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# The involvement of NADP(H) binding and release in energy transduction by proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli*

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**Abstract**

Proton-translocating transhydrogenase was solubilised and purified from membranes of *Escherichia coli*. Consistent with recent evidence [Hutton, M., Day, J., Bizouarn, T. and Jackson, J.B. (1994) Eur. J. Biochem. 219, 1041–1051], at low pH and salt concentration, the enzyme catalysed rapid reduction of the $NAD^+$ analogue $AcPdAD^+$ by a combination of NADH and NADPH. At saturating concentrations of NADPH, the dependence of the steady-state rate on the concentrations of NADH and $AcPdAD^+$ indicated that, with respect to these two nucleotides, the reaction proceeds by a ping-pong mechanism. High concentrations of either NADH or $AcPdAD^+$ led to substrate inhibition. These observations support the view that, in this reaction, NADP(H) remains bound to the enzyme: $AcPdAD^+$ is reduced by enzyme-bound NADPH, and NADH is oxidised by enzyme-bound $NADP^+$, in a cyclic process. When this reaction was carried out with [4A-$^2$H]NADH replacing [4A-$^1$H]NADH, the rate was decreased by 46%, suggesting that the $H^-$ transfer steps are rate-limiting. In simple 'reverse' transhydrogenation, the reduction of $AcPdAD^+$ was 46% slower with [4B-$^2$H]NADPH than with [4B-$^1$H]NADPH when the reaction was performed at pH 8.0, but there was no deuterium isotope effect at pH 6.0. This indicates that $H^-$ transfer is rate-limiting at pH 8.0 and supports our earlier suggestion that $NADP^+$ release from the enzyme is rate-limiting at low pH. The lack of a deuterium isotope effect in the reduction of thio-$NADP^+$ by NADH at low pH is also consistent with the view that NADPH release from the enzyme is slow under these conditions. A steady-state rate equation is derived for the reduction of $AcPdAD^+$ by NADPH *plus* NADH, assuming operation of the cyclic pathway. It adequately accounts for the pH dependence of the enzyme, for the features described above and for kinetic characteristics of *E. coli* transhydrogenase described in the literature.

*Keywords:* Transhydrogenase; Proton translocation; Nicotinamide nucleotide; Energy transduction; (Bacterial membrane); (*E. coli*)

## 1. Introduction

Nicotinamide nucleotide transhydrogenase ($H^+$-Thase) couples the transfer of reducing equivalents (as $H^-$ equivalents) between NAD(H) and NADP(H) to the translocation of protons across a membrane (reviewed [1–3]).

$$NADH + NADP^+ + H^+_{out} \leftrightarrow NAD^+ + NADPH + H^+_{in}$$

The enzyme is found in the inner mitochondrial membrane of animal cells, where it might serve in the control of flux through the tricarboxylic acid cycle [4], and in the cytoplasmic membranes of many bacteria, where one of its functions could be to augment the supply of NADPH for biosynthesis [5]. Thus, 'forward' transhydrogenation (the reduction of $NADP^+$ by NADH), is envisaged as a reaction that consumes the proton electrochemical gradient generated by respiratory or photosynthetic electron transport.

The amino acid sequences of three $H^+$-Thases (from *Escherichia coli* [6,7], bovine mitochondria [8] and *Rhodospirillum rubrum* [9]) indicate that the enzyme is made up of three large domains. The relatively hydrophilic domains I and III probably protrude on the same side of the membrane (the matrix side in mitochondria and the cytoplasmic side in bacteria) [10,11], and carry the binding

Abbreviations: $AcPdAD^+$, acetylpyridine adenine dinucleotide; $\Delta p$, transmembrane proton electrochemical gradient; $\Delta$pH, transmembrane pH gradient.

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sites for NAD(H) and for NADP(H), respectively [12]. Domain II is strongly hydrophobic and spans the membrane [6–9]. An important question, which is largely unanswered, is how the transfer of $H^-$ equivalents between nicotinamide nucleotides bound to domains I and III is coupled to the translocation of protons through domain II and thus across the membrane.

Acetyl pyridine adenine dinucleotide ($AcPdAD^+$) is a close analogue of $NAD^+$. The reduction of $AcPdAD^+$ by NADPH catalysed by $H^+$-Thase (equivalent to 'reverse' transhydrogenation) is well characterised [13–16]. Recently, we showed that solubilised and purified $H^+$-Thase from *E. coli* at low pH and salt concentrations, catalysed the reduction of $AcPdAD^+$ by NADH, but only in the presence of either $NADP^+$ or NADPH, at rates about 50-times faster than the reduction of $AcPdAD^+$ by NADPH [17]. It was argued that, under these conditions, the rates of release of $NADP^+$ and of NADPH from the enzyme are extremely slow and thus limit the rate of simple 'forward' and 'reverse' transhydrogenation. However, NADP(H)-dependent reduction of $AcPdAD^+$ by NADH was thought to arise from a cyclical reaction in which the NADP moiety remained bound to the enzyme during catalysis: NADH would be oxidised by enzyme-bound $NADP^+$, and then $AcPdAD^+$ would be reduced by enzyme-bound NADPH. The dependence on pH of these transhydrogenation reactions indicated that release of $NADP^+$ and of NADPH from the enzyme are accompanied by release of a proton. It was considered that, in membrane-bound transhydrogenase, the proton binding/release accompanying NADP(H) binding/release might be components of the proton translocation reaction. The symmetrical effects on the rate of dissociation of both [enzyme-$NADP^+$] and [enzyme-NADPH] of pH, salt concentration and treatment with dicyclohexylcarbodiimide [17] suggested that a single acidic group undergoes a decrease in $pK_a$ as a consequence of the release of either $NADP^+$ or NADPH. This acidic group, perhaps located in the proton channel would be expected to play a critical role in proton translocation.

In 1973 it was reported that replacement of $^1H$ with $^2H$ at the 4A position of the nicotinamide ring of NADH had no effect on the rate of reduction of $NADP^+$ catalysed by mitochondrial $H^+$-Thase [18]. This observation has been widely quoted and yet has remained unchallenged, despite its causing some problems for the view that interconversion of the ternary complexes of enzyme and nucleotide substrates is rate-limiting during catalysis [13,16].

In this report we describe the results of experiments on the solubilised and purified $H^+$-Thase from *E. coli* which further test the predictions of the model proposed [17]. We establish that, in some conditions, there is a deuterium isotope effect in some of the reactions catalysed by the enzyme, and that this provides further evidence for our hypothetical reaction mechanism. Finally, we derive a steady-state rate equation that satisfactorily describes the kinetic behaviour of $H^+$-Thase from *E. coli* with respect to simple transhydrogenation and to NADP(H)-dependent reduction of $AcPdAD^+$ by NADH.

## 2. Methods

*E. coli* strain JM109 harboring the transhydrogenase gene on a high copy-number plasmid, pDC21 [19], was grown and membranes were prepared as described [17]. $H^+$-Thase was solubilised and purified by the method [11,19] as modified [17]. Specific activities, measured under the conditions described [17], were routinely about 7 $\mu mol \cdot mg\ protein^{-1} \cdot min^{-1}$.

Transhydrogenation was measured on a dual wavelength Shimadzu UV3000 spectrophotometer. Reduction of $AcPdAD^+$ was recorded at 375–450 nm using an absorbance coefficient of 6.10 $mM^{-1}\ cm^{-1}$ [20]. Reduction of thio-$NADP^+$ was measured at 395–470 nm using an absorbance coefficient of 10.6 $mM^{-1}\ cm^{-1}$ [21].

[4A-$^2$H]NADH with deuterium at the 4A position of the nicotinamide ring was synthesised as described [22]. $NAD^+$ (5.6 mM), 48 mM deuterated ethanol-$d_6$ (Aldrich, 99 + atom% $^2H$), 14.7 mg (25 units) horse-liver alcohol dehydrogenase and 2.9 mg (17 units) yeast aldehyde dehydrogenase were added to 6 mM Ches-KOH buffer (pH 9.0), final volume 5.0 ml, at 25° C. The pH was maintained at pH 9.0 with periodic additions of dilute KOH and the reaction was monitored at 340 nm until it reached completion (about 1 h). The temperature was decreased to 4° C and the reaction mixture was centrifuged for 45 min through Microsep™ 10 kDa filters (Filtron Technology Corporation) to remove the enzymes (> 99%). The $A_{260}/A_{340}$ ratio of the product was 2.36. It was used within 5 h. In measurements of deuterium isotope effects, [4A-$^1$H]NADH was prepared by a similar procedure but using undeuterated ethanol, and experiments were performed in parallel.

[4B-$^2$H]NADPH with deuterium in the 4B position was prepared as described [23]. $NADP^+$ (4.5 mM), 5.0 mM ATP, 6.0 mM deuterated D-glucose-*1-d* (Aldrich, 98 atom % $^2H$), 10 mM $MgSO_4$, 0.13 mg (3.5 units) yeast hexokinase and 0.014 mg (4 units) yeast glucose-6-phosphate dehydrogenase were added to 6 mM Tris-HCl buffer (pH 8.0), final volume 5.0 ml, at 25° C. The pH was maintained at pH 8.0 with periodic additions of dilute KOH and the reaction was monitored at 340 nm until it reached completion (about 1 h). The temperature was decreased to 4° C and enzymes were removed from the reaction mixture as described above. Experiments were performed within 5 h of preparation and, again, control experiments were carried out in parallel with [4B-$^1$H]NADPH prepared by a similar procedure but using undeuterated D-glucose.

Enzymes and nucleotides were purchased from Sigma.

Rate constants were fitted to the steady-state rate equation with Microsoft Excel, which uses minimisation routines based on the Marquardt algorithm.

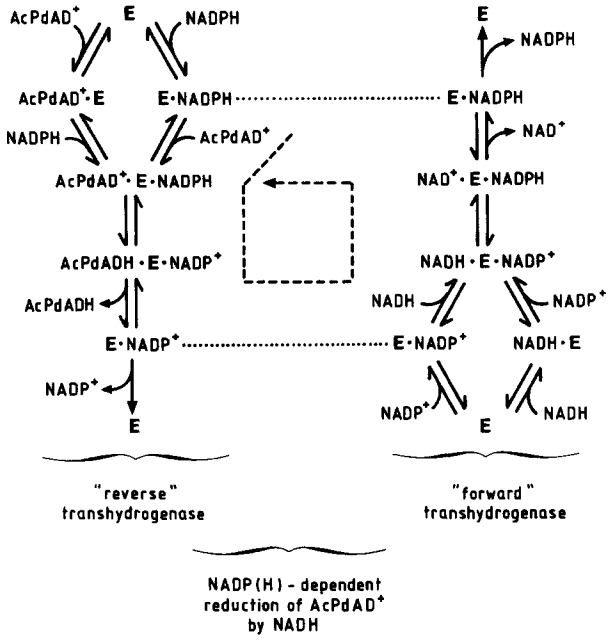
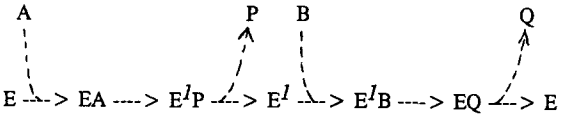



Fig. 1. Reactions catalysed by transhydrogenase. The reduction of AcPdAD$^+$ by NADPH (a 'reverse' transhydrogenation) shown on the left, proceeds from top to bottom. The reduction of NADP$^+$ by NADH ('forward' transhydrogenation), shown on the right, proceeds from bottom to top. Binary complexes that are common to both 'reverse' and 'forward' transhydrogenation are linked thus (· · · ·). NADP$^+$-dependent and NADPH-dependent reduction of AcPdAD$^+$ by NADH proceed in the direction shown (← —). The NAD$^+$/NADH binding site is to the left of the enzyme (E) and the NADP$^+$/NADPH binding site is to the right.

## 3. Results

### *3.1. Ping-pong kinetics in NADPH-dependent reduction of AcPdAD$^+$ by NADH*

It is well established that, during the transhydrogenase-catalysed reduction of AcPdAD$^+$ by NADPH, the nucleotides add randomly to give a ternary complex with the enzyme [13–16] (the left side of Fig. 1, proceeding from top to bottom). However, according to the proposal [17], it is evident that NADPH-dependent reduction of AcPdAD$^+$ by NADH (dashed lines in Fig. 1) shares some characteristics with a 'ping-pong', or substituted enzyme mechanism [24]. A ping-pong mechanism is one in which the first substrate (A) reacts to give a covalently modified enzyme (E$^I$) and the release of one product (P). The second substrate reacts with the covalent enzyme intermediate to regenerate the unmodified enzyme and release the second product (Q):




In NADPH-dependent reduction of AcPdAD$^+$ by NADH catalysed by H$^+$-Thase, A, P, B and Q are equivalent to AcPdAD$^+$, AcPdADH, NADH, and NAD$^+$, respectively, E is equivalent to [enzyme-NADPH] and E$^I$ to [enzyme-NADP$^+$] (compare Fig. 1). Thus, covalent modification of the enzyme in this context is represented by alternating oxidation and reduction of the enzyme-bound NADP moiety. A distinguishing feature of ping-pong reaction mechanisms is that when, for example, the concentration of A is varied at a fixed concentration of B, the value of $V^{app}/K_m^{app}$ is constant ($= V/K_m^A$). Therefore (in the absence of products), plots of $s/v$ against $s$ for substrate A at different, fixed concentrations of substrate B should give straight lines converging on the $s/v$ axis at $K_m^A/V$. The data in Fig. 2 are in agreement with the prediction. Thus, although there are three substrates in these experiments, because the rate of release of NADP$^+$ is very slow compared with the rate of AcPdAD$^+$ reduction, the reaction can be satisfactorily described as a ping-pong, bi:bi reaction. No deviation from this was observed even at the lowest concentration of AcPdAD$^+$ used.

Another implication of the proposal shown in Fig. 1 is that AcPdAD$^+$ and NADH should bind to the same site on the enzyme. The behaviour shown in Fig. 2 was therefore, as expected, only observed at low substrate concentrations. At high concentrations of either AcPdAD$^+$ or NADH, competitive effects became evident, presumably as the formation of 'dead-end' complexes, [AcPdAD$^+$-enzyme-NADP$^+$] and [NADH-enzyme-NADPH], became favourable (Fig. 3). At a fixed low concentration (13.3 $\mu$M) of AcPdAD$^+$, concentrations of NADH greater than about 20 $\mu$M clearly began to inhibit the reaction (Fig. 3A). At a higher fixed concentration of AcPdAD$^+$ (133

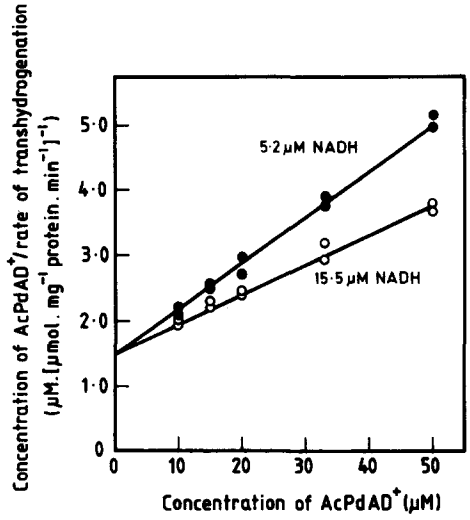



Fig. 2. NADPH-dependent reduction of AcPdAD$^+$ by NADH catalysed by solubilised H$^+$-Thase from *E. coli* displays ping-pong kinetics. Reduction of AcPdAD$^+$ was measured as described in the Methods in a medium containing 10 mM Mes (pH 6.0), 99.8 $\mu$M NADPH, 2 mM $MgSO_4$, 0.25 mM EDTA, 0.5 mg ml$^{-1}$ lysophosphatidylcholine, 1 mM dithiothreitol, 0.05% (mass/vol) Brij 35, 0.15 $\mu$g ml$^{-1}$ purified H$^+$-Thase, at 30° C. For the closed symbols the concentration of NADH was 5.2 $\mu$M, and for the open symbols, 15.5 $\mu$M. The concentration of AcPdAD$^+$ was varied as shown.

$\mu$M), more NADH was required before the inhibitory effect became evident. Fig. 3B shows that plots of [NADH]/$v$ versus [NADH] were parabolic, intersecting at [NADH] = 0, precisely as expected for a ping-pong reaction mechanism subject to substrate inhibition [24]. In Fig. 3C, the complementary effect, inhibition by high concentrations of AcPdAD$^+$ at a fixed, low concentration of NADH, is illustrated.

### 3.2. A deuterium isotope effect in transhydrogenase

Isotopic labelling experiments have shown that, in the reaction catalysed by $H^+$-Thase, $H^-$ is transferred to or from the 4A position of the nicotinamide ring of NAD(H), and to or from the 4B position of the nicotinamide ring of NADP(H) [25–27].

The effects of replacing with deuterium the 4A hydrogen of NADH, and the 4B hydrogen of NADPH, on the rate of the key reactions of $H^+$-Thase as shown in Fig. 1 are shown in Table 1. Two pH values, either side of the pH optimum (about pH 7.2 [17]) for 'forward' and 'reverse' transhydrogenation, were selected for these experiments. At pH 6.0, 'forward' transhydrogenation is predicted to be limited by release of NADPH from the enzyme, and 'reverse' transhydrogenation by the release of NADP$^+$ [17]. At pH 8.0, provided the substrates are saturating, 'forward' and 'reverse' transhydrogenation are probably limited by the rate of interconversion of the ternary complexes. In accordance with these expectations, at pH 8.0, simple 'reverse' transhydrogenation between [4B-$^2$H]NADPH and AcPdAD$^+$ was substantially slower (46%) than the corresponding reaction between [4B-$^1$H]NADPH and AcPdAD$^+$ (experiment B), but at pH 6.0 there was no isotope effect for this reaction (experiment A). The rate of simple 'forward' transhydrogenation between [4A-$^2$H]NADH and thio-NADP$^+$ at pH 8.0 was only slightly slower (10%) than that between [4A-$^1$H]NADH and thio-NADP$^+$ (experiment E), but, as expected, there was no significant difference between the $^1$H and the $^2$H reduced nucleotides at pH 6.0 (experiment D).

The relationship with pH of NADP(H)-dependent reduction of AcPdAD$^+$ by NADH is completely different to those for simple 'forward' and 'reverse' transhydrogenation [17]. This is because the reaction is most favourable when NADP$^+$ and NADPH remain bound to the enzyme, i.e., at low pH; at saturating concentrations of nucleotide substrates, NADP(H)-dependent reduction of AcPdAD$^+$ is probably limited by the interconversion of the ternary complexes (see Fig. 1). In support of this view, (i) the rate of AcPdAD$^+$ reduction with a combination of [4A-$^2$H]NADH and [4B-$^1$H]NADPH was considerably slower (46%) than with a combination of [4A-$^1$H] NADH and [4B-$^1$H]NADPH (Table 1, experiment F), and (ii) the rate of AcPdAD$^+$ reduction with a combination of [4A-$^1$H]-NADH and [4B-$^2$H]NADPH was similar to that with a combination of [4A-$^1$H]NADH and [4B-$^1$H]NADPH (experiment C). In the latter situation, reducing equivalents for AcPdAD$^+$ reduction are expected (Fig. 1) to be derived from the [4B-$^2$H]NADPH or [4B-$^1$H]NADPH only during the first catalytic turnover of the enzyme, and

Table 1
Deuterium isotope effects in solubilised, purified $H^+$-Thase from *Escherichia coli*

| Experiment | pH | Oxidised nucleotide | Reduced nucleotide(s) | Rate ($\mu$mol (mg protein)$^{-1}$ min$^{-1}$) |
|---|---|---|---|---|
| A: | 6.0 | AcPdAD$^+$ | | |
| (i) | | | [4B-$^1$H]NADPH | 1.65 ± 0.04 |
| (ii) | | | [4B-$^2$H]NADPH | 1.55 ± 0.03 |
| B: | 8.0 | AcPdAD$^+$ | | |
| (i) | | | [4B-$^1$H]NADPH | 2.98 ± 0.07 |
| (ii) | | | [4B-$^2$H]NADPH | 1.60 ± 0.03 |
| C: | 6.0 | AcPdAD$^+$ | | |
| (i) | | | [4B-$^1$H]NADPH *plus* [4A-$^1$H]NADH | 25.8 ± 0.5 |
| (ii) | | | [4B-$^2$H]NADPH *plus* [4A-$^1$H]NADH | 27.0 ± 1.4 |
| D: | 6.0 | thio-NADP$^+$ | | |
| (i) | | | [4A-$^1$H]NADH | 0.117 ± 0.002 |
| (ii) | | | [4A-$^2$H]NADH | 0.120 ± 0.007 |
| E: | 8.0 | thio-NADP$^+$ | | |
| (i) | | | [4A-$^1$H]NADH | 0.067 ± 0.003 |
| (ii) | | | [4A-$^2$H]NADH | 0.060 ± 0.002 |
| F: | 6.0 | AcPdAD$^+$ | | |
| (i) | | | [4B-$^1$H]NADPH *plus* [4A-$^1$H]NADH | 29.0 ± 0.9 |
| (ii) | | | [4B-$^1$H]NADPH *plus* [4A-$^2$H]NADH | 15.7 ± 0.2 |

The reduced nucleotides were prepared and reaction rates were measured as described in the Materials and Methods, except that in experiment C the [4A-$^1$H]NADH, and in experiment F, the [4B-$^1$H]NADPH were commercial samples. The experimental media are described in Fig. 2. In experiments at pH 8.0, Mes was replaced by Tricine. The nucleotide concentrations were as follows: AcPdAD$^+$, 133 $\mu$M; thio-NADP$^+$ 100 $\mu$M; NADPH, 100 $\mu$M; NADH, 100 $\mu$M (experiments C, D and E) and 40 $\mu$M (experiment F). The enzyme concentration (in $\mu$g ml$^{-1}$) was 3.9 (in experiments A and B), 0.48 (in C and F) and 4.8 (in D and E). All rates are mean values of four recordings with the standard deviation as shown.

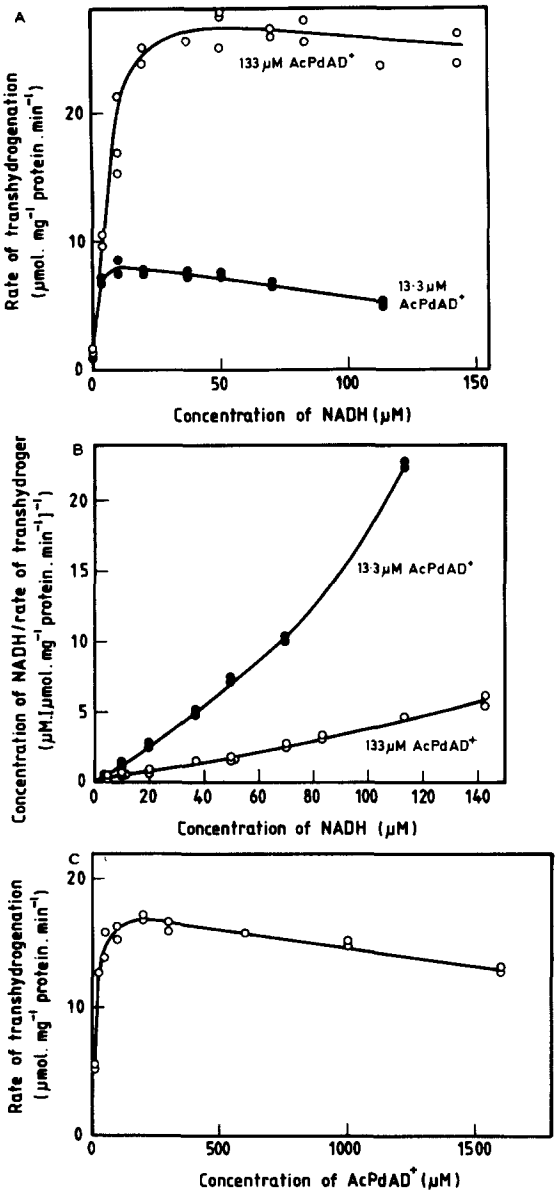



Fig. 3. Substrate inhibition during NADPH-dependent reduction of $AcPdAD^+$ by NADH catalysed by solubilised $H^+$-Thase from *E. coli*. Experiments were carried out as described in Fig. 2, using 0.24 μg $ml^{-1}$ $H^+$-Thase. (A,B) The NADPH concentration was 86.3 μM; for the closed symbols the concentration of $AcPdAD^+$ was 13.3 μM, and for the open symbols, 133 μM; the concentration of NADH was varied as shown. (C) The NADPH and NADH concentrations were 93.0 and 5.0 μM, respectively, and the $AcPdAD^+$ concentration was varied as shown.

therefore the deuterium isotope effect will not be resolved on the time scale of measurement; during the steady state, the [4A-$^1$H]NADH will, in both cases, supply the $H^-$.

Note that, in the above experiments, apart from removal of enzymes employed in synthesis, the reduced nicotinamide nucleotides were, as recommended [22], used without purification. The main reason for this is that they are labile in solution at neutral pH. However, conditions during synthesis were chosen to ensure that the reactions proceeded to completion [22,23] and this was confirmed by monitoring the course of the reaction. Even so it was separately checked that reagents which would be carried over into the reaction medium were without significant effect on transhydrogenation (not shown). Furthermore, for each deuterated derivative, control experiments were always performed with the hydrogenated derivative prepared by the same method at the same time (see Table 1).

## 4. Discussion

### *4.1. The deuterium isotope effect in the solubilised, purified transhydrogenase of E. coli*

The data described above are consistent with the idea that during (a) reduction of $AcPdAD^+$ by NADPH at pH 8.0, and (b) NADP(H)-dependent reduction of $AcPdAD^+$by NADH, the step involving the transfer of $H^-$ contributes towards rate limitation of the reactions. On the basis of evidence presently available, it is not possible to determine whether the rate-limiting transfer of $H^-$ is directly from one nucleotide to the other, or if it is between nucleotide and amino acid residues of the protein within the ternary complex. Conformational rearrangements of the protein during interconversion of the ternary complexes might also contribute to the limitation of rate. The fact that the isotope effect for the reduction of thio-$NADP^+$ by NADH at pH 8.0 is only about 10%, which may not be significant, suggests, either that these conformational rearrangements are particularly critical for the analogue nucleotide, or that, in contrast to NADPH (see below), release of thio-NADPH from the enzyme is slow even at alkaline pH. The latter possibility was excluded by the following experiment (not shown). It was first confirmed that, at pH 6.0, the rate of oxidation of NADH by thio-$NADP^+$ was enhanced by the addition of $AcPdAD^+$, one of the observations which led to the suggestion that 'forward' transhydrogenation from NADH to thio-$NADP^+$ is limited by the rate of release of thio-NADPH from the enzyme (see [17]). However, at pH 8.0 and otherwise similar conditions there was no stimulation by $AcPdAD^+$; evidently, as with the equivalent physiological nucleotide, the rate of release of thio-NADPH from the enzyme increases substantially with pH.

At low pH, there was no deuterium isotope effect either for the reduction of $AcPdAD^+$ by NADPH ('reverse' transhydrogenation), or for the reduction of thio-$NADP^+$ by NADH ('forward' transhydrogenation) indicating that, under these conditions, a step in the turnover of the enzyme, other than the interconversion of the ternary complexes, is rate-limiting: we proposed [17] that, in fact, the rate-limiting steps are release from the enzyme of $NADP^+$ and of thio-NADPH, respectively.

In the light of these findings on solubilised *E. coli* $H^+$-Thase, there is a need to repeat, with purified enzyme

and at a wide range of pH values, the early experiments performed on submitochondrial particles, in which no deuterium isotope effect was observed [18].

### 4.2. Steady-state kinetics of transhydrogenase

In the Appendix a steady-state rate equation is derived for the reduction of $AcPdAD^+$ in the presence of both NADH and NADPH. Experiments described here and elsewhere indicate that under such conditions, $AcPdAD^+$ reduction can proceed either by simple 'reverse' transhydrogenation (top left to bottom left in Fig. 1) or by the cyclic pathway involving bound $NADP^+$ and NADPH (dashed lines in Fig. 1). Because a rather large number of enzyme intermediates has to be considered in the reaction mechanism (see also Fig. 5), and especially because it was desirable to include the protonation reactions, the equation is complex. However, to produce a fit with experimental data, minimisation routines indicate that it can only be satisfied by a narrow range of rate constants and dissociation constants.

A primary objective of the analysis was to determine whether the complex pH dependences of the reactions catalysed by $H^+$-Thase [17] could be quantitatively explained by two simple assumptions, (i) that the binding of $NADP^+$ and of NADPH are accompanied by an increase in the $pK_a$ of a functional group on the enzyme, and therefore, at optimal values of pH, by proton binding, and (ii) that interconversion of the ternary complexes can only take place when the enzyme is protonated. Thus, first note that when the concentrations of NADPH and $AcPdAD^+$ are saturating and in the absence of NADH, the rate equation from the Appendix reduces to:

$$v = \frac{k_3 k_4 K_H [H^+][E_0]}{k_4 K_H^2 + (k_4 + k_3) K_H [H^+] + k_3 [H^+]^2}$$

which predicts a bell-shaped pH dependence with a maximum rate at $pH = pK_a - \log\sqrt{k_4/k_3}$ (where $pK_a = -\log K_H$). At pH values less than the optimum, the reaction is limited predominantly by $NADP^+$ release, and above the optimum, by the interconversion of the ternary complexes. Fitting with experimental data (Fig. 4) gives a first approximation for the values of $k_3$ and $k_4$. In an equivalent way, the pH dependence for 'forward' transhydrogenation (in this case the reduction of thio-$NADP^+$ by NADH) can also be precisely fitted to experimental data (Fig. 4).

When NADH (also at saturating concentrations) is present in addition to NADPH and $AcPdAD^+$, the equation from the Appendix reduces to

$$v = \frac{k_3 (k_4 K_H + k_{-8}[H^+])[H^+]}{\{k_4 K_H + (k_{-8} + k_3)[H^+]\}(K_H + [H^+])}$$

Now, the substantially higher rates of $AcPdAD^+$ reduction observed with a combination of NADH and NADPH, compared with NADPH alone, indicate that $k_4$ is less than $k_3$ and $k_{-8}$ [17], and therefore, this equation predicts that the pH dependence of the reaction should resemble an acid-base titration curve, in excellent agreement with the data, see Fig. 4. The inflexion point on the curve is the $pK_a$ of the group on the enzyme which binds a proton as a consequence of either $NADP^+$ or NADPH binding. The fitting process also leads to a refinement of the values of $k_3$ and $k_4$ and to an estimate of $k_{-8}$.

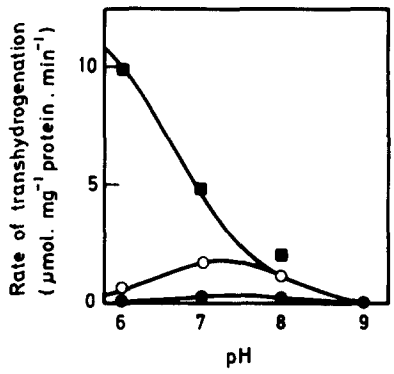



Fig. 4. Fitting of the steady-state rate equation to experimental data on the pH dependence of reactions catalysed by transhydrogenase. Data (from [17]) are shown (i) for reduction of $AcPdAD^+$ by NADPH (open circles), (ii) for reduction of thio-$NADP^+$ by NADH (closed circles), and (iii) for the NADPH-dependent reduction of $AcPdAD^+$ by NADH (squares). The curves are constructed as described in the text, using the parameters listed in Table 2.

The agreement, between the predictions of the rate equation and the observed pH dependences of transhydrogenase, provides support for the two key assumptions mentioned above. The first assumption underpins the model proposed for proton translocation [17], the second is critical, within the framework of the proposed model, to prevent the enzyme from 'slipping' (catalysing $H^-$ transfer without proton transfer).

As well as providing a good fit for the pH dependences of $H^+$-Thase, the rate equation also leads to the following observations:

(1) When the concentration of NADH approaches zero, only simple 'reverse' transhydrogenation is possible. At less than saturating concentrations of nucleotides, the equation then simplifies to that for a reaction following a random order ternary complex mechanism having Michaelis-Menten kinetics with respect to both $AcPdAD^+$ and NADPH. Such properties are observed experimentally [13–16].

(2) At saturating concentrations of NADPH, the equation reduces to a form equivalent to that for a ping-pong reaction mechanism with respect to $AcPdAD^+$ and NADH. For example, in this situation it can be shown that, for $AcPdAD^+$:

$$K_m^{app}/V^{app} = \frac{K_2(K_H + [H^+])}{k_3 [H^+][E_0]}$$

and therefore the ratio, $K_m^{app}/V^{app}$, is independent of the concentration of the variable substrate concentration (NADH), a characteristic of ping-pong reactions. The result was experimentally demonstrated for NADPH-dependent reduction of AcPdAD$^+$ by NADH catalysed by H$^+$-Thase (Fig. 2). On this basis, the value of $K_2$ can be calculated. When terms for the formation of dead-end complexes are included in the derivation (not shown), the equation takes the form of one appropriate for a ping-pong mechanism with substrate inhibition (see Fig. 3), and hence, the value of $K_7$ can be deduced. To maintain Michaelis-Menten behaviour, in this derivation it was necessary to assume that there is some interaction between the nucleotide binding sites; specifically the rate constant for NADPH binding is smaller in the dead-end complex, [NADH-enzyme-NADPH], than in the catalytic complex, [AcPdAD$^+$-enzyme-NADPH].

(3) At this point it will be clear that many of the values of the constants for the complete equation derived in the Appendix have been obtained from experimental data (Table 2), and therefore, the equation can be used in a more predictive manner. For example, (a) for the reduction of AcPdAD$^+$ by NADPH in simple 'reverse' transhydrogenation (pH 6.0, 2 mM $MgSO_4$), the predicted $K_m$ values for AcPdAD$^+$ and NADPH are 1.0 and 0.2 $\mu$M, respectively, in reasonable agreement with experimentally obtained values of 1.0 and 0.8 $\mu$M (unpublished observations), (b) for NADPH-dependent reduction of AcPdAD$^+$ by NADH (pH 6.0, 2 mM $MgSO_4$) the predicted $K_m$ for NADPH is 0.6 $\mu$M in good agreement with the measured value of 0.1 $\mu$M [17], and (c) at pH 7.5 the binding constants for NADH ($K_7$) and NADPH ($\{k_{-1}/k_1\}\{K_H/(K_H + [H^+])\}$) are predicted to be 11.0 $\mu$M and 1.8 $\mu$M, respectively; measured values for mitochondrial H$^+$-Thase at this pH were 9.5 and 5.7 $\mu$M, respectively [12].

Table 2
Estimated values of constants for the steady-state rate equation

| | |
|---|---|
| $k_1$ | $1.0 \cdot 10^9$ M$^{-1}$ min$^{-1}$ $K = k_{-1}/k_1 = 2.0 \cdot 10^{-6}$ M |
| $k_{-1}$ | $2.0 \cdot 10^3$ min$^{-1}$ |
| $K_2$ | $2.1 \cdot 10^{-4}$ M |
| $k_3$ | $1.1 \cdot 10^5$ min$^{-1}$ |
| $k_4$ | $3.2 \cdot 10^3$ min$^{-1}$ |
| $K_7$ | $1.1 \cdot 10^{-5}$ M |
| $k_{-8}$ | $2.0 \cdot 10^4$ min$^{-1}$ |
| $K_H$, $K_H^1$ | $10^{-6.7}$ M |

Notes: (1) the values of $K_H$, and $K_H^1$ are similar at approx. 2 mM $Mg^{2+}$ but diverge at higher and low concentrations of the cation (unpublished observations); (2) with thio-NADP$^+$ replacing NADP$^+$, the value of $k_{-1}$ is $4.1 \cdot 10^2$ min$^{-1}$ and $k_{-8}$ is $2.0 \cdot 10^4$ min$^{-1}$.

(4) In the model described [17], proton translocation by transhydrogenase is coupled to the alternate binding of NADPH and release of NADP$^+$ to and from the enzyme – a barrier, whose operation is controlled by the occupancy of the NADP(H) site, directs proton access to one side of the membrane or the other. It was based on the conclusion, for which further evidence is presented in this paper, that

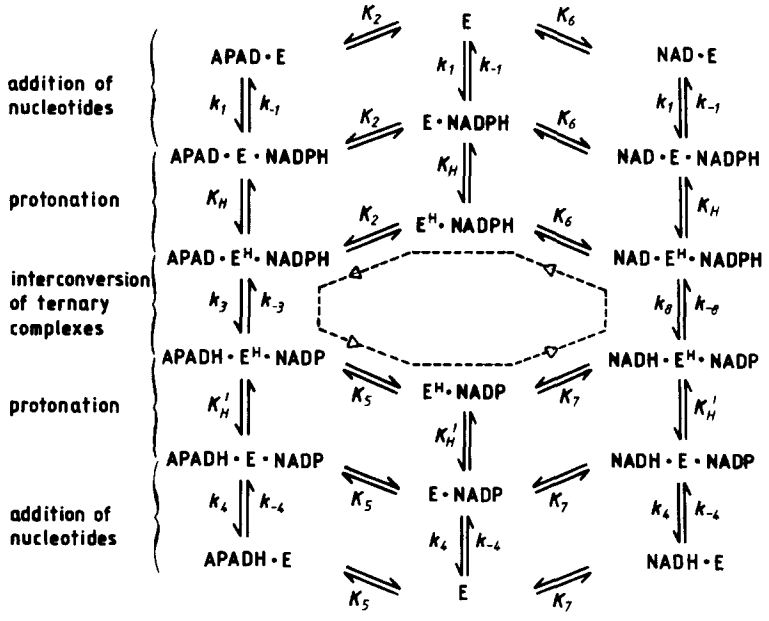



Fig. 5. A scheme for the reactions catalysed by transhydrogenase. For simplicity, free nucleotides and free protons are omitted from the figure. The NADP(H) binding site is positioned on the right of the enzyme, E, the NAD(H) binding site on the left. $E^H$ indicates that the enzyme is protonated. The symbol $K$ indicates a dissociation constant, and $k$ indicates a rate constant. Rate constants are designated positive in the direction of AcPdAD$^+$ reduction. The proton dissociation constants for [enzyme-NADPH] and [enzyme-NADP$^+$] are distinguished from one another ($K_H$ and $K_H^1$, respectively), but they might reflect protonation/deprotonation from the same functional group and, at least under some conditions, have identical values (see text). Following the conclusion [17], the free enzyme (or the enzyme with only the NAD(H) site occupied) is unprotonated between pH 6 and 8 because, when the NADP(H) site is unoccupied, the value of $K_H$ (and $K_H^1$) is presumed to be very large. It is recognised that dead-end complexes can form, and are permitted in the analysis (see text), but are not shown in the figure. The dashed line indicates the pathway for NADP(H)-dependent reduction of AcPdAD$^+$ by NADH (see text).

the binding constants for $NADP^+$ and NADPH are strongly dependent on pH. In earlier reports, the importance of substrate binding energy to the coupling mechanism has been considered, mainly on the basis of changes in $K_m$ induced by energisation of membrane preparations or changes in pH of solubilised or uncoupled enzyme [14,28]. Thus, changes in the $K_m$ for $NAD^+$ and NADH (or their analogues) upon energisation have been described and cited in support of the view that changes in the binding affinity of these nucleotides are central to the process of coupling with proton translocation. However, the equation derived in the Appendix illustrates the fact that, also within the framework of our model, in which the affinities for $NAD^+$ and NADH (and their analogues) are independent of pH (and $\Delta p$), and therefore do not significantly contribute to the energetics of the reaction, large changes in their $K_m$ values with pH (or $\Delta p$) can be expected. For example, even for the solubilised enzyme, the expression for the $K_m$ for $AcPdAD^+$ during simple 'reverse' transhydrogenation includes a term in $[H^+]$ (not shown); thus, a decrease in solution pH of one unit can lead to a decrease in the $K_m$ for $AcPdAD^+$ of up to 5-fold. A feature of the equation derived in the Appendix is that it can be readily adapted to a form which is appropriate for $H^+$-Thase located in a vesicle membrane. For example, during the reduction of $AcPdAD^+$ by NADPH in everted *E. coli* membrane vesicles (or reconstituted proteoliposomes), NADPH binding will be accompanied by $H^+$ binding on the outside of the vesicle (at the reaction described by the $K_H$ equilibrium in Fig. 5), and $NADP^+$ release will be accompanied by $H^+$ release on the inside of the vesicle (at the $K'_H$ reaction). The generation of a proton electrochemical gradient across the membrane (e.g., by respiration) will influence these protonation and deprotonation equilibria through the effect of the bulk phase $pH_{OUT}$ and $pH_{IN}$, respectively, and through the conversion of the electrical membrane potential into the respective pH components by proton channels or wells which link the bulk phases with the sites of the protolytic reactions [31]. Thus, a form of the equation can be derived (not shown) in which protonation and deprotonation are separated by a membrane. Different values of pH can be chosen for the inside and outside aqueous phases to represent $\Delta p$. Even though, within the constrains of our model, the true *affinities* ($K_D$ values) of $NAD^+$, NADH and their analogues $AcPdAD^+$ and AcPdADH do not change with $\Delta p$, the apparent $K_m$ values do. Because terms in $[H^+]$ appear in both the nominator and denominator of the equation, the direction and magnitude of the predicted changes in apparent $K_m$ with $\Delta p$ are complex, a fact which is reflected in experimental observation. Thus, the addition of protonophore to proteoliposomes inlaid with mitochondrial transhydrogenase led to a decrease in the apparent $K_m$ for $NAD^+$ but an increase in the $K_m$ for $AcPdAD^+$, during reduction by NADPH [14]. In absolute terms the predicted change in $K_m$ can be quite large. For example, during reduction of $NADP^+$ by NADH in everted transhydrogenase vesicles, the equation predicts that the $K_m$ for NADH is 5-fold greater when $pH_{OUT} = pH_{IN} = 7.0$ than when $pH_{OUT} = 7.0$ and $pH_{IN} = 6.0$. The conclusion is that, in the absence of a detailed understanding of the reaction mechanism, a change in $K_m$ can not be taken as evidence for a change in nucleotide binding affinity or provide information on the nature of the coupling reactions.

## Appendix 1

*A rate equation for the reactions catalysed by transhydrogenase*

The essential features of the reaction scheme, shown in Fig. 5, are:

(i) There are separate sites on the enzyme for $NAD^+$ and NADH (and their analogues), and for $NADP^+$ and NADPH (and their analogues) [12].

(ii) Nucleotide substrates bind randomly to the enzyme; $H^-$ transfer to or from a nucleotide can only occur within a ternary complex [13–16]. For example, during the reduction of $AcPdAD^+$ by NADPH, the reaction proceeds downwards from the upper left quadrant of Fig. 5. The binding of $AcPdAD^+$ is described by a dissociation constant but, because under some conditions NADPH binding and release can be slow, these reactions are described by individual rate constants. It is assumed that, in the formation of catalytic ternary complexes, there is no interaction between the nucleotide binding sites; for example the binding constant ($K_2$) for $AcPdAD^+$ is similar for the free enzyme and for [enzyme-NADPH]. Equivalent considerations apply to the binding of substrates in the reduction of (a) $NADP^+$ by AcPdADH (lower left quadrant of Fig. 5), (b) $NAD^+$ by NADPH (upper right quadrant), and (c) $NADP^+$ by NADH (lower right quadrant). The formation of dead-end complexes (both bound nucleotides oxidised or both reduced) is not shown in Fig. 5.

(iii) Depending on pH, the binding of either $NADP^+$ or NADPH to any species of the enzyme may result in protonation of the enzyme [17] (or of the bound NADP(H) – see [1]). As discussed [17], this can be viewed as a change in the $pK_a$ from a low value ($\ll 5.0$) when the NADP(H) site is unoccupied, to a value in the region of 6.0–7.0 (dependent on salt concentration) when the site is occupied. Protonation is assumed to be fast. For the equation derived below, the same acid dissociation constant is employed for [enzyme-$NADP^+$] and for [enzyme-NADPH], i.e., $K_H = K^1_H$, since it was suggested that the same dissociable group on the enzyme is responsible for both protonation reactions [17]. In fact, if the derivation is re-formulated to include different acid dissociation constants, its fit with experimental data indicates that, in the experimental conditions described for Fig. 4, the constants must have a very similar value (not shown). Occupation of the NAD(H) site does not influence protonation.

(iv) As suggested from the pH dependences of the rates of $AcPdAD^+$ reduction by NADH in the presence of either $NADP^+$ or of NADPH, at saturating concentrations of nucleotides, there is a step in the interconversion of the ternary complexes which can only occur when the enzyme is protonated [17].

(v) The reduction of $AcPdAD^+$ in the presence of both NADH and NADPH can proceed, either by simple 'reverse' transhydrogenation (from top left to bottom left in Figs. 1 and 5), or by the cyclic mechanism involving permanently bound NADP(H) (indicated by the dashed lines in Figs. 1 and 5). The pathway taken will depend on the rate of release of NADP(H) from the enzyme. For both reactions, the rate in steady state (for $[NADP^+] = [NAD^+] = [AcPdADH] = 0$) is given by:

$$v = k_3 \cdot [AcPdAD^+ \cdot E^H \cdot NADPH]$$

The complete equation can be derived from this by application of the King-Altman method [29] using the modification of Cha [30], to incorporate the steps rapidly reaching equilibrium, as outlined by Cornish-Bowden [24]. In the treatment leading to the equation presented below, the formation of dead-end complexes was ignored: during the reduction of $AcPdAD^+$ by NADPH in the absence of products, their concentration is negligible; during NADPH-dependent reduction of $AcPdAD^+$ by NADH, dead-end complexes are responsible for substrate inhibition (see Discussion) but experiments suggest that this only becomes significant at high concentrations of either $AcPdAD^+$ or NADH. The relevant equation is:

$$\begin{aligned} v = {} & c_{22}\{c_{23} + c_{24}[NADH]\} \\ & \times [NADPH][AcPdAD^+][H^+][E_0] \\ & / \{c_0 + c_1[NADPH] + c_2[AcPdAD^+] + c_3[NADH] \\ & + c_4[NADPH][H^+] \\ & + c_5[AcPdAD^+][H^+] + c_6[NADH][H^+] \\ & + c_7[NADPH][AcPdAD^+] \\ & + c_8[NADPH][NADH] + c_9[AcPdAD^+][NADH] \\ & + c_{10}[NADPH][NADH][H^+] \\ & + c_{11}[AcPdAD^+][NADH][H^+] \\ & + c_{12}[NADPH][AcPdAD^+][H^+] \\ & + c_{13}[NADH]^2 + c_{14}[NADH]^2[H^+] \\ & + c_{15}[NADPH][NADH][H^+]^2 \\ & + c_{16}[NADPH][AcPdAD^+][H^+]^2 \\ & + c_{17}[NADPH][AcPdAD^+][NADH] \\ & + c_{18}[NADPH][AcPdAD^+][NADH][H^+] \\ & + c_{19}[NADPH][AcPdAD^+][NADH][H^+]^2 \\ & + c_{20}[AcPdAD^+][NADH][H^+] \\ & / (K_2 + [AcPdAD^+]) + c_{21}[AcPdAD^+][NADH]^2[H^+] \\ & / (K_2 + [AcPdAD^+])\} \end{aligned}$$

where,

$$\begin{aligned} c_0 &= k_{-1}K_2k_4K_H^2K_7^2 & : c_1 &= k_1K_2k_4K_H^2K_7^2 \\ c_2 &= k_{-1}k_4K_7^2K_H^2 & : c_3 &= 2k_{-1}K_2k_4K_7K_H^2 \\ c_4 &= k_1K_2k_4K_7^2K_H & : c_5 &= k_3k_4K_7^2K_H \\ c_6 &= k_{-1}K_2K_7k_{-8}K_H & : c_7 &= k_1k_4K_7^2K_H^2 \\ c_8 &= k_1K_2k_4K_7K_H^2 & : c_9 &= k_{-1}k_4K_7K_H^2 \\ c_{10} &= k_1K_2K_7K_H(k_4 + k_{-8}) & : c_{11} &= (k_{-1}k_{-8} + k_3k_4) K_7K_H \\ c_{12} &= k_1K_7^2K_H(k_3 + k_4) & : c_{13} &= k_{-1}K_2k_4K_H^2 \\ c_{14} &= k_{-1}K_2k_{-8}K_H & : c_{15} &= k_1K_2K_7k_{-8} \\ c_{16} &= k_1k_3K_7^2 & : c_{17} &= k_1k_4K_7K_H^2 \\ c_{18} &= k_1K_7K_H(k_3 + k_4 + k_{-8}) & : c_{19} &= k_1K_7(k_3 + k_{-8}) \\ c_{20} &= K_2k_3k_4K_7K_H & : c_{21} &= K_2k_3k_4K_H \\ c_{22} &= k_1k_3K_7 & : c_{23} &= k_4K_7K_H \\ c_{24} &= k_4K_H + k_{-8}[H^+] & & \end{aligned}$$

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