Amino acid residues associated with cluster N3 in the NuoF subunit of the proton-translocating NADH-quinone oxidoreductase from *Escherichia coli*

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Abstract The NuoF subunit, which harbors NADH-binding site, of *Escherichia coli* NADH-quinone oxidoreductase (NDH-1) contains five conserved cysteine residues, four of which are predicted to ligate cluster N3. To determine this coordination, we overexpressed and purified the NuoF subunit and NuoF + E subcomplex in *E. coli*. We detected two distinct EPR spectra, arising from a [4Fe–4S] cluster ($g_{x,y,z}$ = 1.90, 1.95, and 2.05) in NuoF, and a [2Fe–2S] cluster ($g_{x,y,z}$ = 1.92, 1.95, and 2.01) in NuoE subunit. These clusters were assigned to clusters N3 and N1a, respectively. Based on the site-directed mutagenesis experiments, we identified that cluster N3 is ligated to the $^{351}Cx_2Cx_2Cx_{40}C^{398}$ motif.

Keywords: Complex I; NuoF; Iron–sulfur cluster; NADH-quinone oxidoreductase; Electron paramagnetic resonance; *Escherichia coli*

1. Introduction

Bacterial proton-translocating NADH-quinone oxidoreductase (NDH-1) oxidizes NADH and transfers two electrons to quinone with concurrent proton translocation [1–5]. In general, bacterial NDH-1 is composed of 14 different subunits and contains one FMN and eight iron–sulfur clusters as prosthetic groups and considered as a minimal structural form of mitochondrial counterpart, complex I [2]. In the *Escherichia coli* enzyme, subunit C and D are fused as single subunit (C · D), and 9th iron–sulfur cluster (N7 [3]) is housed. This enzyme complex is composed of three parts: the NADH dehydrogenase, the connecting, and the membrane parts. The hydrophilic NADH dehydrogenase part contains the NuoE, F, and G subunits and harbors one FMN, two [2Fe–2S] clusters (N1a and N1b), and four [4Fe–4S] clusters (N3, N4, N5, and N7) [3,6–10]. Although cluster N3 is housed in the NuoF subunit, ligand residues of this cluster have not yet been rigorously determined. Therefore, we overexpressed the NuoF subunit alone or as the NuoF + E subcomplex and characterized the purified proteins. Using site-directed mutagenesis approach, we have demonstrated that C351, C354, C357, and C398 in the NuoF subunit are involved in the ligation of cluster N3.

2. Materials and methods

The *E. coli* nuoF gene was amplified by PCR from genomic DNA using two primers, GGAGCGGTACATATGAAAAACATTATCCGTATCCC (sense) and GTTAATCGAACTCGAGTTACCAGCGCTCTTTCAGC (antisense). The underlined bases were altered from *E. coli* DNA to introduce a *Nde*I and a *Xho*I site, respectively. The PCR product was first cloned in pCRSCRIPT (Stratagene) and then ligated into the pET16b expression vector (pET16/nuoF). For the NuoF + E subcomplex expression, a DNA fragment encompassing the nuoE and nuoF genes was amplified by PCR with the following two primers: CCGCTACATATGCACGAGAATCAACAACCAC (sense) and AGCGTTAATCGGATCCGGTTACCAGCGTC (antisense). The final construct was designated as pET16/nuoEF. Site-directed mutagenesis was performed by using QuikChange II XL site-Directed Mutagenesis Kit from Stratagene. The following 10 primers (forward and reverse) were used: For the C180A mutation, (1) GGGCGCTACATCGCCGGGGAAGAAACAGC and (2) GCTGTTTCTTCCCCGGCGATGTAGCGCCC; for the C351A mutation, (3) GCCCGTGAGTCCGCCGGCTGGTGTACG and (4) CGTACACCAGCCGGCGGACTC ACGGGC; for the C354A mutation, (5) CCTGCGGCTGGGCTACGCCGTGCCG and (6) CGGCACGGCGTAGCCCAGCCGCAGG; for the C357A mutation, (7) TGGTGTACGCCGGCCCGCGACGGTCTG and (8) CAGACCGTCGCGGGCCGGCGT ACACCA; for the C398A mutation, (9) CCGGGTAAAACTTTCGCTGCCCACGCACCTG and (10) CAGGTGCGTGGGCAGCGAAAGTTTTACCCGG. All the constructs were verified by sequence analyses before use.

E. coli strain BL21(DE3) transformed with pET16b/nuoF or pET16b/nuoEF were cultured aerobically in 1 L TB medium containing 100 μg/ml ampicillin at 37 °C until A_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to be 0.4 mM and the cells were grown at 25 °C for 4 h. Expressed proteins were purified from the cytoplasmic fraction according to [6] with minor modifications. The isolated proteins were immediately used for experiments. In some experiments, the isolated proteins were reconstituted with FMN basically according to [8] and used.

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[3] Cluster N7 is present only in the limited numbers of bacterial NDH-1.

Abbreviations: NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; EPR, electron paramagnetic resonance

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NADH-ferricyanide reductase activity was measured in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA at 30 °C by following absorbance at 420 nm. Electron paramagnetic resonance (EPR) spectra were measured as described in [8]. Native gel electrophoresis [11] and NADH dehydrogenase activity staining [12] were carried out as before. SDS–polyacrylamide gel electrophoresis was performed by a modified method of Laemmli [13]. The antibody against the *E. coli* NuoF subunit was raised in a rabbit [14]. Immunoblotting was conducted as described previously [15]. Protein concentration was determined by Coomassie Protein Assay Kit from Pierce [16]. Contents of flavin, non-heme iron and acid-labile sulfide were determined according to [16–18], respectively.

3. Results

The His-tagged NuoF subunit was overexpressed in *E. coli* and was purified with a nickel chelation affinity column. As shown in Fig. 1A, the NuoF protein was co-purified with a small amount of the NuoE subunit. The His-tagged NuoF subunit (52 kDa) and the NuoE subunit (25 kDa) were recognized by anti-NuoF and anti-NuoE antibodies, respectively (data not shown). A molar ratio of the co-purified NuoE subunit relative to NuoF was estimated to be approximately1:10. The contents of FMN, non-heme Fe, and S^{2-} in the purified NuoF preparation (called NuoF preparation hereafter), were 0.904 ± 0.48, [4] 21.5 ± 4.2, and 21.3 ± 7.9 nmol/mg protein, respectively. Iron–sulfur clusters were partially (approximately 30%) incorporated in vivo. Our attempt to increase cofactor contents by supplementing culture media with either riboflavin, FMN, or ferrous ammonium sulfate, sodium sulfide was unsuccessful. However, NADH dehydrogenase activity staining revealed a distinctive violet-blue band that corresponds to the position of NuoF on the native gel (Fig. 1B), indicating that a trace amount of FMN still remained in this NuoF preparation.

The EPR spectrum of the dithionite-reduced NuoF preparation showed the presence of [4Fe–4S] and [2Fe–2S] clusters (Fig. 2). The $[4Fe–4S]^{1+}$ cluster exhibited a rhombic EPR spectrum with g values of $g_{x,y,z}$ = 1.90, 1.95, and 2.05 (Fig. 2A). This species was detectable within a temperature range between 6 and 30 K. The EPR properties of this cluster are very similar to those of cluster N3 in the *E. coli* NDH-1 ($g_{x,y,z}$ = 1.88, 1.94, 2.044) [2] and those of the $[4Fe–4S]^{1+}$ cluster ($g_{x,y,z}$ = 1.87, 1.94, and 2.035) housed in a NuoF homologue, the Nqo1 subunit from *Paracoccus denitrificans* [8]. The $[2Fe–2S]^{1+}$ cluster exhibited a rhombic EPR spectrum with g values of $g_{x,y,z}$ = 1.92, 1.95, and 2.01 (Fig. 2B) and was detectable within a wide temperature range from 10 up to 80 K examined in this study. The EPR spectrum is almost identical to those of clusters N1a of the *E. coli* NuoE subunit and of the *P. denitrificans*/*Thermus thermophilus* Nqo2 subunits [6,19].

Since the isolated NuoF preparation was very unstable and easily aggregated even in the presence of 30–50% glycerol, we employed the co-expression of the two genes, *nuoE* and *nuoF*, which was successful for the *Paracoccus* Nqo1 + 2 subcomplex. In fact, this system improved the stability of the subcomplex, resulting in a higher yield. After purification, the molar ratio of NuoF + E was higher than 2:1 on the SDS gel (data not shown). The contents of FMN, non-heme Fe, and S^{2-} in the purified NuoF + E preparation, were 0.651 ± 0.5, [4] 38.1 ± 3.1, and 32.8 ± 8.6 nmol/mg protein, respectively. It indicated that the incorporation of FMN in

[4] Accuracy of the measurements of FMN content is greatly diminished mostly because of its extremely low content.

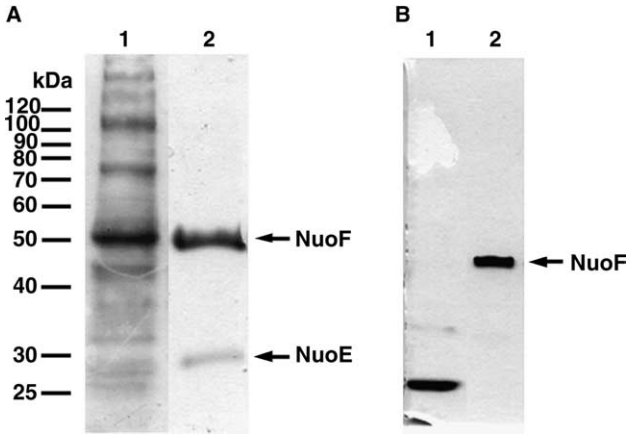

Fig. 1. Overexpression and purification of the NuoF subunit of *E. coli* NDH-1. (A) CBB-stained SDS–polyacrylamide gel (10%); *lane 1*, soluble fraction (20 μg) and *lane 2*, His-tagged NuoF subunits (5 μg). (B) NADH dehydrogenase activity staining of the native polyacrylamide gel; *lane 1*, soluble fraction (20 μg) and *lane 2*, His-tagged NuoF subunit (5 μg). The significantly stained band in the soluble fraction (B, lane 1) is not due to expressed NuoF because the NuoF or His-tag antibody does not react with this band.

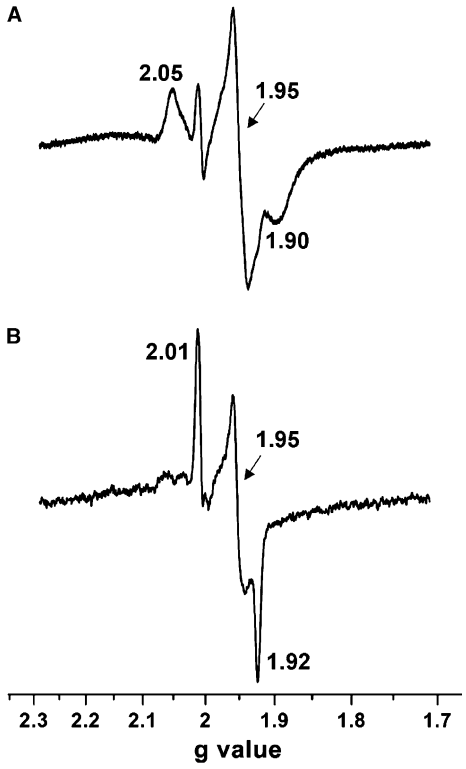

Fig. 2. EPR spectra of the iron–sulfur clusters in the expressed NuoF subunit at 12 K (A) and 45 K (B) in the presence of 10 mM sodium dithionite. EPR spectra were recorded under the following conditions: microwave frequency, 9.44 GHz; microwave power, 5 mW; modulation amplitude, 10.115 G; modulation frequency, 100 kHz; time constant, 164 ms. Principal g values were indicated.

this preparation was also very low (around 5% of the expected amount). As shown in Fig. 3A, the purified NuoF + E subcomplex exhibited a UV–visible spectrum with absorption peaks at 425, 460, and 560 nm, which is characteristic of a [2Fe–2S] cluster [6]. Upon the addition of 2 mM NADH, the absorbance spectrum only slightly changed whereas the reduction with 10 mM sodium dithionite caused significant bleaching (Fig. 3A). When the NuoF + E subcomplex was reconstituted in vitro with FMN and then incubated with 5 mM NADH, a significant reduction of the absorbance was observed (Fig. 3B), indicating that the iron–sulfur clusters were reducible with NADH. EPR spectroscopic analyses revealed that the NuoF + E subcomplex contained a [2Fe–2S] (N1a) cluster and a [4Fe–4S] (N3) cluster at an approximate ratio of 4:1, and that only cluster N1a was reducible by NADH.

To verify the cysteine coordination of cluster N3, the five fully conserved cysteine residues in the NuoF subunit were individually mutated to Ala, and each mutated *nuoF* gene was co-expressed with the wild type *nuoE* gene. UV–visible spectra of the purified mutated NuoF + E subcomplex were shown in Fig. 3C. Generally, the mutated subcomplexes exhibited lower absorbance in a visible region than the wild type, indicating lower amounts of the iron–sulfur clusters. C180A mutant subcomplex showed the absorption spectrum almost identical to that of the wild type. In the case of mutants C351A, C354A, C357A, and C398A, the absorption spectra were different from that of the wild type in which the absorbance at 420 nm was lower than the peak at 460 nm. The absorption spectra of the latter four mutants were almost identical to those of solely expressed Nqo2 subunits of *Paracoccus* and *Thermus*, which contained only a [2Fe–2S] cluster [6,19].

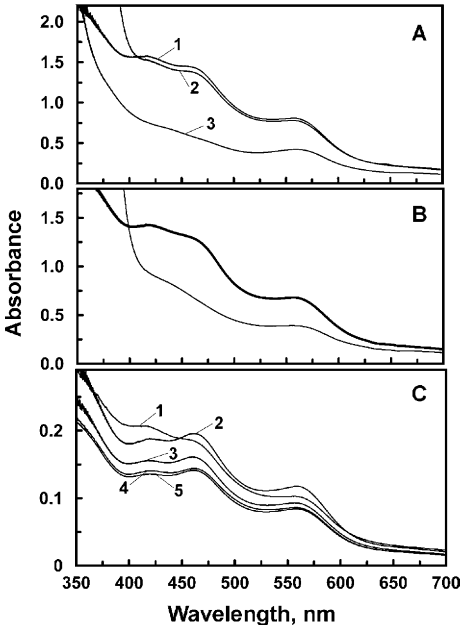

Fig. 3. UV–visible absorption spectra of the purified NuoF + E subcomplex (A), the NuoF + E subcomplex reconstituted with FMN (B), and the mutated NuoF + E subcomplexes (C). In (A), 1, the oxidized form; 2, the reduced form with 2 mM NADH; 3, the reduced form with 10 mM sodium dithionite. In (B), *thick solid line*, the oxidized form; *thin solid line*, the reduced form with 5 mM NADH. Protein concentrations were 5.9 and 4.6 mg/ml in (A) and (B), respectively. In (C), 1, C180A; 2, C398A; 3, C357A; 4, C351A; 5, C354A. The absorbance was normalized to 1 mg/ml of protein concentration. All the spectra were recorded at room temperature.

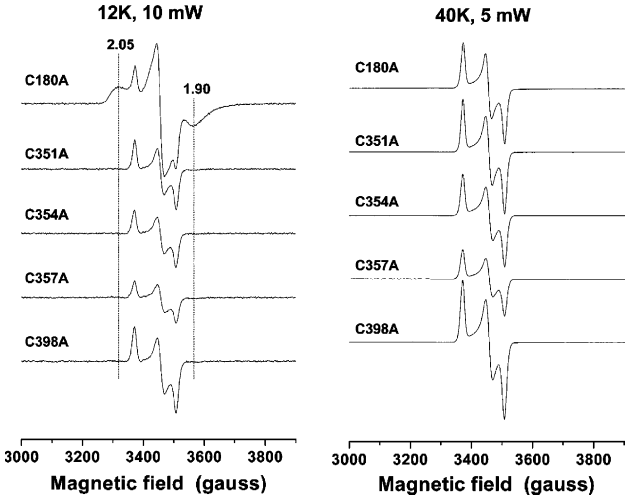

Fig. 4. EPR spectra of the mutated NuoF + E subcomplexes at 12 K, 10 mW (*left*), and at 40 K, 5 mW (*right*). Protein concentrations were 15.2, 15.0, 6.7, 8.4, and 11.2 mg/ml for C180A, C351A, C354A, C357A, and C398A, respectively. The samples were reduced with 10 mM sodium dithionite. EPR conditions are the same as in Fig. 2.

As shown in Fig. 4 left, the EPR signal arising from a [4Fe–4S] cluster (N3) was detected only in C180A mutant but not in all other mutant subcomplexes reduced with dithionite while the EPR signal from a [2Fe–2S] cluster (N1a) was observed in all the mutants. We did not detect any iron–sulfur cluster EPR signals in their oxidized forms, excluding the possibility that a [3Fe–4S] cluster was formed instead of a [4Fe–4S] cluster N3 due to the removal of a cysteine residue. Based on these analyses, it can be concluded that C351, C354, C357, and C398 are involved in the coordination of the [4Fe–4S] cluster in the NuoF subunit.

Despite the loss of cluster N3, the mutant subcomplexes, C351A, C354A, C357A, and C398A showed NADH-ferricyanide reductase activities. Apparent K_m values for NADH of mutant subcomplexes were 2.9–6.6 μM, which were similar to that of the wild type (5.2 μM, on average) and to the reported value of *Paracoccus* Nqo1+2 subcomplex (8.2 μM) [8]. The V_{max} values were all within 1.7–3.3 μmol of NADH oxidized min^{-1} mg^{-1} protein, and there were no significant differences. Interestingly, after FMN reconstitution, both K_m and V_{max} of the wild type subcomplex were increased to 35.4 μM and 32.3 μmol min^{-1} mg^{-1} protein, respectively, while only V_{max} value was increased to 17.6 μmol min^{-1} mg^{-1} protein for the sole NuoF preparation.

4. Discussion

In the present study, we have clearly shown that the four conserved cysteine residues (C351, C354, C357, and C398) coordinate the [4Fe–4S] cluster, N3, in the NuoF subunit of *E. coli* NDH-1. The fifth conserved cysteine, C180, is present in a putative FMN-binding site, ^{174}GAGRYICGEETAL^{186}

(*E. coli* numbering), and was apparently not involved in the coordination of the iron–sulfur cluster because C180A mutant exhibited the EPR signals from a [4Fe–4S] cluster, N3.

In contrast to the NuoE, the single NuoF expression in *E. coli* was problematic as described previously for the *Paracoccus* Nqo1 subunit [8]. Although the NuoF subunit is hydrophilic as deduced from its primary sequence and cellular location, it tends to become very unstable after the isolation. The NuoF subunit was stabilized when co-expressed with the NuoE subunit, but the FMN content remained low. It seems likely that yet another subunit or protein is required for the cofactor incorporation and the formation of a stable NADH dehydrogenase subcomplex. In fact, Friedrich's group obtained the NADH dehydrogenase subcomplex containing the complete set of cofactors by overexpression of NuoB-G subunits [9]. Also, it has been reported that the subunits α, β, γ, and δ of the NAD^+-reducing hydrogenase from *Rastonia eutropha* are divided into two segments, in which one segment composed of the α and γ subunits is regarded as diaphorase, containing homologous sequence to NuoE, NuoF, and NuoG subunits [20,21]. Another notable outcome in this study is that only cluster N1a in the purified NuoF + E subcomplex was reduced by NADH after FMN reconstitution. It indicated that cluster N1a has a high midpoint redox potential and can directly accept an electron from FMN without cluster N3. In fact, we observed cluster N1a in the FMN-reconstituted mutant NuoF + E subcomplex devoid of cluster N3 (e.g., C398A mutant) was also reducible by NADH. These results are consistent with the previous study showing that the redox potential of cluster N1a in the *E. coli* NuoE subunit was −280 mV in the presence of 2 M NaCl by using protein-film voltammetry, which was 90–135 mV higher than those of its counterparts of *Paracoccus*, *Thermus*, and bovine [22]. In addition, previously assigned [2Fe–2S] cluster "N1c" signals were shown to arise from cluster N1a signals [10,23], which was reducible by NADH in both the purified *E. coli* NDH-1 [24] and the overproduced NADH dehydrogenase fragment [9]. This property of cluster N1a is unique to *E. coli*, and is not observed in the bovine FP subcomplex [25] or the Nqo1+2 subcomplex from *Paracoccus* [8].

The [4Fe–4S] cluster binding motif "$CxxCxxCx_{40}C$" in the *E. coli* NuoF subunit is strikingly conserved not only among complex I but also in a variety of enzymes including the β subunit of Fe-hydrogenase from *Thermotoga maritima* (NP_229225), subunit C of NADP-reducing hydrogenase from *Desulfovibrio fructosovorans* (C57150), the HoxF subunit of NAD^+-reducing [NiFe] hydrogenase from *Synechocystis sp*. Strain PCC 6803 (CAA66209), and the β subunit of NAD^+-dependent formate dehydrogenase from *R. eutropha* (CAA11234). Recently, the complex I-like protein Ndh51, which is 57% identical to the NuoF subunit, was found in a soluble hydrogenosomal fraction from *Trichomonas vaginalis* [26,27]. Ndh51 formed stable complex with Ndh24, a NuoE homologue, and was coupled to the central fermentative pathway in hydrogenosomes. It is remarkable that similar domains and structures are shared between the NuoF + E subcomplex and other redox enzyme complexes. More detailed studies are needed to elucidate the roles of clusters N1a and N3 in the electron transfer pathway from NADH.

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