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The reduction of acetylpyridine adenine dinucleotide by NADH: is it a significant reaction of proton-translocating transhydrogenase, or an artefact?

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Abstract

Transhydrogenase is a proton pump. It has separate binding sites for NAD^+/NADH (on domain I of the protein) and for $NADP^+$/NADPH (on domain III). Purified, detergent-dispersed transhydrogenase from *Escherichia coli* catalyses the reduction of the NAD^+ analogue, acetylpyridine adenine dinucleotide ($AcPdAD^+$), by NADH at a slow rate in the absence of added $NADP^+$ or NADPH. Although it is slow, this reaction is surprizing, since transhydrogenase is generally thought to catalyse hydride transfer between NAD(H) – or its analogues and NADP(H) – or its analogues, by a ternary complex mechanism. It is shown that hydride transfer occurs between the 4*A* position on the nicotinamide ring of NADH and the 4*A* position of $AcPdAD^+$. On the basis of the known stereospecificity of the enzyme, this eliminates the possibilities of transhydrogenation (a) from NADH in domain I to $AcPdAD^+$ wrongly located in domain III; and (b) from NADH wrongly located in domain III to $AcPdAD^+$ in domain I. In the presence of low concentrations of added $NADP^+$ or NADPH, detergent-dispersed *E. coli* transhydrogenase catalyses the very rapid reduction of $AcPdAD^+$ by NADH. This reaction is cyclic; it takes place via the alternate oxidation of NADPH by $AcPdAD^+$ and the reduction of $NADP^+$ by NADH, while the NADPH and $NADP^+$ remain tightly bound to the enzyme. In the present work, it is shown that the rate of the cyclic reaction and the rate of reduction of $AcPdAD^+$ by NADH in the absence of added $NADP^+$/NADPH, have similar dependences on pH and on $MgSO_4$ concentration and that they have a similar kinetic character. It is therefore suggested that the reduction of $AcPdAD^+$ by NADH is actually a cyclic reaction operating, either with tightly bound $NADP^+$/NADPH on a small fraction ($< 5\%$) of the enzyme, or with NAD^+/NADH (or $AcPdAD^+$/AcPdADH) unnaturally occluded within the domain III site. Transhydrogenase associated with membrane vesicles (chromatophores) of *Rhodospirillum rubrum* also catalyses the reduction of $AcPdAD^+$ by NADH in the absence of added $NADP^+$/NADPH. When the chromatophores were stripped of transhydrogenase domain I, that reaction was lost in parallel with 'normal reverse' transhydrogenation (e.g., the reduction of $AcPdAD^+$ by NADPH). The two reactions were fully recovered upon reconstitution with recombinant domain I protein. However, after repeated washing of the domain I-depleted chromatophores, reverse transhydrogenation activity (when assayed in the presence of domain I) was retained, whereas the reduction of $AcPdAD^+$ by NADH declined in activity. Addition of low concentrations of $NADP^+$ or NADPH always supported the same high rate of the NADH → $AcPdAD^+$ reaction independently of how often the membranes were washed. It is concluded that, as with the purified *E. coli* enzyme, the reduction of $AcPdAD^+$ by NADH in chromatophores is a cyclic reaction involving nucleotides that are

Abbreviations: $AcPdAD^+$, acetylpyridine adenine dinucleotide (oxidized form).

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tightly bound in the domain III site of transhydrogenase. However, in the case of *R. rubrum* membranes it can be shown with some certainty that the bound nucleotides are $NADP^+$ or NADPH. The data are thus adequately explained without recourse to suggestions of multiple nucleotide-binding sites on transhydrogenase.

Keywords: Transhydrogenase; Proton pump; Nicotinamide nucleotide; (*Rhodospirillum rubrum*); (*Escherichia coli*)

1. Introduction

Transhydrogenase, which is found in the inner membranes of animal mitochondria and in the cytoplasmic membranes of bacteria, couples the transfer of hydride equivalents between NAD(H) and NADP(H) to the translocation of protons. Thus, in the probable physiological direction:

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

where H^+_{out} and H^+_{in} represent hydrogen ions in the external and internal aqueous phases, respectively, and where $n = 1$ [1].

Transhydrogenase has no known redox centres or prosthetic groups. Extensive kinetic analyses of the membrane-bound [2–4] and detergent-dispersed [5] protein indicate that the reaction proceeds by a ternary complex mechanism; there are separate binding sites on transhydrogenase for NAD^+/NADH and for $NADP^+$/NADPH. Measurements of isotopic exchange [6] indicate that the transfer of the hydride equivalent between the nicotinamide rings of the nucleotides is direct, from the *A* (or *pro-R*) position on NADH to the *B* (or *pro-S*) position on NADPH.

The amino acid sequences of a number of transhydrogenases indicate a tridomain structure. Domains I and III are relatively hydrophilic and protrude from the membrane (on the matrix side in mitochondria and on the cytoplasmic side in bacteria). The NAD^+/NADH binding site is located in domain I and the $NADP^+$/NADPH binding site in domain III [7–10]. The very hydrophobic domain II spans the membrane.

$AcPdAD^+$ is an analogue of NAD^+. 'Reverse' transhydrogenation (see Eqn. 1) is conventionally assayed as the reduction of $AcPdAD^+$ by NADPH (the absorbance maximum of AcPdADH is shifted relative to that of NAD(P)H). Transhydrogenase from various sources, *in the presence of either* $NADP^+$ *or NADPH*, can also catalyse the reduction of $AcPdAD^+$ by NADH (sometimes at very high rates). Detailed analysis of this reaction by solubilized *E. coli* transhydrogenase [11,12] showed that it is cyclic ($NADP^+$ and NADPH remain tightly bound to domain III of the enzyme, while being alternately reduced and oxidized by NADH and $AcPdAD^+$ located on domain I, see Fig. 1). There are some differences in the character of the cyclic reaction in transhydrogenases isolated from different species. Solubilized bovine mitochondrial transhydrogenase catalyses the cyclic reaction with added $NADP^+$, but not with added NADPH (evidently, $NADP^+$ binds only rather weakly), unless the enzyme is first partially inactivated by prolonged exposure to the nucleotide [13]. A mixture of the recombinant domains I and III of *R. rubrum* transhydrogenase catalyses the cyclic reaction by way of the very tightly bound $NADP^+$ and NADPH that are associated with the domain III protein even after extensive purification [10]. We proposed that the cyclic reaction does not involve the pumping of protons [11,12], whereas Rydström and colleagues have maintained that oxidation of bound NADPH causes proton pumping in one direction and reduction of bound $NADP^+$ causes proton pumping in the opposite direction [14].

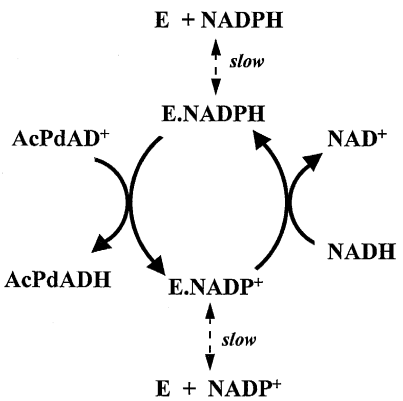

Fig. 1. The cyclic reduction of $AcPdAD^+$ by NADH involving $NADP^+$ and NADPH tightly bound to transhydrogenase E = transhydrogenase. See text.

Many years ago Fisher's group [15,16] claimed that transhydrogenase on *R. rubrum* chromatophores could catalyse $AcPdAD^+$ reduction by NADH in the absence of added NADP(H). They took this as evidence for the existence of a reduced enzyme intermediate. Although this conclusion does not fit with the modern view of the mechanism of action of this enzyme – there was some concern about the validity of the experimental controls [5] – the original observation has still not been satisfactorily explained. We reported a very slow reduction of $AcPdAD^+$ by NADH by purified *E. coli* transhydrogenase in the absence of added NADP(H) [11] and, more recently, Bragg and colleagues have reported conditions where this reaction takes place at a higher rate (though it is still slow compared with the NADP(H)-dependent reaction) [17]. From our present knowledge, four possible explanations might account for the reduction of $AcPdAD^+$ by NADH in the absence of added NADP(H).

1. There might be (some) tightly bound NADP(H) associated with the domain III of transhydrogenase and this would permit the cyclic reaction to operate.
2. On the basis that transhydrogenase can bind the nucleotides very tightly [11,12], there might be enough contaminating NADP(H) (e.g., $< 0.1\%$) in commercial solutions of $AcPdAD^+$ or NADH to permit the cyclic reaction to operate.
3. Either $AcPdAD^+$ or NADH might be able to enter the wrong site (i.e., the NADP(H) site on domain III) at a sufficient rate to allow transhydrogenation to take place.
4. There is more than one pathway for hydride transfer on transhydrogenase and/or more than a single site for NAD(H) binding and for NADP(H) binding. For example, it might be possible, (a) that NADH bound into the domain I of one partner of the transhydrogenase dimer can reduce $AcPdAD^+$ located in domain I in the other partner [17] by a pathway that does not operate during normal forward/reverse transhydrogenation, or (b) there is a second nucleotide-binding site on domain III (see [17–20]) that can bind NADH to serve as hydride donor for $AcPdAD^+$ on domain I (P.D. Bragg, personal communication).

This report describes the results of experiments to distinguish between these possibilities.

2. Materials and methods

E. coli strain JM109, bearing plasmid pSA2 [21] and *R. rubrum* strain RTB2 [1] were grown and membranes prepared as described [22,23]. Membranes of *R. rubrum* depleted of their transhydrogenase domain I protein were prepared by washing once by centrifugation in 2M NaCl, 20 mM Tris-Cl, pH 8.0. Further washing was carried out in 100 mM Tris-HCl, pH 8.0, 10% sucrose, 1 mM dithiothreitol, as described in Table 2. The membranes were stored in this medium, with 50% glycerol at -20°C. The bacteriochlorophyll content of the *R. rubrum* membranes was estimated using the in vivo extinction coefficient given [24]. *E. coli* transhydrogenase (1 mg per ml protein) was solubilized in 10% Triton X-100 and purified by column chromatography, essentially by the procedure of Tong et al. Solubilized transhydrogenase was loaded on to a 25×3cm column of DEAE Trisacryl (LKB) pre-equilibrated with TED buffer (50 mM Tris-Cl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol). The column was washed with TED buffer containing 0.05% Brij and 70 mM NaCl and then transhydrogenase was eluted with TED buffer containing 0.05% Brij and 300 mM NaCl. Pooled active fractions were loaded on to a 15×1.7 cm column of Q-Sepharose HP (Pharmacia) pre-equilibrated with MED buffer (20 mM Mops, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Brij). Transhydrogenase was eluted with a 0–500 mM gradient of NaCl in MED buffer. Pooled active fractions were further chromatographed on a MonoQ HR5/5 column in MED buffer with a 250–500 mM gradient of NaCl using a Pharmacia FPLC system. The concentration of protein was determined by the bicinchoninic acid assay [25]. The domain I protein of *R. rubrum* transhydrogenase was expressed in *E. coli* and purifed by column chromatography [8,23]; its concentration was determined by the microtannin assay [26].

The reduction of $AcPdAD^+$ by NADH and by NADPH was monitored, either with a Shimadzu UV3000 dual-wavelength spectrophotometer at 375–450 nm, or with a Perkin Elmer Lambda 16 double-beam spectrophotometer at 375 nm, using extinction coefficients given [27] and in experimental media described in the figure legends.

In some experiments solutions of commercial

$AcPdAD^+$ and NADH (from Sigma) were purified by chromatography on a Mono-Q HR5/5 column fitted to a Pharmacia FPLC system using a modification of the procedure described by Orr and Blanchard [28]. The column was equilibrated with 20 mM triethanolamine-HCl, pH 7.7 and developed with a gradient of 0–200 mM KCl in 20 mM triethanolamine-HCl. Control experiments showed that this elution system gave very good separation of $AcPdAD^+$ and of NADH from $NADP^+$ and NADPH.

The stereospecificity of hydride transfer from NADH to $AcPdAD^+$ was performed using both [4*A*-^{3}H]NADH and [4*B*-^{3}H]NADH, prepared from [4-^{3}H]NAD^+ (Amersham) essentially as described by Wu et al. [29], but using different procedures for the separation of the nucleotides. [4*A*-^{3}H]NADH was generated using glutamate dehydrogenase, type III from bovine liver (4*B*-specific, Sigma). After 15 min of reaction at 22°C (> 90% conversion) using the conditions described [29], the mixture was cooled to 4°C passed through a 10k Centricon tube (Amicon) and loaded on a Mono-Q HR5/5 column (Pharmacia). Unreacted NAD^+ was eluted with 10 mM NH_4HCO_3 and labelled NADH was eluted with a 10–500 mM gradient of NH_4HCO_3. [4*B*-^{3}H]NADH was prepared using alcohol dehydrogenase from yeast (4*A*-specific, Sigma). After 30 min of reaction at 22°C (> 90% conversion) using the conditions described [29], the radiolabelled nucleotide was purified as above. Freshly prepared NADH (either 4*A* or 4*B* labelled) was added (final concentration, 200 μM, and approximately 1 $\mu Ci \cdot ml^{-1}$) to a solution containing 50 mM Mes, pH 6.0, 0.5 mM EDTA, 0.01% Brij 35, 2 mM dithiothreitol, 200 μM $AcPdAD^+$ and 2 μg purified *E. coli* transhydrogenase in a volume of 1ml at 30°C. The reduction of $AcPdAD^+$ was monitored at 375 nm on a Kontron double-beam spectrophotometer, and, after 10min, the mixture was cooled to 4°C and passed through a 10k Centricon filter, before loading on a Mono-Q column equilibrated with 20 mM triethanolamine, pH 7.7 [28]. NAD^+ and unreacted $AcPdAD^+$ were eluted from the column with 20 mM triethanolamine, pH 7.7. A gradient of 0–200 mM KCl in 20 mM triethanolamine, pH 7.7 was applied to the column. AcPdADH was eluted as a single peak which overlapped slightly with the unreacted NADH. Pooled peak fractions of the AcPdADH were re-chromatographed under the same conditions; this time the nucleotide was eluted as a single sharp peak. After removing a sample for counting, the radiolabelled AcPdADH was re-oxidised with 4*A*-specific yeast alcohol dehydrogenase, as described for the re-oxidation of NADH [29]. After 10 min the AcPdADH was largely oxidized (as judged by the absorbance at 375 nm), the reaction mixture was loaded on a Dowex 1 column (mesh 200–400, Sigma), pre-equilibrated with water, and the $AcPdAD^+$ was eluted with a gradient of 0–1M formic acid/sodium formate [30]. Radioactivity was determined using an LKB-Wallac 1217 counter with Wallac Hisafe II scintillation fluid.

3. Results

3.1. The reduction of $AcPdAD^+$ by NADH by purified transhydrogenase from E. coli

We reported that purified *E. coli* transhydrogenase catalyses a very low rate of $AcPdAD^+$ reduction by NADH [11]. Glavas and Bragg reported a higher rate [17]. The discrepancy between the two sets of data arises partly as a result of different Brij 35 concentrations (data not shown), partly as a result of different concentrations of lysophosphatidyl choline (data not shown), and partly as a result of differences in the ionic strength, in the experimental media. A detailed description of the effects of ionic strength ($MgSO_4$) on the reaction will be given below (Fig. 2).

3.2. The stereospecificity of the reduction of $AcPdAD^+$ by NADH by purified transhydrogenase from E. coli

The nucleotide-binding site on domain I of transhydrogenase is specific for NAD(H) [7–9], and that on domain III is specific for NADP(H) [7,10]. The transfer of the hydride equivalents between nucleotides bound to transhydrogenase is also stereospecific. Thus, with physiological substrates, hydride transfer to and from NAD(H) is *A*-specific, whereas hydride transfer to and from NADP(H) is *B*-specific [6]. Therefore, if the NADH → $AcPdAD^+$ reaction results from nucleotides entering the 'wrong' sites (either the NADH entering domain III and being oxidized by $AcPdAD^+$ in domain I, or $AcPdAD^+$

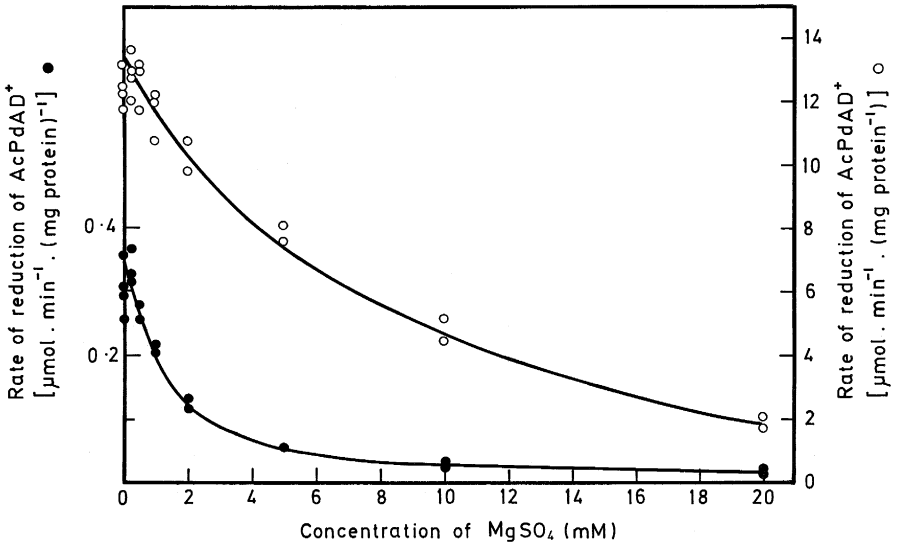

Fig. 2. The dependence on $MgSO_4$ concentration of the rate of reduction of $AcPdAD^+$ by NADH in the absence, and in the presence of NADPH, by *E. coli* transhydrogenase. Experiments were performed on a dual-wavelength spectrophotometer in a medium containing 20 mM Mes, 0.5 mg · ml^{-1} lysophosphatidyl choline, 0.05% Brij, 1 mM dithiothreitol, 0.25 mM EDTA, 1.3 μg · ml^{-1} purified *E. coli* transhydrogenase at 30°C, pH 6.0, and at the $MgSO_4$ concentration shown. ●, 200 μM NADH and 200 μM $AcPdAD^+$; ○, 200 μM NADH, 200 μM $AcPdAD^+$ and 10 μM NADPH.

entering domain III and being reduced by NADH in domain I), and if, as expected, it is the binding-site pocket which determines the stereospecificity, then the hydride transfer would be *AB* (or *BA*). The results described in Table 1 show that this is not the case; the hydride is transfer is *AA*. In the first set of experiments, $AcPdAD^+$ was reduced with NADH tritiated at the 4*A* position of the nicotinamide ring. At the end of the experiment the specific activity of the product AcPdADH was similar to that of the starting activity of the NADH. This indicates that hydride transfer was *from* the *A* position of the latter. When a sample of the product AcPdADH was treated with 4*A*-specific yeast alcohol dehydrogenase, the nucleotide lost its radioactivity, showing that hydride transfer had been *into* the *A* position of the $AcPdAD^+$.

In separate experiments, $AcPdAD^+$ was reduced with NADH tritiated in the 4*B* position of the nicotinamide ring. In this case, the resulting AcPdADH was not significantly labelled (Table 1), again indicating that hydride transfer is from the 4*A* position of NADH.

Table 1
Stereospecificity of hydride transfer during the reduction of $AcPdAD^+$ by NADH by purified *E. coli* transhydrogenase (see Section 2)

Reduction of $AcPdAD^+$ by [4A^{3}H]NADH [a]:	Specific activity (Ci · mol^{-1})
Initial NADH	11.3
Resultant AcPdADH	11.2
$AcPdAD^+$ after treatment of resultant AcPdADH with ADH [b]	0.07
Reduction of $AcPdAD^+$ by [4B^{3}H]NADH [c]:	
Initial NADH	0.77
Resultant AcPdADH	0.01

[a] The experiment was performed three times, and the results averaged.
[b] ADH, alcohol dehydrogenase (see Section 2).
[c] Each specific activity is the mean of three measurements from one experiment.

3.3. If the reduction of $AcPdAD^+$ by NADH by purified transhydrogenase from E. coli is a cyclic reaction involving bound $NADP^+$/NADPH, what is the source of that $NADP^+$/NADPH?

The stereospecificity of hydride transfer revealed by these experiments is consistent with the idea [11]

that the reduction of $AcPdAD^+$ by NADH is a consequence of a cyclic transhydrogenation reaction, in which contaminating levels of $NADP^+$ or NADPH bind tightly to domain III of the enzyme, and are alternately reduced and oxidized by NADH and $AcPdAD^+$, respectively, on domain I. It was observed that, at pH 6.0, extremely low concentrations (< 0.1 μM) of either $NADP^+$ or NADPH can promote the cyclic reaction [11]. Thus, contamination of the commercial solutions of NADH/$AcPdAD^+$, by either $NADP^+$ or NADPH even at levels of $< 0.1\%$, would be enough to support substantial rates of the cyclic reaction. However, this possibility was eliminated by experiments in which the commercial stock solutions of NADH and $AcPdAD^+$ were subjected to ion exchange chromatography under conditions that led to good separation from any contaminating $NADP^+$ or NADPH. The rates of NADH → $AcPdAD^+$ catalysed by *E. coli* transhydrogenase using the purified nucleotides were not significantly decreased (data not shown).

There remains the possibility that, even after extensive purification, the *E. coli* transhydrogenase might still possess tightly bound $NADP^+$/NADPH. Certainly, some mutant transhydrogenases in membrane vesicles of *E. coli* appear to be associated with bound $NADP^+$ [17], and, even after ion exchange and size exclusion chromatography, wild-type recombinant domain III protein of *R. rubrum* transhydrogenase retains substantial levels of tightly bound $NADP^+$ and NADPH, which can participate in a cyclic reaction with NADH, $AcPdAD^+$ and recombinant domain I protein ([10], and see the results of experiments described below on *R. rubrum* membranes). The rate of the NADH → $AcPdAD^+$ reaction catalysed by our purified *E. coli* transhydrogenase is less than 5% of that of the cyclic reaction (see Fig. 2), and the detection of such low levels of bound $NADP^+$ and NADPH was below the resolution of our analysis (for example, see [10]). We therefore attempted to remove any residual $NADP^+$/NADPH by extensively washing the transhydrogenase while it was bound to an ion exchange column, but this failed to lower the rate of the NADH → $AcPdAD^+$ reaction by the subsequently eluted enzyme. In further separate experiments, the washing procedure was performed at high pH (8.0) and at elevated concentrations of $MgSO_4$ (10 mM) in an attempt to displace bound NADP(H) (see [11] and below), but still there was no decrease in the rate of the NADH → $AcPdAD^+$ reaction. On the basis that $NADP^+$ might have a greater 'off' rate from the detergent-dispersed enzyme than NADPH [11,12], we preincubated purified *E. coli* transhydrogenase with $AcPdAD^+$ with the view to oxidizing any bound NADPH to $NADP^+$ before subjecting it to column washing, but again this treatment did not lower the rate of the subsequently measured NADH → $AcPdAD^+$ reaction (data not shown).

3.4. Comparison of the properties of the NADH → $AcPdAD^+$ reaction catalysed by E. coli transhydrogenase with the cyclic reaction

Although proof could not be obtained, the above experiments are consistent with the possibility that the NADH → $AcPdAD^+$ reaction is in reality the cyclic reaction operating with a low level of $NADP^+$ or NADPH tightly bound to the enzyme. We therefore carried out a series of experiments to compare the properties of the two reactions: if they are essentially the same process (only differing in the effective $NADP^+$/NADPH concentration), they should have similar properties.

Fig. 3 shows that, when measured under identical conditions, the pH dependence of the NADH → $AcPdAD^+$ reaction is similar to that of the cyclic reaction. Note that the latter was measured at a suboptimal concentration of NADPH (1.0 μM), but that the data had a similar profile to that shown previously at a higher concentration of the nucleotide [11]. In all cases the rates of reaction were at a maximum at pH < 6.0, and fell monotonically as the pH of the medium was increased. The results are in marked contrast to the pH dependence of simple, reverse transhydrogenation, NADPH → $AcPdAD^+$ and simple, forward transhydrogenation, NADH → thio-$NADP^+$, which both have bell-shaped profiles with optima at approximately pH 7.3 [11].

Fig. 2 shows that the dependences of the NADH → $AcPdAD^+$ reaction, and of the cyclic reaction, on $MgSO_4$ concentration are also very similar. In both cases, the reactions proceeded at substantial rates in the absence of added $MgSO_4$, and were strongly inhibited in the range 1–10 mM salt. Note that a slight stimulation of the cyclic reaction seen under

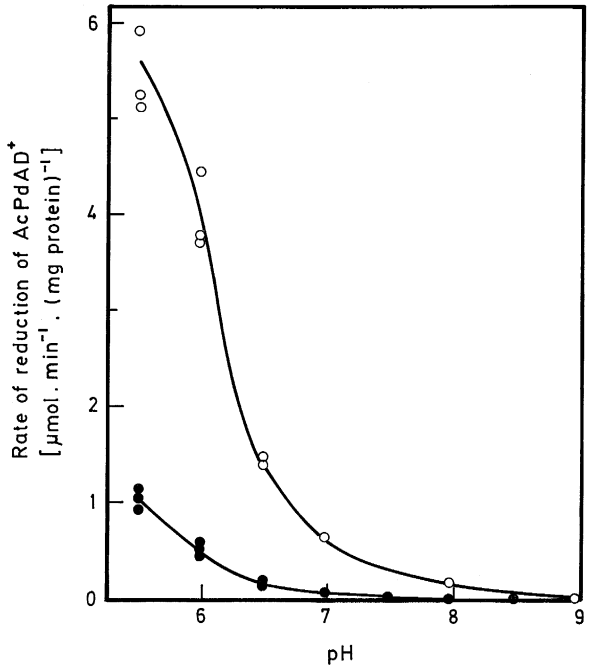

Fig. 3. The dependence on pH of the rate of reduction of $AcPdAD^+$ by NADH in the absence, and in the presence of NADPH, by *E. coli* transhydrogenase. Experiments were performed on a dual-wavelength spectrophotometer in a medium containing 12 mM Mes, 12 mM Mops, 12 mM Ches, 12 mM Tricine, 0.5 $mg \cdot ml^{-1}$ lysophosphatidyl choline, 0.05% Brij, 1 mM dithiothreitol, 0.25 mM EDTA, 0.5 $\mu g \cdot ml^{-1}$ purified *E. coli* transhydrogenase at 30°C and at the pH shown. ●, 200 μM NADH and 200 μM $AcPdAD^+$; ○, 200 μM NADH, 200 μM $AcPdAD^+$ and 1.0 μM NADPH.

different solution conditions [11] was barely evident in Fig. 2. As with the pH dependences, these results are also in marked contrast to those obtained for simple, reverse and simple, forward transhydrogenation; those reactions at pH 6.0 are *stimulated* by $MgSO_4$ across the entire concentration range [11].

As part of the evidence that the NADP(H)-dependent reduction of $AcPdAD^+$ by NADH is indeed a cyclic reaction, we showed that NADH and $AcPdAD^+$ display 'ping-pong' kinetics [12]. Thus, $AcPdAD^+$ reacts with the [enzyme-NADPH] binary complex and generates [enzyme-$NADP^+$] and AcPdADH. In a subsequent step, NADH reacts with the [enzyme-$NADP^+$] intermediate and regenerates [enzyme-NADPH] – see Fig. 1. As in a classical ping-pong mechanism, the NADH and the $AcPdAD^+$ interact at the same site on the enzyme, whereas the [enzyme-$NADP^+$] and [enzyme-NADPH] binary complexes behave as the alternate, modified forms of the enzyme. Fig. 4 shows that, in the NADH → $AcPdAD^+$ reaction, the NADH and the $AcPdAD^+$ also show ping-pong kinetics. Notably, (a) at low nucleotide concentrations the s/v against s curves converge on the s/v axis, and (b) at high concentrations of NADH, there is a distinct upward curvature, which indicates substrate inhibition (the NADH and the $AcPdAD^+$ compete for the domain I site. The data are similar to those previously described for the cyclic reaction, and again, this behaviour contrasts with that of simple, reverse transhydrogenation, which shows that that reaction proceeds through a random order ternary complex mechanism [2–5]; at low pH the reduction of $AcPdAD^+$ by NADPH has Michaelis–Menten kinetics with a K_m for NADPH of approximately 0.8 μM, and a K_m for $AcPdAD^+$ of approximately 1.0 μM [12].

3.5. The reduction of $AcPdAD^+$ by NADH by transhydrogenase on R. rubrum chromatophores.

The ability of chromatophore membranes to catalyse the reduction of $AcPdAD^+$ by NADPH ('reverse' transhydrogenation), and the reduction of $AcPdAD^+$ by NADH was established many years ago [15,16]. Like the equivalent reactions in purified *E. coli* transhydrogenase, the former is an *AB* transfer, and the latter *AA* [16]. Fisher and colleagues argued that both reactions are due to transhydrogenase by performing experiments in which domain I (the 'soluble transhydrogenase factor', as it was then known) was removed by washing, was partially purified, and then reconstituted with the depleted membranes [15,16]. Restoration of $AcPdAD^+$ reduction by NADH took place in parallel with $AcPdAD^+$ reduction by NADPH. However, a problem in interpreting those data was that the impure 'soluble factor' itself has the capacity to perform $AcPdAD^+$ reduction by NADH, probably the result of contamination by other extrinsic chromatophore enzymes such as lipoamide dehydrogenase [5].

With the development of a procedure to prepare highly purified, recombinant domain I protein [8], that controversy can now be settled. Chromatophores were isolated from a strain of *R. rubrum* which over-expresses transhydrogenase [1]. These chromatophores catalysed: (a) reverse transhydrogenation

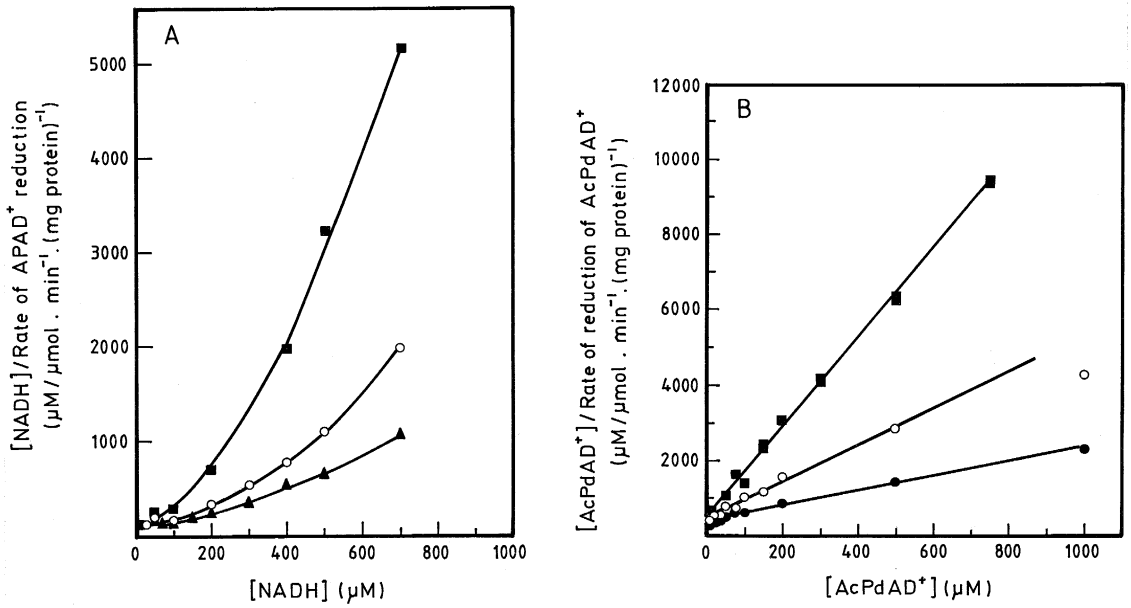

Fig. 4. A kinetic analysis of the rate of $AcPdAD^+$ reduction by NADH in the absence of NADPH, by *E. coli* transhydrogenase. Experiments were performed on a double-beam spectrophotometer in a medium containing 10 mM Mes, 0.5 mg · ml^{-1} lysophosphatidylcholine, 0.05% Brij, 1 mM dithiothreitol, 0.25 mM EDTA, and either 11.5 μg · ml^{-1} (A), or 5.0 μg · ml^{-1} (B), purified *E. coli* transhydrogenase at 30°C, pH 6.0. In (A) the fixed concentrations of $AcPdAD^+$ were: ■, 50 μM; ○, 200 μM; ▲, 500 μM, and in (B) the fixed NADH concentrations were: ■, 50 μM; ○, 100 μM; ●, 200 μM.

(NADPH → $AcPdAD^+$); (b) the reduction of $AcPdAD^+$ by NADH in the absence of NADPH; and (c) at an enhanced rate, the reduction of $AcPdAD^+$ by NADH in the presence of NADPH (Table 2). It may be noted that the addition of purified recombinant domain I protein led to a small increase in each of these rates – as previously explained the chromatophore preparation procedure results in a small loss of the native domain I protein from transhydrogenase [23]. The chromatophores were subsequently washed in concentrated salt solution (wash 1), and this led to a large decrease in the rates of all three of the test reactions. However, each of the reactions was recovered when the assay media were supplemented

Table 2
Evidence that the reduction of $AcPdAD^+$ by NADH by transhydrogenase of *R. rubrum* membranes is the cyclic reaction operating by way of tightly bound NADP(H) (see text)

	NADPH → $AcPdAD^+$		NADH → $AcPdAD^+$ (minus NADPH)		NADH → $AcPdAD^+$ (plus NADPH)	
	− DI	+ DI	− DI	+ DI	− DI	+ DI
Chromatophores	3.9	4.4	10.1	13.4	18.9	26.4
After wash 1	0.3	5.5	0.2	4.2	1.8	25.3
After wash 2	0.0	4.2	0.0	1.5	0.0	26.2

Wash 1 was in a medium containing 2 M NaCl, 20 mM Tris-Cl, pH 8.0, at a bacteriochlorophyll concentration of 30 μM. Wash 2 was in a medium of 100 mM TrisCl, pH 8.0, 10% sucrose, 1 mM dithiothreitol at a bacteriochlorophyll concentration of 30 μM. During each wash the membranes (from the over-expressing strain) were sedimented by centrifugation at 100 000 *g* for 120 min, and then resuspended in the medium used in the wash. The assay conditions were 50 mM Tris-HCl, pH 8.0, 50 mM KCl. $AcPdAD^+$ was present at 200 μM throughout, and NADH (where shown) was 100 μM. In the measurement of the NADPH → $AcPdAD^+$ activity, the NADPH concentration was 200 μM, and for the NADH → $AcPdAD^+$ (plus NADPH) reaction, it was 20 μM. Rates are given as μmol $AcPdAD^+$ reduced · μmol^{-1} bacterichlorophyll · min^{-1}.

with purified recombinant domain I protein (the domain I protein had no transhydrogenation activity). This is completely in accordance with the experiments and conclusions of Fisher and colleagues using 'soluble transhydrogenase factor'. Thus, the washing process removes native domain I from the membranes, but added recombinant domain I readily reassociates with the membrane-located domain II/III components to restore transhydrogenation. Importantly from the present perspective, it shows that all three of the test reactions are indeed transhydrogenase related. It is evident, however, that whereas reverse transhydrogenation, and the reduction of $AcPdAD^+$ by NADH in the presence of NADPH, recovered completely after wash 1 upon addition of domain I protein, the reduction of $AcPdAD^+$ by NADH in the absence of NADPH recovered only partly. This was even clearer after a further wash, this time in a low ionic strength buffer. The rates of all three reactions decreased to zero in the absence of supplementary domain I. In the presence of domain I there was almost complete recovery of reverse transhydrogenation, and of the reduction of $AcPdAD^+$ by NADH in the presence of NADPH, but the reduction of $AcPdAD^+$ by NADH in the absence of NADPH remained at a very low rate. The washings (supernatant obtained after centrifugation) also partly restored the rate of $AcPdAD^+$ reduction by NADH (not shown). These experiments show that the capacity of domain II/III in the membranes to re-assemble with domain I protein into a complete transhydrogenase was not influenced by the washing procedure, but that some other component, that is additionally responsible for the reduction of $AcPdAD^+$ by NADH in the absence of added NADPH, is lost during washing. It is very likely that the lost component is NADPH (or $NADP^+$). The simple interpretation is that chromatophore domain III is associated with tightly bound NADP(H), which can support the *cyclic* reduction of $AcPdAD^+$ by NADH, even in the absence of added NADP(H). The tightly bound nucleotide is progressively displaced during the washing steps, and therefore the chromatophores lose their ability to catalyse $AcPdAD^+$ by NADH, unless supplementary NADP(H) is added.

To establish that this has the same character as the cyclic reaction catalysed by detergent-dispersed *E. coli* transhydrogenase [11] (i.e., *NADPH-dependent* reduction of $AcPdAD^+$ by NADH), it was subsequently shown (Fig. 5, top) that the extent of $AcPdAD^+$ reduction by domain I-reconstituted washed chromatophore membranes (in the presence of NADPH) was equivalent to the amount of NADH added; evidently the hydride ion equivalents required for the reduction are derived from the latter nucleotide. Note that, as the NADH was exhausted, the rate of reduction of $AcPdAD^+$ subsided back to the slower rate of reduction by NADPH (i.e., simple, reverse transhydrogenation). As with the *E. coli* enzyme, the fact that only extremely low concentrations of NADPH were needed to support the reaction (approximately 10 nM, data not shown), reflects the high affinity between nucleotide and enzyme.

Again, as observed for the *E. coli* enzyme [11], the domain I-reconstituted, washed chromatophore membranes also catalysed the complementary, $NADP^+$-dependent reduction of $AcPdAD^+$ by NADH (Fig. 5, bottom). Each burst of reduction corresponded ap-

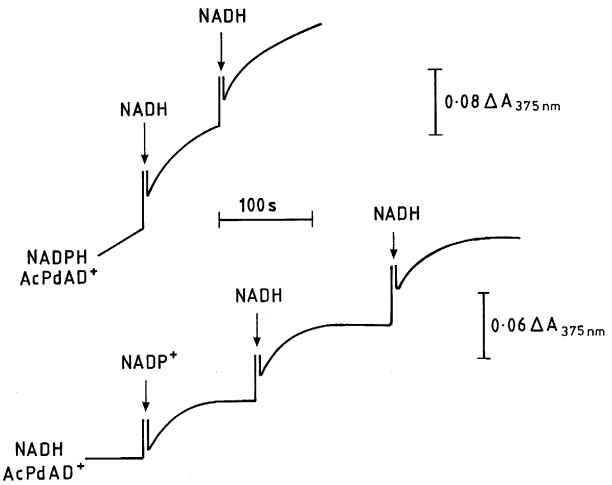

Fig. 5. The reduction of $AcPdAD^+$ by NADH by *R. rubrum* transhydrogenase. Experiments were performed on a double-beam spectrophotometer in 50 mM KCl, 50 mM Mes, pH 6.0, chromatophores (from the over-expressing strain), washed twice as described in Section 2, at a bacteriochlorophyll concentration of 0.6 μM, and purified recombinant domain I (25 nM). In the top trace, $AcPdAD^+$ (200 μM) and NADPH (50 μM) were present at the start, and NADH (8.6 μM) was added twice, where shown. In the bottom trace, $AcPdAD^+$ (200 μM) and NADH (8.6 μM) were present at the start. Then $NADP^+$ (100 μM) and more NADH (8.6 μM, twice) were added where shown. In both traces the extents of the bursts of $AcPdAD^+$ reduction were approximately equivalent to the amount of NADH added. Note that on this instrument the recording goes off-scale during an addition.

proximately to the amount of NADH added. The rate was similar to that of the NADPH-dependent reaction. Only very low concentrations of $NADP^+$ were required to support the reaction (approximately 100 nM, data not shown), reflecting the high affinity of the enzyme also for that nucleotide.

4. Discussion

There seems little doubt that the NADH → $AcPdAD^+$ reaction catalysed by everted membrane vesicles from *R. rubrum* is actually the 'cyclic' reaction operating with tightly bound $NADP^+$ or NADPH on domain III of transhydrogenase (Table 2, and see above). We can be less certain about the NADH → $AcPdAD^+$ reaction catalysed by purified *E. coli* transhydrogenase. A number of lines of evidence point to the conclusion that, also in the *E. coli* enzyme, it results from the cyclic reaction. (1) The pH and the $MgSO_4$ concentration dependences of the NADH → $AcPdAD^+$ reaction are very similar to those for the cyclic reaction (Fig. 2 and Fig. 3), and are distinctly different to those of the simple forward and reverse reactions [11,12]. (2) Both reactions have ping-pong kinetics ([12] and Fig. 4). (3) The hydride transfer of the NADH → $AcPdAD^+$ reaction is *AA* specific (Table 1), as expected for the cyclic reaction.

The last two observations tend to rule out another, otherwise plausible, explanation for the NADH → $AcPdAD^+$ reaction in *E. coli* transhydrogenase, that it results from nucleotide binding into the 'wrong' site – either $AcPdAD^+$ or NADH into domain III. Certainly the nucleotide-binding sites of the enzyme are not absolutely specific, for example it is clear from NMR and equilibrium dialysis experiments that NADPH can bind into recombinant domain I (probably $K_d > 1$ mM, whereas for NADH $K_d \approx 30$ μM [9,31]). However, it is to be expected, if a nucleotide does enter the wrong site, and is then capable of donating or accepting a hydride equivalent, that it will do so with the stereospecificity dictated by that site. Therefore, this hypothesis predicts, in contradiction with the experimental result (Table 1), an *AB* transfer.

A weakness in our suggestion that the NADH → $AcPdAD^+$ reaction in *E. coli* transhydrogenase results from a cyclic reaction operating in a small fraction (< 5%) of the enzyme having bound $NADP^+$ or NADPH is that we were unable, by washing, to remove this nucleotide (see Section 3). Certainly the rate of release of NADPH and $NADP^+$ from transhydrogenase can be extremely slow, and can limit the rates of forward and reverse transhydrogenation [11,12]. But, on the basis that the rate of nucleotide release must be at least as fast as the k_{cat} for the overall reaction, we calculated that, in the conditions used for washing, a substantial part of the residual bound nucleotide should have been released.

We offer two suggestions to resolve this problem. (i) A subpopulation (< 5%) of the enzyme exists in a state in which rapid release of $NADP^+$/NADPH from domain III is blocked. This state (an 'occluded' state [10,32]) would be similar to that adopted by the isolated recombinant domain III of *R. rubrum* transhydrogenase (we believe that it is an intermediate conformation in the turnover of the enzyme under physiological conditions [10,32]). Thus, it was shown that the rates of release of $NADP^+$ and NADPH from *R. rubrum* recombinant domain III are about three orders of magnitude slower than from the complete enzyme, and that the tightly bound $NADP^+$ and NADPH are not removed from the recombinant protein by column chromatography [10]. Why a small fraction of the isolated *E. coli* enzyme should be locked in this configuration is not known, but it might result from damage at the interface between domains II and III (presumably, it is the absence of interactions with domain II that leads to the tight binding of $NADP^+$ and NADPH to recombinant *R. rubrum* domain III [10]). (ii) Another possibility is that $AcPdAD^+$ or NADH can enter the domain III site and serve instead of $NADP^+$/NADPH to support the cyclic reaction. Even though the affinity of the domain III site for AcPdAD(H) and NAD(H) might be very low, once they were bound, like NADP(H), they would be occluded during a subsequent conformational change of the binding site, and then released at only a low rate. The bound nucleotide (either $AcPdAD^+$ or NADH) on domain III could therefore catalyse multiple turns of the cyclic oxidation of NADH and reduction of $AcPdAD^+$ at the domain I site. Note that this would give predominantly an *AA* transfer for NADH → $AcPdAD^+$ (depending on how many turns of the cycle took place before nucleotide release from domain III). This is in contrast with

simple 'wrong' site transhydrogenation as defined in the Introduction (either NADH on domain I → $AcPdAD^+$ on domain III, or NADH on domain III → $AcPdAD^+$ on domain I) which predicts *AB* transfer (see above).

In summary, the evidence is consistent with the NADH → $AcPdAD^+$ reaction being a form of cyclic transhydrogenation. It involves only a single pathway for hydride transfer between the nucleotide bound on domain I and the nucleotide bound on domain III (NADP(H), or NAD(H) or AcPdAD(H)). It is unnecessary to propose, either another pathway for hydride transfer (e.g., from domain I to domain I [17]), or an additional (regulatory or catalytic) nucleotide binding site. Evidence for a third nucleotide binding site on transhydrogenase in our opinion remains unconvincing. (1) Two regions identified in the amino-acid sequences of domain III were only very weakly homologous with the G-X-G-X-X-G/A motif of the nucleotide-binding Rossman fold [19], and more recently published transhydrogenase sequences suggest that homology is even less likely. (2) The results of experiments with dicyclohexyl carbodiimide, which were taken as an indication that there is a third, regulatory nucleotide-binding site on transhydrogenase [18], can be explained more simply on the basis of a cyclic reaction involving a single site on domain I and a single site on domain III [33]. (3) It is yet to be established that the reported interaction between NAD^+-agarose and the β-subunit of *E. coli* transhydrogenase [20] is only mediated by specific interactions; it is well-documented that so-called affinity ligands can bind proteins non-specifically [34]. On the contrary, the evidence for only two nucleotide-binding sites on transhydrogenase is rather strong. (1) Direct binding studies on the complete mitochondrial enzyme have revealed only one class of NAD(H)-binding site and one class of NADP(H)-binding site [7]. (2) There is only one NAD(H)-specific binding site on recombinant domain I from *R. rubrum* and *E. coli* transhydrogenases [9,35]. (3) Recombinant domain III from the *R. rubrum* enzyme is associated with tightly bound $NADP^+$ (at a molar ratio of 0.1–0.5) and NADPH (molar ratio aproximately 0.5), but not NAD^+ or NADH [10]. (4) Extensive kinetic analyses of transhydrogenase indicate only a single site for NAD(H) and a separate single site for NADP(H) [2–5].

Since submitting the first version of this manuscript, a new paper by Bragg was published on the mechanism of $AcPdAD^+$ reduction by NADH by *E. coli* transhydrogenase [36]. On the basis of K_m measurements he concludes that the reaction follows as a consequence of $AcPdAD^+$ entering what is normally the NADP(H) site (the third alternative listed in our Section 1). However, as we show above, the stereospecificity of hydride transfer, and the ping-pong kinetics, eliminate this possibility. Non-linearity in the dependence of the rate of the reaction on $AcPdAD^+$ concentration, which was taken as an indication of an extra nucleotide-binding site [36], actually results from competition between $AcPdAD^+$ and NADH for the *same* site (i.e., 'substrate inhibition') [12]. We must also emphasize (see [12]) that K_m values rarely reflect binding-site affinities. Thus, in forward and reverse transhydrogenation the rate constants for release of NADPH and $NADP^+$, respectively, make a significant contribution to the expressions for K_m, but not in the cyclic reaction, where the domain III nucleotides remain bound during catalysis.

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