Purification of the 45 kDa, membrane bound NADH dehydrogenase of *Escherichia coli* (NDH-2) and analysis of its interaction with ubiquinone analogues

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Abstract The NADH:ubiquinone reductase (NDH-2) of *Escherichia coli* was expressed as a His-tagged protein, extracted from the membrane fraction using detergent and purified by chromatography. The His-tagged NDH-2 was highly active and catalyzed NADH oxidation by ubiquinone-1 at rates over two orders of magnitude higher than previously reported. The purified, His-tagged NDH-2, like native NDH-2, did not oxidize deamino-NADH. Steady-state kinetics were used to analyze the enzyme's activity in the presence of different electron acceptors. High V_{max} and low K_m values were only found for hydrophobic ubiquinone analogues, particularly ubiquinone-2. These findings strongly support the notion that NDH-2 is a membrane bound enzyme, despite the absence of predicted transmembrane segments in its primary structure. The latter observation is in agreement with possible evolutionary relation between NDH-2 and water-soluble enzymes such as dihydrolipoamide dehydrogenase. There is currently no clear indication of how NDH-2 binds to biological membranes.

Key words: Deamino-NADH; Idebenone; NDH-2; NADH:quinone reductase; Ubiquinone; NAD(P)H-(disulfide)-oxidoredeductase

1. Introduction

The respiratory NADH dehydrogenase of *Escherichia coli*, the product of the *ndh* gene, was first purified and partially characterized about 20 years ago [1–3]. It was then considered to be the sole NADH:quinone reductase of *E. coli*, but it was later discovered that the bacterium also has a Complex I-type NADH dehydrogenase [4]. The bacterial Complex I is often called NDH-1, and the 'alternative' enzyme that does not pump protons across the membrane and does not oxidize deamino-NADH is called NDH-2 [4]. NDH-1 is a multi subunit, H^+-translocating NADH:quinone reductase that contains FMN and many iron-sulfur clusters [5]. NDH-2, on the other hand, contains neither Fe–S clusters nor FMN, and its sole redox center is the flavin cofactor FAD [1–3,6].

NDH-2 was not detected in animal mitochondria, but similar enzymes are present in fungi mitochondria. In *Saccharomyces cerevisiae*, a fungus that lacks Complex I, there is at least one NDH-2 that faces the mitochondrial matrix, and another one that faces the cytoplasmic side of the inner membrane, i.e. the inter-membrane space [7]. *Yarrowia lipolytica* is a fungus that has Complex I and also expresses an outward facing NDH-2 [8]. An NDH-2 with a putative Ca binding site was recently characterized in mitochondria of *Neurospora crassa*, another Complex I-containing fungus [9].

Paracoccus denitrificans is a soil bacterium that has NDH-1, but no NDH-2. In order to enable genetic inactivation of this NDH-1, the *ndh* gene of *E. coli* was inserted into the *nqo8* gene of *P. denitrificans*, leading to the expression of an active and membrane bound NDH-2 in *P. denitrificans* [10]. In the course of that work, it was noticed that the *ndh* gene product lacks predicted transmembrane segments, and the question how this membrane protein binds to biological membrane was raised. One segment of this protein, from L242 to T247, was previously suggested to bind to the membrane [10]. However, when more NDH-2 sequences became available, it turned out that this segment is not highly conserved, thus rendering that suggestion less likely.

The purified NDH-2, as originally prepared, was almost inactive. Its activity was somewhat improved by the addition of FAD to the reaction mixture, but it was still very low [1,2]. It might be speculated that both the low activity and the absence of predicted transmembrane helices in NDH-2 stem from the loss of a not yet discovered second subunit. Perhaps native NDH-2 is made of two subunits, only one of which is encoded by *ndh*, and the other one is involved in binding to the membrane. At present, however, there is no evidence that NDH-2 contains another protein in addition to the *ndh* gene product.

If NDH-2 is indeed a single-subunit NADH:ubiquinone reductase, it may serve as an interesting model system for studies on ubiquinone binding sites, and on protein interactions with biological membranes. In order to gain insight into the structure and function of NDH-2, one must develop a purification method that yields highly active enzyme in considerable amounts. To this end, we have expressed the *ndh*

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Abbreviations: DB, decylbenzoquinone (decylubiquinone); DCIP, dichlorophenolindophenol; Idebenone, hydroxidecyl benzoquinone; IPTG, isopropylthio-β-D-galactoside; NDH-1, H^+-translocating NADH:quinone oxidoreductase (Complex I); NDH-2, the 'alternative', non-H^+-pumping NADH:quinone reductase; PMSF, phenylmethylsulfonyl fluoride; Q_1, ubiquinone-1; Q_2, ubiquinone-2

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gene in *E. coli* as a His-tagged protein and purified the enzyme in the presence of mild detergent. The effect of the induced expression on the morphology of the cells was examined, and the activity of the purified enzyme was analyzed. The results are described and discussed below.

2. Materials and methods

2.1. Materials

Ubiquinone-1 (Q_1) was a generous gift from Hofmann-La Roche, Switzerland; ubiquinone-2 (Q_2) was a generous gift from Eisai, Japan; hydroxidecyl benzoquinone (Idebenone) was a generous gift from Dr. Degli Esposti, Monash University; decylubiquinone (DB) and deamino-NADH were purchased from Sigma. NADH was purchased from Boehringer, dodecyl sucrose (DS) was from Novabiochem, and dodecyl maltoside (DM) from Anatrace.

2.2. Cloning and expression of His-tagged NDH-2

The *ndh* gene [3] was amplified from genomic DNA, using a forward primer that contained a *Bgl*II site downstream the first codon of the gene, and a reverse primer that contained an *Xho*I site immediately downstream the stop codon. The amplified DNA was digested by *Bgl*II and *Xho*I and ligated into the expression plasmid pQE-31 (Qiagen) that was digested with *Bam*HI and *Sal*I. The resultant cloned *ndh* encodes NDH-2 with an N-terminal extension of 13 amino acids, MRGSHHHHHHTDL. The plasmid was named pHN3, and it was used to transformed *E. coli* XL1 cells (Stratagene). Cells carrying pHN3 were named HN3-XL1. Induced expression of His-tagged NDH-2 was done in LB medium (1 l in a 2.5 l bottle) that was inoculated with 20 ml of overnight culture of HN3-XL1, and placed on a shaker (200 rpm) at 37°C. About 3 h after inoculation, the cells reached the early log phase and NDH-2 expression was initiated by the addition of isopropylthio-β-D-galactoside (IPTG, 0.5 mM). The cells were harvested 2 h after induction, washed with a solution containing 500 mM KCl, 10 mM Tris–Cl, pH 7.5, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and stored at −20°C.

2.3. Membrane isolation

Cells were thawed on ice and suspended in 2.5 mM EDTA, 0.2 mM PMSF, 200 mM Tris–Cl, pH 8.0, to about 1 g per 10 ml. Lysozyme was added and the mixture was stirred for 1 h on ice. The suspension was centrifuged at $120\,000 \times g$ for 60 min. The pellet was subjected to osmotic shock by suspension in 10 mM potassium phosphate, 2 mM EDTA, 0.2 mM PMSF, pH 8.0, and vigorous mixing by a tissue homogenizer (Ultra Turrax), followed by a second ultracentrifugation. The membrane fraction was homogenized in 10 mM potassium phosphate, 2 mM EDTA, 0.2 mM PMSF, pH 8.0, and stored at −80°C.

2.4. Affinity purification of His-tagged NDH-2

Membranes were thawed on ice and extracted by the addition of detergent, either DS or DM, to a final concentration of 0.2% (w/v), and a detergent to a protein weight ratio of 0.1. NaCl was added to a final concentration of 500 mM, and the suspension was mixed well, on ice, for 30 min followed by 60 min centrifugation at $120\,000 \times g$. His-tagged NDH-2 was purified from the detergent extract of the membranes by metal-chelating chromatography using Ni-NTA resin (Qiagen) at 4°C. The column (1 ml per 0.5 mg protein in the supernatant) was pre-equilibrated with buffer containing 50 mM potassium phosphate, pH 7.5, 500 mM NaCl, 0.2% (w/v) DS or DM, and 20 mM imidazole (buffer A). After loading the sample, the column was washed with two column volumes of buffer A, followed by a wash of a similar volume of 50 mM Tris–Cl, pH 7.5, 0.2% (w/v) DS or DM (buffer B) and then with one column volume of 50 mM Tris–Cl, pH 7.5, 0.2% (w/v) DS or DM, and 10 mM $CaCl_2$ (buffer C). After this 'calcium wash', the column was washed again with three volumes of buffer B and two column volumes of buffer A. The bound NDH-2 was then eluted using an imidazole gradient in the presence of 500 mM NaCl and 50 mM potassium phosphate, pH 7.5. His-tagged NDH-2 eluted from the column at about 200 mM imidazole, and was concentrated to about 2 mg/ml using either a 'centricon' or a 'centriprep' system (Amicon) with a pore size of 50 kDa. Glycerol was added to a final concentration of 20% (v/v), and the enzyme was stored at −80°C.

2.5. Activity assays

The enzymatic activity of purified His-tagged NDH-2 was measured spectrophotometrically. The purified enzyme was thawed and diluted to 40 μg protein/ml in a solution containing 12 mg/ml sonicated phospholipid (6 mg/ml soybean and 6 mg/ml *E. coli* phospholipid), 150 mM NaCl, 50 mM potassium phosphate, pH 7.5. The diluted enzyme was placed on ice for 10 min followed by a 10-fold dilution (second dilution) using the same phospholipid-containing solution, and kept on ice for 20 min (and no longer than 3 h) before the assays that were carried out at 30°C in a final volume of 1 ml. The enzyme (10 μl from the second dilution mixture) was added into the buffer (50 mM potassium phosphate, pH 7.5, 30°C), followed by the addition of acceptor to the desired concentration. After 3 min incubation at 30°C, the reaction was started by the addition of NADH. The substrate concentration was measured spectrophotometrically using the following extinction coefficients (mM^{-1}, cm^{-1}): NADH, 6.2 at 340 nm; Q_1 and Q_2, 13.7 at 275 nm; DB, 14.0 at 278 nm; duroquinone, 19.2 at 270 nm; ferricyanide, 1.0 at 420 nm; dichlorophenolindophenol (DCIP), 22.0 at 600 nm.

3. Results and discussion

NDH-2 of *E. coli* is an interesting enzyme, particularly for studies on ubiquinone reduction sites and the interactions of proteins with biological membranes. The enzyme was first purified and partially characterized about 20 years ago, and found to contain FAD but no Fe–S clusters or hemes [1–3]. Unfortunately, the activity of the enzyme, as originally purified, was poor, thus severely limiting further studies on its structure and function. In order to overcome this problem, we have designed a new purification protocol, taking advantage of tools that were not available at the time of the previous purification, e.g. the addition of an affinity tag to the N-terminus of the protein.

A glycine-rich sequence motif is present very near the N-terminus of NDH-2 [3]. This segment is probably involved in binding the ADP part of the FAD cofactor ([7] and see below) and its N-terminal location might render the bound flavin more susceptible to dissociation during purification, as reportedly occurred in the past [1,2]. Due to the proximity of the FAD binding site to the N-terminus, the addition of a 13 amino acid extension at this end of the protein might interfere with FAD insertion into the enzyme. However, if assembly was unaffected, the additional stretch of amino acids could contribute positively to the stability of the enzyme during purification. The high specific activity of the purified His-tagged NDH-2 (Table 1) demonstrates that the N-terminal extension did not prevent correct FAD binding to the enzyme.

The expression of His-tagged NDH-2 was induced by the addition of IPTG to a culture of HN3-XL1 cells at the early logarithmic growth phase. The induced cells had a pale green color that was not seen in the control cells that harbor the pQE-31 plasmid without any insert. The membranes fraction of induced HN3-XL1 cells, i.e. the top layer of the pellet after ultracentrifugation of lysozyme-treated and osmotically disrupted cells, had a distinct yellowish color. The greenish color of the harvested cells and the yellowish color of the membrane, as well as the absence of a large fraction of inclusion bodies, suggested that the expression of His-tagged NDH-2 was both high and efficient.

Detergents play a major role in purification of integral membrane proteins. In the previous NDH-2 purification, the membranes were solubilized using high concentrations of cholate (3% in the presence of 1 M KCl). That solubilization was followed by chromatography on hydroxylapatite in the

presence of merely 0.1% cholate at very low ionic strength [1]. In the present work, DM (or DS, a practically identical detergent as far as this purification is concerned) was used. In addition, we took care not to lower the detergent concentration below its critical micellar concentration (cmc), as might have happened during the chromatography step in the previous works [1,2].

His-tagged NDH-2 was purified by metal-chelating chromatography in the presence of detergent. Bound enzyme was thoroughly washed and then eluted by an imidazole gradient in the presence of high ionic strength (Fig. 1). The fractions exhibiting high NADH:Q_1 reductase activity were immediately pooled, concentrated and, after addition of glycerol, stored at −80°. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the pooled fractions shows a single band, the M_r of which is, as expected, about 47 kDa (Fig. 1).

The NADH:Q_1 reductase activity of the purified NDH-2, after dilution and incubation in the presence of sonicated phospholipid, was very high (Table 1). For comparison, the activity of the previously purified enzyme was about 0.5 mmol NADH oxidized per min per mg protein, and in the present case, it was about 100 mmol NADH per min per mg protein (Table 1). The reasons for this dramatic difference in specific activity are not fully clear. It may partly be due to the differences in either the detergent used during solubilization and purification, the chromatography step, or the storage of the purified enzyme. We have noticed that activity declines rapidly if the NDH-2 is not concentrated soon after chromatography and stored at −80°C in the presence of glycerol and high ionic strength. In addition, part of the difference in specific activity may be due to improvements in the assay conditions, and in the preparation of the enzyme for the assay, i.e. the dilution and pre-incubation in the presence of phospholipid. In any case, the high activity of the purified His-tagged NDH-2 indicates that the *ndh* gene product is the sole protein component of this enzyme.

Table 1
Kinetic parameters for the activity of NDH-2 in the presence of different substrates

	K_m (apparent, μM)	V_{max} (mmol NADH/mg protein/min)
NADH[a] (Q_1 reduction)	34	106
Q_1[b]	5.9	106
Q_2[b]	2.3	190
DB[b]	18.7	191
Idebenone[b]	1.8	107
Duroquinone[b]	n.d.	5.5
Ferricyanide[b]	n.d.	6.5
DCIP[b]	20	19.5

[a]In measurements of the K_m and V_{max} for NADH, the acceptor was 45 μM Q_1.
[b]The NADH concentration in the assays for different electron acceptors was 0.15 mM.

The K_m and V_{max} values for the reaction of NDH-2 with NADH were determined (Table 1). The acceptor in these experiments was Q_1, and the apparent K_m for NADH was about half the value determined previously [1]. No turnover was detected when deamino-NADH was the electron donor. The latter is in full agreement with the substrate specificity of the unmodified enzyme in the native membrane [4], and these results suggest that the NADH binding site of the His-tagged NDH-2 is very similar to the donor binding site in native NDH-2.

In studies on Complex I and subcomplexes that were isolated from it, we have found that the turnover rate of the intact enzyme in the presence of DB was higher than with Q_1. In contrast, subcomplexes Iλ and IS that lack several of

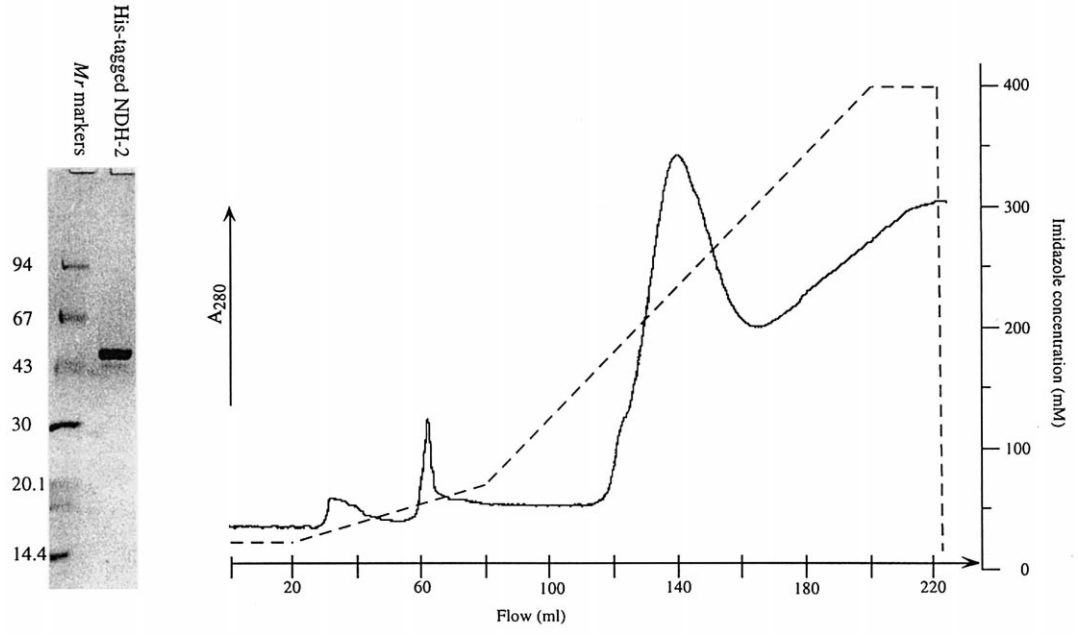

Fig. 1. Purification of His-tagged NDH-2 by metal-chelating chromatography. Solubilized NDH-2 was loaded onto the column, washed well and eluted using an imidazole gradient in the presence of 500 mM NaCl and 0.1% DM. The chromatogram shown here starts after the washes (see Section 2) and the imidazole gradient is indicated by the dashed line. It may be noted that imidazole contributes to the absorbance at 280 nm. The fraction eluted at about 200 mM imidazole contained the His-tagged NDH-2. An SDS–PAGE analysis (10–20% acrylamide gradient) of a pool of these fractions is shown on the left. 10 μg His-tagged NDH-2 was loaded on the gel and it was stained with Coomassie blue.

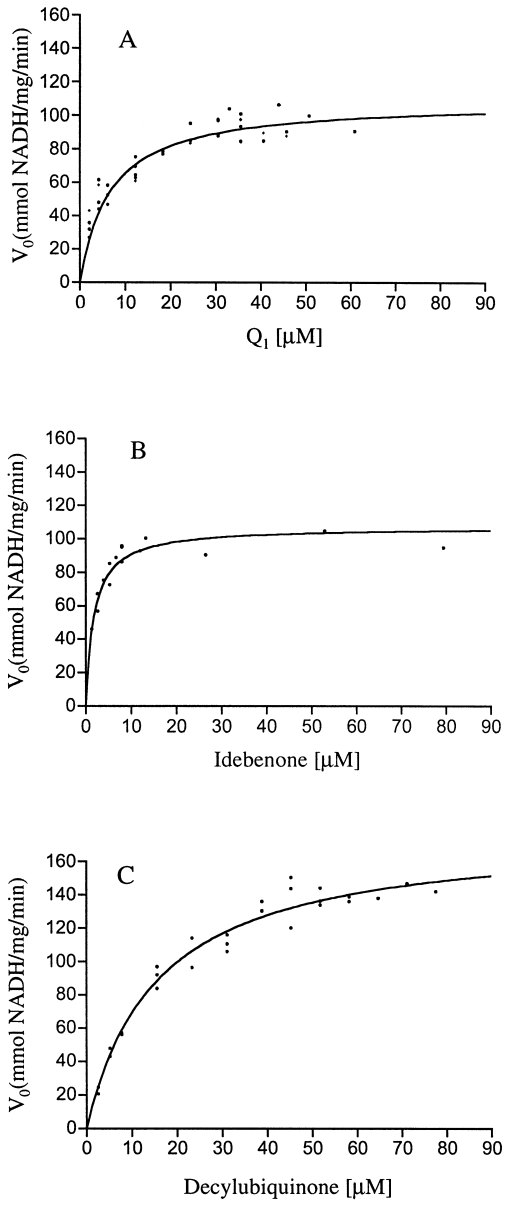

Fig. 2. Kinetic analyses of the NADH:quinone reductase activity of His-tagged NDH-2 in the presence of Q_1, Idebenone and DB. The titrations were carried out after short incubation of the purified enzyme in the presence of sonicated phospholipid (see Section 2). Solid lines represent best fits according to the Michaelis–Menten equation.

the integral membrane subunits of Complex I were almost inactive in the presence of DB, but reduced Q_1 at similar rates to that of intact Complex I [11]. These results may suggest that high NADH:Q_1 reductase activity by itself is not sufficient evidence that the entire integral membrane part of the enzyme is present in the purified NDH-2. Considering that the physiological electron acceptor for NDH-2 in *E. coli* membranes is ubiquinone-8, it was interesting to measure the activity of the purified enzyme in the presence of more hydrophobic acceptors than Q_1. Q_2 is an inhibitor of the mitochondrial Complex I, but with bacterial NDH-1 from *P. denitrificans*, it exhibits similar properties to Q_1 [12]. The V_{max} of the latter bacterial NDH-1 in the presence of DB is very similar to that in the presence of Q_1 or Q_2, but the K_m for DB is higher than for the quinone analogues that side chain of which is isoprenoid [12]. In light of these results with mitochondrial Complex I, some of its subcomplexes, and *P. denitrificans* NDH-1, it was interesting to examine the activity of NDH-2 in the presence of ubiquinone analogues that are more hydrophobic than Q_1.

The activity of purified NDH-2 was examined in the presence of several electron acceptors. In all cases where a significant turnover rate was detected, the steady-state kinetics of the reaction were analyzed, as shown for Q_1, Idebenone and DB (Fig. 2). The K_m and V_{max} values that were derived from such titrations are summarized in Table 1. One of the clear outcomes of these experiments is the high V_{max} of NDH-2 in the presence of all the hydrophobic quinone analogues that were used in this work, namely Q_1, Q_2, DB and Idebenone. In particular, the high V_{max}/K_m ratio for Q_2 (Table 1) indicates that this is a very good electron acceptor for NDH-2 of *E. coli*.

Idebenone is an inhibitor of mitochondrial Complex I [13], and we have initially examined its potency as an inhibitor of the Q_1 reductase activity of NDH-2. It soon became clear, however, that Idebenone is a better acceptor for NDH-2 than Q_1 (Fig. 2 and Table 1). It may be noted that Idebenone also functions as an electron acceptor in the case of the mitochondrial succinate:ubiquinone reductase (Complex II) [13].

In addition to hydrophobic ubiquinone analogues, we have also examined the kinetic properties of several water-soluble acceptors. The turnover rate of NDH-2 with the water-soluble quinone analogue duroquinone was very low, as was the activity in the presence of the single electron acceptor ferricyanide (Table 1). DCIP is a slightly better acceptor than either duroquinone or ferricyanide, but in comparison to the hydrophobic quinones that were examined in this study, it is also a poor acceptor for NDH-2 (Table 1). It thus appears that the quinone reduction site of NDH-2 is not easily accessible for water-soluble acceptors. These results also demonstrate that NDH-2 does not exhibit a typical diaphorase activity. This does not exclude, however, evolutionary relationships between NDH-2 and diaphorases such as lipoamide dehydrogenase (see below).

The homology between *E. coli* NDH-2 and similar enzymes in fungi has been analyzed elsewhere, and a detailed alignment of the putative FAD and NADH binding segments in NDH-2 can be found in [7]. It has recently been reported that the p64 protein of *N. crassa* is homologous to the NDH-2-like enzymes of *S. cerevisiae*, with the exception that it contains an additional internal segment of about 85 amino acids that includes a Ca binding motif [8]. Such a domain is not present in NDH-2 of *E. coli* (not shown).

Alignments of NDH-2-like enzymes from different organisms may indicate which amino acids in these proteins are conserved and thus might play a role in their structure or function [7,8]. Nevertheless, until now, knowledge about the structure and function of NDH-2-like enzyme has been scarce, hence the information that can be derived from such alignments is limited. Due to this, we have tried to identify either flavoproteins or NAD(P)H oxidoreductases among the proteins of low homology to NDH-2 in a regular GenBank search, and examine them carefully. Such a search strategy revealed that NDH-2 may be related to lipoamide dehydrogenase (Fig. 3) and other members of the FAD-dependent

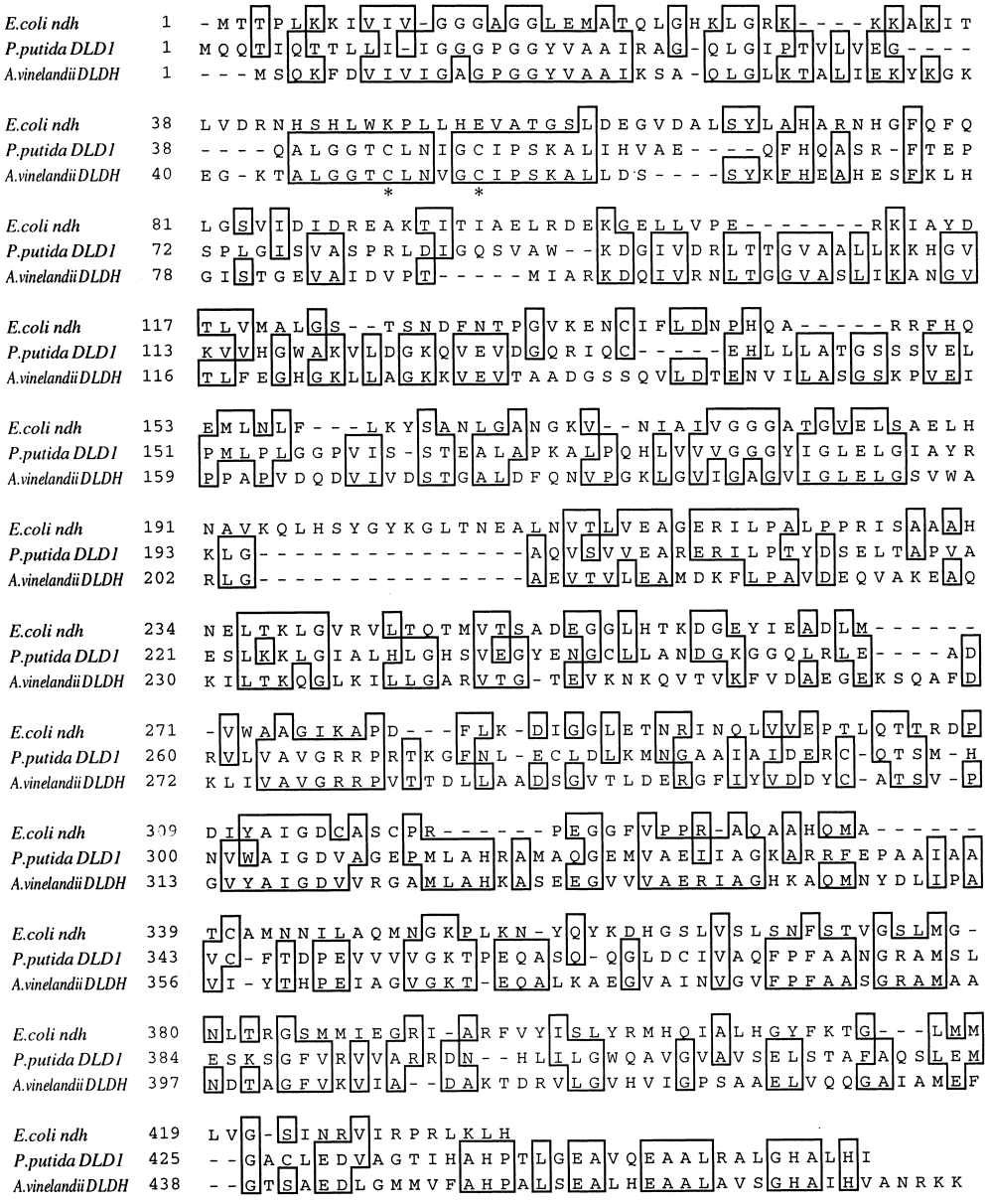

Fig. 3. Sequence alignments of NDH-2 of *E. coli* (*ndh*) with lipoamide dehydrogenase from *P. putida* (DLD1) and *A. vinelandii* (DLDH). Accession numbers for the genes used in this figure are: *ndh*, P00393; *DLD1*, P09063; *DLDH*, P18925. The two highly conserved cysteines in members of the FAD-dependent NAD(P)H-(disulfide)-oxidoredeductases protein family are marked by asterisks.

NAD(P)H-(disulfide)-oxidoredeductase protein family. The members of this family are water-soluble FAD-containing enzymes that have a pair of highly conserved cysteine residues that form a disulfide bridge that may be reduced by NAD(P)H [14,15].

An alignment of NDH-2 with lipoamide dehydrogenase from two bacteria, *Pseudomonas putida* and *Azotobacter vinelandii*, is shown in Fig. 3. The three-dimensional structures of these two lipoamide dehydrogenases have been solved by X-ray crystallography [16,17], and thus may be useful in future studies on NDH-2. The homology between NDH-2 and lipoamide dehydrogenases is high at the protein regions that are involved in binding FAD and NADH, e.g. the first 30 amino acids, and the segments between amino acids 175–186, 210–240 and 297–317 (*ndh* sequence) (Fig. 3). A interesting exception to the similarity between NDH-2 and lipoamide dehydrogenase is the absence of the conserved cysteine pair in NDH-2. These cysteines (marked by asterisks in Fig. 3) are one of the major hallmarks of the FAD-dependent NAD(P)H-(disulfide)-oxidoredeductases protein family. Nevertheless, the similarity between NDH-2 and members of this protein family may be significant since in addition to sequence homology it also includes non-covalently bound FAD and NAD(P)$^+$ or NAD(P)H as a substrate of the enzyme. It may thus be suggested that NDH-2 shares a distant common ancestor with members of the FAD-dependent NAD(P)H-(disulfide)-oxidoredeductases protein family.

The homology between NDH-2 and lipoamide dehydrogenase, a water-soluble enzyme, raises again the question how NDH-2 binds to biological membranes. We have not yet

found the answer to this question. Nevertheless, it is hoped that the availability of a large amount of pure and active NDH-2 (Figs. 1, 2 and Table 1) will be instrumental in addressing this as well as other questions about the structure and catalytic mechanism of NDH-2.

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