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The pH dependences of reactions catalyzed by the complete proton-translocating transhydrogenase from *Rhodospirillum rubrum*, and by the complex formed from its recombinant nucleotide-binding domains

Tania Bizouarn, Shaun Stilwell, Jamie Venning, Nick P.J. Cotton, J. Baz Jackson *

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

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Abstract

Transhydrogenase couples the translocation of protons across a membrane to the transfer of reducing equivalents between NAD(H) and NADP(H). Using transhydrogenase from *Rhodospirillum rubrum* we have examined the pH dependences of the 'forward' and 'reverse' reactions, and of the 'cyclic' reaction (NADP(H)-dependent reduction of the analogue, acetyl pyridine adenine dinucleotide, by NADH). In the case of the membrane-bound protein in chromatophores, the imposition of a protonmotive force through the action of the light-driven electron-transport system, stimulated forward transhydrogenation, inhibited reverse transhydrogenation, but had no effect on the cyclic reaction. The differential response at a range of pH values provides evidence that hydride transfer per se is not coupled to proton translocation and supports the view that energy transduction occurs at the level of NADP(H) binding. Chromatophore transhydrogenase and the detergent-dispersed enzyme both have bell-shaped pH dependences for forward and reverse transhydrogenation. The cyclic reaction, however, is rapid at low and neutral pH, and is attenuated only at high pH. A mixture of recombinant purified NAD(H)-binding domain I, and NADP(H)-binding domain III, of *R. rubrum* transhydrogenase carry out the cyclic reaction with a similar pH profile to that of the complete enzyme, but the forward and reverse reactions were much less pH dependent. The rates of release of $NADP^+$ and of NADPH from isolated domain III were pH independent. The results are consistent with a model for transhydrogenation, in which proton binding from one side of the membrane is consequent upon the binding of $NADP^+$ to the enzyme, and then proton release on the other side of the membrane precedes NADPH release.

Keywords: pH dependence; Transhydrogenase; (*Rhodospirillum rubrum*)

1. Introduction

Transhydrogenase is a proton pump found in the inner mitochondrial membranes of animal cells and the cytoplasmic membranes of bacteria. Under most physiological conditions it probably functions in the direction of $NADP^+$ reduction by NADH driven by the proton electrochemical gradient generated by respiratory (or photosynthetic) electron transport. For reviews, see [1–3].

$$NADH + NADP^+ + H^+_p \rightleftarrows NAD^+ + NADPH + H^+_n \quad (1)$$

Abbreviations: FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; $AcPdAD^+$, acetyl pyridine adenine dinucleotide (oxidized form)

* Corresponding author. Fax: +44-121-4143982; E-mail: j.b.jackson@bham.ac.uk

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where H_p^+ and H_n^+ (corresponding to H_{out}^+ and H_{in}^+ in an alternative terminology) represent hydrogen ions in the p and n (outside and inside) phases, respectively.

Experiments on chromatophores from photosynthetic bacteria, and on bovine heart transhydrogenase reconstituted into liposomes, indicate that one proton is translocated across the membrane per hydride equivalent transferred between the nucleotide substrates [4].

The enzyme has separate sites, for NAD^+/NADH on domain I of the protein, and for $NADP^+$/NADPH on domain III. These two domains protrude from the membrane, the third (domain II) spans the membrane.

A consensus view is emerging that proton translocation by transhydrogenase is closely linked to nucleotide binding and release [2,5–7]; we have developed the case that proton binding and release are coupled specifically to events at the NADP(H) site on domain III [5]. The pH dependence of forward and reverse transhydrogenation by the detergent-dispersed enzyme from *E. coli* is bell-shaped [5,8]. It was proposed that on the acid side of the pH optimum the reactions are limited by deprotonation steps that accompany the release of product NADPH (or $NADP^+$). On the alkaline side, failure of transhydrogenase to protonate following the binding of $NADP^+$ (or NADPH) prevents the hydride transfer reaction.

Not only will transhydrogenase catalyze the forward and reverse reactions described by Eq. (1), in some conditions it will also perform a rapid cyclic reaction, the reduction of $AcPdAD^+$ (acetyl pyridine adenine dinucleotide, an analogue of NAD^+) by NADH in the presence of either $NADP^+$ or NADPH. Kinetic analysis of this reaction in detergent-solubilized *E. coli* transhydrogenase [5,8], and subsequently in other systems [9–11], indicated that the NADP(H) remains bound to the domain III site of the protein, and is alternately oxidized by $AcPdAD^+$, and then reduced by NADH in the domain I site.

$$AcPdAD^+ + E.NADPH \rightarrow AcPdADH + E.NADP^+ \quad (2)$$

$$NADH + E.NADP^+ \rightarrow NAD^+ + E.NADPH \quad (3)$$

where E.NADP(H) represent enzyme-bound forms. The pH dependence of cyclic transhydrogenation in *E. coli* transhydrogenase reflects the linkage between the binding of NADP(H) and the binding of protons.

Rhodospirillum rubrum transhydrogenase is a particularly useful system for experimentation. Firstly, everted membrane vesicles (chromatophores) from this organism have an active transhydrogenase with both nucleotide-binding sites exposed to the external medium [12]. The proton electrochemical gradient (Δp) across the chromatophore membrane can be readily manipulated by activating the photosynthetic electron transport chain with light energy; for example, illumination of chromatophore membranes increases the rate of forward transhydrogenation by as much 20–50 fold [13]. Secondly, domain I of *R. rubrum* transhydrogenase uniquely exists as a separate polypeptide, which can be easily stripped from the chromatophore membranes, and then reconstituted with full restoration of activity [14,15]. Finally, recombinant forms of domain I and domain III of the enzyme are now available; a mixture of the two proteins catalyses high rates of hydride transfer on the enzyme [16,17]. In the domain I:III complex the rates of forward and reverse transhydrogenation are heavily limited by the rates of release of NADPH and $NADP^+$, respectively, but the cyclic reaction is extremely rapid [17,18].

The conclusion that emerges from the pH dependences of the ‘reverse’, ‘forward’ and ‘cyclic’ reactions of the *E. coli* enzyme [5,8], and the observations on catalysis by the mixture of *R. rubrum* domain I and domain III proteins [17], is that, in the complete enzyme, changes in the binding energy of $NADP^+$ and NADPH brought about by protonation and deprotonation, are central to the coupling mechanism; it was proposed that the protonation–deprotonation components of H^+ translocation through domain II are linked specifically to changes in the affinity of domain III for $NADP^+$ and NADPH. In this report, that view is tested through an examination of the pH dependences of transhydrogenation reactions in the complete membrane-bound *R. rubrum* enzyme, in the complete detergent-dispersed enzyme, and in the domain I:III complex.

2. Experimental

Chromatophores from a transhydrogenase-overexpressing strain of *R. rubrum* were isolated as described [4]. Bacteriochlorophyll concentration was

measured using an in vivo extinction coefficient of 140 mM^{-1} cm^{-1} at 880 nm [19]. Native domain I was removed from the membranes by washing [4,14]. Recombinant domains I and III of *R. rubrum* transhydrogenase were separately expressed in *E. coli* and purified [16,17]. Their protein concentrations were measured by the microtannin procedure using bovine serum albumin as standard [20]. Reconstitution of the complete enzyme (domain I-depleted chromatophores plus recombinant domain I), and of the domain I:III complex, was by simple mixing of components [14,17]. In experiments with unwashed chromatophores, the media were supplemented with recombinant domain I protein to maximize transhydrogenation rates [14].

The membrane-bound components of *R. rubrum* transhydrogenase (originally called Th_m [21] and now known to comprise domain II, and the associated domain III [15]) were isolated from domain I-depleted membranes and partially purified by a modification of a published procedure [21]. Chromatophore membranes from the over-expressing strain of *R. rubrum* (100 mg protein) were washed at 4°C with 2 M NaCl, 3% sucrose, 30 mM Tris–HCl, pH 8.0 and resuspended in 3% sucrose, 30 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride plus lysolecithin such that the final detergent and protein concentrations were 5 mg ml^{-1} and 2 mg ml^{-1}, respectively. The suspension was stirred for 10 min and then incubated for a further 20 min on ice before centrifuging at $200\,000 \times g$ for 90 min. The supernatant (48 ml) was applied to a 6 ml column of Q-Sepharose HP, which had been pre-equilibrated in 20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 0.01% dodecyl maltoside (buffer A). The column was then washed, first with 50 ml of buffer A, followed by 50 ml 250 mM NaCl in buffer A, before applying a gradient (250 ml) of 250–500 mM NaCl in buffer A. One third (40 ml) of the pooled active fractions were dialysed against 500 ml of 20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 10% glycerol, 0.2% Mega 9 and then applied to a 6 ml Q-Sepharose HP column pre-equilibrated in 20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 0.2% Mega 9 (buffer B). The sample was washed in with 20 ml buffer B and protein was eluted with a 0–0.6 M gradient of NaCl in buffer B. Active fractions were pooled and dialysed against 500 ml of 20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 10% glycerol, 0.01% dodecyl maltoside and applied to a 1 ml Mono Q column pre-equilibrated with 20 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol, 0.01% dodecyl maltoside (buffer C). The column was developed, first with 5 ml buffer C, followed by a 20 ml gradient of 0–0.5 M NaCl in buffer C. Fractions from the most active of the two peaks were pooled and used for experiments. Throughout this procedure, activity of the domain II/III complex was assayed by measuring the rate of reduction of $AcPdAD^+$ by NADPH in samples supplemented with a saturating amount of purified recombinant domain I ([17] and see below). The protein concentration of the domain II/III complex was assayed using the bicinchoninic acid method using bovine serum albumin as a standard [22].

Transhydrogenase from *E. coli* was isolated from an inner membrane fraction essentially as described [5,23]. The enzyme was incorporated into phospholipid vesicles by a modification of the method of Paternostre et al. [24]. Phosphatidylethanolamine, phosphatidylcholine and phosphatidic acid (Lipid Products) in a ratio of 5:5:1 (by weight) were subjected to reverse-phase evaporation and the resulting liposomes were resuspended in 50 mM KCl, 1 mM EDTA, 50 mM Na–phosphate, pH 7.0, and filtered sequentially through two polycarbonate filters (Costar) of pore diameter 0.4 and 0.2 μm. The lipid concentration was adjusted to 3.6 mg ml^{-1}, and recrystallized Na–cholate was added to 15 mM. *E. coli* transhydrogenase was added under continuous stirring to the detergent–lipid mixture, at 4°C, to give a final concentration of 24 μg ml^{-1}. The suspension was diluted 100 fold into the above buffer immediately prior to assay.

Transhydrogenation was measured spectrophotometrically, either as the reduction of $AcPdAD^+$, or as the reduction of thio–$NADP^+$, in media described in the figure legends, and using absorbance coefficients given [25] in either a dual beam (Perkin Elmer Lambda 16) or a dual wavelength instrument (Shimadzu UV3000). The latter was fitted with a GaAlAs emitter (OD100, Optodiode) at right angles to the measuring beam to provide saturating photosynthetic illumination where needed [26].

To determine the contributions of cyclic and of

reverse transhydrogenation to $AcPdAD^+$ reduction in the presence of NADH and NADPH, the $NADP^+$ production was measured fluorimetrically after denaturing the protein (see [17,27]). *R. rubrum* chromatophores (or reconstituted *E. coli* transhydrogenase liposomes) were resuspended in the media described in the figure legends and incubated at 25°C (or 30°C) for 5 min. A 0.6 ml sample was removed and added to 0.3 ml ice-cold 14% perchloric acid. The transhydrogenation reaction was initiated in the remaining solution by addition of $AcPdAD^+$ (see figure legends), and further samples were collected at 60 s intervals and added to perchloric acid. The acidified samples were stored on ice for at least 10 min before neutralizing with 0.45 ml 1 M KOH–1 M $KHCO_3$. They were returned to ice for a further 15 min and then frozen at −20°C for 1 h. After thawing, the samples were centrifuged at $8000 \times g$ for 5 min to remove the precipitate and then assayed for $NADP^+$ with isocitrate dehydrogenase using a Spex Fluoromax, essentially as described [17].

The rates of $NADP^+$ and NADPH release from recombinant domain III were measured by fluorescence as described [17] in the conditions given in the legend to Table 2.

3. Results

3.1. The pH dependences of forward, reverse and cyclic transhydrogenation in chromatophore membranes from R. rubrum in the presence and absence of Δp

pH dependences of complete transhydrogenase were studied with everted membranes (chromatophores) prepared from a strain of the bacterium which over-expresses the enzyme. In one set of experiments, the membranes were treated with the ionophore, FCCP, to prevent any build-up of Δp during proton translocation by the enzyme. Under these conditions, forward transhydrogenation (the reduction of thio–$NADP^+$ by NADH) proceeded slowly; it had a bell-shaped pH dependence with an optimum at approximately pH 6.3 (Fig. 1). Reverse transhydogenation (the reduction of $AcPdAD^+$ by NADPH) was much faster than the forward reaction, but it also had a bell-shaped pH dependence with an optimum at pH 7.1 (Fig. 2). As previously reported, the reduction of $AcPdAD^+$ with a combination of NADPH and NADH was faster still. In principle, the reduction of $AcPdAD^+$ under these conditions might result from both simple reverse transhydrogenation and from the cyclic reaction [10,11]. To estimate the relative contributions, the rate of $NADP^+$ formation was measured at the same time as the net rate of $AcPdAD^+$ reduction. Fig. 3A shows that the rate of NADPH oxidation was insignificant relative to the rate of AcPdADH formation, i.e., NADH was the source of reducing equivalents for the $AcPdAD^+$ reduction, the cyclic reaction was totally dominant. The reduction of $AcPdAD^+$ by this route had a much broader pH optimum than either of the simple, linear

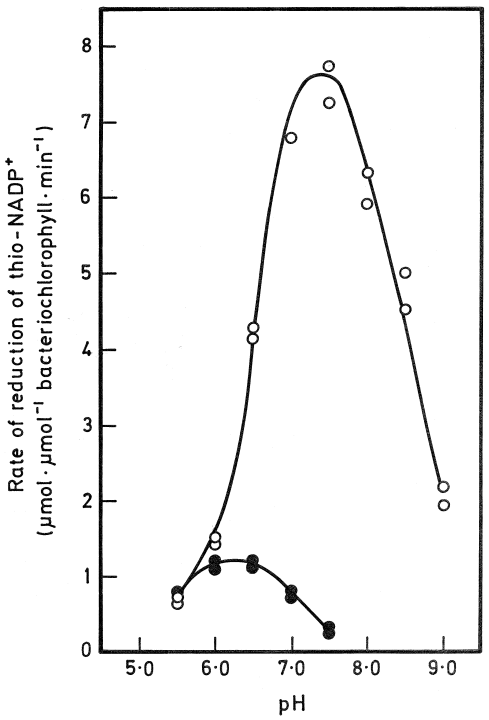

Fig. 1. The pH dependence of forward transhydrogenation in uncoupled chromatophores, and in illuminated, coupled chromatophores, from *R. rubrum*. Experiments were performed at 25°C in 50 mM NaCl, 2 mM Na–succinate, 20 mM Mes, 20 mM Mops, 20 mM Tris, 20 mM Ches at the pH values shown (using KOH). Oligomycin was present at 1 μg ml^{-1}, NADH at 167 μM, thio–$NADP^+$ at 162 μM, transhydrogenase-overexpressing chromatophores at 0.73 μM bacteriochlorophyll, purified recombinant domain I at 21 nM. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (1 μM) was added to assay media used with darkened chromatophores (●) but not those with illuminated chromatophores (○).

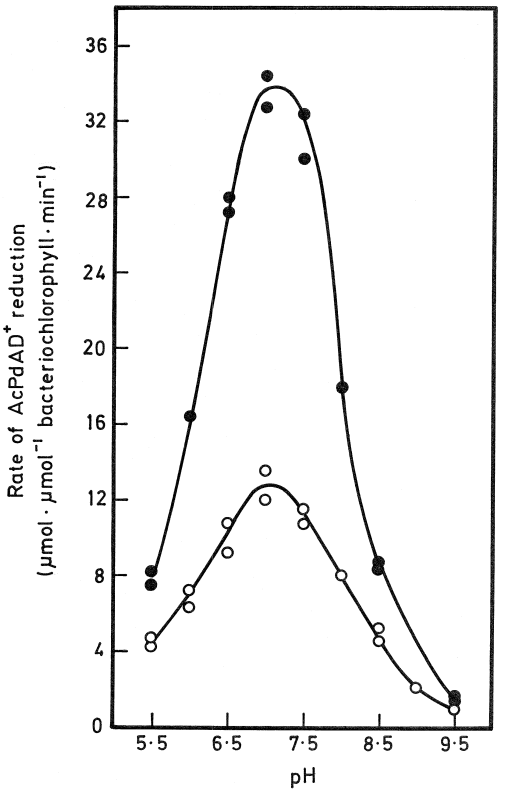

Fig. 2. The pH dependence of reverse transhydrogenation in uncoupled chromatophores, and in illuminated, coupled chromatophores, from *R. rubrum*. Conditions as in Fig. 1, except that the chromatophores were present at 0.83 μM bacteriochlorophyll, and the nucleotides were 204 μM NADPH and 200 μM $AcPdAD^+$.

reactions (Fig. 4). The rate was maximal at pH 6.0–6.5. At higher pH values, it decreased a little over the range 7.0–8.0 and then declined further at pH > 8.5.

In Section 4, we shall put forward the proposition that the relationship between the cyclic reaction and the simple forward and reverse transhydrogenation reactions is essentially similar in the *R. rubrum* and *E. coli* enzymes, but that different values of some rate constants dramatically change the apparent behavior. As an illustration of this, Fig. 3B (cf. Fig. 3A) shows that in *E. coli* transhydrogenase the reduction of $AcPdAD^+$ by a combination of NADPH and NADH was dominated by the cyclic reaction only at low pH (the rate of $NADP^+$ formation was low relative to the rate of $AcPdAD^+$ reduction). At around pH 7, the rate of $NADP^+$ formation was equivalent to the rate of $AcPdAD^+$ reduced, i.e., the reaction proceeds via simple reverse transhydrogenation. This figure also confirms earlier results [5] in that it shows that, with *E. coli* transhydrogenase, the reduction of $AcPdAD^+$ by the combination of NADPH and NADH is half-attenuated at about 6.5, some 2–2.5 units less than with the *R. rubrum* enzyme (Fig. 4).

Note, that for the reverse and forward transhydrogenation reactions described in Figs. 1 and 2, the nucleotide concentrations were close to saturation. Approximate K_m^{app} values obtained at selected values of the medium pH for *R. rubrum* transhydrogenase are given Table 1. As discussed [11], it is difficult to determine the true K_m^{app} of substrates for the cyclic reaction because of substrate inhibition. However, the profiles of the rate of the cyclic reaction versus either NADH or $AcPdAD^+$ concentration changed very little with pH (data not shown), and we can therefore assume that the rates measured in Fig. 4 are a reliable reflection of V_{max} values. To confirm that the reactions measured in Figs. 1–4 originated only from transhydrogenase, some experiments at key pH values were performed using domain I-depleted chromatophores in the absence, and in the presence, of recombinant domain I (data not shown).

Figs. 1, 2 and 4 also include data from parallel experiments recorded with illuminated chromatophore membranes in the absence of FCCP. The resultant Δp under these conditions had a pronounced stimulatory effect on the rate of forward transhydrogenation (Fig. 1). The resulting profile remained bell-shaped, but its maximum was shifted to about pH 7.4. The imposition of a Δp led to strong inhibition of reverse transhydrogenation (Fig. 2), but the effect was almost independent of the pH of the medium, with the optimum remaining at about pH 7.1. Presumably, inhibition by Δp of $AcPdAD^+$ reduction by NADPH is a simple reflection of the thermodynamic reversibility of the pump. In contrast to forward and reverse transhydrogenation, the cyclic reaction was entirely unaffected by illumination at any value of the medium pH (Fig. 4).

3.2. The pH dependences of detergent-dispersed transhydrogenase from R. rubrum

To date, we have been unable to obtain a pure, detergent-dispersed preparation of the domain II/III protein (i.e., the PntAB/PntB protein [15]) of trans-

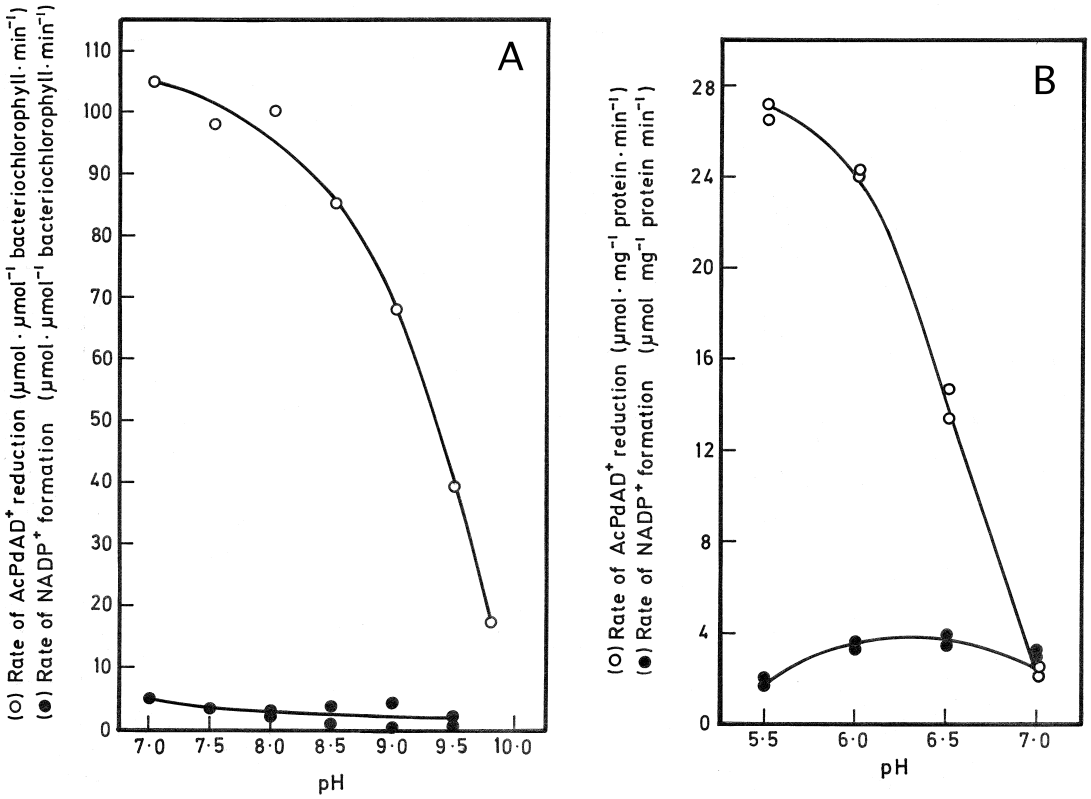

Fig. 3. Contributions from reverse and from cyclic transhydrogenation to the reduction of $AcPdAD^+$ in the presence of both NADH and NADPH by (A) *R. rubrum* and (B) *E. coli* transhydrogenase. (A) Conditions as in Fig. 1, except that the chromatophores were present at 0.35 μM bacteriochlorophyll, and the nucleotides were 100 μM NADH, 50 μM NADPH and 200 μM $AcPdAD^+$. The experiment was performed in the dark in the presence of the uncoupler. The reduction of $AcPdAD^+$ (○) was measured spectrophotometrically in real time, and the formation of $NADP^+$ (●) was measured after perchloric acid precipitation of the enzyme followed by fluorimetric assay, as described in the Section 2. (B) The experiment was performed at 30°C in 20 mM Mes, 20 mM Mops at the pH values shown, and the nucleotides were 200 μM NADPH, 50 μM NADH, 120 μM $AcPdAD^+$. Liposomes inlaid with purified *E. coli* transhydrogenase were added to give a protein concentration of 2.4 μg ml^{-1} and uncoupled with 5 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. $AcPdAD^+$ reduction and $NADP^+$ formation were measured as in (A) (see Section 2).

Table 1
The pH dependence of the Michaelis constants of transhydrogenase preparations from *R. rubrum*

Preparation	Reaction	pH	K_m NADP(H) species or analogue (μM)	K_m NAD(H) species or analogue (μM)
Chromatophores	NADH → $sNADP^+$	6.0	5	9
Chromatophores	NADH → $sNADP^+$	6.5	4	10
Chromatophores	NADPH → $AcPdAD^+$	7.0	17	26
Chromatophores	NADPH → $AcPdAD^+$	8.5	80	25
I:III complex	NADPH → $AcPdAD^+$	6.1	< 5	7
I:III complex	NADPH → $AcPdAD^+$	7.0	< 5	5
I:III complex	NADPH → $AcPdAD^+$	8.1	< 5	10

The chromatophore experiments were performed as described in Figs. 1 and 2, and the I:III complex experiments as in Fig. 7. In each experiment, the concentration of one nucleotide substrate was varied while the other was maintained at a saturating concentration at the required pH. K_m values were calculated using Enzfitter (Elsevier, Cambridge). $sNADP^+$ = thio–$NADP^+$.

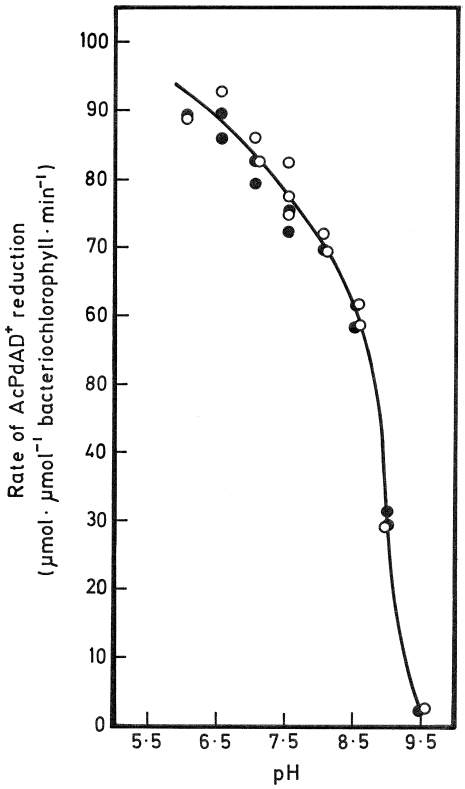

Fig. 4. The pH dependence of cyclic transhydrogenation in uncoupled chromatophores, and in illuminated, coupled chromatophores, from *R. rubrum*. Conditions as in Fig. 1, except that the chromatophores were present at 1.0 μM bacteriochlorophyll, the oligomycin was 0.66 μg ml^{-1} and the nucleotides were 100 μM NADH, 136 μM NADPH and 200 μM $AcPdAD^+$.

hydrogenase from membranes of *R. rubrum* (see [21]). As explained on the earlier occasion, solubilization of the protein can be achieved with a surprisingly limited number of detergents (and see also [28]). The purification procedure described in Section 2 represents an improvement on our earlier attempts, but the expected observation of PntAB (14.9 kDa) and PntB (47.8 kDa) on SDS–PAGE was complicated by the presence of contaminating polypeptides (results not shown). Even so, the partially-purified material, when reconstituted with purified recombinant domain I protein, catalyzed forward, reverse and cyclic transhydrogenation at substantial rates. Fig. 5 shows the rates of reduction of $AcPdAD^+$ with NADPH during a titration.

The pH dependences of the forward, reverse and cyclic transhydrogenase activities of the reconstituted, detergent-dispersed protein (Fig. 6) were similar in character to those found for uncoupled chromatophore membranes (see above). Thus, forward and reverse transhydrogenation had bell-shaped profiles with maxima at pH 6.7 and 7.3, respectively, and the rate of the cyclic reaction was greatest at low pH and declined monotonically with increasing pH. It is also significant that in both chromatophore membranes, and in the reconstituted, detergent-dispersed enzyme, the reverse and forward reactions were only several-fold slower than the cyclic reaction (Fig. 6), whereas in the domain I:III complex ([17] and see below) simple linear transhydrogenation was > 1000-fold slower than the cyclic reaction.

3.3. The pH dependences of the I:III complex of transhydrogenase from R. rubrum

The pH dependences of the reverse and cyclic reactions catalyzed by the domain I:III complex of *R. rubrum* transhydrogenase are shown in Fig. 7. Experiments with this complex must be carried out with due regard to its transitory nature. Thus, during the reverse reaction under standard conditions [17] the rate of association and dissociation of the two subunits, and the rate of hydride transfer, are considerably faster than the rate-limiting step in catalysis of $NADP^+$ release (0.03 s^{-1}). That is, domain I bearing

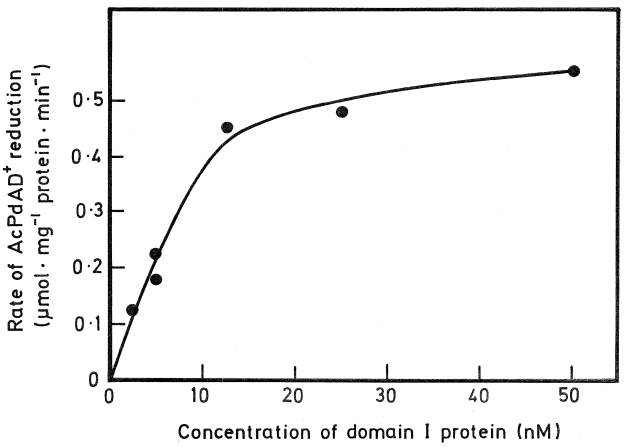

Fig. 5. The rate of $AcPdAD^+$ reduction by NADPH during a titration of partially purified domain II/III complex with recombinant domain I. Experiments were performed at 25°C in a solution containing 50 mM KCl, 2 mM $MgCl_2$, 20 mM Mops, pH 7.0, 200 μM NADPH, 200 μM $AcPdAD^+$. The concentration of partially purified domain II/III protein was 2.4 μg ml^{-1}, and that of purified recombinant domain I protein was as shown.

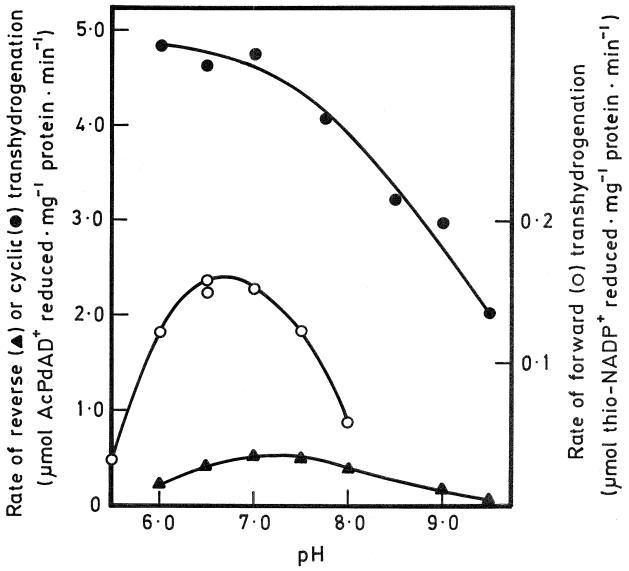

Fig. 6. The pH dependences of forward, reverse and cyclic transhydrogenation in detergent dispersed preparations of *R. rubrum* transhydrogenase. The reverse and cyclic reactions were measured at 25°C in a medium containing 50 mM NaCl, 2 mM $MgCl_2$, 20 mM Mes, 20 mM Tris, 20 mM Mops, 20 mM Ches, at the pH values shown (using KOH). Reverse transhydrogenation (▲) was measured with 200 μM $AcPdAD^+$, 200 μM NADPH, 2.4 μg ml^{-1} partially purified domain II/III protein and 64 nM purified domain I. Cyclic transhydrogenation (●) was measured with 200 μM $AcPdAD^+$, 100 μM NADH and 20 μM NADPH, 1.2 μg ml^{-1} partially purified domain II/III protein and 32 nM purified domain I. Forward transhydrogenation (○) was measured in a medium containing 4 mM $MgCl_2$, 50 mM Mops, 50 mM Mes, at the pH shown, and 100 μM NADH, 100 μM thio–$NADP^+$, 12 μg ml^{-1} partially purified domain II/III protein and 160 nM purified domain I.

fresh $AcPdAD^+$ can visit many domain III molecules during the time taken for the release of $NADP^+$ from domain III. For the reverse reaction, throughout the pH range investigated in Fig. 7, domain I was present in excess, and therefore rates are expressed relative to the turnover of domain III. Furthermore, the rates were measured following the pre-steady-state burst of transhydrogenation (<1 s), a phenomenon which also results from the disparity between the rapid rates of docking and of hydride transfer, and the slow rate of $NADP^+$ release [17]. The bell-shaped pH dependence of the reverse reaction, as catalyzed by the preparations of complete enzyme (see above), was not evident with the domain I:III complex. Instead the reaction was much less dependent on pH; the rate was highest at pH 6.0 (below which protein aggregation limited further measurements) and declined only by about 50% over the pH range 6.0–10.0.

In contrast to the situation with the reverse reaction, the pH dependence of the cyclic reaction catalyzed by the domain I:III complexes (Fig. 7) bore a resemblance to that observed for the complete enzyme (Fig. 4); the rate was maximal between pH 6.0–7.5 and was suppressed at high pH as a group with an apparent $pK_a \approx 9.1$ was deprotonated. The cyclic reaction in Fig. 7 was measured with domain III in excess, and rates are expressed relative to the turnover of domain I.

The domain I:III complex catalyses forward transhydrogenation only at extremely low rates; it is lim-

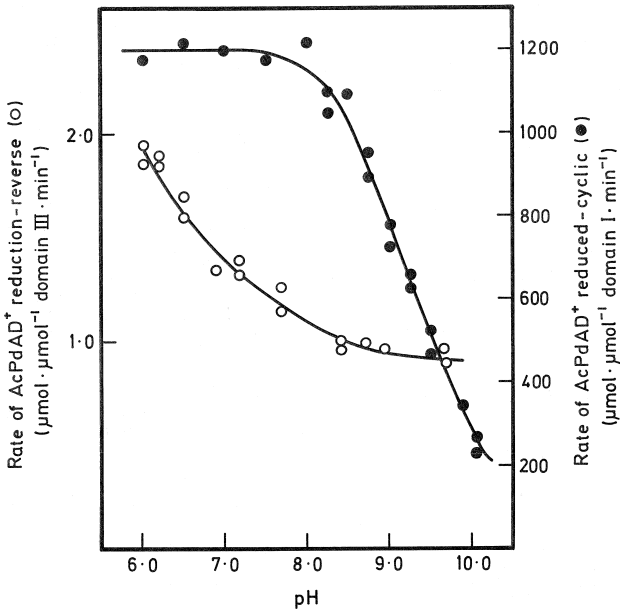

Fig. 7. The pH dependences of the reverse and cyclic reactions in recombinant domain I:III complexes of *R. rubrum* transhydrogenase. Experiments were performed at 25°C in 50 mM KCl, 2 mM $MgCl_2$, 50 mM K^+–acetate, 20 mM Mes, 20 mM Mops, 20 mM Tris, 20 mM Ches; the pH was adjusted with either HCl or NaOH to the value shown. The reverse reaction was measured in the presence of 100 μM $AcPdAD^+$ and 100 μM NADPH. The domain I concentration was 325 nM, and domain III, 1427 nM; under these conditions domain III was saturated with domain I [17]. The cyclic reaction was measured in the presence of 200 μM $AcPdAD^+$, 100 μM NADH and 20 μM $NADP^+$. The domain I concentration was 7.16 nM, and domain III, 1142 nM; under these conditions domain I was saturated with domain III at all values of the medium pH (see text and [17]).

ited by the very slow release of NADPH (or thio–NADPH) from domain III [17]. The large amounts of protein required to measure the rate of the forward reaction and the large experimental error preclude an analysis of the pH dependence at present. However, it was shown that at pH 7.0 and 8.0 the rates of thio–$NADP^+$ reduction by NADH were similar, at ca. 0.02 μmol μmol^{-1} domain III min^{-1}.

Controls with the domain I:III complex again established that the nucleotide substrates in the linear transhydrogenation reactions in the above experiments were close to saturation; K_m^{app} values are given in Table 1.

3.4. The pH dependences of $NADP^+$ and NADPH release from isolated recombinant domain III of transhydrogenase from R. rubrum

It was shown that $NADP^+$ and NADPH bind very tightly to isolated domain III of *R. rubrum* transhydrogenase [17]. Even the purified recombinant protein is associated with tightly-bound $NADP^+$ and NADPH. The bound nucleotide is catalytically active as shown by the fluorescence decrease at 450–460 nm following addition of domain I plus $AcPdAD^+$. The first order rate constants for the release of the tightly bound NADPH and $NADP^+$ at neutral pH are ca. 6×10^{-4} and 0.03 s^{-1}, respectively. The similarity of these values to the k_{cat} values for forward and reverse transhydrogenation by the domain I:III complex, indicates that the linear transhydrogenation reactions are limited mainly by the rate of release of NADPH and $NADP^+$, respectively. Unfortunately, the method for measuring the rate of nucleotide release from domain III is constrained by the limitations of the trapping systems used (see Section 2), i.e., by the pH dependences of the trapping enzymes. However, Table 2 shows that within the measurable range (pH 7.0–9.0) the rate constants for the release of $NADP^+$ and NADPH did not change significantly.

Table 2
The pH dependence of the rates of release of $NADP^+$ and NADPH from recombinant domain III of transhydrogenase from *R. rubrum*

pH	$NADP^+$ release (s^{-1})	NADPH release (s^{-1})
7.0	2.7×10^{-2}	7.7×10^{-4}
9.0	2.2×10^{-2}	5.4×10^{-4}

Experiments were performed at 25°C in 3 mM $MgCl_2$, 20 mM Mops, 20 mM Tris at the pH shown (NaOH). $NADP^+$ release was measured from the fluorescence change resulting from addition of domain III protein (1 μM) to the above medium, supplemented with 2 mM *iso*-citrate and 0.1 U *iso*-citrate dehydrogenase (NADP-linked porcine heart, type IV, Sigma). NADPH release was measured from the fluorescence change resulting from addition of domain III protein (1 μM) to the same medium, but supplemented with 2 mM oxidized glutathione and 0.6 U glutathione reductase. Control experiments showed that, in each case, the enzyme system used for oxidizing or reducing the NADP(H) did not limit the change in fluorescence.

4. Discussion

4.1. Cyclic transhydrogenation is not coupled to proton translocation

We proposed that cyclic transhydrogenation takes place without any accompanying proton translocation [5,8]. Our model is illustrated in the scheme shown in Fig. 8, where the cyclic reaction proceeds only by way of the E^{**} states. This view contrasts with an alternative proposal [7] in which it was suggested that, during the cyclic reaction, reduction of $AcPdAD^+$ by NADPH (on the enzyme) is coupled to proton translocation from the n-phase to the p-phase, and then oxidation of NADH by $NADP^+$ (on the enzyme) is coupled to proton translocation in the opposite direction, from the p-phase to the n-phase. If the latter view were correct, then imposition of Δp during the cyclic reaction would lead to inhibition of the steps involving the reduction of $AcPdAD^+$ by NADPH, and to stimulation of those involving the oxidation of NADH by $NADP^+$. Unless, fortuitously, inhibition of the one were precisely complemented by stimulation of the other, then there should be a net effect of Δp (either inhibitory or stimulatory) on the overall rate of the cyclic reaction. In fact, upon imposition of Δp (i.e., upon illumination) neither inhibition nor stimulation of the cyclic transhydrogenation reaction was observed, whatever the pH (Fig. 4). The possibility of fortuitous cancellation of inhibition and stimulation in different parts of the cyclic pathway can be eliminated. Firstly, note that steady-state forward and steady-state reverse transhydrogenation are strongly stimulated (thio–$NADP^+$

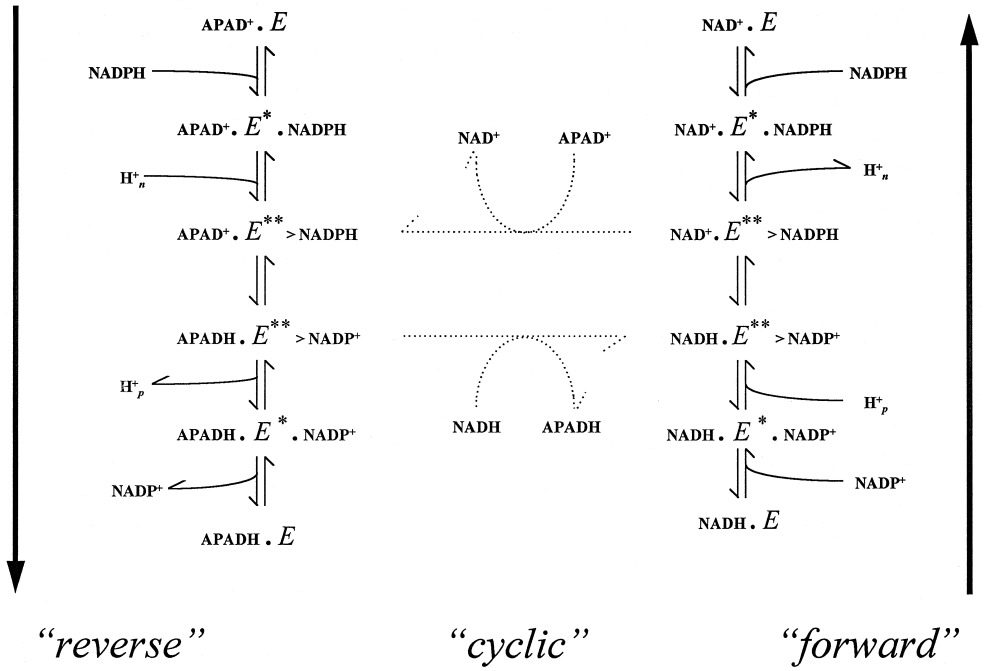

Fig. 8. Hypothetical mechanism of coupling between transhydrogenation and proton translocation. 'Reverse' transhydrogenation (with the NAD^+ analogue, acetylpyridine adenine dinucleotide, $APAD^+ = AcPdAD^+$) is shown on the left, proceeding from top to bottom, and 'forward' transhydrogenation on the right, proceeding from bottom to top. Hydride transfer can take place in ternary complexes involving the E^{**} states (e.g., $AcPdAD^+.E^{**} > NADPH$ and $NADH.E^{**} > NADP^+$), but not in ternary complexes involving E^* states (e.g., $AcPdAD^+.E^*.NADPH$ and $NADH.E^*.NADP^+$). The 'cyclic reaction' proceeds via the E^{**} states and the dotted lines across the center of the scheme. In the E state (no bound $NADP^+$ or NADPH) the protein cannot bind protons at its catalytic center, either because the pK_a is very low [5], or because of physical barriers to proton conduction. In the E^* state the protein can bind protons from the solvent (from the n-phase in $E^*.NADPH$, and from the p-phase in $E^*.NADP^+$). The E^{**} state is generated from E^* as a result of protonation and the associated conformational rearrangement. H^+_n and H^+_p represent protons in the negative and positive aqueous phases on either side of the membrane (in chromatophores, n is the outside, p, the inside). The domain I nucleotide-binding site is on the left of the E symbols, the domain III binding site on the right. The · symbol signifies that a nucleotide ligand is in rapid exchange with the enzyme; the > symbol signifies very slow exchange. Note that the NAD^+/NADH and $APAD^+$/APADH are in fast exchange with all intermediate states of the enzyme (only the bound states are shown), but $NADP^+$/NADPH are in fast exchange with the enzyme in its E/E^* configuration, and in very slow exchange with the E^{**} state. This is fully consistent with experiments indicating that nucleotide addition during reverse transhydrogenation is random [38–40]. The scheme differs from one presented earlier [5]; we now recognize that the protonation step is not fast in transhydrogenase from all species (see text). Thus, in the *R. rubrum* enzyme, especially in the isolated I:III complex, the conformational change and accompanying protonation step, $E^* \rightleftarrows E^{**}$ are relatively slow.

reduction by NADH, Fig. 1), or strongly inhibited ($AcPdAD^+$ reduction by NADPH, Fig. 2), by Δp. This indicates that the proton translocating steps are not far from equilibrium with Δp. Now observe that the pH dependence of the stimulation of forward transhydrogenation by Δp (Fig. 1) differs from that for the inhibition of reverse transhydrogenation by Δp (Fig. 2). Thus, according to the alternative model [7] we should expect some differential effects of Δp on cyclic transhydrogenation at different pH values. There were none, and we conclude that the cyclic reaction, with its two associated hydride transfer steps, is not accompanied by proton translocation. These observations provide the first experimental evidence that hydride transfer in transhydrogenase is not directly involved in the energy coupling mechanism.

Note that the argument advanced above is not affected by the fact that the value of Δp achieved during illumination is pH-dependent (that relationship will be determined by the dependences on pH of the proton leak and the light-driven electron transport

reactions). The crucial observation is the differential effect of Δp at some values of the suspension pH on forward and reverse transhydrogenation, compared with the complete lack of effect of Δp on the cyclic reaction at any pH.

4.2. pH dependences of the reactions of the complete transhydrogenase from R. rubrum, and a comparison with the enzymes from E. coli and mitochondria

It was suggested that the bell-shaped curves of the pH dependences of the reverse and forward reactions of the complete detergent-dispersed *E. coli* transhydrogenase might reflect titration curves of the proton-binding/proton-release components of the translocation process [5,8]. The pH dependences of forward and reverse transhydrogenation in the complete *R. rubrum* enzyme (both uncoupled, membrane-bound, Figs. 1 and 2, and detergent-solubilized, Fig. 6), are similar to those for the *E. coli* enzyme, and can be explained in the same way (see Fig. 8). At low pH, the reactions are attenuated as proton release accompanying the $E^{**} \rightarrow E^{*}$ conversion is suppressed, thus decreasing the rate of NADPH release during forward transhydrogenation, and of $NADP^+$ release during the reverse reaction. At high pH (during either forward or reverse transhydrogenation), failure to protonate E^{*} blocks formation of the E^{**} state.

In contrast, the pH dependences of the cyclic reaction are quite different in the transhydrogenases of *E. coli* [5] and *R. rubrum* (Figs. 3 and 4). In the former the cyclic reaction is attenuated with an apparent pK_a at about 6.5, which coincides with the apparent pK_a values measured during forward and reverse transhydrogenation, but in the latter the titration is distorted, and the reaction disappears with an apparent pK_a (ca. 8.3), much greater than those observed for the forward and reverse reactions. Presumably the coupling mechanism is essentially the same in the two enzymes, and identical functional groups with similar pK_a values, are responsible for the central proton binding–release reactions. The difference in behavior can be explained if the conformational rearrangement (as distinct from the protonation reaction), which is involved in the $E^{*} \rightleftarrows E^{**}$ interconversion has a different equilibrium constant (this will affect the observed pK_a [29]), and is slower relative to H^- transfer in *R. rubrum* transhydrogenase than in the *E. coli* enzyme. The different K_{eq} would explain why the pK_a associated with the cyclic reaction is higher in *R. rubrum* than in *E. coli* transhydrogenase: since there is no proton binding/release during cyclic transhydrogenation (see above), the rate is only dependent on the ratio of E^{**}/E^{*} at equilibrium. The notion that the conformational rearrangement and associated protonation/deprotonation are relatively slow in *R. rubrum* transhydrogenase and fast in the *E. coli* enzyme would explain why, in the former, the apparent pK_a's for cyclic and for forward/reverse are different (E^{*} and E^{**} do not reach equilibrium during catalysis), but in the latter they are the same (E^{*} and E^{**} do reach equilibrium). Giving support to this idea, Fig. 3 shows that with *R. rubrum* transhydrogenase in the presence of NADPH and NADH, the reduction of $AcPdAD^+$ is completely cyclic, there is no significant release of $NADP^+$. However, with the *E. coli* enzyme in similar conditions, an appreciable proportion of the $AcPdAD^+$ reduction (e.g., ca. 30% at pH 6.5) is accompanied by $NADP^+$ release.

Available information on mitochondrial transhydrogenase is also accommodated by the scheme shown in Fig. 8, but in this enzyme the balance of the rate constants is a little different to that for the bacterial proteins, and for reasons that are not yet clear, the reactions catalyzed by isolated mitochondrial transhydrogenase are more sensitive to specific ion effects, and to inhibition during prolonged incubation with NADPH [9]. A notable feature is that the processes involved in the binding and release of $NADP^+$ from mitochondrial transhydrogenase are faster relative to the hydride transfer reactions, and thus the interconversion of intermediates such as $E^{**}.NADP^+$ and $E^{*}.NADP^+$ is more sensitive to the proton electrochemical potential of the p-phase, than the equivalent process in the *E. coli* and *R. rubrum* enzymes. This has several consequences. (i) The detergent-dispersed mitochondrial enzyme (when not subjected to prolonged incubation with NADPH) catalyses an $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH, but not an NADPH-dependent reaction, i.e., the cyclic reaction is incomplete due to relatively fast $NADP^+$ release [9]. (ii) The rate of reverse transhydrogenation in liposomes inlaid with the mitochondrial enzyme is subjected to very exten-

sive control by Δp (> 50 fold changes in rate upon addition of uncoupler [9,30] compared with about 1.5 fold in darkened *R. rubrum* chromatophores (Bizouarn, unpublished results), and at most 3 fold in proteoliposomes prepared with *E. coli* transhydrogenase ([7] and Stilwell, unpublished results). (iii) With NADH, NADPH and $AcPdAD^+$ as substrates, there is a pronounced shift from the cyclic reaction to simple reverse transhydrogenation when uncoupler is added to the mitochondrial proteoliposomes [30,31]. (iv) With NADH, $NADP^+$ and $AcPdAD^+$ as substrates the cyclic reaction cannot proceed unless Δp (negative inside) is dissipated by ionophore [9]. We make a distinction between the lack of effect of Δp on the rate of the cyclic reaction in *R. rubrum* transhydrogenase (see above and Fig. 4), and the switching effect of Δp in the mitochondrial enzyme [9]. In the case of mitochondrial transhydrogenase, the NADPH-dependent cyclic reaction requires a high proton potential inside the liposome (resulting from inward proton translocation accompanying NADPH $\rightarrow AcPdAD^+$) to prevent release of $NADP^+$ and to maintain the enzyme in the E^{**} state. Equivalently, the $NADP^+$-dependent cyclic reaction requires dissipation of the negative internal proton potential (resulting from outward proton translocation accompanying NADH $\rightarrow NADP^+$) to promote formation of the E^{**} state. The rates of the hydride transfer reactions per se also of mitochondrial transhydrogenase are still predicted to be unaffected by Δp (see above), but this remains to be established.

4.3. pH dependences of the reactions catalyzed by the domain I:III complex of R. rubrum transhydrogenase

The fact that the rates of the cyclic reaction are similar in the complete *R. rubrum* transhydrogenase, and in the domain I:III complex, indicates that the reaction proceeds by the same pathway in both [17]. The observations on the pH dependences, described in this communication, can be explained in two general ways.

Firstly, the similarity between the pH dependences of the cyclic reaction in the complete enzyme (Figs. 4 and 6) and in the I:III complex (Fig. 7) might suggest that the same acid–base group regulates the reaction in both systems. In the context of the model (Fig. 8), this implies that the protonation/deprotonation reactions accompanying the $E^* \rightleftarrows E^{**}$ transition take place in the domain I:III complex. Domain II would be essentially a channel or 'wire' to conduct protons across the membrane to or from the energy transducing reactions located in the peripheral subunits. Note, however, that since $NADP^+$ and NADPH are bound much more tightly in the I:III complex than in the deenergized complete enzyme, it would be a requirement that interactions with domain II change the ground-state (i.e., uncoupled) affinity of domain III for these nucleotides [17]. The fact that the bell-shaped dependence of the rates of reverse transhydrogenation in the complete enzyme is lost in the mixture of the recombinant domains I and III (Fig. 7), the reaction catalyzed by the I:III complex is only weakly dependent on pH, and the fact that within the accessible range the rates of $NADP^+$ and NADPH release from isolated domain III are also pH independent (Table 2), are clearly consistent with one another, because the rates of forward and reverse transhydrogenation by the I:III complex are strongly limited by the rates of release of NADPH and $NADP^+$, respectively [17]. The loss of pH dependence is explained if the retarded NADP(H) release (from isolated domain III relative to that from the complete enzyme) results from a decrease in the rate of a component step in nucleotide release that does not involve deprotonation (e.g., one of the conformational rearrangements at the level of E^{**}).

Alternatively, the substantial loss of pH dependence for simple reverse transhydrogenation in the I:III complex, compared with that in the complete enzyme (Fig. 7 vs. Figs. 2 and 6), might imply that the reaction in the complex is not associated with the input and output of protons. Either proton input and output pathways to domains I:III are available only through specific interactions with domain II, or free energy released during H^+ flux through domain II is conformationally transmitted to domains I/III to drive the transhydrogenation reaction. In either of these cases, the apparent pK_a of the cyclic reaction observed in the I:III complex (Fig. 7) would not be associated with translocated protons, but with scalar protonation. The extremely slow release of $NADP^+$ and NADPH from isolated domain III would not occur by the physiological pathway (which is perhaps blocked in the absence of domain II), but rather it would proceed directly from the E^{**} state without

the enzyme first passing through the proton release step. However, also in this view of coupling the cyclic reaction would still occur only by way of E^{**}.

An important goal will be to discriminate between these two alternatives, and establish whether domain II serves only to direct the transmembrane proton current into or out of the I:III complex, or whether energy coupling takes place through conformational interactions between the membranous and extramembranous regions of the protein.

4.4. The energetics of proton translocation by transhydrogenase

Some aspects of the scheme shown in Fig. 8 which relate to the energy coupling efficiency of transhydrogenase require comment. We have previously proposed that NAD(H) and domain I play only a passive role in transhydrogenation [5,8]. The nucleotide simply acts as a hydride donor or acceptor, its redox potential does not change during catalysis. The K_d of isolated domain I for NADH is about 30 μM, independent of pH, and that for NAD^+ is about 300 μM [32]. We suppose that, throughout catalytic turnover, these values (and those for $AcPdAD^+$/AcPdADH) do not change, in Fig. 8 the domain I nucleotides (NAD^+, NADH, $AcPdAD^+$ and AcPdADH) are shown as being in rapid exchange with the protein in all the reaction intermediates. A surface loop of ca. 20 amino acid residues is thought to close down on the nucleotide in domain I and provide the appropriate environment for hydride transfer, but this conformational change is clearly linked to the binding of nucleotide, rather than proton translocation [32–35]. The available evidence suggests that the energy of Δp is focused at the NADP(H)-binding site. In forward transhydrogenation the high proton potential of the p-phase, generated under physiological conditions by the action of the respiratory or photosynthetic electron-transport chains, leads to increased levels of $NADH.E^{**} > NADP^+$, and equivalently, the low proton potential of the n-phase leads to depletion of $NAD^+.E^{**} >$ NADPH (Fig. 8), thus providing a driving force for the hydride transfer reaction. In addition, the standard redox potential of the $NADP^+$/NADPH couple could be raised within the E^{**} state by the destabilization of bound $NADP^+$ relative to bound NADPH. For example, if the protonation site were close, its interaction with the positive charge of the nicotinamide ring of $NADP^+$ would favor reduction of the nucleotide [1]. This would (a) smooth out the energy profile of the reaction by minimizing differences in the concentrations of reaction intermediates [36], and (b) might provide the necessary chemical pathway between the protonation reactions and the process of NADP(H) binding.

The mechanism summarized in Fig. 8 neatly accounts for a ca. 5 ms burst of forward transhydrogenation that occurs at the onset of energization of *Rhodobacter capsulatus* membrane vesicles [37]. Thus, before energization, the enzyme exists predominantly in the state, $NADH.E^{*}.NADP^+$, and further reaction is limited by the low value of Δp. As Δp is driven to an elevated value, the resulting protonation of the enzyme promotes the hydride transfer reaction to give $NADH.E^{**} > NADP^+$, and hence to the formation of NADPH on the enzyme (as $NAD^+.E^{**} >$ NADPH), but further turnover is then limited by the rate of release of NADPH or H^+.

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