Biochimica et Biophysica Acta, 722 (1983) 94–101
Elsevier Biomedical Press

BBA 41225

PURIFICATION AND PROPERTIES OF THE MEMBRANE-BOUND NADH DEHYDROGENASE FROM HYDROCARBON-GROWN *ACINETOBACTER CALCOACETICUS*

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(Received September 17th, 1982)

Key words: NADH dehydrogenase; Respiratory chain; (Acinetobacter calcoaceticus)

A single NADH dehydrogenase (EC 1.6.99.3) species which seems to be connected with the respiratory chain was localized in the membrane fraction of hydrocarbon-grown *Acinetobacter calcoaceticus*. The enzyme was completely solubilized by Triton X-100 and purified by chromatography on DEAE-cellulose, dextran blue-Sepharose 6B and Phenylsepharose 4B. SDS-polyacrylamide gel electrophoresis of the purified enzyme showed one band of molecular weight 34 000. The purified enzyme is inactive in the absence of detergent, needs added FMN for activity and is strictly specific for the electron donor NADH. During the course of purification, 0.4 μmol phospholipid per mg protein are retained most of which is cardiolipin with traces of phosphatidylethanolamine and phosphatidylglycerol also being found. The results of lipid substitution experiments using phospholipids purified from *A. calcoaceticus* are indicative of a specificity for phosphatidylglycerol but the possibility of a dual phospholipid requirement cannot be ruled out.

Introduction

Acinetobacter calcoaceticus is a microorganism capable of using long-chain hydrocarbons as the sole source of carbon and energy [1].

Growth on hydrocarbons was reported to result in reduced rates of membrane-bound NADH oxidase activity in *A. calcoaceticus* [2]. On the other hand, growth on hydrocarbons was shown to effect the lipid composition of *Acinetobacter* sp. membranes [3,4].

Phospholipid requirement for several steps of the respiratory chain system seems to be a well established fact by now but the exact nature of phospholipid dependence is still a topic of investigation. We focused our interest on the membrane-bound NADH dehydrogenase (EC 1.6.99.3) of *A. calcoaceticus* located at the portal of the electron-transport chain.

Furthermore, despite intensive investigations of the mitochondrial respiratory chain, the NADH dehydrogenase complex, Complex I, is that part of the system about which very little is known. There are conflicting views on the mechanism of electron transfer, the energy conservation associated with it or even the structure of the enzyme complex [5].

Even less is known about the properties of the corresponding bacterial enzyme and reports on highly purified NADH dehydrogenases of bacterial origin have been presented only for *Escherichia coli* [6–8], *Acholeplasma laidlawii* [9], *Photobacterium phosphoreum* [10] and the thermophilic *Bacillus caldotenax* [11]. In recent reports, the question of the purity or integrity of some of the preparations of the respiratory enzyme was raised

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholineethanesulphonic acid.

[8,12]. The effect of lipids on the bacterial membrane-bound NADH dehydrogenase was studied only for the enzymes isolated from *B. caldotenax* [11] and *E. coli* [13] and with respect to phospholipid specificity in the latter report only.

In the present study, the purification and some properties of the purified membrane-bound NADH dehydrogenase from *A. calcoaceticus* are reported and the effect of some phospholipids purified from this microorganism on the enzyme is examined.

Materials and Methods

Microorganism and culture. A. calcoaceticus 69-V was cultured aerobically at 30°C in a minimal medium as described previously [1]. Hexadecane was added at a concentration of 6 g/l. Cells were routinely harvested during the late exponential phase of growth, washed three times and suspended in buffer A (0.05 M Tris-HCl, pH 7.6).

Preparation of the membrane fraction and the soluble fraction. The membrane fraction was obtained by isolating complete cell envelopes by differential centrifugation closely following a procedure outlined in Ref. 2. Briefly, washed cells were disrupted by ultrasonic treatment (Schoeller Schall ultrasonic disintegrator TG 250, maximum output) for 6 min. Whole cells were removed by centrifuging twice at $4000 \times g$ for 30 min at 4°C. A soluble fraction still containing small membrane particles was obtained as the supernatant after centrifugation at $110000 \times g$ for 60 min at 4°C. The pellet was resuspended in buffer A and centrifuged once more at $4000 \times g$ as above. The supernatant obtained was centrifuged at $110000 \times g$ and the pellet was washed in buffer A three times as above. The membrane fraction thus obtained is free of cytoplasmic contaminations as judged from the absence of malic enzyme activity determined as described by Aurich et al. [2].

From the soluble fraction membrane particles were removed by passage over a Sepharose 2B column. The soluble fraction thus obtained was devoid of NADH oxidase activity. The whole procedure was carried out in buffer A at 4°C.

Solubilization. Solubilization of the membrane-bound NADH dehydrogenase was performed using Triton X-100 for 15 h at 4°C in buffer A at the ratios of detergent to protein indicated in the text. Proteins solubilized are found in the supernatant after centrifugation at $110000 \times g$ as above. Triton X-100 concentrations were determined at 274 nm using the following coefficient: $A_{1\ cm}^{1\ mg/ml} = 2.32$ [14].

Purification of the solubilized NADH dehydrogenase. All purification steps were carried out in buffer B (0.01 M Tris-Mes, pH 7.1, plus 0.05% Triton X-100) at 4°C. During column chromatography the A_{280} absorption was continually monitored by an LKB autoanalyzer (Uvichord LKB 8 300) and recorded. The A_{340} absorption was measured in the fractions using a spectrophotometer (Chiratic IX, CSSR).

Polyacrylamide gel electrophoresis. Electrophoresis in the presence of SDS was performed according to the method of Weber and Osborne [15] in a separating gel of 7% acrylamide containing 0.1% SDS. Samples containing Triton X-100 were treated before electrophoresis as described by Retz and Steele [16]. After migration at 3 mA/tube, proteins were stained with 0.2% Coomassie blue. Molecular weight determinations were performed using the following protein standards: bovine serum albumin, chymotrypsinogen, trypsin and cytochrome *c*.

Electrophoresis in the presence of sodium deoxycholate was performed as described by Neville [17] substituting SDS for sodium deoxycholate with a separating gel of 11% polyacrylamide containing 0.1% sodium deoxycholate. Samples were treated before electrophoresis with 0.05 M Na_2CO_3, 8 mg sodium deoxycholate/mg protein and 10% β-mercaptoethanol [17]. After migration at 1.5 mA/tube, gels were stained for activity with 0.1 mM nitroblue tetrazolium and 3 mM NAD(P)H and scanned using an Eri 65 m densitometer (Zeiss, Jena).

Phospholipid analysis. Identification of phospholipids was carried out by thin-layer chromatography on silica gel plates in the solvent system $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and comparison with authentic standards. Phospholipids were visualized with a phospholipid spray reagent prepared as described by Ryn and MacCoss [18].

Phospholipid concentrations were determined according to the method of Kahovcová and Ovadic [19].

Purification of phospholipids from A. calcoaceti-

cus. The total phospholipid fraction from *A. calcoaceticus* was obtained as described by Law and Essén [20]. Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were purified from this fraction on DEAE-cellulose as described by Rouser et al. [21]. Purity was controlled by thin-layer chromatography as above.

Lipid substitution. Lipid substitution experiments were carried out according to the technique described by Warren et al. [22] using phospholipids purified from *A. calcoaceticus.* Lipid dispersions in cholate were prepared with an 800 kHz bath-type sonicator (own workshop) at half-maximum output for 3 min and incubated with the purified NADH dehydrogenase for 1 h at room temperature and dialyzed against buffer A overnight at 4°C.

Enzyme assays. NADH oxidase activity was determined polarographically using a Clark oxygen electrode (Metra) and a recorder (model M 65 F, Metra) at 30°C. The assay mixture contained in a final volume of 5 ml: buffer C (0.05 M potassium phosphate, pH 7.6), 0.09 mM NADH and enzyme.

NADH dehydrogenase activities were measured by following the absorbance change of DCIP at 600 nm ($\varepsilon_{mM} = 20.6$), ferricyanide at 420 nm ($\varepsilon_{mM} = 1.0$) and cytochrome *c* at 550 nm ($\varepsilon_{mM} = 18.5$). The assay mixture contained in a final volume of 3 ml: buffer C, 0.09 mM NADH and 0.07 mM DCIP or 1.0 mM ferricyanide or 0.02 mM cytochrome *c* and was started with enzyme. One unit of enzyme activity is the amount of enzyme that catalyzes the oxidation of 1 μmol NADH per min. For the assay of the solubilized enzyme a Triton X-100 concentration of 0.05% was maintained in the assay mixture and with the purified enzyme 0.2 mM FMN was added.

Protein was determined by the method of Lowry et al. [23].

Chemicals. Cardiolipin was obtained from Koch-Light. Phosphatidylethanolamine and phosphatidylglycerol (synthetic products) were a kind gift from Dr. Kertscher, Section of Biological Sciences, Department of Biologically Active Compounds. Blue dextran-Sepharose 6B was prepared as described in Ref. 24. Phenylsepharose 4B was prepared as described in Ref. 25. All other reagents used were commercial products of guaranteed grade.

Results

Soluble and membrane-bound NADH dehydrogenases

In *A. calcoaceticus* NADH oxidase activity was located in the membrane fraction only. NADH dehydrogenase activity was found in the membrane fraction as well as in the soluble fraction with about 90% of the total activity associated with the latter.

The NAD(P)H dehydrogenase activity of the soluble fraction seems to be attributed to the presence of several different NAD(P)H : acceptor oxidoreductases. The patterns of polyacrylamide gel electrophoresis and column chromatography are indicative of this hypothesis. By polyacrylamide gel electrophoresis of the soluble fraction under non-denaturating conditions, six bands were detected after staining for activity with NADH

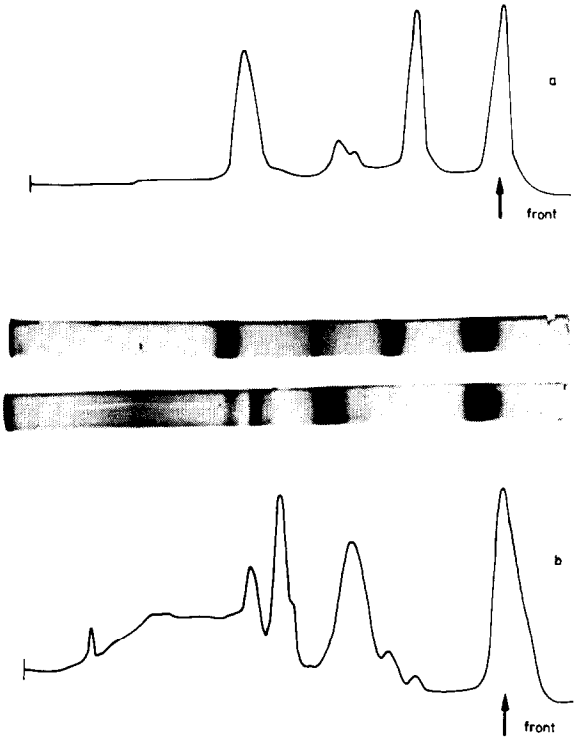

Fig. 1. Polyacrylamide gel electrophoretic analysis of the soluble fraction under non-denaturating conditions. Gels were run and stained for NAD(P)H dehydrogenase activity with nitroblue tetrazolium and NADPH (a) and NADH (b) as described in Materials and Methods and are shown together with the corresponding densitometer scans.

and nitroblue tetrazolium and four bands were detected with NADPH and nitroblue tetrazolium (Fig. 1). DEAE-cellulose chromatography of the soluble fraction resulted in two peaks with NADH : ferricyanide oxidoreductase activity and three peaks with NADH : cytochrome *c* oxidoreductase activity, and dextran blue-Sepharose 6B chromatography of the soluble fraction resulted in four peaks with NADH : ferricyanide oxidoreductase activity.

The NADH dehydrogenase activity of the membrane fraction seems to be due to the presence of a single enzyme species. There was only one band of NADH dehydrogenase activity observed in the sodium deoxycholate-polyacrylamide gel electrophoretic pattern of the membrane fraction while six bands of soluble NADH dehydrogenase activity were detected under the same conditions (Fig. 2). From our investigations it cannot be decided whether or not the one band of the sodium deoxycholate-polyacrylamide gel electrophoretic pattern of the soluble fraction which coincides in migration with the membrane-bound enzyme is a true constituent of the soluble fraction or rather an artefact due to disruption of the cells.

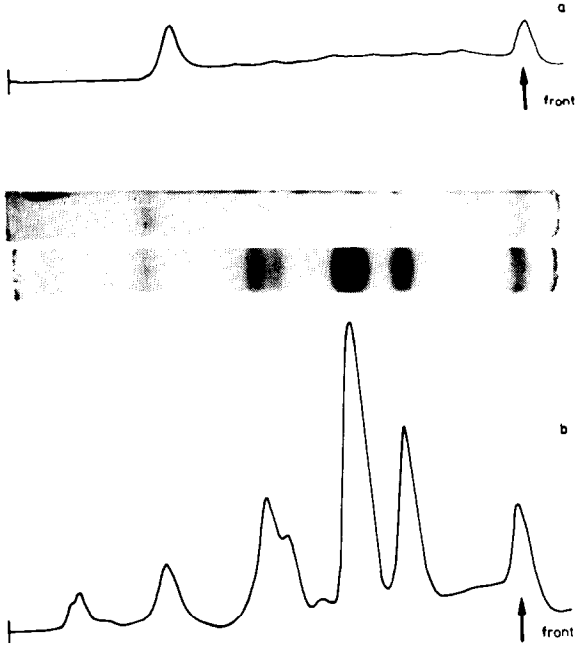

Fig. 2. Deoxycholate-polacrylamide gel electrophoretic analysis of the membrane (a) and soluble (b) fraction. Samples were treated and gels were run and stained for NADH dehydrogenase activity with nitroblue tetrazolium and NADH as described in Materials and Methods and are shown together with the corresponding densitometer scans.

TABLE I

APPARENT MICHAELIS CONSTANTS K_m^{NADH} OF THE NADH DEHYDROGENASES OF THE SOLUBLE AND MEMBRANE FRACTION AND OF THE NADH OXIDASE

Concentrations of electron acceptors: ferricyanide, 1.0 mM; DCIP, 0.07 mM; O_2, 0.23 mM. Values are given in mM.

Electron acceptor	NADH dehydrogenase		NADH oxidase
	Soluble fraction	Membrane fraction	
Ferricyanide	0.55	0.05	–
DCIP	0.07	0.03	–
O_2	–	–	0.05

However, kinetic data also indicate that the NADH dehydrogenase activities of the membrane fraction and the soluble fraction are distinct from each other and that the homogeneous enzyme species located in the membrane should be the respiratory chain-linked enzyme (Table I).

Solubilization of the membrane-bound NADH dehydrogenase

Solubilization of the membrane-bound NADH dehydrogenase was attempted by using the follow-

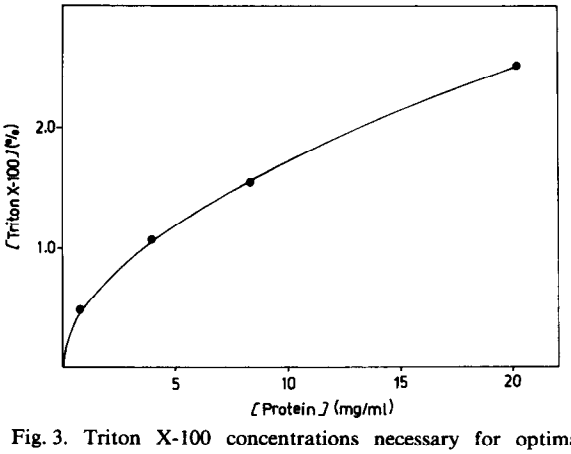

Fig. 3. Triton X-100 concentrations necessary for optimal (100%) solubilization of the membrane-bound NADH dehydrogenase at different membrane protein concentrations.

ing agents: EDTA, SDS, sodium deoxycholate, ethanol, Brij 58, Tween 40, Tween 80 and Triton X-100. Of the detergents tested the best results were obtained with Triton X-100 which was used in the following studies.

The release of NADH dehydrogenase activity from membranes was sensitive to the ratio of detergent to membrane protein displaying differently extended optima for solubilization dependent on the detergent concentration used. Detergent concentrations were determined permitting optimal (100%) solubilization at different membrane protein concentrations (Fig. 3). Solubilization did not cause any loss of NADH dehydrogenase activity but, as expected, addition of detergent completely disrupted the NADH oxidase chain.

Purification of Triton-solubilized NADH dehydrogenase

All purification steps were carried out in the presence of 0.05% Triton X-100 in 10 mM Tris-Mes buffer (pH 7.1). The enzyme solubilized by rather high concentrations of Triton X-100 (usually 1–2%) was first passed through a DEAE-cellulose column (20 ml) mainly to remove excess Triton X-100. After washing with 35 ml buffer and 50 ml buffer containing 0.1 M NaCl, NADH dehydrogenase was eluted with 60 ml buffer containing 0.7 M NaCl at a flow rate of 24 ml/h. Active fractions were pooled, diluted 1 : 15 with buffer and passed through a column of dextran blue-Sepharose 6B (5 ml). At a flow rate of 45 ml/h about 80% of the NADH dehydrogenase activity was bound to the gel and after washing with 30 ml buffer containing 1 mM NADH activity could be eluted with 40 ml buffer containing 6.9 mM NADH. The high concentration of NADH necessary for elution indicates an unspecific interaction between the dextran blue resin and the dehydrogenase.

The fractions with NADH dehydrogenase activity were combined and NaCl was added to a concentration of 3 M to favour hydrophobic interactions during hydrophobic interaction chromatography using Phenylsepharose 4B (column: 2.5 × 50 cm). At a flow rate of 13 ml/h NADH dehydrogenase was not bound to the gel but some of the contaminating proteins were and could be eluted by reducing the salt concentration and by SDS. A second DEAE-cellulose column was used to remove NADH still contained in the enzyme solution and resulted in further purification and concentration of the purified enzyme.

The results of a typical purification procedure are listed in Table II. With DCIP as the electron acceptor the purification factor is 11.6 but using ferricyanide the purification factor is 30. As the ferricyanide assay was reported to be insensitive to lipid depletion of NADH dehydrogenase [26], this finding could be indicative of a loss of phospholipids necessary for optimal activity during the course of purification, presumably due to purification steps making use of hydrophobic interactions. Thus, we estimate that the enzyme was purified about 30-fold over membranes, or about 300-fold over whole cells.

TABLE II

SUMMARY OF PURIFICATION OF NADH DEHYDROGENASE FROM MEMBRANES OF *A. CALCOACETICUS*

The details of each purification step are given in the text and in the legends to the corresponding figures. Activities given are NADH : DCIP oxidoreductase activities.

Purification step	Total protein (mg)	Total activity (U)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
Membrane fraction	160	5.9	100	0.037	–
Solubilization	120	5.9	100	0.049	1.3
DEAE-cellulose chromatography	21	4.6	78	0.220	5.9
Dextran blue-Sepharose 6B chromatography	5.0	1.5	25	0.300	8.1
Phenylsepharose 4B chromatography	1.0	0.38	6.4	0.380	10.3
DEAE-cellulose chromatography	0.7	0.30	5.1	0.430	11.6

SDS-polyacrylamide gel electrophoresis of the purified enzyme revealed one major protein band and some very low molecular weight impurities near the front (Fig. 4).

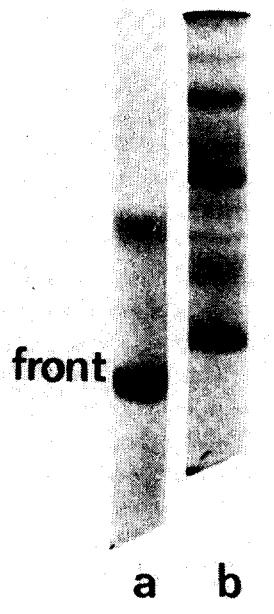

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified enzyme (a) and the protein fraction obtained by solubilization (b). Conditions were as described in Materials and Methods.

Some properties of the purified enzyme

The enzyme is completely inactive in the absence of detergent (Triton X-100) which is characteristic of an integral membrane enzyme. The molecular weight determined by SDS-polyacrylamide gel electrophoresis is 34000. The enzyme preparation requires added FMN for activity. Restoration of NADH : DCIP oxidoreductase activity follows a saturation curve for FMN with saturation attained at 0.2 mM. Riboflavin and FAD had no effect on the enzyme activity.

The purified NADH dehydrogenase is strictly specific for NADH as the electron donor. The enzyme can oxidize NADH with either ferricyanide or DCIP as the electron acceptor but is inactive with cytochrome *c* or O_2.

Effect of phospholipids on the purified NADH dehydrogenase

The NADH dehydrogenase purified from membranes of *A. calcoaceticus* in the presence of the non-ionic detergent Triton X-100 retained 0.4 μmol lipid phosphorus/mg protein during this treatment. Most of it was cardiolipin with small amounts of phosphatidylethanolamine and phosphatidylglycerol also detected. With a molecular weight of the purified enzyme of 34000 this amounts to about six molecules cardiolipin bound per enzyme molecule.

For definitive studies of specificity for phospholipids the use of enzyme complexes containing essentially pure lipid and almost free of detergent is obligatory. An approach to exchange the bound endogenous lipid for added exogenous lipid without the potential problem of irreversible denaturation of the enzyme is the lipid titration technique of Warren et al. [22] originally developed for an ATPase. This technique depends on the equilibration of the enzyme-lipid complex and a large excess of added lipid in the presence of cholate.

We have applied this method to purified NADH

TABLE III

EFFECT OF PHOSPHOLIPIDS FROM *A. CALCOACETICUS* ON THE ACTIVITY OF THE NADH DEHYDROGENASE PURIFIED FROM MEMBRANES

The incubation mixture contained in a final volume of 2 ml: 0.1 mg enzyme, 0.025% Triton X-100, 0.05 M Tris-HCl, pH 7.6, 4 mg sodium cholate and 2 mg phospholipid. Details of the preparation of lipid dispersions in cholate solutions and of the lipid substitution technique are given in Materials and Methods. Activities given are NADH : DCIP oxidoreductase activities.

Phospholipid	Activity (U/mg)
Endogenous phospholipid [a]	0.43
None [b]	0
Total phospholipid fraction	0.24
Phosphatidylethanolamine	0
Phosphatidylglycerol	0.32
Cardiolipin	0

[a] Enzyme preparation without sodium cholate treatment.
[b] Enzyme preparation with cholate treatment only; without the addition of lipid.

dehydrogenase and phospholipids (phosphatidylethanolamine, phosphatidylglycerol and cardiolipin) purified from *A. calcoaceticus* by DEAE-cellulose chromatography. NADH : DCIP oxidoreductase activities obtained in the presence of various phospholipids are listed in Table III.

According to these results, NADH dehydrogenase from membranes of *A. calcoaceticus* seems to be specific for phosphatidylglycerol. Phosphatidylglycerol comprises about 18% of the total phospholipids of *A. calcoaceticus* (data not shown). But it should be noted that we are not sure about complete exchange of endogenous phospholipid for exogenous phospholipid occurring, thus the possibility of a dual phospholipid requirement for enzyme activity cannot be excluded.

Discussion

High activities of soluble NADH dehydrogenases have been described for a variety of bacteria including *E. coli* [27], *Propionibacterium shermanii* [28] and *Bacillus stearothermophilus* [29]. Thorough investigations with the *E. coli* enzymes have established the presence of several different flavoprotein NAD(P)H : acceptor oxidoreductases in the soluble fraction [6]. The same holds true for *A. calcoaceticus* were part of this activity should be attributed to a soluble NADH : rubredoxin oxidoreductase [30].

In the membrane fraction of *A. calcoaceticus*, only one NADH dehydrogenase enzyme species was shown to exist which should thus be linked to the respiratory chain system. Furthermore, the concentration of NADH giving half-maximum activity for the membrane-bound NADH dehydrogenase is close or identical to the K_m for NADH of the membrane-bound NADH oxidase.

The purification of the membrane-bound NADH dehydrogenase of bacterial origin has been claimed to be difficult. The most highly purified enzyme preparations from *E. coli* [6] and *Ach. laidlawii* [9] were purified about 21–30-fold over membrane vesicles. For the *E. coli* enzyme a purification factor over whole cells of about 300 was estimated with a specific activity of 0.32 U/mg attained. Taking into consideration the SDS-polyacrylamide gel electrophoretic pattern, the specific activity of 0.43 U/mg attained and the purification factor in the phospholipid-insensitive ferricyanide assay, the *A. calcoaceticus* NADH dehydrogenase seems to be purified to about the same extent as the *E. coli* enzyme.

However, the molecular weight of the purified enzyme (M_r = 34000) is rather low. In two recent reports [8,12], it was questioned whether the NADH : DCIP oxidoreductase of *E. coli* is really linked to the respiratory chain. The *E. coli* enzyme is very similar in size (M_r = 38000) and catalytic properties (e.g., strictly specific for NADH as the electron donor, effective with DCIP and ferricyanide as electron acceptors) to the enzyme described in this study. An NADH : quinone oxidoreductase with M_r = 45000 [8] or M_r = 46000 [12] could be isolated and was claimed to be the real respiratory chain NADH dehydrogenase. In both reports the possibility was not neglected that the M_r 38000 enzyme is a true constituent of the M_r 45000 (46000) NADH : quinone oxidoreductase. This hypothesis reveals striking similarities to the relations within the mitochondrial system with a high and a low molecular weight form of the NADH dehydrogenase isolated which show rather different catalytic properties.

As to the lipid requirement of Complex I, first demonstrated by Ragan and Racker [31], Heron et al. [26] could establish a dual phospholipid requirement for enzyme activity. Cardiolipin, which was retained during the course of purification, seems to bind to one class of binding sites and is not removed by cholate treatment while phosphatidylethanolamine and phosphatidylcholine bind to another class of sites and rapidly exchange with exogenous lipid at low cholate concentrations.

Our finding that the endogenous lipid retained at the NADH dehydrogenase from *A. calcoaceticus* during purification is comprised mainly of cardiolipin is in good agreement with the above results. According to the results of lipid substitutions in the presence of cholate, the purified NADH dehydrogenase from *A. calcoaceticus* seems to be specific for phosphatidylglycerol. But with respect to a possible incomplete exchange of endogenous for exogenous phospholipid, a dual phospholipid requirement for activity as described for the mitochondrial system [26] cannot be excluded.

For the *E. coli* membrane-bound NADH : DCIP oxidoreductase an about 2-fold stimulation was reported of the Triton X-100-containing enzyme by phosphatidylglycerol and over a more narrow concentration range by cardiolipin. In this study no attention was paid to the problem of endogenous phospholipids bound.

The specificity of the enzyme for the coenzyme FMN seems to be an unusual feature for a NADH dehydrogenase of bacterial origin. Thus far the NADH dehydrogenase from *E. coli* has been reported to require FAD for activity [6] and the NADH dehydrogenase from *P. phosphoreum* was also shown to contain FAD [10].

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