Characterization of the Iron-Sulfur Cluster N7 (N1c) in the Subunit NuoG of the Proton-translocating NADH-quinone Oxidoreductase from Escherichia coli*

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The proton-pumping NADH-quinone oxidoreductase from Escherichia coli houses nine iron-sulfur clusters, eight of which are found in its mitochondrial counterpart, complex I. The extra putative iron-sulfur cluster binding site with a CXXXXXXC motif in the NuoG subunit has been assigned to ligate a [2Fe-2S] (N1c). However, we have shown previously that the Thermus thermophilus N1c fragment containing this motif ligates a [4Fe-4S] (Nakamaru-Ogiso, E., Yano, T., Ohnishi, T., and Yagi, T. (2002) J. Biol. Chem. 277, 1680–1688). In the current study, we individually inactivated four sets of the iron-sulfur binding motifs in the E. coli NuoG subunit by replacing all four ligands with Ala. Each mutant subunit, designated ΔN1b, ΔN1c, ΔN4, and ΔN5, was expressed as maltose-binding protein fusion proteins. After in vitro reconstitution, all mutant subunits were characterized by EPR. Although EPR signals from cluster N1b were not detected in any preparations, we detected two [4Fe-4S] EPR signals with g values of g_{x,y,z} = 1.89, 1.94, and 2.06, and g_{x,y,z} = 1.91, 1.94, and 2.05 at 6–20 K in wild type, ΔN1b, and ΔN5. The former signal was assigned to cluster N4, and the latter signal was assigned to cluster N1c because of their disappearance in ΔN4 and ΔN1c. Confirming that a [4Fe-4S] cluster ligates to the N1c motif, we propose to replace its misleading [2Fe-2S] name, N1c, with “cluster N7.” In addition, because these mutations differently affected the assembly of peripheral subunits by in trans complementation analysis with the nuoG knockout strain, the implicated structural importance of the iron-sulfur binding domains is discussed.

The proton-translocating NADH-quinone oxidoreductase is the first energy-transducing complex in the respiratory chains of many prokaryotes and eukaryotes (1–6). The enzyme complex is one of the most complicated and elaborate iron-sulfur proteins ever known (7). Mitochondrial enzyme (complex I) and its bacterial counterpart (NDH-1)1 contain at least six EPR-detectable iron-sulfur clusters: N1a, N1b, N2, N3, N4, and N5 (4, 8, 9). Clusters N1a and N1b are binuclear ([2Fe-2S]i), and N2, N3, N4, and N5 are tetranuclear ([4Fe-4S]) clusters. Based on the sequence analyses, however, NDH-1/complex I contains up to eight iron-sulfur clusters. Recently, two remaining [4Fe-4S] clusters located in TKY/Nqo9/NuoI subunit (nomenclature used for bovine heart mitochondria/Paracoccus denitrificans/Escherichia coli enzymes), have been detected by means of UV-visible spectroscopy but not by EPR and designated N6a and N6b (10, 11). To elucidate the electron transfer mechanism in the NDH-I/complex I, it is necessary to characterize the individual iron-sulfur clusters and identify their subunit locations. For this purpose, we have overexpressed the putative cofactor-binding subunits of P. denitrificans and Thermus thermophilus NDH-I, such as Nqo1, Nqo2, Nqo3, and Nqo9, in E. coli, and characterized the iron-sulfur clusters in these subunits (10, 12–19).

Interestingly, in some organisms such as E. coli and T. thermophilus, the NDH-I contains an additional iron-sulfur cluster binding motif (20) in the subunit NuoG for E. coli and NuoG3 for T. thermophilus (12, 13, 20). This motif is not found in either P. denitrificans NDH-I or mitochondrial complex I (Fig. 1). It has been proposed that this additional unique cysteine sequence motif is responsible for the EPR signals from the binuclear cluster N1c of the E. coli NDH-I (21–23). In our previous report, however, we have shown that the Thermus N1c fragment containing this motif ligates a [4Fe-4S] cluster, not a [2Fe-2S] cluster, by UV-visible and EPR spectroscopic analyses (12).

In the present study, to characterize the N1c cluster at the subunit level and distinguish it from three other iron-sulfur clusters (N1b, N4, and N5 accommodated in the NuoG subunit), we have individually inactivated four iron-sulfur cluster binding sites by substituting Ala for all four conserved Cys (or His) residues. We overexpressed wild type and the individual iron-sulfur-cluster mutant NuoG subunits, designated ΔN1b, ΔN1c, ΔN4, and ΔN5, as maltose-binding protein (MBP) fusion proteins in E. coli. We compared EPR spectra of the individual mutant NuoG subunits and identified the [4Fe-4S] EPR signals, which could not be assigned to either N4 or N5 cluster. Based on our present analyses and the fact that the

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1 The abbreviations used are: NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; MBP, maltose-binding protein; MBP-NuoG, MBP-fused NuoG; WT, wild type; mW, milliwest.
motif \textit{CXX}, coordinates only [4Fe-4S] clusters in other known enzymes such as the periplasmic nitrate reductase in \textit{P. denitrificans} (24), we concluded that the N1c binding motif undoubtedly ligation a [4Fe-4S]. Therefore, we propose to change its misleading name, N1c, to N7. In addition, we attempted to see the effects of each cluster mutation on the assembly of the peripheral subunits by complementing the \textit{nuoG} knockout \textit{E. coli} mutant with mutated plasmids.

**EXPERIMENTAL PROCEDURES**

**Materials**—The PCRScript Cloning kit was from Stratagene (La Jolla, CA). The expression vector pMALc2g, amylose resin, and anti-MBP serum were from New England Biolabs (Beverly, MA). Materials for PCR product purification, gel extraction, and plasmid preparation were obtained from Qiagen (Valencia, CA). The BCA protein assay kit and SuperSignal West Pico chemiluminescent substrate were from Pierce.

\textit{E. coli} strain BL21(DE3) was purchased from Novagen. The gene replacement vector pKO3 was a generous gift from Dr. George M. Church (Harvard Medical School). The \textit{E. coli} strain MC4100 was a gift from Dr. Mutsuo Yamaguchi (The Scripps Research Institute). All chemicals used were of the highest grade available from Sigma.

**Construction of Expression Vectors for the \textit{E. coli} \textit{NuoG} Subunit**—pET20b/\textit{nuoG} (Ndel/BamHI) (for non-fused \textit{NuoG}) and pET20b/\textit{nuoG} (Ndel/XhoI) (for His-tagged \textit{NuoG}) plasmids were a generous gift from Dr. Marta Perego (The Scripps Research Institute, La Jolla, CA). For ligation into a pMALc2g vector, the forward and reverse oligonucleotide primers, \textit{nuoG}-F (sense) and \textit{nuoG}-R (antisense), were designated to generate EcoRI and BamHI sites near the \textit{nuoG} initiation and stop codons, respectively: \textit{nuoG}-F (sense), 5'/H11032\ AGGA\ AGG\ AGA\ CTG-3'/H11032 and \textit{nuoG}-R (antisense), 5'/H11032\ GGATC\ CTG-3'/H11032. (The italic bases were altered from \textit{E. coli} sequences; the underlined bases indicate the newly introduced restriction sites). The amplified DNA fragment (2700 bp) was subcloned into the pCRscript vector and verified by sequencing. EcoRI/BamHI \textit{nuoG} fragments digested from pCRscript/\textit{nuoG} were ligated into a pMALc2g as pMALc2g/\textit{nuoG}.

**Expression and Purification of the \textit{NuoG} Subunits**—Expression of the \textit{NuoG} subunits with pET20b/\textit{nuoG} (with or without a His tag) and pMALc2g/\textit{nuoG} vectors was conducted basically according to Nakamura-Ogiso et al. (12). Briefly, competent \textit{E. coli} strain BL21(DE3) cells were transformed with expression vectors, and the cells were grown overnight at 30–33 °C until A 600 reached ~2.0. After the cells were cooled down to 20 °C followed by the addition of 0.4 mM isopropyl 1-thio-\textbeta-D-galactopyranoside, the cells were further incubated for 3–5 h. The cells harvested were suspended in 7.5% (w/v) in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol, which had been degassed and purged with oxygen-free argon on ice before use. The cells were broken up by two passages through a French pressure cell (Scientific Instruments, FIG. 1. Multiple sequence alignments of the N-terminal region of the \textit{NuoG} subunit (total 910 amino acids) in \textit{E. coli} NDH-1 with its homologues from various organisms. The numbering is according to the \textit{E. coli} sequences. The putative binding sites for the four iron-sulfur clusters are highlighted by gray shading. The contributions of each cysteine residue to the binding of four iron-sulfur clusters are indicated by arrows. \textit{E. coli} (GenBank accession number AAC75543); \textit{T. ther}, \textit{Thermus thermophilus} (GenBank accession number Q56223); \textit{P. deni}, \textit{Paracoccus denitrificans} (GenBank accession number AAI25587); \textit{N. cras}, \textit{Neurospora crassa} mitochrondria (GenBank accession number CAB91229); \textit{B. taur}, \textit{Bos taurus} mitochondria (GenBank accession number AAA30662).
DNA-purified Knock-out Strain (the MBP-NuoG mutants were conducted according to the same procedure). Expression, purification, and reconstitution of the MBP-NuoG subunits were analyzed for expression levels of each form of the NuoG subunit. The column was washed with 60 ml of the same buffer, and then the MBP-NuoG protein was eluted with the same buffer containing 10 mM maltose and 0.2 mM 4-(2-aminoethyl)-benzenesulfonfonyl fluoride.

In Vitro Reconstitution of the Iron-Sulfur Clusters into the MBP-NuoG Subunits—The reconstitution procedures were carried out in the anaerobic chamber by a modified method of Yano et al. (10, 16). The purified MBP-NuoG subunit was diluted to 10–20 μM with the column buffer containing 10% (v/v) glycerol. β-Mercaptoethanol was added to the protein solution at 1.0% (v/v), and the solution was gently mixed and left for 90 min. Fe(NH₄)₂(SO₄)₆ and Na₂S were added to the solution at a final concentration of ~500 μM. The reconstitution proceeded for 2–3 h. Excess Fe²⁺ and S²⁻ were removed by a desalting column (Bio-Rad, 10-DG). The reconstituted MBP-NuoG protein was applied onto a DEAE (Toyopearl 650 m) column (0.8 × 1.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 15% (w/v) glycercoll. The cell suspension was then passed once in a French press at 20,000 p.s.i. and centrifuged again in the GSA rotor at 12,000 rpm for 1.0 cm) equilibrated with the column buffer, 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM β-mercaptoethanol, 200 mM NaCl, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The column was washed with 60 ml of the same buffer, and then the MBP-NuoG protein was eluted with the same buffer containing 10 mM maltose and 0.2 mM 4-(2-aminoethyl)-benzenesulfonfonyl fluoride.

Site-directed Mutagenesis of Individual Iron-Sulfur Clusters in the MBP-NuoG Subunits—Site-directed mutagenesis was performed by using the GeneEditor™ in vitro site-directed mutagenesis system kit from Promega (Madison, WI). The pCRScript/nuoG (EcoRI/BamHI) plasmid was digested with the DNA template. All four conserved Cys (or His) residues in each iron-sulfur cluster were mutated to Ala subsequently. Mutated DNA fragments, EcoRI/NruI (~1 kb) for ΔN1b(C36A,G47A,C50A, C69A), ΔN5(H101A,C105A,C108A,C114A), and ΔN4(C153A,C156A, C159A,C203A) and BglII/NruI (~1 kb) for ΔN1c(C230A,C233A,C237A, C265A), were ligated to the original pMAL2g2/nuoG plasmid by using intrinsic sites in the nuoG. The final constructs were designated pMAL2g2/nuoGΔ1b, pMAL2g2/nuoGΔ1c, and pMAL2g2/nuoGΔ1c. Expression, purification, and reconstitution of the MBP-NuoG mutants were conducted according to the same procedure as described for the wild type.

Cloning of the E. coli nuoG Gene and Construction of the nuoG Knock-out Strain (ΔnuoG::Spc)—The gene encoding the NuoG subunit was amplified using the DNA fragment upstream and an ~220-bp DNA segment downstream, was cloned by PCR from genomic DNA-purified E. coli DH5α. We used intrinsic sites SmaI for the sense primer, 5′-CTTGGCCCGGTTAATACTTTCGGCCACCGC-3′, and BglII or BamHI for the antisense primers, 5′-CCGCGGATCTGTGAGCTCAGGTAATC-3′ or 5′-CCGAGAATTCGGGATCCAGTCTCTTTTAAAGAC-3′, respectively (the italicized bases represent the restriction site sequence). The spectromycin (sps) gene cassette from the Staphylococcus aureus transposon Ts54 (25) was cloned by PCR. The DNA fragments were assembled together with the spc cassette by using the intrinsic BglII site in pCRScript and finally transferred to pKO3. The resulting construct was designated pKO3 (nuoG-spc). The E. coli strain MC4100 was transformed with the pKO3 (nuoG-spc) plasmid, and homologous recombination was carried out as described in Link et al. (26). The presence of the spc cassette and its location in the genomic nuoG gene were verified by PCR and DNA sequencing.

Membrane Preparation—The cell pellet was resuspended at 10% (w/v) in a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 15% (w/v) glycerol. The cell suspension was then passed once in a French press at 20,000 p.s.i., and centrifuged again in the GSA rotor at 12,000 rpm for 10 min. Cell debris was discarded, and the supernatant was then ultracentrifuged in a 70Ti rotor at 50,000 rpm for 30 min. The pellet was resuspended in the same buffer and used immediately for various analyses.

EPR Spectroscopy—EPR samples were prepared in an anaerobic chamber, and the redox mediators, methyl viologen and benzyl viologen, were added at 5 μM each. Anaerobically prepared neutralized SDS-PAGE and blue native PAGE were carried out according to Laemmli (34) and Schagger (35), respectively. Immunoblotting was conducted as described previously (36). Non-heme iron and acid-labile sulfide were determined according to Refs. 37 and 38, respectively. Any variations from the procedures and other details are described in the figure legends.

RESULTS

Expression, Isolation, and Reconstitution of the MBP-NuoG Subunit and Its Iron-Sulfur Cluster Mutants—The purified wild type, MBP-NuoG(WT), was reddish-brown and exhibited absorption spectra as shown in Fig. 2A, which were similar to those of its homologue, the MBP-fused T. thermophilus Nuo3.
subunit described by Nakamaru-Ogiso et al. (12). The purified MBP-NuoG(WT) contained only 1.9 mol of non-heme Fe and 1.4 mol of $S^2$ /mol of protein, indicating that the iron-sulfur cluster contents were much lower than expected amounts (12 or 14 mol of each Fe and $S^2$ /mol of protein). However, the contents of non-heme iron and acid-labile sulfide in MBP-NuoG(WT) were significantly improved upon the in vitro reconstitution to 7.9 mol of Fe and 5.9 mol of $S^2$ /mol of protein on average. The absorption spectrum of the reconstituted MBP-NuoG(WT) exhibited a broad shoulder around 380–420 nm with a tailing reaching 700 nm. The absorption spectrum did not show peaks at 320, 460, and 560 nm, which are prominent for [2Fe-2S] clusters, suggesting that the reconstitution of a binuclear cluster(s) in the NuoG subunit was not successful (Fig. 2B). UV-visible spectra of the reconstituted MBP-NuoG mutants showed slightly different spectral features (Fig. 2, B and C). A broad absorption peak around 420 nm, which is characteristic of [4Fe-4S]$^{2+}$ clusters, was lower than that of wild type. The absorbance was roughly proportional to the iron-sulfur-cluster contents in the reconstituted subunits, most likely because of the inactivation of the putative iron-sulfur cluster binding motifs (Fig. 2, B and C).

**EPR Characterization of the Iron-Sulfur Clusters in the MBP-NuoG Mutants**—To characterize the bound iron-sulfur clusters in the reconstituted MBP-NuoG mutants, we performed EPR analyses. First, we examined EPR spectra of the dithionite-reduced samples at the $g = 2$ region at 40–60 K. We did not detect any EPR signals in all preparations including MBP-NuoG(WT), indicating that the reconstituted subunits did not contain the binuclear cluster N1b (results not shown). When EPR spectra were measured at 6–30 K, EPR signals arising from [4Fe-4S]$^{2+}$ clusters were detected, and the individual samples exhibited different spectra. As shown in Fig. 3, MBP-NuoG(WT) displayed multiple EPR resonances with $g$ values of 1.89, 1.91, 1.94, and 2.05 at 12 K and 5 mW. At 6 K, EPR signals at $g = 1.91$ and $g = 2.05$ became smaller and broader because of the saturation, whereas the signal at $g = 1.89$ and a broad resonance at $g = 2.06$ became more pronounced. Further inspection of the EPR spectra at 4–50 K and 5 mW showed that the $g = 1.91$ and $g = 2.05$ resonances were best seen at 14–16 K, whereas the EPR signals at $g = 1.89$ and $g = 2.06$ showed the optimal temperature at $-10$ K. These observations indicate that MBP-NuoG(WT) contains at least two distinct [4Fe-4S] clusters. The mutants, MBP-NuoGΔN5 (Fig. 3) and MBP-NuoGΔN1b (data not shown), exhibited almost the same EPR spectra as those of MBP-NuoG(WT) under these conditions except that slight $g$ value shifts were noticed. When MBP-NuoGΔN5 was examined at 4 K and various microwave power levels, the $g = 1.91$ resonance was readily saturated at low power levels ($P_{1/2} = 0.02$ mW at 4 K), whereas the signals at $g = 1.89$ and $g = 2.06$ were saturated at higher power levels ($P_{1/2} = 1.0$ mW at 4 K). In contrast, MBP-NuoGΔN4 showed different EPR spectra. At 12 K, it showed the EPR signal with $g_{x,y,z} = 1.91, 1.94$, and 2.05. However, the EPR signals at $g = 1.89$ and $g = 2.06$ were absent. At 6 K, the EPR signal was partially saturated, and the line width became broader. The behaviors of this EPR signal were similar to the corresponding EPR signals observed in wild type, MBP-NuoGΔN1b, and MBP-NuoGΔN5. From these observations, it was concluded that the EPR signal with $g_{x,y,z} = 1.91, 1.94$, and 2.06 arose from the [4Fe-4S] cluster coordinated in the N4 binding site.

MBP-NuoGΔN1c exhibited very small and broad EPR signals at both 6 and 12 K, which were completely different from the EPR signals arising from the two [4Fe-4S] clusters detected in MBP-NuoG(WT) (Fig. 3). The contents of non-heme iron and acid-labile sulfide in this mutant after in vitro reconstitution were the lowest among all of the preparations, indicating that the inactivation of the N1c cluster-binding motif severely affected in vitro incorporation of the iron-sulfur cluster into this protein. Because the EPR signal with $g_{x,y,z} = 1.91, 1.94$, and 2.05 was detected in wild type and all other mutant subunits
except for MBP-NuoGΔN1c, we concluded that the EPR signal arose from the [4Fe-4S] cluster coordinated in the N1c motif.

To resolve the EPR spectra of these two [4Fe-4S] clusters, we attempted to simulate the EPR spectra of MBP-NuoG/H9004 N5 that contained only two [4Fe-4S], one each in the N4 and N1c binding sites. For the first approximation, we retrieved EPR parameters for the [4Fe-4S] cluster coordinated in the N1c binding site from the EPR spectrum of MBP-NuoG/H9004 N4 measured at 14 K and 5 mW. Under this condition, neither of the EPR signals of the two [4Fe-4S] clusters was saturated, and almost no contribution from other iron-sulfur clusters can be assumed. Then, the second [4Fe-4S] cluster EPR signal was simulated. Fig. 4A shows the EPR spectra of MBP-NuoGΔN5 using the following parameters for the two [4Fe-4S] clusters: for cluster N1c, \( g_{x,y,z} = 1.91, 1.94, \) and \( 2.05 \) and \( L_{x,y,z} = 21, 12, \) and \( 12.5 \) gauss; and for cluster N4, \( g_{x,y,z} = 1.89, 1.94, \) and \( 2.06 \) and \( L_{x,y,z} = 55, 38, \) and \( 65 \) gauss. The ratio of the clusters was estimated to be N1c:N4 = 3:1. Using these parameters, we obtained a reasonably good fit for the EPR spectrum of MBP-NuoG(WT) at 14 K and 5 mW (Fig. 4B).

The EPR Signal Arising from Cluster N5—We attempted to resolve the EPR spectra of cluster N5 in MBP-NuoG proteins. Cluster N5 has been detected in bovine complex I (4), yeast Yarrowia lipolytica complex I (8), and the overexpressed Nqo3 subunit from P. denitrificans (39); however, the EPR signal of cluster N5 has not been reported for the E. coli NDH-1 thus far. In general, the spin relaxation of cluster N5 is so fast that the EPR signal is only detected at 4–6 K. We examined EPR spectra of MBP-NuoGΔN4 at 4 K and at 1 and 10 mW. It is apparent that the \( g = 1.91 \) and \( g = 2.05 \) resonances arising from the [4Fe-4S] cluster in the N1c binding motif were saturated at 10 mW (Fig. 5). At the same time, rather broad resonance became noticeable at the \( g = 1.85–1.88 \) region as well as at \( g = -2.06 \), which seemed likely to be derived from a distinct [4Fe-4S] cluster with much faster spin relaxation properties. In the case of the P. denitrificans Nqo3 subunit, a portion of the electron spin of cluster N5 has been shown to take a \( S = 3/2 \) ground state, giving rise to EPR signals at \( g = -5 \) (39). We also examined a lower magnetic field to search for EPR signals; however, we could not detect any EPR signal at the \( g = -5 \) region in any of NuoG mutants examined. Despite several attempts, we were not able to show conclusively that the EPR signals were arising from cluster N5 in the expressed subunits mainly because of the low concentration of the [4Fe-4S] cluster. However, the broad EPR signals shown in Fig. 5 somehow resemble those of cluster N5 in the overexpressed P. denitrifi-
MBP-NuoGΔN1b, the expression of the NuoB, NuoCD, and NuoF subunits was detected, whereas the NuoE subunit was missing. For the expression of MBP-NuoGΔN1c and MBP-NuoGΔN5, only NuoB and NuoCD were detected. Interestingly, in the case of MBP-NuoGΔN4, the NuoCD subunit was absent. The amount of NuoB subunit was much less in the MBP-NuoGΔN4 and MBP-NuoGΔN5 subunits. These results suggest that the incorporation of the iron-sulfur clusters is important for the assembly of the peripheral subunits and that the individual iron-sulfur clusters are involved in the association with the other peripheral subunits in different manners.

**DISCUSSION**

In this study, we were able to detect at least two [4Fe-4S] clusters and identify their binding sites by EPR analyses. The EPR signal with $g_{x,y,z} = 1.89, 1.94,$ and 2.06 was assigned to cluster N4 and was consistent with the results of the expressed *F. denitrificans* Nuo3 subunit (18). The EPR signal of cluster N4 was similar to that in the entire NDH-1 enzyme from *E. coli* with $g$ values of $g_{x,y,z} = 1.89, 1.93,$ and 2.09 (23) except for the position of the $g_z$ resonance. The second EPR signal with $g_{x,y,z} = 1.91, 1.94,$ and 2.05 was assigned to the [4Fe-4S] cluster coordinated in the N1c binding motif. This observation confirmed that the N1c cluster motif coordinates a [4Fe-4S] cluster. The EPR signal of this [4Fe-4S] cluster showed rhombic symmetry, and the EPR line shape was almost identical among all of the NuoG mutant subunits, suggesting that the [4Fe-4S] cluster is coordinated within the rather stable polypeptide structures. The effects of the protein environments on the EPR properties of the [4Fe-4S] cluster were apparent when the EPR spectra of the [4Fe-4S] cluster in the MBP-NuoG subunits were compared with that of the [4Fe-4S] cluster in the *Thermus* N1c fragment (12). The N1c fragment is composed of only 85 amino acids compared with 910 amino acids for the NuoG subunit, and the bound [4Fe-4S] cluster in the N1c fragment showed a broad EPR signal with nearly axial symmetry ($g_{x,y,z} = 1.94, 1.94, 2.045$). Therefore, it is possible that the [4Fe-4S] cluster in the expressed MBP-NuoG subunits is coordinated in the protein environments that are close to those in the entire enzyme complex. Our observation is also consistent with the fact that the motif CXXX[XXX]C exclusively utilizes to coordinate a [4Fe-4S] cluster in other enzymes such as the formate dehydrogenase H in *E. coli* (CXXX[XXX]C$^{[40]}$) and the periplasmic nitrate reductase in *Desulfovibrio desulfuricans* (ATCC 27774) (CXXX[XXX]C$^{[41]}$). Thus, we propose to rename it cluster N7.

This work enabled us to determine the type and the number of iron-sulfur clusters coordinated in the NDH-1 from *E. coli* and *T. thermophilus*. The NDH-1 of these bacteria contains 2×[2Fe-2S] cluster (N1a and N1b) and 7×[4Fe-4S] clusters (N2, N3, N4, N5, N6a, N6b, and N7). In the *E. coli* NDH-1, clusters N1a, N1b, N2, N3, and N4 have been spectroscopically identified thus far. The EPR signal of cluster N7 ($g_{x,y,z} = 1.91, 1.94,$ and 2.05), however, is not readily recognized in the EPR spectra of the entire *E. coli* NDH-1 enzyme published earlier because of significant overlaps of EPR signals arising from other iron-sulfur clusters, cluster N2 ($g_{x,y,z} = 1.91, 1.91,$ and 2.05), cluster N3 ($g_{x,y,z} = 1.88, 1.92,$ and 2.04), and cluster N4 (23). In the case of the *T. thermophilus* NDH-1, two [2Fe-2S] clusters and four [4Fe-4S] clusters were detected and potentiometrically resolved by EPR spectroscopy$^2$ suggesting that there are no more than two [2Fe-2S] clusters, but at least one more [4Fe-4S] cluster besides N2, N3, and N4. Therefore, it will be necessary to reinvestigate the EPR signals of the iron-sulfur clusters in the *E. coli* NDH-1.

$^2$ T. Yano and T. Ohnishi, unpublished results.
The 75-kDa/Nqo3/NuoG subunit has been suggested to play a structurally important role in connecting the flavoprotein subcomplex, other amphipathic subunits, and hydrophobic subunits together in the NDH-1/complex I (42, 43). Consistently, the knock-out of the nuoG gene resulted in the loss of the NDH-1 assembly in the cytoplasmic membranes. All peripheral subunits were absent except that rather large quantities of NuoA subunit (the gene of which is located upstream of the nuoG gene) was not detected in the membranes, it seems likely that the NuoCD subunit alone is able to directly associate with the cytoplasmic membrane. When the MBP-NuoG(WT) subunit was expressed, we observed significantly different effects on the subunit assembly. Similar results were reported in subunit NuoI (46). These results suggested that the individual iron-sulfur cluster binding domains seem to play roles in interactions with the neighboring subunits, which are essential for the intersubunit electron transfer pathway. Further study is in progress in our laboratories.

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