Reconstituted mitochondrial transhydrogenase is a transmembrane protein

Robin M. Pennington and Ronald R. Fisher*

Department of Chemistry, University of South Carolina, Columbia, SC 29208, USA

Received 5 October 1983

Bovine heart mitochondrial transhydrogenase, a redox-linked proton pump, can be functionally and asymmetrically inserted into liposomes by a cholate-dialysis procedure such that the active site faces the external medium. *N*-(4-Azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine), a membrane-impermeant photoprobe, when encapsulated in the vesicles, covalently modified the enzyme and inhibited transhydrogenation between NADPH and the 3-acetylpyridine analog of NAD^+ ($AcPyAD^+$) in a light-dependent manner. External $AcPyAD^+$ increased the rate of inactivation several fold, whereas NADPH, $NADP^+$, and NADH were without effect. Labeling of the enzyme by intravesicular [^{35}S]NAP-taurine was enhanced by $AcPyAD^+$ and $NADP^+$, decreased by NADH, and not significantly affected by NADPH. These results indicate that transhydrogenase spans the membrane and that substrate binding alters the conformation of that domain of the enzyme protruding from the inner surface of the membrane.

Transhydrogenase *NAP-taurine* *Photochemical modification* *Topography* *Proteoliposome*

1. INTRODUCTION

Mitochondrial transhydrogenase is an inner membrane redox-linked proton pump that catalyzes the reaction:

$$H^+_M + NADPH + NAD^+ \rightleftharpoons H^+_C + NADP^+ + NADH$$

where M and C represent the matrix and cytosolic sides of the membrane [1]. The active site, which faces the matrix, is comprised of separate domains for the binding of NAD and NADP substrates [2]. On reconstitution into phosphatidylcholine liposomes homogeneous bovine heart transhydrogenase orients such that the entire complement of active sites faces the external medium [3,4]. That the enzyme is nearly entirely functionally reconstituted is indicated by:

(i) the high (80–90%) inhibitory effect of the transmembrane proton electrochemical gradient on the NADPH ⟶ NAD^+ reaction [4]:
(ii) the efficient coupling of transhydrogenation to vectorial proton uptake under conditions that dissipate the membrane potential ($\Delta\psi$) [5].

Although the translocation of protons across the lipid bilayer catalyzed by transhydrogenase suggests that the enzyme spans the membrane, other mechanisms involving the participation of phospholipids in this process cannot be disregarded.

The current studies demonstrate not only that the enzyme traverses the liposomal membrane, but also that the binding of substrates alters the conformation of the domain of the protein that extends into the internal space of the vesicles.

2. MATERIALS AND METHODS

Homogeneous bovine heart transhydrogenase

* To whom correspondence should be addressed

Abbreviations: NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; $AcPyAD^+$, 3-acetylpyridine adenine dinucleotide

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

was prepared as in [6]. Reconstituted enzyme was assayed for valinomycin-dependent NADPH ⟶ $AcPyAD^+$ transhydrogenation as in [5].

K^+-loaded proteoliposomes were prepared by a cholate dialysis procedure [5] as modified in [7]. Egg yolk phosphatidylcholine (Sigma type V-E, 4.4 mg) in chloroform, was dried in a 10 × 75 mm glass tube with N_2. The lipid was dissolved in ether and dried again. Potassium phosphate (1.5 mM), pH 7.2, 100 mM K_2SO_4, and 5% sodium cholate (0.12 ml) were added to the lipid and the mixture sonicated in a bath type sonicator (model G1225PL, Laboratory Supplies Co., Hicksville, NY) at 4°C until the solution was clear. Soluble, purified transhydrogenase (12–20 μg), 1.5 mM potassium phosphate (pH 7.2), 100 mM K_2SO_4 and H_2O were added to a final volume of 0.5 ml. The mixture was then dialyzed at 4°C for 5 h against 60 ml of 1.5 mM potassium phosphate (pH 7.2) containing 100 mM K_2SO_4. The buffer was changed and dialysis continued for an additional 16 h. Where NAP-taurine was encapsulated, 50–100 μM of the photoprobe was included in the dialysis buffer and subsequent procedures were performed in the dark. External photoprobe was removed by gel filtration on a 28 × 1.5 cm Sepharose 4B column. The liposome fractions were pooled and concentrated by centrifugation at 164000 × *g* for 16 h at 4°C. The liposomal pellet was resuspended in 0.2 ml of 1 mM Tricine–NaOH (pH 7.2) containing 100 mM choline chloride and stored at 4°C. The liposomes were then dialyzed against 100 ml of 1 mM Tricine–NaOH (pH 7.2) containing 100 mM choline chloride for 6 h with one change of buffer.

Radioactive [^{35}S]NAP-taurine was synthesized as in [8] except that further purification was performed as follows. The NAP-taurine was lyophilized, dissolved in water, and subjected to column chromatography (13 × 3.5 cm) on Sephadex G-10 at 25°C using water as a solvent. Usually two peaks of radioactivity were observed. The fractions of the second peak containing NAP-taurine were pooled, lyophilized, dissolved in water, and stored in the dark at 4°C. The product had a specific activity of 94.7 mCi/mmol. Photolysis of samples containing NAP-taurine was performed in an ice bath at a distance of 4 cm from a 120 W/120 V General Electric photoflood lamp under conditions described in the tables and figures.

3. RESULTS AND DISCUSSION

The asymmetrical orientation and complete functionality of proteoliposomal transhydrogenase suggested that the vesicles could be used as a model system to determine if the enzyme is a transmembraneous protein. The approach used was to entrap the membrane impermeant photoprobe, NAP-taurine [8], in the vesicles during their formation. Subsequent light activation of the encapsulated NAD-taurine to its corresponding aryl nitrene was expected to modify with broad specificity [9] that domain of the enzyme at the intravesicular surface. Modification was monitored either by inhibition of valinomycin-dependent NADPH ⟶ $AcPyAD^+$ transhydrogenation or, when [^{35}S]-probe was used, by the formation of labeled enzyme that was stable to SDS gel electrophoresis.

To demonstrate that NAP-taurine modification of transhydrogenase occurs only during illumination, NAP-taurine-loaded proteoliposomes were preincubated in the light or in the dark and the NADPH ⟶ $AcPyAD^+$ activity assayed. Similarly, vesicles not containing NAP-taurine were preincubated in the light or in the dark in the presence or absence of external NAP-taurine. In table 1, exp.1, it is shown that photolysis of encapsulated NAP-taurine resulted in a time-dependent inactivation of transhydrogenase. No inactivation was observed in dark controls. NAP-taurine also inhibited transhydrogenase activity from the external surface in a light-dependent manner (table 1, exp.2). Furthermore, no inhibition of the enzyme was observed in the light in the absence of NAP-taurine. These results indicate that inactivation of the enzyme results exclusively from a reaction with the light-generated aryl nitrene.

To confirm that the light-dependent inactivation of transhydrogenase results from covalent modification of the enzyme, the external and internal surfaces of the proteoliposomes were reacted with [^{35}S]NAP-taurine in the presence and absence of light. The vesicles were then electrophoresed on SDS polyacrylamide gels, stained for protein, and autoradiographed. In fig.1, lanes 1–4 show the preparations after staining for protein, whereas lanes 5–8 represent an autograph of the gel. As can be seen, no labeling of the enzyme occurred in the dark (lane 6,8), whereas labeling was observed in

Table 1

Light-dependent inactivation of reconstituted transhydrogenase by internal and external NAP-taurine

Conditions	Incubation time			
	30 min		60 min	
	Specific activity (units/mg)	% Control activity	Specific activity (units/mg)	% Control activity
Expt 1 (internal NAP-taurine)[a]				
Dark	13.3	98	14.2	104
Light	9.9	74	5.9	41
Expt 2 (external NAP-taurine)[b]				
Light + NAP-taurine	3.5	20	0	0
Dark + NAP-taurine	17.4	102	19.6	115
Dark − NAP-taurine	17.9	105	18.9	110
Light − NAP-taurine	17.3	102	17.9	105

[a] Proteoliposomes (4.3 μg protein) loaded with 100 μM NAP-taurine were incubated at 0°C in the light or dark in a 0.14 ml reaction mixture containing 1 mM Tricine–NaOH (pH 7.2) and 100 mM choline chloride. After times incubated, 0.05 ml was assayed for NADPH ⟶ AcPyAD$^+$ activity. The control specific activity was 13.6 units/mg

[b] Proteoliposomes (4.3 μg protein) were incubated in the light or dark in a 0.2 ml reaction mixture containing 1 mM Tricine–NADH (pH 7.2), 100 mM choline chloride in the presence or absence of 100 μM NAP-taurine at 0°C. After times indicated, 0.05 ml was assayed for NADPH ⟶ AcPyAD$^+$ activity. The control specific activity was 17.0 units/mg

the light (lane 5,7). It is concluded that NAP-taurine covalently modifies transhydrogenase only in the light.

Inhibition and labeling of liposomal transhydrogenase by internal NAP-taurine provides substantial evidence for the transmembranous orientation of the enzyme. This result suggested the possibility that photolysis of internalized NAP-taurine might also be used as a probe to assess substrate-induced conformational changes of the enzyme domain that is present at the inner membrane surface. Accordingly, NAP-taurine loaded vesicles were illuminated in the presence and absence of each substrate. As shown in fig.2, NADPH, NADP$^+$ and NADH had little influence on the rate of transhydrogenase inactivation, whereas AcPyAD$^+$ significantly stimulated inhibition. This result indicates that AcPyAD$^+$-binding to the active site induces a conformational change that exposes an essential site or sites for reaction with internal NAP-taurine. The effects of substrates on enzyme inhibition by external NAP-taurine were quite different (fig.3). Here, AcPyAD$^+$ had no effect on the rate of NAP-taurine inactivation and the other 3 substrates provided considerable protection. The differences in the effect of substrates on internal and external NAP-taurine inactivation provide evidence that the inhibition and labeling of the enzyme by internal NAP-taurine is not an artifact resulting either by the leakage of the probe from the vesicles followed by modification at the external surface or from the incomplete removal of external probe during liposome preparation.

Internalized [^{35}S]NAP-taurine was used as a more general probe of substrate-induced conformational changes in the inner surface domain of the enzyme. After illumination of the proteoliposomes in the presence and absence of substrates, liposomes were subjected to SDS–polyacrylamide gel electrophoresis and the transhydrogenase band was counted after excision from the gel. Table 2 shows that the extent of labeling of transhydrogenase did not correlate with the extent of inactivation (fig.2). As predicted from the inhibition studies, AcPyAD$^+$ promoted

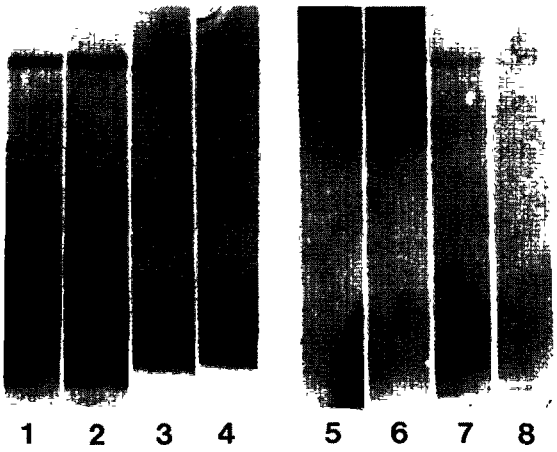

Fig.1. Labeling of reconstituted transhydrogenase by internal and external [^{35}S]NAP-taurine. Proteoliposomes (9 μg protein) were incubated in a 0.08 ml reaction mixture containing 1 mM Tricine–NaOH (pH 7.2), 100 mM choline chloride and 50 μM external [^{35}S]NAP-taurine (0.2 μCi) for 15 min at 4°C in the presence (lane 1,5) and absence (lane 2,6) of light. [^{35}S]NAP-taurine-loaded proteoliposomes (9 μg) were incubated in a 0.075 ml reaction mixture of 1 mM Tricine–NaOH (pH 7.2) and 100 mM choline chloride for 3 h at 4°C in the presence (lane 3,7) and absence (lane 4,8) of light. The samples were electrophoresed in a 10–20% gradient slab gel for 2.5 h, stained, destained, dried and exposed to X-ray film for 2 months at −70°C. Lanes 1–4 are stained for protein with Coomassie blue while lanes 5–8 are the autoradiograph.

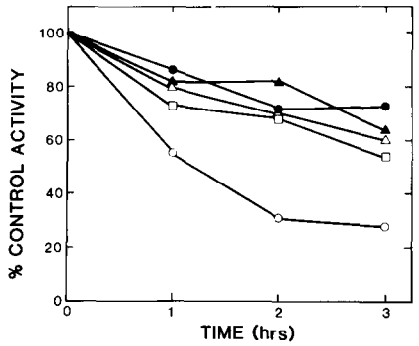

Fig.2. Effect of substrates on inhibition of liposomal transhydrogenase by internal NAP-taurine. Proteoliposomes (1.7 μg protein) loaded with 100 μM NAP-taurine as in section 2 were incubated in the light in a 0.2 ml reaction mixture with 1 mM Tricine–NaOH (pH 7.2), 100 mM choline chloride, and 1 mM of the indicated substrates: none (●); NADPH (△); NADH (□); NADP$^+$ (▲); AcPyAD$^+$ (○). At the indicated times, 0.06 ml was assayed for NADPH ⟶ AcPyAD$^+$ activity.

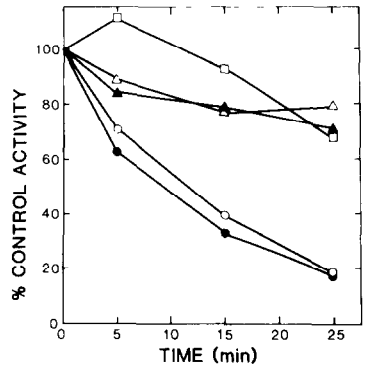

Fig.3. Effect of substrates on inhibition of liposomal transhydrogenase by external NAP-taurine. Proteoliposomes (1.7 μg) were incubated in the light at 4°C in a 0.2 ml reaction mixture containing 1 mM Tricine–NaOH (pH 7.2), 100 mM choline chloride, 50 μM NAP-taurine, and 1 mM of the indicated substrates: none (●); NADPH (△); NADH (□); NADP$^+$ (▲); AcPyAD$^+$ (○). After various times, 0.06 ml was assayed for NADPH ⟶ AcPyAD$^+$ activity.

and NADPH gave little effect on labeling. However, NADP$^+$ significantly stimulated labeling, and NADH significantly protected against labeling although neither of these substrates affected inactivation. These results indicate that in

Table 2

Effect of substrates on transhydrogenase labeling by internal [^{35}S]NAP-taurine[a]

Additions	dpm	% Control
None	264	100
AcPyAD$^+$	373	141
NADH	136	52
NADP$^+$	363	137
NADPH	228	86

[a] Proteoliposomes (10 μg protein) loaded with 50 μM [^{35}S]NAP-taurine were incubated in a 0.11 ml reaction mixture containing 1 mM Tricine–NaOH (pH 7.2), 100 mM choline chloride, and 1 mM of the indicated substrates. After photolysis for 3 h at 0°C, the samples were electrophoresed on 7.5% SDS polyacrylamide cylindrical gels, stained for protein, sliced, and counted for [^{35}S] in the transhydrogenase band. The data given are accurate to within ± 10% as calculated from 3 experiments

addition to $AcPyAD^+$, the binding of both $NADP^+$ and NADH alter the conformation of the enzyme at the internal surface. Presumably, the NAP-taurine reactive sites exposed on $NADP^+$-binding or protected on NADH-binding represent amino acid residues that are not essential for the expression of enzymatic activity.

Transmembrane conformational changes are common features of most pump-type mechanisms proposed for proton translocation by transhydrogenase (cf. [10,11]). It is anticipated that differential labeling studies, such as those reported here, coupled with an analysis of the specific residues modified will more fully define that domain of transhydrogenase which traverses the membrane and is functional in proton translocation.

ACKNOWLEDGEMENT

This work was supported in part by National Institutes of Health Grant GM 22070.

REFERENCES

[1] Fisher, R.R. and Earle, S.R. (1982) in: The Pyridine Nucleotide Coenzymes (Everse, J. et al. eds) pp.279–324, Academic Press, New York.
[2] Rydström, J. (1977) Biochim. Biophys. Acta 463, 155–184.
[3] Rydström, J. (1979) J. Biol. Chem. 254, 8611–8619.
[4] Earle, S.R. and Fisher, R.R. (1980) Biochemistry 19, 561–569.
[5] Earle, S.R. and Fisher, R.R. (1980) J. Biol. Chem. 255, 827–830.
[6] Wu, L.N.Y., Pennington, R.M., Everett, T.D. and Fisher, R.R. (1982) J. Biol. Chem. 257, 4052–4055.
[7] Anderson, W.M., Fowler, W.T., Pennington, R.M. and Fisher, R.R. (1981) J. Biol. Chem. 256, 1888–1895.
[8] Staros, J.V., Richards, F.M. and Haley, B.E. (1975) J. Biol. Chem. 250, 8174–8178.
[9] Staros, J.V. and Richards, F.M. (1974) Biochemistry 13, 2720–2726.
[10] Pennington, R.M. and Fisher, R.R. (1981) J. Biol. Chem. 256, 8963–8969.
[11] Enander, K. and Rydström, J. (1982) J. Biol. Chem. 257, 14760–14766.