FEBS Letters 545 (2003) 18–24 FEBS 27210

Minireview

Proton translocation by transhydrogenase

J. Baz Jackson*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Received 13 January 2003; accepted 17 January 2003

First published online 22 April 2003

Edited by Bernard L. Trumpower

Abstract Transhydrogenase, in animal mitochondria and bacteria, couples hydride transfer between NADH and $NADP^+$ to proton translocation across a membrane. Within the protein, the redox reaction occurs at some distance from the proton translocation pathway and coupling is achieved through conformational changes. In an 'open' conformation of transhydrogenase, in which substrate nucleotides bind and product nucleotides dissociate, the dihydronicotinamide and nicotinamide rings are held apart to block hydride transfer; in an 'occluded' conformation, they are moved into apposition to permit the redox chemistry. In the two monomers of transhydrogenase, there is a reciprocating, out-of-phase alternation of these conformations during turnover.

Key words: Transhydrogenase; Membrane protein; Ion translocation; Nicotinamide nucleotide; Redox reaction

1. Introduction

Transhydrogenase operates at an important three-way interface between the two major, soluble redox cofactors in the cell – NAD(H) and NADP(H) – and the proton electrochemical gradient (Δp). Under most physiological conditions in animal mitochondria and bacteria, the enzyme is a consumer of Δp:

$$NADH + NADP^+ + H^+_{out} \Leftrightarrow NAD^+ + NADPH + H^+_{in} \quad (1)$$

The energy of the gradient can drive the [NADPH][NAD^+]/[$NADP^+$][NADH] ratio to values >400. The resulting NADPH is used for biosynthesis and for reduction of glutathione (needed, for example, to limit damage caused by free radicals generated in the respiratory chain) [1]. The physiological role of transhydrogenase has a more subtle aspect in mitochondria from some animal tissues (e.g. heart and muscle) where, in principle, the NADP-linked isocitrate dehydrogenase has a greater capacity to produce NADPH than transhydrogenase. Here, it is thought that transhydrogenase and the two isocitrate dehydrogenases (the NAD- and NADP-linked enzymes) together catalyse a 'mini-cycle' between isocitrate and α-ketoglutarate that fine-tunes the regulation of the Krebs tricarboxylic acid pathway. An increased rate of consumption of Δp is the metabolic price to pay for the improved control [2].

Although transhydrogenase catalyses a redox reaction, its energy-coupling processes probably have more in common with those of F_1F_o-ATP synthase than with those of the respiratory chain complexes: (a) in transhydrogenase and the F_1F_o-ATP synthase, nucleotide-binding changes at the catalytic site are central to the mechanism, and (b) in both proteins the nucleotide-binding site is located some distance from the proton translocation pathway and linkage is achieved by conformational change. In this article, information on the coupling mechanism that emerges from recent kinetic and thermodynamic experiments and from considerations of recent X-ray structures of transhydrogenase components will be reviewed. The main theme is that coupling depends crucially on movements of the nicotinamide rings of the nucleotides either to block or to permit hydride transfer.

2. Transhydrogenase basics (for earlier reviews, see [3–5])

1. Transhydrogenase is the only ion translocator that we are aware of in which the standard free energies of the products of the scalar chemical reaction are similar to those of the reactants.
2. One proton is translocated per hydride ion equivalent transferred from NADH to $NADP^+$.
3. The transhydrogenase reaction is stereospecific for the C4*A* (*pro-R*) hydrogen of the dihydronicotinamide ring of NADH and the C4*B* (*si*) face of the nicotinamide ring of $NADP^+$.
4. There is no intrinsic hydrogen exchange between the reduced nucleotide and water protons during enzyme turnover.

3. Transhydrogenase architecture

The global structure of transhydrogenase is very similar in a wide variety of organisms. The dI component, which binds NAD^+/NADH, and the dIII component, which binds $NADP^+$/NADPH, protrude from the membrane – on the matrix side in mitochondria and on the cytoplasmic side in bacteria (Fig. 1). The dII component spans the membrane. The intact enzyme is essentially a dimer of two dI+dII+dIII 'monomers' (designated A and B), although there is variability in the arrangement of the polypeptides amongst species.

The dI component has two domains, dI.1 and dI.2 [6–8]. Both comprise parallel twisted β-sheets flanked by helices and have the form and connectivity of the Rossmann fold. They

*Fax: (44)-121-414 5925.
E-mail address: j.b.jackson@bham.ac.uk (J.B. Jackson).

Abbreviations: $AcPdAD^+$, acetylpyridine adenine dinucleotide; TM, transmembrane

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00388-0

are separated by a deep cleft and linked by two long helices (α6 and α11). The NAD^+/NADH-binding site is located within the cleft. A GXGXXG 'fingerprint', characteristic of a class of proteins that bind nicotinamide nucleotides [9], is located in the loop at the N-terminus of the 'pyrophosphate-binding' helix in dI.2. The specificity of NAD^+/NADH over $NADP^+$/NADPH probably derives from the H-bond interaction between Asp202 (*Rhodospirillum rubrum* numbering[1]) and the 2′-OH of the adenosine ribose. The dI polypeptides that form the physiological dimer are related by a two-fold axis of pseudo-symmetry that runs through a central 'core' formed by the dI.2 domains. A β-hairpin structure of some 13 residues extends from each dI.2 domain across the back of the symmetry-related subunit and contributes to the wall of its nucleotide-binding site. The fold of dI is similar to that of alanine dehydrogenase, a water-soluble bacterial enzyme, which catalyses the reductive amination of pyruvate to alanine. This raises the possibility that an ancestor of alanine dehydrogenase was recruited by a membrane protein in the molecular evolution of transhydrogenase.

Transhydrogenase dIII has a single domain which also has the form and connectivity of the Rossmann fold [10–12]. A very unusual feature is that the bound $NADP^+$ has a flipped orientation relative to that commonly found with nucleotides in the Rossmann fold. Thus, the adenosine moiety is located over the second βαβαβ motif of the fold and the nicotinamide mononucleotide over the first. A GXGXXA sequence matches the fingerprint for NADP-dependent enzymes (compare above) but, remarkably, it appears to have a different functional relationship with the bound nucleotide to that in other proteins. Specificity for $NADP^+$/NADPH over NAD^+/NADH results from a complex nest of H-bonds between Lys164, Arg165 and Ser166 (*R. rubrum* isolated dIII numbering) and the 2′-phosphate group on the adenosine ribose. Transhydrogenase dIII has a similar fold to that of the FAD-binding domain of the soluble protein, pyruvate oxidase, and here too the adenosine moiety of the FAD is bound to the second βαβαβ motif.

A stable dI_2dIII_1 complex forms spontaneously in mixtures of separately prepared, recombinant dI and dIII [12,13]. The crystal structure of the complex shows that the single dIII polypeptide is associated with the cleft of just one of the two dI polypeptides (designated the B polypeptide – see Fig. 1). The dIII polypeptide makes extensive contacts with the dI.2 core of the dI dimer. Though they are few, the contacts between dIII and dI.1(B) involve highly conserved regions of the protein and are probably important in the transmission of conformational changes during the coupling of proton translocation to the redox reaction (see later). The single hydride transfer site in the complex is at the dI/dIII interface.

There is no high-resolution structure of the membrane-spanning dII component but hydropathy analysis of amino acid sequences [3–5] and biochemical experiments, particularly after engineering unique Cys residues into the protein [14,15], have led to the conclusion that the single-subunit, mammalian transhydrogenases have 14 transmembrane (TM) helices (28 per dimer), the two-subunit enzymes from bacteria (e.g. *Escherichia coli*) have 13 (26 per dimer) and the three-subunit enzymes from bacteria (e.g. *R. rubrum*) have 12 (24 per dimer). Helices 3, 4, 9, 10, 13 and 14 (mammalian numbering) are the most conserved. There is little experimental information to indicate how the helices are clustered, though there are predictions based on the locations of conserved amino acid residues (see [15,16]). The loops between TM helices are thought to be quite short (< 14 residues on the cytoplasmic side of the membrane in bacteria, and < 25 residues on the periplasmic side). The cytoplasmic loops, some of which must interact with dIII, are more conserved.

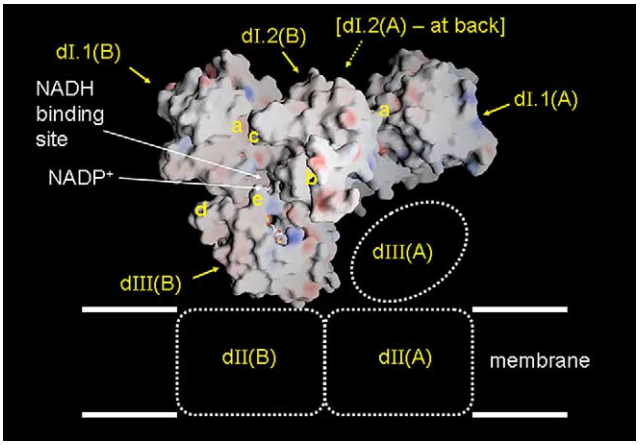

Fig. 1. The domain structure and some important secondary-structure features in transhydrogenase. The upper part of the figure is a surface representation of the crystal structure of the dI_2dIII_1 complex of *R. rubrum* transhydrogenase [7] showing positive and negative Coulombic fields in blue and red, calculated by the program GRASP [47]. Bound $NADP^+$, partly obscured by loop E, can be seen (in stick format) in the dIII component but dI(B) lacks NAD(H) in the crystal structure. Features referred to in the text are: *a*, the cleft between domains dI.1 and dI.2; *b*, the mobile loop of dI (truncated in this structure due to weak electron density); *c*, the TAGP loop of dI; *d*, helix D/loop D of dIII; *e*, the loop E 'lid' of dIII. The lower part of the figure shows the probable position of the second dIII component and the two dII components of the intact enzyme in the membrane.

The overall arrangement of components in the intact enzyme can be deduced from the crystal structure of the dI_2dIII_1 complex (Fig. 1) [7]. A second dIII (probably in a different conformational state to that observed in dI_2dIII_1 – see below) must be located at or close to the vacant cleft of dI(A). The two dII components are probably associated mainly with the two dIII components. In the three-subunit transhydrogenases, it is likely that there is no contact between dI and dII – thus dI can be easily displaced from (and then reconstituted to reform) the intact enzyme [17]. In the one- and two-subunit enzymes, there is a direct connection between dI and dII but only through a single strand of unconserved polypeptide chain.

4. Direct hydride transfer in the enzyme catalytic site

dI_2dIII_1 complexes catalyse a rapid single-turnover burst of transhydrogenation [18–21]. In the absence of dII, further turnover is limited by the extremely slow rate of release of the product nucleotide ($NADP^+$ or NADPH, depending on direction) from dIII. Acetylpyridine adenine dinucleotide ($AcPdAD^+$) and AcPdADH are good analogues of NAD^+ and NADH in transhydrogenation but the optical absorbance

[1] Unless otherwise stated, the amino acid numbering system is for *Rhodospirillum rubrum* transhydrogenase. For the dIII component of the enzyme, the sequence number of the isolated recombinant protein is given.

of AcPdADH is shifted relative to that of NADH and NADPH. Thus it was shown, for the reaction between AcPdAD$^+$ and NADPH on dI_2dIII_1 complexes, that the kinetics of AcPdAD$^+$ reduction and of NADPH oxidation are identical [18]. Similarly, with AcPdADH and NADP$^+$, the AcPdADH oxidation kinetics precisely match those of NADP$^+$ reduction [20]. This proves that hydride transfer is direct; there are no redox intermediates in transhydrogenase. During the physiological reaction in the intact enzyme, the *pro-R* C4H of the dihydronicotinamide ring of NADH must come into apposition with the *si* face of C4 of the NADP$^+$ nicotinamide at the redox step.

This simple conclusion must be considered alongside the fact that dihydronicotinamide and nicotinamide rings are highly reactive. Even in aqueous solution in the absence of enzymes, there can be a considerable rate of reaction between reduced and oxidised nicotinamide nucleotides and their analogues: second-order rate constants are in the order 10^{-2}–10^{-3} M^{-1} s^{-1} [22,23]. At the low concentration of nicotinamide nucleotides in the living cell, and in most experiments with transhydrogenase, the non-enzymic rate is insignificant. However, there is an important consequence of these substantial reactivities for the coupling mechanism of transhydrogenase: the dihydro- and nicotinamide rings of the NADH and the NADP$^+$, respectively, must be kept apart when the nucleotides bind to the enzyme. If NADH and NADP$^+$ were permitted to bind with their nicotinamide C4 atoms in apposition, a disastrous redox reaction would ensue uncoupled from proton translocation and driven by the solution ΔG of the nucleotides.

5. The binding-change mechanism

In the framework of the 'binding-change' model of transhydrogenase [3,24], the requirement to move the dihydro- and the nicotinamide rings towards and away from one another becomes central in the mechanism of coupling. Two conformational states of each monomer in the intact enzyme are recognised (see Fig. 2, and note that the transhydrogenase is shown with its monomers in alternating conformations, as will be discussed later). In the 'open' state, bound nucleotides can rapidly exchange with those in the solvent but the nicotinamide rings of the bound nucleotides are held apart to prevent redox reaction. It is from the open state that product nucleotides can dissociate and fresh substrates can bind. In the 'occluded' state, bound nucleotides cannot exchange with those in the solvent and their nicotinamide rings are held together to favour hydride transfer. The open and occluded states are inter-converted by protonation and deprotonation reactions associated with translocation of protons through dII. Directionality is imposed by changing access of the translocation 'device' in dII (about which very little is known – see below) to protons from either one side of the membrane or the other, depending on the nucleotide occupancy of dIII.

At neutral pH, dI_2dIII_1 complexes are locked in a conformation resembling the occluded state [25]. They catalyse very fast hydride transfer at their single dI/dIII interface but release NADP$^+$ and NADPH from dIII only very slowly. The crystal structures indicate that the extended 'loop E', arching over and enclosing the pyrophosphate group of the bound NADP$^+$, is at least partly responsible for the occlusion of the nucleotide from the solvent (Fig. 1) [11,12]. The tyrosyl

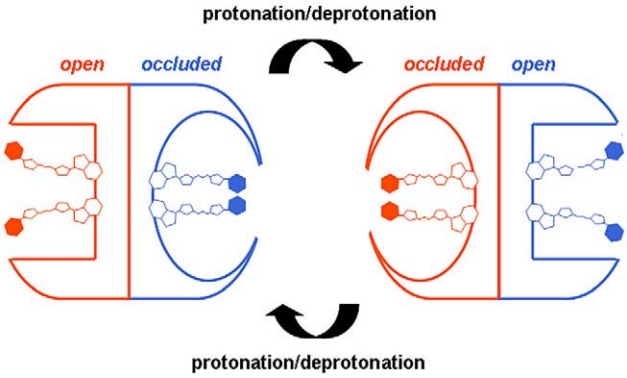

Fig. 2. The binding-change mechanism of transhydrogenase. In the 'open' state, bound nucleotides can exchange with those in the solvent, and hydride transfer between NADH and NADP$^+$ is blocked – the dihydronicotinamide and nicotinamide rings (shaded) are kept apart. In the 'occluded' state, bound nucleotides cannot exchange with those in the solvent and hydride transfer is allowed – the two rings are brought together. The conformational states of the two monomers of transhydrogenase (one monomer in red and the other in blue) alternate during turnover.

ring of the highly conserved Tyr171 in a GYA sequence at the apex of the loop interacts with the NADP$^+$ nicotinamide ring and with the invariant Arg90. In turn, this arginine is in van der Waals contact with the nicotinamide ring and its guanidinium group makes an H-bond with the nucleotide pyrophosphate. After hydride transfer in the intact enzyme, there must be a loosening of these interactions during conversion to the open state to allow retraction of loop E and release of the NADPH product into the solvent. In isolated dIII and in dI_2dIII_1 complexes, loop E is retracted when the proteins are exposed to low pH [26].

The so-called mobile loop of dI, first identified and characterised by nuclear magnetic resonance (NMR) spectroscopy [27,28], has some similarities with loop E of dIII. It too has a highly conserved tyrosine (Tyr235) in a GYA sequence, and the tyrosyl ring of this residue can make van der Waals contact with an invariant arginine (Arg127). As with its dIII counterpart, this arginine is in contact with the nicotinamide ring (but of NAD$^+$) and its guanidinium group H-bonds to the nucleotide pyrophosphate. NMR experiments show that the loop closes down during NAD(H) binding to dI. In X-ray diffraction data, the electron density for the loop is well-defined in some subunits of isolated dI (where it interacts with the strongly conserved TAGP loop – see Fig. 1), but not at the hydride transfer site of dI_2dIII_1 complexes. Like loop E in dIII, it might have a function in the occlusion of nucleotide from the solvent during hydride transfer.

The crystal structure of isolated dI with bound NAD$^+$ provides a clear indication that the nicotinamide ring of the bound nucleotides in transhydrogenase can move across significant distances, as proposed in the binding-change mechanism [6]. There are four polypeptides in the asymmetric unit of the crystals. In two of these, the electron density for the NAD$^+$ nicotinamide is well-defined and reveals distinctively different NAD$^+$ conformations (Fig. 3). In one polypeptide, the nicotinamide is *syn* relative to the ribose and is located at the back of the cleft between domains dI.1 and dI.2. In the other, rigid-body movement of dI.1 relative to d1.2 has caused the nicotinamide group to rotate into the *anti* conformer and to be expelled towards the rim of the cleft. The possible sig-

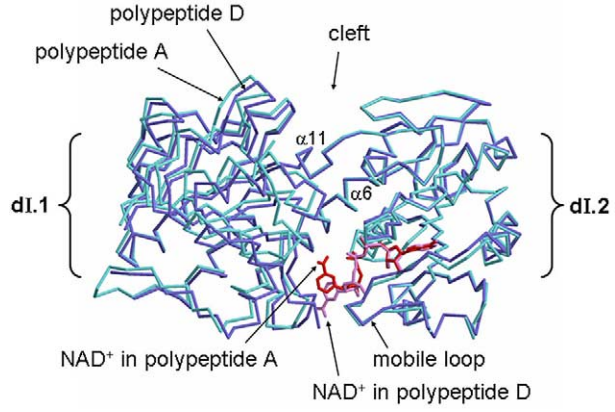

Fig. 3. Different NAD^+ conformations in the subunits of isolated dI. The A (pale blue) and D (dark blue) polypeptides from the asymmetric unit of isolated dI of *R. rubrum* transhydrogenase were superposed using amino acid residues in the β-sheets of the dI.2 domains and displayed as C_α traces using MOLSCRIPT [48]. Note the resulting misalignment of the dI.1 domains of polypeptides A and D, due to hinge movement in helices α6 and α11 (which run across the clefts), and the pronounced movements in the nicotinamide mononucleotide moieties of the bound NAD^+. The nucleotide of polypeptide A is shown in red, and that of D is shown in violet. Note that the polypeptide labelling of isolated dI does not correspond to that in the dIdIII complex (Figs. 1 and 4).

nificance of these conformational changes in promoting or blocking the redox reaction was revealed by the subsequent structural analysis of dI_2dIII_1 complexes [7]. The structure of the complexes was solved for protein having bound NAD^+ and $NADP^+$ (to ensure that there was no redox reaction during crystallisation). The dIII site is fully occupied by $NADP^+$ but only the dI(A) polypeptide (whose cleft is not associated with dIII) has strong electron density for bound NAD^+. Therefore to visualise the hydride transfer reaction, an NADH molecule was modelled into dI(B) in the same conformation as, and in the equivalent binding site to, the NAD^+ in dI(A). In this ('distal') state, C4 of the NADH dihydronicotinamide is 6.5 Å from C4 of the $NADP^+$ nicotinamide – too far for hydride transfer (see Fig. 4). However, by applying small rotations equivalent to those described above in isolated dI, the two rings can be moved into apposition, thereby bringing the two C4 atoms into van der Waals contact (the 'proximal' state).

This organisation will thus set the trajectory for the formation of the transition state for hydride transfer and will lead to the correct stereochemistry. It is proposed that the movements between the distal (redox-inactive) and proximal (redox-active) states in the model reflect real events in the intact enzyme during inter-conversion of the open and occluded conformations, as illustrated by the Fig. 2 cartoon. There is a suggestion of a corresponding conformational shift in the $NADP^+$ molecule – a pocket in dIII (occupied by glycerol in the X-ray structure) is appropriately positioned to house $NADP^+$-nicotinamide rotated away from the NADH in the open state [11]. The relative motions of dI.1 and dI.2 required to move the NADH dihydronicotinamide, the opening and closing of loop E and the movements of the $NADP^+$ nicotinamide are all thought to result from conformational changes in the helix D region of dIII which are driven by proton translocation though dII (see below).

The invariant dI residue, Gln132, appears to have an important role in maintaining the proximal state for hydride transfer (Fig. 4). In the dI_2dI_1 crystal structure, the side-chain amide of this residue extends across the interface with dIII and makes H-bond contact with the 2′-OH group of the $NADP^+$ ribose. In the modelled proximal state, Gln132 can make an additional H-bond with the 3-carboxamide group of the NAD nicotinamide, which led to the suggestion that the residue is a 'molecular tether' to hold the two nucleotides together in the pre-transition state for hydride transfer [29]. When Gln132 was replaced by Asn, the NADH-binding affinity of the isolated dI was unchanged but hydride transfer in complexes formed from the mutant protein and wild-type dIII was very strongly inhibited. X-ray crystallography shows that these complexes fold correctly but the shorter side chain of residue 132 results in a failure of the tether. In the structure of dI.NADH [8] (but not in dI.NAD^+ [6]), the invariant Arg204 makes H-bond contact with hydroxyl groups in both the nicotinamide ribose and the adenosine ribose – this bridge might also be important to constrain the structure of the nucleotide during the shift between the distal and proximal positions at the hydride transfer site.

6. Changes in nucleotide binding energy

The on-enzyme equilibrium constant of the hydride transfer step of transhydrogenase is >36-fold greater than that in free solution [20,21]. This must be the result of a stabilisation of NADPH (relative to $NADP^+$) and/or of NAD^+ (relative to NADH) in the occluded state, and its significance requires comment. The catalytic site of transhydrogenase operates in a solution environment (the bacterial cytoplasm and the mi-

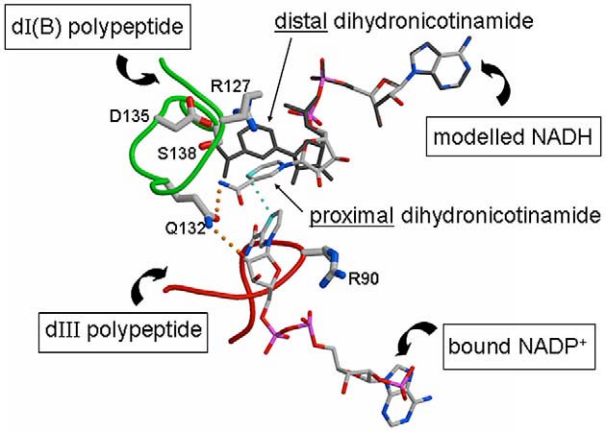

Fig. 4. The hydride transfer site of transhydrogenase. A MOLSCRIPT [48] cartoon taken from the crystal structure of the dI_2dIII_1 complex of *R. rubrum* transhydrogenase [7]. A segment of the dI(B) polypeptide is shown in green and an adjacent segment of the dIII polypeptide in red. The bound $NADP^+$ molecule and some of the invariant amino acid residues in the active site are in their conventional atom colours. An NADH molecule (dark grey) is modelled into dI(B) in the same conformation as, and at the equivalent site to, the NAD^+ in dI(A) – this is denoted the 'distal' position (see text). The nicotinamide mononucleotide moiety of the NADH was then rotated (compare Fig. 3) into the 'proximal' position as shown by the nucleotide molecule in conventional atom colours: in this conformation the C4 atoms of the NADH and $NADP^+$ (both coloured light blue) are within hydride transfer distance (light blue dots). The tethering function of Gln132 in stabilising the proximal arrangement is illustrated: hydrogen bonds between this residue and both the bound $NADP^+$ and the modelled NADH are shown as orange dots.

tochondrial matrix) where NAD^+/NADH is somewhat oxidised and $NADP^+$/NADPH is (usually) very reduced. Although K_d values are unavailable, the enzyme in its open state is therefore expected to bind NADH and $NADP^+$ with high affinity relative to NAD^+ and NADPH (thus promoting substrate binding and product release). Were these relative affinities to remain unchanged during conversion to the occluded state, then the K_{eq} for hydride transfer would be unfavourable and the enzyme would be trapped in a 'thermodynamic pit' – in principle, the transhydrogenase would still work and still be fully coupled to proton translocation but it would operate at a low rate (see [30]). A change in binding energies of the two nucleotides during inter-conversion of the open and occluded states avoids the problem. Note that nucleotide occlusion, which, as explained above, is essential to prevent the redox reaction becoming uncoupled from proton translocation, is also necessary for this change in binding affinities; for example to allow destabilisation of the newly bound substrates, $NADP^+$ and NADH, without their dissociating from the protein before hydride transfer.

7. The hydride transfer reaction

Quantum-mechanical (AM1) calculations show that the energy cost to pucker the planar 1,4-dihydronicotinamide ring into a quasi-boat form ($\alpha_N = \alpha_C = 10°$) is only 1.7 kcal/mol. This is small relative to the decrease in energy of the transition state for hydride transfer of dogfish lactate dehydrogenase that can be achieved by the deformation [31]. The ring pucker puts the *pro-R* atom at C4 into a pseudoaxial position and this is thought to increase the driving force for hydride transfer by allowing greater orbital overlap during the reaction. That this effect might be kinetically significant was shown by molecular dynamics calculations on several dehydrogenases – there was a preference for the C4H atom of the dihydronicotinamide ring to be transferred to pucker towards the substrate-binding pocket. In transhydrogenase, the rate of hydride transfer between NADH and $NADP^+$ is extremely rapid ($k_{app} \approx 21\,000\ s^{-1}$ in dI_2dIII_1 complexes [21]) – significantly more rapid than substrate oxidation/reduction by NAD(P)(H) in the soluble dehydrogenases. Although we expect at least modest rates of hydride transfer in transhydrogenase simply as a result of binding and orientation of the nucleotides (see above), activation of the nicotinamide rings, for example by ring puckering, is not ruled out.

The conserved amino acid residues in the hydride transfer site were identified in the crystal structures [6–8,10–12]. Of particular note is the almost invariant run of 10 residues (Ile84–Gly93) in the binding loop for the $NADP^+$ nicotinamide in dIII, the invariant polar/charged residues, Arg127, Gln132, Asp135 and Ser138 (in the 'RQD' loop) of dI, which can all interact with the NAD(H) nicotinamide (see Fig. 4), and the conserved tyrosines (Tyr235 in dI, and Tyr54 and Tyr171 in dIII). These residues will participate in the positioning of the dihydronicotinamide ring of NADH and the nicotinamide ring of $NADP^+$ for hydride transfer during formation of the occluded state, as described above. After closure of the site to expel water, they will also be responsible for the polarisation that is evidenced by the elevated equilibrium constant for the redox reaction.

Strictly, a true hydride transfer mechanism (as distinct from a sequential electron/proton/electron transfer) remains unproven for transhydrogenase although experiments with nucleotide analogues appear to rule out the formation of 'high-energy intermediates' (single-electron oxidation products of dihydronicotinamide and single-electron reduction products of nicotinamide) in the reaction in solution [32]. The energy required simultaneously to generate $NADP^{+\bullet}$ and $NADH^{\bullet}$ by a single-electron transfer would probably be prohibitive (see [33]) and hydride transfer is therefore the likely mechanism. The temperature dependences of the primary kinetic isotope effect for $AcPdAD^+$ reduction by $NADP^2H/NADP^1H$ in dI_2dIII_1 complexes indicate that there may be a contribution to the reaction from quantum-mechanical tunnelling of the hydride ion through the activation barrier [19]. Hydrogen tunnelling has been studied in detail in some soluble enzymes. One attractive view [34] is that thermal fluctuations of the ground state of the enzyme–substrate complex decrease the distance over which the hydrogen must tunnel and thus accelerate the rate of reaction (and see review [35]).

8. Force generation by proton translocation through dII, and energy transmission to the hydride transfer site

The balance of evidence suggests that proton translocation through dII leads to conformational changes that are transmitted across dIII to the hydride transfer site at the interface of the latter with dI. Transhydrogenation activity is partially inhibited when the dII residues, βHis91, βAsn222, βAsp213 or those in the sequence βCys260–βSer266, of the *E. coli* enzyme are substituted by mutagenesis [36–40], and these residues might therefore be involved either in the conduction of protons through dII or in the energy-transduction device itself. Presumably the propagation of the all-important conformational change will involve relative motions of rigid elements of dII – most probably the TM helices – but the nature of these motions is not known and, for clues, a high-resolution structure of the intact enzyme is awaited.

The core of dIII is β-sheet and, as such, is likely to be a rather inflexible structure unsuitable for transmitting long-distance conformational changes between dII and the hydride transfer site. The feature most likely to serve in energy transmission across dIII is helix D/loop D [7,11]. This emerges at 90° from the C-terminus of the β-sheet. It passes close to the $NADP^+$ pyrophosphate group and then runs through a series of H-bonded turns before rejoining the next strand in the sheet. Helix D/loop D contains a large number of invariant amino acid residues and, crucially, interacts with *both* the loop E 'lid' of dIII and the RQD loop of dI (see above). It is proposed that conformational changes in helix D/loop D, driven by the events in dII that are associated with proton translocation, regulate the opening of loop E (and thus the exchange of NADP(H) with the solvent) and the width of the dI cleft (and hence the movement of the NADH nicotinamide ring that is required for hydride transfer). The invariant Asp132 in helix D/loop D is located under the loop E lid. Mutagenesis studies (in *E. coli* transhydrogenase) indicate the importance of this residue and, though it was proposed to be involved in the proton translocation pathway [40,41], we can find no evidence of this from the X-ray structure. Rather, we suggest that Asp132, which calculation indicates has a substantially elevated pK_a in isolated (occluded) dIII [3], is involved in stabilising the structure of the lid in its closed and retracted conformations [42].

9. Signalling to the proton translocation gate

Since coupling is indirect, the proton translocation device must be synchronised with the progress of the hydride transfer reaction: access of the device to protons from one side of the membrane or the other must be switched by the redox state of the nucleotides – most probably of NADP(H). It is not clear how this is achieved but an NMR experiment with isolated dIII proteins reveals a highly localised region of magnetisation change in the nicotinamide-binding pocket, in helix D/loop D and in loop E, when $NADP^+$ is replaced with NADPH. The (heteronuclear single-quantum coherence) experiment, in which the ^1H and ^{15}N spins of the amide groups in the protein are correlated, has been performed with both *R. rubrum* [43] and *E. coli* dIII [44]. It is not yet clear to what extent the observed chemical shift changes represent atomic displacements or electric polarisations in the protein but the localised nature of the response in these important secondary structures suggested to us that it might correspond to the signal transmitted from the nucleotide-binding site to the membrane-spanning dII to inform on the progress of the redox reaction [12].

10. The alternation of sites in transhydrogenase

Intact transhydrogenase is essentially a dimer of two dI+dII+dIII 'monomers' (reviewed in [45]). However, in solution [13] and in the crystalline state [7], only one isolated dIII stably associates with the isolated dI dimer – but, nevertheless, the single catalytic site between the single dI/dIII interface is fully active. The symmetry of the dI_2dIII_1 complex clearly reveals the position at which a second dIII can bind – into the side of the cleft of the 'vacant' dI (see Fig. 1) – but inserting a modelled copy of the first dIII into this site leads to severe side-chain clashing between the two dIII polypeptides [7]. NMR experiments show that a second dIII can interact with the dI_2dIII_1 'heterotrimer' though with very low affinity [43]. These observations suggest that, in the intact enzyme, the two dI/dIII sites must be alternately brought together to allow hydride transfer. It was proposed that the conformational changes responsible for coupling transhydrogenation to proton translocation relate to the site alternation [7]. Thus, as dI(A)/dIII(A) enters the open state to permit product release and substrate binding, dI(B)/dIII(B) enters the occluded state to permit hydride transfer (Fig. 2). The conclusions from the structural work are strongly supported by earlier covalent-modification and nucleotide-binding experiments which revealed half-of-the-sites reactivity in intact mitochondrial transhydrogenase [5].

The alternating-site mechanism might enable transhydrogenase better to balance its internal equilibria (compare the principle enunciated for enzyme catalysis [46]). Invariant amino acid residues and mutations that disrupt transhydrogenase function (reviewed in [4]) are located mainly on the cytosolic side of dII (in bacteria). This suggests that the transduction device lies close to the dIII/dII interface, and therefore that the dielectric drop for proton binding from the periplasm will be larger than that for proton release to the cytoplasm. By coupling together the A and the B sides of the protein dimer, the energies available to drive the open ↔ occluded conformational changes would be equalised, promoting catalytic efficiency. To achieve this, the two dII components would operate as a single integrated device in proton translocation – reciprocating motions between symmetry-related TM helices in the two dII monomers would simultaneously drive dI(A)/dIII(A) into the open state and dI(B)/dIII(B) into the occluded state (a 'push-pull' effect). It is even conceivable that, as in the F_1F_O-ATP synthase, there is just a single proton channel with structural contributions from both dII(A) and dII(B).

Acknowledgements: The author is grateful to the Wellcome Trust and the Biotechnology and Biological Sciences Research Council for financial support and is indebted to colleagues, Scott White, Nick Cotton, Jamie Venning, Philip Quirk, Avtar Singh, Harma Brondijk, Gijs van Boxel, Owen Mather and Andy White for their discussion and advice.

References

[1] Rydstrom, J. and Hoek, J.B. (1988) Biochem. J. 254, 1–10.
[2] Sazanov, L.A. and Jackson, J.B. (1994) FEBS Lett. 344, 109–116.
[3] Jackson, J.B., White, S.A., Quirk, P.G. and Venning, J.D. (2002) Biochemistry 41, 4173–4185.
[4] Bizouarn, T., Fjellstrom, O., Meuller, J., Axelsson, M., Bergkvist, A., Johansson, C., Karlsson, G. and Rydstrom, J. (2000) Biochim. Biophys. Acta 1457, 211–218.
[5] Hatefi, Y. and Yamaguchi, M. (1996) FASEB J. 10, 444–452.
[6] Buckley, P.A., Jackson, J.B., Schneider, T., White, S.A., Rice, D.W. and Baker, P.J. (2000) Structure 8, 809–815.
[7] Cotton, N.P.J., White, S.A., Peake, S.J., McSweeney, S. and Jackson, J.B. (2001) Structure 9, 165–176.
[8] Prasad, G.S., Wahlberg, M., Sridhar, V., Sundaresan, V., Yamaguchi, M., Hatefi, Y. and Stout, C.D. (2002) Biochemistry 41, 12745–12754.
[9] Wierenga, R.K., De Maeyer, M.C.H. and Hol, W.G.J. (1985) Biochemistry 24, 1346–1357.
[10] Prasad, G.S., Sridhar, V., Yamaguchi, M., Hatefi, Y. and Stout, C.D. (1999) Nat. Struct. Biol. 6, 1126–1131.
[11] Jackson, J.B., Peake, S.J. and White, S.A. (1999) FEBS Lett. 464, 1–8.
[12] White, S.A., Peake, S.J., McSweeney, S., Leonard, G., Cotton, N.N.J. and Jackson, J.B. (2000) Structure 8, 1–12.
[13] Venning, J.D., Rodrigues, D.J., Weston, C.J., Cotton, N.P.J., Quirk, P.G., Errington, N., Finet, S., White, S.A. and Jackson, J.B. (2001) J. Biol. Chem. 276, 30678–30685.
[14] Meuller, J. and Rydstrom, J. (1999) J. Biol. Chem. 274, 19072–19080.
[15] Bragg, P.D. and Hou, C. (2000) Biochem. Biophys. Res. Commun. 273, 955–959.
[16] Studley, W.K., Yamaguchi, M., Hatefi, Y. and Saier, M.H. (1999) Microb. Comp. Genomics 4, 173–186.
[17] Diggle, C., Hutton, M., Jones, G.R., Thomas, C.M. and Jackson, J.B. (1995) Eur. J. Biochem. 228, 719–726.
[18] Venning, J.D., Grimley, R.L., Bizouarn, T., Cotton, N.P.J. and Jackson, J.B. (1997) J. Biol. Chem. 272, 27535–27538.
[19] Venning, J.D., Bizouarn, T., Cotton, N.P.J., Quirk, P.G. and Jackson, J.B. (1998) Eur. J. Biochem. 257, 202–209.
[20] Venning, J.D. and Jackson, J.B. (1999) Biochem. J. 341, 329–337.
[21] Pinheiro, T.J.T., Venning, J.D. and Jackson, J.B. (2001) J. Biol. Chem. 276, 44757–44761.
[22] Spiegel, M.J. and Drysdale, G.R. (1960) J. Biol. Chem. 235, 2498–2501.
[23] van Eikeren, P. and Grier, D.L. (1977) J. Am. Chem. Soc. 99, 8057–8060.
[24] Hutton, M.N., Day, J.M., Bizouarn, T. and Jackson, J.B. (1994) Eur. J. Biochem. 219, 1041–1051.
[25] Diggle, C., Bizouarn, T., Cotton, N.P.J. and Jackson, J.B. (1996) Eur. J. Biochem. 241, 162–170.
[26] Rodrigues, D.J. and Jackson, J.B. (2002) Biochim. Biophys. Acta 1555, 8–13.
[27] Diggle, C., Cotton, N.P.J., Grimley, R.L., Quirk, P.G., Thomas, C.M. and Jackson, J.B. (1995) Eur. J. Biochem. 232, 315–326.
[28] Quirk, P.G., Smith, K.J., Thomas, C.M. and Jackson, J.B. (1999) Biochim. Biophys. Acta 1412, 139–148.

[29] van Boxel, G.I., Quirk, P., Cotton, N.J.P., White, S.A. and Jackson, J.B. (2003) Biochemistry 42, 1217–1226.
[30] Jenks, W.P. (1983) Curr. Top. Membr. Transp. 19, 1–18.
[31] Almarrson, O. and Bruice, T.C. (1993) J. Am. Chem. Soc. 115, 2125–2138.
[32] Lee, I.-S.H., Jeoung, E.H. and Kreevoy, M.M. (1997) J. Am. Chem. Soc. 119, 2722–2728.
[33] Page, C.C., Moser, C.C., Chen, X. and Dutton, P.L. (1999) Nature 402, 47–52.
[34] Bruno, W.J. and Bialek, B. (1992) Biophys. J. 63, 689–699.
[35] Sutcliffe, M.J. and Scrutton, N.S. (2002) Eur. J. Biochem. 269, 3096–3102.
[36] Glavas, N.A., Hou, C. and Bragg, P.D. (1995) Biochemistry 34, 7694–7702.
[37] Bragg, P.D. and Hou, C. (1999) Arch. Biochem. Biophys. 363, 182–190.
[38] Althage, M., Bizouarn, T. and Rydstrom, J. (2001) Biochemistry 40, 9968–9976.
[39] Yamaguchi, M., Stout, C.D. and Hatefi, Y. (2002) J. Biol. Chem. 277, 33670–33675.
[40] Hu, X., Zhang, J.W., Fjellstrom, O., Bizouarn, T. and Rydstrom, J. (1999) Biochemistry 38, 1652–1658.
[41] Meuller, J., Hu, X., Buntoff, C., Olausson, T. and Rydstrom, J. (1996) Biochim. Biophys. Acta 1273, 191–194.
[42] Rodrigues, D.J., Venning, J.D. and Jackson, J.B. (2001) Eur. J. Biochem. 268, 1430–1438.
[43] Quirk, P.G., Jeeves, M., Cotton, N.P.J., Smith, K.J. and Jackson, J.B. (1999) FEBS Lett. 446, 127–132.
[44] Bergkvist, A., Johansson, C., Johansson, T., Rydstrom, J. and Karlsson, B.G. (2000) Biochemistry 39, 12595–12605.
[45] Jackson, J.B. (1991) J. Bioenerg. Biomembr. 23, 715–741.
[46] Albery, W.J. and Knowles, J.R. (1976) Biochemistry 15, 5627–6531.
[47] Nicholls, A., Sharp, K.A. and Honig, B. (1991) Proteins 11, 281–296.
[48] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.