Expression of the 25-Kilodalton Iron–Sulfur Subunit of the Energy-Transducing NADH–Ubiquinone Oxidoreductase of *Paracoccus denitrificans*†

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ABSTRACT: The energy-transducing NADH–ubiquinone (Q) oxidoreductase of *Paracoccus denitrificans* is composed of 14 dissimilar subunits and contains at least four iron–sulfur clusters [Yagi, T. (1993) *Biochim. Biophys. Acta 1141*, 1–17]. The complete DNA sequence of the gene cluster encoding the energy-transducing NADH–Q oxidoreductase of *P. denitrificans* has been determined. This paper reports the expression of the 25-kilodalton (kDa) (NQO2) subunit of the *P. denitrificans* enzyme complex in *Escherichia coli* and the characterization of the iron–sulfur cluster bound to the expressed subunit. The 25-kDa subunit was expressed in the cytoplasmic phase but not in the membrane fraction of *E. coli* cells and then purified using an affinity nickel chelation column. The purified subunit contains 1.44 mol of non-heme iron and 1.33 mol of acid-labile sulfide/mol of subunit. EPR analysis of the reduced form of this subunit indicates that the expressed subunit contains a single binuclear [2Fe–2S] cluster. This cluster exhibits a spectrum of rhombic symmetry with g values of $g_{x,y,z}$ = 1.913, 1.942, and 1.996, which is very similar to the spectrum of the [2Fe–2S] cluster in the resolved flavoprotein II subfraction (subunit 24 + 9 kDa) of bovine heart complex I [Ragan, C. I., Galante, Y. M., Hatefi, Y., & Ohnishi, T. (1982) *Biochemistry 21*, 590–594; Ohnishi, T., Ragan, C. I., & Hatefi, Y. (1985) *J. Biol. Chem. 260*, 2782–2788]. The assignment of the binuclear iron–sulfur cluster of the 25-kDa subunit to an EPR-visible iron–sulfur cluster in the *Paracoccus* NADH–Q oxidoreductase *in situ* is discussed.

Paracoccus denitrificans is a Gram-negative soil bacterium and has been called "a free-living mitochondrion" (Steinrücke & Ludwig, 1993; Kurowski & Ludwig, 1987; Ludwig, 1987; Stouthamer, 1980). Aerobically grown *P. denitrificans* expresses a mammalian mitochondrial-type respiratory chain (Stouthamer, 1992) which contains the energy-transducing NADH–ubiquinone oxidoreductase (NDH-1)[1] (Yagi, 1991, 1993). The *Paracoccus* NDH-1 contains noncovalently bound FMN and 4–5 EPR-visible iron–sulfur clusters and appears to be composed of at least 14 unlike subunits (Yagi, 1986; Meinhardt et al., 1987; Yagi et al., 1992). Recently, by Xu et al. (1991a,b, 1992a,b, 1993), cloning and DNA sequencing of a gene cluster encoding the *Paracoccus* NDH-1 have been carried out. The gene cluster of the *Paracoccus* NDH-1 was found to contain 14 structural genes and 6 URFs, designated *NQO1–14* and URF1–6, respectively (Yagi et al., 1992, 1993; Yagi, 1993).

By comparing the deduced primary structures of the *Paracoccus* NDH-1 subunits against the consensus sequence known for different types of iron–sulfur clusters (Matsubara & Saeki, 1992), it was possible to speculate as to which NDH-1 subunits bind iron–sulfur clusters. The NQO1, NQO3, and NQO9 subunits all contain at least one consensus sequence for tetranuclear iron–sulfur cluster binding sites (Yagi et al., 1993). The NQO9 subunit bears two putative tetranuclear cluster binding sites. Furthermore, the NQO2 and NQO3 subunits bear possible binuclear iron–sulfur cluster binding sites, although the consensus sequence for binuclear iron–sulfur binding sites is not as well established as that for tetranuclear iron–sulfur cluster binding sites (Yagi, 1993). The resolution experiments utilizing bovine complex I (Ragan et al., 1982; Hatefi et al., 1985; Hatefi, 1985; Ohnishi et al., 1985; Ragan, 1987) support the hypothesis that the NQO1, 2, 3, and 9 subunits are probably associated with the iron–sulfur clusters. Therefore, it is important to clarify whether these subunits actually ligate the iron–sulfur clusters and, if so, to assign the EPR-visible iron–sulfur clusters to individual subunits.

For this purpose, two approaches appear to be applicable. One is resolution of the NDH-1 and complex I. As described in Ragan et al. (1982) and Ohnishi et al. (1985), separation of each subunit from these enzyme complexes without damaging iron–sulfur clusters appears difficult because harsh conditions are required to separate each subunit. In addition, the limits of available amounts of subunits hamper progress in their structural studies. The other approach is to express the gene encoding each subunit. If overexpression of the individual subunits is successful, problems caused by limitation

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[1] Abbreviations: Q, ubiquinone; NDH-1, bacterial energy-tarnsducing NADH–quinone oxidoreductase(s); complex I, mitochondrial energy-transducing NADH–quinone oxidoreductase(s); FP, flavoprotein fraction of bovine complex I; EPR, electron paramagnetic resonance; [2Fe–2S], binuclear iron–sulfur cluster; NQO2f subunit, fused NQO2 subunit bearing sequence $MGH_{10}S_2HIEGRH$ as the leading sequence; bp, base pair(s); kDa, kilodalton(s); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; mT, millitesla; URF, unidentified reading frame.

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of material available will vanish. If host cells were equipped with the machinery required for incorporation of cofactors and native structural folding of the subunits, difficulties with which resolution experiments of complex I and NDH-1 have been confronted might be circumvented. Furthermore, once native subunits are expressed, determination of residues ligating cofactors may be possible utilizing site-directed mutagenesis. Therefore, we attempted to express genes encoding putative subunits bearing cofactors of the *Paracoccus* NDH-1 in *Escherichia coli* which is also known to contain NDH-1 enzyme (Matsushita et al., 1987; Yagi, 1991, 1993; Weidner et al., 1992; Sled' et al., 1993).

This paper describes the expression of the *NQO2* gene encoding the *Paracoccus* 25-kDa subunit in *E. coli* using pET16b and pKK233-2 vectors and characterization of the expressed NQO2 subunit. The expressed NQO2 subunit was located in the cytoplasmic phase but not in the membrane fraction. EPR measurements of the expressed subunit indicated that this subunit is associated with a binuclear FeS cluster which exhibits a spectrum of rhombic symmetry. Furthermore, assignment of this binuclear cluster to the cluster N1b of the *Paracoccus* NDH-1 has been discussed.

MATERIALS AND METHODS

Construction of Expression Vector. A 1450-bp *Eco*RI/*Bsm*AI DNA fragment bearing the full-length NQO2 was excised from pXT-1 (Xu et al., 1991b), blunt-ended with DNA polymerase I Klenow fragment, and ligated into *Sma*I-cut cloning vector pTZ19U. The resulting plasmid is designated pTZ19(NQO2).

An oligonucleotide primer was designed to generate a *Kpn*I recognition site at the protein initiation codon: 5'-AGGATAAGAGGTACCTGA-3' encoding the *Kpn*I site (the underlined residues are modified from the *Paracoccus* DNA). A plasmid containing *Kpn*I site was synthesized using pTZ19(NQO2) as template. The resulting plasmid is designated pTZ19(NQO2KpnI). Plasmid pTZ19(NQO2KpnI) was digested with *Kpn*I, blunt-ended with T4 DNA polymerase, and then digested with *Hin*dIII. The resulting DNA fragment was ligated into pKK233-2 vector which was digested with *Nco*I, blunt-ended with Klenow fragment, and cut with *Hin*dIII. The resulting plasmid is designated pKK(NQO2).

Another oligonucleotide primer was synthesized to introduce *Nde*I site at the 5' end of the NQO2 gene (5'-GGCGCAGCATATGCGGTCAACC-3') into pTZ19(NQO2) (the underlined bases are changed from the *Paracoccus* DNA). The resulting plasmid is designated pTZ19(NQO2NdeI). As described above, pTZ19(NQO2NdeI) was cut with *Nde*I and *Bam*HI. The *Nde*I/*Bam*HI DNA fragment was ligated into an *Nde*I/*Bam*HI site in pET16b vector which was designed for expression of the fused protein. The resulting expression plasmid is designated pET16b(NQO2).

Both constructs were verified by single-stranded DNA sequencing of both strands by dideoxy chain-termination as previously described (Sanger et al., 1977).

Expression of NQO2 and NQO2f Subunits. Competent *E. coli* strains BL21(DE3)pLysS and XL1-Blue were transformed with pET16b(NQO2) and pKK(NQO2), respectively. Colonies transformed with pET16b(NQO2) lifted from the 2 × YT agar plate containing 50 μg/mL carbenicillin were grown at 37 °C in 10 mL of 2x YT medium plus ampicillin (100 μg/mL) to the stationary phase. This suspension was inoculated in 500 mL of TB medium in the presence of 100 μg/mL ampicillin. Ferric ammonium citrate (10 μg/mL) and/or sodium sulfide (10 μM) was added as required. Cells were grown at 37 °C to an absorbance of approximately 0.4 at 600 nm. IPTG was then added (final concentration 0.5 mM) and the cells were grown for 4 h at 25 °C. The cells were harvested by centrifugation at 6000 rpm for 10 min in a GSA rotor. The expression of NQO2 subunit with pKK(NQO2) was similar to that of NQO2f subunit with pET16b(NQO2) except that IPTG was added to a final concentration of 1 mM when the culture was grown to an absorbance of 0.2–0.3 and then cells were grown for 48 h at 28 °C.

Purification of the NQO2f Subunit. The cells harvested were suspended in buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF to 20% (w/v). The cell suspension was freeze–thawed twice using liquid nitrogen and a water bath at 37 °C and disrupted by passing twice through Parr cell disruption bombs. The suspension was centrifuged at 6000 rpm for 10 min in SS34 rotor in a Sorvall centrifuge to remove undisrupted cells. The supernatant was centrifuged at 50 000 rpm for 60 min in a 60 Ti rotor to remove the membrane fraction, dialyzed against 1 L of buffer containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM PMSF for 3 h, and then loaded on an affinity nickel chelation column (Novagen). The column was washed with dialyzing buffer containing 0.5 M NaCl and 40 mM imidazole. The NQO2f subunit was eluted with dialyzing buffer containing 0.5 M NaCl and 400 mM imidazole. The eluted subunit was dialyzed against buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM DTT. The purified subunit was immediately subjected to various assays.

Other Analytical Procedures. Protein was estimated by the method of Lowry et al. (1951) or by the biuret method in the presence of sodium deoxycholate at 1 mg/mL (Gornall et al., 1949). SDS–polyacrylamide gel electrophoresis was carried out by modified methods of Laemmli (1970). Immunoblotting experiments were conducted as described previously (Han et al., 1988, 1989; Hekman et al., 1991). Cloning techniques were as described by Sambrook et al. (1989). Nonheme iron and acid-labile sulfide were determined according to Doeg and Ziegler (1962) and Forgo and Popowski (1949), respectively. Amino acid composition (Yagi & Dinh, 1990; Xu & Yagi, 1991), amino acid sequence (Matsudaira, 1987; Yagi & Hatefi, 1988), and DNA sequences (Sanger et al., 1977; Xu et al., 1991b) were determined according to the references cited. Any variations from the procedures and other details are described in the figure legends.

Materials. Acrylamide, *N,N'*-methylenebis(acrylamide), SDS, prestained low-range marker proteins, and Coomassie brilliant blue R-250 were from Bio-Rad; DNA sequencing kit was from U.S. Biochemical Corp; [α-^{35}S]thio-dATP was from Amersham; alkaline phosphatase-conjugated affinity-purified antibodies to rabbit IgG were from Calbiochem; expression vector pET16b, competent *E. coli* strain BL21(DE3)pLysS, and His:Bind metal chelation resin were from Novagen; expression vector pKK233-2 was from Pharmacia-LKB; carbenicillin was from ICN. The monospecific antiserum to the bovine FP 24-kDa subunit was a generous gift from Drs. Ai-Li Han and Youssef Hatefi (The Scripps Research Institute). The affinity-purified antibody to the *Paracoccus* NQO2 (25 kDa) subunit was isolated from the antiserum to the *Paracoccus* NADH dehydrogenase complex (Yagi, 1986; Han et al., 1988).

RESULTS

Expression of the NQO2 Subunit of Paracoccus NDH-1 in E. coli. Figure 1 shows SDS–polyacrylamide gel patterns of *E. coli* strain hosting plasmid pKK(NQO2) containing the

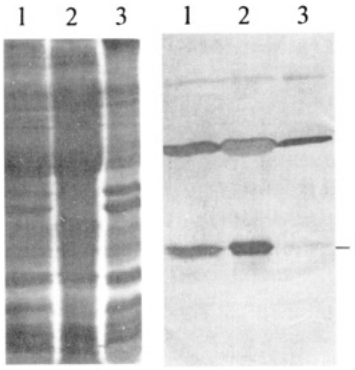

FIGURE 1: SDS–polyacrylamide gel pattern (left) and immunoblotting (right) showing the localization of the full-length, non-fused 25-kDa (NQO2) subunit. (1) Cell lysate of *E. coli* pKK(NQO2) (20 μg); (2) soluble fraction (20 μg); (3) membrane fraction (20 μg). The samples were loaded on a mini slab gel (55 × 95 × 0.75 mm) composed of 12% acrylamide. The gel was electrophoresed for 50 min at 195 V. The procedures for staining and destaining of the gel were described in Yagi (1987). Immunoblotting was carried out using affinity-purified antibody to the *Paracoccus* 25-kDa subunit and alkaline phosphatase-conjugated anti-rabbit IgG antibody as described previously (Han et al., 1988, 1989; Hekman et al., 1991). The bar in the figure indicates the expressed NQO2 (25-kDa) subunit.

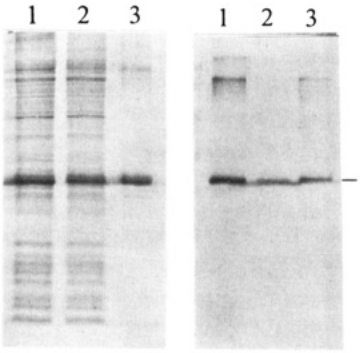

FIGURE 2: Purification of the expressed fused 25-kDa (NQO2) subunit from *E. coli*. Left panel is an SDS–polyacrylamide gel and right panel is immunoblotting using affinity-purified antibody to the *Paracoccus* 25-kDa subunit. (1) Lysate of *E. coli* [pET16b(NQO2)] cells (10 μg); (2) soluble fraction of *E. coli* [pET16b(NQO2)] cells (10 μg); (3) purified fused 25-kDa subunit (1 μg). The bar in the figure displays the expressed fused NQO2 (27-kDa) subunit. The SDS–polyacrylamide gel electrophoresis and immunoblotting experiments are the same as Figure 1.

NQO2 gene encoding the full-length, unfused *Paracoccus* NQO2 (25-kDa) subunit, and immunoblotting results using the affinity-purified antibody to the *Paracoccus* 25-kDa subunit. The antibody reacted with two bands. One band migrated with an apparent molecular weight of 50 000 and the other band exhibited an apparent molecular weight of 25 000. The antibody against the bovine complex I 24-kDa subunit, which is a bovine counterpart of the *Paracoccus* NQO2 (25-kDa) subunit, cross-reacted with the M_r 25 000 band but not with the M_r 50 000 band. In addition, the M_r 50 000 band was expressed in *E. coli* lacking plasmid pKK(NQO2). Furthermore, the competent *E. coli* BL21(DE3)-pLysS lacks the M_r 50 000 polypeptide which cross-reacted with the *Paracoccus* 25-kDa subunit antibody (see Figure 2). Therefore, it is conceivable that the M_r 50 000 polypeptide in *E. coli* is not related to the NQO2 subunit, but it might contain an epitope that is recognized by the antibody to the *Paracoccus* NQO2 subunit. This is not surprising because similar results have been reported for expression of the *Paracoccus* methanol dehydrogenase in *E. coli* (Harms et al., 1987). The expressed 25-kDa polypeptide was subjected to the gas-phase amino acid sequencer. The sequence MLRRL was detected, which perfectly matches N-terminal partial sequence of the NQO2 subunit isolated from the *Paracoccus* NADH dehydrogenase complex (Xu et al., 1991a). In view of this fact, it is concluded that the NQO2 subunit is correctly expressed in the *E. coli* strain harboring plasmid pKK(NQO2).

Table 1: Amino Acid Composition of the Expressed Fused 25-kDa (NQO2) Subunit of *Paracoccus* NDH-1

amino acid	by amino acid analysis	from DNA sequence
alanine	28.8	30
arginine	12.7	15
aspartic acid	16.3	15
glutamic acid	36.7	32
glycine	23.9	22
histidine	15.0	15
isoleucine	12.3	16
leucine	18.2	21
lysine	7.0	7
methionine	6.4	7
phenylalanine	7.8	9
proline	19.0	17
serine	12.1	12
threonine	14.0	13
tyrosine	4.4	5
valine	14.2	12

As seen in Figure 1, the expressed NQO2 subunit was detected in the cytoplasmic phase but not in the membrane fraction, although some of the expressed subunits are located in inclusion bodies (data not shown). When *Paracoccus* NDH-1 genes located downstream of the *NQO1* gene have been knocked out, the NQO2 subunit was detected only in the cytoplasmic phase of *P. denitrificans* (T. Yano and T. Yagi, unpublished data). These results strongly support our expectation that the NQO2 subunit and its bovine counterpart are soluble polypeptides and lack any membrane-spanning part as predicted on the basis of hydropathy plots of the deduced primary structure (Xu et al., 1991a) and also topological studies of bovine complex I using a monospecific antibody to 24-kDa subunit (Han et al., 1988). It is known that expressed polypeptides located in inclusion bodies tend to be denatured (Nilsson & Anderson, 1991). In fact, the content of non-heme iron in the NQO2 subunit present in the inclusion bodies was significantly low (data not shown). Therefore, we have attempted to purify the expressed subunit from the cytoplasmic phase. Unfortunately, there is no simple procedure available for isolation of the expressed subunit at the present time. On the other hand, fused polypeptides are known to be readily purified. Therefore, the expression of the fused NQO2 subunit has been carried out using pET16b vector encoding the leading oligopeptide ($MGH_{10}S_2GHIEGRH$) and bearing a T7 promoter. The expressed fused NQO2 subunit was located in the cytoplasmic phase, but (as with expression of the full-length NQO2 subunit) not in membrane fraction (data not shown). Using a His:Bind nickel chelation resin column, the fused NQO2 subunit was easily purified (see Figure 2). The expressed fused NQO2 subunit migrated with approximately M_r 27 000 on the SDS–polyacrylamide gels as anticipated (25 kDa + 2.2 kDa for the leading oligopeptide). As shown in Table 1, the amino acid composition of the purified fused NQO2 subunit is consistent with that calculated from its deduced primary structure. Furthermore, the affinity-purified antibody to the *Paracoccus* NQO2 subunit reacted with the expressed fused polypeptide, assuring that the correct polypeptide was indeed expressed in *E. coli*. Factor Xa, which is reported to cleave the fused protein, does not cleave the proteolysis site engineered into the fusion protein but does cleave it elsewhere. Similar phenomena have been reported for the fused Rieske iron–sulfur polypeptide (Van Doren et

Table 2: Effects of Ferric Ammonium Citrate and Sodium Sulfide in the Growth Medium on the Content of Non-Heme Iron and Acid-Labile Sulfide of the Isolated Fused 25-kDa (NQO2) Subunit of *Paracoccus* NDH-1

additions	non-heme iron (mol/mol of subunit)	acid-labile sulfide (mol/mol of subunit)
none	0.41	0.33
10 μg/mL ferric ammonium citrate	0.83	0.58
10 μg/mL ferric ammonium citrate and 10 μM sodium sulfide	1.44	1.33

al., 1993), suggesting that the substrate-binding site of Factor Xa might be less specific and the proteolysis site engineered into fusion protein might be unexposed.

Iron–Sulfur Cluster. The *Paracoccus* NDH-1 bears five EPR-visible FeS clusters similar to bovine complex I (Meinhardt et al., 1987; Albracht et al., 1980; Yagi, 1991, 1993). Those are designated clusters N1a, N1b, N2, N3, and N4 (Meinhardt et al., 1987). Clusters N1a and N1b are binuclear and clusters N2, N3, and N4 are tetranuclear (Meinhardt et al., 1987). We have predicted from the primary structure that the NQO2 subunit has a putative iron–sulfur binding site (Xu et al., 1991a). Therefore, we analyzed the expressed *Paracoccus* NQO2 subunit for non-heme iron and acid-labile sulfide and determined their content. As shown in Table 2, the purified subunit contained low amounts of non-heme iron and acid-labile sulfide. When cells were cultured in the presence of ferric ammonium citrate, the content of non-heme iron and acid-labile sulfide of the purified NQO2 subunit was increased approximately 2-fold. Furthermore, when supplemented with sodium sulfide, the content increased to 1.44 mol of Fe and 1.33 mol of S^{2-}/mol of subunit. These results suggest that the expressed fused NQO2 subunit carries an iron–sulfur cluster as we anticipated (Xu et al., 1991a; Yagi et al., 1993).

Iron–sulfur clusters can be divided into two groups depending on whether or not they transfer an electron (Cammack, 1992). Figure 3 shows the optical absorption spectra of the expressed *Paracoccus* NQO2 subunit. There are three broad peaks (at approximately 430, 470, and 560 nm). This absorption spectrum appears to be similar to that of *Clostridium pasteurianum* ferredoxin containing a binuclear cluster (Meyer et al., 1992). Addition of sodium dithionite significantly quenched the absorption of the expressed NQO2 subunit throughout the entire wavelength range as shown in Figure 3. These results support the hypothesis that the expressed NQO2 subunit ligates redox-functional iron–sulfur cluster(s).

In order to not only confirm the presence of iron–sulfur cluster(s) in the expressed NQO2 subunit but also determine the type of iron–sulfur cluster(s), the EPR spectrum of the expressed NQO2 subunit was examined with the concentrated cytosolic fraction of the expressed *E. coli* cells. After reduction with dithionite, the iron–sulfur cluster bound to the expressed fused NQO2 subunit exhibits an EPR spectrum of rhombic symmetry (Figure 4, top). The spectrum is simulated with a reasonable fit as a single component using the following parameters: $g_{x,y,z}$ = 1.913, 1.942, and 1.996 and $L_{x,y,z}$ = 1.42, 1.45, and 0.95 mT. Identical EPR spectra were obtained using the purified preparation of the expressed fused NQO2 subunit. The EPR spectrum of the expressed fused subunit has not been affected by the presence or absence of ferric ammonium citrate and sodium sulfide in the growth medium. Furthermore, concentrated cytoplasmic phase of *E. coli* cells

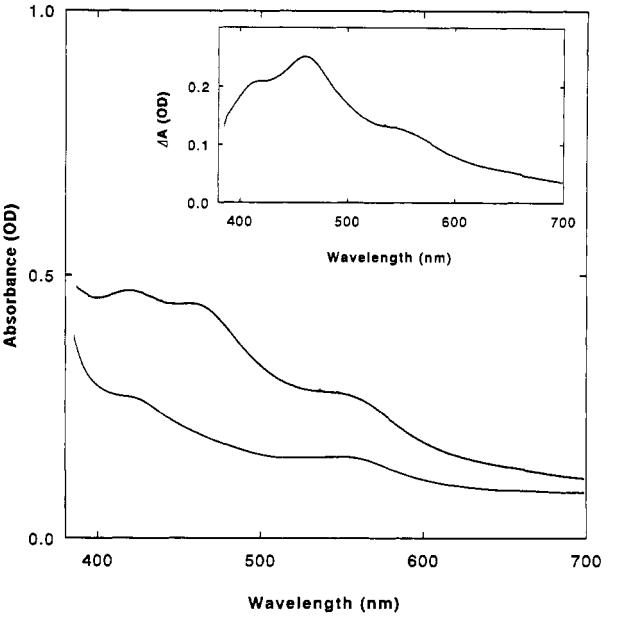

FIGURE 3: Absorption spectra of the purified fused 25-kDa (NQO2) subunit. The isolated subunit was diluted in 10 mM Tris-HCl (pH 8.0) containing 1 mM DTT to 9 mg of protein/mL. Upper curve, oxidized form of the 25-kDa subunit; lower curve, after addition of 1 M dithionite solution (pH 8.0) to a final concentration of 10 mM; curve of the inset, difference spectrum between oxidized and reduced forms. The spectra were measured at room temperature using an SLM-Aminco DW-2000 spectrophotometer.

containing the expressed full-length nonfused subunit also shows the same EPR line shape. The EPR signal of this cluster can be detected under a wide range of temperature (up to 77 K), and the relaxation behavior of this FeS species exemplified at 57 K and 13 K (see Figure 4, bottom) strongly indicates that the expressed NQO2 subunit ligates a single EPR-visible binuclear iron–sulfur cluster. As seen in Table 3, spectral and relaxation characteristics of the expressed *Paracoccus* NQO2 subunit are very similar to those of the [2Fe–2S] cluster component of FP fraction of mammalian complex I and FP subfraction, obtained by further resolution of FP which contains the 24-kDa subunit, a homolog of *Paracoccus* NQO2 (25-kDa) subunit (Xu et al., 1991a). Potentiometric titration of the [2Fe–2S] cluster of the expressed NQO2 subunit has been conducted using the g = 1.92 signal (pH 8.0, 29 K) according to Ohnishi et al. (1985). The [2Fe–2S] cluster of the expressed subunit titrated with E_m = –456 mV (n = 1). This value is consistent with the E_m = –465 mV (n = 1) of the [2Fe–2S] cluster of bovine heart FP fraction (Ohnishi et al., 1985).

DISCUSSION

Albracht et al. (1980) have reported that the membrane-bound NDH-1 segment of *P. denitrificans* contains one EPR-visible binuclear iron–sulfur cluster. Recently, Ohnishi and her colleagues (Meinhardt et al., 1987) have detected two distinct EPR-visible binuclear clusters in *Paracoccus* membrane-bound NDH-1 segment, which are designated N1a and N1b (Meinhardt et al., 1987). The cluster N1a, however, is labile and may give rise to variable spin concentrations and extremely low E_m values due to the facile modifications of the microenvironment around the cluster. In addition, rotenone-sensitive NADH–Q_1 reductase activity of *Paracoccus* membranes remains even after the signals of cluster N1a become completely EPR-undetectable (Yagi, 1991). Therefore, it

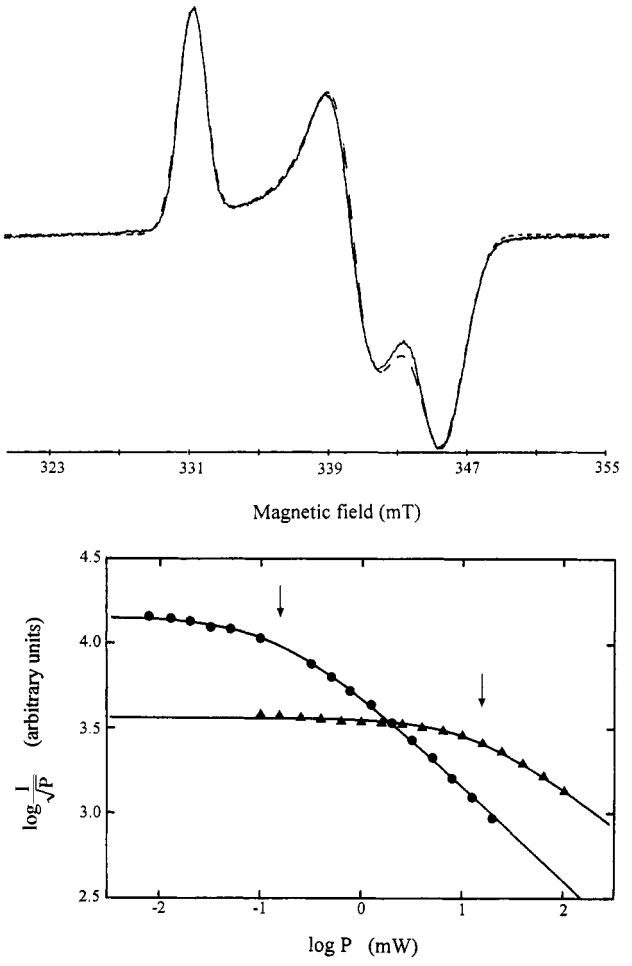

FIGURE 4: (Top) EPR spectrum of the expressed fused 25-kDa (NQO2) subunit of *Paracoccus* NDH-1. The soluble fraction containing the fused NQO2 subunit was concentrated to 44 mg of protein/mL and reduced with dithionite. EPR spectrum was recorded under the following conditions: microwave frequency 9.25 GHz, microwave power 1 mW, modulation amplitude 0.8 mT, time constant 0.128 s, sample temperature 29 K. Computer simulation of the spectrum (dashed line) was conducted as described in Blum and Ohnishi (1980); parameters used were $g_{x,y,z}$ = 1.913, 1.942, and 1.996 and $L_{x,y,z}$ = 1.42, 1.45, and 0.95 mT. The spin concentration of the cluster was determined as 102 μM (or 2.3 nmol/mg of protein) using 0.5 mM Cu(II)-EDTA as a standard. (Bottom) Power saturation behavior of the binuclear iron–sulfate cluster in the fused NQO2 subunit at 57 K (▲) and 13 K (●). The vertical arrows show values of half-saturation parameters ($P_{1/2}$). Those values were 15.5 and 0.16 mW at 57 and 13 K, respectively. EPR analysis was conducted as described in the top panel. A half-saturation parameter was determined according to the quantitative procedure reported by Blum and Ohnishi (1980). Through the experimental points theoretical best-fit curves were drawn. *I*, g = 1.91 trough amplitudes relative to base line in the higher magnetic field.

remains to be seen whether cluster N1a is an essential component of the *Paracoccus* NDH-1 or not.

Bovine heart complex I can be resolved into a water-soluble fraction and a water-insoluble fraction (Hatefi et al., 1985; Hatefi, 1985). The water-soluble fraction can be separated into a flavoprotein fraction (FP) and an iron–sulfur protein fraction by ammonium sulfate fractionation. FP is composed of three subunits of M_r 51 000, 24 000, and 9 000, which can be further resolved into a 51-kDa subunit fraction and a 24-kDa + 9-kDa subunit fraction. As described above, the bovine heart FP 24-kDa subunit is a homologue of the *Paracoccus* NQO2 (25-kDa) subunit. Bovine heart FP isolated rapidly on a small scale retains unmodified spectral characteristics of a binuclear cluster N1b ($g_{x,y,z}$ = 1.92, 1.94, and 2.03) and a

Table 3: Half-Saturation ($P_{1/2}$) Parameters of EPR Signals of FP and FP Subfraction from Bovine Heart Complex I[a] and of Expressed Fused *Paracoccus* NQO2 Subunit

fraction	signal (g-factor)	$P_{1/2}$ (mW) 13 K	$P_{1/2}$ (mW) 57 K
FP (51 + 24 + 9 kDa)	2.04 (N3)	7.2	>2500
	2.00 (N1b)	0.05	10.0
FP (24 + 9 kDa)	1.92	0.16	9.4
NQO2 subunit (25 kDa)	1.91	0.16	15.5

[a] Bovine heart complex I can be resolved into a water-soluble fraction and a water-insoluble fraction (Hatefi, 1985; Hatefi et al., 1985; Ragan, 1987). The water-soluble fraction can be divided into a flavoprotein fraction (FP) and an iron–sulfur protein fraction by ammonium sulfate fractionation. FP is composed of three subunits (51, 24, and 9 kDa) and contains noncovalently bound FMN and two EPR-visible iron–sulfur clusters (tetranuclear cluster N3 and binuclear cluster N1b). FP can be further resolved into a 51-kDa subunit fraction and a 24-kDa + 9-kDa subunit fraction. Values of half-saturation parameters of EPR signals concerning FP and FP subfraction (24-kDa + 9-kDa subunit) of bovine heart complex I were from Ohnishi et al. (1985).

tetranuclear center N3 ($g_{x,y,z}$ = 1.87, 1.95, and 2.04) (Ohnishi et al., 1981). However, the large-scale preparation of the whole FP as well as FP subfraction (24-kDa + 9-kDa subunit) gave rise to partially modified N1b signals ($g_{x,y,z}$ = 1.92, 1.95, and 2.00), suggesting a relatively sensitive nature of this binuclear cluster to its microenvironment. Since the 9-kDa subunit does not contain any cysteines, the 24-kDa subunit was suggested to harbor a [2Fe–2S] cluster N1b (Ragan et al., 1982; Ragan, 1987). The g values ($g_{x,y,z}$ = 1.913, 1.942, and 1.996), very similar to the latter N1b signals, were observed with the binuclear cluster present in the expressed NQO2 subunit. In terms of the E_m values, the binuclear cluster of the expressed NQO2 subunit is similar to the [2Fe–2S] cluster of bovine heart FP fraction. EPR signals from cluster N1b of the *Paracoccus* membrane-bound NDH-1 segment was simulated as an axial-type cluster with the g values of $g_{x,y,z}$ = 1.929, 1.941, and 2.019 (Meinhardt et al., 1987). These data suggest that the protein environment of the binuclear cluster in the expressed NQO2 subunit seems to be similar to that of the large-scale prepared FP or the resolved FP subfraction (24-kDa + 9-kDa subunit) of bovine heart complex I, whereas the environment around the binuclear cluster in the expressed NQO2 subunit appears to be slightly distinct from that around *Paracoccus* cluster N1b *in situ*. It should be noted that overlapping various EPR signals present in the membranes frequently makes it difficult to obtain an exact profile of EPR signals of the *in situ* iron–sulfur cluster of interest, although various attempts to extract the precise EPR feature of the iron–sulfur cluster of the membrane-bound NDH-1 segment have been made (Meinhardt et al., 1987, 1989, 1990). This is the case especially in bacterial membranes where NDH-1 is present in relatively low content as compared to mitochondria. Therefore, we conclude that a binuclear cluster N1 is present in the *Paracoccus* NDH-1 25-kDa subunit, but its final identity as N1a or N1b remains for further scrutiny.

As described previously (Xu et al., 1991a; Yagi et al., 1993), by searching through the amino acid residues that are capable of binding Fe and are conserved between the NQO2 subunit and its mitochondrial homologues, we assumed that His_{92}, Cys_{96}, Cys_{101}, Cys_{137}, and Cys_{141} are the candidates for binding of the iron–sulfur cluster, although four residues are enough for ligation of a binuclear iron–sulfur cluster. As shown in Figure 4, strong EPR signals can be detected in the expressed fused NQO2 subunit. Therefore, it may be possible to determine the residues of the NQO2 subunit involved in

ligation of iron–sulfur cluster utilizing site-directed mutagenesis and spectroscopic techniques.

The isolated fused NQO2 subunit contains 1.44 mol of Fe and 1.33 mol of S^{2-}/mol of subunit. In addition, EPR studies of the fused NQO2 subunit indicate that the subunit carries a single binuclear cluster. Taken together, approximately 70% of the fused NQO2 subunit expressed in the cytoplasmic phase may ligate the EPR-visible binuclear cluster. In the future, we will attempt to express 100% of iron–sulfur cluster-ligated NQO2 subunit in host cells by improving conditions of cell culture and the isolation procedure and/or to conduct purification of iron–sulfur cluster-ligated NQO2 subunit. Such a homogeneous iron–sulfur cluster-ligated NQO2 subunit will provide a promising useful material for various physicochemical measurements which can clarify the fine structure of this subunit. This type of approach should shed light on investigation of the entire structure of the energy-transducing NADH–quinone oxidoreductase (NDH-1 and complex I), which is referred to as the most structurally intricate membrane-bound enzyme complex (Ragan, 1987; Weiss et al., 1991; Walker, 1992; Yagi, 1993).

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