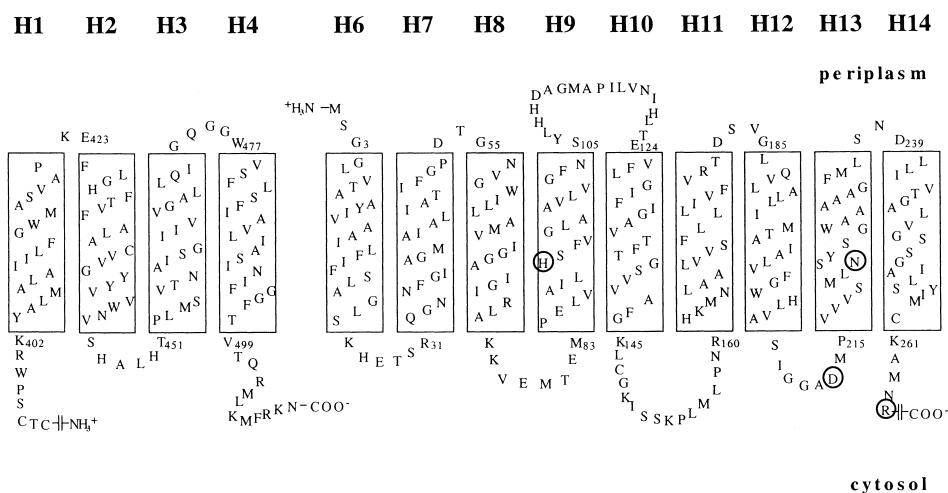


Fig. 5. Membrane topology of the *E. coli* transhydrogenase domain II. Transmembrane α -helices are numbered according to [60], as indicated above the sequence. The residues mentioned as important in the text, β H91, β D213, β N222 and β R265 are encircled.

Fig. 5, with an additional helix (helix 5) in the mitochondrial enzymes, applies to all membrane bound transhydrogenases.

By this cysteine labelling method [21,61], it has been shown that each transmembrane helix contains between 19 and 22 amino acids, the average length being what is required to span the non-polar region of the membrane. Thus, even taking into account the uncertainty about the proposed lengths, especially for helices 4, 10 and 11, this means that the helices should have a rather small tilt relative the membrane plane. The loops on the periplasmic side were identified as short and containing mainly unconserved residues (six residues or less except for the highly variable loop between helices 9 and 10), suggesting a lack of peripheral structural elements which could have had regulatory effects on the entry and exit point of the proton pathway.

The loops on the cytosolic side are generally longer and relatively well conserved; four of the eight loops contain at least 50% conserved residues². Surprisingly, most of the cytosolic loops are very accessible to large molecules such as fluorescein maleimide, indicating a rather flexible contact surface between the hydrophilic domains and dII [21].

The translocation of protons through transhydrogenases is very likely to directly involve the membrane domain and is therefore probably coupled to the catalytic events in domains I and III through long range conformational changes (see Section 5). Site directed mutagenesis has been performed on the *E. coli* enzyme in order to find the residues involved in the translocation/coupling mechanism. Nearly all conserved charged and polar residues have been mutated and not a single amino acid that is truly essential has been found, although some positions are severely affected by substitution [62,63].

Except for β H91N, mutations of β H91, located in the well conserved helix 9, have been found to result in a decrease of the reverse reaction to 2–20% of the wild-type enzyme activity [58,62,64,65]. The effects of the mutations were highly variable. Some mutants exhibited only a relatively high cyclic reaction, while others were mainly affected in their ability to pump

protons and, finally, a few were strongly inhibited with regards to all reactions. The mutations also affected the NADP(H)-dependent trypsin sensitivity of the β -subunit indicating that the effect of dII mutation was transmitted to dIII and, more specifically, to the NADP(H) binding site.

Mutations of the residue β N222 situated in another well conserved helix, helix 13, were found to inhibit all activities to a great extent and to have effects on the NADP(H) site [63]. Residue β D213 which, according to the 13-helix model, is situated in a very conserved cytosolic loop, has been suggested to be involved in proton translocation [13,58]. β D213 mutants exhibited similar features as β N222 although the importance of this residue is not clear. The remaining conserved charged and polar residues have been assigned as less important. However, since for most of them only one substitution had been performed and not always thoroughly analyzed, it is not unlikely that some may still participate actively in the proton pathway. For instance, it is noteworthy that α H450T retained only 17% activity of the reverse reaction which may be compared to the 19% activity remaining in β H91S. A more detailed investigation of this particular residue could reveal interesting properties.

The lack of protonable groups within the membrane that when mutated, specifically and severely affect proton translocation, suggests a proton pathway of polar residues and/or water molecules, with β H91 as the regulatory site, possibly in conjunction with β N222. These two residues were proposed to be located on very conserved sides of helices 9 and 13 respectively at a distance of 10–12 Å from the cytosol–membrane interface [21,63]. β D213 could be in the entry/exit port of the proton pathway.

The organization of the 26 transmembrane α -helices in the *E. coli* transhydrogenase tetramer (28 in the mitochondrial dimeric enzymes) is not well known. Examination of the residue variability to assign the functionally and/or structurally important helices, highlights helices 3, 9, 10, 13 and 14, which all possess more than 50% conserved residues, as candidates for subunit interactions and for constituting the framework of the proton translocating pathway. Indeed, these helices have a relatively high content of conserved Ala and Gly mixed with conserved polar residues, a suitable situation for a water-filled

² A residue was considered conserved when 80% of the aligned residues from 11 sequences were identical.

pathway. Proximity of helices 9, 10, 13 and 14 has not yet been shown but it seems likely that they together contribute to the putative proton pathway.

By constructing a set of deletion mutants, it was shown that helices 1, 2 and 7–11 were not essential for assembly although they do affect the stability and activity of the enzyme [8]. Crosslinking experiment using Cu-phenantroline gave α_2 dimers as the major product. It was shown that this dimerization was due to disulfide crosslinking between α C395 and α C397, i.e. the residues immediately preceding helix 1 [66]. It is thus likely that the transmembrane region of the α -subunits forms an α -helical bundle. Interestingly, in contrast to ecIII, the NADH binding domain of *E. coli* (ecI) was isolated as a dimer apparently with a very high affinity between the subunits [10].

Considering the two-fold symmetry that should apply for the *E. coli* transhydrogenase tetramer, it is therefore most likely that each β -subunit is associated primarily with the α -subunit, probably stabilized by interactions between the transmembrane α -helices. The binding of an antibody to dI, or of *R. rubrum* dI (rrI) to dIII did not separate the α - β -subunits, suggesting a strong binding between the two membrane parts (T. Bizouarn, unpublished). Contacts between β -subunits cannot be excluded. Furthermore, no evidence has been presented to suggest one common proton translocating channel per tetramer $\alpha_2\beta_2$.

4. Reconstitution of the dI-dIII complex from isolated domains I and III

4.1. General features of the dI+dIII system

Domain II in transhydrogenase is not required for the reversible transfer of hydride equivalents between NAD(H) and NADP(H). This fact was first reported by Yamaguchi and Hatefi who showed that mixtures of bovine dI+bovine dIII or *R. rubrum* dI+bovine dIII catalyzed reverse and cyclic transhydrogenation in the absence of dII [67]. However, the ability of a complex to transfer hydride equivalents between NAD(H) and NADP(H) seems to depend largely on dI. For example, rrI+ecIII mixture catalyzes a cyclic reaction of the same order of magnitude as

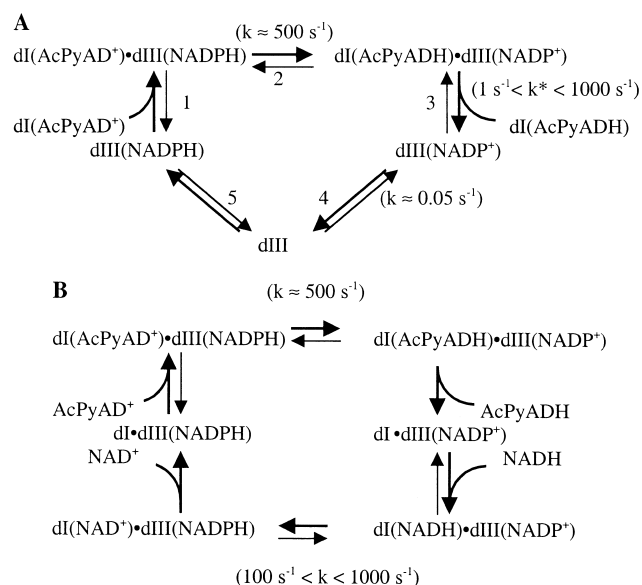


Fig. 6. (A) Scheme of the reverse reaction catalyzed by rrI-dIII complexes. At fixed rrI concentration, the reaction is composed of steps 1–3 and includes also steps of association/dissociation of nucleotides from dI. At fixed dIII concentration, the reaction is composed of the steps 1–5. The rate constant k^* is indirectly estimated and depends largely on the complex present [14,16,17]. (B) Scheme of the cyclic reaction catalyzed by rrI-ecIII complex. The rate constants of the four steps of association/dissociation of nucleotides from dI are considered to be non-limiting during this process [16,17]. From the rate of the global reaction, the rate of hydride ion equivalent transfer is predicted to be between 100 and 1000 s^{-1} .

that observed with the native enzymes. On the other hand, ecI and ecIII can also form a complex but with catalytic activities much lower than those of the intact enzyme, which may indicate, in this particular case, a role of dII in orienting the two domains relative to each other [17].

In the first detailed analysis of the catalytic properties of a dI+dIII mixture [11] it was demonstrated that, after purification and dialysis, *R. rubrum* dIII (rrIII) still contained tightly bound NADP(H), suggesting a high affinity of isolated rrIII for substrate, much higher than that in the intact enzyme. This property was found to be common to all wild-type domain III isolated so far [10,11,14], with the exception of the bovine domain III generated by cleavage of the whole enzyme by papain [67]. This latter result may be due to an experimental difficulty in either purifying the fragment or in separating nucleotides from the protein, which is necessary for dissociation constant measurements [68].

The tight NADP(H) binding has dramatic consequences for transhydrogenation activities catalyzed by dI+dIII mixtures. For example, at fixed rrIII and saturating levels of rrI, the forward and the reverse reaction rates were limited by the slow release rates of thio-NADPH ($6 \times 10^{-4} \text{ s}^{-1}$) and NADP^+ (0.03 s^{-1}), respectively [11] (Fig. 6A). EcIII contains a single tryptophan residue in position βW415 , and its fluorescence quantum yield is lowered when NADPH binds to ecIII, at least partially through an energy transfer process [10]. This feature was utilized to determine that ecIII, like rrIII [11], displayed a 50-fold higher affinity for NADPH than for NADP^+ . The values for the individual dissociation constants could not be determined with great accuracy, but estimates suggested a K_d^{NADPH} in the nM range [10].

Protein–protein titrations, where one domain concentration is kept fixed and the second domain concentration is varied, are essential for relevant comparisons of the various dI+dIII systems. In the following discussion, rates will be referred to as the specific activity of the fixed domain, at saturating concentrations of the second domain.

4.2. Cyclic and reverse reactions catalyzed by dI+dIII mixtures

The ability of some dI-dIII complexes to catalyze a cyclic reaction similar to that of the intact enzyme (Fig. 6B) is of great interest. Four sets of experiments may be pointed out. First, a similar deuterium isotope effect was observed for the hydride transfer step measured by stopped flow [16] and for the whole cyclic reaction, both catalyzed by the rrI+rrIII mixture [17]. Second, mutations inhibiting specifically hydride transfer, such as Y235N in rrI, decreased the cyclic reaction in the same proportion for the rrI+rrIII mixture as for the whole enzyme [69,70]. All these data show that in all cases the same step, namely the hydride equivalent transfer, is rate limiting. Third, the concentration dependence for NAD(H) and analogues of the cyclic reaction were identical for both the rrI+rrIII mixture and the whole enzyme [69]. Fourth, the pH dependences of the cyclic reaction catalyzed by rrI+rrIII mixtures and the intact *R. rubrum* enzyme were similar [71]. Therefore, dII has little effect on the NAD(H) bind-

ing site of rrI and on the interactions between the two domains. Thus, and as has been mentioned earlier [67,70], the complex formed with the rrI+rrIII mixtures and also with the rrI+ecIII mixtures and the intact enzyme during hydride transfer may be considered as nearly identical. Therefore, the information obtained with dI+dIII mixtures could be used to understand part of the mechanism of the reaction catalyzed by the wild-type enzyme in physiological conditions.

Because of the tight nucleotide binding on dIII and absence of regulation by protonation/deprotonation events, measurement of rate constants in pre-steady state conditions using dI+dIII mixtures have been accessible (see review [70] for a detailed analysis). Recent stopped flow experiments showed that H^- -transfer between NADPH bound to rrIII and AcPyAD^+ bound to rrI is direct [15] and occurs at a rate of about 500 s^{-1} , approximately one order of magnitude faster than the cyclic reaction rates observed for the same complex. Even though this calls for a word of caution, the difference is not enormous. It is possible that the H^- -transfer from NADH to NADP^+ is slower than that from NADPH to AcPyAD^+ . Also, the formation of dead-end complexes, i.e. dI(NADH)·dIII(NADPH) and dI(AcPyAD^+)·dIII(NADP^+) will decrease the effective concentration of the catalytically competent complex, thus rendering the estimation of V_{max} difficult.

A specific problem with isolated domains is related to their association and dissociation and in identifying the consequences of these events on the rate of the reactions. As discussed [17], protein–protein titrations for the cyclic reaction can be regarded as apparent binding curves. Regardless of which domain was kept constant, the maximal rates and the concentration of protein needed to obtain $1/2 V_{\text{max}}$ were similar. Therefore, the maximal cyclic reaction rates measured give crude estimates of hydride equivalent transfer rates. From the titration curves, it can be concluded that the affinities of rrI for both rrIII and ecIII are high [11,17] but low for human dIII [14], and that the cyclic reaction rates catalyzed by those complexes are similar. These results were confirmed by stopped flow experiments [14,16]. On the other hand, ecI displays a low affinity for ecIII and essentially no affinity for rrIII [17]. That rrI has a high affinity for dIII is presumably a consequence of

the fact that it is naturally expressed as a separate subunit, lacking transmembrane helices. Thus, it must have a high affinity for either the loops extruding from dII or for dIII in order to form a functional enzyme. In the intact *E. coli* enzyme, ecI is a part of the α -subunit which contains four transmembrane helices that are firmly anchored to the membrane spanning portion of the β -subunit. Consequently, the low cyclic reaction rates catalyzed by the ecI-ecIII complex may be due to improper contacts between the two domains. The dissociation of the two domains does not seem to contribute to the mechanism of the cyclic reaction.

The reverse and forward reaction catalyzed by dI+dIII mixtures is of somewhat less interest for understanding the catalytic process by which the intact enzyme works. However, it gives valuable information concerning the properties of the binding between the two domains. As mentioned above, the release rate of NADP^+ limits the reverse reaction as calculated per domain III with excess dI. The concentration of dI required for half maximal rates will depend on the number of NADPH-containing dIII molecules that each dI can visit and successfully oxidize during the time it takes for one dIII to release its NADP^+ product [11]. It is reasonable to assume that binding and release rates of the dI substrates, i.e. NADH and AcPyAD^+ , are not limiting, neither in the reverse nor in the cyclic reaction catalyzed by dI+dIII mixtures or intact transhydrogenase from *E. coli* and *R. rubrum*. This assumption is based on the kinetics of the transhydrogenase reactions measured by stopped flow techniques [16], NMR data on domain I from *E. coli* or *R. rubrum* [72], and on the kinetic isotope effects observed for the cyclic reaction [24]. Therefore, it is conceivable that the maximal reverse rate observed with fixed rrI and variable rrIII is mainly limited by the lifetime of the complex. However, the much higher rates seen with constant rrI and variable ecIII [17] than with constant rrI and variable rrIII [11] suggest a shorter lifetime of the rrI-ecIII complex compared to the rrI-rrIII complex. The maximal reverse reaction rate calculated per rrI with excess ecIII is of similar order as the maximal cyclic reaction rate observed for rrI+ecIII mixtures. Therefore, it is possible that H^- -transfer is also partly rate limiting for the reverse reaction catalyzed by this mixture.

5. Conformational changes

5.1. Indirect versus direct coupling mechanism

The view that proton translocation is coupled to events taking place in the NAD(P)(H) binding site through conformational changes has become a common working model [70,73,74]. The directionality of proton translocation must be tightly regulated to avoid slips and maintain the experimentally observed H^+/H^- ratio of unity. That is, the binding of the reactant, NADP^+ , must be associated with protonation of a residue, or possibly a tightly bound water molecule, from the periplasmic side, and the release of product, NADPH, must be accompanied by proton release to the cytoplasm. One hypothesis (indirect coupling mechanism) is that this is achieved by redox-driven conformational changes, perhaps by a regulation of pK_a values and/or a change of accessibility of protonable groups in the proton pathway. An alternative hypothesis (direct mechanism) is that the difference in charge of the substrates, following hydride equivalent transfer, directly influences the pK_a of groups in the immediate vicinity of the nicotinamide moiety to affect directionality (see review [2] for more details). Conformational changes indeed occur during turnover of the enzyme, which will be described below. In order to better understand the transhydrogenase mechanism, it is important to determine both the number of conformational states involved in the coupling mechanism and their nature.

5.2. NAD(H)-induced conformational changes

Substrate binding is often accompanied by structural changes. For transhydrogenases, it has been reported that NAD(P)(H)-induced conformational changes influenced chemical modification reactions [75–82], proteolysis [25,76,83], and thermostability [76,84].

Conformational changes following NAD(H) binding were proposed to be important parts of the catalytic mechanism of NAD-dependent dehydrogenase [85]. This has found support in crystal structures of formate dehydrogenase [86], where NAD^+ binding caused a closure of the active site cleft mediated by alterations in the hinge region connecting domains A and B. The flexibility of the hinge was also observed

in phosphoglycerate dehydrogenase [87], and is probably a general feature of enzymes in this superfamily [30,88]. That domain I of transhydrogenase belongs to this group of proteins raises several intriguing issues. For example, since hydride equivalent transfer is stereospecific and direct [15], the 4A hydrogen on the C4 position of NADH must be near the si-face of the NADP⁺ nicotinamide moiety at the moment of redox exchange. The cleft between dIA and dIB must be flexible enough to accommodate sufficient space for NADP(H) to approach NAD(H) for direct hydride equivalent transfer, while still bound to dIII. The design of the hinge region may be one factor that is important in this regard. It is interesting to note that there is, in comparison to the L-AlaDH template, an insertion of two strictly conserved residues, α A124 and α Q125, in the predicted hinge region. These two residues may influence the orientation of the two domains relative to one another.

Another region of interest is the stretch of approximately 20 amino acids following β C in dIB which is unique to transhydrogenases [89]. NMR experiments performed on both *R. rubrum* and *E. coli* domain I, showed that this region constitutes a flexible loop [28]. Upon NAD(H) binding, the mobility of the loop decreased. From the effect of mutation of *R. rubrum* Y235 and M239 located in this segment [69,70,72], they proposed that the loop closure would be important for positioning the nicotinamide ring but would be involved neither in discrimination between NADH and NAD⁺ nor in the binding of dI to dIII. NOE signals between the A8 protons of the adenine ring and the C α protons of Y235 and A236 indicate that the loop does not lock the nicotinamide moiety of NAD(H) per se. Rather, indirect effects of the loop closure to facilitate hydride equivalent transfer may be envisaged, e.g. by rearrangement of the NAD(H) orientation. This could be critical for allowing the C4 positions of the two nicotinamide moieties to end up within 1–2 Å from one another. It is interesting to note that reduced nicotinamide (NMNH) is a substrate with a poor affinity for dI and producing a low but still significant cyclic activity [49]. Even though the NAD(H)-induced conformational change facilitates efficient hydride transfer, there is no evidence that this process directly influences proton conduction. Further-

more, there is no indication that NAD⁺ and NADH induce different conformations [39,70].

5.3. NADP(H)-induced conformational changes

It is becoming increasingly evident that NADP(H) binding and release are important events in the coupling mechanism [10,24,64,90]. Tong et al. demonstrated that the β -subunit is only sensitive to trypsin digestion in the presence of NADP(H), suggesting a substrate-induced conformational change [57]. This has also been observed for the *R. rubrum* [25] and to some extent, the bovine transhydrogenases [83]. The cleavage site in the *E. coli* enzyme was localized to β R265 [57]. This characteristic feature is not dependent on interactions with dI, since the same cleavage occurs upon addition of NADP(H) even after digestion of dI. Note that β R265 is only five residues from the C-terminal end of what is thought to be the last transmembrane helix. It is thus possible that the structural changes caused by NADP(H) binding are not confined to the binding pocket but propagated to dII as well. They could, for example, move dIII in closer contact with the membrane and the proton channel. This view is supported by the resistance of β M214C to modification by fluorescein maleimide in the presence of NADP(H) (J. Meuller, unpublished). Indeed, a communication between the NADP(H) site and domain II has been validated by several observations. For example, the β -subunit in the mutants β H91C, β H91S and β H91T was no longer digestible by trypsin in the presence or in the absence of NADP(H) [64]. In contrast, the β H91K mutant was trypsin-sensitive even in the absence of added NADP(H) probably due to substoichiometric amounts of tightly bound NADP⁺ [65]. Also, a mutant lacking the six C-terminal residues in the α -subunit contained NADP⁺ and was cleaved by trypsin [64,65].

Interestingly, the isolated ecIII [10] and rrIII [9] have much higher affinities for NADP(H) than the intact enzymes, e.g. the NADPH dissociation constant is in the range of nM for ecIII, whereas it is about 2 μ M in the intact *E. coli* transhydrogenase [18]. It has been suggested that the conformation of isolated dIII mimics those of the mutants with elevated NADPH affinities, e.g. β H91K, and that this

conformation is transiently present during turnover of the enzyme [10,71].

From a preparation where the α -subunit was specifically degraded by trypsin, but where the β -subunit and probably the whole dII were intact, activities could be recovered by the addition of rrI (T. Bizouarn, unpublished). The catalytic properties of this preparation were comparable to those obtained with the intact enzyme. More specifically, the much higher reverse reaction rate of trypsin-digested *E. coli* enzyme with excess rrI compared to ecIII revealed a rather high release rate of NADP⁺. Also, the K_m^{NADPH} for this reaction was about 15 μM , a value very similar to the one obtained with intact enzyme [17]. Taken together, these results clearly demonstrate a pronounced interdependence between the NADP(H) site and dII.

5.4. Redox-dependent conformational changes

That NADPH and NADP⁺ induce different conformations has been observed from their differential effects on thermostability and/or proteolysis, in rat liver [84] and bovine transhydrogenase [75,76]. Similar observations have been found for the *E. coli* enzyme. The sensitivities to trypsin cleavage are slightly different depending on the redox state of the substrate bound to dIII. Circular dichroism measurements on the isolated ecIII indicated a structural change as NADP⁺ was exchanged for NADPH [10]. CD also showed that the NADPH bound structure was more resistant to thermal denaturation, in agreement with thermal inactivation data reported for the mitochondrial enzyme. It is clear that the NADP⁺ and NADPH bound forms of ecIII are physically different [10]. In order to determine which residues and regions of ecIII are structurally altered, NMR techniques were used. A number of chemical shifts in an HSQC spectrum were altered upon exchange of bound NADP⁺ for NADPH both for ecIII [50] and rrIII [19]. The sequential assignment of the peaks allowed identification of residues that were affected. Many of these residues were found in regions located in the immediate vicinity of the NADP(H) binding site, suggesting either a different conformation of the substrate, an altered structure of the site, or both. However, the high number of shifted peaks is consistent with a conformational change of a larger part

of dIII. From a similar comparison performed on rrIII it was concluded that substitution of NADP⁺ for NADPH might induce structural changes in both the adenosine and nicotinamide binding parts of dIII but also along αE and βE [19].

An interesting observation that may be linked to the redox-dependent conformational states of the enzyme is the difference in cyclic reaction rates observed in presence of NADP⁺ versus NADPH [62]. It is possible that the conformation of the enzyme caused by NADP⁺ binding, and the NADPH conformation, differ in either their hydride equivalent transfer rates or the pK_a of the groups involved.

6. pH dependences and mechanisms

6.1. pH dependences of forward and reverse transhydrogenation

The coupled transhydrogenase reactions involve protonation and deprotonation events. Thus, studies on the pH dependences on forward, reverse, and cyclic reactions in wild-type and mutant transhydrogenases as well as in a reconstituted system of dI·dIII, may provide useful information concerning the catalytic/coupling mechanism. As will become clear in the following sections, it is not trivial to arrive at a unifying mechanism that can account for all observed pH dependence profiles. The forward and reverse reactions display bell-shaped pH dependences in both the *E. coli* and the *R. rubrum* enzymes [62,71,90]. Maximal turnover is observed at about pH 6.5 and 7.0 for the forward and the reverse reactions, respectively. Utilizing [4B-²H]NADPH, a kinetic isotope effect was observed for the reverse reaction catalyzed by the *E. coli* enzyme at pH 8.0 but not at 6.0, and it was suggested that hydride transfer is limiting at high pH, whereas NADP⁺ release is limiting at low pH [24]. Similar experiments concerning the forward reaction were also consistent with a slow release of NADPH at pH 6.0, but the minimal isotope effect using [4A-²H]NADH indicated that in this reaction other steps than hydride equivalent transfer were limiting at high pH, e.g. conformational changes. In order to develop a mechanism for the coupled transhydrogenase reaction, it is important to identify the protonable residues that are responsible

for the regulation of substrate release and hydride equivalent transfer rates.

Studies of pH dependences obtained with mutant enzymes may provide some information. Unfortunately, very few mutants have been studied in detail in this context. However, the general observation is that mutation of residues located in dII and dIII which were found to be important for the binding of NADP(H), either directly such as β K424 or indirectly such as β H91, changed the pH profile of the reverse reaction in similar ways. Reverse transhydrogenation catalyzed by these mutants increased as pH was lowered from 10 to 7, like the wild-type enzyme. However, further lowering of pH was not accompanied by the normal decrease in activity. It varied from a less pronounced decrease between 7.0 and 5.5, in the case of β H91E [62], β K424C and β R425C mutants [36], to an increase of activity in the case of β K424R [62] (and also the complex $ec\beta+rrI$ [17]). This suggests that the pH dependence of the reverse reaction may not truly reflect the titration curve of a protonable group(s) responsible for the proton translocation as proposed [71], but may reflect a more complex chain of events, including conformational changes. The release of NADP(H) from dIII probably requires a finely regulated system to obtain a tightly coupled reaction. Nevertheless, the proposition that in bacteria, NADP⁺ binding and release are associated with protonation/deprotonation from the periplasm and NADPH binding and release are associated with protonation/deprotonation from the cytoplasm is still valid. This is supported by the stimulation of the forward reaction at high pH and the inhibition of the reverse reaction mainly at low pH by the presence of a proton gradient, when catalyzed by the membrane bound enzyme [71].

6.2. pH dependence of transhydrogenase reactions catalyzed by dI+dIII mixtures

Studies of pH dependences of the reconstituted dI+dIII system have been performed with the aim to determine the roles of the various domains with regard to protonation events important for the various reactions. In contrast to the intact *E. coli* and *R. rubrum* enzymes, the reverse reaction catalyzed by the dI+dIII mixtures investigated [14,17,71] is slow

and relatively pH independent. These measurements were performed with fixed dIII and dI in excess, and therefore indicate that release rates of NADP⁺ are not greatly influenced by pH in the range of 6.0 to 9.5, in contrast to what is believed to be the case in the intact enzyme. This is interpreted as a decoupling of the transhydrogenase reaction from the translocation of protons when dII was absent, which strengthens the view of an indirect coupling mechanism. It also suggests that in order to increase the rate of NADP(H) release from the very low value observed with dI+dIII mixtures to the high value of the intact enzyme, a proton has to be translocated through dII, thus creating a bell-shaped pH dependence of the reverse reaction with the intact enzymes.

Interestingly, the cyclic reaction displayed a similar pH dependence regardless of the presence or absence of dII, which was true both for the *E. coli* and *R. rubrum* enzymes [17,71]. It seems likely that the group(s) that is (are) important for the pH dependence of the cyclic reaction reside(s) in the extramembraneous domains. Furthermore, the pH dependences of the cyclic reaction catalyzed by $rrI\cdot rrIII$, $rrI\cdot ecIII$ and $rrI\cdot hIII$ complexes are very similar ($pK_a \approx 9.5$), which suggests a location of the group(s) in dI. As suggested [17,70], this probably represents a scalar protonation event not involved in the energy coupling process.

6.3. Is the coupling dependent on one protonable site?

The generally accepted basis of the coupling mechanism is that binding and release of NADP(H) are accompanied by binding and release of protons. Protonation of the enzyme dramatically increases the affinity of dIII for nucleotides and positions the molecule in order to allow hydride transfer to take place, a condition necessary for a tight coupling between the transhydrogenation reaction and the proton translocation. If it is assumed that only one key residue is involved, this protonable group has protonic access to the periplasmic side if NADP⁺ is bound to the protein, and to the cytosolic side if the enzyme contains bound NADPH (Fig. 7). In the initial studies, the existence of only one protonable group seemed sufficient to explain the pH dependences of all the reactions catalyzed by wild-type *E. coli* transhydrogenase [24,90,91]. A more sophisti-

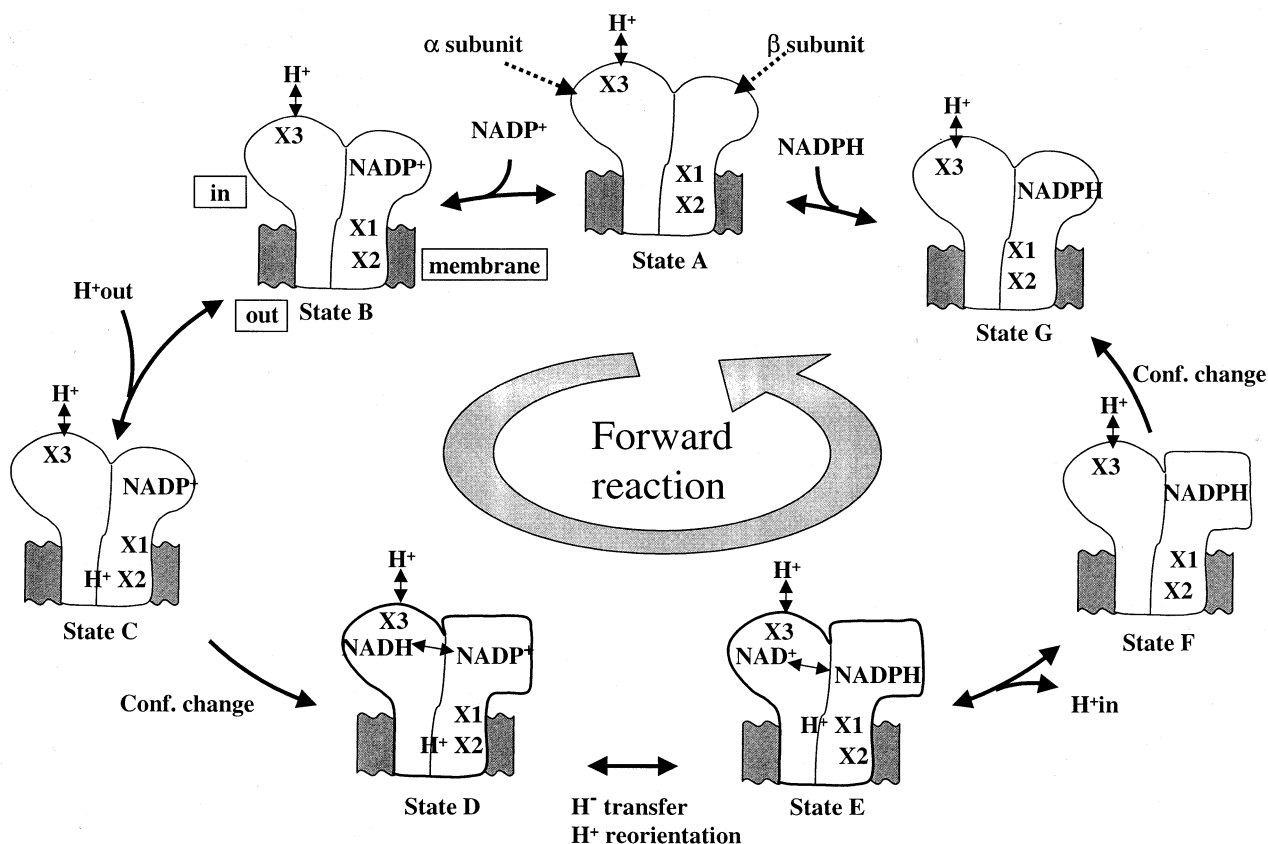


Fig. 7. Schematic representation of the binding change mechanism during the forward reaction catalyzed by the *E. coli* transhydrogenase monomer. For clarity, NAD⁺ and NADH have been drawn only for the states D and E but they both bind in rapid exchange to all conformational states of the enzyme. X1 and X2 represent the two putative groups involved in the proton translocating process and are accessible to the bulk only in the presence of NADPH and NADP⁺, respectively. X3 represents the protonable group located in dI and is responsible for pH dependence of the cyclic reaction. See text (Sections 6.3, 6.4) for further details.

cated model including steps of conformational changes was proposed in order to be able to include the pH dependences observed with the *R. rubrum* enzyme [71].

From the site directed mutagenesis experiments carried out so far, β H91, the only conserved charged residue located in a transmembraneous helix, emerges as a highly important residue, which has properties suggesting that it is involved in the coupling process. For example, all mutations of β H91 severely inhibited proton translocation. NADP(H) binding and the associated conformational changes, observed in trypsin cleavage experiments, were differently affected depending on the type of mutation [64]. The replacement of β H91 with a positively charged residue such as lysine, increased the affinity for nucleotides to a level comparable to that ob-

served for isolated dIII [65], which has been proposed to mimic the protonated (or 'occluded') state of the intact enzyme [17,70]. On the other hand, replacement with a small non-polar residue like threonine or cysteine seemed to lock the site in a conformation insensitive to trypsin cleavage which may be similar to the unprotonated state [64]. It is therefore clear that this residue is part of the proton translocation pathway involved in the coupling process.

The accumulation of data concerning the pH dependences of the cyclic reaction favors the involvement of a second group located in dI, regulating the hydride equivalent transfer step and is suggested not to be part of the proton translocation pathway. Therefore, this group would not take part in the regulation of NADP(H) binding and/or release, which would explain the lack of pH dependence of

the reverse reaction catalyzed by the dI+dIII mixtures. Furthermore, the cyclic reaction catalyzed by β H91K still displayed an apparent pK_a similar to that of the wild-type enzyme. This observation makes it unlikely that β H91 alone regulates the hydride equivalent transfer rates of the cyclic reaction, even in *E. coli* enzyme (cf. Section 6.1).

Some observations are not readily accounted for by the hypothesis of one protonable group responsible for the coupling mechanism. For example, proton pumping activities are generally not completely abolished by mutations of β H91 residue. The mutant β H91N was capable of catalyzing the reverse reaction to a significant extent but with a strongly inhibited proton translocating activity. Furthermore, the conformational change induced by binding of NADP(H), which increases the sensitivity to trypsin, still occurred [64]. Finally, the pH profile of the reverse reaction catalyzed by β H91E mutant did not only display a shift of the peak towards lower pH, but the peak was also broadened [62]. These observations may indicate the involvement of other protonable groups contributing to the coupling process, either indirectly by stabilizing the proton on β H91, or directly, by participating in the orientation of the vectorial proton towards the cytoplasm or the periplasm. However, all but one of the charged and conserved polar residues situated in dII have been studied. Except for β H91, none has been found to have properties that correspond to what is expected from a residue involved in the coupling mechanism. Interestingly, β N222, probably located at around the same distance from the periplasm as β H91, seems to be specifically important for the NADP⁺-induced conformational changes [63]. This residue may play a role in the coupling between the redox state of the nucleotide and the proton movement in the protein. β D213 is probably located in or close to the entry/exit of the proton pathway, with a possible role in its opening or closing.

6.4. Outline of a hypothetical reaction mechanism

A hypothetical mechanism for the transhydrogenase reaction is schematized in Fig. 7. It is a development of previously presented schemes [24,71]. At the outset, it is assumed that NAD⁺ and NADH are in rapid exchange with all the conformational states of

transhydrogenase along the reaction pathway. Thus, the energy transducing steps involve the NADP(H) binding site. This scheme is more general and is not restricted by the assumption that only one residue is responsible for coupling. For example, one residue may be responsible for the communication with the periplasm (X2) and another with the cytoplasm (X1). It also includes a protonation of a group on dI necessary for hydride equivalent transfer. Taking the forward reaction as an example, the critical point is that hydride transfer is not allowed until four events (states A to D) have occurred, i.e.: (A) binding of NADP⁺ succeeded by (B) binding of a proton from the periplasmic side, which results in a change of affinity for nucleotides in dIII, (C) binding of NADH and (D) protonation of a group in dI. This last condition is not an event in the cycle but a state required in order to reach its maximal activity. The forward reaction proceeds through hydride equivalent transfer from NADH to NADP⁺, yielding NADPH and NAD⁺. This step is accompanied either by a reorientation of the protonated group (in dII) or an internal movement of the vectorial proton, and is not necessarily as rapid as the hydride transfer (states D to E). It is therefore crucial for a tight coupling that the vectorial proton is released to the cytoplasm only in the NADPH bound state of the enzyme. The catalytic cycle is completed when both the proton and NADPH have departed, and the associated conformational changes have taken place. It has been previously suggested that isolated dIII is trapped in the states where hydride equivalent transfer is fast and release of nucleotide is slow. In dIII, the structures have been found to be different depending on the redox state of NADP(H) [10,19,50]. By extrapolation, this would then support the proposition that the states D and E in the intact enzyme are structurally different (Fig. 7). The finding that the affinity of dIII for NADPH is approximately 50-fold higher than for NADP⁺ may imply that in the hydride equivalent transfer competent intermediates, formation of NADPH is thermodynamically favored. This was also suggested by a comparison of pre-steady state kinetic measurements of NADP⁺ reduction by AcPyADH with NADPH oxidation by AcPyAD⁺ which again indicated a shift of the equilibrium constant of the hydride equivalent transfer step on the enzyme compared to in solution [92].

Then, the energization of the membrane is used not at the hydride equivalent transfer step but first to increase the affinity of dIII for nucleotides, in a state where the formation of NADPH is favored and, second, to facilitate the release of the product NADPH from dIII. Clearly, a binding change mechanism is utilized by transhydrogenase following the same principles as those for the mitochondrial H^+ -ATP synthase [93].

7. Conclusion

A large amount of structural information has been unearthed for transhydrogenases during the last few years. The numerous site directed mutagenesis experiments combined with the recently solved structure of dIII, have provided important general knowledge of the structure function of most of the regions of the enzyme. However, even though key residues in the membrane domain have been identified, the complete proton pathway is still unknown and the residues or regions involved in the long range conformational changes are uncertain.

A very exciting time lies ahead in the field of transhydrogenase research, where advances of structural information are anticipated to boost the level of understanding of the mechanism of hydride equivalent transfer and, in the longer perspective, how this process is linked to proton translocation.

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