Expression and Characterization of the Flavoprotein Subcomplex Composed of 50-kDa (NQO1) and 25-kDa (NQO2) Subunits of the Proton-translocating NADH-Quinone Oxidoreductase of Paracoccus denitrificans*

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Takahiro Yanó, Vladimir D. Sled’, Tomoko Ohnishi, and Takao Yagi†‡

From the †Division of Biochemistry, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037 and the ‡Johnson Research Foundation, Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

This study reports the expression of the flavoprotein (FP) subcomplex of the proton-translocating NADH-quinone oxidoreductase (NDH-1) from Paracoccus denitrificans, which is composed of the NQO1 (50 kDa) and the NQO2 (25 kDa) subunits. The two subunits are co-expressed in Escherichia coli using a double expression plasmid system. The expressed subunits form a water-soluble heterodimer complex with 1:1 stoichiometry. The expressed complex contained one [2Fe–2S] cluster but almost no FMN or [4Fe–4S] cluster. The two latter prosthetic groups could be partially reconstituted with FMN, Na₂S, and (NH₄)₂Fe(SO₄)₂ in vitro under anaerobic conditions. The reconstituted FP subcomplex showed EPR signals from two distinct species of iron-sulfur cluster. One resonance transition originates from a [2Fe–2S] cluster with g values of gₓᵧᵧ = 1.92, 1.95, and 2.00 and slow spin relaxation, which was tentatively assigned to the cluster N1a. These EPR properties are very similar to those reported for the NQO2 subunit expressed alone (Yano, T., Sled’, V. D., Ohnishi, T., and Yagi, T. (1994) Biochemistry 33, 494–499). The other originates from a [4Fe–4S] cluster with g values of gₓᵧᵧ = 1.87, 1.94, and 2.04 and fast relaxing behavior, which are reminiscent of the cluster N3 in the membrane bound enzyme complex. After reconstitution with FMN, the FP subcomplex catalyzed electron transfer from NADH and from deaminoo-NADH to a variety of electron acceptors. The enzymatic properties of the FP subcomplex, reconstituted with FMN and iron-sulfur, correspond to those of the isolated P. denitrificans NADH-dehydrogenase complex.

The respiratory chain of aerobically grown Paracoccus denitrificans consists of NADH-quinone oxidoreductase (designated as Complex I or NDH-1),† succinate-quinone oxidoreductase, quinol-cytochrome c oxidoreductase, and cytochrome c oxidase (1, 2), and thus resembles the mitochondrial respiratory chain. Complex I from mammalian mitochondria is composed of at least 41 unlike subunits (3). The simplest bacterial NDH-1 described thus far consists of 14 subunits and can be regarded as a minimal functional unit required to drive the oxidation-reduction reaction coupled to the proton-translocating activity of coupling site 1 (4, 5). As has been revealed from the EPR analysis of membrane preparations, the P. denitrificans NDH-1 contains at least 5 EPR-detectable iron-sulfur clusters (6) with EPR and redox properties very similar to those of the bovine enzyme (7, 8). The five EPR visible iron-sulfur clusters are designated as N1a, N1b, N2, N3, and N4. The clusters N1a and N1b are binuclear and the clusters N2, N3, and N4 are tetranuclear.

The NADH-quinone oxidoreductase has been isolated and characterized (9). The gene cluster encoding the P. denitrificans NDH-1 enzyme has been cloned and sequenced (10–14). This gene cluster is composed of 14 structural genes and 6 unidentified reading frames which are designated as NQO1–14 and URF1–6, respectively (15). It was demonstrated that the P. denitrificans genes encoding NDH-1 show striking sequence identities to their mammalian counterparts. The P. denitrificans and the bovine Complex I polypeptides show immunoreactivity (9). Furthermore, enzyme activity and sensitivity to specific inhibitors are very similar in the two complexes. Therefore, P. denitrificans NDH-1 is a suitable model for further understanding of the mitochondrial Complex I.

To understand the electron transfer mechanism of the NDH-1 enzyme complex, it is important to locate and identify all redox components at the molecular level. Sequence analysis allows us to predict that NQO1, NQO2, NQO3, NQO9, and possibly NQO6, are iron-sulfur cluster ligating subunits and that the NQO1 subunit contains a FMN-binding site (16, 17). In addition, it has been shown that the NQO1 subunit bears the NADH-binding site (18). These subunits seem to be all located in the peripheral part of the enzyme (5, 19).

In previous work we have expressed single P. denitrificans NDH-1 subunits in Escherichia coli and characterized their iron-sulfur cluster(s). These studies showed that the NQO2 subunit contains a [2Fe–2S] cluster which is most likely N1a (20, 21). Furthermore, by expressing and characterizing NQO2 altered by site-directed mutagenesis, we have shown that the [2Fe–2S] cluster is coordinated by four conserved cysteine residues; Complex I, mitochondrial proton-translocating NADH-ubiquinone oxidoreductase; FP, flavoprotein; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.
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ides (C96, C101, C137, and C141) (22). Recently, the expression and characterization of the NQO3 (66 kDa) subunit (equivalent to the bovine 75-kDa subunit) was carried out. The expressed NQO3 subunit contained a [2Fe-2S] cluster (N1b), a [4Fe-4S] cluster (N4), and possibly an additional iron-sulfur cluster (N23). These experiments have demonstrated that the strategy of expressing individual subunits is useful for investigating the location of individual redox components and refines previous studies in which mitochondrial Complex I or bacterial NDH-1 were split into different subfractions (5, 19, 24, 25). We have attempted to express the NQO1 subunit in E. coli to characterize its properties. When the NQO1 subunit was solubly expressed in E. coli, the subunit was produced as aggregated form (inclusion body) under any conditions used. Any attempts to refold the NQO1 subunit have not been successful so far. Therefore, we attempted to co-express the NQO1 and NQO2 subunits in E. coli. The co-expression strategy may be an alternative way to express intact subunits on the basis of the following facts. 1) Bovine flavoprotein (FP) subcomplex is composed of the 51-kDa subunit (homologue of the NQO1 subunit), 24-kDa subunit (homologue of the NQO2 subunit), and 9-kDa subunit (the P. denitrificans NDH-1 lacks a 9-kDa subunit counterpart), and is water-soluble (26, 27). (2) The FP subcomplex is homologous to the α-subunit of NAD⁺-reducing hydrogenase of Alcaligenes eutrophus (10, 28–30).

In this study the two FP subunits, NQO1 and NQO2, were co-expressed as soluble proteins in E. coli, forming a complex with 1:1 stoichiometry in situ. The isolated subcomplex did not contain FMN or any [4Fe-4S] cluster, but did contain a [2Fe-2S] cluster (N1a) which had EPR properties almost identical to those previously reported for the NQO2 subunit expressed alone. The FMN and the [4Fe-4S] cluster could be successfully reconstituted in vitro. EPR studies of the reconstituted FP subcomplex revealed that the subcomplex exhibits two distinct EPR signals: one arises from the [2Fe-2S] cluster (N1a), whereas the other, rapidly relaxing, signal originates from a [4Fe-4S] cluster. Furthermore, the reconstitution of FMN and [4Fe-4S] cluster into the subcomplex restored NADH-oxidizing activity with a variety of soluble electron acceptors to the level of that of the isolated P. denitrificans NADH dehydrogenase complex. The molecular and enzymatic properties of the expressed and reconstituted FP subcomplex as well as the location and assignment of the cofactors are discussed.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors for NQO1 and NQO2 Subunits—A 1.5-kilobase pair BamHI fragment containing the full-length NQO1 gene was digested from pX-T1 (10), blunt-ended with a DNA polymerase Kliewon fragment, and then ligated into Smal-digested cloning vector pTZ19U. The resulting vector was designated pTZ(NQO1). In order to generate a NdeI restriction site at the translation initiation site, an oligonucleotide was synthesized: 5′-CAAGAGTAACGATATTGCTGAACGCA-3′. The underlined bases were altered from P. denitrificans DNA, and italic bases indicate the NdeI site. Site-specific mutagenesis was carried out by using the Bio-Rad in vitro mutagenesis kit, which is based on the method of Kunkel et al. (31). The mutated plasmids were isolated and checked by restriction enzyme analysis. The mutated plasmid thus obtained was designated pTZ(NQO1). pTZ(NQO1) plasmid was digested with BglII/ EcoRI. The BglII/EcoRI fragments, which contain the NQO1 gene under the T7 transcription/translation region, were ligated into the BamHI/EcoRI site of pKT230. The resulting expression vectors were designated pET11a(NQO1) and pET16b(NQO1). The former is a nonfused NQO1 subunit and the latter produces a fused NQO1 subunit.

Expression of FP Subcomplex in E. coli—Competent E. coli strain BL21(DE3)LyS strain was transformed with both pKT16b(NQO1) and pET11a(NQO1). A well-isolated single colony transformed with both plasmids was retrieved from the 2xYT agar plate containing 100 μg/ml tetracycline and 50 μg/ml of kanamycin, and inoculated into 10 ml of 2xYT medium containing 100 μg/ml ampicillin and 50 μg/ml of kanamycin. Cells were aerobically grown to the stationary phase at 37°C and then transferred into 500 ml of TB medium containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, 100 μg/ml ferric ammonium citrate, 100 μM Na₂S, and 100 μg/ml FMSF, and then allowed to grow at 25°C. When cells were grown to an absorbance of approximately 0.1 at 600 nm, isopropyl-1-thio-D-galactopyranoside was added at final concentration of 0.4 mM, and then the cells were grown for 18 h at 25°C. The cells were harvested by centrifugation at 5,900 × g for 10 min in a Sorvall GSA rotor. The cell pellets were suspended in 50 ml Tris-HCl buffer (pH 8.5) containing 1 mM DTT, 0.1 mM PMSF, and 0.15 mM NaCl to 0.2 g of cells (wet weight) per ml. The cell suspension was immediately frozen in liquid nitrogen and stored at -80°C. Any attempts to thaw the soluble proteins from the membrane fraction. The supernatant was dialyzed against 2.5 liters of 10 mM Tris-HCl buffer (pH 8.5) containing 0.1 mM PMSF and 0.5 M NaCl for 4 h with one buffer exchange. The dialyzed protein solution was loaded on a nickel-nitrate affinity column (3.0 × 5.0 cm) which had been equilibrated with the same buffer for dialysis. The column was washed with 100 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 0.1 mM PMSF and 0.5 mM NaCl followed by 200 ml of the same buffer plus 40 mM imidazole to remove nonspecifically bound proteins. The absorbed target proteins were eluted with 10 ml Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl and 400 mM imidazole. The fractions containing the expressed FP subcomplex were combined and dialyzed against 1 liter of 10 mM Tris-HCl buffer (pH 8.5) containing 5.0 mM DTT for 6 h. After concentration with Amicon mini Prep-30 to appropriate concentration, the FP subcomplex was used for further experiments immediately.

In Vitro Reconstitution of FMN and Iron-Sulfur Cluster—Standard procedure for reconstitution of FMN and iron-sulfur clusters are as follows unless otherwise indicated: 1.0 ml of purified FP subcomplex solution (1.0–5.0 mg/ml) in 50 mM Tris-HCl buffer (pH 8.5) buffer containing 5.0 mM DTT were degassed and purged with oxygen-free argon. Under anaerobic conditions we added: 5 μl of β-mercaptoethanol, 10 μl of 300 mM (NH₄)₂Fe(III)(SO₄)₂, 10 μl of 300 mM Na₂S, and 8 μl of 250 mM FMSF. The suspension was incubated at 4°C in the dark overnight. The reconstituted proteins were centrifuged in a microtube at 14,000 × g for 10 min to remove precipitates which formed during incubation. The clarified supernatant was applied to a Sephadex G-50 gel filtration column (1.0 × 30 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.5) containing 5.0 mM DTT in order to remove excess FMN, iron, and PMSF. The fractions containing the NQO1 subunit were collected and concentrated with Amicon mini Prep-30 to appropriate concentrations and then subjected to several gel filtration analyses immediately.

Gel Filtration Analysis—The purified reconstituted FP subcomplex (1.0 mg) was applied to a gel filtration column (Bio-Rad A-5m, 1.0 × 45 cm), equilibrated with 30 mM Tris-HCl buffer (pH 8.5), containing 5.0 mM DTT. The column was eluted with a flow rate of 2 ml/min. The protein was monitored at 280 nm with a Pharmacia LKB Uvicord S1 monitor. Molecular size standards used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). EPR Measurements—EPR spectra were recorded by a Bruker ESP300 E spectrometer operating at X-band (9.2 GHz). The sample temperature was varied using an Oxford Instrument ESR-9 helium flow cryostat. The magnetic field was calibrated using a strong or weak pitch standard. Spin quantitations were performed under non-power-satu-
rated conditions using 500 μM Cu-EDTA as a standard. 

Enzyme Assay—NADH-K₃(Fe(CN)₆) and deamino-NADH-K₃(Fe(CN)₆) reductase activities were assayed spectrophotometrically at 420 nm in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA at 30 °C. NADH-2,6-dichlorophenolindophenol reductase activity and NADH-ubiquinone-1 reductase were followed at 600 and 340 nm, respectively. Concentrations of substrates are indicated in the figure legends. The extinction coefficients used were Δε₅₄₀nm = 6,220 M⁻¹ cm⁻¹ for NADH, Δε₆₂₀nm = 1,000 M⁻¹ cm⁻¹ for K₃(Fe(CN)₆), and Δε₆₅₀nm = 16,100 M⁻¹ cm⁻¹ for 2,6-dichlorophenolindophenol.

Sequence Analysis—GCCG software programs were used to analyze the amino acid sequence (34). Sequence comparison of the polypeptides were conducted with BESTFIT and PILEUP programs. The FASTA and PROFILESSEARCH programs were used to search the GenBank™/EMBL sequence data bases for proteins having some homology to the polypeptides. Homology search was also carried out by using the BLAST program running at the National Center for Biotechnology Information (35).

Other Analytical Procedures—UV-visible absorption spectra were recorded on an SLM-Aminco DW-2000 spectrophotometer at room temperature. Protein was estimated by the method of Lowry et al. (36) in the presence of 1 mg/ml sodium deoxycholate (37). SDS-polyacrylamide gel electrophoresis was carried out by a modified method of Laemmli (38). Immunoblotting was conducted as described previously (39–41).

The specific antibodies against the NQO1 and NQO2 subunits were raised in rabbits using the purified recombinant proteins. The antibodies were affinity-purified from the antiserum against the NQO1 and NQO2 subunits (38). Immunoblotting was conducted as described previously (39–41). The arrows at the right indicate the expressed proteins. The molecular marker (lane A, lane M) includes myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

RESULTS

Expression of the P. denitrificans FP Subcomplex—Composed of the NQO1 and NQO2 Subunits

In order to co-express the NQO1 and NQO2 subunits in the same E. coli cell, we employed two different types of plasmid vectors: one is a pET plasmid expressing NQO1 and NQO2 genes (50) and the other is a ptk16b plasmid expressing NQO2 (25 kDa). These constructs were subcloned into different plasmid vectors. The NQO1 and NQO2 genes were placed under the T7 promoter and the respective plasmid vectors. These constructs were subsequently transferred into competent E. coli strain BL21 (DE3) pLyS. The two plasmids contain different drug resistance marker genes so that the cells transformed with both plasmids could be selected on 2xYT agar plates containing both ampicillin and kanamycin. Expression of the NQO1 + NQO2 complex in E. coli varied depending on culture media, growth temperature, induction time, and miscellaneous supplements. The optimal conditions for expression are described under “Experimental Procedures.”

To examine whether the NQO1 and NQO2 subunits expressed in trans from different plasmids could form a FP subcomplex in situ, we employed a fusion protein expression strategy where a His-Tag sequence was attached to the N-terminal region of either the NQO1 or the NQO2 subunit. His-fusion proteins have the advantage of easy purification of target proteins using nickel chelation column chromatography. If the two subunits formed a complex in situ, it should be possible to purify the NQO1 + NQO2 subcomplex using this affinity chromatography. We found that when the His-Tag was fused to the N terminus of the NQO1 subunit, the nonfused NQO2 subunit was co-purified with the NQO1 subunit (Fig. 1, lane 2). Immuno blots were taken in the presence of 1 mg/ml sodium deoxycholate. The specific antibodies against the NQO1 and NQO2 subunits were affinity-purified from the antiserum against the NQO1 and NQO2 subunits (38). Immunoblotting was conducted as described previously (39–41). The arrows at the right indicate the expressed NQO1 (50 kDa) (upper) and NQO2 (25 kDa) subunits (lower). The molecular marker (lane A, lane M) includes myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

In Vitro Reconstitution of FMN and Iron-Sulfur Clusters into Subcomplex—The FMN, non-heme iron, and acid-labile sulfide
contents of the purified subcomplex were determined as shown in Table II. The purified subcomplex contains only 0.01–0.05 mol of FMN and approximately 1.8 mol of individual iron and S²⁻ per mole of subcomplex. The subcomplex as isolated showed an absorption spectrum which was very similar to that of the solely expressed NQO2 (Fig. 2, spectrum A) (20). The dithionite reduced subcomplex exclusively exhibited an EPR signal with rhombic symmetry, arising from a [2Fe–2S] cluster, with g values similar to those reported for the expressed NQO2 subunit (data not shown). These results show that the purified subcomplex contained only one [2Fe–2S] cluster with neither FMN nor [4Fe–4S] cluster. Moreover, almost the same EPR results were obtained with crude preparations (data not shown), suggesting that little incorporation of FMN and [4Fe–4S] cluster occurred in situ. The contents of cofactors could not be improved by changing expression conditions (e.g. by supplement of riboflavin or FMN into culture medium).

Therefore, we attempted to reconstitute FMN and iron-sulfur clusters in vitro. The standard reconstitution procedures were described under “Experimental Procedures.” When iron and S²⁻ but no FMN were added in the reaction mixture, both non-heme iron and acid-labile sulfide contents increased to 4.1–4.2 mol/mol (Table II). The reconstituted subcomplex showed broad absorption peaks in the visible region which were due to the iron-sulfur clusters (Fig. 2, spectrum B). EPR analysis suggested that the reconstituted iron-sulfur cluster was a [4Fe–4S] cluster (data not shown). This demonstrates that an iron-sulfur cluster was reconstituted in vitro. The [4Fe–4S] cluster-reconstituted subcomplex was unable to oxidize NADH (Table II). When only FMN was added to the reconstitution mixture, FMN was incorporated into the subcomplex up to 0.25 mol/mol whereas the iron-sulfur cluster contents were unaltered (Table II). The subcomplex reconstituted with only FMN showed an absorption spectrum in which the absorbance characteristic for the FMN moiety could be seen (Fig. 2, spectrum C). Incorporation of FMN into the subcomplex partially restored the enzymatic activity (Table II). These results clearly indicated that the [4Fe–4S] cluster and the FMN, to some extent can be incorporated independently and that the FMN molecule is an essential component for the NADH-oxidizing activity of the subcomplex. When the reconstitution was performed in the presence of FMN, iron, and S²⁻, both FMN and iron-sulfur clusters were incorporated into the subcomplex (Table II). This FMN + FeS cluster-reconstituted subcomplex (referred to as the FP subcomplex) showed an absorption spectrum in which the absorption peaks attributable to FMN and iron-sulfur clusters could be seen (Fig. 2, spectrum D). Fig. 3 shows the absorption spectra of the oxidized, NADH-reduced, and dithionite-reduced forms of the reconstituted FP subcomplex. Upon addition of NADH, the absorbance was slightly diminished, similar to that in the isolated NDH-1 complex. The difference spectra, oxidized form minus NADH-reduced form, showed that the spectral changes could be attributed to the reduction of FMN (data not shown). As described below, none of the iron-sulfur clusters were reduced by NADH. Upon addition of dithionite, the absorbance was bleached in the entire visible region, indicating that all redox components in the reconstituted FP subcomplex, FMN, [2Fe–2S], and [4Fe–4S] clusters were reduced. In the reconstituted FP subcomplex, the NADH-oxidizing activity with several artificial electron acceptors was restored. Turnover number (FMN basis) of the reconstituted FP subcomplex was approximately 2-fold higher than that of the subcomplex reconstituted with only FMN (Table II). This suggests that the presence of the [4Fe–4S] cluster is an important factor for effective flavination and for the complete resta-
ration of NADH-oxidizing activity. It may be suggested that the [4Fe-4S] cluster affects the structure or interacts with the FMN- and/or the NADH-binding site. It should also be noted that flavination of the subcomplex seems to be FMN specific. FAD was not incorporated under any conditions we tested (data not shown). Moreover, incorporation of FMN and the iron-sulfur cluster into the expressed subcomplex required a prolonged incubation time. In case of other flavoproteins, FMN incorporation into apoproteins has been reported to take place within a few minutes of incubation (47).

EPR Measurements of the Reconstituted FP Subcomplex—In order to characterize the iron-sulfur clusters in the reconstituted FP subcomplex, we performed EPR spectroscopic analysis (Fig. 4). EPR signals from the reconstituted FP subcomplex reduced with dithionite revealed the presence of two distinct iron-sulfur clusters. One is a [2Fe-2S] cluster with EPR signals of rhombic symmetry and g values of $g_{x,y,z} = 1.92, 1.95,$ and 2.00, which are almost identical to those of the [2Fe-2S] cluster in the expressed NQO2 subunit (20) (Table III and Fig. 5). The other arises from a rapidly relaxing species with g values of $g_{x,y,z} = 1.87, 1.94,$ and 2.04, indicating that this cluster is a [4Fe-4S] cluster, most likely N3. The stoichiometry of the two clusters was [2Fe-2S]:[4Fe-4S] $= 1:0.25$ as determined by spin quantitation. As mentioned before, neither the [2Fe-2S] nor the [4Fe-4S] cluster was reduced by NADH. As discussed in previous reports concerning NQO2 and NQO3 subunit expression (20, 23), it seems likely that the midpoint redox potentials of the iron-sulfur clusters are generally lowered when subunits are expressed as single, soluble subunits (data not shown). Furthermore, the phenomenon is analogous to that of the bovine FP subcomplex, where the midpoint redox potentials of the two iron-sulfur clusters were greatly lowered after resolution of Complex I into FP subcomplex using chaotropic reagents (48).

Enzymatic Activity of the Reconstituted FP Subcomplex—As mentioned above, the NADH-oxidizing activity with each of several artificial electron acceptors was restored in the reconstituted FP subcomplex. The reconstituted FP subcomplex specifically oxidized NADH as a substrate but scarcely NADPH. The FP subcomplex could also utilize deamino-NADH (dNADH), which is known to be a specific substrate for the E. coli NDH-1, but not for the NDH-2 (49). This activity was about 50% compared to that with NADH. The enzymatic properties of the reconstituted FP subcomplex are summarized in Table IV. The enzymatic activities discussed were inhibited by NAD$^+$ in a competitive manner ($K_i = 0.85$ mM). These enzymatic properties are very similar to those of the isolated P. denitrificans NADH-dehydrogenase complex (9) and to those of the bovine

Table III

Half-saturation ($P_{1/2}$) parameters of EPR signals of the [2Fe-2S] and [4Fe-4S] clusters of the expressed NQO2 subunit and the expressed FP subcomplex (NQO1 + NQO2)

| Expressed proteins | Cluster | $P_{1/2}$ | 13 K | 57 K |
|--------------------|---------|-----------|------|------|
| NQO2               | N1a (g = 2.00) | 0.18 | 22.1 |
| NQO1 + NQO2        | N1a (g = 2.00) | 0.15 | 34.1 |
|                    | N3 (g = 2.04)  | 37.5 | ND$^{b}$ |

$^{a}$ Yano et al. (20).  
$^{b}$ Not determined.

Fig. 3. Absorption spectra of the expressed-reconstituted FP subcomplex of P. denitrificans NDH-1. The expressed-reconstituted FP subcomplex was diluted in 50 mM Tris-HCl (pH 8.5) containing 5.0 mM DTT and 0.1 mM PMSF to 0.7 mg/ml. The spectra were recorded at room temperature in the oxidized form (as prepared) (line A), in the NADH-reduced form (line B), and in the dithionite-reduced form (line C). The final concentrations of NADH and dithionite were 5 mM and 10 mM, respectively.

Fig. 4. Resolved EPR spectra of the iron-sulfur clusters of the expressed-reconstituted FP subcomplex of P. denitrificans NDH-1. Spectrum of [2Fe-2S] cluster was recorded at microwave power 25.3 $\mu$W (1); in order to resolve the spectrum of [4Fe-4S] cluster, the contribution of saturated binuclear cluster was eliminated by recording the weighted difference between spectra at 100 $\mu$W and 12.7 $\mu$W (2). EPR condition: sample temperature, 13 K; microwave field frequency, 9.449 GHz; modulation amplitude, 1 mT; modulation frequency, 100 kHz; time constant, 0.16 s; scan rate, 0.596 mT/s. Principal g values for the clusters are indicated by arrows.
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Fig. 5. Power saturation behavior of the iron-sulfur clusters in the reconstituted FP subcomplex and the [2Fe–25] cluster in the expressed NQO2 subunit. Panel A, power saturation of the g = 2.00 signal (g component of [2Fe–25] cluster) in isolated NQO2 subunit (1) and in reconstituted FP subcomplex (2). Sample temperature was 57 K. Panel B, power saturation profiles of the g = 2.04 signal (g component of [4Fe–4S] cluster) in reconstituted FP subcomplex (1), and of the g = 2.00 signal (g component of [2Fe–25] cluster) in isolated NQO2 subunit (2) and in reconstituted FP subcomplex (3). Sample temperature was 13 K. Other EPR conditions were as in Fig. 4. Half-saturation parameters were determined according to Blum and Ohnishi (53) and $P_{1/2}$ values obtained are indicated in the figures.

| Reaction        | $v_{max}$ | $K_m$ | $v_{max}$ | $K_m$ |
|-----------------|----------|-------|----------|-------|
|                 | units/s/mg | $\mu$M | units/s/mg | $\mu$M |
| NADH $\rightarrow$ Fe(CN)$_6$ | 29.2 | 8.2 | 31.5 | 358 |
| NADH $\rightarrow$ DCIP | 9.5 | 5.4 | 17.4 | 56.6 |
| NADH $\rightarrow$ Q$_1$ | 22.0 | 18.5 | 20.1 | 100 |
| dNADH $\rightarrow$ Fe(CN)$_6$ | 15.7 | 7.3 | ND$^c$ | ND$^c$ |

$^a$ DCIP, 2,6-dichlorophenolindophenol; $Q_1$, ubiquinone-1.

The present expression study demonstrated that the NQO1 and NQO2 subunits, produced in trans from different plasmids, can form a subcomplex in E. coli. Although the two subunits form a subcomplex in 1:1 stoichiometry, with seemingly native conformation, the incorporation of FMN and [4Fe–4S] cluster does not take place in situ. These data imply that either the P. denitrificans-specific machinery or interaction with other neighboring subunits, such as the NQO3 subunit, may be required for the cofactor incorporation and to form a more stable subcomplex. These issues will be addressed in future studies.

The reconstitution experiments described herein provided some insight into the structural location and function of the cofactors in the FP subcomplex. The subcomplex as isolated contains almost equimolar amounts of the [2Fe–25] cluster and its EPR properties are identical to those of the previously studied NQO2 subunit by itself (20), which was assigned as cluster N1a (23). The NQO2 subunit does not contain any additional conserved cysteine residues except for the 4 cysteines that were shown to ligate the [2Fe–25] cluster in our previous study (22). Thus, the reconstituted [4Fe–4S] cluster most likely resides in the NQO1 subunit. Since the NQO1 subunit contains a conserved sequence motif for [4Fe–4S] cluster ligation, C$^{347}$XXC$^{350}$XXC$^{353}$, C$^{393}$ (P. denitrificans numbering), the [4Fe–4S] cluster is probably coordinated by these cysteine residues. Considering the EPR properties of the reconstituted [4Fe–4S] cluster, it seems to correspond to cluster N3 in P. denitrificans NDH-1 (6). Our results from the present study provided experimental evidence that the FMN molecule is an indispensable component for NADH-oxidizing activity, suggesting that the FMN molecule is the primary electron acceptor from NADH. Nevertheless, we have no experimental evidence for the exact location of the FMN-binding site. Either FMN is embedded within the NQO1 subunit or both the NQO1 and the NQO2 subunits contribute to the FMN-binding site.

Since flavin could be incorporated into the expressed subcomplex in vitro, we will attempt to employ direct labeling techniques with FMN analogues as has been done with other flavoproteins (47). Identification of amino acid residues in the binding pocket by labeling experiments should be followed by site-directed mutagenesis in order to probe the specific functional role of the targeted residues. These experiments will help us to further understand the molecular mechanism of the NADH dehydrogenase section of NDH-1 enzyme complex.

Mitochondrial and bacterial NADH-ubiquinone oxidoreductases consist of two distinct parts, one peripheral arm protruding into the matrix or cytoplasm, and one hydrophobic arm spanning the membrane. Our recent immunological studies on subunit location within the P. denitrificans NDH-1 enzyme complex using specific antibodies against each subunit have suggested that the NQO1-6 and 9 subunits constitute the peripheral part of the enzyme complex. None of these subunits seem to penetrate through the cytoplasmic membrane. A series of expression studies of the putative cofactor ligation subunits of the P. denitrificans NDH-1 have shown that the NQO2 subunit contains cluster N1a (20) and that the NQO3 subunit bears cluster N1b and N4, and possibly an additional iron-sulfur cluster (23). The present study suggests that the NQO1 subunit contains cluster N3 and at least contributes to the FMN-binding site, which agrees with data on the disruption of Nuo51 gene encoding the 51-kDa subunit (NQO1 homologue) of Neurospora crassa Complex I (52). Sequence analysis regarding iron-sulfur cluster ligation sites predicts that only the NQO9 and NQO6 subunits may contain additional iron-sulfur clusters. Thus, all prosthetic groups seem to be located in the peripheral part of the enzyme. A major future challenge is to elucidate how electron transfer through the

**DISCUSSION**

The present expression study demonstrated that the NQO1 and NQO2 subunits, produced in trans from different plasmids, can form a subcomplex in E. coli. Although the two subunits...
peripheral part of NDH-1 is coupled to proton translocation across the membrane.

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