Mechanism of hydride transfer during the reduction of 3-acetylpyridine adenine dinucleotide by NADH catalyzed by the pyridine nucleotide transhydrogenase of *Escherichia coli*

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Abstract **The pyridine nucleotide transhydrogenase is a proton pump which catalyzes the reversible transfer of a hydride ion equivalent between NAD^+ and $NADP^+$ coupled to translocation of protons across the cytoplasmic membrane. The enzyme also catalyzes the reduction of the NAD^+ analog 3-acetylpyridine adenine dinucleotide ($AcPyAD^+$) by NADH. It has been proposed (Hutton et al. (1994) Eur. J. Biochem. 219, 1041–1051) that this reaction requires NADP(H) as an intermediate. Thus, $NADP^+$ bound at the NADP(H)-binding site on the transhydrogenase would be reduced by NADH and reoxidized by $AcPyAD^+$ binding alternately to the NAD(H)-binding site. The reduction of $AcPyAD^+$ by NADPH would be a partial reaction in the reduction of $AcPyAD^+$ by NADH. Using cytoplasmic membrane vesicles from mutants having elevated activities for transhydrogenation of $AcPyAD^+$ by NADH in the absence of added NADP(H), the kinetics of reduction of $AcPyAD^+$ by NADH and NADPH have been compared. The K_m values for the reductants NADPH and NADH over a range of mutants, and for the non-mutant enzyme, differed to a much lesser degree than the K_m for $AcPyAD^+$ in the two reactions. The K_m^{AcPyAD} values for the transhydrogenation of $AcPyAD^+$ by NADH were over an order of magnitude greater than those for the transhydrogenation of $AcPyAD^+$ by NADPH. It is unlikely that $AcPyAD^+$ binds at the same site in both reactions. A plausible explanation is that this substrate binds to the NADP(H)-binding site for transhydrogenation by NADH. Thus, a hydride equivalent can be transferred directly between NADH and $AcPyAD^+$ under these conditions.**

Key words: Transhydrogenase; Pyridine nucleotide; Hydride transfer; Enzyme mechanism; *Escherichia coli*

1. Introduction

Pyridine nucleotide transhydrogenase, found in the cytoplasmic membrane of *Escherichia coli* and other bacteria, and in the inner mitochondrial membrane, catalyzes transmembrane proton translocation coupled to transfer of a hydride ion equivalent between NAD^+ and $NADP^+$ [1–5]. The enzyme from *E. coli* is composed of two subunits, α and β, organized as an $\alpha_2\beta_2$ structure [6,7]. The NAD(H) and NADP(H)-binding sites are on the α and β subunits, respectively [8,9]. Equivalent sites are present on the mitochondrial enzyme [10–12]. The relative simplicity of the transhydrogenase makes it an ideal enzyme with which to study the mechanism of transmembrane proton pumping.

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Abbreviations: $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide; Mes, 2-[*N*-morpholino]ethanesulfonic acid.

Transhydrogenase activity is usually measured by following the reduction of $AcPyAD^+$, an NAD^+ analog, by NADPH ('NADPH → $AcPyAD^+$ transhydrogenation'). Recently, Hutton et al. [13] showed that $AcPyAD^+$ can be reduced by NADH ('NADH → $AcPyAD^+$ transhydrogenation') in a solubilized preparation of the *E. coli* transhydrogenase. Wu et al. [14] had previously described this reaction with the mitochondrial enzyme. As with the mitochondrial enzyme, this reaction depended on the presence of NADP(H). Jackson and coworkers [13,15,16] have proposed that $NADP^+$ at the NADP(H)-binding site is reduced by NADH bound at the NAD(H)-binding site. The NADPH so formed is then reoxidized by the $AcPyAD^+$ subsequently occupying the NAD(H)-binding site (the 'cyclic' mechanism). In contrast to this mechanism, we have shown with membrane-bound transhydrogenase that reduction of $AcPyAD^+$ by NADH will occur at pH 6 in the absence of NADP(H), either enzyme-bound or added [17]. The reaction was accelerated by NADP(H). We proposed that NADP(H) and/or pH 6 cause the enzyme to adopt a conformation favouring the reduction of $AcPyAD^+$ by NADH. In the current paper we present evidence suggesting that $AcPyAD^+$ is bound to a different site when reduced by NADH than when reduced by NADPH. The most plausible site for $AcPyAD^+$ binding in the former reaction is the NADP(H) site. Thus, transfer of a hydride equivalent could occur directly between NADH and $AcPyAD^+$ without the involvement of NADPH.

2. Materials and methods

2.1. Bacterial strain, plasmids and mutagenesis

E. coli JM109 cells containing wild-type (pSA2) or mutant plasmids were grown overnight at 37°C in LB broth. The medium was shaken at 250 rpm in a New Brunswick Scientific Controlled Environment Incubator Shaker. Plasmid pSA2 contains the *pnt* genes of the pyridine nucleotide transhydrogenase of *E. coli* introduced into the pGEM-7Zf(+) plasmid [6].

Plasmid pSA2 was used to isolate single-stranded phagemid DNA. Site-directed mutagenesis to convert selected residues was performed according to the method of Taylor et al. [18] using degenerate primers. The reagents and protocols as outlined in the Amersham mutagenesis kit were followed, except that competent *E. coli* JM109 cells were used for transformation. Plasmid DNA was prepared from individual colonies, and the mutants were identified by double-stranded DNA sequencing. The entire coding region of the *pnt* genes from each mutant was completely resequenced.

The α-carboxy-terminus deletion mutant had the six carboxy-terminal amino acid residues of the α subunit removed by mutation [19].

2.2. Preparation of membrane vesicles containing transhydrogenase

The cell cultures were harvested by centrifugation at 4400 × *g* for 20 min. The cell pellets were washed by resuspension in 0.9% NaCl followed by centrifugation at 12000 × *g* for 15 min. Cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 7.8, 1 mM DTT, 1 mM

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EDTA) at 1 g wet weight/5 ml. All steps were performed at 0–4°C. The cells were lysed by passage through an AMINCO French Pressure Cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12000×*g* for 10 min. The supernatant was centrifuged at 252000×*g* for 2 h, and the membrane pellet containing everted membrane vesicles was suspended in buffer A at 1 g wet weight/5 ml. Membrane vesicles (1.5 ml) were layered on a 6 ml sucrose cushion [45% sucrose (w/w) in buffer A] and centrifuged in a Beckman type 65 fixed angle rotor at 40000 rpm (139000×*g*) for 1 h. The outer membrane fraction pelleted to the bottom of the tube and was discarded while the everted membrane vesicles banded at the interface and were removed by a syringe. The vesicles were diluted with buffer A then centrifuged at 252000×*g* for 3 h. The washed membrane pellet was suspended in buffer A for use.

2.3. Measurement of NADPH→AcPyAD$^+$ and NADH→AcPyAD$^+$ transhydogenation activities

Transhydrogenation of AcPyAD$^+$ by NADPH was measured as described previously [20]. An appropriate amount of washed membrane (20–100 μg protein) was added to 1 ml of 50 mM sodium phosphate buffer (pH 7), 0.5 mM EDTA, 2 mM DTT, 0.01% Brij 35 containing AcPyAD$^+$ at 1 mM and NADPH at 0.5 mM. Reduction of AcPyAD$^+$ was followed at 375 nm using a Perkin-Elmer Lambda 3A UV/VIS spectrophotometer. For assay of transhydrogenase activities at pH 6 the buffer was 50 mM Mes-KOH (pH 6.0) containing 0.5 mM EDTA, 2 mM DTT and 0.01% Brij 35. For transhydrogenation of AcPyAD$^+$ by NADH, 1 mM NADH was added instead of NADPH. Protein concentration was determined by the method of Lowry et al. [21].

2.4. Materials

All biochemicals including NADH, AcPyAD$^+$, NADP$^+$ and NADPH were obtained from Sigma Chemical Co. LB broth was supplied by Difco.

3. Results

3.1. Enzyme preparations used

Buffer-washed cytoplasmic membrane vesicles were chosen for this study since the enzyme was in its native milieu. Furthermore, there was a very high level of enzyme expression from the plasmids encoding the transhydrogenase such that the plasmid-encoded transhydrogenase was the major protein in the cytoplasmic membrane [6,20]. Various mutant transhydrogenases were selected for study. These were characterized by having higher than normal activity for the reduction of AcPyAD$^+$ by NADH in the absence of added NADP(H). They were incorporated to normal non-mutant levels in the cytoplasmic membrane.

The V_{max} values for the reduction of AcPyAD$^+$ by NADPH and by NADH using washed membranes of normal and mutant cells are shown in Table 1. Although showing lower activities than the non-mutant enzyme in NADPH→AcPyAD$^+$ transhydrogenation, the βH91K, βH345N, αH450T mutants and the α-carboxy-terminus deletion mutant were very much more active at pH 7 than the non-mutant enzyme in NADH→AcPyAD$^+$ transhydrogenation in the absence of added NADP(H). The latter activities were increased when measurements were made at pH 6. The rate of reduction of AcPyAD$^+$ by NADPH is lower at pH 6 [15,17,22].

3.2. Kinetics of NADPH→AcPyAD$^+$ and NADH→AcPyAD$^+$ transhydrogenations

The K_m values for the reduction of AcPyAD$^+$ by NADPH and NADH are listed in Table 2. Both non-mutant and mutant enzymes gave linear Lineweaver-Burk and Eadie-Hofstee plots [23] in the NADPH→AcPyAD$^+$ transhydrogenation (Fig. 1). The K_m values for the non-mutant enzyme were of a similar order to those measured previously (K_m^{NADPH}, 52 μM; K_m^{AcPyAD}, 83 μM) [9]. The K_m values for the mutant enzymes were lower than those of the non-mutant transhydrogenase but the extent of the decreases did not parallel the lowered enzyme activities of the mutants. Similarly, the differences in the K_m values for substrates in the NADH→AcPyAD$^+$ reaction did not match the very large differences between the activities of the different mutants. A major difference was observed when comparing the K_m for AcPyAD$^+$ between NADPH→AcPyAD$^+$ and the NADH→AcPyAD$^+$ transhydrogenations. The K_m^{AcPyAD} at pH 7 was at least 10-fold higher for NADH→AcPyAD$^+$ transhydrogenation. The K_m^{AcPyAD} for the non-mutant enzyme

Table 1
V_{max} values for transhydrogenase activities of washed membrane vesicles from non-mutant and mutant strains measured at pH 6 and 7

Strain	V_{max} (μmol/min per mg protein)		
	NADPH→AcPyAD$^+$	NADH→AcPyAD$^+$	
	pH 7	pH 7	pH 6
Non-mutant	9.3	0.49	9.8
βH91K	0.22	14.4	52.3
βH345N	1.6	3.6	4.4
αH450T	2.3	5.2	32.7
α-Carboxy-terminus deletion mutant	0.42	19.3	35.4

Transhydrogenase activities were measured as described in Section 2. The data were plotted by the method of Eadie and Hofstee [23].

Table 2
K_m values (μM) for transhydrogenase activities of washed membrane vesicles from non-mutant and mutant strains measured at pH 6 and 7

Strain	pH 7				pH 6	
	K_m^{NADPH}	K_m^{AcPyAD}	K_m^{NADH}	K_m^{AcPyAD}	K_m^{NADH}	K_m^{AcPyAD}
Non-mutant	62	66	61	non-linear	36	449
βH91K	40	38	31	521	44	604
βH345N	15	37	100	884	33	1847
αH450T	19	31	58	419	65	730
α-Carboxy-terminus mutant	49	23	38	819	30	414

Transhydrogenase activities were determined as described in Section 2. The data were plotted by the method of Eadie and Hofstee [23].

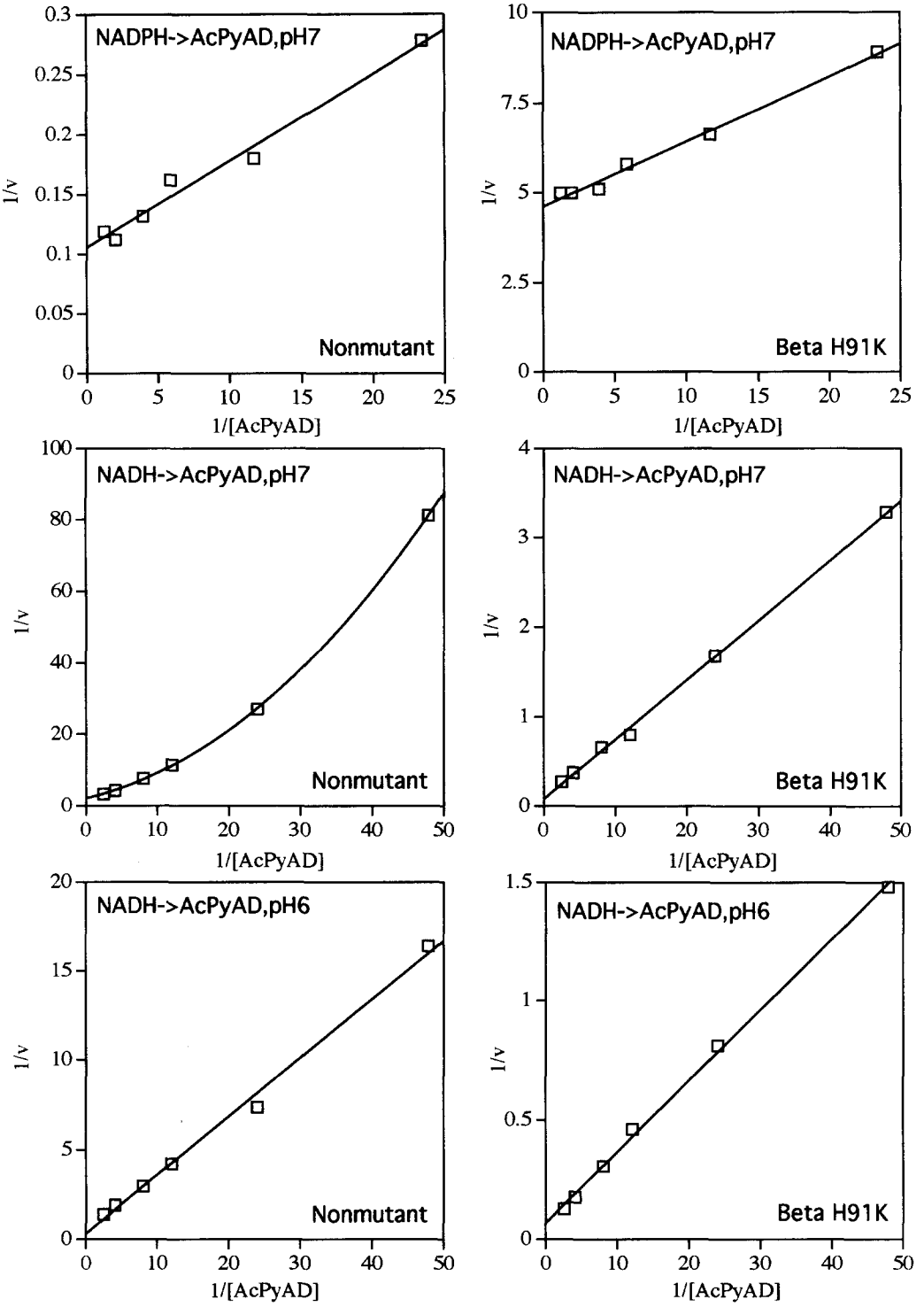

Fig. 1. Representative kinetic plots for NADPH→AcPyAD$^+$ transhydrogenation at pH 7 and NADH→AcPyAD$^+$ transhydrogenation at pH 6 and 7 by cytoplasmic membrane vesicles from non-mutant and βH91K mutant strains. υ is expressed as μmol AcPyADH formed/min per mg protein. The concentration of AcPyAD$^+$ is expressed as mM.

could not be evaluated at pH 7 since curved Lineweaver-Burk and Eadie-Hofstee plots were obtained (Fig. 1). However, the data gave linear plots for the Hill equation [23]

$$\log(\upsilon/V_m-\upsilon) = n \log S-\log K^1$$

A value of 12 μM was calculated for K^1. The Hill coefficient n was 2.02. This suggested that the substrate AcPyAD$^+$ was binding at more than one site on the transhydrogenase. Linear plots were obtained from the kinetic data measured at pH 6 (Fig. 1). The K_m^{AcPyAD} for the non-mutant enzyme at pH 6 was of the same order of magnitude as the values for the mutant enzymes (Table 2).

4. Discussion

The mechanism of reduction of $AcPyAD^+$ by NADH is controversial. Jackson and co-workers [13,15,16] have proposed that $NADP^+$ bound to the NADP(H)-binding site on the enzyme is reduced by NADH and reoxidized by $AcPyAD^+$, with these two substrates alternately occupying the NAD(H)-binding site (the cyclic mechanism). By contrast, we [17] have suggested that NADP(H) is not an intermediate in the transfer of a hydride equivalent between NADH and $AcPyAD^+$ but promotes this reaction by inducing the enzyme to adopt a favourable conformation. Although recognizing the limitations in using K_m values to predict enzyme mechanisms (e.g. see [15]), it would be reasonable to expect for the mechanism proposed by Jackson and co-workers [13,15,16] that the K_m^{AcPyAD} should be somewhat similar in both $NADPH \rightarrow AcPyAD^+$ and $NADH \rightarrow AcPyAD^+$ transhydrogenations, since $AcPyAD^+$ would be occupying the NAD(H) site in both reactions and the first reaction is considered to be a partial reaction of the second reaction. The order of magnitude difference between the K_m^{AcPyAD} found here for the two reactions is more consistent with $AcPyAD^+$ occupying a site other than the NAD(H) site in $NADH \rightarrow AcPyAD^+$ transhydrogenation. NADH presumably occupies this site in the $NADH \rightarrow AcPyAD^+$ reaction, whereas it is occupied by $AcPyAD^+$ in $NADPH \rightarrow AcPyAD^+$ transhydrogenation. The relative similarity between K_m^{NADH} and K_m^{AcPyAD} for $NADH \rightarrow AcPyAD^+$ and $NADPH \rightarrow AcPyAD^+$ reactions, respectively (Table 2), supports this view. Furthermore, the Hill coefficient of 2.0 determined for the non-mutant enzyme suggests that $AcPyAD^+$ can bind at more than one site on the transhydrogenase at pH 7.

The location of the binding site for $AcPyAD^+$ in the $NADH \rightarrow AcPyAD^+$ reaction has not been determined. The most likely possibility is that $AcPyAD^+$ occupies the NADP(H) site. The high K_m^{AcPyAD} would reflect unfavourable binding interactions at this site. Under the conditions of our experiment (pH 6, low salt [13], presence of Brij 35 [17]) the specificity of the site for NADP(H) may have been relaxed to permit binding and reduction of $AcPyAD^+$ in the absence of NADP(H). A similar effect may have been induced by the mutations in the mutant enzymes. Binding of $AcPyAD^+$ to the NADP(H) site would permit direct reduction by NADH without the involvement of NADPH as an intermediate in the reaction.

The reduction of $AcPyAD^+$ by NADH was increased significantly in the presence of 0.5 mM $NADP^+$. This may be due to $NADP^+$ binding at a second site on the enzyme to induce a conformational change favouring the $NADH \rightarrow AcPyAD^+$ reaction, as we have proposed previously [17]. Alternatively, $NADP^+$ could bind at the NADP(H) site with displacement of $AcPyAD^+$. (Half-maximal rates with the non-mutant enzyme were obtained at 5–7 μM NADP(H) (unpublished results). This concentration is much higher than that (0.25 μM $NADP^+$ or 0.1 μM NADPH) found by Hutton et al. [13] and presumably is due to the different types of enzyme preparations used.) The reduction of $AcPyAD^+$ by NADPH could then occur by the cyclic mechanism of Jackson and co-workers [13,15,16]. It is unlikely that the reaction we observe at pH 6 in the absence of added NADP(H) is due to contamination of $AcPyAD^+$ or NADH by these nucleotides. $AcPyAD^+$ contained no NADP(H). Analysis of NADH gave a maximum level of contamination by $NADP^+$ of 0.1%. A similar value was obtained for NADP(H). Thus, the maximum level of contamination in the reaction mixture would be 1 μM NADP(H) [17]. This amount would not lead to the levels of activity observed in our experiments.

We conclude that the cyclic mechanism does not occur under the conditions of our experiments when NADP(H) is absent and that the transfer of a hydride equivalent between NADH and $AcPyAD^+$ is direct. Cyclic reduction may occur when NADP(H) is present but a valid explanation is that this nucleotide induces a conformational change in the enzyme facilitating reduction of $AcPyAD^+$ by NADPH [17].

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