A shift in the equilibrium constant at the catalytic site of proton-translocating transhydrogenase: significance for a 'binding-change' mechanism

Jamie D. VENNING and J. Baz JACKSON¹

School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

In mitochondria and bacteria, transhydrogenase uses the transmembrane proton gradient (Δp) to drive reduction of NADP^+ by NADH. We have investigated the pre-steady-state kinetics of NADP^+ reduction by acetylpyridine adenine dinucleotide (AcPdADH, an analogue of NADH) in complexes formed from the two, separately prepared, recombinant, peripheral subunits of the enzyme: the dI component, which binds NAD^+ and NADH, and the dIII component, which binds NADP^+ and NADPH. In the stopped-flow spectrophotometer the reaction proceeds as a single-turnover burst of hydride transfer to NADP^+ on dIII before product NADPH release becomes limiting in steady state. The burst is biphasic. The results indicate that the fast phase represents direct hydride transfer from AcPdADH to

NADP^+ in dI:dIII complexes, and that the slow phase, which predominates when $[\text{dI}] < [\text{dIII}]$, corresponds to dissociation of the protein complexes during multiple turnovers of dI. Measurements on the amplitude of the burst, and on the apparent first-order rate constant of the fast phase, indicate that the equilibrium constant of the hydride-transfer step on the enzyme is shifted relative to that in solution. This has consequences for a model proposed earlier, in which Δp is used, not at the hydride-transfer step, but to change the binding affinities of NADP^+ and NADPH.

Key words: membrane protein, nicotinamide nucleotide, proton pump, *Rhodospirillum rubrum*, stopped-flow kinetics.

INTRODUCTION

Transhydrogenase is found in the cytoplasmic membranes of bacteria, and in the inner membranes of mitochondria from animal cells. It couples the transfer of reducing (hydride ion) equivalents between $\text{NAD}(\text{H})$ and $\text{NADP}(\text{H})$ to the translocation of protons across the membrane.

Under most physiological conditions the reaction is driven from left to right by the proton electrochemical gradient (Δp) that results from the activity of respiratory (or photosynthetic) electron-transport systems. The NADPH generated by transhydrogenase is used for the reduction of glutathione and in biosynthesis and, in mitochondria, the enzyme might be involved in the regulation of the tricarboxylic acid cycle [1,2].

Transhydrogenase has a tripartite structure, reviewed in [3–5]. The dI component (which contains the NAD^+/NADH -binding site) and the dIII component (which contains the $\text{NADP}^+/\text{NADPH}$ -binding site) protrude from the membrane, on the cytoplasmic side in bacteria, and on the matrix side in mitochondria. The dI component probably comprises two Rossmann folds, one of which binds the NAD^+/NADH (P. J. Baker, P. A. Buckley, D. W. Rice, P. G. Quirk and J. B. Jackson, unpublished work), the other having an unknown function. The dIII component, a single Rossmann fold, binds the $\text{NADP}^+/\text{NADPH}$ [7,8]. The dII component spans the membrane. There is relatively little known about the structure of dII; it might comprise 10–14 transmembrane helices [9,10].

Using recombinant-DNA procedures, isolated protein fragments, equivalent to dI and dIII, have been prepared from the transhydrogenases of several species [11–17]. The dI proteins

from *Rhodospirillum rubrum* and *Escherichia coli* bind NADH fairly tightly ($K_d \approx 30 \mu\text{M}$), and NAD^+ more weakly ($K_d \approx 300 \mu\text{M}$), one nucleotide per monomeric unit [11,12,18,19]. Even after chromatographic purification, the dIII proteins from these bacterial species [14,16], the equivalent protein from *Homo sapiens* [17], and probably that from *Bos taurus* [14,15], are associated with tightly bound NADP^+ and NADPH. It has been demonstrated that, at least for *R. rubrum* dIII, the bound nucleotides (one per monomeric unit) reside in the active site of the enzyme [14]. Dissociation constants are unavailable, but for both of the bacterial dIII proteins the rates of nucleotide release are extremely slow: for NADP^+ , $k_{\text{off}} \approx 0.03 \text{ s}^{-1}$, and for NADPH, $k_{\text{off}} \approx 6 \times 10^{-4} \text{ s}^{-1}$ [14,16]; they are 'occluded' from the solvent.

Simple mixtures of separately prepared recombinant dI and dIII, including hybrids from different species, give rise spontaneously to protein complexes (dI:dIII complexes), which catalyse transhydrogenation in either the 'forward' or 'reverse' directions (eqn. 1), depending on the nucleotides supplied [13–17,20]. However, the steady-state rates of reaction are about 1000-fold slower than the equivalent reactions in the complete membrane-bound enzyme [14]. Significantly, the k_{cat} values for forward and reverse transhydrogenation catalysed by dI:dIII complexes are very similar to the k_{off} values for the release from dIII of NADPH and NADP^+ , respectively (see above). This suggests that the steady-state rates of transhydrogenation by dI:dIII complexes are limited by the slow release of product, NADP^+ or NADPH [14–17,20]. These observations, and others, gave rise to the suggestion that, in the complete enzyme, interactions with the membrane-spanning dII are crucially important in promoting $\text{NADP}(\text{H})$ release from dIII [14,21].

Mixtures of the dI and dIII proteins also catalyse 'cyclic transhydrogenation' [14–17,20], the reduction of AcPdAD⁺ (the oxidized form of acetylpyridine adenine dinucleotide, an NAD^+

Abbreviations used: AcPdAD⁺, AcPdADH, oxidized and reduced forms of acetylpyridine adenine dinucleotide, respectively.

¹ To whom correspondence should be addressed (e-mail j.b.jackson@bham.ac.uk).

analogue) by NADH in the presence of NADP⁺ or NADPH. In this case, steady-state rates of reaction are very high, comparable with those measured for the complete enzyme [14]. This is explained readily, because, during the course of the cyclic reaction, NADP⁺ and NADPH can remain bound to dIII (e.g. [22]). The measurement also indicates that the hydride-transfer steps during the cyclic reaction on the dI:dIII complex (NADPH → AcPdAD⁺ and NADH → NADP⁺), the binding of AcPdAD⁺ and NADH, and the release of AcPdADH (the reduced form of acetylpyridine adenine dinucleotide) and NAD⁺ are all rapid [14].

That hydride transfer is fast in dI:dIII complexes was substantiated by studies on a reverse transhydrogenation reaction, the reduction of AcPdAD⁺ by NADPH, using stopped-flow spectrophotometry [23,24]. Rapid hydride transfer ($k \approx 550 \text{ s}^{-1}$) during the first turnover was detected as a 'burst' before release of NADP⁺ became rate-limiting in the steady state. Two important conclusions arose from these experiments. First, the apparatus for hydride transfer resides entirely within dI and dIII; the membrane-spanning component, dII, does not participate in the process. Secondly, hydride transfer from NADPH to AcPdAD⁺ is probably direct; there seem to be no intermediate redox acceptors.

There are a number of indications that, in the complete enzyme, the energy released in the translocation of protons is used, not at the level of the hydride-transfer step, but to change the binding affinity of NADP⁺ and NADPH [14,21,22], hence a 'binding-change' mechanism, in some ways analogous to that proposed for H⁺-ATPase (see [25]). In the dI:dIII complex, hydride transfer probably takes place by the same mechanism as that in the complete enzyme but, in the absence of interactions with dII (and, of course, Δp), dIII is locked into a conformation (the 'occluded state') in which bound NADP⁺ and NADPH cannot rapidly exchange with the solvent.

In this study we describe the stopped-flow kinetics of AcPdADH oxidation by NADP⁺ (forward transhydrogenation), catalysed by mixtures of dI and dIII. We show that the equilibrium constant of the hydride-transfer step in the dI:dIII complex is shifted considerably from that in free solution, and this provides a new perspective on our binding-change mechanism.

EXPERIMENTAL

The dI and dIII proteins of *R. rubrum* transhydrogenase were expressed in *E. coli* from the constructs pCD1 and pCD2, and purified by column chromatography, as described previously [11,14]. The purities of the dI and dIII preparations were checked routinely by SDS/PAGE [26], and protein concentrations were determined by the microtannin procedure [27].

As prepared, the dI preparation is normally free of nucleotide, but the dIII protein is associated with stoichiometric quantities of tightly bound NAD(P)H, mainly NADP⁺ [14]. For stopped-flow experiments, dIII protein was treated to ensure full occupation of the nucleotide-binding site with either NADPH, as described [23,24], or with NADP⁺. In the latter case, typically 15 mg of purified dIII and 0.6 mg of purified dI were incubated in 75 ml of 20 mM Tris/HCl (pH 8.0)/200 μM AcPdAD⁺ at 4 °C for 1 h. The AcPdAD(H) and dI protein were then removed from the dIII by chromatography on Q-Sepharose HP using a 200–400 mM gradient of NaCl in 20 mM Tris/HCl (pH 8.0).

Stopped-flow spectrophotometry was performed on an Applied Photophysics DX-17MV operating in its absorbance mode with an optical pathlength of 2 mm. The slit widths of the monochromator were set at 5 nm. Absorbance changes were

Table 1 The extinction coefficients for the reduction of AcPdAD⁺ by NAD(P)H at 375 nm

dI protein (50 nM), dIII protein in its NADPH form (100 nM) and 2 mM AcPdAD⁺ were pre-incubated in 10 mM (NH₄)₂SO₄/20 mM Hepes (pH 8.0) at 30 °C in a 1-cm pathlength cuvette in a Perkin-Elmer Lambda 16 double-beam spectrophotometer. NADH, from a stock solution of 1.70 mM (checked independently from the absorbance at 340 nm, using an extinction coefficient of $6.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$), was added to give the concentrations shown, and the ensuing absorbance change at 375 nm was measured over a period of ≈ 10 min. The reaction was about 95% complete in ≈ 3 min. Separate experiments were performed in the absence of AcPdAD⁺ to determine the contribution to the initial absorbance change, at 375 nm, of the added NADH solution. The extinction coefficient was calculated from the absorbance change due to simultaneous reduction of AcPdAD⁺ and oxidation of NADH, assuming that the reaction went to completion. The results of four separate experiments are presented.

Concentration of NADH (μM)	17.0	17.0	17.0	34.0
Extinction coefficient ($\text{mM}^{-1} \cdot \text{cm}^{-1}$)	6.021	6.080	6.051	6.130

measured either at 320 or 375 nm. The data were analysed with the Applied Photophysics software, which uses the Marquardt–Levenberg algorithm of non-linear regression. Curves were fitted to the sum of two exponentials; residuals for representative data sets are shown in Figure 3 (see below). Routinely, 6–8 experiments were performed in succession. The curves were either averaged before curve fitting (as shown in Figures 1–5, see below), or were analysed and plotted individually (see the upper panels of Figures 6 and 7, below); the latter provide a good indication of reproducibility. The mixing dead time of the instrument, measured as described previously [23,28], was 1.31 ms. In calculating the amplitude of absorbance changes, due correction for the signal undetected in the dead time was made using the instrument software.

There are different values in the literature [29–33] for the extinction coefficient at 375 nm for the reduction of the NAD⁺ analogue, AcPdAD⁺, by NAD(P)H. They range from 5.1 to $6.38 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, and the two extreme values, differing by 25%, still appear to be in current use. Because the value is especially critical to the analysis described in this study, an independent determination of the extinction coefficient was carried out. We made use of the fact that a mixture of transhydrogenase dI and dIII catalyses an extremely rapid 'cyclic reaction' [the reduction of AcPdAD⁺ by NADH in the presence of NAD(P)H]. The reaction should lead to almost complete reduction of AcPdAD⁺. Table 1 shows that the extinction coefficient measured in this way yielded a mean value of $6.07 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, which is similar to the value of $6.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ that we calculated earlier, based on different experimental data [33].

RESULTS

The transient-state kinetics of hydride transfer catalysed by a mixture of recombinant dI and dIII proteins of *R. rubrum* transhydrogenase

In the experiments shown in Figure 1, the first drive syringe of the stopped-flow spectrophotometer was filled with a solution containing nucleotide-free dI plus dIII with its binding sites fully loaded with NADP⁺. The second drive syringe was filled with an AcPdADH solution. Upon mixing the contents of the two syringes, a burst of hydride transfer was observed in the direction of forward transhydrogenation. The kinetics of AcPdADH oxidation (an absorbance decrease at 375 nm) were better resolved than those of NADP⁺ reduction (an absorbance increase at 320 nm) due to the greater background absorbance in the latter but, within the limits of error, the reactions took place at

Figure 1 The stopped-flow kinetics of AcPdADH oxidation and NADP⁺ reduction catalysed by a mixture of recombinant dI and dIII of *R. rubrum* transhydrogenase

The first drive syringe contained 50 μM dI protein and 50 μM dIII protein loaded with NADP⁺ (see the Experimental section) in a buffer containing 10 mM $(\text{NH}_4)_2\text{SO}_4$ /20 mM Hepes/KOH (pH 8.0). The second drive syringe contained 200 μM AcPdADH in the same buffer. The two solutions were mixed in a 1:1 ratio. The upper panel shows the reduction of NADP⁺ measured at 320 nm; the lower panel shows the oxidation of AcPdADH measured at 375 nm. Both represent an average of six separate recordings. The mean values ($\pm$ S.D.) of the phase A and B amplitudes, from the set of recordings in the lower panel were $4.0(\pm 1.2) \times 10^{-3}$ A and $2.6(\pm 1.7) \times 10^{-3}$ A, respectively. The corresponding rate constants were 105 ± 14 and $23 \pm 11 \text{ s}^{-1}$, respectively. The errors are greater than those for reverse transhydrogenation [23,24] because of the higher background absorbance of AcPdADH relative to AcPdAD⁺. Temperature = 21 °C.

similar rates. This adds strong support to the suggestion, arising from the equivalent observation on the complementary reverse transhydrogenation (AcPdAD⁺ reduction by NADPH [23,24]), that hydride transfer between nucleotides is direct, and does not involve intermediate redox reactions. In the earlier work, the possibility that an optically invisible intermediate acceptor is reduced relatively slowly by the donor nucleotide, and then very rapidly oxidized by the acceptor nucleotide could not be eliminated entirely. However, the fact that in both directions the hydride equivalent is seen to leave one nucleotide at the same rate that it arrives at the other now makes this possibility extremely unlikely.

The burst kinetics in Figure 1 arise as a consequence of rapid hydride transfer from AcPdADH to NADP⁺, and from the fact that product NADPH is released only very slowly from the

Figure 2 The effect of AcPdADH concentration on the stopped-flow kinetics of AcPdADH oxidation by NADP⁺ catalysed by a mixture of recombinant dI and dIII of *R. rubrum* transhydrogenase

Experiments were performed at 375 nm as in Figure 1, but with the final AcPdADH concentration shown. Upper panel, the amplitude of the burst; lower panel, the apparent first-order rate constant. Data points represent an average of, typically, six separate recordings.

dI:dIII complex ($k \approx 6 \times 10^{-4} \text{ s}^{-1}$ [14]); the burst corresponds to a single turnover of NADP⁺ reduction on dIII.

Like the burst of hydride transfer from NADPH to AcPdAD⁺ [23,24], that from AcPdADH to NADP⁺ was bi-exponential (Figure 1); the faster of the two components is defined as phase A, and the slower as phase B. Figure 2 (lower panel) shows that the apparent first-order rate constants of the two phases were independent of AcPdADH concentration between 40 and 250 μM . This indicates that, in this range, neither phase is kinetically limited by the rate of AcPdADH binding. Below 40 μM the apparent first-order rate constant of phase A decreased substantially, and it became difficult to resolve the two phases (results not shown). The amplitudes of phases A and B were constant between 60 and 250 μM AcPdADH (Figure 2, upper panel). Below 60 μM there was a decrease in the amplitude of phase B of the burst. Events that occur at low concentrations of AcPdADH are probably influenced by the fact that, during the burst, the reaction is approaching equilibrium (see below). To date, we have been unable to obtain active, stable, NADP(H)-free dIII; we can only exchange the nucleotides. It has, therefore, not yet been possible to examine the dependence of the components of the pre-steady-state transhydrogenation reactions on the concentrations of either NADP⁺ or NADPH.

Bi-exponential behaviour in pre-steady-state kinetics can arise for a number of reasons. From the results of experiments in which the burst of AcPdAD⁺ reduction by NADPH was re-

Figure 3 The kinetics of AcPdADH oxidation by NADP^+ at different concentrations of dI protein

Experiments were performed at 375 nm as in Figure 1, except that the final concentration of dI was 10 (left), 17.5 (centre) and 100 μM (right). The upper panels show the experimental traces (each an average of six sequential recordings), and the bi-exponential fits (offset by +0.005 A). Residuals for mono-exponential fits are shown in the middle panels, for the bi-exponential fits in the lower panels. Note that, when phases A and B are equally dominant (centre panels), only the bi-exponential fit was adequate, but at low dI (left), where phase B dominates, and at high dI (right), where phase A dominates, the mono-exponential fits were equally satisfactory.

corded, and in which the dI concentration was varied at fixed dIII, it was argued that phase A corresponds to intrinsic hydride transfer in dI:dIII complexes, whereas phase B reflects the multiple turnovers of dI that are required for a single turnover of dIII [24]. If this hypothesis is correct, we should be able to identify a similar pattern of behaviour in the results of equivalent experiments on the oxidation of AcPdADH by NADP^+ . That this is the case is shown by the data in Figures 3 and 4 (upper panel). At low concentrations of dI, phase B predominated. As the dI concentration was increased, while the total amplitude of the burst did not change, phase B was replaced by phase A. Thus when the dIII is effectively saturated with dI, the major component, phase A, must correspond to intra-complex hydride transfer. However, when dI is less than saturating, the turnover of unreacted dIII (i.e. protein still occupied by bound NADP^+) requires multiple turnover of dI protein. This involves dissociation of the reacted complexes, exchange of AcPdAD $^+$ for AcPdADH on dI, and re-association of the proteins (not necessarily in that order) to give dI·AcPdADH:dIII· NADP^+ , from which subsequent hydride transfer can take place. The question arises as to which of these steps is primarily responsible for phase B. Now, Figure 4 shows that similar results were obtained when the experiments were performed with a different protocol; dI plus AcPdADH from one drive syringe were mixed with NADP^+ -loaded dIII from the other. The similarity between the two sets of data shows that equilibrium between complexes and isolated proteins ($\text{dI} + \text{dIII} \leftrightarrow \text{dI}:\text{dIII}$) is reached within the dead time of the apparatus (≈ 1.31 ms), and this establishes clearly that complexes are formed very rapidly. The rate of AcPdADH binding (see above) and AcPdAD $^+$ release (see below and [34]) are also believed to be relatively fast, and we conclude, therefore, that phase B corresponds mainly to the dissociation of dI:dIII complexes. In support of this, it may be observed that the value of k^{app} for phase B is similar in measurements of AcPdAD $^+$

reduction by NADPH [24], and in measurements of AcPdADH oxidation by NADP^+ (Figure 4, lower panel); it is independent of the redox states of the nucleotides. The increase in the value of k^{app} for phase B (from 3 to 25 s^{-1}) with the dI concentration (Figure 4, lower panel) can be explained by the more probable formation of 'dead-end' complexes (in this case, dI·AcPdAD $^+$:dIII· NADP^+) at low dI concentrations. These complexes tie up the dI protein, and are catalytically unproductive; they must dissociate before dI can interact productively with dIII· NADP^+ . The true rate constant for dI:dIII dissociation is therefore approached only at high concentrations of dI. In contrast, the value of the apparent first-order rate constant for phase A was independent of the dI concentration (Figure 4, lower panel), consistent with its correspondence to the hydride-transfer step in dI:dIII complexes.

The working scheme also leads to predictions about the kinetic character of the hydride-transfer reaction when, at fixed dI, the concentration of dIII is varied. This is illustrated in Figure 5 for the reduction of AcPdAD $^+$ by NADPH. When the dIII concentration was increased progressively over that of dI, the amplitude of phase A remained constant, but the amplitude of phase B increased in an almost linear fashion (Figure 5, upper panel). These observations are consistent with the conclusions (i) that phase A corresponds to intra-complex hydride transfer, and (ii) that dI protein can subsequently exchange its product, AcPdADH, for substrate, AcPdAD $^+$, and repeatedly dissociate from, and reassociate with, dIII to catalyse further reaction as part of phase B. The total amplitude of the burst (phase A plus phase B) is proportional to the amount of dIII; it is this protein that undergoes the single turnover. The lower panel of Figure 5 shows that, again, k^{app} for phase A was not affected by the relative concentration of protein (see Figure 4, and see above), but that k^{app} for the slow phase decreased at elevated concentrations of dIII: as the dI/dIII ratio decreases, the probability of

Figure 4 The effect of the dl concentration, at fixed dlII, on the stopped-flow kinetics of AcPdADH oxidation by NADP⁺

Data points represented by ●, for phase A, and ■, for phase B, were obtained from experiments performed at 375 nm as in Figures 1 and 3, except that the final concentration of dl was varied as shown. Data points represented by ○, for phase A, and □, for phase B, were obtained in similar experiments, but with the first drive syringe containing 50 μM dl protein plus 200 μM AcPdADH, and the second containing 50 μM dlII protein loaded with NADP⁺. Upper panel, the amplitude of the burst; lower panel, the apparent first-order rate constant. Note that the k_{app} for phase B becomes difficult to measure at increasing concentrations of dl, since the amplitude of the phase becomes very small. All data points represent an average of, typically, six separate recordings.

the formation of unproductive dead-end complexes increases (see above).

The extent of the hydride-transfer reaction between AcPdADH and NADP⁺

The equilibrium constant for the reduction of NADP⁺ by AcPdADH in aqueous solution, calculated from the standard redox potentials [35], is 3.8×10^{-3} . Starting from the nucleotide concentrations of the experiment shown in Figure 1, solution equilibrium would have been reached when only 2.86 μM NADP⁺ and AcPdADH had reacted. However, the measured extent of AcPdADH oxidation shows that the reaction progressed substantially beyond this point; $\approx 12.3 \mu\text{M}$ nucleotides had reacted by the end of the burst. Thus the value of the mass-action ratio, $\Gamma = [\text{AcPdAD}^+][\text{NADPH}]/[\text{AcPdADH}][\text{NADP}^+]$ at the end of

the burst (0.135), was much greater than the solution equilibrium constant. This can be explained on the basis (i) that (some of) the reacting nucleotides remain predominantly enzyme-bound during the entire period of the burst, and (ii) that the equilibrium constant on the enzyme is larger than that for the reaction in free solution. Since NADP⁺ and NADPH remain very tightly bound to dlII [14], and AcPdAD⁺ and AcPdADH can exchange quite rapidly with dl (at least in its isolated form [34]), the measured value of Γ at the end of the burst reflects an equilibrium between the bound species of NADP(H) and the bound and free species of AcPdAD(H). For the equivalent reverse-transhydrogenation reaction [24], the measured mass-action ratio, $\Gamma = [\text{AcPdADH}][\text{NADP}^+]/[\text{AcPdAD}^+][\text{NADPH}]$ at the end of the burst was typically about 0.015. Under the experimental conditions in which they were measured (notably at high [AcPdAD⁺] during reverse transhydrogenation, at high [AcPdADH] during

Figure 5 The effect of varying the dIII concentration, at fixed dI, on the stopped-flow kinetics of AcPdAD⁺ reduction by NADPH

The first drive syringe contained 50 μM dI protein and a variable concentration of dIII protein loaded with NADPH (see the Experimental section) in a buffer containing 10 mM $(\text{NH}_4)_2\text{SO}_4$ and 20 mM Hepes/KOH (pH 8.0). The second drive syringe contained 2 mM AcPdAD⁺ in the same buffer. The two solutions were mixed in a 1:1 ratio with the final dIII concentration as shown. ●, Phase A, and ■, phase B. Upper panel, the amplitude of the burst; lower panel, the apparent first-order rate constant. All data points represent an average of, typically, six separate recordings.

the forward reaction, and at high dI concentrations for both), there is considerable experimental error on the two Γ values but, even so, it is clear that they are not reciprocally related, and this is because the distribution of the AcPdAD(H)-binding species during the burst is complex and cannot be defined.

The experiments shown in Figure 6 (forward reaction) and Figure 7 (reverse reaction) were performed at low concentrations of dI protein, for two reasons. (i) To ensure domination of the burst by phase B (see Figure 4); this gives a more accurate determination of the burst amplitude, since less signal is lost in the instrument dead time, and the necessary correction is reduced (see the Experimental section). (ii) To keep the concentrations of bound AcPdAD⁺ and AcPdADH low relative to the concentration of free nucleotides. The reactions during the burst periods are, therefore, approximated by the equation:

The dI molecules can repeatedly visit dIII during the burst, which is thus predominantly composed of phase B (e.g. see Figure 4). The upper panels of Figures 6 and 7 show that, under these conditions, the amplitudes of the single-turnover bursts of forward and reverse transhydrogenation were depressed by the

Figure 6 The effect of AcPdAD⁺ on AcPdADH oxidation by NADP⁺ during the stopped-flow burst of hydride transfer

The first drive syringe contained 4 μM dI protein and 50 μM dIII protein loaded with NADP⁺ (see the Experimental section) in a buffer containing 10 mM $(\text{NH}_4)_2\text{SO}_4$ and 20 mM Hepes/KOH (pH 8.0). The second drive syringe contained, in the same buffer, 100 μM AcPdADH plus AcPdAD⁺ to give the final concentration shown, after mixing in a 1:1 ratio. Upper panel, the amplitude of the burst (data points are shown for individual separate recordings); lower panel, the mass-action ratio at the end of the burst (≈ 2 s after mixing, average of eight data points).

prior addition of product AcPdAD⁺ and AcPdADH, respectively, indicating that the reactions were operating not far from equilibrium. In the lower panels of Figures 6 and 7, we have calculated Γ values at the end of the bursts from these data. The value of Γ for the forward reaction converged towards the reciprocal of Γ for the reverse reaction when the concentrations of product AcPdAD(H) were increased. We assume, therefore, from Figures 6 and 7, that the equilibrium constant of the reaction given by eqn. (2) is in the region of convergence, i.e. in the range 0.25–0.6.

Since the affinity of dI protein for AcPdADH is ≈ 10 times that for AcPdAD⁺ [18,34], then the equilibrium constant for hydride transfer between AcPdAD(H) and NADP(H) on the enzyme (given by the product of the equilibrium constant of the reaction in eqn. 2, and the ratio of the binding constants for AcPdADH and AcPdAD⁺) should be in the range 0.025–0.06.

Interpretation of the rate constants of phase A

Only the following reaction components need be considered for phase A (see above):

Figure 7 The effect of AcPdADH on AcPdAD⁺ reduction by NADPH during the stopped-flow burst of hydride transfer

The first drive syringe contained 4 μM dI protein and 50 μM dIII protein loaded with NADPH (see the Experimental section) in a buffer containing 10 mM (NH₄)₂SO₄ and 20 mM Hepes/KOH (pH 8.0). The second drive syringe contained, in the same buffer, 100 μM AcPdAD⁺ plus AcPdADH to give the final concentration shown, after mixing in a 1:1 ratio. Upper panel, the amplitude of the burst (data points are shown for individual separate recordings); lower panel, the mass-action ratio at the end of the burst (≈ 2 s after mixing, average of eight data points).

The concentrations of AcPdADH (in the forward reaction) and AcPdAD⁺ (in the reverse reaction) can be made high enough experimentally to ensure that the nucleotides remain at constant concentrations throughout the burst (see Figure 2 above, and [24]). However, the system is still kinetically complex. Two different limiting situations can be envisaged for phase A, depending on the rates of product AcPdAD⁺ or AcPdADH release.

(i) If products AcPdAD⁺ (forward) and AcPdADH (reverse) were slow to dissociate from dI·AcPdAD⁺:dIII·NADPH and

Table 2 The dependence of k^{app} for phase A on the concentration of AcPdAD⁺ during the oxidation of AcPdADH by NADP⁺

Experiments were performed as described in Figure 6, except that the dI concentration was 100 μM; under these conditions, essentially only phase A of the burst was observed.

Concentration of AcPdAD ⁺ after mixing (μM)	0	10	50
Apparent first-order rate constant (s ⁻¹)	69	84	178
Amplitude of the burst (ΔA)	7.3 × 10 ⁻³	6.5 × 10 ⁻³	4.2 × 10 ⁻³

dI·AcPdADH:dIII·NADP⁺, respectively, relative to the rate of the hydride-transfer reactions, then phase A would be governed by reversibility at the hydride-transfer step and, in the limit, k^{app} in both directions would be equal to the sum of k_f and k_r (e.g. see [36]). This cannot be the case since the k^{app} for the forward transhydrogenation is much greater than that for the reverse reaction.

(ii) If, as we expect from NMR data on isolated dI [34], the binding and release of AcPdAD⁺ and AcPdADH were fast relative to hydride transfer, then the exchange of these nucleotides between dI:dIII complexes and solution would remain close to equilibrium throughout the reaction. Since the binding affinities are rather weak (K_d for AcPdAD⁺ ≈ 1 mM, and K_d for AcPdADH ≈ 120 μM [18,34]), the concentrations of the intermediates dI·AcPdAD⁺:dIII·NADPH for the forward reaction, and dI·AcPdADH:dIII·NADP⁺ for reverse, would remain low throughout the burst. Under these conditions, the hydride-transfer steps would be essentially irreversible and k^{app} for the forward reaction (92 ± 17 s⁻¹ from 11 separate sets of experiments) would give k_f , and k^{app} for the reverse reaction (560 ± 62 s⁻¹ from 17 separate sets of experiments [24]) would give k_r . An illustration of the effect of the concentration of dI·AcPdAD⁺:dIII·NADPH on k^{app} for the forward reaction is presented in Table 2. The measurements were performed with an excess of dI over dIII to ensure predominance of phase A. The nucleotide substrate concentrations were held constant but the concentration of product AcPdAD⁺ was increased progressively. In agreement with the earlier experiments (carried out under somewhat different conditions, Figure 6), the amplitude of the pre-steady-state burst decreased as the initial mass-action ratio was set closer to the equilibrium position but, crucially, the k^{app} of phase A increased from 69 to 178 s⁻¹, indicating a greater contribution from k_f when the concentration of dI·AcPdAD⁺:dIII·NADPH was maintained at a higher level. Within the constraints of this set of assumptions, the equilibrium constant for hydride transfer on the enzyme ($K_{\text{eq}} = k_f/k_r$) would be ≈ 0.17. This is almost 3-fold higher than the upper limit of the range of values calculated from the mass-action ratios (see above), and might indicate that there is, in fact, some contribution to k^{app} from the respective back reactions.

DISCUSSION

The facts that there is a similar pattern of behaviour in the pre-steady-state kinetics of AcPdAD⁺ reduction by NADPH [24], and of AcPdADH oxidation by NADP⁺ (this study), as catalysed by mixtures of recombinant dI and dIII proteins of transhydrogenase, provide strong support for a simple model; phase A arises from rapid hydride transfer in dI:dIII complexes, and phase B, evident at low concentrations of dI, results from dissociation of dI:dIII complexes prior to multiple turnover of dI as it visits unreacted dIII·NADP⁺. Redox intermediates are

Scheme 1 The coupling of transhydrogenation to proton translocation

Based on [3], and see [22] and text. Forward transhydrogenation is depicted as if proceeding from top left to lower right. In the E state (no bound NADP^+ or NADPH) the protein cannot interact with Δp at its catalytic centre, either because the pK_a is very low, or because of physical barriers to proton conduction. In the E^* state (with bound NADP^+ or NADPH) the catalytic centre of the protein is accessible to protons in the solvent (the n -phase in $E^* \cdot \text{NADPH}$, and the p -phase in $E^* \cdot \text{NADP}^+$). In the complete enzyme, $E^{**} \cdot \text{NADP}^+$ is generated from $E^* \cdot \text{NADP}^+$ as a result of protonation and associated conformational rearrangements. Equivalently, $E^* \cdot \text{NADPH}$ is generated from $E^{**} \cdot \text{NADPH}$ through deprotonation and associated conformational rearrangements. The dI:dIII complex, however, is locked in the E^{**} state (within the dashed box). The $\cdot$ symbol signifies that a nucleotide ligand is in rapid exchange with the enzyme; the $>$ (tight-binding) and $>>$ (very-tight-binding) symbols signify slow exchange. Thus although the reaction is shown with an ordered addition of substrates (and release of products), NADH and NAD^+ can exchange rapidly with the dI-binding site in all intermediate states; NADP^+ and NADPH can only exchange rapidly with the dIII-binding site in the E and E^* states of the enzyme.

not detected during either the forward or reverse reactions; hydride transfer proceeds directly from one nucleotide to the other.

A comparison of the forward and reverse transhydrogenation reactions shows that the equilibrium position of the hydride-transfer step on the dI:dIII complex is perturbed relative to that for nucleotides in free solution. The results show that, for hydride transfer between NADP(H) and AcPdAD(H) , the equilibrium constant on the enzyme is in the range 0.025–0.06 compared with a solution value of 3.8×10^{-3} . Since the standard redox potential of $\text{AcPdAD}^+/\text{AcPdADH}$ in solution is ≈ 72 mV more positive than that of NAD^+/NADH , we can calculate that, for hydride transfer between the physiological nucleotides on dI:dIII complexes,

the equilibrium constant is in the range 6–16, a significant shift from the value of 1.0 in aqueous solution. This shows that the hydride-transfer step in transhydrogenase is biased in the forward direction of the enzyme by the relative binding affinities of the substrate nucleotides. The binding affinity of dI for NADH is ≈ 10 -fold greater than that for NAD^+ [11,18,19]. This, in itself, would lead to a decrease in the equilibrium constant, and it must be offset by favoured binding of NADPH relative to NADP^+ on dIII by a factor in the range 60–160 (equivalent to a standard redox potential, E^0 , of between -0.268 and -0.254 V, compared with a solution value of -0.320 V). Incidentally, it may be noted that the degree of stabilization of NADPH relative to NADP^+ during hydride transfer corresponds approximately to the ratio of the dissociation rate constants for the two nucleotides ($\approx$

1/54) from isolated *R. rubrum* dIII [14] (and a similar figure was later determined [16] for the equivalent *E. coli* protein), indicating that the association rate constants are similar for the two nucleotides. Actual values are not known, but since extensive dialysis does not readily remove NADP(H) from dIII protein [14], they are very large, probably close to the diffusion limit.

These conclusions have interesting implications for the mechanism of action of transhydrogenase, and they fit comfortably within the framework of a minimal model, proposed in [4,22] (Scheme 1). The model rests on evidence that Δp interacts with the enzyme at the level of $\text{NADP}^+/\text{NADPH}$ binding and release, and not directly, either at the hydride-transfer step, or during NAD^+/NADH binding and release. Thus the complete transhydrogenase is shifted between an 'open state' (E and E^* , from which NADP^+ and NADPH can exchange rapidly with the solvent) and an 'occluded state' (E^{**} , from which NADP^+ and NADPH cannot exchange with the solvent) by way of conformational changes that are coupled to proton translocation through dII. Hydride transfer takes place only in the E^{**} state; the new result shows that hydride transfer within this state is favoured in the direction of NADP^+ reduction. That is, the enzyme maintains a positive driving force on the hydride-transfer step, even though that step is not coupled to Δp . In the absence of dII (i.e. in isolated dIII and in dI:dIII complexes), dIII is trapped in the E^{**} state.

It is revealing to consider the changes in nucleotide redox potential during the course of catalysis by the complete transhydrogenase. Note first that, because of the favoured binding of NADH relative to NAD^+ by a factor of 10, the standard potential of this nucleotide will be 0.030 V greater in its binding site on dI ($E^0 = -0.290$ V) than in free solution ($E^0 = -0.320$ V [35]). However, relative to the turnover rate, the bound and the free NAD^+ and NADH can exchange rapidly from all the catalytic intermediates and, therefore, the operating redox potential (E_n) of this nucleotide will be similar in the solvent, and on the enzyme, and will remain so throughout turnover. Thus even under physiological conditions (where the concentration of NADH is relatively low, and that of NAD^+ is relatively high), dI favours the binding of NADH , and the release of NAD^+ , both of which are advantageous for forward catalysis, but the bound NADH is consequently a rather poor reducing agent. For the open state of the complete enzyme (E and E^*), complementary arguments apply to the binding of NADP^+ and NADPH to dIII. That is, even when the ambient NADP^+ concentration is lower than the NADPH concentration, the binding of the former nucleotide is favoured relative to that of the latter (promotes forward catalysis), but the bound NADP^+ is consequently a rather poor oxidizing agent (E^0 is less than -0.320 V). Under these conditions then, hydride transfer from NADH to NADP^+ would be unfavourable; to prevent slip in the mechanism at some nucleotide concentrations, hydride transfer is assumed to be inhibited in this state, probably by keeping apart the C_4 atoms of the two nicotinamide rings. The energy of Δp (probably through H^+ binding from the p -phase [22]) now drives the enzyme into its occluded conformation ($E^* \cdot \text{NADP}^+$ to $E^{**} \cdot \text{NADP}^+$) in which the E^0 of the $\text{NADP}^+/\text{NADPH}$ in the dIII-binding pocket is increased to between -0.267 and -0.254 V (this study), and in which hydride transfer is allowed, probably by bringing the two nicotinamide rings into apposition. The bound NADP^+ on dIII is now a better oxidizing agent; it can abstract H^- even from the weakly reducing NADH on dI (K_{eq} between 6 and 16, see above). Another input of energy from Δp (probably H^+ release to the n -phase [22]) is used to drive the enzyme conformation from $E^{**} \cdot \text{NADPH}$ to $E^* \cdot \text{NADPH}$, from which the reduced nucleotide is readily released.

In summary, transhydrogenase achieves a thermodynamically uphill reduction of NADP^+ by NADH using a rather simple device. In the open state, the enzyme binds the substrates, NADH and NADP^+ , favourably relative to the products, NAD^+ and NADPH. It then uses the energy of Δp to switch to the occluded state, in which exchange of NADP^+ and NADPH with the solvent is blocked, bound NADPH is stabilized relative to bound NADP^+ , and hydride transfer is permitted. A second input of energy switches the enzyme back into its open state.

It will be important to our understanding of the transhydrogenase mechanism to determine the factors responsible for favoured binding to the enzyme of either oxidized or reduced nucleotides. Particularly interesting will be the structural changes that accompany the postulated Δp -driven switch that shifts the protein from the state which binds NADP^+ more favourably to the state which binds NADPH more favourably. Possible reasons for the stabilization of NADPH relative to NADP^+ on dIII are (i) a stereochemical distortion of the binding pocket to favour the puckered (quasi-boat) conformation of the dihydronicotinamide ring of NADPH relative to the almost planar pyridinium ring of NADP^+ [37], and (ii) unfavourable charge interactions between amino acid side chains of the protein and the cationic and aromatic pyridinium ring relative to the electrically neutral and non-aromatic dihydronicotinamide ring. Essential to the switch is the process of occlusion; without this, substrate NADP^+ would simply dissociate from the protein during the structural changes that lead to stabilization of NADPH.

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