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EVIDENCE FOR A LIPID DEPENDENCE OF MITOCHONDRIAL NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE

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SUMMARY

1. The lipid dependence of mitochondrial nicotinamide nucleotide transhydrogenase from beef heart was investigated. With submitochondrial particles digestion of phospholipids by phospholipases A and C led to a partial inhibition that could not be readily reversed by phospholipids.

2. Extraction of neutral lipids including ubiquinone from lyophilized submitochondrial particles with pentane did not inhibit the transhydrogenase, whereas further extraction with water/acetone led to a complete and apparently irreversible inhibition.

3. A partially purified preparation of transhydrogenase, depleted of lipids (and inactivated) by treatment with cholate and ammonium sulphate, was reactivated by various purified phospholipids but not by detergents or triacylglycerols.

4. It is concluded that mitochondrial transhydrogenase, catalyzing the non-energy-linked transhydrogenase reaction, requires phospholipids specifically for its catalytic activity and not as dispersing agents. A mixture of phospholipids appears to fulfill this requirement better than the individual phospholipids.

INTRODUCTION

Nicotinamide nucleotide transhydrogenase in submitochondrial particles derived from beef heart catalyzes the reversible reduction of NAD^+ by NADPH (cf. ref. 1 for a review). Partially purified transhydrogenase as well as transhydrogenase in submitochondrial particles is highly sensitive to agents that either remove or alter the phospholipids of the various preparations, e.g. organic solvents [2–4], detergents [2–5] and phospholipases [3, 4, 6, 7]. However, no direct evidence for a reversible lipid dependence of the enzyme has as yet been presented. In this communication, the conditions for delipidating the transhydrogenase have been investigated and it is shown that a lipid-depleted and inactive transhydrogenase was reactivated by addition of phospholipids. This activation was relatively unspecific and could be achieved with several purified phospholipids. The lack of activation by detergents suggests that the

effect of the phospholipids may be attributed to a hydrophobic interaction between the protein and the phospholipids, rather than a dispersion phenomenon.

MATERIALS AND METHODS

EDTA-submitochondrial particles were prepared as described by Lee and Ernster [8]. The particles were washed twice with 0.25 M sucrose and twice with distilled water. The final pellet was suspended in double-distilled water at a concentration of 20 mg/ml and stored at −15 °C. Particle protein was determined according to the biuret method. In case of highly diluted samples, or samples containing high concentrations of lipids, protein was determined according to Schaffner and Weissman [9]. Lyophilization of the water-washed particles in the absence of salts, subsequent pentane extraction and assay of succinoxidase was carried out as described by Ernster et al. [10]. A partial purification of transhydrogenase from EDTA-particles using lysophosphatidylcholine was carried out as described by Rydström et al. [5]. Lipid depletion of partially purified transhydrogenase was accomplished by addition of crystalline ammonium sulphate to obtain 10 % saturation, followed by addition of sodium cholate to a final concentration of 0.5 %. After 20 min at 0–4 °C the extract was centrifuged for 30 min at 100 000 × g. The white pellet on the bottom and the wall of the tube was collected in 20 mM tricine (pH 8.0) containing 3 mM EDTA and 1 mM dithiothreitol. Cholic acid was purified as described by Schneider et al. [11]. Reduction of NAD^+ by NADPH and the assay of cytochrome oxidase and NADH dehydrogenase were carried out as described previously [5]. Free fatty acids, total phospholipid and P_i were determined by the methods of Ho [12], Folch et al. [13] and Bartlett [14], respectively. Phospholipase A (*Crotalus terrificus terrificus*) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany), phospholipase C (*Clostridium welchii*) from Sigma Chemical Co. (St. Louis, U.S.A.), chromatographically pure lysophosphatidylcholine (egg) and cardiolipin (beef heart) from Grand Island Biological Company (Grand Island, N.Y., U.S.A.), and monogalactosyl diacylglycerol (plant) from Applied Science Laboratories, Inc. (State College, Pa., U.S.A.). Phospholipase C was purified on Sephadex A-50 using 10 mM Tris · HCl (pH 8.4) as eluant. Mitochondrial phospholipids were extracted, purified and dispersed by sonication as described by Kagawa et al. [15]. Other biochemicals were purchased from Sigma Chem. Co. or from Boehringer Mannheim.

RESULTS

Digestion of the phospholipids of submitochondrial particles was accomplished by the action of phospholipases A and C. As demonstrated in Table I, treatment with either phospholipase A or phospholipase C inhibited transhydrogenase. The extent of inhibition amounted to about 74 % in the case of phospholipase A and about 10–20 % in the case of phospholipase C. In both cases a time course of the phospholipase treatment revealed that the inhibition was rapid initially (not shown) but levelled off to the values indicated in Table I. The inhibition could not be ascribed to possible contamination by proteolytic enzymes (cf. ref. 16) since EDTA completely prevented the effect of both phospholipase A and phospholipase C (Table I). Analysis of the reaction products after digestion showed that the extent of hydrolysis was 90 % and

TABLE I

EFFECT OF PHOSPHOLIPASE TREATMENT ON TRANSHYDROGENASE IN SUBMITOCHONDRIAL PARTICLES

The reaction mixture contained 0.1 M Tris/acetate (pH 7.4), 1 mM dithiothreitol, 30 mg bovine serum albumin (defatted), 1 mM $CaCl_2$ and 5 μg phospholipase A or 120 μg phospholipase C in a final volume of 1.5 ml. The reaction was started by the addition of 2 mg submitochondrial particles and stopped after 30 min by the addition of 0.05 ml 0.25 M EDTA. Temperature was 30 °C. After incubation the mixture was kept on ice and assayed for transhydrogenase activity, free fatty acids and phospholipids as described in Methods.

Treatment	Digestion of phospholipids (%)	Inhibition of transhydrogenase activity (%)
Phospholipase A	90	74
+EDTA	4	5
Phospholipase C	80	10–20
+EDTA	3	5

80 % for phospholipase A and phospholipase C, respectively (Table I). Occasionally, a partial reactivation of the phospholipase A-treated particles could be achieved by sonication in the presence of various phospholipids. However, the extent of activation was highly variable, and appeared to be related to the time of incubation with phospholipase (not shown). The reason for this variability is as yet unknown.

Lyophilization and subsequent pentane extraction of submitochondrial particles (Table II) led to an inhibition of succinoxidase due to removal of ubiquinone and other neutral lipids, as described earlier [17, 18, 10]. By using water-washed submitochondrial particles, the transhydrogenase activity was preserved during the lyophilization procedure. Pentane extraction did not lead to an inhibition of transhydrogenase but rather involved a significant increase in specific activity of the enzyme, indicating that ubiquinone and presumably other neutral lipids are not essential for its activity.

The pentane-extracted, neutral lipid-depleted particles were then subjected to extraction of the phospholipids by water/acetone mixtures (Fig. 1). Increasing water/

TABLE II

EFFECT OF LYOPHILIZATION AND PENTANE EXTRACTION ON TRANSHYDROGENASE AND SUCCINOXIDASE IN SUBMITOCHONDRIAL PARTICLES

Lyophilization, pentane extraction and assays of transhydrogenase and succinoxidase were carried out as described in Methods.

Activity	Treatment		
	None (nmol NADPH or 0/min per mg protein)	Lyophilization (nmol NADPH or 0/min per mg protein)	Pentane extraction (nmol NADPH or 0/min per mg protein)
Succinoxidase	190	234	20
Transhydrogenase	148	151	219

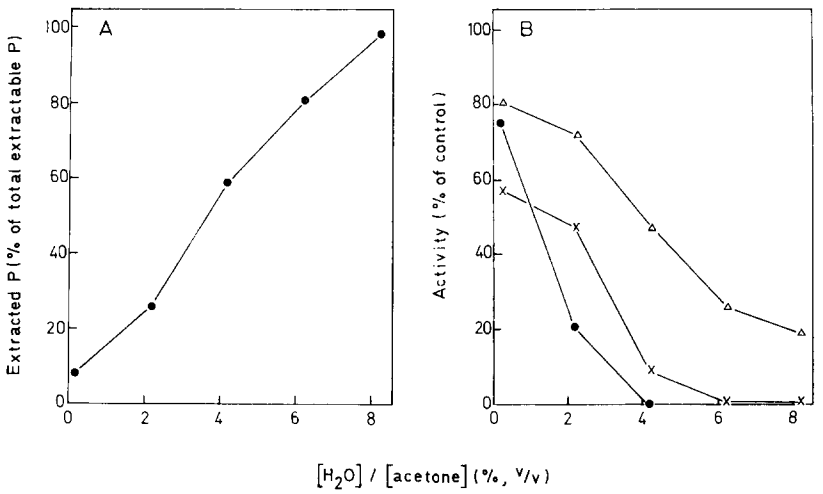

Fig. 1. Effect of acetone extraction of lipids on various activities in submitochondrial particles. (A) Phosphate extracted. (B) The enzyme activities assayed were: ●, transhydrogenase; X, cytochrome oxidase; △, NADH dehydrogenase. 120 mg lyophilized submitochondrial particles were repeatedly extracted with acetone containing increasing amounts of water. After each extraction the mixture was centrifuged for 30 min at 10 000 × *g*, the supernatant was then decanted and assayed for organic phosphate. The pellet was dried under a stream of N_2. A small sample (about 2 mg) of the pellet was withdrawn for enzyme assay and the remaining protein resuspended in the next water/acetone mixture. Each extraction was carried out with stirring for 15 min at 0–4 °C.

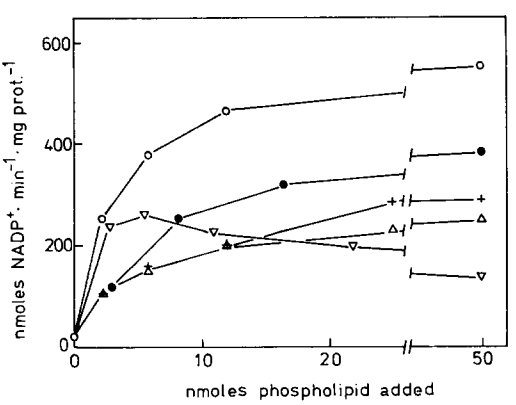

Fig. 2. Activation of lipid-depleted transhydrogenase by phospholipids. The phospholipids tested were: △, phosphatidylethanolamine; +, phosphatidylcholine; ▽, cardiolipin; ●, lysophosphatidylcholine; ○, mixed mitochondrial phospholipids. The indicated amounts of phospholipids were sonicated for 5 min at 10 °C under N_2 (cf. Methods) in a medium containing 50 mM Tris/acetate (pH 7.4), 3 mM EDTA and 1 mM dithiothreitol in a final volume of 1 ml. 20 μg lipid-depleted transhydrogenase (cf. Methods) were added and the mixture was resonicated for 1 min at 10 °C under N_2, and assayed for transhydrogenase activity.

acetone ratios resulted in an increased extraction of phospholipids, approaching 100 % at a water content of 8 % (Fig. 1A, cf. ref. 19). Water-free acetone extracted less than 10 % phospholipids (Fig. 1A) and led to only a slight inhibition of transhydrogenase (Fig. 1B). At higher water/acetone ratios the increased lipid depletion was accompanied by a marked inhibition that was close to 100 % at a water content of 4 %, corresponding to a phospholipid extraction of about 60 %. Other enzymes tested, i.e. cytochrome oxidase and NADH dehydrogenase, showed similar delipidation-inhibition patterns (Fig. 1). Attempts at reactivating the water/acetone-treated transhydrogenase by phospholipids were without success.

Conclusive evidence for a lipid dependence of the transhydrogenase emerged from experiments with lipid depletion of a partially purified enzyme (cf. Methods). In this case protein was precipitated with ammonium sulphate in the presence of cholate, yielding an inactive preparation that contained 0.04 μmol phospholipid per mg protein as compared to 0.57 μmol phospholipid per mg submitochondrial particle protein. Reactivation of this preparation by adding phospholipid was reproducible. As may be seen in Fig. 2, various pure mitochondrial and egg phospholipids were efficient activators, the order of decreasing efficiency being cardiolipin, lysophosphatidylcholine, phosphatidylcholine and phosphatidylethanolamine. At high concentrations, cardiolipin was inhibitory. A mixture of mitochondrial phospholipids was considerably more efficient than the individual pure phospholipids tested with respect to the amount required for half-maximal activation. With a mixture of mitochondrial phospholipids the maximal extent of reactivation of the lipid-depleted transhydrogenase was about 90 %. The activating effect of lysophosphatidylcholine explains the successful use of this "phospholipid detergent" in the solubilization of transhydrogenase from submitochondrial particles [5]. Sonication in the absence of phospholipids or in the presence of triacylglycerols (e.g. glyceryl tripalmitate) was without effect. Diacylglycerols (e.g. 1,2-dipalmitoyl glycerol and monogalactosyl diacylglycerols) were slightly activating, whereas the detergents cholate, deoxycholate and Triton X-100 were without effect and at higher concentrations rendered the transhydrogenase irreversibly inactive. Lipid depletion of submitochondrial particles by cholate treatment alone did not give a preparation that showed a reproducible lipid activation, possibly due to a coprecipitation of transhydrogenase with non-transhydrogenase protein.

DISCUSSION

The present data indicate that a lipid-depleted, inactive mitochondrial transhydrogenase may be reactivated by lipids, preferentially phospholipids, but not by detergents. No pronounced specific phospholipid dependence was apparent, although cardiolipin was most efficient at low concentrations. Higher concentrations of cardiolipin were inhibitory, presumably because of the strongly increased negative charge of the environment. The amount of phospholipid required for half-maximal activation varied between 0.2 and 0.5 μmol phospholipid per mg protein, which is close to the value found with the intact mitochondrial inner membrane, 0.57. A mixture of mitochondrial phospholipids appeared to be more efficient with respect to the amount required for half-maximal activation than any of the individual pure phospholipids tested, indicating that the activating effects of the individual phospholipids

were at least partially additive. It should be pointed out that the phospholipid dependence demonstrated here concerns only the nonenergy-linked transhydrogenase reaction, and is therefore likely to reflect a direct interaction between the transhydrogenase and the phospholipid. In contrast, previously demonstrated effects of ubiquinone [20] and phospholipids [21] on the energy-linked transhydrogenase reaction may be attributed to effects on the energy-generating system rather than on the transhydrogenase per se. Alternatively, the recently described reconstitution of the energy-linked transhydrogenase per se, in the absence of auxiliary energy-generating systems, which has an apparent requirement for phosphatidylcholine [22, 23], suggests that the transhydrogenase may have different lipid requirements depending on whether the enzyme catalyzes the nonenergy-linked reaction or the energy-linked reaction.

Lipid depletion using cholate plus ammonium sulphate was found to be the only reproducible procedure for preparing a phospholipid-activated enzyme. Digestion of the phospholipids of submitochondrial particles by phospholipases A and C gave preparations that were composed mainly of lysophosphatides and diacylglycerols, respectively. In the former case the inhibition of transhydrogenase is likely to result from the lower activating effect of lysophosphatides (mainly lysophosphatidylcholine) in combination with the inhibitory effect of cardiolipin, which is not readily digested by phospholipase A [24]. Similarly, in the latter case, the inhibition can be attributed to the relatively poor activating effect of diacylglycerols. Inhibition of transhydrogenase following phospholipid depletion by water/acetone apparently involves a subsequent denaturation by acetone.

Phospholipid-dependent enzymes may be divided into two types; those that require a specific phospholipid (e.g. β-hydroxybutyrate dehydrogenase [25, 26]) and those that are activated by a number of phospholipids, as well as by detergents (e.g. the oligomycin-sensitive ATPase [27]). The mitochondrial transhydrogenase seems to be different from both of these types of enzymes in the sense that its activation by phospholipids appears to be relatively unspecific and that phospholipids cannot be replaced by detergents. Therefore, it seems unlikely that the activation of transhydrogenase by phospholipids is due to dispersion, as has been proposed for the oligomycin-sensitive ATPase [28]. More likely, by occupying hydrophobic surfaces on the molecule which normally are exposed to the hydrophobic interior of the intact membrane, the phospholipids may serve as agents stabilizing an active conformation of the transhydrogenase. The amount of phospholipid required for half-maximal activation of mitochondrial transhydrogenase is of the same order of magnitude as compared to those found for half-maximal activation of β-hydroxybutyrate dehydrogenase [25, 26] and oligomycin-sensitive ATPase [17]. Similar to the oligomycin-sensitive ATPase that requires about 10 times excess of phospholipid to catalyze energy-linked reactions in reconstituted systems, i.e. $^{32}P_i$-ATP exchange [29], the transhydrogenase thus also requires about 10 times excess of phospholipid to catalyze energy-linked uptake of lipophilic anions [23].

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