The Kinetic Mechanism of Pyridine Nucleotide Transhydrogenase from *Escherichia coli*®

Ronald L. Hanson‡

From the Department of Biochemistry, Columbia University, New York, New York 10027

Pyridine nucleotide transhydrogenase was extracted from the membrane fraction of *Escherichia coli* with Triton X-100 and purified 71-fold by chromatography on DEAE-cellulose and Sepharose 4B in buffers containing detergent. The kinetic mechanism for reduction of 3-acetylpyridine adenine dinucleotide (AcPyAD) by NADP was determined. Plots of reciprocal velocity versus the reciprocal concentration of either substrate at different fixed concentrations of the other substrate were intersecting, indicating a sequential mechanism. 2'-AMP was a competitive inhibitor versus AcPyAD and was noncompetitive versus NADPH, whereas 2'-AMP was competitive versus NADPH and noncompetitive versus AcPyAD. These results establish that the mechanism is random. In product inhibition experiments, NADP was competitive with NADPH and noncompetitive versus AcPyAD; AcPyADH was competitive with AcPyAD and noncompetitive versus NADPH. These patterns are consistent with a random mechanism in which dead-end complexes of E-NADP•AcPyADH and E-NADP•AcPyAD can form. NAD, an alternative substrate, is competitive with AcPyAD with respect to AcPyADH production and noncompetitive with NADPH, which is also consistent with a random mechanism. In the reverse direction, reciprocal plots were concave downward.

The effect of MgATP on the kinetics of transhydrogenase was studied with membrane preparations from *E. coli*. At 0.1 mM substrate concentrations, MgATP increased by at least 3-fold the rate of reduction of NADP and analogs by NADH and analogs. V_max for reduction of NADP by NADH was increased 2.2-fold by MgATP. Reciprocal plots were concave downward, both in the presence and absence of MgATP.

Pyridine nucleotide transhydrogenase (EC 1.6.1.1), found in the cytoplasmic membrane of *Escherichia coli* and in the inner membrane of mitochondria, catalyzes the reduction of NAD by NADPH. In the absence of energy the rate of the reverse reaction is low, but addition of ATP or substrates for respiration increases this rate severalfold. The equilibrium for the reaction is also shifted by energy toward reduction of NADP by NADH. The effect of ATP is mediated by the energy-transducing ATPase. In mutants of *E. coli* lacking ATPase, ATP does not drive the transhydrogenase reaction (1), although respiration is still effective (2). An antibody to purified ATPase inhibits the stimulation by ATP of transhydrogenase as well as inhibiting ATPase (3). In addition, ATPase can be extracted from the membrane with loss of the ATP effect and reconstituted to restore the effect (4, 5).

A single enzyme is apparently responsible for hydride transfer by both the energy-linked and non-energy-linked transhydrogenase activities in *E. coli* and is distinct from NADH dehydrogenase. Thus, mutants have been isolated which have simultaneously lost both transhydrogenase activities (6, 7) but retain NADH dehydrogenase and ATPase (7). A study of the mechanism of transhydrogenase offers an opportunity to determine how a membrane protein catalyzing a well defined enzymatic reaction is able to use the energized state of the membrane.

From kinetic studies of the transhydrogenase in beef heart submitochondrial particles (which is similar in many respects to the *E. coli* enzyme), Rydström et al. suggested a Theorell-Chance mechanism and calculated the effects of an energy source on the individual rate constants for the proposed mechanism (8–10). Kinetic studies have also been carried out on soluble energy-independent bacterial transhydrogenases containing FAD. Cohen and Kaplan proposed a ping-pong mechanism for a transhydrogenase from *Pseudomonas aeruginosa* (11), whereas van den Broek and Veeger suggested a rapid equilibrium random mechanism for the enzyme from *Azotobacter vinelandii* (12).

This paper describes the detergent extraction and partial purification of transhydrogenase from *E. coli* and studies of the kinetic mechanism of the purified enzyme using initial velocity patterns, dead-end and product inhibitors, and a competing alternative substrate. The effects of ATP on the kinetics of the membrane-bound enzyme are also described.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were obtained from the following sources: affinity reics and nucleotides, P L Biochemicals; 2-heptyl-4-hydroxyquinoline-N-oxide, Sigma; yeast alcohol dehydrogenase, Boehringer, DEAE-cellulose (DE52), Whatman; Sepharose 4B, Pharmacia.

**Bacterial Strains and Growth Conditions**—*E. coli* strain A-5245 was used as starting material for purification of transhydrogenase. It was grown to late log phase (Klett 200 with Filter 66) in a Brunswick 12-liter fermenter at 39°C with stirring and aeration at 4 liters/min. Strain AE62 (his-1, arg G6, trp-31, thy A) was used for preparation of membranes, because we obtained more complete inhibition of NADH oxidation with this strain, and was grown on a Brunswick rotary shaker at 37°C. Both of these K12 strains were grown on mineral medium 63 (13) plus 1% glucose and thiamin (2 mg/liter). For AE62, required amino acids (40 mg/liter) and thymine (50 mg/liter) were also added.

**Preparation of Membranes**—Strain AE62 was grown in 40 ml of medium to Klett 100. All subsequent operations were at 4°C. Cells were harvested by centrifugation, suspended in 5 ml (50 mM Tris/sulfate (pH 7.8), 10 mM MgSO4, 10 mM mercaptoethanol), and disrupted with a Branson W185 sonifier. Membranes were pelleted by centrifuging 1 h at 105,000 × g. Pellets were resuspended with a Potter-Elvehjem homogenizer in 1 ml (60 mM Tris/sulfate (pH 7.8),
10 mM MgSO₄, 10 mM mercaptoethanol) to give a protein concentration of about 3 mg/ml.

Enzyme Assays—For following the purification of transhydrogenase (Table I), the assay solution contained, in 1 ml: 0.1 M potassium phosphate (pH 7), 0.1 mM NADPH, 0.1 mM AcPyAD, 10 mM mercaptoethanol, and 0.01% Brij 35. The kinetic experiments were performed at pH 7.8 which is optimum (14) for the energy-linked reaction but above the optimum for the energy-independent reaction. The assay solution for the purified enzyme contained, in 1 ml: 0.1 M Tris-chloride (pH 7.8), 10 mM mercaptoethanol, 0.01% Brij 35, and the indicated concentrations of pyridine nucleotides. The addition of 0.01% Brij 35 did not change the initial rate, but initial rates rapidly declined in the absence of detergent. Addition of 0.1% or 1% Brij 35 did not change the initial rate, but initial rates rapidly declined in the absence of detergent. Addition of 0.1% or 1% Brij 35 inhibited 40%; up to 1% Triton X-100 had little effect; 0.1% sodium cholate inhibited 50%, and 1% inhibited almost completely.

Most of the assays were performed at 30°C in 1-cm cuvettes in a Gilford 240 spectrophotometer with a model 6050 recorder, using 0.1 absorbance as full scale. For the experiments at low substrate concentrations shown in Figs. 3 and 5A, the reactions were carried out in cells of 10-cm path length containing 28 ml in a Cary model 15 spectrophotometer, using 0.1 absorbance as full scale.

The reduction of AcPyAD by NADPH was followed at 380 nm. At substrate concentrations higher than 0.8 mM, nonenzymatic reduction was detectable, but concentrations used in our experiments did not exceed 0.4 mM. The reduction of NADP by NADH or AcPyADH was followed at 340 nm and the assay mixture also contained 3 μg (0.9 unit) of yeast alcohol dehydrogenase plus 0.27 mM ethanol. For the experiments at low substrate concentrations shown in Figs. 3 and 5A, the reactions were carried out in cells of 10-cm path length containing 28 ml in a Cary model 15 spectrophotometer, using 0.1 absorbance as full scale.

Results

Purification of Transhydrogenase—Because of competing NADH oxidation and turbidity of membrane suspensions, it is somewhat difficult to study the kinetic mechanism of transhydrogenase in crude membrane suspensions. Therefore, the enzyme was extracted and purified 71-fold in detergent before studying the mechanism of the energy-independent reaction. Then, the effects of ATP on the kinetics were determined by stirring in the Amicon cell for 15 min and then applied to a column (5 cm x 92 cm) containing 460 ml of Sepharose 4B in 0.1 M potassium phosphate (pH 7), 0.1% Brij 35, 10 mM mercaptoethanol (Buffer B). The column was run at room temperature with a flow rate of 2 ml/min and an elution profile is shown in Fig. 1. Seventy milliliters containing the most active fractions were pooled and used for the kinetic studies reported in this paper. The activity was stable for a month or more at 0.5°C in Buffer B.

A typical purification is summarized in Table I. It was necessary to harvest cells before they entered stationary phase in order to get effective extraction of activity. Triton X-100 and sodium cholate were effective for extraction, but Brij 35 was not. The Sepharose 4B column produced good resolution and the most active fractions were pooled and used for the kinetic studies reported in this paper. The activity was stable for a month or more at 0.5°C in Buffer B.

The concentrated enzyme was warmed to room temperature by stirring in the Amicon cell for 15 min and then applied to a column (5 cm x 92 cm) containing 460 ml of Sepharose 4B in 0.1 M potassium phosphate (pH 7), 0.1% Brij 35, 10 mM mercaptoethanol (Buffer B). This column was run at room temperature with a flow rate of 2 ml/min and an elution profile is shown in Fig. 1. Seventy milliliters containing the most active fractions were pooled and used for the kinetic studies reported in this paper. The activity was stable for a month or more at 0.5°C in Buffer B.

A typical purification is summarized in Table I. It was necessary to harvest cells before they entered stationary phase in order to get effective extraction of activity. Triton X-100 and sodium cholate were effective for extraction, but Brij 35 was not. The Sepharose 4B column produced good resolution at room temperature but not at 4°C. It was also necessary to change the detergent from Triton X-100 to Brij 35 in order to prevent aggregation of the enzyme. Fig. 2 shows that when Sepharose 4B chromatography is done twice in Buffer B with Triton X-100 substituted for Brij 35 the size of the enzyme increased, but this did not occur in Buffer B. Gels in sodium dodecyl sulfate showed that the protein is not yet homogeneous after Step 4, but the preparation does not oxidize NADH at this stage.

Initial Velocity Patterns—When AcPyAD was varied at different fixed levels of NADPH, the family of lines in the initial water and disrupted by treatment for 5 min with a Branson W185 sonifier using the large probe at maximum output. The broken cell suspension was centrifuged 1 h at 29,000 rpm in a Beckman 30 rotor. The supernatant was discarded and the membrane pellets were dispersed with the aid of a blender in 200 ml containing 0.01 M potassium phosphate (pH 7), 10 mM mercaptoethanol, and 1% Triton X-100. The suspension was stirred for 1 h and then centrifuged 1 h at 29,000 rpm in a Beckman 30 rotor.

The supernatant containing the extracted enzyme was added to 800 ml of Buffer A (0.02 M potassium phosphate (pH 7), 1% Triton X-100, 10 mM mercaptoethanol) and this was applied to a column (72 cm x 8.3 cm) containing 600 ml of DEAE-cellulose in Buffer A. The column was then washed with 1800 ml of 0.12 M NaCl in Buffer B, and the enzyme was eluted with 0.25 M NaCl in Buffer B. The flow rate of this column was 28 ml/min for sample application and washing but was reduced to 17 ml/min for elution of the enzyme. Activity was recovered in a volume of about 120 ml and the active fractions were yellow. The active fractions were concentrated to 40 ml by ultrafiltration through a 62 nm Amicon PM-10 membrane.

The concentrated enzyme was warmed to room temperature by stirring in the Amicon cell for 15 min and then applied to a column (5 cm x 92 cm) containing 460 ml of Sepharose 4B in 0.1 M potassium phosphate (pH 7), 0.1% Brij 35, 10 mM mercaptoethanol (Buffer B). This column was run at room temperature with a flow rate of 2 ml/min and an elution profile is shown in Fig. 1. Seventy milliliters containing the most active fractions were pooled and used for the kinetic studies reported in this paper. The activity was stable for a month or more at 0.5°C in Buffer B.
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FIG. 3. Initial velocity pattern with AcPyAD and NADPH as substrates. NADPH concentrations were 2.86, 4.00, 6.67, and 20.0 μM, respectively, from top to bottom. Protein concentration was 0.89 μg/ml.

Reciprocal plots (Fig. 3) was convergent, indicating a sequential mechanism. From slope and intercept replots, the Michaelis constants, $K_m$ for NADPH, and $K_s$ for AcPyAD, were 14 and 28 μM, respectively. The inhibition constant $K_i$ was 3.5 μM for NADPH and $K_i$ for AcPyAD was 7 μM.

Dead-end Inhibitors—The effects of a competitive inhibitor for each substrate allow sequential mechanisms to be classed as ordered or random. 5'-AMP was competitive with respect to AcPyAD and noncompetitive versus NADPH; 2'-AMP was competitive with NADPH and noncompetitive with AcPyAD (Fig. 4). These patterns establish that transhydrogenase has a random mechanism. If the mechanism were ordered, a competitive inhibitor for the second substrate to add to the enzyme would be uncompetitive versus the first substrate (18). The inhibition constants from slope and intercept replots are reported in Table II.

From the noncompetitive patterns, the dissociation constants for enzyme-inhibitor complexes were calculated using Equations 1 and 2 which hold for a rapid equilibrium random mechanism (18).

$$K_i = K_s \left(1 + \frac{A}{K_m}\right)$$

(1)

$$K_i = K_i \left(1 + \frac{A}{K_i}\right)$$

(2)

In these equations, $A$ is the fixed substrate ($B$ is the varied substrate); $K_m$ and $K_i$ are the dissociation constants of $A$ from $EA$ and $EAD$, respectively; $K_s$ and $K_i$ are the dissociation constants of inhibitor from $EI$ and $EIB$, respectively. The dissociation constant of 5'-AMP from E · 5'-AMP was 0.47 μM and from E · NADPH · 5'-AMP was 3.7 mM; for 2'-AMP the dissociation constant from E · 2'-AMP was 0.77 mM and from E · APAD · 2'-AMP was 1.2 mM. As was the case for both substrates, binding to the free enzyme is tighter than binding to the binary complexes.

Product Inhibition—NADP was a competitive inhibitor
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versus NADPH and was noncompetitive versus AcPyAD. AcPyADH was competitive with AcPyAD and noncompetitive with NADPH (Fig. 5). The inhibition constants are given in Table II. These patterns are consistent with a random mechanism which can be approximated kinetically by the rapid equilibrium assumption and where both abortive ternary complexes can form. From the noncompetitive patterns, using Equations 1 and 2, the dissociation constants were calculated (19). \( K_{is} = 67 \mu M \), the dissociation constant of NADP from \( E \cdot NADP \) and \( K_{e} = 98 \mu M \), the dissociation constant from \( E \cdot AcPyAD \cdot NADP \). For AcPyADH, \( K_{is} = 12 \mu M \), and \( K_{e} = 0.14 \mu M \), the respective dissociation constants from \( E \cdot AcPyADH \) and \( E \cdot NADPH \cdot AcPyADH \).

Alternative Substrate Inhibition—Transhydrogenase activity was retained on an affinity column containing

**Table III**

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Specific activity (+MgATP)</th>
<th>Specific activity (-MgATP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NADPH, AcPyAD</td>
<td>0.12</td>
<td>0.14</td>
</tr>
<tr>
<td>2. AcPyADH, NADP</td>
<td>0.0051</td>
<td>0.0004</td>
</tr>
<tr>
<td>3. NADH, AcPyAD</td>
<td>0.054</td>
<td>0.001</td>
</tr>
<tr>
<td>4. NADH, thio-NADP</td>
<td>0.039</td>
<td>0.010</td>
</tr>
<tr>
<td>5. NADH, NADP</td>
<td>0.074</td>
<td>0.025</td>
</tr>
</tbody>
</table>

**Fig. 5.** Product inhibition. A, inhibition by NADP with AcPyAD as the variable substrate. NADPH concentration was 10 \( \mu M \). NADP concentrations were 0.30, 0.20, 0.10, and 0 \( \mu M \), respectively, from top to bottom. Protein concentration was 0.89 \( \mu g/ml \). B, inhibition by NADP with NADPH as the variable substrate. AcPyADH concentration was 100 \( \mu M \). NADP concentrations were 1.2, 0.80, 0.40, and 0 \( \mu M \), respectively, from top to bottom. Protein concentration was 2.5 \( \mu g/ml \). C, inhibition by AcPyADH with AcPyAD as the variable substrate. NADPH concentration was 100 \( \mu M \). AcPyADH concentrations were 0.24, 0.12, 0.060, and 0 \( \mu M \), respectively, from top to bottom. Protein concentration was 7.7 \( \mu g/ml \). D, inhibition by AcPyADH with NADPH as the variable substrate. AcPyADH concentration was 100 \( \mu M \). AcPyADH concentrations were 0.24, 0.16, 0.080, and 0 \( \mu M \), respectively, from top to bottom. Protein concentration was 7.7 \( \mu g/ml \).

**Fig. 6 (left).** Alternative substrate inhibition. A, inhibition by NAD with AcPyAD as the variable substrate. NADPH concentration was 50 \( \mu M \). NAD concentrations were 2.0, 1.0, and 0 \( \mu M \), respectively, from top to bottom. Protein concentration was 2.0 \( \mu g/ml \). B, inhibition by NAD with NADPH as the variable substrate. AcPyAD concentration was 50 \( \mu M \). NADH concentration was 7.7 \( \mu g/ml \). C, inhibition by AcPyADH with NAD as the variable substrate. AcPyAD concentration was 50 \( \mu M \). AcPyADH concentrations were 0.24, 0.12, 0.060, and 0 \( \mu M \), respectively, from top to bottom. Protein concentration was 2.5 \( \mu g/ml \).

**Fig. 7 (center).** Reciprocal plots for the reverse reaction. A, NADH as the variable substrate. NADP concentration was 0.4 \( \mu M \). Protein concentration was 7.7 \( \mu g/ml \). B, NADP as the variable substrate. NADH concentration was 20 \( \mu M \). Protein concentration was 7.7 \( \mu g/ml \).

**Fig. 8 (right).** Reciprocal plots for the reverse reaction, catalyzed by membrane preparations, in the presence and absence of MgATP. A, NADP as the variable substrate. NADH concentration was 20 \( \mu M \). Protein concentration was 41 \( \mu g/ml \). Upper lines, no MgATP; lower lines, assav with 5 \( mM \) MgCl\(_2\) and 5 \( mM \) ATP.
agaro/se/hexane/NAD, type 1 (NAD is linked by a carbodi-
imide coupling reaction), and was eluted with NADH. Binding
to agaro/NDAP would have confirmed the random mech-
bism, but the activity was not retained by agaro/NDAP,
type 3 (linked through the C of adenine), or type 4 (linked to
ribose). However, transhydrogenase also did not bind to aga-
rose/NAD with these two types of linkage. As further evidence
for a random mechanism, the effects of NAD, an alternative
substrate for AcPyAAD, were examined.

When AcPyADH production was followed, NAD was com-
petitive with AcPyAD and noncompetitive with NADPH (Fig.
6), as expected for a random mechanism (18). For an ordered
mechanism, with NAD as the first substrate bound, nonlinear
reciprocal plots would be expected in Fig. 6B. Since nonlinear-
arity may not be observed for an ordered mechanism, depend-
ing on the kinetic constants, this is not conclusive proof for a
random mechanism but is supporting evidence.

**Kinetics of Reverse Reaction**—In the reverse direction,
reciprocal plots were concave downward and appeared bi-
phasic for both substrates. These plots are shown for reduction
of NADP by NADH in Fig. 7. The Hill coefficients from these
data were 0.78 for NADP and 0.61 for NADH. Similar nonlinear
plots resulted when the reduction of NADP by AcPyADH
or the reduction of thionicotinamide adenine dinucleotide
phosphate by NADH were studied (data not shown). These
nonlinear plots precluded a successful analysis of the kinetic
mechanism in the reverse direction but results were qualita-
tively similar to those in the forward direction. For reduction
of NADP by NADH or AcPyADH, the initial velocity patterns
appeared intersecting. Patterns for inhibition by 2′-AMP and
5′-AMP of reduction of NADP by NADH also appeared
similar to the patterns in the forward direction.

**Membrane Transhydrogenase**—The effect of MgATP on
the rates of transhydrogenase reactions catalyzed by mem-
brane preparations is shown in Table III. The rate of the
forward reaction was decreased slightly and the rates of the
reverse reactions are increased by 3-fold or more. The reduc-
tion potential, E₅₀, of AcPyAD has been determined to be
–0.25 V (20), and AcPyADP would be expected to have a
similar potential. Thus, MgATP increases the rates of both
energetically favorable and unfavorable reductions in the
reverse reaction.

The membrane-bound enzyme showed the same behavior
as the purified enzyme, with NADP and NADH giving recip-
rocral plots concave down, both in the presence and absence of
MgATP (Fig. 8). When both substrates were varied together in
a constant ratio and the plots were extrapolated to the
ordinate, MgATP increased V_max 2.2-fold. When the data
represented in Fig. 8 were treated as biphasic plots, the only
consistent changes in apparent Kₘ values for several experi-
ments were that the lower apparent Kₘ for NADP was de-
creased by MgATP (from 45 to 13 µM in Fig. 8A) and the
higher apparent Kₘ for NADH was increased by MgATP
(from 10 to 19 µM in Fig. 8B). For both substrates, MgATP
decreased both slopes and intercepts of reciprocal plots.

**DISCUSSION**

The results of the kinetic studies in this report are consistent
with a random mechanism for *E. coli* transhydrogenase, which
can be approximated kinetically by the rapid equilibrium
assumption. Most of the data are similar to the results of
Rydström et al. (8–10) for mitochondrial transhydrogenase,
although a Theorell-Chance mechanism, with NAD as the
first substrate to bind, was proposed for the mitochondrial
enzyme. The product inhibition patterns can be explained by
either mechanism, but the inhibition patterns for 2′-AMP and
5′-AMP show that the mechanism is random for the *E. coli*
enzyme. Although the plots (10) for 2′-AMP inhibition of the
mitochondrial enzyme versus NAD appear to show slope
effects, Rydström concluded that the inhibition was close to
uncompetitive and therefore proposed a Theorell-Chance
mechanism. Recently Lueck et al. showed how the ordinate
at the point of convergence of initial velocity patterns in the
forward and reverse directions can be used as a criterion of
kinetic mechanism (21). For a Theorell-Chance mechanism,
if patterns intersect below the abscissa in the forward direc-
tion, they must intersect at an equal distance above the
abscissa in the reverse direction. For the mitochondrial trans-
hydrogenase, the plots intersect below the abscissa in the
forward direction and on the abscissa in the reverse direction
(8), which clearly rules out a Theorell-Chance mechanism but
is consistent with a random sequential mechanism.

Fisher and Kaplan have shown for the mitochondrial trans-
hydrogenase, that there is no kinetic isotope effect when [4A-
H]NADH reduces NADP or thionicotinamide adenine di-
nucleotide phosphate, with or without ATP (22). Therefore
hydride transfer cannot be the rate-limiting step. However,
even when the interconversion of ternary complexes is not
rate-limiting, the kinetics for a random mechanism may ap-
pear similar to those expected for a rapid equilibrium random
mechanism (18).

In the forward direction, reciprocal plots for the *E. coli*
transhydrogenase appeared linear. In the reverse direction,
however, the plots were concave downward. Product inhibi-
tion of the forward reaction appeared linear, but higher prod-
uct concentrations were used in the inhibition experiments
than were used as substrates for the reverse reaction. Al-
though steady state random mechanisms are predicted to
have nonlinear reciprocal plots, the rapid equilibrium assum-
ption should be better satisfied in the slower reverse direction
than in the forward direction (23). A more likely explanation
for the nonlinearity of the plots is that transhydrogenase has
more than one active site. These sites, if independent, have
different kinetic parameters, or, if the sites are identical, they
exhibit negative cooperativity.

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R L Hanson