EVIDENCE THAT RECONSTITUTED BOVINE HEART MITOCHONDRIAL TRANSHYDROGENASE FUNCTIONS AS A PROTON PUMP

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1. Introduction

Inner mitochondrial membrane-bound pyridine dinucleotide transhydrogenase (EC 1.6.1.1) comprises an energy-coupling site of the respiratory chain [1]. Mitchell and Moyle [2] demonstrated an uptake of protons into bovine-heart submitochondrial particles coupled to the reduction of NAD^+ by NADPH, while Skulachev et al. [3] found the reaction to be coupled to the generation of a membrane potential having the same polarity (positive inside the vesicles) as that generated by respiration or ATP hydrolysis. At high [NADPH] [NAD^+]/[$NADP^+$] [NADH] ratios, transhydrogenation is coupled to ATP synthesis from ADP and P_i [4]. It has been proposed that transhydrogenase functions directly as a reversible proton pump catalyzing the following reaction in submitochondrial particles [1,5–7]:

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

Recently, transhydrogenase has been independently purified to homogeneity [8,9], and shown to be composed of a single polypeptide chain having mol. wt 97 000–120 000. When reconstituted into synthetic liposomes, the preparation from [8] catalyzed an uncoupler-sensitive uptake of lipophilic anions coupled to the reduction of NAD^+ by NADPH, suggesting that the enzyme promotes the influx of a positively-charged species during reverse transhydrogenation.

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We report here on the incorporation of our transhydrogenase preparation into liposomes. Experiments described demonstrate that reconstituted transhydrogenation is:

(i) In the reverse direction coupled to the acidification of the vesicle internal space.
(ii) Stimulated several-fold in both directions on addition of uncoupler.

It is concluded that transhydrogenase functions as a proton pump.

2. Experimental

Transhydrogenase was purified to homogeneity from bovine heart mitochondria as in [9]. The forward transhydrogenase reaction was assayed by the reduction of thio-$NADP^+$ by NADH [10]; reaction mixtures (3 ml) at 25°C contained 80 mM potassium phosphate, pH 6.8, 0.5 μM rotenone, 175 μM NADH and 117 μM thio-$NADP^+$. The reverse reaction was assayed by the reduction of 3-acetylpyridine adenine dinucleotide by NADPH as in [7].

Transhydrogenase was incorporated into liposomes by the general cholate-dialysis procedure in [11]. Dioleoyl-L-α-phosphatidylcholine (4.3 mg/0.22 ml chloroform) in a 10 × 75 mm glass test tube was taken to dryness under N_2. The lipid was dissolved in a drop of diethylether and taken again to dryness. The residue was suspended in 0.12 ml solution containing 5% sodium cholate, 5 mM Tricine–KOH, pH 8.3, 2 mM dithiothreitol, 0.1 mM EDTA, and 25 mM sucrose, and the tube, positioned in a bath-

type sonicator (Model G1225P1, Laboratory Supplies Company, Hicksville, NY) at 4°, was sonicated for 7 min. A solution of transhydrogenase (0.08 ml) containing 31 μg protein, 0.1 M sodium phosphate, pH 7.5, 5 mM β-mercaptoethanol, and 0.5% sodium cholate was mixed with the sonicated phospholipid. Cold water (0.3 ml) was added and the mixture was dialyzed for 19 h at 4°C against 65 ml 10 mM Tricine–KOH, pH 8, 0.2 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) methanol. The buffer was changed once after the initial 4 h dialysis.

3-Acetylpyridine adenine dinucleotide was prepared by the method in [12]. All other pyridine dinucleotides were products of P-L Biochemicals. Lactate dehydrogenase (Type XI), glucose 6-phosphate dehydrogenase (type XII), pyruvate, glucose 6-phosphate, 9-aminoacridine, and dioleoyl-L-α-phosphatidylcholine were from Sigma Chemical Co. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Pierce Chemical Co.

3. Results and discussion

A SDS-polyacrylamide gel of the purified transhydrogenase used in this study is presented in fig.1. Homogeneous transhydrogenase was incorporated into dioleoyl-L-α-phosphatidylcholine liposomes as described in section 2. Figure 2 illustrates the rate of transhydrogenation in the forward and reverse directions catalyzed by identical quantities of purified and reconstituted transhydrogenase. Reconstitution resulted in a 80–85% decrease in enzymatic activity. Addition of the uncoupler, FCCP, enhanced the forward and reverse reconstituted transhydrogenase rates 5.3-fold and 7.3-fold, respectively. Uncoupler did not significantly affect the activity of unreconstituted transhydrogenase. These data suggest that the inhibition of transhydrogenation in both directions derives from a rapid establishment of a pH gradient across the membrane. The 'respiratory control', that is released on addition of uncoupler, presumably results from a balancing of the redox potential against the electrochemical hydrogen ion gradient generated by transhydrogenase-coupled proton translocation. Uncouplers would, by collapsing the pH gradient, provide a continual supply of protons to the vesicle interior to be pumped out during the forward reaction,

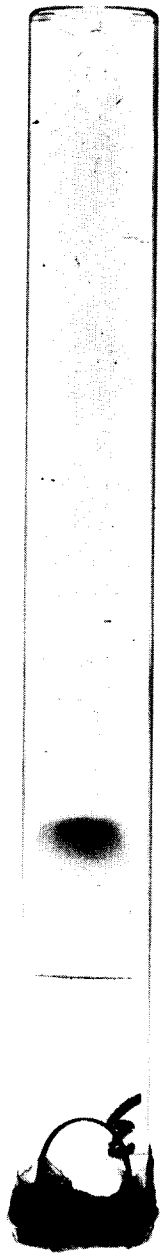

Fig.1. SDS–polyacrylamide gel electrophoresis of purified transhydrogenase. Gel electrophoresis was performed as in [9], with 5 μg of purified transhydrogenase. Protein was stained with Coomassie brilliant blue.

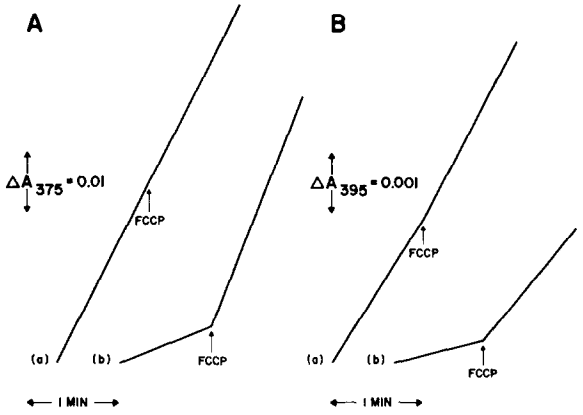

Fig.2. Effect of reconstitution and FCCP on forward and reverse transhydrogenase activities. Reconstitution and assays were performed as in section 2. Exp. (A) reduction of 3-acetylpyridine adenine dinucleotide by NADPH: (a) 3.1 μg unreconstituted transhydrogenase; (b) 3.1 μg reconstituted transhydrogenase. Exp. (B) reduction of thio-$NADP^+$ by NADH: (a) 3.1 μg reconstituted transhydrogenase; (b) 3.1 μg reconstituted transhydrogenase. FCCP (0.13 μM) was added where indicated.

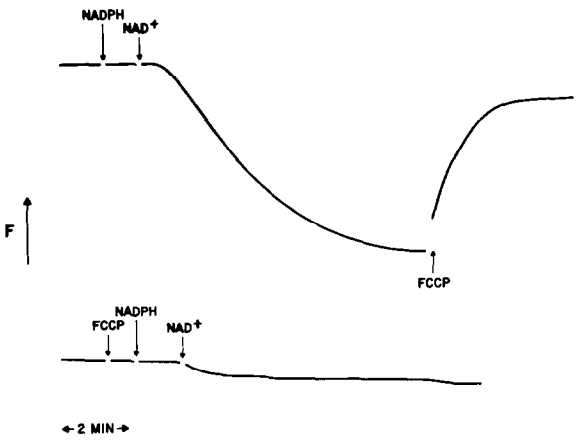

Fig.3. Quenching of 9-aminoacridine fluorescence during transhydrogenation. The reaction mixtures (1.08 ml) at pH 7.5 contained 90 μl proteoliposomes (5.6 μg transhydrogenase), 84 mM choline–Cl, 1.25 mM Tris–HCl, 9.3 mM pyruvate, 0.44 mM glucose 6-phosphate, 9.7 units lactate dehydrogenase, 11 units glucose 6-phosphate dehydrogenase and 1.6 μM 9-aminoacridine. Excitation was at 420 nm and fluorescence emission was determined at 500 nm. Where indicated, 45 μM NADPH, 45 μM NAD^+ and 0.13 μM FCCP were added.

and relieve the proton back-pressure formed during the reverse reaction.

To provide more direct evidence that reconstituted transhydrogenation is coupled to proton translocation, 9-aminoacridine was employed to assess changes in intravesicular pH [13–15]. Fluorescence of 9-aminoacridine was monitored during the reduction of NAD^+ by NADPH with reconstituted transhydrogenase (fig.3). Following addition of both substrates, 9-aminoacridine fluorescence was substantially quenched, indicating an uptake of protons into the vesicles. Equilibration of pH across the membrane on addition of FCCP restored 9-aminoacridine fluorescence. Addition of uncoupler prior to substrates nearly completely prevented quenching of 9-aminoacridine fluorescence during active transhydrogenation (cf. fig.2).

Taken together these results suggest that reconstituted transhydrogenase acts as a reversible proton pump, consistent with the chemiosmotic hypothesis [5,16]. The fact that transhydrogenase exhibits a single protein band on SDS-gels indicates that it participates in energy transduction in the absence of other coupling factors. It is not known if the functional form of the enzyme is monomeric or oligomeric. However, crosslinking of either unreconstituted or reconstituted transhydrogenase with dimethylsuberimidate does yield a dimeric species on SDS-gels (W. M. A. and R. R. F., unpublished observations).

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