Properties of a cysteine-free proton-pumping nicotinamide nucleotide transhydrogenase

Johan MEULLER*, Junwei ZHANG*, Cynthia HOU†, Philip D. BRAGG† and Jan RYDSTRÖM*‡

*Department of Biochemistry and Biophysics, Göteborg University, S-413 90 Göteborg, Sweden, and †Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

Nicotinamide nucleotide transhydrogenase from *Escherichia coli* was investigated with respect to the roles of its cysteine residues. This enzyme contains seven cysteines, of which five are located in the α subunit and two are in the β subunit. All cysteines were replaced by site-directed mutagenesis. The final construct (αC292T, αC339T, αC395S, αC397T, αC435S, βC147S, βC260S) was inserted normally in the membrane and underwent the normal NADPH-dependent conformational change of the β subunit to a trypsin-sensitive state. Reduction of $NADP^+$ by NADH driven by ATP hydrolysis or respiration was between 32 % and 65 % of the corresponding wild-type activities. Likewise, the catalytic and proton pumping activities of the purified cysteine-free enzyme were at least 30 % of the purified wild-type enzyme activities. The H^+/H^- ratio for both enzymes was 0.5, although the cysteine-free enzyme appeared to be more stable than the wild-type enzyme in proteoliposomes. No bound NADP(H) was detected in the enzymes. Modification of transhydrogenase by diethyl pyrocarbonate and the subsequent inhibition of the enzyme were unaffected by removal of the cysteines, indicating a lack of involvement of cysteines in this process. Replacement of cysteine residues in the α subunit resulted in no or little change in activity, suggesting that the basis for the decreased activity was probably the modification of the conserved β-subunit residue Cys-260 or (less likely) the non-conserved β-subunit residue Cys-147. It is concluded that the cysteine-free transhydrogenase is structurally and mechanistically very similar to the wild-type enzyme, with minor modifications of the properties of the NADP(H) site, possibly mediated by the βC260S mutation. The cysteine-free construct will be a valuable tool for studying structure–function relationships of transhydrogenases.

INTRODUCTION

Nicotinamide nucleotide transhydrogenases (EC 1.6.1.1) are integral membrane proteins and redox-driven proton pumps that catalyse the reduction of $NADP^+$ by NADH and a concomitant translocation of n protons according to the reaction:

$$n\mathrm{H^+_{out}} + \mathrm{NADH} + \mathrm{NADP^+} \rightleftharpoons n\mathrm{H^+_{in}} + \mathrm{NAD^+} + \mathrm{NADPH}$$

Transhydrogenases from several different sources have been isolated and reconstituted in liposomes, and their kinetic and functional properties have been characterized. It is generally concluded that the value of n is 1. The enzymes probably serve as a source of NADPH and as a regulatory system for the redox level of $NADP^+$. Some eight transhydrogenase genes have been cloned. Enzymes within the transhydrogenase group are homologues and the overall amino acid sequence identity is about 30–40 %, with four or five strongly conserved domains. The latter domains are the two proposed substrate-binding sites, one for NAD(H) and one for NADP(H), and parts of the predicted 10–14 transmembrane α-helices presumably forming a proton pathway. All evidence suggests that transhydrogenases are conformationally driven proton pumps in which the conformational states are primarily regulated by the ligands/substrates occupying the NADP(H) site, with the NAD(H) site providing reducing equivalents for $NADP^+$ reduction (for reviews, see [1,2]).

One of the central problems related to transhydrogenase is the mechanism of conformationally driven proton pumping by the enzyme. During the past decade, information regarding the structure–function relationships of transhydrogenases has accumulated rapidly, and it is now possible to probe the roles and functions of individual domains/residues of at least the *Escherichia coli* and *Rhodospirillum rubrum* enzymes by mutagenesis [1,3–8] and NMR [8,9]. In order to probe more specifically the environments of certain residues and their specific roles in, for example, proton pumping, it is of interest to chemically label specific cysteine residues. Obviously, this is difficult in a protein with several cysteine residues, and cysteine-free mutant proteins are therefore generated, in which a cysteine residue can subsequently be inserted and labelled by SH-reactive agents in any desired position, provided that the construct gives a reasonably active enzyme. In addition, a cysteine-free enzyme provides a better alternative to toplogical studies than, for example, gene fusion techniques. Apart from bacteriorhodopsin, which does not contain cysteine residues [10], membrane proteins with a transport function that have so far been mutated to cysteine-free variants are very few, e.g. lactose permease [11] and yeast plasma membrane H^+-ATPase [12]. Cysteine-free lactose permease is about 50 % as active as the wild-type enzyme, whereas cysteine-free yeast plasma membrane H^+-ATPase is severely inhibited.

E. coli transhydrogenase contains seven cysteine residues, of which five reside in the α subunit and two are in the β subunit [13]. In the present investigation, the cysteine residues of this transhydrogenase were simultaneously replaced by site-directed mutagenesis and the properties of the cysteine-free enzyme were determined. The results show that the cysteine-free enzyme behaves essentially like the wild-type enzyme with regard to membrane insertion and NADPH-induced conformational changes, and retains at least 30 % of catalytic and proton pumping activities.

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; $AcPyAD^+$, 3-acetylpyridine-NAD^+; 2′-AMP-5′-DPR, adenosine 2′-monophosphate 5′-diphosphate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

‡ To whom correspondence should be addressed.

MATERIALS AND METHODS

Bacterial strains and plasmid

The *pnt* gene was introduced into the pGEM-7Zf(+) plasmid, resulting in the construct denoted pSA2. pSA2 was subsequently used to transform the *E. coli* K12 strain JM109 and used as a source of wild-type and mutant transhydrogenases [14].

Mutagenesis

E. coli transhydrogenase contains seven cysteine residues, i.e. at positions 292, 339, 395, 397 and 435 in the α subunit and positions 147 and 260 in the β subunit. Mutants in the α subunit were made by PCR-mutagenesis [3–5,15], using a *Sal*I/*Bst*EII cassette to avoid amplification of the whole gene, in which the cysteine residues were replaced with Ala, Thr and/or Ser. The most active substituted enzymes were chosen for further manipulations.

The β-subunit cysteines were replaced with Ser as described [14]. The final construct, pCL (i.e. αC292T, αC339T, αC395S, αC397T, αC435S, βC147S, βC260S), was made by replacing a *Bst*BI/*Bss*HII restriction fragment of pSA2 lacking α-subunit cysteines with the corresponding fragment from pSA2 lacking β-subunit cysteines. This construct codes for a transhydrogenase without cysteines, i.e. cysteine-free transhydrogenase. Gene sequences of mutants were verified by sequencing the manipulated subfragment of the transhydrogenase gene as well as the whole gene of the final construct.

Preparation of cytoplasmic membrane vesicles

Inside-out cytoplasmic vesicles were prepared as described previously [3]. When indicated, washed cytoplasmic membrane vesicles were used [6].

Purification of wild-type and cysteine-free *E. coli* transhydrogenases

Wild-type transhydrogenase was purified as described previously [16], except that a Pharmacia Resource Q column was used in the ion-exchange chromatography step. Cysteine-free transhydrogenase was purified as for the wild-type enzyme, except for the final affinity chromatography step which was replaced by a second anion-exchange step. The peak from the first ion-exchange step was diluted 2.5-fold with equilibration buffer (50 mM sodium phosphate, pH 7.5, 0.1 % sodium cholate) and loaded on to the column. After washing with 160 mM NaCl in equilibration buffer, cysteine-free transhydrogenase was eluted with a 160–460 mM NaCl gradient in equilibration buffer. This final chromatography step was included mainly as a detergent-exchange step. For catalytic measurements, transhydrogenase from the late fractions of the first ion-exchange chromatography step was used.

The cysteine-free transhydrogenase purified in this way contained some minor additional bands as compared with the wild-type preparation, but was nevertheless 90 % pure as judged by SDS/PAGE in the presence of urea (results not shown).

Catalytic activity

The catalytic activity of wild-type and cysteine-free transhydrogenases was measured spectrophotometrically at 375 nm or 375–410 nm as the reduction of 3-acetylpyridine-NAD^+ ($AcPyAD^+$) by NADPH, using molar absorption coefficients of 5100 and 4200 $M^{-1} \cdot cm^{-1}$ respectively [17]. The reduction of thio-$NADP^+$ by NADH was measured at 400–460 nm using a molar absorption coefficient of 10700 $M^{-1} \cdot cm^{-1}$ [17]. Unless otherwise indicated, these assays were carried out in 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.01 % Brij 35 at 25 °C with 0.5 mM substrate.

The reduction of $AcPyAD^+$ by NADPH and the reduction of $NADP^+$ by NADH, driven by a proton motive force generated by electron transport or ATP hydrolysis, were assayed with cytoplasmic membrane vesicles [18]. The cyclic reduction of $AcPyAD^+$ by NADH in the absence and in the presence of NADP(H), catalysed by the purified enzyme, was assayed as described [16,19] using Mes buffer (25 mM Mes/KOH, pH 6.0, 1 mM EDTA, 1 mM DTT, 0.01 % Brij 35) or MesK buffer (25 mM Mes/KOH, pH 6.0, 1 mM EDTA, 1 mM DTT, 0.01 % Brij 35, 300 mM KCl, 5 mM $MgCl_2$).

Preparation of proteoliposomes

Reconstitution was based on a method developed by Richard et al. [20]. A solution of phospholipids (45 % phosphatidylcholine, 50 % phosphatidylethanolamine and 5 % phosphatidylserine, by wt.) was dried under nitrogen, twice dissolved in diethyl ether and dried, and finally resuspended in 100 mM potassium phosphate/50 mM KCl (pH 7.2) at a concentration of 11 mg/ml. Liposomes were prepared by sonicating the solution for 12 min in a bath-type sonicator. All further steps were performed at 4 °C. Transhydrogenase, from the second chromatographic step, was diluted 15–20-fold with 5 mM sodium phosphate (pH 7.2), 1 mM EDTA, 1 mM DTT and 0.025 % Triton X-100 to a concentration of 50 μg/ml. A mixture of 3 ml of this enzyme solution, 0.3 ml of 10 % (v/v) Triton X-100 and 4.5 ml of liposomes was prepared and incubated for 45 min with gentle mixing. To remove the detergent, Biobeads SM2 (Bio-Rad), washed in methanol and water, were added. Three additions of 0.3 g of Biobeads were carried out, with 90 min of incubation for each of the first two additions and 60 min of incubation for the final addition. After removal of the beads, the proteoliposomes were dialysed against 600 vol. of 50 mM KCl/75 mM sucrose/25 μM potassium phosphate (pH 7.2) for 14 h. The activity of the cysteine-free transhydrogenase was essentially unaffected by the reconstitution procedure. In contrast, the wild-type enzyme was partially inactivated by the reconstitution. Undialysed proteoliposomes were stable for at least 4 days when kept on ice. Proteoliposomes used for 9-amino-6-chloro-2-methoxyacridine (ACMA) quenching were prepared as described by Eytan et al. [21], using the same lipid composition as above and transhydrogenase from the second chromatography step/detergent-exchange step (see section on purification of transhydrogenase).

H^+/H^- ratio measurements

Experiments to measure the rate of transhydrogenation and proton transfer were performed using a Shimadzu UV-3000 dual-wavelength spectrophotometer equipped with a magnetic stirrer. Rapid mixing was achieved by injection into the cuvette with a Hamilton syringe while stirring with a magnetic bar. Mixing was more than 90 % complete in 0.5 s. The rate of hydride-ion transfer from NADPH to $AcPyAD^+$ was measured spectrophotometrically at 375–410 nm using a molar absorption coefficient of 4200 $M^{-1} \cdot cm^{-1}$ [17]. The accompanying proton transfer was measured by following the absorbance change of Phenol Red at 560–640 nm, essentially as described [22] but with some modifications. The buffer used was 5 mM $MgCl_2$, 40 mM choline cloride and 30 mM KCl (pH 7.2). For each measurement, 0.4 ml of vesicles was used in a 2 ml assay with 60 μM Phenol

Red, 2.5 μg/ml valinomycin, 100 μM AcPyAD$^+$ and 75 μM NADPH. For H^- measurements the Phenol Red was omitted, since the absorbance spectrum of Phenol Red overlaps with the wavelengths used in H^- measurements; this is justified, since addition of 0.25 mM Hepes (pH 7.2) did not affect the H^- rate. Calibration of the Phenol Red signal was performed by triplicate additions of HCl and NaOH, prepared from standard solutions (Merck). Calculations of the rates were carried out from slopes drawn between 0.8 and 3 s, where the traces were approximately linear.

Determination of bound NADP(H)

Tightly bound NADP(H) was determined as described [7], with a detection limit of 0.015 mol of NADP(H)/mol of $\alpha_2\beta_2$ transhydrogenase. An attempt was made to remove possibly bound NADP(H) by treating the wild-type and cysteine-free purified transhydrogenases with adenosine 2′-monophosphate 5′-diphosphate (2′-AMP-5′-DPR), an NADP(H)-specific inhibitor [23]. Purified transhydrogenase (1 mg) obtained directly from the FPLC step was mixed with 2.5 mM 2′-AMP-5′-DPR, incubated at pH 7.0 for 30 min at 4 °C and subjected to gel filtration on a Pharmacia Fast desalting FPLC HR10 column. The transhydrogenase treated in this manner was separated from the inhibitor and possibly bound and released NADP(H).

Inhibition by *N*-ethylmaleimide (NEM)

Wild-type or cysteine-free transhydrogenase, at a concentration of 80–110 μg/ml, was incubated with or without 1 mM NEM in 50 mM sodium phosphate (pH 7.5) and 0.1 % sodium cholate at 23 °C. At appropriate time intervals, aliquots were assayed for catalytic activity, i.e. the reduction of AcPyAD$^+$ by NADPH. In these studies, wild-type and cysteine-free transhydrogenases were concentrated and desalted before use to remove the DTT and to change the buffer to pH 7.5.

SDS/PAGE analysis

Proteins were separated on an SDS/10 % (w/v)-acrylamide gel in the absence or presence of 3 M urea, and stained with Coomassie Blue [24].

Protein determination

Protein was measured by the method of Peterson [25], with BSA as standard.

Chemicals

Oligonucleotides were purchased from SGS (Scandinavian Diagnostic Services, Falkenberg, Sweden), and enzymes were from Life Technologies or Boehringer-Mannheim. All other biochemicals were of analytical grade and were purchased from Sigma or Boehringer-Mannheim. Phospholipids were purchased from Lipid Products (S. Nutfield, Surrey, U.K.) or Sigma.

RESULTS

Catalytic activities of cysteine-free transhydrogenase

Figure 1 shows the distribution of cysteine residues in *E. coli* transhydrogenase. Residues Cys-292, Cys-339, Cys-395, Cys-397 and Cys-435 are located in the α subunit, whereas residues Cys-147 and Cys-260 are located in the β subunit. The domains that have been predicted as being membrane-spanning contain residues αCys-435, βCys-147 and βCys-260 [5].

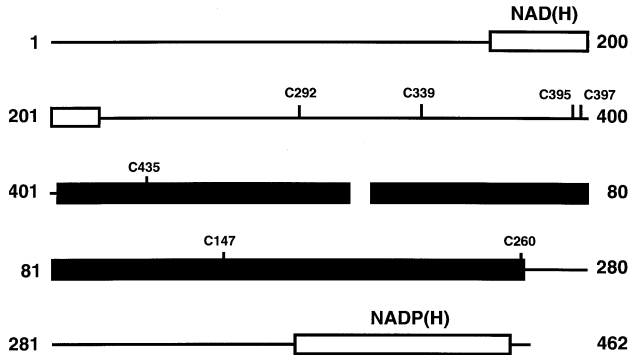

Figure 1 Distribution of cysteine residues in *E. coli* transhydrogenase

Table 1 Catalytic activities of cytosolic membrane vesicles, and of washed cytosolic membrane vesicles, containing wild-type and cysteine-free transhydrogenases

The conditions for assaying the activities were as described in the Materials and methods section. For the NADH + NADP$^+$ reactions, $n = 3$ and values are means ± S.D. Values in parentheses are the activity of the cysteine-free enzyme as a percentage of the wild-type activity.

	Activity (μmol/min per mg)	
Reaction	Wild-type	Cysteine-free
Vesicles		
AcPyAD$^+$ + NADPH	3.0	1.9 (65 %)
NADP$^+$ + NADH (aerobic)	0.42 ± 0.14	0.20 ± 0.02 (48 %)
NADP$^+$ + NADH (ATP)	0.74 ± 0.10	0.24 ± 0.03 (32 %)
Washed vesicles	7.5	2.4 (32 %)
AcPyAD$^+$ + NADPH		

As shown in Table 1, cytoplasmic membrane vesicles containing cysteine-free transhydrogenase catalysed the reduction of AcPyAD$^+$ by NADPH at a level that was approx. 65 % of the corresponding wild-type activity. The activities for the reduction of NADP$^+$ by NADH driven by electron transport or ATP hydrolysis catalysed by the same preparation were approx. 48 % and 32 % respectively. As shown previously, the catalytic activity of vesicles that had been subjected to washing (cf. [6]) was significantly higher than that of non-washed vesicles (Table 1). The activity of cysteine-free transhydrogenase in washed vesicles, assayed as the reduction of AcPyAD$^+$ by NADPH, was about 32 % of the wild-type activity (Table 1). Thus the cysteine-free transhydrogenase retained a significant level of all catalytic activities tested.

Bovine heart mitochondrial transhydrogenase is inhibited by modification of histidine residues by diethyl pyrocarbonate, possibly via cysteines [26,27]. Similar results were obtained with the *E. coli* transhydrogenase (P. D. Bragg and C. Hou, unpublished work). All groups have found that the rate of inactivation by these agents was accelerated by NADP(H), but not by NAD(H), suggesting that the site of interaction is outside the NADP(H) site. NADP(H) induces a conformational change in the β subunit which may be detected by proteolysis by trypsin [28]. Indeed, mutants have been constructed that are locked in the NADP(H)-induced conformation in the absence of these substrates [6]. Therefore it was of interest to investigate whether the cysteine-free *E. coli* enzyme behaved in a wild-type manner when exposed to diethyl pyrocarbonate. As shown in Figure 2,

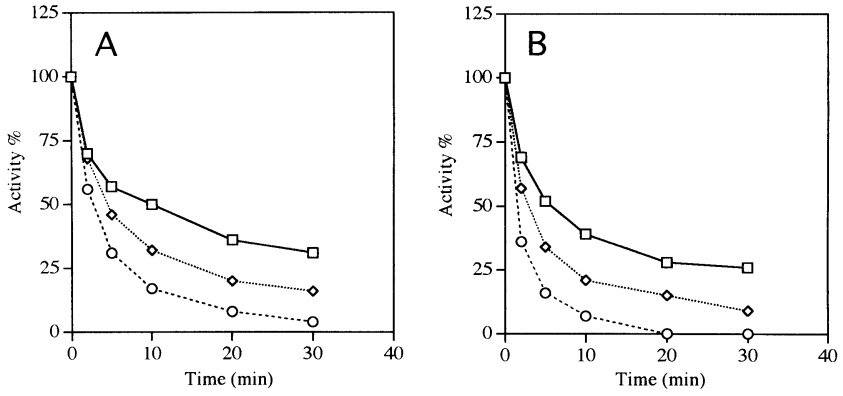

Figure 2 Inhibition of catalytic activity by diethyl pyrocarbonate

Washed cytosolic membrane vesicles (0.8 mg/ml) containing wild-type (**A**) or cysteine-free (**B**) transhydrogenase were incubated with diethyl pyrocarbonate in 50 mM Mes/KOH buffer (pH 6.0) containing 0.5 mM EDTA, 2 mM DTT and 0.01% Brij 35. The concentrations of diethyl pyrocarbonate were: □, 1 mM; ◇, 2 mM; ○, 3 mM. The catalytic activity was measured at intervals, as indicated.

the behaviour of the cysteine-free enzyme was essentially identical to that of the wild-type enzyme. In addition, trypsin digestion in the presence and absence of substrates gave essentially identical degradation patterns for the wild-type (Figure 3A) and cysteine-free (Figure 3B) enzymes. The results in Figure 3 also demonstrate that the expression levels were similar for the wild-type and cysteine-free transhydrogenases, and that the latter was incorporated normally into the membrane.

The catalytic and proton pumping activities of the αC292T, αC339T, αC395S, αC397T, αC435S mutant in cytoplasmic membrane vesicles were 88% and 98% respectively of the corresponding wild-type activities, suggesting that the decreased activities of the cysteine-free transhydrogenase are due to the βC147S and/or βC260S mutations (results not shown). This is in agreement with previously reported data for the βC260S mutant, which showed between 60 and 80% of wild-type activity [5].

Various kinetic parameters, proton pumping and sensitivity to NEM were assayed using the purified cysteine-free transhydrogenase and the purified wild-type enzyme as a control. The determination of K_m(app) values for the reduction of AcPyAD$^+$ by NADPH and the reduction of thio-NADP$^+$ by NADH is shown in Table 2. Removal of the cysteine residues did not affect significantly the K_m(app) values for NADH or AcPyAD$^+$. Under the conditions used in this investigation, these K_m values were approx. 1.7–2.0 μM and 20–26 μM respectively. However, the corresponding values for NADPH and thio-NADP$^+$ were significantly different for the wild-type and cysteine-free enzymes. With the wild-type enzyme, K_m(app) values were 15 ± 0.5 μM and 8 ± 3.0 μM respectively for NADPH and thio-NADP$^+$. With the cysteine-free enzyme these values were 5.1 ± 1.9 μM and 18 ± 2.0 μM respectively. Thus removal of the cysteines led to a change in the affinity of the NADP(H) site for its substrates, favouring binding of the reduced substrate.

Activities of purified and reconstituted wild-type and cysteine-free transhydrogenases

Table 3 shows the effect of removal of the cysteines on the reduction of AcPyAD$^+$ by NADH in the absence and in the presence of NADP$^+$, and on the reduction of AcPyAD$^+$ by NADPH in the absence and in the presence of NADH. The reduction of AcPyAD$^+$ by NADH was about 5-fold more active when catalysed by the purifed cysteine-free transhydrogenase, a difference that was eliminated by the presence of salt, i.e. in the presence of MesK buffer. In the presence of NADP$^+$ the difference was decreased to about 4-fold, a difference that again was eliminated by the presence of salt. The so-called cyclic reduction of AcPyAD$^+$ by NADH via NADPH was between 2.0- and 2.5-fold more active when catalysed by the cysteine-free enzyme, a difference that was independent of the absence or presence of salt.

Reconstitution of the purified transhydrogenase leads to diminished activity in the presence of valinomycin, due to a limiting flux of protons. Under these conditions the activity for the reduction of AcPyAD$^+$ by NADPH catalysed by the wild-type and cysteine-free enzymes was 1.6 ± 0.2 and 5.1 ± 0.5 μmol/min per mg respectively (Table 3). The associated proton pumping activities were 0.8 ± 0.1 and 2.5 ± 0.4 μmol/min per mg respectively. These rates gave a mean H^+/H^- ratio (based on separate sets of ratios calculated for each individual pair of H^+ and H^- determinations) for both the wild-type and the cysteine-free enzymes of 0.5. Maximal individual ratio values were 0.6 and 0.8 for the wild-type and cysteine-free enzymes respectively (results not shown). The degree of stimulation of catalytic activities in dialysed proteoliposomes by carbonyl cyanide *m*-chlorophenylhydrazone was routinely more than 10-fold for the cysteine-free enzyme, but about 3-fold for the wild-type enzyme (results not shown).

The wild-type and cysteine-free transhydrogenases contained less than 0.015 mol of NADP(H)/mol of tetramer when determined in two separate washed cytoplasmic membrane vesicle preparations (see the Materials and methods section). Also, somewhat surprisingly, the cyclic transhydrogenase activities catalysed by the 2′-AMP-5′-DPR-treated purified enzymes were at least 100% of those of the untreated enzymes (results not shown).

Proton pumping activity catalysed by the wild-type and cysteine-free reconstituted transhydrogenase proteoliposomes, assayed as quenching of ACMA, is shown in Figure 4. Pumping is initiated with the second substrate, i.e. AcPyAD$^+$. No proton pumping was observed after adding the first substrate, NADPH. Wild-type transhydrogenase caused a final extent of ACMA quenching of 80 ± 1%, compared with 70 ± 1% for the cysteine-free enzyme. The degree of quenching for the cysteine-free enzyme was 87 ± 1% of that of the wild-type. Thus, assayed by this method, the cysteine-free transhydrogenase was approx.

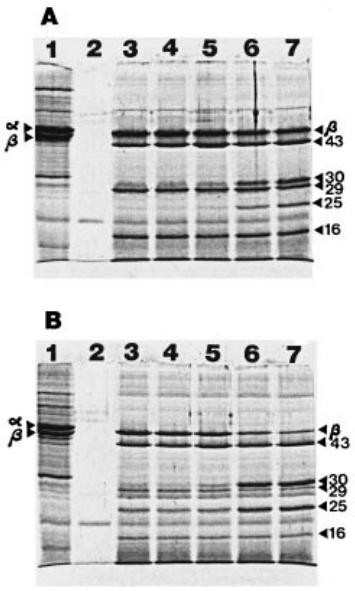

Figure 3 Trypsin digestion of cytosolic membrane vesicles

Washed cytosolic membrane vesicles (1 mg/ml) containing wild-type (**A**) or cysteine-free (**B**) transhydrogenase were incubated with diphenyl carbamyl chloride (DPPC) treated trypsin at a trypsin/transhydrogenase ratio of 1:100 (w/w) for 60 min at room temperature in the absence (lane 3) or the presence of 0.5 mM NAD^+ (lane 4), NADH (lane 5), $NADP^+$ (lane 6) or NADPH (lane 7). The medium contained 50 mM Tris/HCl buffer (pH 7.8), 1 mM DTT and 1 mM EDTA. The reactions were terminated by the addition of soybean trypsin inhibitor at a weight twice that of the trypsin. The samples were examined by SDS/PAGE on 13% (w/v) gels. Lane 1, untreated vesicles; lane 2, vesicles omitted. The positions of migration of the α and β subunits and of the trypsin cleavage fragments (in kDa) are indicated.

Table 2 Determination of K_m(app) values of purified wild-type and cysteine-free transhydrogenase catalysing the reduction of $AcPyAD^+$ by NADPH, and the reduction of thio-$NADP^+$ by NADH

K_m(app) values were determined using between five and seven different concentrations of the limiting substrate and 0.5 mM of the saturated substrate. Each concentration was assayed three times with two different preparations. Results are means ± S.D.

	K_m(app) (μM)	
Substrate	Wild-type	Cysteine-free
NADH	1.7 ± 0.1	2.0 ± 1.0
$AcPyAD^+$	26.0 ± 3.0	20.5 ± 3.5
NADPH	15.0 ± 0.5	5.1 ± 1.9
Thio-$NADP^+$	8.0 ± 3.0	18.0 ± 2.0

10–20% less effective than the wild-type enzyme in proton pumping. All transhydrogenase-dependent quenching of ACMA fluorescence was abolished by uncouplers (results not shown).

Modification by NEM

Modification of cysteine residues in *E. coli* transhydrogenase by NEM leads to inhibition of activity, and the rate of this inhibition is enhanced by NADPH and inhibited by $NADP^+$ and NAD^+ [29,30]. The cysteine-free transhydrogenase would be expected to be insensitive to NEM. This is indeed shown in Figure 5. In contrast to the wild-type enzyme, which was strongly inhibited by NEM (Figure 5A), no noticeable inhibition by NEM was detected with the cysteine-free enzyme (Figure 5B).

Table 3 Determination of the rates of reduction of $AcPyAD^+$ by NADH in the absence and in the presence of $NADP^+$, and the reduction of $AcPyAD^+$ by NADPH in the absence and in the presence of NADH, catalysed by purified and reconstituted wild-type and cysteine-free transhydrogenases

The concentrations of NADH, $AcPyAD^+$ and NADPH were each 200 μM. Additions were 20 μM $NADP^+$ or 40 μM NADH. Other conditions were as described in the Materials and methods section. Assays were carried out using two different preparations ($n = 4$ for assays of soluble enzymes; $n = 5$ for H^--transfer catalysed by reconstituted wild-type enzyme; $n = 3$ for H^+ pumping catalysed by reconstituted wild-type enzyme; $n = 12$ for H^--transfer catalysed by reconstituted cysteine-free enzyme; $n = 10$ for H^+ pumping catalysed by cysteine-free reconstituted enzyme). Results are means ± S.D.

		Activity (μmol/min per mg)	
Reaction	Addition	Wild-type	Cysteine-free
Soluble enzyme; Mes buffer			
NADH + $AcPyAD^+$	–	11.5 ± 3.9	61.1 ± 11.8
NADH + $AcPyAD^+$	$NADP^+$	37.8 ± 10.1	131.1 ± 55.1
NADPH + $AcPyAD^+$	–	1.3 ± 0.6	2.3 ± 1.2
NADPH + $AcPyAD^+$	NADH	50.4 ± 3.7	129.9 ± 21.0
Soluble enzyme; MesK buffer			
NADH + $AcPyAD^+$	–	1.1 ± 0.4	1.2 ± 0.2
NADH + $AcPyAD^+$	$NADP^+$	7.4 ± 5.0	12.0 ± 1.7
NADPH + $AcPyAD^+$	–	6.3 ± 0.2	9.1 ± 0.2
NADPH + $AcPyAD^+$	NADH	20.7 ± 1.5	65.9 ± 10.0
Reconstituted enzyme (+valinomycin)			
NADPH + $AcPyAD^+$ (H^- transfer)		1.6 ± 0.2	5.1 ± 0.5
NADPH + $AcPyAD^+$ (H^+ pumping)		0.8 ± 0.1 (ratio = 0.5)	2.5 ± 0.4 (ratio = 0.5)

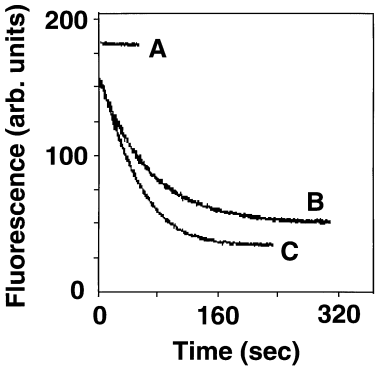

Figure 4 Quenching of ACMA fluorescence by reconstituted transhydrogenase proteoliposomes

Proton pumping was driven by the reduction of $AcPyAD^+$ by NADPH, catalysed by reconstituted wild-type (curve C) or cysteine-free (curve B) transhydrogenase. No quenching was observed before addition of the final substrate, i.e. $AcPyAD^+$ (curve A). Conditions were as described in the Materials and methods section.

DISCUSSION

The present data show that replacement by site-directed mutagenesis of all cysteine residues in *E. coli* transhydrogenase with serine or threonine residues, generating an αC292T, αC339T, αC395S, αC397T, αC435S, βC147S, βC260S mutant transhydro-

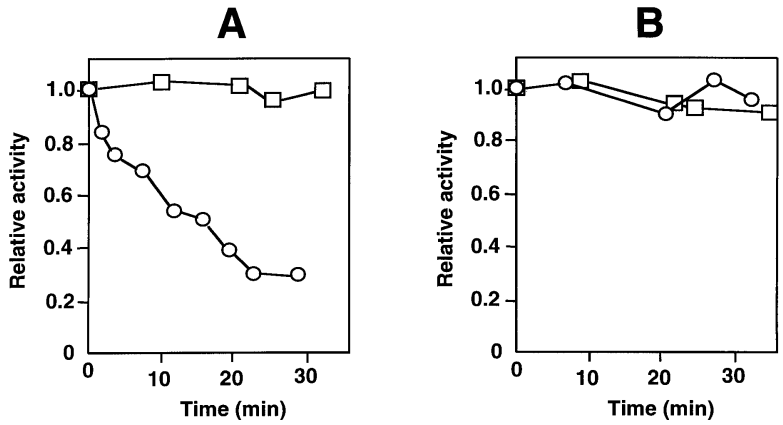

Figure 5 Effect of NEM on the purified wild-type (A) and cysteine-free (B) transhydrogenases

Additions were: ○, 1 mM NEM; □, control. Conditions were as described in the Materials and methods section.

genase, can be carried out, giving an enzyme with partially retained activity. This mutant transhydrogenase is inserted normally in the cytoplasmic membrane, undergoes the normal NADPH-dependent conformational change to a trypsin-sensitive state, and catalyses hydride-ion transfer and proton pumping at rates corresponding to at least 30% of those of the wild-type enzyme.

Most of the decrease in catalytic activity seems to be due to mutagenesis of the Cys-147 and Cys-260 residues of the β subunit, since mutagenesis of the cysteines in the α subunit gave no or small changes in activity. This is consistent with the altered K_m(app) values for NADP(H), but not for NAD(H) (and/or derivatives thereof), observed for the cysteine-free transhydrogenase. Likewise, the reduction of AcPyAD$^+$ by NADH at low pH and low salt concentrations [which may involve the NADP(H) site], as well as the cyclic reduction of AcPyAD$^+$ by NADH in the presence of NADP(H) (cf. [7]), are all catalysed with significantly higher activities by the cysteine-free enzyme. This may be related to altered properties of the NADP(H) site, possibly mediated by the βC260S mutation. It has previously been shown by site-directed mutagenesis that βCys-260, the only conserved cysteine, is not essential for activity [5]. The previous conclusion that βCys-260 does not undergo redox changes essential for transhydrogenase activity [5] can now be extended to include all cysteine residues in the *E. coli* enzyme.

Determination of bound NADP(H) gave negative results, and washing the enzymes with an NADP(H)-specific inhibitor did not influence the cyclic reduction of AcPyAD$^+$ by NADH in the absence of added NADP(H), suggesting that this reaction does not involve bound NADP(H), as was concluded previously [7,23]. In contrast, the soluble β-domain of the *Rhodospirillum rubrum* transhydrogenase contains bound NADP(H) which probably is involved in this cyclic reaction [31]. Jackson and co-workers [32] have previously demonstrated the pronounced dependence of this same reaction on micromolar concentrations of added NADP(H) in the wild-type *E. coli* enzyme. However, bound NADP(H) in the *E. coli* transhydrogenase has so far not been demonstrated, a fact that is further emphasized by the present findings. These discrepancies suggest that there may be a qualitative difference between the *R. rubrum* and *E. coli* enzymes in this regard.

The activities for the ATP-driven reduction of NADP$^+$ by NADH differed by some 3-fold, and ACMA quenching differed by 10–20%; in both cases the cysteine-free transhydrogenase was the less active enzyme. ACMA quenching is a very sensitive assay for the generation of an electrochemical proton gradient, i.e. the sum of the pH gradient and the membrane potential. Elimination of the membrane potential by the addition of valinomycin to K$^+$-loaded vesicles gave initial rates that were too high to be measured (results not shown). It was therefore of interest to determine the actual ratio of H$^-$ transfer and H$^+$ pumping, also in the presence of valinomycin. The H$^+$/H$^-$ ratio was 0.5 for both the wild-type and the cysteine-free enzymes, which may be compared with values of approx. 0.5–1.0 found for other transhydrogenases [1,22]. Since the wild-type enzyme was more unstable/uncoupled than the cysteine-free enzyme during the particular reconstitution procedure used, it is possible that the ratio for the former enzyme was underestimated to a greater extent than that for the latter enzyme. Indeed, maximal individual ratio values reached 0.6 and 0.8 for the wild-type and cysteine-free enzymes respectively. It is therefore possible that the true ratio for the wild-type enzyme is 1.0, in accordance with earlier and more recent results obtained with reconstituted transhydrogenases [1,22]. However, a possible slightly lower value for the cysteine-free enzyme cannot be excluded at present.

The modification of histidine residues by diethyl pyrocarbonate [26,27] strongly inhibits transhydrogenase activity, and this process may involve cysteines. However, the rates of inhibition caused by diethyl pyrocarbonate treatment of the wild-type and cysteine-free transhydrogenases were almost identical. It is therefore concluded that cysteines are not involved in this modification process.

The modification of cysteine residues in transhydrogenases has been studied both with the *E. coli* enzyme [29,30] and with the bovine enzyme [33–36]. Comparisons are difficult to make, since the residue corresponding to βCys-260 in *E. coli* transhydrogenase (residue 834 in the bovine enzyme) is the only conserved cysteine residue located in a domain composed of a sequence of about 12 conserved residues. However, in view of the localization of several cysteine residues in the *E. coli* transhydrogenase relatively close to the binding sites for NAD(H) and NADP(H), it is not surprising that chemical modification of the enzyme is influenced by both of these substrates [29].

Bacteriorhodopsin is a cysteine-free enzyme in the wild-type form. This proton pump has been subjected to a number of interesting modifications based on the introduction of cysteine

residues by site-directed mutagenesis followed by labelling of the residue by a suitable probe for studying local pK_a changes, proton movements during the photocycle and membrane structure [37–41]. Likewise, the generation of cysteine-free lactose permease [11,42–47] was used for studies of α-helix packing. Also, in the permease, so-called cysteine scanning mutagenesis was used to investigate the importance of each individual residue in a predicted α-helix. Transmembrane helix packing studies have also been carried out with the *E. coli* leader peptidase protein [48].

Transhydrogenases represent unique redox-driven proton pumps. The generation of the cysteine-free αC292T, αC339T, αC395S, αC397T, αC435S, βC147S, βC260S mutant of *E. coli* transhydrogenase will make it possible to carry out essential experiments with this redox-driven proton pump which otherwise would be difficult or impossible to perform. Through site-directed mutagenesis, single cysteine residues can be introduced at will into the cysteine-free background, which then can be modified or chemically labelled as outlined above.

In conclusion, a cysteine-free mutant of *E. coli* transhydrogenase has been produced. The properties of the mutant have been thoroughly investigated and found to be essentially normal with regard to structure and function.

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