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Biochimica et Biophysica Acta 1229 (1995) 64–72

BBA Biochimica et Biophysica Acta

Characterization of the interaction of NADH with proton pumping *E. coli* transhydrogenase reconstituted in the absence and in the presence of bacteriorhodopsin

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Received 6 July 1994; accepted 7 December 1944

Abstract

(1) Proton-pumping nicotinamide nucleotide transhydrogenase from *Escherichia coli* was purified in a reconstitutively active form employing affinity chromatography on immobilized palmitoyl-Coenzyme A. Reconstituted transhydrogenase showed an active proton pumping and a stimulation of the rate of reduction of 3-acetylpyridine-NAD^+ by NADPH by uncouplers. Reconstitution in the absence of a thiol-reducing agent, e.g. dithiothreitol, abolished proton pumping without affecting catalytic activity, giving a decoupled transhydrogenase. (2) Co-reconstitution of transhydrogenase with bacteriorhodopsin gave vesicles which catalyzed a 5–10-fold increased rate of reduction of thio-$NADP^+$ by NADH in the light. The K_m for NADH, but not that for thio-$NADP^+$, decreased markedly in the light, indicating an effect of the electrochemical proton potential on the affinity of the enzyme for NADH. Inhibition by substrate derivatives in the absence or presence of light supported this conclusion. Replacement of NADH with 2′-deoxy-NADH gave a strongly sigmoidal concentration dependence, indicating an allosteric change induced by binding to the NAD(H)-site. (3) Reduction of 3-acetylpyridine-NAD^+ by NADH in the presence of NADPH, previously demonstrated to be catalyzed by both reconstitued bovine transhydrogenase and detergent-dispersed *E. coli* transhydrogenase, occurred at a pH below 6.5. This reaction did not pump protons. Proton pumping by 3-acetylpyridine-NAD^+ plus NADPH occurred at a pH above 5.5. The two reactions were thus close to mutually exclusive, with a cross point at pH 5.8. Assuming a helix bundle structure of the membrane domain of transhydrogenase, a model is proposed involving histidine 91 of the β subunit which previously was shown to be essential by site-directed mutagenesis. According to the model the extent of protonation of this histidine determines whether proton pumping or the NADH-3-acetylpyridine-NAD^+ reaction takes place.

Keywords: Transhydrogenase; Proton pumping; Nucleotide; NAD; NADP; Membrane protein

1. Introduction

Membrane-bound nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) is a proton pump found in e.g. mitochondria, *E. coli*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum*, which catalyzes the reversible reduction of $NADP^+$ by NADH and the concomitant translocation of 0.5–1 protons according to the reaction

$$nH^+_{out} + NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH + nH^+_{in}$$

Most of the proton-pumping transhydrogenases have been characterized extensively both in their pure forms and reconstituted in liposomes (for reviews see [1–3]). The genes for the enzymes from *E. coli* [4], bovine mitochondria [5] and *Rhodospirillum rubrum* [6] have been cloned and their cDNA has been sequenced. However, of the latter cloned transhydrogenases, only the *E. coli* transhydrogenase has been overexpressed [4,7] and this has made it possible to replace specific amino acid residues, i.e., FSBA and DCCD-reactive residues, by site-directed

Abbreviations: FSBA, *p*-fluorosulfonylbenzoyladenosine; DCCD, *N′,N*-dicyclohexylcarbodiimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ACMA, 9-amino-6-chloro-2-methoxyacridine; DTT, dithiothreitol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; t$NADP^+$, oxidized thionicotinamide adenine dinucleotide; Ac$PyAD^+$, 3-acetylpyridine adenine dinucleotide; 5′-ADR, 5′-adenosine diphosphate ribose; NMNH, reduced nicotinamide nucleotide.

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SSDI 0005-2728(94)00187-1

mutagenesis [8,9]. Neither of these residues was found to be essential for catalytic or proton-pumping activity [8,9].

All proton-pumping transhydrogenases characterized sofar are composed of two hydrophilic domains, containing the NAD(H) and NADP(H)-binding domains, respectively, and a hydrophobic, probably membrane-spanning, domain localized in between the two hydrophilic domains. Recently, the secondary structure of the membrane-spanning domain of the *E. coli* transhydrogenase was predicted to contain 10 α-helices but no conserved acidic residues and only one conserved positively charged residue, i.e., histidine β91 of helix 7 [10]. This histidine was concluded to be essential by site-directed mutagenesis, and may constitute a key component of a proton-conducting pathway [10].

Reconstitution of purified mitochondrial transhydrogenase from bovine heart in phospholipid vesicles, originally developed in collaboration with the late Ephraim Racker [11], has provided an essential basis for understanding the kinetics of interaction of the substrates with transhydrogenase exposed to an electrochemical proton gradient [12–15]. An important part of the characterization of purified and reconstituted mitochondrial transhydrogenase from bovine heart with regard to effects of an external electrochemical proton gradient, was the co-reconstitution with ATPase or bacteriorhodopsin [16–18]. In the presence of ATP or light these vesicles catalyzed a dramatically increased rate of reduction of $NADP^+$ by NADH, similar to or exceeding that found with intact membrane vesicles from michondria or *E. coli* upon energization. Using these co-reconstituted vesicles, a kinetic characterization of reconstituted transhydrogenase was then employed as a means for investigating the mechanism of utlilization of a electrochemical proton gradient by transhydrogenase, allowing the conclusion that the interaction was entirely chemiosmotic [16–20].

Coupled reconstituted purified bovine transhydrogenase was earlier found to catalyze a reduction of $AcPyAD^+$ by NADH in the presence of NADPH [13,21], which was interpreted to involve a cyclic reduction/oxidation cycle of bound NADP(H) [13]. Interestingly, a similar reaction was recently found to be catalyzed also by detergent-solubilized *E. coli* transhydrogenase at a low pH, e.g., 6.0 [22]. This striking pH-dependence was proposed to involve a critical histidine [22], in agreement with a recent conclusion based on site-directed mutagenesis that histidine β91 is essential for both catalytic activity and proton pumping [10].

Previous results obtained with reconstituted purified bovine transhydrogenase indicated that the generation of an electrochemical proton gradient strongly affected primarely binding of NAD(H) [1,2,13]. In order to characterize the effect of an electrochemical proton gradient on NAD(H) binding at the level of the structure of the NAD(H)-binding site using *E. coli* transhydrogenase, as well as mutated forms of this enzyme produced in ongoing studies, methods were developed to purify *E. coli* transhydrogenases and to reconstitute them in the absence and in the presence of bacteriorhodopsin. Using the wildtype enzyme, the present report shows that the kinetics of the light-driven transhydrogenase reaction is strongly affected by the electrochemical proton gradient generated by bacteriorhodopsin. The results suggest that the NAD(H)-binding site, possibly the nicotinamide nucleotide-binding domain, is primarily affected. A reaction scheme is proposed for the reduction of $AcPyAD^+$ by NADH in the presence of NADPH catalyzed by reconstituted vesicles, which involves bound NADP(H), a protonated histidine β91 and no net proton pumping.

2. Materials and methods

2.1. Bacterial strain and plasmid

The *pnt* gene was introduced in the pGEM-7Zf(+) plasmid, giving the construct pSA2, and subsequently transformed into *E. coli* K12 strain JM109 [7]. Normally, a 40–80-fold overexpression was obtained.

2.2. Preparation of membrane vesicles

E. coli cells carrying wild-type pSA2 were grown in LB medium supplemented with 0.1 g/l ampicillin, to an A_{550} of 1.0 before harvest essentially as described [7,23]. Cells were sedimented at $4400 \times g$ for 20 min and washed as described [23], after which they were frozen below $-20°$ C.

2.3. Purification of E. coli transhydrogenase

E. coli transhydrogenase was purified according to a modified procedure based on previously described methods [23,24]. About 8 g *E. coli* cells were suspended in 40 ml TED buffer composed of 50 mM Tris-HCl, 1 mM EDTA and 1 mM DTT (pH 7.8) and disrupted by passage through a French press at 1400 kg/square cm. Unbroken cells were removed by low speed centrifugation and the membrane vesicles precipitated by centrifuging the supernatant for 2.5 h at $250000 \times g$ in a Beckman 55.2 Ti rotor. The membrane vesicles were suspended at 1 g/5 ml TED buffer to which was added 1 M KCl, 30 mM sodium cholate and 30 mM sodium deoxycholate. The mixture was stirred at 0° C for 20 min and then centrifuged at 45000 rpm in a Beckman 55.2 Ti rotor for 2.5 h. The supernatant was rapidly desalted by application to a Phenyl-Sepharose column (2.5 × 4 cm), after which the column was washed with 20 ml TED buffer containing 1 mg/ml Brij 35. The enzyme was then eluted with TED buffer containing 20 mg/ml Triton X-100 at a flow rate of 1 ml/min. Fractions of 4 ml were collected.

Active fractions were applied to a FPLC column (Mono Q HR 10/10), equilibrated with 50 mM Tris-H_2SO_4 (pH

7.8), 2 mM DTT, 1 mg/ml Brij 35 and 1 mg/ml Thesit at room temperature. Immediately before use, the medium was filtered through a 0.22 μm Millipore filter and degassed. After washing with 10 ml of equilibration buffer, transhydrogenase was eluted with a 0–0.5 M linear gradient of potassium chloride in equilibration buffer with a relative gradient slope of 5 mM potassium chloride per ml of buffer. The flow rate was 4 ml/min. Fractions of 2 ml were collected. Fractions collected at the peak and thereafter of the eluted transhydrogenase showed the highest purity. However, pre-peak fractions were less pure but also reconstitutively active.

Active fractions were pooled and diluted 4-times, then applied to an immobilized Palmitoyl Coenzyme A column (1 × 7 cm), equilibrated with 10 ml buffer B (10 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM DTT, 0.5 mg/ml Brij 35) at 0–4° C. The column was washed with 10 ml of buffer C (10 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM DTT and 1% sodium cholate) containing 100 mM NaCl, then 10 ml of buffer C. The enzyme was finally eluted with a 0–200 mM linear gradient of 2′,3′-AMP in buffer C in which the concentration of cholate had been increased to 3%. The relative gradient slope was 2.5 mM/ml, and the flow rate was 0.5 ml/min. Fractions of 2 ml were collected. The details of the affinity chromatography of transhydrogenase on immobilized palmitoyl Coenzyme A are described elsewhere [25].

2.4. Transhydrogenase vesicles

Transhydrogenase vesicles were prepared essentially as described for co-reconstituted transhydrogenase-ATPase vesicles except that ATPase was not added [16].

2.5. Transhydrogenase-bacteriorhodopsin vesicles

Transhydrogenase-bacteriorhodopsin vesicles were prepared essentially as described previously [17].

2.6. Catalytic activity

The catalytic activity of transhydrogenase was measured spectrophotometrically at 375 nm (split beam spectrophotometer) or 375–420 nm (dual wavelength spectrophotometer) as reduction of $AcPyAD^+$ by NADPH as described [10]. Unless otherwise indicated, the medium was 50 mM potassium phosphate (pH 7.0).

Oxidation of tNADPH by $AcPyAD^+$ was followed at 400–460 nm. Other conditions were the same as described above. The variability of this assay as well as those of the light-driven and proton pumping reactions described below was less than $\pm 10\%$.

2.7. Light-driven transhydrogenase reaction

The medium used in this assay was 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$ and 300 mM KCl. Other conditions were essentially as described previously [17].

2.8. Proton pump activity

The initial relative rate of proton translocation across the liposomal membrane driven by the reduction of $AcPyAD^+$ by NADPH was assayed fluorimetrically by quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence as described [10]. Medium was composed of 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 300 mM KCl, 2 mM DTT and 3.6 mM sodium nitrate.

All activity-dependent quenching was sensitive to uncouplers, e.g., CCCP. Rates were expressed in terms of a decrease in arbitrary fluorescence units/min or % extent of quenching.

2.9. Synthesis and purification of 2′-deoxy-NADH

2′-Deoxy-NADH was synthesized by a procedure described as an assay for NAD-pyrophosphorylase, which converts β-NMN and deoxy-ATP to 2′-deoxy-NAD^+ and pyrophosphate (Sigma quality control test procedure: Enzyme assay of NAD-pyrophosphorylase). The reaction mixture consisted of 100 mM glycylglycine (pH 7.4), 2.2 mM 2′-deoxy-ATP, 12 mM β-NMN, 15 mM $MgCl_2$ and 0.02 units of NAD-pyrophosphorylase, which was incubated at 37° C overnight. An excess of pyrophosphatase was also added. After completion of the reaction the mixture was boiled for 10 min, allowed to cool to room temperature, and then filtered by centrifugation in a Centricon-30. The filtrate so obtained was reduced by the addition of a few grains of $NaBH_4$. The 2′-deoxy-NADH synthesized was then purified on a Waters HPLC using a μ-Bondapak C_{18} column [26]. The fractions containing 2′-deoxy-NADH were collected, pH was adjusted to 7.5–7.8, and the preparation was frozen at −20° C. For technical reasons, a concentration of 2′-deoxy-NADH exceeding about 70–80 μM could not easily be obtained.

2.10. Chemicals

Biochemicals were of analytical grade and purchased from Sigma Chem. Co., or Boehringer-Mannheim. Phospholipids were purchased from Lipid Products (Nutfield Nurseries, Crabhill Lane, S. Nutfield, RH1 5PG, UK). Bacteriorhodopsin was obtained from Consortium für Elektrochemische Industrie (Zielstattstrasse 20, 8000 München 70, Germany).

3. Results

3.1. Purification and reconstitution of E. coli transhydrogenase

The purification of *E. coli* transhydrogenase by the present method provided a preparation that was at least 90% pure as judged by SDS-PAGE and had a specific

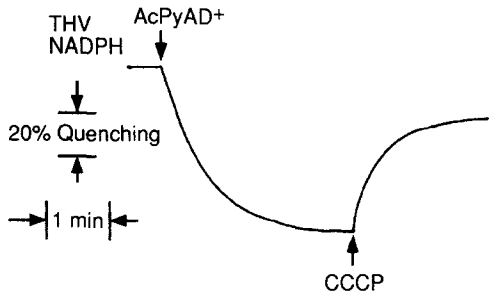

Fig. 1. Proton pumping driven by the reduction of $AcPyAD^+$ by NADPH catalyzed by reconstitited *E. coli* transhydrogenase vesicles. Conditions were as described in Materials and Methods. Additions were: 100 μl THV and 9 μM CCCP.

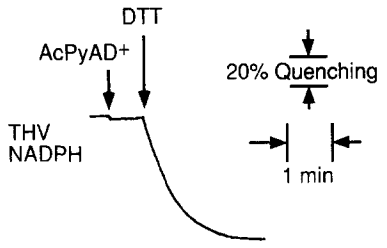

Fig. 2. Effect of DTT on proton pumping driven by reduction of $AcPyAD^+$ by NADPH catalyzed by reconstituted transhydrogenase vesicles. Conditions were as in Fig. 1, except that 2 mM DTT was initially omitted and subsequently added.

activity of 12–15 μmol/min per mg protein (not shown), i.e. comparable to previous preparations [22–24]. However, the introduction of FPLC and the use of a mixture of Brij 35 and Thesit gave a substantially increased resolution of the major chromatography step. In addition, the strong affinity to the Palmitoyl-CoA affinity column used in the last purification step also provided a means for an efficient exchange of the detergents Triton X-100 and Brij-35 for cholate (or any other detergent), which subsequently could be removed conveniantly by dialysis. A complete exchange of Triton X-100 and Brij-35 for cholate is essential for obtaining optimally coupled reconstituted vesicles. Transhydrogenase vesicles catalyzed a reduction of $AcPyAD^+$ by NADPH (not shown), and a concomitant proton pumping as indicated by an extensive quenching of ACMA (Fig. 1). The uncoupler CCCP stimulated the reduction of $AcPyAD^+$ by NADPH about 2-fold and inhibited ACMA quenching completely (not shown). The phospholipid mixture used in the reconstitution of the above activities as well as the light-driven activity described below, i.e., 47% PC, 47% PE and 6% PS, was found to be optimal when all individual phospholipids were varied; lysolecithin used previously [17,18] did not improve the vesicles (not shown).

The presence of DTT in the reconstitution buffer was important for obtaining active transhydrogenase vesicles. Omission of DTT had little or no effect on the catalytic activity (not shown), but impaired the proton pumping activity (Fig. 2). Addition of DTT directly to the cuvette restored proton pumping (Fig. 2). The response of the ACMA to an electrochemical proton gradient was not affected by the absence of DTT as judged from ACMA quenching induced by light-exposed bacteriorhodopsin vesicles (not shown). Titration of the requirement of transhydrogenase for DTT showed a half-maximal concentration of about 1 mM DTT (Fig. 3). Surprisingly, the light-driven reduction of $tNADP^+$ by NADH, assumed to be driven by a electrochemical proton gradient generated by bacteriorhodopsin, was not influenced by a lack of DTT in the dialysis buffer (not shown). Thus, these results indicate that the catalytic reduction of $AcPyAD^+$ by NADPH and the concomitant proton pumping may be decoupled completely.

3.2. Reconstitution and properties of transhydrogenase-bacteriorhodopsin vesicles

Co-reconstitution of transhydrogenase with bacteriorhodopsin was carried out in order to obtain transhydrogenase-bacteriorhodopsin vesicles. In the absence of light (510–650 nm) the reduction of $tNADP^+$ by NADH proceeded at the low rate normally obeserved with this direction of the reaction. However, in the presence of light the rate of the reaction was stimulated 5–10-fold (Fig. 4). In the presence of the uncoupler CCCP, the stimulation by light was abolished (Fig. 4). Addition of an uncoupler in the dark gave a stimulation of less than 2-fold (not shown).

Fig. 5 shows a double-reciprocal plot of the initial rate of reduction of $tNADP^+$ by NADH in the dark and in the light, as a function of the concentration of NADH (Fig. 5A) and $tNADP^+$ (Fig. 5B), respectively, at saturating concentrations of the second substrate. The various Michaelis-Menten constants were calculated and are shown in Table 1. Apparently, light caused a 7-fold decrease in the K_m of the enzyme for NADH but little change in the K_m for $tNADP^+$ as compared with the corresponding values in the dark. CCCP caused an increase in the K_m for both NADH and $tNADP^+$.

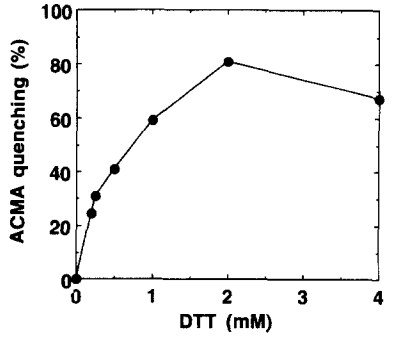

Fig. 3. Effect of varying concentrations of DTT on proton pumping by reconstituted transhydrogenase. Conditions were as in Fig. 2 except that the concentrations of DTT indicated were added 1 min prior to the initiation of proton pumping.

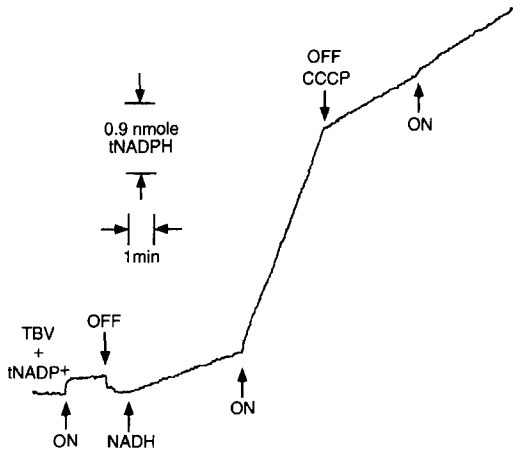

Fig. 4. Light-driven reduction of $tNADP^+$ by NADH catalyzed by reconstituted transhydrogenase-bacteriorhodopsin vesicles. Conditions were as described in Materials and Methods. Additions were: 10 μl transhydrogenase-bacteriorhodopsin vesicles (TBV) and 9 μM CCCP.

3.3. Effect of an electrochemical proton gradient on the interaction of NADH and 2'-deoxy-NADH with transhydrogenase

Binding of NAD(H) to various dehydrogenases and other NAD-dependent enzymes is assumed to involve

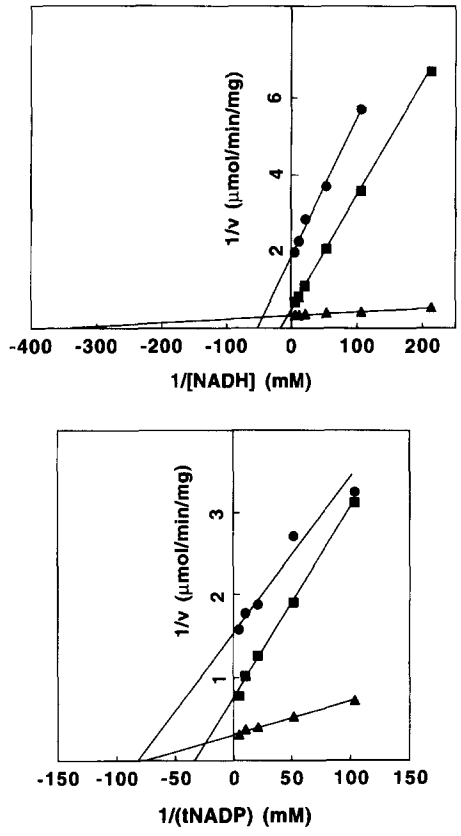

Fig. 5. Reciprocal plots of the light-driven reduction of $tNADP^+$ by NADH as a function of the concentration of NADH (A, upper figure) and $tNADP^+$ (B, lower figure), in the dark or light and ithe presence of an uncoupler. Conditions were as described in Fig. 4 except that the concentration of the fixed substrate was 200 μM. (l), dark; (s), light; (n), 5 μM CCCP added.

Table 1
K_m values of *E. coli* transhydrogenase co-reconstituted with bacteriorhodopsin in the dark and light, and in the presence of an uncoupler

Condition	K_m (μM)	
	NADH	$tNADP^+$
Dark	19	12
Light	3	13
CCCP	63	31

Values were derived from the plots in Figs. 5A and B.

among other residues the 2'-hydroxy moiety of NAD(H) [27]. This moiety in turn is assumed to interact with a conserved acidic side chain, in *E. coli* transhydrogenase aspartic acid α195 (cf. [10]). In order to test whether this interaction is essential for the dark or light-driven transhydrogenase reaction catalyzed by transhydrogenase-bacteriorhodopsin vesicles, NADH was replaced by 2'-deoxy-NADH. The dark-light transition at a concentration of deoxy-NADH of 50 μM resulted in a similar although less extensive increase in rate as that obtained with NADH (not shown, cf. Fig. 4). However, in contrast to NADH (cf. Fig. 5) the concentration dependence of the light-driven transhydrogenase reaction for 2'-deoxy-NADH was strongly sigmoidal (Fig. 6), tentatively suggesting that 2'-deoxy-NADH induced an heterotrophic allosteric response. Essentially the same concentration dependence was found for 2'-deoxy-NADH and the reaction in the absence of light (not shown). Thus, it may be concluded that aspartic acid α195 indeed is involved in NAD(H)-binding but probably not directly in proton translocation, and that 2'-deoxy-NADH is allosterically active.

3.4. Effect of an electrochemical proton potential on the interaction of substrate analogues of NAD(H) with transhydrogenase

The effects of the electrochemical proton gradient on the affinity of the transhydrogenase for NADH raises the question as to which part(s) of the NADH molecule are

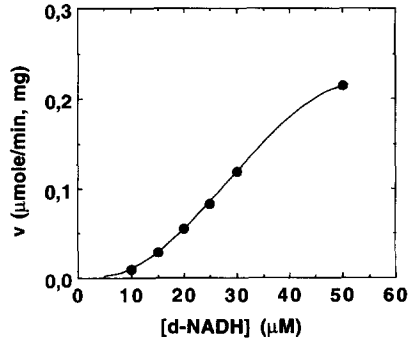

Fig. 6. Light-driven reduction of $tNADP^+$ by 2'-deoxy-NADH as a function of the concentration of 2'-deoxy-NADH catalyzed by reconstituted transhydrogenase-bacteriorhodopsin vesicles. Conditions were essentially as in Fig. 5 except that the concentration of 2'-deoxy-NADH was varied as indicated.

Table 2
Effect of substrate-derived inhibitors on the rate of reduction of $tNADP^+$ by NADH catalyzed by transhydrogenase-bacteriorhodopsin vesicles in the dark and in the light

Inhibitor	Inhibition (%)	
	dark	light
NAD(H) inhib		
NAD^+	61.5	18.4
NMNH	−15.8	−3.3
5′-ADR	29.6	13.2
5′-AMP	54.6	24.3
Adenosine	54.2	16.0
NADP(H) inhib		
NADPH	76.9	57.4
NMNH	−4.3	0
2′-AMP	36.8	31.2
2′,5′-ADP	36.8	34.4

The reaction medium contained either 200 μM $tNADP^+$ and 16.7 μM NADH in the casae of NAD(H)-derivatives, and 200 μM NADH and 15 μM $tNADP^+$ in the case of NADP(H)-derivatives. Additions were 2 mM derivative and 10 μl transhydrogenase-bacteriorhodopsin vesicles. Other conditions were as described in Materials and Methods

involved. In order to clarify this question, NAD^+, NADPH and various derivatives of NAD(P) were tested as inhibitors of reconstituted transhydrogenase in transhydrogenase-bacteriorhodopsin vesicles, in the absence and in the presence of light, and at non-saturating concentrations of the parent substrate. As shown in Table 2, NAD^+ was about 3-fold more inhibitory in the dark than in the light. NADPH showed only about 25% difference between dark and light. The NAD(H)-derivatives Adenosine, 5′-AMP, ADP and 5′-ADR were considerably less efficient as inhibitors in the light than in the dark, whereas NMNH was even stimulatory in the dark and essentially inactive in the light. In contrast, the NADPH-derivatives tested, i.e., 2′-AMP, 2′-5′-ADP and NMNH, showed a slight difference between light and dark. These results, which agree with similar results obtained with submitochondrial membranes [28], are consistent with an increased affinity of the enzyme for NADH in the light as compared to the dark, and only a slight change in the affinity for $tNADP^+$.

3.5. Reduction of $AcPyAD^+$ by NADH in the presence of NADPH

In the presence of NADPH, NADH has previously been shown to reduce $AcPyAD^+$, catalyzed by reconstituted bovine transhydrogenase [13,21] or detergent-dispersed *E. coli* transhydrogenase [22]. In the former case coupled vesicles were required, whereas in the latter case the reaction obviously did not require a proton gradient. Reconstituted *E. coli* transhydrogenase vesicles catalyzed a similar reaction, the final rate of which was similar in uncoupled and coupled vesicles (not shown). When tested with ACMA this reaction did not pump protons, and NADH could not be replaced by 2′-deoxy-NADH (not

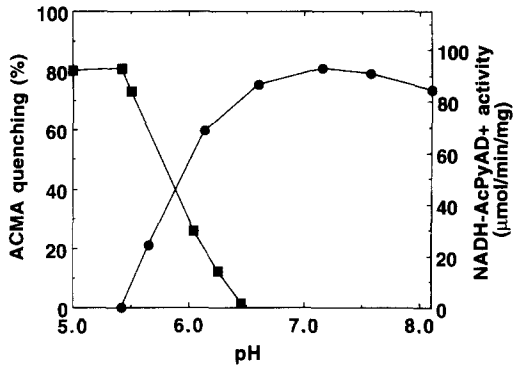

Fig. 7. pH-Dependencies of the reduction of $AcPyAD^+$ by NADH in the presence of NADPH and proton pumping driven by reduction of $AcPyAD^+$ by NADPH, catalyzed by reconstituted transhydrogenase vesicels. Conditions were as in Figs. 8 and 1B except that medium and pH varied. Medium was 20 mM potassium phosphate at pH 6.0 and above, and 20 mM sodium acetate at pH values below 6.0. (n), reduction of AcPyAD+ by NADH; (l), proton pumping.

shown). In order to further characterize the NADH-$AcPyAD^+$ reaction and its possible relationship with proton pumping, the pH dependences of the NADH-$AcPyAD^+$ reaction and $AcPyAD^+$ plus NADPH-dependent proton pumping were investigated (Fig. 7). In agreement with previous findings [22], the NADH-$AcPyAD^+$ reaction was active below pH 6.5, whereas proton pumping showed the reverse pH dependence, i.e. it was active above pH 5.5. Thus, when catalyzed by *E. coli* transhydrogenase the two reactions were apparently mutually exclusive and overlapping only around pH 5.5–6.5.

Proton pumping was then measured at a pH where the NADH-$AcPyAD^+$ reaction and proton pumping driven by reduction of $AcPyAD^+$ by NADPH were both active, i.e. at pH 6.0. Under these conditions low concentrations of NADH added during proton pumping were strongly inhibitory (Fig. 8). Replacement of NADH with AcPyADH, a substrate which can only slowly be oxidized because of its high redox potential [29], gave only a slight inhibition (Fig. 8). Since both NADH and AcPyADH were assumed to bind to transhydrogenase with approximately equal efficiencies, the discrepancy between the two substrates may depend on the ability of NADH to be oxidized.

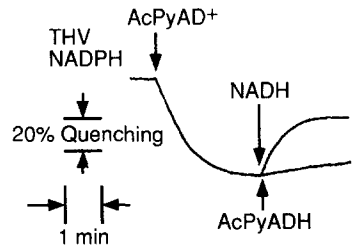

Fig. 8. Effect of NADH and AcPyADH on proton pumping driven by reduction of $AcPyAD^+$ by NADPH, catalyzed by reconstituted transhydrogenase vesicles. Conditions were as in Fig. 1B except that the buffer was 20 mM Bistris (pH 6.0). Additions were: 20 μM NADH (upper curve) and 20 μM AcPyADH (lower curve).

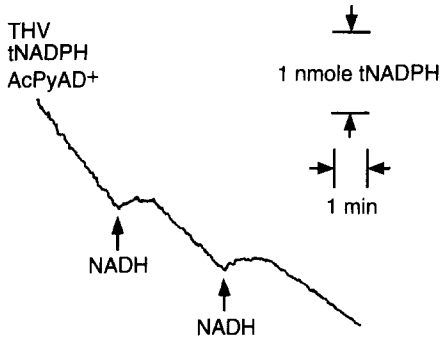

Fig. 9. Inhibition by NADH of the reduction of AcPyAD$^+$ by tNADPH catalyzed by reconstituted transhydrogenase vesicles. Conditions were as in Fig. 10 except that 200 μM tNADPH, 200 μM AcPyAD$^+$ and 10 μl transhydrogenase vesicles (THV) were used. Addition was: 8 μM NADH.

The results obtained in the experiment of Fig. 8 suggest that proton pumping was inhibited by NADH by inhibiting the reduction of NADPH by AcPyAD$^+$ which provides the redox energy for proton pumping. This possibility was tested by measuring the effect of NADH on the oxidation of tNADPH by AcPyAD$^+$. Indeed, as shown in Fig. 9, NADH had a strong transient inhibitory effect on the reduction of AcPyAD$^+$ by tNADPH, indicating that the inhibition only took place during the NADH-AcPyAD$^+$ reaction.

4. Discussion

The present results show that *E. coli* transhydrogenase may be reconstituted efficiently by using a detergent-exchange procedure based on affinity chromatography on immobilized palmitoyl-Coenzyme A. Removal of non-dialyzable detergents is essential for generating tightly coupled vesicles containing only transhydrogenase, or both transhydrogenase and a second protein, e.g., bacteriorhodopsin. A previously employed method used for bovine transhydrogenase, based on hydroxy apatite [14], proved to be inhibitory in the case of the *E. coli* transhydrogenase (not shown). Transhydrogenase vesicles and transhydrogenase-bacteriorhodopsin vesicles prepared by this new method were then used for investigating various properties of *E. coli* transhydrogenase.

Transhydrogenase vesicles were actively pumping protons as judged by quenching of ACMA fluorescence and an uncoupler-stimulated reduction of AcPyAD$^+$ by NADPH. A decoupled AcPyAD$^+$-NADPH transhydrogenase reaction, but not the light-driven NADH-tNADP$^+$ reaction, was obtained by omitting DTT from the dialysis medium indicating that, indeed, proton pumping and catalytic activity may be separated. That the two directions of the reaction behave differently in this regard can presently not be explained. Either cysteine or methionine residues are likely to be oxidized in the absence of DTT, but cysteine β260 is the only conserved cysteine in transhydrogenases which, however, is not essential for activity [10]. If oxidation of the critical residue involves a conserved residue, this leaves one or more methionines as possible activity-regulating residues. The identity of these is presently being investigated.

E. coli transhydrogenase-bacteriorhodopsin vesicles were strongly influenced by the electrochemical proton gradient generated by bacteriorhodopsin in the light. The rate of reduction of tNADP$^+$ by NADH was increased some 5–10 fold, similar to that catalyzed by e.g., bovine transhydrogenase-bacteriorhodopsin vesicles, submitochondrial particles or *E. coli* membrane vesicles upon addition of a respiratory substrate or ATP [1–3]. The pronounced light-depent decrease in the K_m for NADH rather than tNADP$^+$ may suggest that the NAD(H)-binding site is primarely affected by the utilization of the electrochemical proton gradient generated by bacteriorhodopsin. The decreased inhibition in the presence of light by derivatives based on the adenosine-ribose-phosphate part of NADH suggests that this moiety is not involved in the shift to a high-affinity conformation and therefore behaves as NAD$^+$. Thus, the increased affinity for NADH probably reflects an increased binding of NADH rather than an increased dissociation of NAD$^+$. However, the less dramatic dark/light change in the effect of NMNH may indicate that the latter may play a role in the light-dependent change in the affinity for NADH, and that the substrate/inhibitor must have a structure similar or identical to that of NADH in order to bind with a high affinity.

Replacement of NADH with 2′-deoxy-NADH gave an activity which was strongly sigmoidal as a function of the concentration of 2′-deoxy-NADH. In addition, the activity ratio in the dark/light for reduction of tNADP$^+$ by NADH was approximately the same as that obtained with NADH, suggesting that aspartic acid α195, which is involved in the binding of the 2′-hydroxy moiety of NAD(H) [28], is not involved in proton pumping. The sigmoidal correlation between activity and 2′-deoxy-NADH, but not NADH, suggests that *E. coli* transhydrogenase is an allosteric enzyme. The interaction of 2′-deoxy-NADH with transhydrogenase is presently being investigated in more detail.

Coupled reconstituted bovine transhydrogenase vesicles were earlier shown to catalyze a rapid and quantitative reduction of AcPyAD$^+$ by NADH in the presence of NADPH [13,21]. The reaction may be conveniantly demonstrated by adding a small amount of NADH to a reaction catalyzing the slow reduction of AcPyAD$^+$ by NADPH under coupled conditions. Uncouplers abolished the reaction. It was concluded that NADH reduced bound NADP$^+$ (formed in the reduction of AcPyAD$^+$ by NADPH), which then in turn reduced a new molecule of AcPyAD$^+$. An essential feature of this mechanism was the proton gradient-dependent affinity change for NAD(H). Subsequently, it was shown by Jackson and coworkers [22] that a similar reaction was catalyzed by purified and detergent-dispersed *E. coli* transhydrogenase at a low pH,

especially at pH 6.0 and below. It was concluded that the NADH-AcPyAD$^+$ reaction ocurred in the presence of catalytic amounts of bound NADP(H), mediated by a residue with a pK_a of about 6 [22].

The pH-dependencies of proton pumping driven by the reduction of AcPyAD$^+$ by NADPH, and the NADH-AcPyAD$^+$ reaction, respectively, obtained in the present investigation are striking in the sense that they are virtual mirror images, the former reaction being active at a pH above 5.5 and the latter reaction being active at a pH below 6.5. Thus, the two reactions are overlapping, which has been used here to study the interactions between the reactions. Also, with reconstituted vesicles and the conditions used in this investigation the pH dependencies were found to be considerably more narrow as compared to those for the detergent-dispersed enzyme which showed a much broader pH dependence [22].

Fig. 10 constitutes a working model of the partial reactions and conformational states involved in the overall proton pumping transhydrogenase reaction, and may explain previous as well as present observations regarding the NADH-AcPyAD$^+$ reaction catalyzed by *E. coli* transhydrogenase. The model assumes a helical bundle structure of the membrane domain of transhydrogenase similar to that of bacteriorhodopsin [30], i.e., secondary structures which are not membrane helices are not located in the membrane. An essential component of the model is histidine β91 which recently has been shown by site-specific mutagenesis to be the only conserved and essential charged residue in the predicted membrane sector of the enzyme [10]. Starting with transhydrogenase in the resting state (state 0) and AcPyAD$^+$ plus NADPH, these substrates bind to transhydrogenase (state 1A), they cause an increased pK_a of histidine β91 exposed on the cytosolic side and subsequently a protonation of this histidine (state 2A), the redox step then takes place generating a constrained state (state 3), the protonated histidine is shifted from the cytosolic space to the periplasmic space (state 2B) driven by the redox energy generated, the pK_a of the histidine is normalized and the proton dissociates (state 1B), followed by the release of the products and return to the resting state (state 0).

Reduction of AcPyAD$^+$ by NADH via NADP(H) is assumed to proceed by replacing AcPyADH with NADH in state 1B and subsequently to reverse the reaction sequence to state 1A. State 1A will then replace its NAD$^+$ formed with a new molecule of AcPyAD$^+$ and the normal sequence of reactions proceed through states 2A, 3, 2B and 1B. The release of AcPyADH then completes the cycle. This model predicts that the net oxidation of NADPH should be strongly inhibited during the NADH-AcPyAD$^+$ cycle. Indeed, when following the oxidation of tNADPH directly at pH 6.0, addition of small amounts of NADH transiently inhibited the rate of tNADPH oxidation, which agrees with earlier findings that the conversion of NADH into AcPyADH is stoichiometric [21,22]. As expected, proton pumping driven by the reduction of AcPyAD$^+$ by NADPH was thus found to be strongly inhibited under similar conditions by NADH but not by AcPyADH, indicating that inhibition requires an initial oxidation of NADH. Of course, in agreement with the model, the NADH-AcPyADH cycle itself involves two opposing steps of proton pumping and therefore no net proton pumping.

The model in Fig. 10 also provides an explanation for the dramatic affinity change for NADH associated with an electrochemical proton gradient as in the reduction of tNADP$^+$ by NADH driven by light catalyzed by the transhydrogenase-bacteriorhodopsin vesicles. In state 1B the histidine β91 is exposed to the periplasmic space, which is triggered by binding of NADH and tNADP$^+$. However, it is proposed that an increased extent of protonation of histidine β91 on the periplasmic side increases the affinity of the enzyme for NADH.

An interesting problem concerns the relationship between the NADH-AcPyAD$^+$ reaction catalyzed by reconstituted bovine transhydrogenase, and the corresponding NADH-AcPyAD$^+$ reaction catalyzed by detergent-dispersed or reconstituted *E. coli* transhydrogenase. It is reasonable to assume that these reactions are all closely related. However, the key question is why the NADH-AcPyAD$^+$ reaction takes place in the first place. A suggestion based on the model in Fig. 10 is that when histidine β91 is exposed to a pH well below its pK_a, the pK_a change that normally allows the deprotonation of state 2B is no longer sufficient for this step to take place. As a consequence, a conformational change associated with deprotonation, which is assumed to be rate-limiting, is no longer required/limiting. A similar reasoning may apply to the

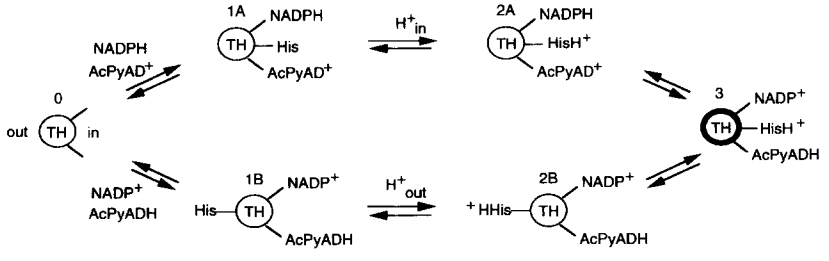

Fig. 10. Model of the intermediate conformational states of the transhydrogenase reaction "Out" and "In" denote the periplasmic space and the cytosol, respectively. The shift of the histidine residue should be regarded as an accessibility to either side of the membrane rather than a physical movement.

reaction catalyzed by reconstituted bovine transhydrogenase, although the NADH-AcPyAD$^+$ activity is some 10–20-fold lower as compared to that of the corresponding *E. coli* transhydrogenase reaction and requires coupled vesicles [13,21]. In this case, the NADH-AcPyAD$^+$ reaction may be visualized to occur because a lowered internal pH caused by the proton pumping NADPH-AcPyAD$^+$ reaction has to replace a low medium pH. However, the pH dependence of the NADH-AcPyAD$^+$ reaction catalyzed by the reconstituted bovine transhydrogenase is essentially indistinguishable from those of other transhydrogenase reactions catalyzed by the bovine transhydrogenase [21]. Therefore, the affinity changes of transhydrogenase for e.g NADH that accompany proton pumping in both *E. coli* and bovine transhydrogenases may be especially important for the NADH-AcPyAD$^+$ reaction catalyzed by reconstituted bovine transhydrogenase. In this context it should be pointed out that the NADH-AcPyAD$^+$ reaction of course is driven by the redox potential difference between NADH and AcPyADH, and therefore does not occur naturally except as a result of exchange reactions.

In conclusion, the successful reconstitution of *E. coli* transhydrogenase in phospholipid vesicles without or with bacteriorhodopsin has provided a valuable system for studying the mechanism of the transhydrogenase reaction. Using reconstituted *E. coli* transhydrogenase, the rapid and electroneutral reduction of AcPyAD$^+$ by NADH has been explained in terms of a high extent of protonation of a critical histidine residue, possibly histidine $\beta 91$, which normally undergoes a protonation/deprotonation cycle in the proton pumping or proton gradient utilizing transhydrogenase reactions.

Acknowledgements

This work was supported by the Swedish Natural Sciences Research Council.

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