Characterization of the Putative 2×[4Fe-4S]-binding NQO9 Subunit of the Proton-translocating NADH-Quinone Oxido-reductase (NDH-1) of Paracoccus denitrificans

EXPRESSION, RECONSTITUTION, AND EPR CHARACTERIZATION*

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Molecular properties of the NQO9 subunit of Paracoccus denitrificans NDH-1, which is predicted to contain 2×[4Fe-4S] clusters, were investigated using recombination expression techniques and EPR spectroscopy. The full-length form of NQO9 subunit co-expressed with thioredoxin in Escherichia coli at ambient temperature was found to be stable in the cysteine-rich membranes with low amplification. Genetic deletion of relatively hydrophobic and less conserved N-terminal stretches (30 or 40 amino acid residues long) of the NQO9 subunit resulted in the overexpression of the truncated soluble form of the subunit in a high yield in the cytoplasm. The purified soluble form of the NQO9 subunit contained only a small quantity of Fe and S²⁻ (2.0–2.2 mol each per mol of subunit). However, the iron-sulfur content was considerably increased by in vitro reconstitution. The reconstituted NQO9 subunit contained 7.6–7.7 mol each of Fe and S²⁻ per molecule and exhibited optical absorption spectra similar to those of 2×[4Fe-4S] ferredoxins. Two sets of relatively broad axial-type EPR signals with different temperature dependence and power saturation profile were detected in the dithionite-reduced preparations at a low temperature range (8–18 K). Due to a negative shift (<600 mV) of the apparent redox midpoint potential of the iron-sulfur clusters in the soluble form of the truncated NQO9 subunit, the following two possible causes could not be discriminated: (i) two sets of EPR signals arise from two distinct species of tetranuclear iron-sulfur clusters with two intrinsically different spectral parameters g∥ = 2.05, ~1.93, and g⊥ = 2.08, ~1.90, and respective slow (P∥ = 8 milliwatts) and fast (P∥ = 342 milliwatts) spin relaxation; (ii) two clusters exhibit similar intrinsic EPR spectra (g∥ = 2.05, ~1.93) with slow spin relaxation. When both clusters in the same subunit are concomitantly paramagnetic, their spin-spin interactions cause a shift of spectra to g∥ = 2.08, ~1.90, with enhanced spin relaxation. In either case, our EPR data provide the first experimental evidence for the presence of two [4Fe-4S] iron-sulfur clusters in the NQO9 subunit.

The proton-translocating NADH-quinone (Q)₁ oxidoreductase is one of five enzyme complexes in the oxidative phosphorylation system that is located in the inner membrane of mitochondrion and in the cytoplasmic membrane of bacteria. The mitochondrial enzyme is termed complex I, and the bacterial enzyme is called NDH-1. Both enzyme complexes catalyze the Reaction 1.

\[
\text{NADH} + \text{Q} + \text{H}^+ + n\text{H}^+ + \text{NAD}^+ \rightarrow \text{NAD}^+ + \text{QH}_2 + n\text{H}^\text{out} \quad (n = 3–5)
\]

The reduction of Q by NADH is coupled to proton translocation across the membrane generating an electrochemical potential. The number (n) of pumped proton coupled to the electron transfer from NADH to Q has not yet been conclusively determined. Reaction 1 is reversible such that under certain conditions the enzyme complex is capable of catalyzing the reversed reaction (reverse electron flow). It is generally accepted that mitochondrial complex I and bacterial NDH-1 share many molecular and enzymatic properties. Both enzyme complexes are composed of multiple subunits. Mitochondrial complex I is composed of at least 42 dissimilar subunits (1), whereas the bacterial NDH-1 is much simpler (2). Genetic studies of bacterial NDH-1 from Paracoccus denitrificans (3–7), Escherichia coli (8, 9), Rhodobacter capsulatus (10), and Thermus thermophilus HB-8 (11) have revealed that the NDH-1 enzymes are composed of 13–14 subunits, and all of their homologues are present in mitochondrial complex I (Fig. 1) (12). Both complex I and NDH-1 contain a non-covalently bound FMN and a number of iron-sulfur clusters as electron transfer components. Both enzyme complexes are inhibited by a number of inhibitors (rotenone, piericidin A, capsaicin, pyridaben, etc.) in a similar fashion (13–15). These results indicate that bacterial NDH-1 are counterparts of mitochondrial complex I. Little is known about mechanism of action of these enzyme complexes.

In order to clarify the mechanism, it is necessary to identify the number and type of iron-sulfur clusters and their locations. Previously, EPR spectroscopic studies have revealed that mitochondrial complex I and several bacterial NDH-1 contain at least five EPR-detectable clusters (designated N1a, N1b, N2, N3, and N4) (16). N1a and N1b are [2Fe-2S] clusters, and all others are [4Fe-4S] clusters (17). The cluster N2 has pH-dependent Eₘ in the physiological pH range and the highest mid-point redox potential among clusters in the complex I (18). It has been suggested, therefore, that the cluster N2 plays an
important role in electron transfer to quinone and probably in the proton translocation (19–21).

We attempted to individually express the putative cofactor-binding subunits of the *P. denitrificans* NDH-1 to identify the location of the cofactors. Expression study of the flavoprotein subcomplex that is composed of the NQO1 and NQO2 subunits has shown that the FMN molecule, a primary electron acceptor from NADH, seems to be located in the NQO1 or between the NQO1 and NQO2 subunits (22). Tetranuclear cluster N3 is housed in NQO1 subunit. Expression study of the sole NQO2 subunit has provided the supporting evidence that the cluster N1a is located in the NQO2 subunit (23, 24) with a novel binding motif (C\(^{99}X_{\text{5}}C^{101-137}X_{\text{C}}^{141}\)) (25). Location of clusters N1b and N4 have tentatively been assigned to the NQO3 subunit (26). The subunit contains 11 fully conserved cysteine residues among its homologues including one [4Fe-4S] cluster-binding motif. It has also been suggested that the NQO3 subunit contains an additional [4Fe-4S] cluster with a unique spin state (26). *E. coli* and *T. thermophilus* NDH-1 subunits seem to contain an additional [2Fe-2S] cluster (tentatively designated cluster N1c) (11, 27). Consequently, our subunit localization analysis of these redox components has led us to propose that the iron-sulfur cluster N2 may be associated to propose that the iron-sulfur cluster N2 may be associated to the proton translocation (19–21).

In the present study, the NQO9 subunit of the *P. denitrificans* NDH-1 was characterized by homologous expression techniques and EPR spectroscopy. When relatively hydrophobic and less conserved stretches were removed from its N and C termini, the truncated forms of the subunits could be expressed as soluble proteins in the cytoplasm of *E. coli*. Although the purified subunit contained substoichiometric amounts of iron-sulfur clusters, they were chemically reconstituted into the subunit. Chemical and physicochemical analyses of the reconstituted NQO9 subunit demonstrated that the subunit contains \(2 \times [4\text{Fe}-4\text{S}]\) clusters.

**EXPERIMENTAL PROCEDURES**

**Construction of the Full-length and Truncated Forms of the NQO9 Subunit**—For the full-length NQO9 subunit expression, the nqo9 gene was mutated by oligonucleotide mutagenesis by Kunkel et al. (35). Briefly, an Nhel/NotI DNA fragment (800 base pairs) that contained the entire region of nqo9 gene was digested from p9T2-b, blunt-ended with a Klenow fragment, and then subcloned in Smal site of pTZ18I cloning vector. A clone that contained the nqo9 gene in a particular orientation was selected and designated as pTZ18(NQO9). An NdeI restriction recognition site was introduced around the translation initation site with an oligonucleotide NQO9/NdeI, 5'–GGG GCG TTT CAT ATG GCC TTC GAT TTC GCC CCG CTG TCG CCG CGC TTC–3' (where italics indicate changes from *P. denitrificans* DNA and underlines indicate the newly introduced restriction site). The construct containing the NdeI site was verified by sequencing and was designated as pTZ18(NQO9/NdeI). The NdeI/BamHI fragment (520 base pairs) was retrieved from pTZ18(NQO9/NdeI) and ligated with pET11a or pET16b expression vectors, respectively. The final constructs were confirmed by restriction enzyme digestion and were named as pET11a(NQO9) and pET16b(NQO9). We employed PCR methods for the construction of the truncated forms of the NQO9 subunit. The following oligonucleotides were synthesized: NQO9F1, 5'–TTT CAT ATG GCC TTC GAT TTC GCC CCG CTG TCG CCG CGC TTC–3'; NQO9F2, 5'–CGG GCC ATG GAA AAG GGC CCG CTG TCG CCG CGC TTC–3'; NQO9R1, 5'–GGA GCC TCC TCA TCT GTA GGG CCC ATC CAG TTG C–3'; NQO9R2, 5'–CGG GCC ATG GAA AAG GGC CCG CTG TCG CCG CGC TTC–3'. New ly introduced restriction enzyme recognition sites have been underlined (CATATG for NdeI and GGATCC for BamHI). Italicized letters are the nucleotides that had been altered from the original sequences. pTZ18(NQO9) plasmid was used as a template. DNA was amplified in 50 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.01% (w/v) gelatin, 200 μM each of dNTPs (dATP, dCTP, dTTP, and dGTP), 1.0 mM each of sense and antisense primers, 100 ng/ml pTZ18(NQO9) plasmid, and 2.5 units of Pfu polymerase (Stratagene). The amplification was performed in a thermocycler with a reaction program that consisted of one cycle of hot start (94 °C for 10 min and 60 °C for 5 min), 35 amplification cycles that were composed of denaturing (94 °C for 1 min), annealing (50 °C for 1.5 min), and elongation (72 °C for 2 min), and finally one cycle of termination (72 °C for 10 min). Amplified DNA fragments were purified with Qiagen PCR purification kit (Qiagen) and run on a 1.0% (w/v) agarose gel. DNA fragments with expected molecular size were excised and purified from the gel with Qiagen Gel Extraction kit (Qiagen) and then subcloned into pCR-script II vector (Stratagene) according to the manufacturer’s protocol. The cloned DNAs were verified by sequencing of both strands. Each construct was designated pCR(NQO9F1–40), pCR(NQO9F2–40), pCR(NQO9R1–151–163), pCR(NQO9R2–151–163), pCR(NQO9R1–151–163), pCR(NQO9R2–151–163), and pCR(NQO9R1–151–163), respectively. NdeI/BamHI fragments were retrieved from each construct and then ligated with pET11a and pET16b expression plasmids. The final constructs were designated pET11a(NQO9F1–40), pET16b(NQO9F1–40), pET11a(NQO9R1–151–163), pET16b(NQO9R1–151–163), pET11a(NQO9R2–151–163), pET16b(NQO9R2–151–163), pET11a(NQO9F1–40), pET16b(NQO9F1–40), pET11a(NQO9R1–151–163), pET16b(NQO9R1–151–163), pET11a(NQO9R2–151–163), pET16b(NQO9R2–151–163), pET11a(NQO9F1–40), and pET16b(NQO9F1–40).
Subunit in E. coli—Expression procedures were based on Takano et al. (28). E. coli strain BL21(DE3)pTrx was transformed with the respective expression vectors. A single, well isolated colony was picked up and inoculated in 2 ml of 2x YT medium containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol and cultivated at 37 °C. The grown culture was transferred to 500 ml of TB medium containing 100 µg/ml carbenicillin and 35 µg/ml chloramphenicol, 100 µg/ml ammonium ferric citrate, and 100 mM Na₃S, and the cells were cultivated at 37 °C until A₆₀₀nm reached 0.5. Then the medium was further shaken at 25 °C for 2 h. Isopropyl-1-thio-β-D-galactopyranoside was added at a final concentration of 0.5 mM to induce expression, and the cells were grown at 20 °C for 6 h. The cells were harvested by centrifugation in a GSA rotor at 6,000 rpm for 10 min at 4 °C. The cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Purification of the His-tagged Truncated Forms of the P. denitrificans NQO9 Subunits from E. coli—Purification procedures were basically the same as in the previous study (23). E. coli cells were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 0.5 mM DTT. The supernatant was degassed and purged with oxygen-free argon and equilibrated in the anaerobic chamber at room temperature.

In Vitro Reconstitution of the Iron-Sulfur Clusters into the Truncated NQO9 Subunits—The purified soluble NQO9 subunits were dialyzed against 50 mM HEPES buffer (pH 8.0) containing 1.0 mM DTT overnight. If necessary, the proteins were concentrated with Microcon-10 to an appropriate concentration.

Expression of the Full-length and Truncated Forms of the P. denitrificans NQO9 Subunit in E. coli—A 10 µg of pET16b, and jugulating affinity purified antibodies to rabbit IgG and ECL kit were purchased from Novagen. NTA-agarose was from Qiagen. Brilliant Blue R-250 were from Bio-Rad. Horseradish-peroxidase-conjugating affinity purified antibodies to rabbit IgG and ECL kit were purchased from Novagen.

Materials—Acrylamide, N,N'-methylenebis(acrylamide), SDS, SDS-polyacrylamide gel electrophoresis marker proteins, and Coomassie Brilliant Blue R-250 were from Bio-Rad. Horseradish-peroxidase-conjugating affinity purified antibodies to rabbit IgG and ECL kit were purchased from Amersham Pharmacia Biotech. A faint band marked by an asterisk is a dimer.
strains, BL21(DE3) and BL21(DE3)pLysS, were used, the products were expressed only as inclusion bodies under all conditions tested. The formed inclusion bodies exhibited a dark brown color. The inclusion body fraction was found to contain relatively large amounts of non-heme iron and acid-labile sulfide (35–40 μmol each of Fe and S₂⁻/mg of protein), suggesting that the subunits may contain iron-sulfur cluster(s). However, the materials were not suitable for further characterization. We decided to test the co-expression system with thioredoxin since it is known that co-expression with thioredoxin not only minimizes formation of inclusion body but also stimulates proper polypeptide folding (28, 50). We found that the full-length NQO9 subunit was located only in the membrane fraction without forming inclusion bodies (Fig. 2). These results suggest that the NQO9 subunit is more hydrophobic than other peripheral subunits (NQO1, NQO2, NQO3, NQO4, and NQO5), which have been expressed as soluble proteins in *E. coli* (22, 23, 26, 28). However, the low quantity of the membrane-localized subunit did not allow us to characterize molecular properties of the subunit. These results led us to consider that the NQO9 subunit may contain particular sequences that are responsible for its hydrophobic properties. Fig. 3A depicts the sequence alignments of the NQO9 subunit with its homologues from different species. Sequence analysis indicates that the sequence identity index among these organisms fell into a relatively wide range (37–87%). When the sequences were scrutinized in detail, it is clear that the N-terminal and C-terminal regions are less conserved, whereas the middle region where two [4Fe-4S] cluster-binding motifs are present contains a number of conserved amino acid residues (Fig. 3A). When comparison of the sequence identity of the middle regions (corresponding to the region from 31 to 140 of *P. denitrificans* subunit) was made among these homologues, the score of sequence identity increased to a range of 45–91%. When hydrophobicity of the NQO9 subunit was taken into consideration, it could be seen that the N-terminal region contains a relatively hydrophobic stretch (Fig. 3B). Similar features were also seen among its homologues (data not shown). These analyses suggested that the middle region plays an essential role for holding iron-sulfur clusters, whereas the N-terminal hydrophobic stretches

![Fig. 3](image-url)

**Fig. 3.** Sequence alignments of the NQO9 subunit (A) and its homologues and hydropathy plot of the NQO9 subunit (B). A, sequences of NQO9 subunit and its homologues were lined up by the PILEUP program. *P. d.*, NQO9 subunit of *P. denitrificans* NDH-1; *R. c.*, NQO9 subunit of *Rhodobacter capsulatus* NDH-1; *Bovine*, TYKY subunit of bovine heart Complex I; *T. th.*, NQO9 subunit of *Thermus thermophilus* NDH-1; *E. coli*, NQO9 subunit of *E. coli* NDH-1. Invariant residues are indicated by asterisks. Fully conserved cysteine and proline residues are indicated by shadowed boxes. Broken lines with scissors indicate the positions where the N- and C-terminal stretches were genetically deleted. Possible coordination of the 2×[4Fe-4S] clusters by the cysteine residues is suggested by analogy to 2×[4Fe-4S] ferredoxins (33). B, the hydropathy plot was analyzed with a window size of 11 residues by the HYDROPLOT program that is based on the data of Kyte and Doolittle (53). Shadowed boxes at the bottom are the N- and C-terminal stretches deleted in this study.
Therefore, we tried to reconstitute the iron-sulfur clusters. The formed iron-sulfur clusters were significantly distorted and only weak EPR signals (data not shown). It seemed likely that the truncated forms of the subunit, indicating that the truncated forms of the subunit could be removed, respectively. Genetically engineered nqo9 genes were placed under T7 promoter and were tested for expression in E. coli. When 30 amino acid residues were deleted from the N terminus, the expression level of the soluble subunit in the cytoplasm dramatically increased (Fig. 5, A and B). Further deletion of up to 40 amino acid residues, which included three fully conserved amino acid residues, resulted in the same amount of soluble product as in the case of NQO9(Δ1–30). Deletions of the C-terminal part (Δ151–163 and Δ141–163) were less effective, resulting in products detectable only in the membrane fraction. When both the N-terminal and C-terminal stretches were deleted, the expression level of the soluble form of the subunits was as high as or slightly less than those of NQO9(Δ1–30) and NQO9(Δ1–40). The same results were obtained in both His-tagged and non-fused forms. These results suggest that the N-terminal hydrophobic and less conserved region is responsible for the hydrophobic properties of the NQO9 subunit and that the removal of the N-terminal stretches makes the NQO9 water-soluble. It should be noted that the NQO9(Δ1–30) and NQO9(Δ1–40) are prone to aggregate more than the subunits whose N- and C-terminal regions have both been deleted.

Purification of the Expressed Truncated Forms of the NQO9 Subunit and in Vitro Reconstitution of Iron-Sulfur Clusters—The truncated forms of the NQO9 subunit expressed in the cytoplasm of E. coli were affinity purified with Ni-NTA agarose (Fig. 6). Precautions were taken to perform most procedures in oxygen-free environments utilizing buffers containing DTT. All the truncated forms of the NQO9 subunit exhibited a dark brown color and were found to contain certain amounts of Fe and S^2- (chemically determined to be 2.0–2.5 mol each/mol of subunit), indicating that the truncated forms of the subunit house iron-sulfur clusters. However, the subunits exhibited only weak EPR signals (data not shown). It seemed likely that the iron-sulfur clusters were significantly distorted. Therefore, we tried to reconstitute the iron-sulfur clusters in vitro. We tested several different conditions in terms of buffer pH, protein concentration, reducing reagents, Fe and S^2- concentrations, incubation time, and so forth. The best result was obtained under the condition as described in detail under “Experimental Procedures.” Although the truncated NQO9 subunits are water-soluble and stable at room temperature, the proteins are prone to aggregate during the reconstitution when the protein concentration is higher than 20 μM. Furthermore, higher concentrations of Fe and S^2- also cause the aggregation of the subunits. Therefore, it is necessary to maintain the protein, Fe, and S^2- concentrations lower. However, once the iron-sulfur clusters are reconstituted in the subunit, the holocomplexes become stable and stay water-soluble even at a higher protein concentration. The reconstituted subunit was surprisingly stable at room temperature. No significant change in the absorbance was observed for 2–3 weeks under anaerobic conditions (oxygen <1 ppm). The His tag of the expressed proteins allowed us to collect and concentrate the reconstituted product on a nickel chelation column. It was found that the iron-sulfur clusters were readily reconstituted into the subunits in vitro, resulting in 7.6–7.7 mol each of Fe and S^2-/mol of subunit. The absorption spectra of the reconstituted subunits are similar to those of 2×[4Fe-4S] ferredoxins (51) (Fig. 7). The reconstituted NQO9 subunit as prepared exhibited absorption peaks at 278 and 410 nm with a broad shoulder at 310 nm. Addition of 10 mM sodium dithionite resulted in a decrease of the absorbance in the visible region, demonstrating that the reconstituted iron-sulfur clusters were redox-active.

EPR Spectroscopic Analysis of the Reconstituted NQO9 Subunits—A representative EPR spectrum of iron-sulfur clusters in the reconstituted NQO9 subunit is shown in Fig. 8. This preparation over 90% of the iron-sulfur clusters were reconstituted. Non-heme iron content in this sample was 590 μg. Iron-sulfur clusters were reduced with 10 mM dithionite. In this spectrum we clearly discern multiple peaks at g = 2.08, 2.05, 1.95, and 1.89 and a shoulder around 1.92, which cannot be attributed to EPR signals arising from a single cluster. Our attempt to resolve these EPR signals by the potentiometric titration analysis was unsuccessful. The E_m values of both iron-sulfur clusters in the overexpressed truncated NQO9 subunit seemed to be too low (<600 mV) to resolve by the conventional potentiometric technique. Perhaps the protein environment around the clusters in the truncated hydrophilic NQO9 subunits differs considerably from that in the original subunit, which caused a negative shift of E_m values (22, 26). Temperature profile of the signal amplitudes at g = 2.08 and 2.05 as well as at g = 1.92 and 1.89 relative to the base line are presented in Fig. 9, A and B, respectively. Similar temperature dependence was observed between relative amplitudes of the g_{i+} = 2.05 and 1.92 signals and between g_{i-} = 2.08 and 1.89 signals; the latter two signals exhibit much faster spin relax-
membrane (48). Immunoblotting procedures were the same as in Fig. 2. SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (48). Molecular sizes are indicated on the left. B, 1 µg of the cytoplasmic fraction was electrophoresed on Schägger's SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane and the gel was stained with Coomassie Brilliant Blue. Molecular sizes are indicated on the left.

**Fig. 5.** SDS-polyacrylamide gel pattern (A) and immunoblotting (B) of the full-length and truncated forms of His-tagged NQO9 subunit expressed in E. coli cytoplasmic fractions. Lane 1, NQO9 (full-length); lane 2, NQO9Δ1–30; lane 3, NQO9Δ1–40; lane 4, NQO9Δ1–141–163; lane 5, NQO9Δ141–163; lane 6, NQO9Δ1–30,Δ151–163; lane 7, NQO9Δ1–30,Δ141–163; lane 8, NQO9Δ1–40,Δ151–163; and lane 9, NQO9Δ1–40,Δ141–163. A, 10 µg each of the cytoplasmic fraction were loaded on 12% Schägger's SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Molecular sizes are indicated on the left. B, 1 µg of the cytoplasmic fraction was electrophoresed on Schägger's SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (48). Immunoblotting procedures were described in the legend of Fig. 2. Molecular sizes are indicated on the left.

**Fig. 6.** SDS-polyacrylamide gel pattern (A) and immunoblotting (B) of the truncated forms of NQO9 subunit purified from E. coli cytoplasm. A, the purified subunits (2.5 µg) were loaded on a 13% Schägger's SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Lane 1, NQO9Δ1–30,Δ151–163; lane 2, NQO9Δ1–30,Δ141–163; lane 3, NQO9Δ1–40,Δ141–163; and lane M, molecular size markers (97 kDa, 66 kDa, 45 kDa, 31 kDa, 21.5 kDa, and 14.5 kDa). B, the purified subunits (0.5 µg) were loaded on a 13% Schägger's SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (48). Immunoblotting procedures were the same as in Fig. 2.

**Fig. 7.** Absorption spectra of the reconstituted truncated form of NQO9 subunit. The purified and chemically reconstituted truncated form of NQO9 subunit, NQO9Δ1–30,Δ151–163, was suspended at 0.33 mg/ml in 10 mM HEPES buffer (pH 8.0) containing 1.0 mM DTT. Absorption spectra of the subunit as prepared (line 1) and in 5 mM dithionite-reduced form (line 2) were recorded at room temperature.

**Fig. 8.** EPR spectra of 90% reconstituted iron-sulfur clusters to the NQO9 subunit reduced by 10 mM dithionite at 14 K. EPR conditions are as follows: microwave power, 10 mW; microwave frequency, 9.43 GHz; modulation amplitude, 1 millitesla (mTesla); time constant, 163 ms.

...ion than the former two. The rate of spin relaxation defines interaction of electron spins of a paramagnetic center with its surrounding protein environment (defined as $T_1$, spin-lattice relaxation time) and with neighboring spins ($T_2$, spin-spin relaxation time). This spin relaxation property was analyzed by power saturation experiment and expressed as a half-saturation parameter ($P_{0.5} = 1/T_2$). As presented in Fig. 10, the signal amplitude between $g = 1.95$ and $1.89$ peaks of [4Fe-4S] spectra of NQO9 subunits showed a biphasic profile with an extremely fast-relaxing component with $P_{0.5} = 327$ mW and a slow-relaxing component with $P_{0.5} = 8$ mW at the sample temperature of 14 K. These data can be interpreted in the following two different cases. (i) Two tetranuclear iron-sulfur clusters residing in the NQO9 subunit exhibit broad near axial-type spectra with distinct EPR properties, namely ($g_{||,\perp} = 2.08$, $\sim 1.90$, with an intrinsically high $P_{0.5}$ value) and ($g_{||,\perp} = 2.05$, $\sim 1.93$, with low $P_{0.5}$). These two iron-sulfur clusters are not significantly spin-coupled. (ii) Two tetranuclear iron-sulfur clusters exhibit almost the same EPR spectra with a broad near-axial symmetry with $g_{||,\perp} = 2.05$, $1.93$, with slow spin relaxation ($P_{0.5} = 8$ mW at 14 K). When two clusters in the same subunit are concurrently paramagnetic, they are spin-coupled and signals are shifted to $g_{||,\perp} = 2.08$, $1.90$, and their spin relaxation rate is greatly enhanced to $P_{0.5} = 327$ mW. Currently we cannot discriminate these two possibilities, namely whether these two iron-sulfur clusters have different intrinsic spin relaxation properties or the fast and slow relaxing components arise from one subunit population containing only a single cluster reduced per subunit and the other with both clusters reduced. In the latter case, magnetic interaction between two paramagnetic clusters in the same molecule may have resulted in the apparently much faster spin relaxation.

In either case, our EPR data on the overexpressed and re-
constituted \textit{P. denitrificans} NQO9 subunit provide the first experimental evidence demonstrating the presence of two distinct tetranuclear iron-sulfur clusters in this subunit, as suggested by its stereotypical cysteinyl-rich sequence motifs.

**DISCUSSION**

In order to characterize the putative cluster N2 binding subunits, we have first attempted to express the NQO9 subunit of the \textit{P. denitrificans} NDH-1 in \textit{E. coli}. By co-expressing with thioredoxin, which is known to help proteins of interest undergo proper folding \textit{in vivo}, we obtained the full-length NQO9 subunit exclusively in the membrane fraction. The same phenomenon was observed previously, \textit{i.e.} the NQO6 subunit was expressed only in the membrane, whereas the other peripheral subunits, NQO1, NQO2, NQO3, NQO4, and NQO5, were expressed as soluble proteins (22, 23, 26, 28). These results implied that the NQO6 and NQO9 subunits are more hydrophobic in nature than the other peripheral subunits. Amino acid sequence analyses provided us with a clue as to the membrane-associated segment of the NQO9 subunit. The NQO9 subunit and its homologues have relatively hydrophobic and less conserved amino acid sequences in the N-terminal region and less invariant sequences in the C-terminal region, whereas the middle region, where two [4Fe-4S] cluster binding motifs are present, is highly conserved. The latter undoubtedly plays an important role in providing the scaffold for the two [4Fe-4S] clusters. By deleting hydrophobic sequences from its N-terminal region, the truncated subunits were expressed as soluble proteins in \textit{E. coli} as well as in \textit{P. denitrificans} (see the accompanying paper). As described in the accompanying paper, the NQO9 and NQO6 subunits have been suggested to be associated with the membrane arm of the NDH-1 enzyme complex. In this connection, it is conceivable that the N-terminal stretch of the NQO9 subunit functions as a membrane anchor of the subunit.

Although the NQO9 subunit is believed to contain 2\(\times\)[4Fe-4S] clusters, no experimental evidence has been available. In this study, we have succeeded in expressing the soluble forms of the NQO9 subunit in \textit{E. coli}. The redox-active iron-sulfur clusters were readily reconstituted into the subunits under anaerobic conditions with high reconstitution efficiency (7.6–7.7 atom of Fe and \(S^2-/\text{mol of subunit}\), more than 95\% of the expected amounts) in this study. The reconstituted subunit exhibited the optical absorption spectra similar to those of some bacterial 2\(\times\)[4Fe-4S] ferredoxins (51). The EPR data indicated that the NQO9 subunit ligates two [4Fe-4S] clusters. Taken together, it seems likely that the two [4Fe-4S] clusters are coordinated to a polypeptide in a similar manner as shown in Fig. 3. This is the first report showing that the NQO9 subunit actually harbors two [4Fe-4S] clusters.

In order to determine the subunit location of the cluster N2, several criteria should be taken into consideration. Given its plausible role in electron transfer as a component directly reducing quinone, the cluster N2, with the highest \(E_m\) value among iron-sulfur clusters in complex, is considered to be located in a hydrophobic environment. In this regard, both NQO6 and NQO9 subunits are the most likely candidates because of their direct association with the membrane part of the enzyme complex (see also the accompanying paper) (27). A fingerprint for identification of cluster N2 is its axial-type EPR spectral line shape (\(g_i = 2.05\) and \(g_j = 1.93\)). All other tetranuclear clusters in complex I exhibit rhombic spectra. We have shown that dithionite-reduced NQO9 subunit exhibited two distinct species of EPR signals with broad near-axial-type symmetry with (\(g_i = 2.05\) and \(g_j = −1.93\)) and (\(g_i = 2.08\) and \(g_j = −1.90\)). The latter spectrum can be the intrinsic spectrum of the second [4Fe-4S] cluster in the NQO9 subunit or can arise from the spectral shift of the former spectrum due to the spin-spin

\textbf{FIG. 9.} Temperature dependence of the normalized EPR signal amplitudes of the \(g_x = 2.08\) (■) and \(g_x = 2.05\) (●) peaks (A) and the \(g_x = 1.92\) (●) and \(g_x = 1.89\) (■) troughs (B) from the 90\% reconstituted iron-sulfur clusters to the NQO9 subunit, which were reduced with 10 mM dithionite. EPR conditions are the same as Fig. 8 except for the sample temperature.

\textbf{FIG. 10.} Microwave power dependence of the EPR signals from the 90\% reconstituted iron-sulfur clusters in the NQO9 subunit. Signal amplitude was measured between \(g_x = 1.95\) peak and 1.89 trough. \(P_{1/2} \pm 3.3\) represents the relative concentration of fast-relaxing (\(P_{1/2} = 327\ mW\)) component, \(P_{1/2} \pm 2.05\) represents the relative concentration of slow-relaxing (\(P_{1/2} = 8.0\ mW\)) component. Sample temperature is 14 K and other conditions are the same as in Fig. 8.

\begin{tabular}{|c|c|}
\hline C1 & 2.96038 \\
\hline P_{1/2} & 327.257 \\
\hline C2 & 3.32039 \\
\hline P_{1/2} & 7.96908 \\
\hline
\end{tabular}
interaction between two paramagnetic clusters in the same NQO9 subunit. In any case, we still favor the notion that NQO6 is the N2-containing subunit rather than NQO9 since all known 2×[4Fe-4S] ferredoxins have low redox potentials at around −400 mV (33, 34). However, this question still remains unanswered to date. Currently EPR analysis of an iron-sulfur cluster in the overexpressed and purified P. denitrificans NQO6 subunit is in progress in our laboratories. Recent findings that pyridaben (a specific potent inhibitor for electron transfer between cluster N2 and Q pool) binds specifically to the NQO6 subunit (15) makes this line of work even more intriguing. On the other hand, it has been reported that the two [4Fe-4S] clusters in the PsAC subunit exhibit changes in both their EPR properties and redox midpoint potentials upon binding onto photosystem I core (52). Therefore, we cannot completely exclude the possibility that the two [4Fe-4S] clusters of the NQO9 subunit might show cluster N2 features in situ, namely the slowest spin relaxation among [4Fe-4S] clusters in complex I and narrower axial spectrum than that seen in the truncated hydrophilic NQO9 subunit. Therefore, the final assessment of the location of the cluster N2 remains for further scrutiny (13).

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REFERENCES

1. Walker, J. E. (1992) Q. Rev. Biophys. 25, 233–324
2. Yagi, T. (1993) Biochim. Biophys. Acta 1141, 1–17
3. Xu, X., Matsumo-Yagi, A., and Yagi, T. (1991) Biochemistry 30, 6422–6428
4. Xu, X., Matsumo-Yagi, A., and Yagi, T. (1991) Biochemistry 30, 8678–8684
5. Xu, X., Matsumo-Yagi, A., and Yagi, T. (1992) Biochemistry 31, 6925–6932
6. Xu, X., Matsumo-Yagi, A., and Yagi, T. (1992) Arch. Biochem. Biophys. 296, 40–48
7. Xu, X., Matsumo-Yagi, A., and Yagi, T. (1993) Biochemistry 32, 968–981
8. Weidner, U., Geier, S., Picck, A., Friedrich, T., Leif, H., and Weiss, H. (1993) J. Mol. Biol. 233, 109–122
9. Blättnre, F., Luska, G., Klom, C., Pernina, N., Burland, V., Riley, M., Collado-Vides, J., Glaser, J., Bode, O. R., Mayew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Gooden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) Science 277, 1448–1474
10. Dupuis, A., Peinequin, A., Chevallet, M., Lunardi, J., Darrouzet, E., Pierriard, B., Procaccio, V., and Issartel, J. P. (1995) Gene (Amst.) 167, 99–104
11. Yano, T., Chu, S. S., Sled, V. D., Ohnishi, T., and Yagi, T. (1997) J. Biol. Chem. 272, 4201–4211
12. Yagi, T., Yano, T., Di Bernardo, S., and Matsumo-Yagi, A. (1998) Biochim. Biophys. Acta 1364, 125–133
13. Miyoshi, H. (1998) Biochim. Biophys. Acta 1364, 236–244
14. Degli Esposti, M. (1998) Biochim. Biophys. Acta 1364, 222–235
15. Schuler, F., Yano, T., Di Bernardo, S., Yagi, T., Yankovskaya, V., Singer, T. P., and Casida, J. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4149–4153
16. Ohnishi, T. (1998) Biochim. Biophys. Acta 1364, 186–206
17. Ohnishi, T. (1993) J. Bioenerg. Biomembr. 25, 325–329
18. Ingledew, W. J., and Ohnishi, T. (1998) Biochem. J. 196, 911–197
19. Ohnishi, T., and Salerno, J. C. (1982) in Iron-Sulfur Proteins (Spiro, T. G., ed) 186, 245–277
20. Baltz, U. (1997) Biochim. Biophys. Acta 1318, 79–91
21. Yano, T., Sled, V. D., Ohnishi, T., and Yagi, T. (1996) J. Biol. Chem. 271, 5907–5913
22. Yano, T., Sled, V. D., Ohnishi, T., and Yagi, T. (1994) Biochemistry 33, 494–499
23. Crouse, B. R., Yano, T., Finnegean, M. G., Yagi, T., and Johnson, M. K. (1994) J. Biol. Chem. 269, 21020–21026
24. Yano, T., Sled, V. D., Ohnishi, T., and Yagi, T. (1994) FEBS Lett. 354, 160–164
25. Yano, T., Yagi, T., Sled, V. D., and Ohnishi, T. (1995) J. Biol. Chem. 270, 18246–18250
26. Leif, H., Sled, V. D., Ohnishi, T., Weiss, H., and Friedrich, T. (1995) Eur. J. Biochem. 230, 538–548
27. Takano, S., Yano, T., and Yagi, T. (1996) Biochemistry 35, 9120–9127
28. Masui, R., Wakabayashi, S., Matsubara, H., and Hathei, Y. (1991) J. Biol. Chem. (Tokyo) 100, 575–582
29. Albracht, S. P. J. (1993) Biochim. Biophys. Acta 12. Biochemistry 35, 222–244
30. Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1995) Nature 373, 580–589
31. Yagi, T., Yano, T., and Matsuno-Yagi, A. (1993) J. Bioenerg. Biomembr. 23, 339–345
32. Matsubara, H., and Sacki, K. (1992) Adv. Inorg. Chem. 38, 223–280
33. Johnson, M. K. (1994) in Encyclopedia of Inorganic Chemistry (King, R. B., ed) pp. 1896–1915, John Wiley & Sons Ltd., Chichester, UK
34. Runkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
35. Han, A.-L., Yagi, T., and Hathei, Y. (1988) Arch. Biochem. Biophys. 267, 490–496
36. Han, A.-L., Yagi, T., and Hathei, Y. (1989) Arch. Biochem. Biophys. 275, 166–173
37. Heiman, C., and Hathei, Y. (1991) Arch. Biochem. Biophys. 284, 90–97
38. Yagi, T. (1996) Arch. Biochem. Biophys. 350, 302–311
39. Deveraux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395
40. Lawry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
41. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70, 241–250
42. Schagger, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379
43. Laemmli, U. K. (1970) Nature 227, 680–685
44. Fogo, J. K., and Popowski, M. (1949) Arch. Biochem. Biophys. 97, 37–40
45. Doeg, K. A., and Ziegler, D. M. (1962) Anal. Biochem. 97, 214–220
46. Matsuda, P. (1967) J. Biol. Chem. 242, 10035–10038
47. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
48. Yasukawa, T., Kaneshi, C., Makawa, T., Fujimoto, J., Yamamoto, T., and Ishii, S. (1990) J. Biol. Chem. 270, 25328–25331
49. Malkin, R. (1973) in Iron-Sulfur Proteins (Lovenberg, W., ed) pp. 1–26, Academic Press, New York
50. Golbeck, J. H. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 293–324
51. Ryte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132