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Interaction of purified NDH-1 from *Escherichia coli* with ubiquinone analogues

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Abstract

The NADH:ubiquinone oxidoreductase (NDH-1 or Complex I) of *Escherichia coli* is a smaller version of the mitochondrial enzyme, being composed of 13 protein subunits in comparison to the 43 of bovine heart complex I. The bacterial NDH-1 from an NDH-2-deficient strain was purified using a combination of anion exchange chromatography and sucrose gradient centrifugation. All 13 different subunits were detected in the purified enzyme by either N-terminal sequencing or matrix-assisted laser desorption/ionization time-of-flight mass spectral analysis. In addition, some minor contaminants were observed and identified. The activity of the enzyme was studied and the effects of phospholipid and dodecyl maltoside were characterized. Kinetic analyses were performed for the enzyme in the native membrane as well as for the purified NDH-1, using ubiquinone-1, ubiquinone-2 or decylubiquinone as the electron acceptors. The purified enzyme exhibited between 1.5- and 4-fold increase in the apparent K_m for these acceptors. Both ubiquinone-2 and decylubiquinone are good acceptors for this enzyme, while affinity of NDH-1 for ubiquinone-1 is clearly lower than for the other two, particularly in the purified state. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Complex I; NADH dehydrogenase; Matrix assisted laser desorption/ionization time of flight; Ubiquinone-2; Respiratory chain; Phospholipid

1. Introduction

NADH:ubiquinone oxidoreductase (also called Complex I or NDH-1) is a large and complex integral membrane protein that forms the first segment of the respiratory chain of most eukaryotes and several prokaryotes. Complex I catalyses the oxidation of NADH to NAD^+ and the reduction of ubiquinone to ubiquinol. The electron transfer reaction of the enzyme is coupled to proton translocation across the membrane, and against the electro-chemical concentration gradient (for reviews see [1,2]). Mammalian mitochondrial Complex I is composed of 43 or even 44 different protein subunits [3], seven of which are encoded and synthesized within the organelle [4,5]. Bacterial Complexes I (NDH-1) are composed of 'only' 14 subunits (13 subunits in bacteria such as

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DBQ, decylubiquinone (decylbenzylquinone); DM, dodecylmaltoside; HAR, hexammineruthenium; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; Q_1, ubiquinone-1; Q_2, ubiquinone-2; P_i, PO_4

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Escherichia coli in which the nuoC and nuoD genes are fused and encode a single protein), and are more easily accessible to genetic manipulation [2,6–8]. However, purification of intact, active and stable NDH-1 from bacteria is not a trivial task, and many trials in several different laboratories have ended without success. On the other hand, the *E. coli* NDH-1 has been purified by Friedrich and coworkers, but not without difficulties pertaining to the completeness of the enzyme and its stability in the purified state [9–11].

In this paper we describe an improved purification method for *E. coli* NDH-1 that allows a greater yield of purified, functional NDH-1 than previously described. The purification does not depend on the use of a strain that overproduces NDH-1, and we have employed a strain that lacks NDH-2, the alternative NADH:ubiquinone reductase. The subunit composition and the activities of the newly purified NDH-1 were studied in greater detail than previously. We took special care to verify that all the subunits, i.e., the products of the genes found in the *nuo* operon of *E. coli*, are indeed present in the purified NDH-1. Kinetic analyses of the enzyme activities were conducted, and the activity with three different short-chain ubiquinone analogues was characterized.

2. Materials and methods

2.1. Materials

The ubiquinone analogue ubiquinone-2 was a generous gift from Eisai Corp., Japan. Ubiquinone 1 was a generous gift from Hoffman-La Roche, Switzerland. Silver stain for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Kaleidoscope pre-stained markers were obtained from BioRad. GelCode Blue Stain Reagent and Bichinconninic Acid Protein Assay Reagent were purchased from Pierce. Soybean phospholipid (L-α-phosphatidylcholine, type II-S), CCCP, valinomycin and decylubiquinone were purchased from Sigma. Complete protease inhibitor was from Boehringer Mannheim, Sequencing Grade Modified Trypsin from Promega and dodecyl maltoside (*n*-dodecyl-β-D-maltopyranoside) was purchased from Anatrace (Maumee, OH, USA). Piericidin A was a generous gift from Dr. Alain Dupuis, Grenoble, France and the Anonine mixture was purchased from Dr Y.K. Gupta, Varanasi, India. Q-Sepharose and Source 15Q ion exchange resin were from Amersham Pharmacia Biotech.

2.2. Bacterial growth and membrane preparation

E. coli strain MWC215 [12] was grown aerobically in 2 L baffled shake flasks containing 1 L of Malate media at 250 rpm, 37°C to a Klett value of at least 200 (filter number 69). The Malate medium composition is: 60 mM NaH_2PO_4•$2H_20$, 76 mM $(NH_4)_2SO_4$, 67 mM DL-malate, 59 μM $MgCl_2$, 1 mM citric acid, 0.1% (w/v) yeast extract, and 1 ml/l salt solution (18.7 mM NH_4Cl, 600 μM Na_2MoO_4•$2H_2O$, 13 μM $MnSO_4$•H_2O, 127 μM EDTA). Cells were harvested by centrifuging (10 min, 5000×*g*, 4°C), washed in 50 mM MES/NaOH, pH 6.0 and stored at −80°C until use. Unless otherwise stated, all subsequent steps were carried out in 50 mM MES/NaOH, pH 6.0 at 4°C. Thawed cells were suspended to a concentration of 250 g cells/l of buffer supplemented with either 1 mM EDTA and 0.2 mM PMSF or Complete tablets, used according to the manufacturer's instructions, as protease inhibitors. Cell membranes were isolated using an APV Gaulin Homogenizer (cell disrupter) at a pressure of 500 bar for 3–4 min. The resulting suspension was centrifuged at 125 000×*g* for 1 h. Cell membranes formed the upper layer of the pellet. The membranes were collected, washed in buffer with 200 mM NaCl and centrifuged. The washed membranes were homogenized, frozen in liquid nitrogen and stored at −80°C until use.

2.3. NDH-1 purification

Thawed *E. coli* membranes were diluted to a protein concentration of 6 mg/ml in 0.5% dodecyl maltoside (DM)-containing buffer, and centrifuged for 1 h at 125 000×*g*. The supernatant was adjusted to 100 mM NaCl and applied to a 135 ml bed volume Q-Sepharose anion exchange column. The anion exchange chromatography steps were carried out using a Gradifrac system (Pharmacia), equipped with a conductivity meter and monitored at 280 nm. A mul-

ti-step gradient from 100 to 300 mM NaCl in 50 mM MES/NaOH (pH 6.0), 0.05% DM was used for elution. Column fractions exhibiting maximal NADH/hexamine ruthenium (HAR) activity were pooled and concentrated approximately 20-fold using Amicon spin concentrators (M_r cut-off 50 000 Da). The concentrated sample was applied onto 15–40% sucrose gradients in 50 mM MES/NaOH (pH 6.0), 0.05% DM and centrifuged for a minimum of 20 h at 198 000 × *g*. The NADH/HAR active fractions harvested from the sucrose gradients were combined and transferred to 50 mM MES/NaOH (pH 6.0), 0.05% DM by cycles of dilution and concentration using Amicon spin concentrators until the sucrose concentration was 3% or less and the volume decreased by 4-fold. Finally, the NDH-1 containing sample was adjusted to 100 mM NaCl and applied to a Source 15Q column that was eluted with a multi-step gradient from 100 to 300 mM NaCl in 50 mM MES/NaOH (pH 6.0), 0.05% DM. NDH-1 fractions were desalted by dilution and concentration, concentrated between 10- and 20-fold and stored at −80°C. Throughout the purification the NDH-1 containing samples were kept at 4°C.

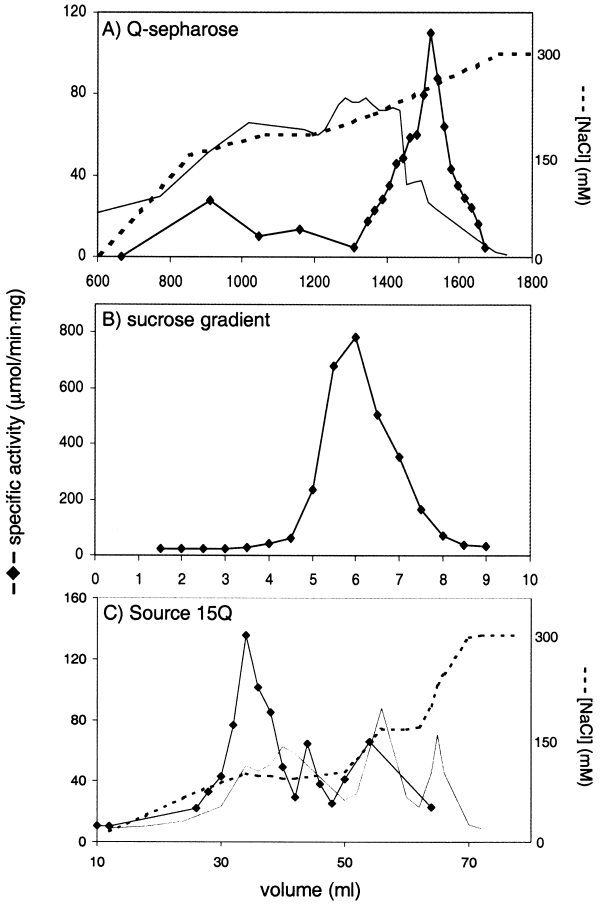

Fig. 1. Purification of *E. coli* NDH-1. (A) Elution profile of *E. coli* MWC215 membrane extract from Q-Sepharose; (B) sucrose gradient ultracentrifugation; (C) elution profile from Source 15Q. (◆) NADH/HAR activity (µmol/min per mg); (———) absorbance at 280 nm; (- - -) NaCl (mM).

2.4. Activity measurements

NADH/HAR oxidoreductase activity was routinely measured at each stage of the purification. The assay (developed from earlier versions [8,13]) was conducted at 30°C in 20 mM HEPES (pH 8.0), 0.05% DM, 600 µM HAR, 300 µM NADH, and initiated by addition of the enzyme sample. To measure the NADH/ubiquinone oxidoreductase activity, the enzyme was first transferred into 50 mM MES/NaOH (pH 6.0), 0.05% DM and only then diluted into the reaction mixture. The assay was conducted at 30°C in 50 mM KPO_4, pH 6.5 (or 50 mM KCl, as indicated), with 0.25 mg/ml sonicated soybean lipids, 60 µM ubiquinone, 15 µg protein, and initiated by the addition of 100 µM NADH (modified from [8]). The assay mixture was incubated for 5 min (3 min at room temperature and 2 min at 30°C) prior to initiation of the assay with NADH. The assay was monitored for 10 min. When necessary, the amount of protein was decreased while maintaining the lipid to protein ratio at 20 (g/g). In experiments where the enzyme sample and lipid were incubated on ice prior to measurement of the ubiquinone reductase activity, the lipid to protein ratio was maintained at 20 (g/g) with a protein concentration of 0.3 mg/ml in the assay buffer. The first assays were conducted after 5 min incubation on ice. When indicated the inhibitors piericidin A or anonine were added after the addition of the ubiquinone analogue. Assays were carried out on a Cary 1C UV/vis spectrophotometer. For V_{max} and K_m measurements on *E. coli* membranes, the NADH/Q assays were carried out in the presence of 12 mM KCN which was added after the ubiquinone analogue and before the addition of membranes.

2.5. SDS–PAGE

Purified NDH-1 was electrophoresed on 15% Laemmli SDS–PAGE [14] or Schägger SDS–PAGE [15] as previously modified [16] in the mini-gel format (BioRad). Protein bands were detected by staining with GelCode Blue Stain Reagent or Silver Stain according to the manufacturer's direction. The sample loading buffer for Laemmli PAGE was prepared with and without β-mercaptoethanol, as indicated. To enhance sensitivity and decrease background, silver-stained PAGEs were re-stained as outlined previously [17]. Protein electrophoresis for mass spectral analysis utilized a larger gel format, 16×14 cm. The protein concentration was determined by the Bichinconninic Assay using BSA as the standard.

2.6. Identification of NDH-1 subunits

Purified NDH-1 was electrophoresed on either Schägger or Laemmli SDS–PAGE as described above. For N-terminal sequencing the NDH-1 protein subunits were electrophoretically transferred from Laemmli SDS–PAGE to PVDF membrane. N-Terminal sequencing was performed on an Applied Biosystems 477A/120A automated sequencer, in the gas phase mode, following the manufacturers supplied procedure. For mass spectral analysis, the gels were silver stained following the procedure of [18], with the modification that the BioRad Silver stain reagent and developer were employed. All bands were excised from the gel and stored at −20°C until further processing. The protein in the excised bands was subjected to tryptic digestion, extracted from the gel slices and prepared for mass spectral analysis as previously described [19]. The tryptic peptides were desalted prior to mass spectral analysis using either C4 or C18 ZipTip desalting tips (Millipore). MALDI-TOF mass spectral analysis was performed on a Bruker Daltonics Biflex III system using a 2 GHz digitizer and the results were visualized using a Sun Microsystems workstation. The NDH-1 subunits were identified by their tryptic peptide masses using either the ProteinProspector (version 3.4.1) or ProFound (version 4.10.5) peptide mass database searching software. The following search criteria were used: taxonomic category, *E. coli* genome, NCBInr database, acrylamide modified cysteine, charge state; MH+, size in Daltons, mass tolerance; ±0.1 Da, protein mass range; ±20% of SDS–PAGE determined mass, p*I* range 0–14, missed trypsin cleavages; 1, monoisotopic masses. When necessary, the search criteria were altered by narrowing the protein mass range and/or p*I* range, or relaxing the mass tolerance to no greater than ±1Da to allow rank number one identification of the subunit.

2.7. Storage and stability of the purified NDH-1

Partially purified NDH-1 in NaCl concentrations higher than 200 mM proved to be unstable at either +4°C, −20°C or −80°C. For storage longer than 18 h, purified or partially purified NDH-1 was exchanged into 50 mM MES/NaOH (pH 6.0), 0.05% DM and stored at −80°C. When stored at −80°C, purified *E. coli* NDH-1 (average concentration 4 mg/ml) is stable to repeated weekly freezing and thawing for at least 6 weeks, as determined by the NADH/HAR and NADH/Q_2 activities. After this period, the NADH/HAR activity increases and the NADH/Q_2 activity decreases. Undisturbed, purified NDH-1

Table 1
Purification of *E. coli* NDH-1

Sample	Volume (ml)	Total protein (mg)	Total activity (mmol/min)	NADH/HAR Specific activity (μmol/min per mg)
Membranes	262	8691	8.1	0.9
Membrane extract	775	2630	12.5	4.8
Q-Sepharose	537	374	3.3	8.9
Sucrose gradient	53	63	2.7	41.9
Source 15Q	55	20	1.1	58.2

Values based on 100 g wet weight of cells.

was found to be stable at −80°C for a period of 6 months or longer.

3. Results

3.1. Enzyme purification

NDH-1 was purified from dodecyl maltoside solubilized *E. coli* membrane proteins using three main steps: anion exchange chromatography on Q-Sepharose, sucrose gradient ultracentrifugation and anion exchange chromatography on Source 15Q. This purification procedure routinely yields about 20 mg of >95% pure NDH-1 from 100 g wet weight of cells (Table 1).

The method development was initiated with membrane protein extraction experiments that were conducted by measuring the rate of NADH/HAR activity in the supernatant after centrifugation. The results indicated that 0.5% (w/v) DM at a protein concentration of 6 mg/ml was optimal for NDH-1 extraction from the membranes prepared as described (not shown). The first anion exchange column, a self-packed, Q-Sepharose column, was eluted with a multi-step salt gradient in order to obtain good separation between the fractions containing relatively high enzymatic activity and those containing the majority of the other proteins (Fig. 1A). The sucrose gradient ultracentrifugation step of the new procedure combines purification of NDH-1 with an estimate of its size and by inference, a measure of its intactness. The NDH-1 containing sample typically migrated two-thirds of the way down a 15–40% sucrose gradient (Fig. 1B), indicating that the enzyme had not separated into subcomplexes. The data in the purification table (Table 1) reveal that sucrose gradient centrifugation, as carried out in this work, was an effective way to remove protein contaminants with minimal loss of the enzyme (total activity), resulting in a large increase in specific activity. The latter is not true for the final step, chromatography over the Source 15Q column. The purified NDH-1 bound weakly to this column, eluting at 130 mM NaCl. Interestingly, a second peak of less pure NDH-1 eluted at a higher NaCl concentration, and it was necessary to optimize the NaCl gradient in order to obtain good separation between the two peaks (Fig. 1C).

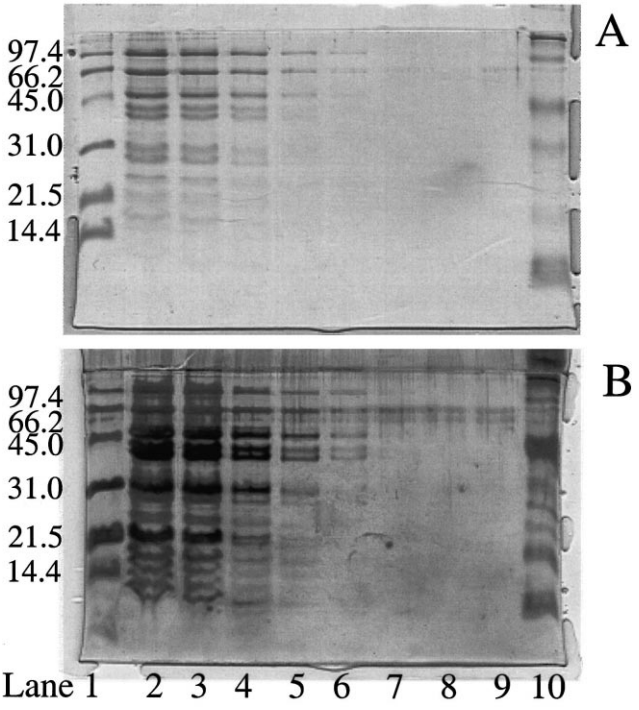

Fig. 2. Duplicate SDS–PAGE of a dilution series of purified *E. coli* NDH-1 in the presence of β-mercaptoethanol. (A) GelCode Blue-stained SDS–PAGE; (B) Silver-stained SDS–PAGE. Lane 1, Molecular mass markers (kDa); lanes 2–9, decreasing amounts of NDH-1: 15 μg (2), 10 μg (3), 5 μg (4), 1 μg (5), 500 ng (6), 100 ng (7), 10 ng (8), 5 ng (9); lane 10, Kaleidoscope pre-stained molecular mass markers.

3.2. Isolation and identification of subunits in E. coli NDH-1

To ensure that the purified NDH-1 contained all 13 expected protein subunits we employed two methods to identify individual subunits after separation by SDS–PAGE. Initially N-terminal sequencing was attempted and four subunits were identified, namely NuoCD, NuoF, NuoB and NuoE. Interestingly, the N-terminal sequence obtained for NuoCD was TDL and not MVNNMTDL as previously suggested by translation of the DNA sequence and N-terminal sequencing [9,20]. However, the protein sequence MVNN was obtained from a protein that is now known to be of the incorrect size, hence its significance is questionable. Our result suggests that the

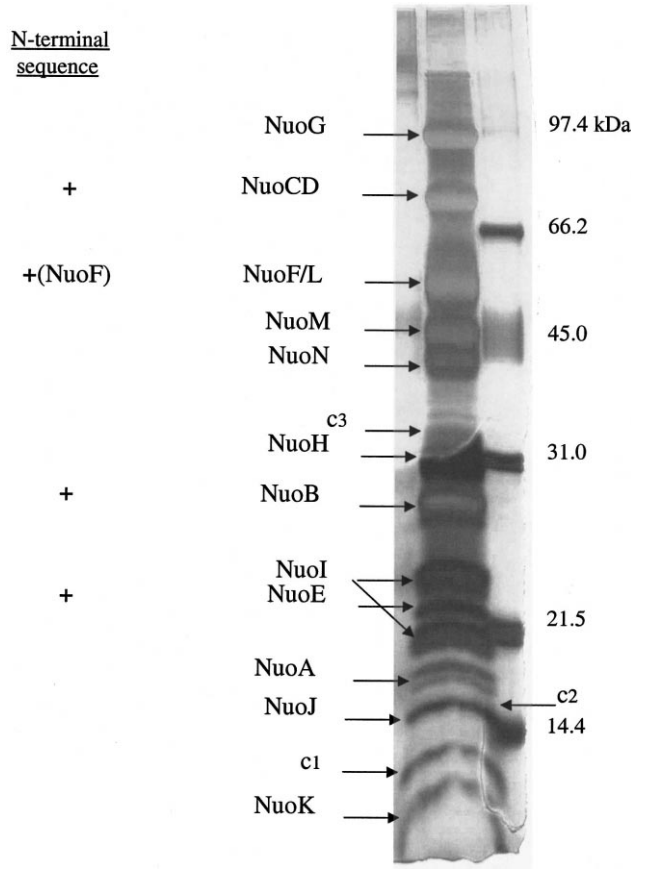

Fig. 3. Identification of the *E. coli* NDH-1 subunits by MALDI-TOF mass spectral analysis. Silver-stained Laemmli SDS–PAGE with 40 μg of protein per lane. The protein bands identified as the 13 NDH-1 subunits and three contaminating proteins are indicated by the arrows. The NDH-1 subunits identified by N-terminal sequencing are also indicated (+). NuoF/L indicates co-migration of these subunits. NuoI was identified at two different positions as indicated by the arrows. C1–C3 indicate the contaminating protein bands (see text for more details). In addition, the succinate dehydrogenase iron–sulphur protein was found to co-migrate with NuoB.

second methionine codon of the *nuoCD* gene is the initiation codon, unless the putative MVNNM peptide is removed from the N-terminus of NuoCD post-translationally. If the second Met is indeed the initiation codon, the molecular mass of the mature NuoCD protein would be 68 061 Da and not 68 650 Da.

Many bacterial proteins have blocked N-termini. Due to this fact, we decided to turn to mass spectroscopy rather than attempt to obtain N-terminal protein sequences from subunits that did not readily yield data in our first trial or in previous works [9]. To make full use of the high sensitivity of mass spectral analysis by MALDI-TOF, an improved method of visualization of the NDH-1 protein bands separated by PAGE was required. Duplicate Laemmli SDS–PAGE of a dilution series of purified NDH-1 were used to compare the detection limit of the highly sensitive, silver staining method to Coomassie-based staining using the GelCode Blue reagent (Fig. 2). The reported, over 100-fold greater sensitivity of silver stain as compared to Coomassie blue-based staining techniques [21] is readily discernible in the case of *E. coli* NDH-1 when comparing Fig. 2A to B. Using silver staining, all 13 subunits of NDH-1 and in particular subunits smaller than 14 kDa in size were easily identifiable from a total of 5 μg of protein per lane (Fig. 2B, lane 4). A less complete identification of the NDH-1 subunits by Coomassie blue staining required a minimum of 15 μg of protein per lane (Fig. 2A, lane 2). The drawbacks to silver staining are artefacts caused by surface deposition of silver ions, and the artefactual bands at 60 and 67 kDa that are due to the presence of β-mercaptoethanol in the sample buffer (Fig. 2B, lanes 7–9) [22]. The latter bands, however, are often seen in Coomassie-stained gels as well (not shown).

The use of MALDI-TOF mass spectroscopy enabled positive identification of all the expected 13 subunits of *E. coli* NDH-1 in the purified enzyme (Fig. 3). It should be noted, however, that identification of NuoM and NuoJ required relaxation of the search criteria before being ranked as the number one match by the peptide database search program ProFound (see Section 2 for more details). Interestingly, the NuoI subunit was identified in two different positions corresponding to masses of around 24 and 20 kDa (Fig. 3). Since the mass spectroscopy analyses were done on tryptic peptides rather than on intact proteins, it is not possible at this stage to determine the reason for the split migration of NuoI. However, this finding may be linked to the omission of β-mercaptoethanol in this gel. Using the identified NDH-1 subunits as a measure of its mobility, the slower migrating NuoI subunit, of approximately 24 kDa, is likely a truer representation of the mass of the SDS denatured, cysteine-rich subunit. The faster migrating NuoI, at approximately 20 kDa, may represent a less unfolded, more compact and

therefore faster migrating protein. MALDI-TOF analyses were also employed for the subunits that were separated by SDS–PAGE according to Schägger. This enabled identification of (only) eight subunits, NuoG, NuoCD, NuoF/NuoL (these two subunits co-migrate in both gel systems), NuoN, NuoB, NuoE and NuoA.

Mass spectral analyses of the protein bands in purified *E. coli* NDH-1 also identified some of the contaminants that are still present in this preparation (Fig. 3). It turns out that the iron–sulphur protein (IP) of succinate dehydrogenase co-migrates with NuoB on Laemmli-type but not Schägger-type SDS–PAGE. In addition, three of the excised bands were contaminants. Two of them were positively identified using the strictest search criteria, and the third with a relaxed stringency. The protein band labelled 'C1' (Fig. 3) is the *cyaY* product, a member of the frataxin family. Mutations in the mitochondrial homologue of this protein may lead to the autosomal recessive neurodegenerative disease Friedreich ataxia [23]. 'C2' is the beta chain of DMRL (6,7-dimethyl-8-ribityllumazine) synthase, which catalyses the final step of riboflavin synthesis [24], and 'C3' is dihydroorotate dehydrogenase [25,26]. It remains to be studied (e.g., by gene deletion) whether or not any of these additional proteins plays a role in the synthesis, assembly or activity of NDH-1.

3.3. Ubiquinone reductase activity

The assay conditions for purified *E. coli* NDH-1 were optimized from the assay conditions used for *Paracoccus denitrificans* NDH-1 in its native membrane [8]. One outcome of these experiments was the determination of an optimum weight ratio of added lipid to protein of 20 g/g. Addition of sonicated soybean phospholipid to more than 20 g lipid per g protein inhibited the rate of ubiquinone reduction. The effects of pH, salt and the detergent DM on the activity were also tested. It was found that DM is a potent inhibitor of the Q_2 reductase activity of purified NDH-1 and due to this finding concentrated NDH-1 samples were first exchanged into the standard buffer (50 mM MES/NaOH (pH 6.0), 0.05% DM) and then diluted into the assay buffer that contained no detergent.

Purified NDH-1 is sensitive to the ionic strength of the assay solution (Fig. 4). In the presence of 50 mM KCl the rate of Q_2 reduction was more than double the rate measured in the presence of 50 mM KP_i. To exclude a specific potassium effect, the activity was also measured in the presence of 50 mM NaCl and the rate of Q_2 reduction was the same as with 50 mM KCl (Fig. 4). In the case of monovalent ions the activity rate decreased when the salt concentration was raised beyond 100 mM, and the optimal NaCl or KCl concentration for Q_2 reduction was 50 mM (Fig. 4). However, the observed results with MES, and the difference between Na_2SO_4 and $(NH_4)_2SO_4$ when both salts are present at the same ionic strength (Fig. 4) clearly indicate that there are additional factors that affect the activity of purified *E. coli* NDH-1.

The activity assays, NADH/HAR and NADH/Q_2 were conducted on the concentrated enzyme samples after purification (Table 2). Both the ubiquinone reductase and the NADH dehydrogenase specific activ-

Table 2
Enhancement of *E. coli* NDH-1 ubiquinone reductase activity upon incubation at 4°C with sonicated soybean phospholipids

Sample	Incubation with added phospholipid	NADH/Q_2 Specific activity (μmol/min per mg)	Fold of rate increase
Membrane extract	No	0.09 ± 0.05	
	Yes	0.09 ± 0.02	1
Q-Sepharose	No	0.11 ± 0.03	
	Yes	0.37 ± 0.05	3.3
Sucrose gradient	No	0.56 ± 0.09	
	Yes	1.53 ± 0.23	2.8
Source 15Q	No	0.78 ± 0.16	
	Yes	1.16 ± 0.07	1.5[a]

[a]Time-dependent rate enhancement.

ity increase throughout the purification until the penultimate step. However, in the initial trials the ubiquinone reductase specific activity after chromatography on Source 15Q has decreased from the previous step, whereas the NADH/HAR activity continued to increase. To test whether this loss of ubiquinone reductase activity was due to the removal of lipids during the final step of purification, the NADH/Q_2 assay was altered to allow interaction of lipids with the purified protein prior to measurement. It was found that at all stages of the purification, except for the membrane extract, incubation of the protein and lipid prior to initiation of the assay lead to an increase in the measured ubiquinone reductase rate (Table 3). The rate enhancement of the ubiquinone reductase activity due to incubation of lipid and NDH-1 is similar at the Q-Sepharose and sucrose gradient steps of the purification and it is time independent, occurring at the first time point after incubation (5 min). The rate enhancement upon lipid and protein incubation is lower for NDH-1 at the S 15Q stage of the purification and in this case, it is time dependent, requiring 100 min of 4°C incubation with lipid to reach the maximal measured ubiquinone reductase rate. This level of activity was maintained for a period of 2–3 h.

Each preparation of purified NDH-1 was tested for the sensitivity of its NADH/Q_2 activity to the inhibitors annonine and piericidin A. On average, a maximum of 30% inhibition was observed by 1 nmol of piericidin A per μg protein, and complete inhibition by 5 nmol annonine per μg protein. The I_{50} value for annonine was found to be 50 pmol/μg protein.

3.4. Kinetic analyses of the interaction of E. coli NDH-1 with ubiquinone analogues

The interaction of both membrane-bound and purified NDH-1 with three different short-chain ubiquinone analogues was studied (Fig. 5). The V_{max} and K_m values for Q_1, Q_2 and DBQ were determined for the two states of the enzyme and the results are summarized in Table 3. The assays conducted on plasma membranes were in the presence of 12 mM KCN.

Initial experiments to reconstitute the purified *E. coli* NDH-1 into coupled proteoliposomes were carried out using a protocol developed for the

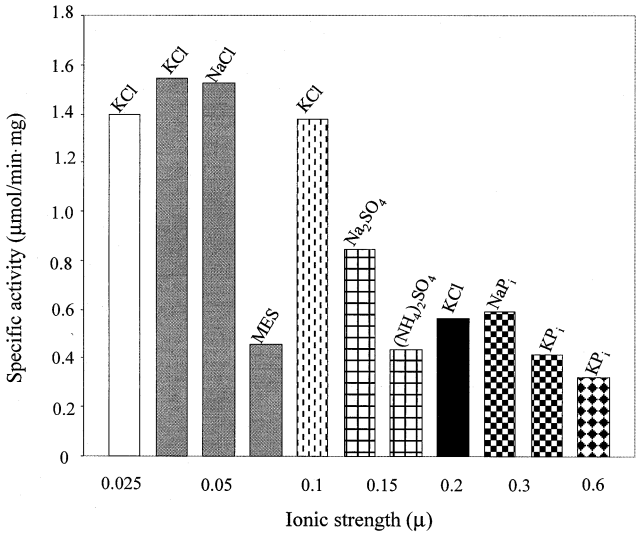

Fig. 4. The effect of ionic strength on the ubiquinone reductase activity of purified *E. coli* NDH-1. The indicated salt or buffer solutions replaced 50 mM KPO_4 that was used in the regular NADH/Q assay (see Section 2). Same column pattern indicates same ionic strength.

E. coli ubiquinol oxidase cytochrome bo_3 [27]. The preliminary results indicate that the enzyme can generate a membrane potential, as determined by stimulation of the rate of NADH/Q_2 activity by the proton ionophore CCCP (so called respiratory control). However, there was high variability between different proteoliposome preparations with regard to the sidedness of the enzyme in the vesicle, and the degree of respiratory control (from 1.7 to 5.8). It is thus necessary to further develop the method for generation of coupled phospholipid vesicles containing *E. coli* NDH-1 before further characterization of the proton-translocating activity of this enzyme could be assayed directly.

4. Discussion

In this paper, we have presented a method for the purification of NDH-1 from *E. coli* that may be viewed as an improvement of the chromatography-based protocol described by Spehr et al. [11]. One of the main benefits of the new protocol is probably that it renders the NaBr washing step unnecessary. This may be the reason that we were able to obtain a higher yield of purified protein from a lower amount

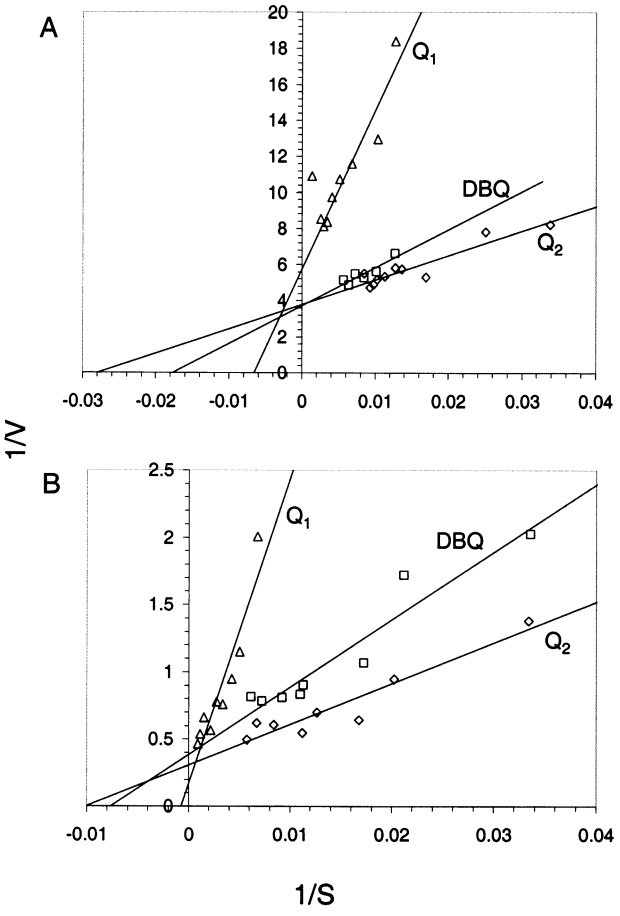

Fig. 5. Kinetic analyses of the ubiquinone reductase activity of *E. coli* NDH-1, both in the native membrane (A), and in the purified state (B). The double reciprocal analyses of the initial rates (Lineweaver–Burk plots) are presented. The units of V are μmol/min per mg, and those of S, μM ubiquinone analogue.

of starting material. In addition, we took special care to verify that all the subunits of *E. coli* NDH-1 were present in the final preparation, rather than rely on the number of visible protein bands in the sample. In order to be confident of the identification of all the subunits (and possible contaminants) visualized by the sensitive silver staining method, 22 bands were excised from a large format Laemmli SDS–PAGE for MALDI-TOF mass spectral analysis. The results of this analysis indicated that the purified sample retains all 13 NDH-1 subunits and contains four contaminant protein bands. The remaining five excised bands (as one contaminant co-migrates with NDH-1 subunit NuoB) were identified to be the leading and/or lagging edges of the adjacent protein band due to overloading of the lane. Of the identified contaminants ('C1', 'C2' and 'C3', see Fig. 3 and Section 3.2), none were the ATPase subunits that Spehr et al. [11] indicate the NaBr wash is required to remove. Furthermore, one or more of these proteins might be important for the assembly of *E. coli* NDH-1.

Before embarking on MALDI-TOF analysis of tryptic peptides, we tried to determined the identification of the subunits by N-terminal sequencing after electro-transfer from acrylamide gel to PVDF membrane. While only four bands yielded protein sequence, the result with NuoCD is worth discussing. The N-terminal sequence of this protein is TDL and not MVNNMTDL, meaning that the protein is four or five amino acids shorter (depending on processing of the initial Met) than previously thought, and deposited in the database. While this difference is rather small, it might be important for researchers who attempt to identify and study this important subunit using peptide-specific antibodies. Short synthetic peptides, the sequence of which is the first 10–15 amino acids of the mature protein, are often selected for the generation of such antibodies. Another interesting observation is that all GenBank sequences that are most closely related to the first 100 amino acids of the *E. coli* NuoCD protein, but from other bacteria (see accession numbers O85275, Q9I0J9,

Table 3
V_{max} and K_m values for purified and native membrane-bound *E. coli* NDH-1 with three ubiquinone analogues

Sample	Q_2		Q_1		DBQ	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Membranes	0.27	30	0.16	97	0.27	46
Purified NDH-1	2.7	83	2.8	400	2	66

The V_{max} units are μmol NADH oxidized/min per mg protein. The K_m units are μM. *E. coli* membranes were assayed in the presence of 12 mM KCN. All values obtained from Eadie–Hofstee double reciprocal plots.

Q9KGW7, and P57254), appear to start between four and six amino acids downstream of the previously published N-terminus of *E. coli* NuoCD. This might mean that although the extreme N-terminal sequence is not highly conserved even among the sequences listed above, there may be a structural importance to its precise size.

The rates of NADH oxidation and reduction of ubiquinone analogues by the isolated enzyme was addressed in this study in several ways. Optimization of the ubiquinone reductase assay system revealed the activity to be ionic strength dependent, and sensitive to low concentrations of DM and high concentrations of soybean phospholipid. The observations as to the importance of these factors to the assay results might indicate that part of the seemingly low activities obtained previously can be attributable to the measurement conditions.

The kinetic analyses of the interaction of *E. coli* NDH-1 with different ubiquinone analogues revealed several interesting observations. The results (Fig. 5 and Table 3) indicate that the purified enzyme exhibits a somewhat altered affinity for the ubiquinone analogues in comparison to the enzyme in the native membrane, although the difference is not very large. In particular, the K_m values for DBQ, and to a lesser degree for Q_2, are hardly affected by the purification. The difference with respect to Q_1 is larger, and since the apparent K_m for this acceptor is already significantly larger in the membrane, it reaches a level that renders this analogue impractical for routine analyses of the purified enzyme. Taken together, the kinetic results suggest that the purified enzyme (at least the fraction obtained at the end of the process) retains quite well the structure of the ubiquinone binding site(s). It is also interesting to compare the *E. coli* NDH-1 in the native membrane to its counterparts in *P. denitrificans* and bovine mitochondria. In agreement with *E. coli*, the latter two enzymes exhibit smaller K_m for Q_2 in comparison to Q_1 [8,28]. However, the enzymes from *P. denitrificans* and bovine mitochondria both exhibit between three and four times higher apparent K_m for decylubiquinone (DBQ or DB) than the enzyme from *E. coli*. Whether or not this difference in affinity to Q_1 (or decylubiquinone, depending on the point of view) is related to differences in inhibitor sensitivity remains to be studied.

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