DEPENDENCE OF ESCHERICHIA COLI PYRIDINE NUCLEOTIDE TRANSHYDROGENASE ON PHOSPHOLIPIDS AND ITS SENSITIVITY TO N-ETHYLMALEIMIDE

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Received September 22,1976

SUMMARY: A partially purified preparation of pyridine nucleotide transhydrogenase (E.C. 1.6.1.1.) (energy-independent) has been obtained from membranes of Escherichia coli by means of deoxycholate extraction and DEAE-cellulose chromatography in the presence of Triton X-100. The enzyme was lipid-depleted by treating with cholate and ammonium sulfate. The preparation was reactivated by various phospholipids, in particular, bacterial cardiolipin and phosphatidyl glycerol. Phosphatidyl ethanolamine, the major phospholipid in the outer membrane of E. coli, was relatively ineffective in stimulating activity. The membrane-bound pyridine nucleotide transhydrogenase is slowly inhibited by N-ethylmaleimide. Protection against inhibition was achieved with NAD^+ and $NADP^+$, but NADPH served to accelerate the rate of inhibition.

INTRODUCTION:

The pyridine nucleotide $NAD(P)^+$ transhydrogenases from beef heart mitochondria and Escherichia coli are as yet unpurified. Attempts have been made by several laboratories to isolate the mitochondrial enzyme (1-4). Purification has, however, been hampered by the high sensitivity of the enzyme to organic solvents, bile salts and phospholipases (2). In this communication we report the partial purification of a lipid-depleted E. coli $NAD(P)^+$ transhydrogenase and its reactivation by purified phospholipids. We also report the sensitivity of the membrane-bound $NAD(P)^+$ transhydrogenase to N-ethylmaleimide and protection against inhibition by various nucleotides.

MATERIALS AND METHODS:

E. coli strain W6 (a proline auxotroph derived from ATCC 96377) was grown

on a minimal salts medium using 30mM glucose as the sole carbon source. Respiratory particles were prepared after breakage in a French pressure cell as previously reported (5) except for one modification, viz., 50 mM Tris-sulfate buffer pH 7.8 containing 1 mM DTT and 1 mM EDTA (TDE) was used as the isolation medium. $NAD(P)^+$ transhydrogenase activity was measured by the reduction of acetyl pyridine NAD^+ by NADPH (5). Crude E. coli phospholipids were obtained from respiratory particles as described by Cunningham and Hager (6). Particles were first extracted with acetone:water (9:1) and then with 100% acetone. The combined extracts were filtered and evaporated to dryness under vacuum. The lipid extract was then redissolved in chloroform-methanol (2:1) and stored under nitrogen at -20°C. Purified E. coli phosphatidyl glycerol, phosphatidyl ethanolamine, cardiolipin, plant lecithin and beef heart mitochondrial cardiolipin were purchased from Supelco Inc., Belfonte, Pa. and used without further purification. Asolectin was obtained from Associated Concentrates, Woodside, N.Y. and purified as described by Kagawa, et al. (7). Water-dispersed phospholipid preparations were obtained by sonicating phospholipids for 5 minutes in TDE in the presence of nitrogen. Suspensions were clarified by centrifugation at 80,000xg for 30 minutes.

RESULTS AND DISCUSSION:

Partial Purification

The membrane preparation (50 ml, 6-8 mg/ml) in TDE was made to 1M KCl and deoxycholate pH 8.0 added to a final concentration of 0.5% (w/v). After stirring for 10 minutes in the cold, the mixture was spun at 180,000xg for thirty minutes. The resulting supernatant was dialyzed overnight against TDE. Triton X-100 was added to a final concentration of 0.5% and the solution was applied to a Whatman DE52 DEAE-cellulose column pre-equilibrated in TDE containing 0.5% Triton X-100 (TDE-T 0.5). The column was washed with 100 ml of TDE-T 0.5 followed by 100 ml of the same made 100 mM in NaCl. Further elution was achieved with a linear salt gradient. The mixing chamber contained 250 ml TDE-T 0.5 with 100 mM NaCl, the reservoir TDE-T 0.5 with 500 mM NaCl. Figure 1 shows a

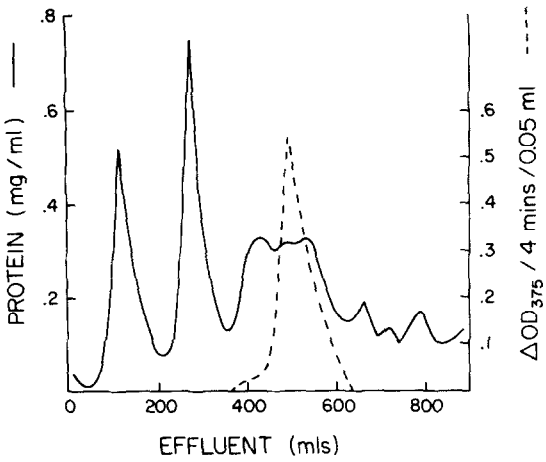

Figure 1 - Elution profile of $NAD(P)^+$ transhydrogenase activity from DEAE-cellulose in the presence of Triton X-100. Fractions (10 ml) were collected and (50λ) samples assayed for enzyme activity as described in the Methods section.

TABLE 1

Typical Purification of Transhydrogenase

Fraction	Total Protein mg	Total Units µmoles/ min	Specific Activity µmoles/min x mg	% Yield	Total Phosphorus nmoles/mg Protein
Membranes	358	483	1.35	100	2.4
Deoxycholate Extract	184	374	2.03	77	-
DE-52 Fraction after Cholate - $(NH_4)_2\ SO_4$	9.2	125	13.6*	26	0.3

* Assayed in the presence of E. coli phospholipids.

typical elution profile of protein and enzyme activity, which was eluted in 0.25-0.30 M salt. Active fractions were combined, and potassium cholate pH 8.0 added to 1% followed by solid ammonium sulfate (250 mg/ml). The mixture was stirred at 4°C and centrifuged at 30,000xg for 15 minutes. The precipitate was resuspended in TDE and dialyzed 1 to 2 hours against the same buffer.

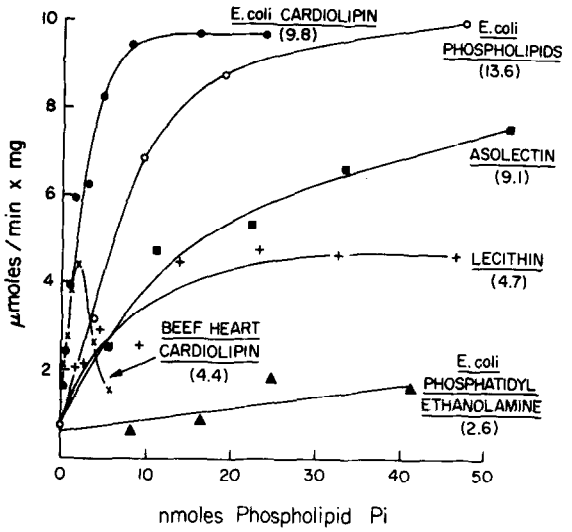

Figure 2 - Reactivation of NAD(P)$^+$ transhydrogenase by various phospholipids. Protein (10 µg) was preincubated in 500 µmoles phosphate buffer pH 6.5 in the presence of 500 nmoles NADPH and 6 µmoles KCN and varying amounts of phospholipids. After 5 min preincubation, the assay medium was made to 3 ml with water and 500 nmoles acetyl pyridine NAD$^+$ added to initiate the reaction. The figures given in brackets are the maximal activity in µmoles/min x mg. The basal activity was 0.72 µmoles/min x mg.

This preparation, the lipid-depleted NAD(P)$^+$ transhydrogenase, was used for subsequent reactivation experiments. A purification outline is shown in Table 1. This preparation which has a low activity of 0.44-0.93 µmoles/min x mg was stimulated by phospholipids.

Reactivation by Phospholipids

The activity of the lipid-depleted fraction could be restored by phospholipids (Fig. 2). A crude E. coli lipid fraction stimulated activity almost 15-fold, giving a maximal activity of 13.6 µmoles/min x mg. The most effective phospholipid was E. coli cardiolipin which stimulated activity ten-fold at a much lower concentration than any other phospholipid. Surprisingly phosphatidylethanolamine, which constitutes 80% of the E. coli inner membrane phospholipids, had little effect, i.e., only a 2- to 3-fold stimulation. Phosphatidylethanolamine is reported to be effective in reconstituting lipid-depleted DCCD-sensitive

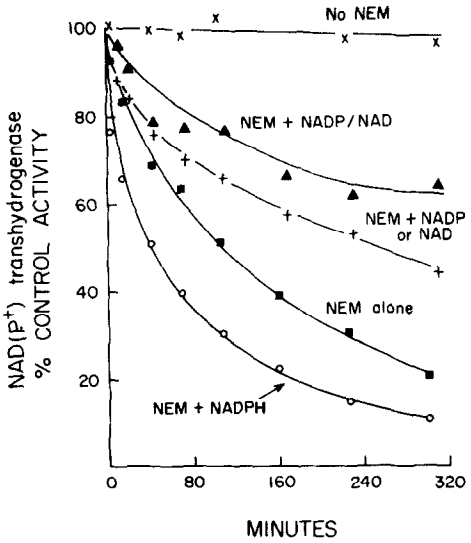

Figure 3 - Time dependent inactivation NAD(P)$^+$ transhydrogenase by N-ethylmaleimide E. coli membranes (0.8 mg/ml) in 0.2 M potassium phosphate buffer pH 7.0 were incubated at 25°C with excess NEM (10 mM) in the presence of various nucleotides (10 mg/ml). Aliquots (25 λ) were removed at the designated times and assayed for enzyme activity. The first order rate constant k for the initial declines in activity were x—x 0, ▲—▲ 9, +—+ 26, ■—■ 46, and o—o 63 min^{-1} (x 10^4). Control activity was 1.63 μmoles/min x mg.

ATPase in E. coli (8). Lecithin, a phospholipid absent from the inner membrane of E. coli, was partially effective in restoring activity but at higher concentrations than bacterial cardiolipin. Asolectin was also effective in the same concentration range as lecithin.

Beef heart cardiolipin also stimulated the enzyme activity at low levels, comparable to E. coli cardiolipin, but at higher concentrations was inhibitory. This inhibition may be related to the different fatty acid side chains present in the beef heart cardiolipin. The latter has unsaturated fatty acids as compared with cyclopropane fatty acids in the side chains of E. coli cardiolipin.

An analagous lipid-depleted preparation of transhydrogenase isolated from beef heart mitochondria (9) was found to be activated by phosphatidylcholine. Beef heart cardiolipin or combinations of this lipid with phosphatidylethano-

lamine or phosphatidylcholine were inhibitory. Phosphatidylglycerol also stimulated the E. coli enzyme activity in a concentration range similar to that of cardiolipin. Half-maximal stimulation with E. coli phospholipid was obtained with roughly 10 nmoles. E. coli cardiolipin and phosphatidyl glycerol, each of which comprise 5-15% of the crude E. coli phospholipid, gave half-maximal stimulation with roughly 1 nmole, suggesting that the stimulation observed with crude E. coli phospholipid is mainly due to its cardiolipin and phosphotidyl glycerol content. The E. coli phospholipids and cardiolipin did not affect the Km for NADPH or acetyl pyridine NAD^+ but only the Vm for the reaction.

During the initial isolation procedures it was found that the enzyme, although stable in the membrane, was more labile when "solubilized". Incorporation of DTT into the medium served to stabilize the activity, implicating the presence of a sensitive sulfhydryl in the enzyme.

Studies on the membrane-bound enzyme showed that the enzyme was indeed sensitive to N-ethylmaleimide (NEM) (Fig. 3). Protection against inhibition was obtained if NAD^+ or $NADP^+$ was present during exposure to NEM. A combination of the two oxidized nucleotides gave an increased protection, and the inhibition did not exceed 40% even after 5 hours. The results suggest the presence of functionally active -SH groups in the vicinity of both the NAD^+ and $NADP^+$ sites. Incorporation of NADPH into the system, instead of protecting against inhibition by NEM, accelerated the rate of inactivation, indicating that the reduced form of the enzyme has more fully exposed -SH.

The presence of arginyl residues at the nucleotide binding sites of the membrane-bound mitochondrial transhydrogenase has already been implicated (10) from studies with butanedione. Similar results with butanedione and phenylglyoxal were observed with the E. coli enzyme (data not shown). It was again found that the enzyme was more susceptible to modification by butanedione or phenylglyoxal in the presence of NADPH, but protected in an additive manner by NAD^+ or $NADP^+$.

The data presented indicate that the E. coli transhydrogenase is phospho-

lipid-dependent and is best reactivated by low levels of E. coli phosphatidylglycerol and cardiolipin. This is in contrast to the beef heart mitochondrial enzyme which was inhibited by mitochondrial cardiolipin at high levels. However, in both cases the specific lipid present in the parent membrane (mitochondrial or the E. coli inner membrane) appeared to produce the highest activity.

Studies with NEM suggest that in addition to the presence of arginyl residues, the E. coli enzyme may contain functionally active sulfhydryl residues in the vicinity of the nucleotide binding sites.

ACKNOWLEDGEMENTS

This research was supported by Grant No. PCM75-03532A01 from the National Science Foundation.

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