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Biochimica et Biophysica Acta 1757 (2006) 215–223
BIOCHIMICA ET BIOPHYSICA ACTA
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A hybrid of the transhydrogenases from *Rhodospirillum rubrum* and *Mycobacterium tuberculosis* catalyses rapid hydride transfer but not the complete, proton-translocating reaction

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Received 20 January 2006; received in revised form 24 February 2006; accepted 5 March 2006
Available online 31 March 2006

Abstract

All transhydrogenases appear to have three components: dI, which binds NAD(H), and dIII, which binds NADP(H), protrude from the membrane, and dII spans the membrane. However, the polypeptide composition of the enzymes varies amongst species. The transhydrogenases of *Mycobacterium tuberculosis* and of *Rhodospirillum rubrum* have three polypeptides. Sequence analysis indicates that an ancestral three-polypeptide enzyme evolved into transhydrogenases with either two polypeptides (such as the *Escherichia coli* enzyme) or one polypeptide (such as the mitochondrial enzyme). The fusion steps in each case probably led to the development of an additional transmembrane helix. A hybrid transhydrogenase was constructed from the dI component of the *M. tuberculosis* enzyme and the dII and dIII components of the *R. rubrum* enzyme. The hybrid catalyses cyclic transhydrogenation but not the proton-translocating, reverse reaction. This shows that nucleotide-binding/release at the NAD(H) site, and hydride transfer, are fully functional but that events associated with NADP(H) binding/release are compromised. It is concluded that sequence mismatch in the hybrid prevents a conformational change between dI and dIII which is essential for the step accompanying proton translocation.

Keywords: Transhydrogenase; *Rhodospirillum rubrum*; *Mycobacterium tuberculosis*; Proton translocation; Nicotinamide nucleotides; Phylogenetic tree

1. Introduction

Transhydrogenases are found in the inner mitochondrial membrane of mammalian cells and in the cytoplasmic membrane of bacteria. The enzymes couple the transfer of hydride-ion equivalents between NAD(H) and NADP(H) to the translocation of protons across the membrane:

$$NADH + NADP^+ + H^+_p \leftrightarrow NAD^+ + NADPH + H^+_n \qquad (1)$$

where H^+_p signifies H^+ on the cytoplasmic side of the membrane in mitochondria and the extra-cellular side in bacteria. The reaction is normally driven from left to right by the inward translocation of H^+ down the proton electrochemical gradient (Δp) generated by the respiratory (or sometimes photosynthetic) electron transport chains. NADPH produced by transhydrogenases is used in biosynthesis reactions [1] and for the reduction of glutathione, needed to limit damage caused by oxidative stress [2,3]. Knock-down of transhydrogenase in MIN6 mouse cells led to a reduction of insulin secretion [4]. In some animal cells, transhydrogenase together with the iso-citrate dehydrogenases, might also participate in the regulation of flux through the tricarboxylic acid cycle [5,6].

The enzyme has three components, dI, dII and dIII (see reviews [7–11]). The dI component, which binds NAD(H), and the dIII component, which binds NADP(H), protrude from the membrane (on the matrix side in mitochondria and on the cytoplasmic side in bacteria). The dII component, which houses the proton-translocation pathway, spans the membrane. Although the global architecture of transhydrogenases appears to be similar, the polypeptide composition varies amongst species

Abbreviations: AcPdAD$^+$, acetylpyridine adenine dinucleotide (oxidised form); Nic$^+$, nicotinamide; NicH, dihydronicotinamide; NAD(H), both NAD$^+$ and NADH

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doi:10.1016/j.bbabio.2006.03.004

(Fig. 1). The enzyme from animal mitochondria comprises a single polypeptide, active as a dimer. Transhydrogenases of this type are placed in the 1P*a* group. There is also a single polypeptide in transhydrogenases from Protoctistal organisms such as *Entamoeba histolytica* (the 1P*b* group) but here the dI–dII–dIII connectivity differs from that in the mitochondrial enzymes. In the eubacteria, there are two different subunit arrangements. In some species (*Escherichia coli* is an example), transhydrogenase has two polypeptides (they are active as an $\alpha_2\beta_2$ dimer)—this is the 2P group. In other species, such as *Rhodospirillum rubrum*, the enzyme has three polypeptides (again probably active in a dimeric form)—the 3P group. To date, sequences for proton-translocating transhydrogenases have not been found in either the archaea or higher plants. Fig. 1a shows how the dI, dII and dIII components are arranged along the polypeptide chains. Note that, in the 3P transhydrogenases, the dI component is located on its own polypeptide (PntAA) whereas, in the 2P group and both the 1P groups, dI is covalently linked through the polypeptide chain to the N-terminal region of dII. There are currently (from September 2005) 14 complete sequences in the database having the 1P*a* organisation, 5 having 1P*b*, 38 having 2P, and 50 sequences having the 3P organisation.

Recombinant forms of dI and dIII have been prepared from several species, where necessary introducing stop and start codons into the DNA sequences [12–18]. The dI and dIII proteins of *R. rubrum* transhydrogenase are amenable to high-resolution structure determinations by X-ray crystallography and NMR [19–21]. Of particular interest is a complex formed between these two components. The "dI_2dIII_1 complex"

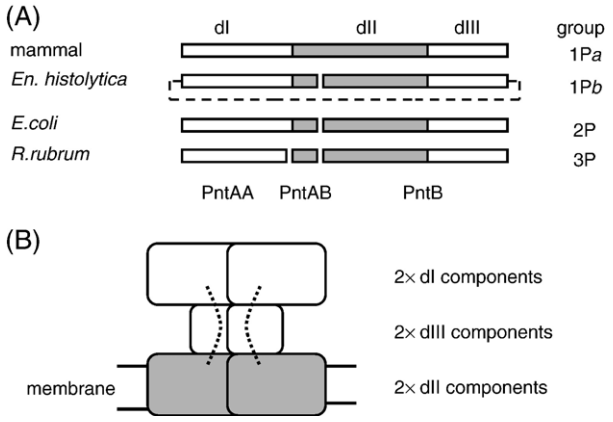

Fig. 1. The structure of transhydrogenase. (A) Variation in polypeptide composition (horizontal bars). Vertical lines delineate the dI, dII and dIII components (see text); vertical gaps delineate the polypeptides. The vertical alignment represents sequence similarity. The dashed line on the *Ent. histolytica* enzyme illustrates that the C-terminus of dIII and the N-terminus of dI are fused. PntAA, PntAB and PntB are the designated names of the three polypeptides of *R. rubrum* transhydrogenase [51] and other enzymes in the 3P group. The group notation, at the right, is defined in the text. (B) The probable component organisation of intact transhydrogenase [24]. The dotted line illustrates the dI–dIII "linker polypeptide", which is present in 1P and 2P transhydrogenases but absent in the 3P group (see text). The grey shade in panels A and B shows the regions comprising trans-membrane helices. For simplicity, the polypeptide separation in the dII component of the 2P and 3P transhydrogenases (see panel A) is not depicted in panel B.

catalyses rapid, direct hydride transfer between bound NAD(H) and bound NADP(H) [22,23]. X-ray structures of the complex show how apposition of the dihydronicotinamide ring of NADH and the nicotinamide ring of $NADP^+$ facilitates hydride transfer with a stereochemistry matching that determined experimentally [24–26]. In the intact enzyme, the hydride-transfer site at the interface between dI and dIII, is probably some >30 Å from the proton translocation pathway through dII. The two processes are thought to be coupled by conformational changes across dIII.

Although their amino acid sequences are only distantly related, the transhydrogenase of *Mycobacterium tuberculosis* like that of *R. rubrum* is in the 3P group (Fig. 1A). As part of a study to investigate the properties of the *M. tuberculosis* transhydrogenase, we have prepared a hybrid of the two enzymes. The hybrid, comprising *M. tuberculosis* dI and *R. rubrum* dII/dIII, catalyses rapid hydride transfer between bound nucleotides but is unable to carry out the complete, proton-translocating transhydrogenase reaction. This finding has implications for the mechanism of energy coupling during transhydrogenation. Previous attempts to construct a hybrid transhydrogenase using *R. rubrum* dII/dIII and the dI from either *E. coli* or *Entamoeba histolytica* were unsuccessful [18,27]. The activity of the *R. rubrum–M. tuberculosis* hybrid, therefore, provides new information about subunit interactions in transhydrogenase.

2. Materials and methods

Cultures of *R. rubrum* strain S1 were grown photo-heterotrophically and chromatophores (inverted cytoplasmic-membrane vesicles) were prepared, as described [28]. The chromatophores were depleted of their transhydrogenase dI by washing repeatedly by centrifugation in 100 mM Tris–HCl, pH 8.0, 10% sucrose. The bacteriochlorophyll content of the membranes was measured using the in vivo extinction coefficient given [29].

The gene encoding the transhydrogenase dI component was isolated by PCR using DNA extracted from *M. tuberculosis* strain H37Rv and the primers, 5′-GGA TCC AAT GAC AGA TCC GCA GAC GCA GAG C-3′ (*Bam*HI site underlined) and 5′-AAG CTT GGA GTC CCT CCC GCG GGT GAC-3′ (*Hin*dIII site underlined). The resulting 1098 base-pair fragment was ligated into the *Sma*I site of pUC18 and the construct was transformed into *E. coli* Top 10 cells (Invitrogen). Subsequently prepared plasmid DNA from these cells was digested with *Bam*HI and *Hin*dIII, and the dI-encoding DNA was isolated and ligated into pET23b to generate pRW1. The fidelity of the PCR was confirmed by DNA sequence analysis. The construct was transformed into *E. coli* C41(DE3) [30]. Overnight pre-cultures were used to inoculate Terrific Broth and the cells were grown at 37 °C to an optical density of 1.2 and then induced with 1 mM isopropyl thiogalactoside. After further incubation for 12–16 h at 16 °C, the cells were harvested by centrifugation. The cell pellet was re-suspended in 0.5 M NaCl, 100 mM imidazole, 20 mM sodium phosphate, pH 7.4 containing DNAse, RNAse, complete protease inhibitor (Roche) and 0.1 mg ml^{-1} lysozyme. The bacteria were disrupted with a Soniprep 150. The extract was centrifuged at 27,000×*g* for 60 min at 4 °C and the supernatant applied to a 1 ml Ni^{2+}-charged His-Trap column (Pharmacia). The column was washed extensively with 0.5 M NaCl, 100 mM imidazole, 20 mM sodium phosphate, pH 7.4, and protein was then eluted by a stepwise increase in imidazole concentration (150, 200, 300 and 500 mM). The dI protein was eluted typically at around 200 mM and was pooled, dialysed against 50 mM Tris–HCl, pH 7.5, containing 10% glycerol and stored at −20 °C. It was approximately 95% pure by analysis on SDS-PAGE. The recombinant dI and dIII components of *R. rubrum* transhydrogenase were expressed from pCD1 and pNIC2 in *E. coli* strains C600 and BL21 (DE3), respectively, and purified by column

chromatography, as described [13,31]. All protein concentrations were determined using the micro-tannin assay [32].

Isothermal-scanning calorimetry was performed on the MicroCal MCS system. The thawed dI was prepared for experiments by concentrating in a 10 kDa Vivaspin filter and then dialysed against 10 mM $(NH_4)_2SO_4$, 20 mM HEPES, pH 8.0. Binding constants were determined using the ORIGIN program as supplied by the instrument manufacturer.

Transhydrogenation rates were measured by following the reduction of acetylpyridine adenine dinucleotide ($AcPdAD^+$) at 375 nm (extinction coefficient, 6.2 mM^{-1} cm^{-1}) in a Shimadzu UV2401 spectrophotometer.

Amino acid sequence analysis was performed using ClustalW [33] within the program, BIOEDIT, and phylogenetic analysis [34,35] was carried out using the program, PAUP (see legend to Fig. 7). Hydropathy profiles with a scan window of 13 [36] were constructed within BIOEDIT. Root mean square deviations between 3D structures of transhydrogenase were calculated using the program SwissPDBViewer.

3. Results

3.1. Interactions between dI of M. tuberculosis and dII/dIII of R. rubrum transhydrogenase

A His-tagged version of the dI component of *M. tuberculosis* transhydrogenase (hereafter called *mt*dI) was expressed at high levels from a pET-based vector in cells of *E. coli* C41 (DE3). After cell breakage, the protein was located in the soluble fraction and was purified by Ni^{2+} chromatography. The yield of the final product (>95% pure from the staining intensity on SDS-PAGE) was approximately 100 mg l^{-1} of bacterial culture. A protein probably equivalent to *mt*dI was previously isolated from a homogenate of *M. tuberculosis* H37Rv and characterised by SDS-PAGE [37].

The binding of NADH to *mt*dI was demonstrated in calorimetry experiments (Fig. 2). The K_d (35 μM) was in the same order as that for *R. rubrum* dI (*rr*dI—20 μM [38]) and *E. coli* dI (*ec*dI—45 μM [39]). However, under similar experimental conditions, the heat change was smaller for *mt*dI ($\Delta H \approx -1.8$ Kcal mol^{-1}) than for *rr*dI.

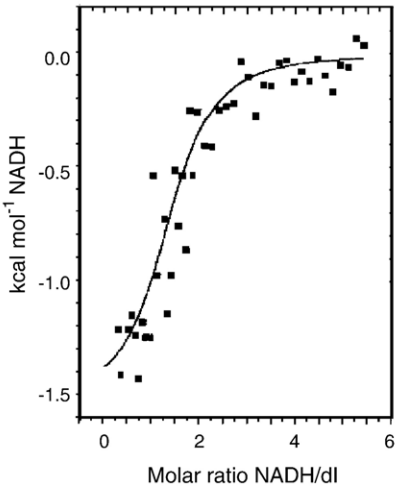

Fig. 2. Heat changes accompanying the binding of NADH to mtdI. The calorimeter cell contained 1.8 ml of *mt*dI (350 μM) in 10 mM $(NH_4)_2SO_4$, 20 mM HEPES, pH 8.0. The injection syringe contained 5.2 mM NADH in the same buffer. Each data point corresponds to a single 1.0 μl injection; the injections were 210 s apart. The temperature was 20 °C. The curve shows the binding isotherm calculated by ORIGIN. K_d=35 μM, ΔH=−1.8 kCal mol^{-1}.

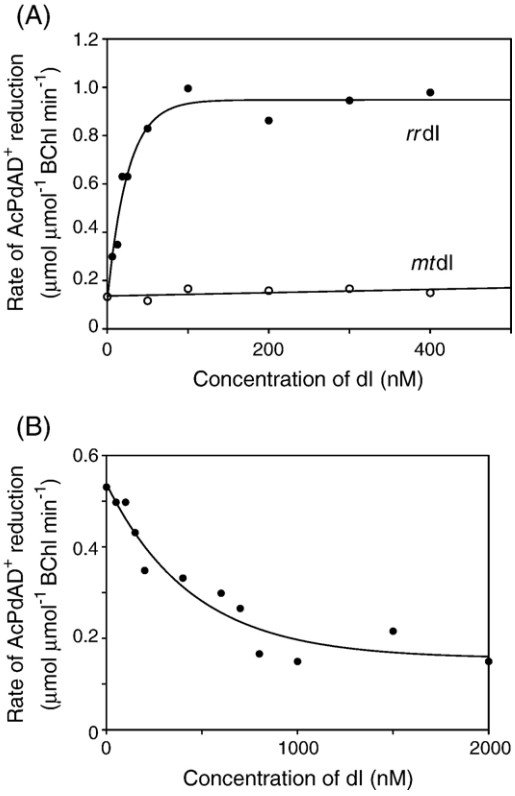

Fig. 3. Rates of transhydrogenation in dI-depleted chromatophore membranes with added rrdI and added mtdI. (A) *R. rubrum* chromatophores were washed to remove dI as described in Materials and methods. The dI-depleted membranes were suspended (to give a bacteriochlorophyll concentration of 10 μM) in 20 mM Tris–HCl, pH 7.2, 50 mM KCl, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 125 mM sucrose, 200 μM NADPH and the dI concentration shown in the figure. The reaction was initiated by addition of $AcPdAD^+$ (final concentration 200 μM). Closed symbols, *rr*dI, open symbols, *mt*dI. Note that the residual transhydrogenation rate observed in the absence of any added dI may be attributable partly to dI that has not been removed in the washing step, and partly to low rates of $AcPdAD^+$ reduction by NADPH catalysed by other enzymes in the chromatophore membranes. (B) As in panel A, but with 10 nM *rr*dI present throughout. The final concentration of *mt*dI was varied as shown. BChl=bacteriochlorophyll.

Chromatophore membranes from *R. rubrum* have an active transhydrogenase. When the membranes are washed by centrifugation in the absence of $NADP^+$ and Mg^{2+}, the transhydrogenase activity is lost [28,40] due to dissociation and removal of the *rr*dI polypeptide. The activity can be fully restored to the depleted membranes by addition of recombinant *rr*dI [13]. In the experiment of Fig. 3A, the transhydrogenation reaction was measured as the reduction of $AcPdAD^+$ by NADPH- essentially the reverse of Eq. (1) but with the analogue substituting for NAD^+. The reaction drives inward proton translocation [41]. In contrast to the experiments with *rr*dI, the addition of *mt*dI to dI-depleted *R. rubrum* membranes had no restorative effect on reverse transhydrogenation. Fig. 3B shows, however, that *mt*dI does bind under these conditions. Enough *rr*dI was added to dI-depleted membranes to give partial restoration of the reverse transhydrogenase activity. Subsequent

addition of *mt*dI then led to inhibition, presumably by competing with *rr*dI for dII/dIII sites in the membrane.

In the presence of low concentrations of either $NADP^+$ or NADPH, transhydrogenase can catalyse the reduction of $AcPdAD^+$ by NADH—the so-called cyclic reaction. This can simply be the sum of reverse transhydrogenation (with $AcPdAD^+$ replacing NAD^+, Eq. (2) and forward transhydrogenation (Eq. (1)):

$$AcPdAD^+ + NADPH + H_n^+ \leftrightarrow AcPdADH + NADP^+ + H_p^+ \quad (2)$$

$$AcPdAD^+ + NADH \leftrightarrow AcPdADH + NAD^+$$

sum of Eqs. (1) and (2).

Here, reverse and forward transhydrogenation drive proton translocation in opposite directions across the membrane and there is no net generation of Δp. However, as shown in the scheme in Fig. 4, the cyclic reaction can take place without either $NADP^+$ or NADPH leaving the enzyme. Thus, steps 1 and 2 in Fig. 4 are entirely sufficient to complete the process. Under these conditions, proton translocation does not occur across the membrane in either direction [42]. This is an important indication that the proton translocation reactions of transhydrogenase are not associated with the redox reaction but are linked specifically to the steps involved in $NADP^+$/NADPH binding and release (the dashed lines in Fig. 4)—and see [43]. The data in Fig. 5 show that, in contrast to the results on reverse transhydrogenation, *mt*dI can restore cyclic transhydrogenation to dI-depleted chromatophores almost as well as *rr*dI. The dependences on dI concentration are similar for the two proteins, indicating a similar affinity for the membrane-located dII/dIII binding sites.

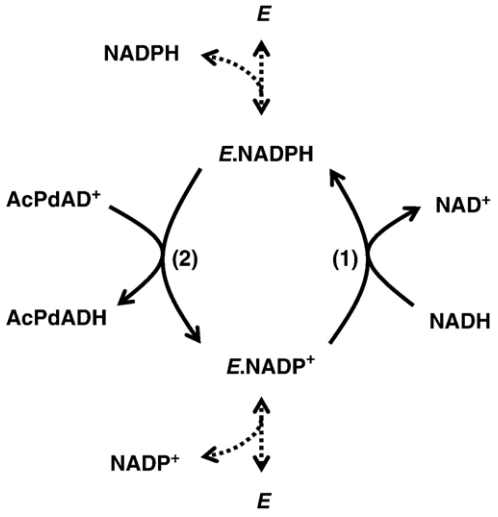

Fig. 4. Scheme for the mechanism of cyclic transhydrogenation. ***E***=transhydrogenase. The solid arrows show the cyclic reaction taking place on the enzyme. The dashed lines show possible association and dissociation of NADP(H) from the enzyme. The latter steps are compulsorily involved in forward and reverse transhydrogenation.

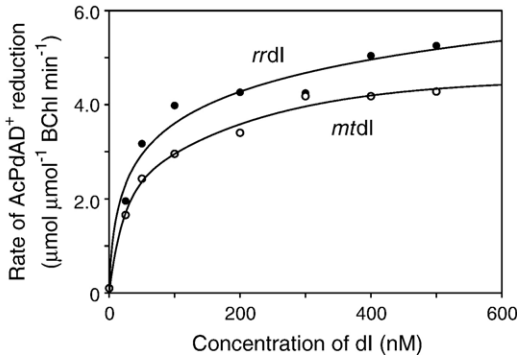

Fig. 5. The mtdI–rrdII–rrdIII hybrid transhydrogenase catalyses rapid cyclic transhydrogenation. Experiments were performed as in Fig. 3A except that 200 μM NADH was additionally present in the assay medium. Closed symbols, *rr*dI, open symbols, *mt*dI.

Mixtures of recombinant *rr*dI and *rr*dIII readily form a dI_2dIII_1 complex. NAD(H) and AcPdAD(H) can bind to, and dissociate from, the dI component of the complex very rapidly, and the bound nucleotides can then undergo fast hydride transfer with NADP(H) bound to the dIII component [23]. However, bound NADP(H) can exchange only very slowly with NADP(H) in the solvent [12]. Thus, in the absence of the membrane-spanning dII, the complex catalyses forward and reverse transhydrogenation only at extremely low rates (limited by the slow release of product NADPH and $NADP^+$, respectively) but it can carry out a rapid cyclic reaction with $NADP^+$ and NADPH remaining tightly bound (Fig. 6). Admixture of *mt*dI and *rr*dIII also led to the formation of a complex capable of catalysing rapid rates of cyclic transhydrogenation. The similar concentration dependences indicate that *rr*dI and *mt*dI bind to *rr*dIII with similar affinities.

3.2. Phylogenetics of transhydrogenase

Fig. 7 shows a phylogenetic tree derived from representative transhydrogenase sequences. As with other proteins [44,45], the tree differs greatly from that determined using sequences of 16s RNA [46] indicating the importance of horizontal gene transfer

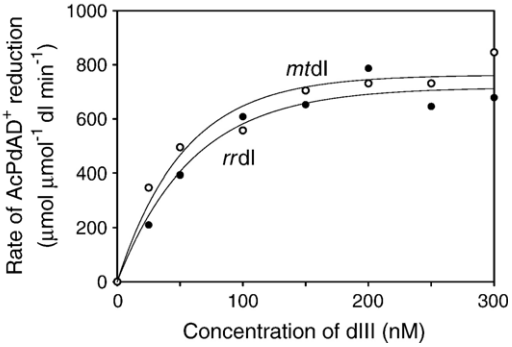

Fig. 6. Rapid cyclic transhydrogenation in complexes of rrdIII and either rrdI or mtdI. Reactions were performed in 50 mM Mops, pH 7.2, 50 mM KCl, 2 mM $MgSO_4$, 200 μM $AcPdAD^+$, 200 μM NADH, and either 50 nM *rr*dI (closed symbols) or 50 nM *mt*dI (open symbols); *rr*dIII was added to give the concentration shown.

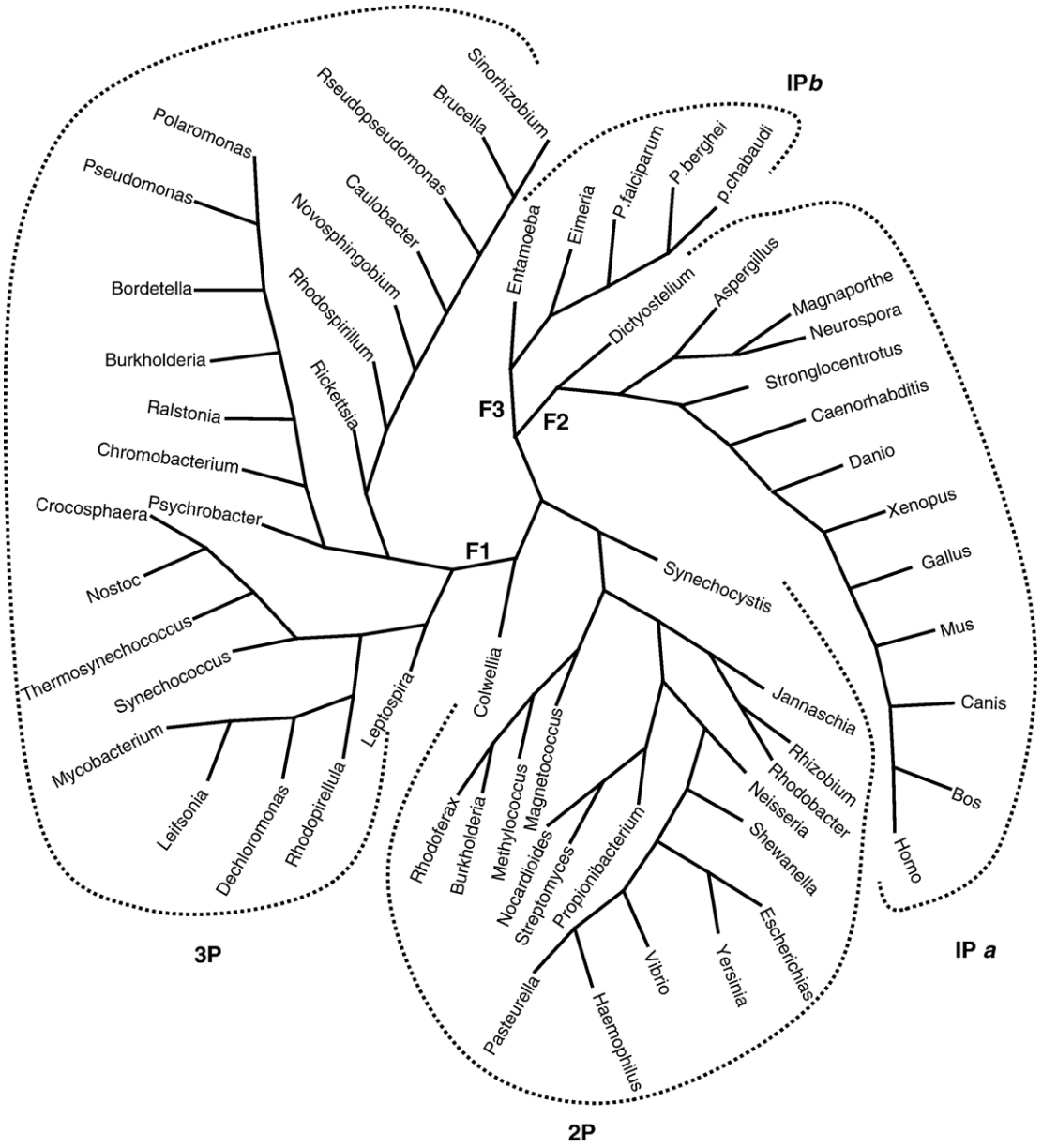

Fig. 7. Phylogenetic tree of transhydrogenase amino acid sequences. Amino acid sequences were aligned using ClustalW [33] and manually adjusted in BIOEDIT. Only representative species from representative genera are shown. The assignment of each transhydrogenase to the 1P*a*, 1P*b*, 2P and 3P groups is based on predictions of polypeptide composition from the gene sequences, as defined in Fig. 1A. Up to September 2005, no transhydrogenase sequence was found that violates the illustrated relationship between the clade organisation and the polypeptide composition. The tree was constructed using a maximum parsimony method [34]. Statistical analysis gave good bootstrap values (>80%). A tree constructed using a neighbour-joining method [35] was almost identical, though it placed the transhydrogenase from *Entamoeba histolytica* on a different branch from those of *Eimeria* and *Plasmodium* species. Regions F1, F2 and F3 are defined in the text.

in the evolution of the enzyme. Strikingly however, the clades defined by the transhydrogenase tree precisely match the groupings defined by polypeptide organisation (Fig. 1)—the 1P*a*, 1P*b*, 2P and 3P groups align with related clades. Horizontal transfer of DNA within the transhydrogenase genes has not significantly disturbed the relationship. We can, therefore, determine where fusions probably led to the emergence of the different groups of transhydrogenases described in Fig. 1A. Thus, a mutation leading to fusion of the C-terminus of PntAA and the N-terminus of PntAB in an ancestral 3P transhydrogenase at a point near F1 in Fig. 7 led to the development of all members of the 2P group. Subsequently to this, two different fusions led to the 1P*a* and the 1P*b* groups from ancestral 2P transhydrogenases. Near F2, a fusion of the C-terminus of (what had been) PntAB and the N-terminus of PntB led to all members of 1P*a*. Similarly near F3, a fusion of the C-terminus of PntB with the N-terminus of (what had been) PntAA led to all members of 1P*b*. Other evolutionary pathways are unlikely because they would have involved either multiple fusion events at a similar position or mutations leading to polypeptide cleavage, which have more demanding requirements to ensure survival of the protein [47]. The proposed pathway places the root of the transhydrogenase tree within the 3P clades of Fig. 7.

A comparison of amino acid sequences indicates that the polypeptide fusion leading to emergence of the 2P group from the 3P group was coupled to the formation of an additional transmembrane helix (TMH). Thus, TMH1, which was predicted from the amino acid sequence of bovine transhydrogenase [48], and whose presence was confirmed experimentally in the *E. coli* enzyme [49], is evident in hydropathy profiles of all 2P, 1P*a* and 1P*b* species (Fig. 8). However, TMH1 is missing from all enzymes in the 3P group. It was noted [50] that the N-terminal amino acid sequence of PntAB of the *M. tuberculosis* enzyme is short and is unlikely to comprise a segment homologous with TMH1. Furthermore, though the equivalent sequence in *R. rubrum* is long enough, it is probably too hydrophilic to form a TMH [9,51]. As more sequences have been deposited in the database, these patterns are repeated across the 3P group—the N-terminal sequence of the PntAB polypeptide is either too short or too hydrophilic to form TMH1 (Fig. 8). Since dI is in the bacterial cytoplasm [28] and the N-terminus of TMH2 is in the periplasm [49], polypeptide fusion during evolution of the 2P group from the 3P group (at F1 in Fig. 7) required passage of the chain across the membrane (eventually) to generate TM1.

Equivalently, it is likely that TMH5 was later formed (at F2 in Fig. 7) during the emergence of the 1P*a* transhydrogenases from their common ancestor in the 2P group by fusion of the C-terminus of (what had been) PntAB and the N-terminus of PntB. These termini are located in the cytoplasm and periplasm,

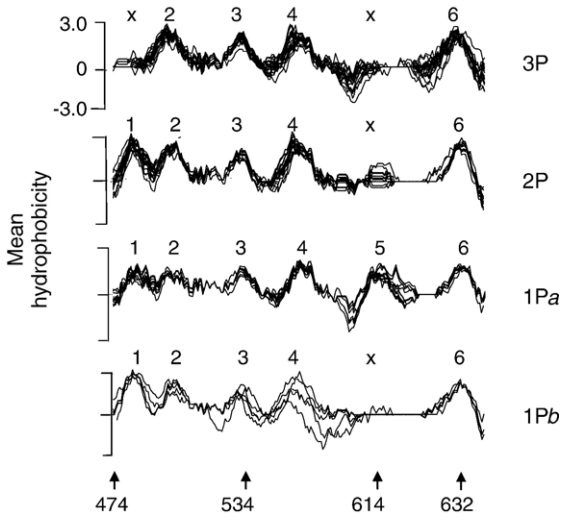

Fig. 8. Hydropathy profiles of amino acid sequences for putative transmembrane helices 1–6 of transhydrogenases. Segments of hydropathy profiles [36] for sequences of all the species in Fig. 7, aligned as described in Materials and methods, are illustrated. The panels show profiles for the 3P, 2P, 1P*a* and 1P*b* groups, as indicated in the right-hand column. The numbers across the top of each panel indicate predicted TMH using the bovine numbering system [48]. "Missing" helices (see text) are marked by a x. The horizontal flat lines arise from gaps introduced during sequence alignment—note that the hydropathy profiles tend to merge at the gaps. The arrows show sequence numbers for the bovine enzyme. Hydrophilic inserts into the amino acid sequences of *Plasmodium falciparum* and *P. berghei* (both from the 1P*b* group) result in distortion of the hydropathy profiles of putative TMH 3 and 4, respectively; the transhydrogenases from these species have not been isolated to verify the properties of the proteins.

respectively [49], and this event too would have required passage of a segment of the polypeptide chain across the membrane. Consistent with this version of events, TMH5 is not predicted by hydropathy profiles in any of the 2P or 3P groups but is strongly predicted in all the 1Pa group (Fig. 8). Interestingly, the insertion of a short polypeptide corresponding to TMH5 of the bovine transhydrogenase between the two polypeptides of the *E. coli* enzyme led to an active protein [52].

In contrast, during the emergence of the 1P*b* group, the mutational event at F3 (Fig. 7) led to fusion of the membrane-peripheral, dI and dIII components of transhydrogenase (for example, see [18]). It did not involve the transmembrane region and, therefore, is predicted not to have resulted in the creation of TMH5. Consistent with this, hydropathy profiles suggest the absence of this helix in the 1Pb group (Fig. 8).

4. Discussion

Isolated *rr*dI forms a high-affinity, redox-active complex with isolated *rr*dIII [12,15], and also somewhat weaker complexes with isolated bovine dIII [14], human dIII [17], *E. coli* dIII [53] and *En. histolytica* dIII [18]. In the intact *R. rubrum* transhydrogenase, dI exists as a separate polypeptide (PntAA in Fig. 1) but the dI components of transhydrogenases from (amongst others) *E. coli* [54] and *En. histolytica* [55] are linked through their respective polypeptide chains to the N-termini of their dII components. The isolated forms of these two latter dI proteins (obtained after appropriately engineering the gene sequences) make only very low-affinity complexes with *rr*dIII and even with their own dIII [16,18]. In the present work, we have expressed and purified dI from *M. tuberculosis* transhydrogenase. We find that, like *rr*dI, *mt*dI readily forms a tight, redox-active complex with *rr*dIII. This is despite the fact that the *R. rubrum* and *M. tuberculosis* transhydrogenases are in widely separated clades of the phylogenetic tree (Fig. 7) and have less sequence identity with one another (41%) than, for example, the *R. rubrum* enzyme has with the *E. coli* enzyme (48%). The results support the suggestion of the Rydstrom group [56,57] that the nature of the interaction between dI and dIII depends on whether or not dI and dII are covalently linked through the polypeptide chain in the intact enzyme. It was envisaged that, because of the structural organisation of the intact enzyme (Fig. 1B), the presence of a dI–dII linker (i.e., in the 1P and 2P transhydrogenases) stabilises the dI–dIII interaction, and that absence of the linker (i.e., in the 3P transhydrogenases) is compensated by tighter binding at the dI/dIII contact surfaces. Thus, in the latter case, isolated dI–dIII complexes are intrinsically more stable. Two conserved basic residues in dI at its interface with dIII in most of the 3P group (Lys192 and Arg193 in *R rubrum* transhydrogenase) but not the 2P group [57] might be partly responsible for the suggested stabilisation.

Evolution of the dI–dII linker probably involved the generation of an extra transmembrane helix (TMH1) during the emergence of the 2P from the 3P group (see above and Fig. 8) as well as a polypeptide segment stretching across the surface of dIII (Fig. 1). These features probably affect the balance of

stability between polypeptides of the enzyme, as indicated above, but we think it is unlikely that they have great mechanistic significance. The view that, in *E. coli*, the dI–dII linker region itself contributes to the coupling mechanism, and that it participates in the transfer of information on nucleotide binding to the membrane-spanning dII [57], seems highly unlikely to us in view of the very similar catalytic properties of the *R. rubrum* and *E. coli* enzymes. Furthermore, the degree of structural similarity between *R. rubrum* dI (PDB, 1F8G [19]) and *E. coli* dI (PDB, 1X13 [57]) is in the range expected for proteins in the same family given their approximately 44% sequence identity: the root mean square deviation between the A-chain of the two proteins is 0.99 Å for 333 pairs of C_α atoms (excluding the "mobile loop" region [58] and the 8 amino acid residues at the C-terminus of *rr*dI)—see [59]. The mechanism of coupling between hydride transfer and proton translocation probably developed before evolutionary separation of the 1P, 2P and 3P groups. We would also anticipate that the development of TMH5 during the emergence of the 1P*a* group and of the fusion of dIII and dI in the formation of the 1P*b* group had little impact on the mechanism of transhydrogenase. The root mean square deviation between *R. rubrum* dIII (PDB, 1HZZ [24] and human dIII (PDB, 1DJL [60]) is only 0.84 Å on 162 pairs of C_α atoms. Given their 38% sequence identity, this is again a typical value for related proteins.

From a mechanistic perspective, the most interesting observation in the present work is that an intact hybrid enzyme has been constructed (*mt*dI–*rr*dII–*rr*dIII) which catalyses a rapid cyclic reaction but not the proton-translocating, reverse transhydrogenase reaction (Figs. 3 and 5). Cyclic and reverse transhydrogenation share several steps. These are the binding of $AcPdAD^+$, hydride transfer from NADPH to $AcPdAD^+$ and the release of AcPdADH. Because the cyclic reaction is fast in *mt*dI–*rr*dII–*rr*dIII, all these individual steps must proceed at a substantial rate in the hybrid enzyme. Reverse transhydrogenation additionally requires an NADPH-binding step and an $NADP^+$-release step (shown in dashed lines in Fig. 4), which are not involved in the cyclic reaction: the defect in reverse transhydrogenation in the hybrid, must result because either one or both of these is inoperative.

The finding that hydride transfer is rapid in a number of inter-species hybrid dIdIII complexes, and now in a hybrid intact enzyme, is remarkable. Stopped-flow experiments showed that hydride transfer between the bound nucleotides in dIdIII complexes is direct – there are no protein-associated redox intermediates [22] – and crystal structures show that the $C4_N$ atoms of the dihydronicotinamide (NicH) and nicotinamide (Nic^+) rings are brought into apposition to effect the reaction [25]. The $C4_N$-to-$C4_N$ distance and the ring orientation is critical in determining the hydride-transfer rate and, therefore, the dI component, bearing NAD(H), and the dIII component, bearing NADP(H), must be precisely aligned in this reaction step. Indeed, amino acid residues in the contact sites between the dI(A), dI(B) and dIII polypeptides of the *R. rubrum* dI_2dIII_1 complex are strongly conserved [24]. The low tolerance towards mutation in the contact regions must partly reflect the importance of preserving the NicH/Nic^+ geometry, and thus the rapid rate of hydride transfer across the domain interface.

The crystal structure of the dI_2dIII_1 complex suggests that dI in the intact enzyme interfaces with dIII but not with dII [24] (see Fig. 1b). The failure in reverse transhydrogenation in the *mt*dI–*rr*dII–*rr*dIII hybrid must, therefore, arise from a mismatch at the mtdI/rrdIII interface. As described above, the defect is attributable to a blockage in the steps associated with NADPH binding and/or $NADP^+$ release. Notably, these are the steps that are thought to be coupled to proton translocation [42,43]. Now NADP(H) binding is to dIII, and proton translocation is through dII. Neither of these processes directly involve dI. Thus, the present results indicate that, in wild-type enzymes, NADP(H) binding/release and H^+ translocation are also coupled to an event that leads to a conformational change across the interface between dI and dIII, and that this conformational change is compromised in the hybrid. The crucial change in interaction between dI and dIII during coupled turnover is that required to enable the hydride-transfer gate as the enzyme moves from an open to an occluded state [7,8]: the gate involves the movement of the Nic(H) ring of NAD(H) from a distal position to a proximal position relative to the Nic(H) ring of NADP(H). The emerging picture is that proton translocation through the membrane-spanning dII leads to coupled conformational changes that spread across both of the peripheral components of the enzyme.

Acknowledgements

We are very grateful to Harma Brondijk, Tina Bhakta, Scott White and Nick Cotton for discussion. The Biotechnology and Biological Sciences Research Council provided financial support.

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