RESOLUTION AND RECONSTITUTION OF MITOCHONDRIAL NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE

J. Rydström[1], N. Kanner and E. Racker

Section of Biochemistry, Molecular and Cell Biology,
Cornell University, N.Y., USA

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Summary: Nicotinamide nucleotide transhydrogenase from bovine heart mitochondria was solubilized with cholate and partially purified by ammonium-sulphate fractionation and density gradient centrifugation. Compared to submitochondrial particles this preparation contained less than 10% of oligomycin-sensitive ATPase and cytochromes. When reconstituted with purified mitochondrial phosphatidylcholine, the enzyme catalyzed a reduction of NAD^+ by NADPH that was stimulated by uncouplers and which showed a concomitent uncoupler-sensitive uptake of the lipophilic anion tetraphenylboron, indicating the generation of a membrane potential. It is concluded that transhydrogenase can energize the vesicles directly without the intervention of ATPase or cytochromes.

INTRODUCTION

Nicotinamide nucleotide transhydrogenase from bovine heart mitochondria, catalyzes the reversible reduction of NAD^+ by NADPH. The reduction of NAD^+ by NADPH energizes the membrane and the reduction of $NADP^+$ by NADH deenergizes the membrane. The presence of an additional energy source, either ATP or respiration, stimulates the rate and extent of $NADP^+$ reduction and inhibits NAD^+ reduction. Several hypothetical mechanisms, similar to those proposed for oxidative and photosynthetic phosphorylation, are currently being considered (cf. ref. 1 for a review) for the mechanism of this interaction.

In the present communication the problem of the coupling mechanism of the energy-linked transhydrogenase was approached by reconstitution of a partially purified transhydrogenase with purified mitochondrial phospholipids,

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Abbreviations: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrzone; TPB^-, sodium tetraphenylboron; 1799, a 2:1 adduct of hexafluoroacetone and acetone.

[1]On leave from the Department of Biochemistry, Arrhenius Laboratory, S-10405 Stockholm, Sweden.

without the addition of an auxilliary energy-generating system, e.g. ATPase or respiratory chain. Reconstitution was monitored by the uncoupler-sensitive uptake of TPB^- as a measure of the membrane potential generated, and by the extent of stimulation of the rate of reduction of NAD^+ by NADPH by uncouplers and ionophores. These observations indicate that a membrane potential is formed in the reconstituted purified transhydrogenase system which involves neither oligomycin-sensitive ATPase nor cytochrome.

MATERIALS AND METHODS

Submitochondrial particles were prepared in the presence of sodium pyrophosphate (SMP) (2), or of Mg^{2+}, Mn^{2+} and ATP (ETPH) (3), and stored at -70°. Protein was determined by the biuret method (4) or by the Lowry method (5). Transhydrogenase was solubilized by incubating SMP for 30 min. at a protein concentration of 20 mg/ml in a medium containing 2% sodium cholate, 10% saturated ammonium sulphate (pH 8.0), 20 mM sodium tricine (pH 8.0), 3 mM EDTA and 2 mM dithiothreitol. After centrifugation for 40 minutes at 100,000 x g (average) the supernatant was decanted and ammonium sulphate was added (dropwise) to obtain 30% saturation. After 20 minutes at 0°C the centrifugation step was repeated and the supernatant was decanted. The saturation of ammonium sulphate was then raised to 38% and the mixture was incubated, centrifuged and decanted as in the previous step. The ammonium sulphate concentration was finally raised to 43% saturation and the precipitate was collected. About 90% of the solubilized transhydrogenase was recovered in this pellet (38-43P) which was suspended in a medium containing 20 mM sodium tricine (pH 8.0), 3 mM EDTA and 2 mM dithiothreitol at a protein concentration of 20 mg/ml. The temperature during the purification procedure was kept at 0-4°. The preparation was stored in batches of 0.5 ml at -70°. Sucrose density gradient centrifugation of 38-43P was carried out in a Beckman SW41 rotor (or a Beckman SW50.2 rotor) with a linear gradient of 20 to 50% sucrose. In addition, the medium contained 0.1% purified mitochondrial phosphatidylcholine, 1.5% sodium cholate, 50 mM tris acetate (pH7.4), 1.5 mM EDTA and 2 mM dithiothreitol. One ml 38-43P was layered on top of the chilled gradient and centrifuged for 12 hours at maximal speed (with the SW 50.2 rotor, 0.5 ml of 38-43P and 6 hours of centrifugation was used). After centrifugation, fractions were collected from the bottom of the tube, and activities were assayed immediately. The most active transhydrogenase fractions were pooled, diluted twice with 50 mM tris acetate (pH 7.4), 1.5 mM EDTA and 2 mM dithiothreitol, and concentrated approximately 10 times in an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass., USA) equipped with a PM 10 filter. Reduction of NAD^+ by NADPH (with lactate-dehydrogenase plus pyruvate), NADH dehydrogenase, succinate-dehydrogenase and ATPase (in the absence and presence of 1 µg oligomycin) were assayed as described earlier (6). Cytochromes were estimated according to Williams (7). Unless otherwise stated, reconstitution of transhydrogenase in liposomes was carried out essentially as described by Ragan and Racker (8) with 4 mg protein and 40 µmoles phospholipid per ml. Uptake of TPB^- was monitored according to Grinius et al (9), with a medium that contained 50 mM tris acetate (pH 7.4), 1.5 mM EDTA and 0.5 µM tetraphenylboron. The uptake is expressed as the diffusion potential (mV) formed across the lipid membrane separating the two compartments of the instrument. The amount of FCCP (1 µg) used for uncoupling did not affect the permeability of the lipid membrane to TPB^-.
Mitochondrial phospholipids were extracted and purified as described by Kagawa et.al. (10). Cardiolipin (bovine heart) was purchased from Grant Island Biological Company (Grand Island, New York, USA). Other biochemicals were purchased from Sigma Chemical Co. (St. Louis, USA) or from Boehringer Mannheim GmbH (Mannheim, Germany).

Table 1

Purification of a reconstitutively active nicotinamide nucleotide transhydrogenase

Assays of the various activities and components were carried out as described in Methods, except that transhydrogenase was assayed in the presence of 0.6 mg lysophosphatidylcholine per ml.

Preparation	Activity				Cytochromes		
	transhydrogenase (nmoles/min/mg)	succinate-dehydrogenase (nmoles/min/mg)	NADH dehydrogenase (μmoles/min/mg)	ATP (μmoles/min/mg)	a	b	c_1 + c (nmoles/mg)
submitochondrial particles	110	200	3.6	1.1	1.20	1.30	0.51
38-43 P	320	71	10.2	1.5	0.19	0.51	0.27
pooled gradient fractions	790	<10	4.5	<0.08	0.08	0.11	0.05

RESULTS

As indicated in Table 1 the precipitate obtained between 38 to 43% saturation of ammonium sulphate in a 2% cholate extract of submitochondrial particles (SMP), contained partially purified transhydrogenase, NADH dehydrogenase and oligomycin-sensitive ATPase, whereas most of cytochrome a and some of cytochromes b and c_1 + c were removed. The activity of succinate dehydrogenase was decreased 3-fold. Further fractionation of the 38-43P precipitate by sucrose density gradient centrifugation in the presence of phosphatidylcholine and cholate resulted in an overall purification of transhydrogenase of more than 7 times. Activities of ATPase and succinate dehydrogenase were lower than

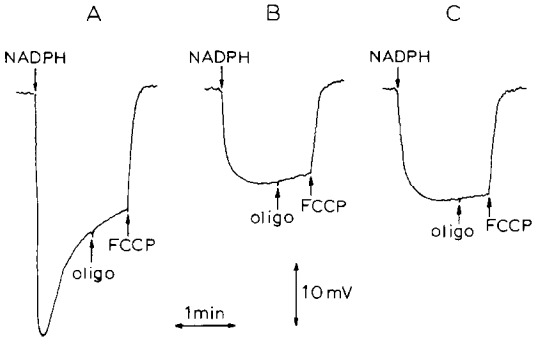

Fig. 1 Uptake of TPB^- catalyzed by submitochondrial particles (A) and reconstituted transhydrogenase (B,C)

The sample compartment of the instrument was filled with 3 ml of medium (cf. Methods), a membrane was applied to the opening connecting the sample compartment and the reference compartment with a 2%decane solution of a partially purified extract of mitochondrial phospholipids, and 0.1 mg submitochondrial particles (ETP_H) (A), 0.12 mg of reconstituted 38-43P (B) or 0.06 mg of reconstituted gradient-purified transhydrogenase (C), was added to the sample compartment. After equilibration for about 2 minutes, the assay system for reduction of NAD^+ by NADPH was added, except either NAD^+ or NADPH. The TPB^- uptake was initiated by the addition of either 0.5 mM NAD^+ or 0.2 mM NADPH. Additions were 1 µg oligomycin and 1 µg FCCP.

10% as compared to submitochondrial particles. Likewise, the amounts of cytochromes a,b and c_1 + c were decreased to less than 10% of those of submitochondrial particles.

When reconstituted with purified mitochondrial phospholipids both the 38-43 P fraction (Fig. 1B) and the gradient-purified preparation (Fig. 1C) catalyzed a reduction of NAD^+ by NADPH which was paralleled by uptake of TPB^-, similar to that observed with submitochondrial particles (ETP_H) (Fig. 1A). With submitochondrial particles the uptake was considerably more transient than with either of the two reconstituted systems, presumably because of the lower ion permeability of the latter systems. Omission of either rotenone or lactate dehydrogenase from the medium, or reversal of the order of addition of substrates, did not alter the uptake of TPB^- significantly (not shown). In all three cases TPB^- uptake was abolished by FCCP whereas oligomycin was without effect (Fig. 1). Addition of 1 mM $NADP^+$ also reversed the uptake of TPB^- by inhibiting the reduction of NAD^+ by NADPH (not shown, cf. ref. 11). Fig. 2

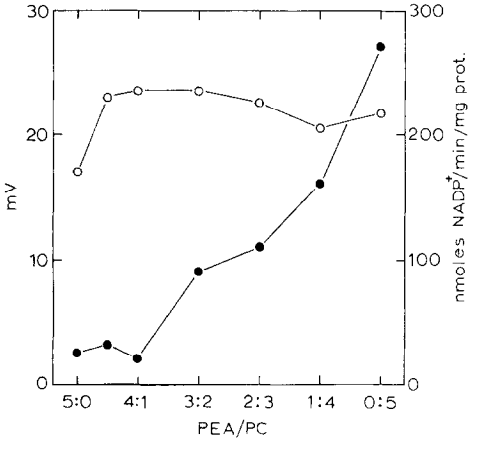

Fig. 2 Effect of different phosphatidylethanolamine/phosphatidylcholine ratios on uptake of TPB^- (●) and on the role of reduction of NAD^+ by NADPH (○) catalyzed by reconstituted transhydrogenase.

TPB^- uptake was assayed essentially as described in the legend of Fig. 1B. The assay of reduction of NAD^+ by NADPH was carried out in the presence of 1 µg FCCP.

shows the effect of phospholipid composition on the extent of TPB^- uptake in reconstituted vesicles. Pure mitochondrial phosphatidylcholine proved to be the best system for reconstitution, whereas pure phosphatidylethanolamine was about 10 times less efficient. Decreasing the phosphatidylethanolamine/phosphatidylcholine ratio lead to increasing TPB^- uptake, essentially without effect on the rate of reduction of NAD^+ by NADPH. Cardiolipin, or combinations of this lipid with either phosphatidylethanolamine or phosphatidylcholine, were inhibitory (not shown). Maximal uptake of TPB^- was achieved with a 5-fold excess of phosphatidylcholine over protein (Fig. 3), whereas no significant uptake was observed in the absence of added phospholipids.

As shown in Table 2 the rate of reduction of NAD^+ by NADPH, catalyzed by reconstituted transhydrogenase was stimulated to various extent by uncouplers, valinomycin, uncouplers plus valinomycin, and valinomycin plus nigericin, in the presence of KCl. No significant effect of these agents was seen with submitochondrial particles (ETP_H) except for a slight stimulation by uncouplers and valinomycin. Similarly, when no phospholipids were added during the reconsti-

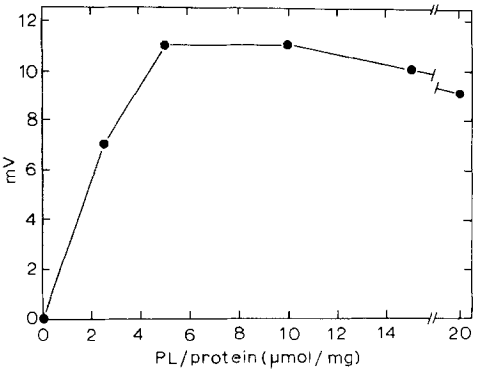

Fig. 3 Effect of different phospholipid (PL)/protein ratios on uptake of TPB^- catalyzed by reconstituted transhydrogenase.

TPB^- uptake was assayed essentially as described in the legend of Fig. 1 with 0.1 mg 38-43P.

Table 2

Effect of uncouplers and ionophores on reduction of NAD^+ by NADPH catalyzed by submitochondrial particles and reconstituted transhydrogenase

Medium contained the assay for reduction of NAD^+ by NADPH (cf. Methods) and 15 mM KCl. Additions were 1 µg FCCP, 0.3 µg valinomycin, 0.1 mM 1799, 0.3 µg nigericin, 0.1 mg submitochondrial particles (ETP_H), 0.12 mg and 0.06 mg reconstituted 38-43 P and gradient-purified fraction, respectively.

Preparation	Addition				
	none	FCCP	val.	val. + nig.	val. + 1799
	nmoles $NADP^+$/min./mg				
Submitochondrial particles	131	133	135	135	145
Reconstituted 38-43 P	61	80	70	101	105
Reconstituted gradient-purified fraction	60	85	72	109	120

tution procedure, transhydrogenase was not stimulated by uncoupling agents and did not catalyze an uptake of TPB^- (cf. Fig. 3). KCl alone at a concentration of 15 mM inhibited the transhydrogenase about 50%, both in the absence and in the presence of FCCP (not shown). However, the activity of the reconstituted transhydrogenase was generally lower than that found with the nonreconstituted transhydrogenase, presumably due to a partial inactivation during the reconstitution procedure. Alternative reconstitution procedures are therefore presently being investigated.

DISCUSSION

The data presented in this communication show that partially purified and reconstituted mitochondrial transhydrogenase catalyzes an uncoupler-stimulated reduction of NAD^+ by NADPH and an uncoupler-sensitive uptake of TPB^-, indicating the generation of a membrane potential. That TPB uptake appears to be related to the generation of membrane potential and energy coupling in membranes in general has already been demonstrated by Skulachev (12). A stimulation of the transhydrogenase reaction by a direct effect of uncoupling agents on the transhydrogenase protein is rendered unlikely by the absence of stimulation when phospholipids were omitted in the reconstitution. Since the specific ATPase activity and the amount of cytochromes in the transhydrogenase preparation used for reconstitution was less than 10% as compared to submitochondrial particles, it seems that these components are not essential for the generation of a membrane potential by the reconstituted transhydrogenase. Still, a possible molecular interaction between a minor component or a subunit of either the ATPase (13,14) or the cytochromes, linked to the generation of a membrane potential, can not be eliminated before a more extensive purification of the reconstitutibly active transhydrogenase has been accomplished. Although not yet shown directly the generation of a membrane potential by the reconstituted system may be indicative of transport of protons linked to the transhydrogenase per se as proposed by Mitchell (15).

Ragan and Widger (16) reported recently the reconstitution of ATP-driven

transhydrogenase using Complex I (NADH-ubiquinone reductase) as the source of transhydrogenase. It was shown that Complex I, reconstituted with a crude preparation of oligomycin-sensitive ATPase (hydrophobic protein) and soybean phospholipids, catalyzed an uncoupler-and oligomycin-sensitive reduction of $NADP^+$ by NADH, driven by ATP. In contrast to the optimal values with phosphatidylcholine demonstrated in this paper, these authors found a maximal activity with a phosphatidylethanolamine to phosphatidylcholine ratio of 4, probably reflecting the lipid requirement of the ATPase system rather than that of the transhydrogenase, since the same phospholipid ratio has been found to be optimal in the reconstitution of the P_i-ATP exchange (10).

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