The proton-translocating NADH-quinone oxidoreductase (NDH-1) of *Thermus thermophilus* HB-8 is composed of 14 subunits (designated Nqo1–14). This NDH-1 houses nine putative iron-sulfur binding sites, eight of which are generally found in bacterial NDH-1 and its mitochondrial counterpart (complex I). The extra site contains a CXXCCXXXCC<sub>27</sub>C motif and is located in the Nqo3 subunit. This motif was originally found in *Escherichia coli* NDH-1 and was assigned to a binuclear cluster (g<sub>⊥,⊥</sub> = 2.00, 1.95, 1.92) and named N1c. In this report, the *Thermus* Nqo3 fragment containing this motif was heterologously overexpressed, using a glutathione S-transferase fusion system. This fragment contained a small amount of iron-sulfur cluster, whose content was significantly increased by in vitro reconstitution. The UV-visible and EPR spectroscopic properties of this fragment indicate that the ligated iron-sulfur cluster is tetranuclear with nearly axial symmetry (g<sub>⊥⊥</sub> = 2.045, −1.94). Site-directed mutants show that all four cysteines participate in the ligation of a [4Fe-4S] cluster. Considering the fact that the same motif coordinates only tetranuclear clusters in other enzymes so far known, we propose that the CXXCCXXXCC<sub>27</sub>C motif in the Nqo3 subunit most likely ligates the [4Fe-4S] cluster.

Bacterial proton-translocating NADH-quinone (Q) oxidoreductase (NDH-1)<sup>1</sup> and its mitochondrial counterpart (complex I) are believed to be the most elaborate iron-sulfur proteins (1–3). It is generally accepted that NDH-1/complex I contains at least six EPR-detectable iron-sulfur clusters; N1a, N1b, N2, N3, N4, and N5 (4–7). Clusters N1a and N1b are binuclear ([2Fe-2S]) and N2, N3, N4, and N5 are tetranuclear ([4Fe-4S]) clusters (4). To decipher the mechanism of electron transfer within the NDH-1/complex I, it is necessary to study physicochemical properties of individual iron-sulfur clusters and their subunit locations. For this purpose, we have attempted to individually express the putative cofactor-binding subunits of the *Paracoccus denitrificans* NDH-1, which is composed of 14 different subunits (designated Nqo1–14) (7–15). It has been indicated that the Nqo1 and Nqo2 subunits harbor clusters N3 and N1a, respectively (11–13). The Nqo3 subunit ligates clusters N1b, N4, and N5 (7, 10). The Nqo9 subunit bears two tetranuclear clusters (N6a and N6b) (16) similar to two [4Fe-4S] ferredoxin-type clusters shown in the overexpressed and purified *P. denitrificans* Nqo9 subunit (8).

*Thermus thermophilus* HB-8, which was isolated from a hot spring in Japan, is an extremely thermophilic, aerobic, Gram-negative bacterium (17). It is known that this bacterium contains NDH-1 in the respiratory chain (18–20) and MQ-8 as the only endogenous quinone (21). The enzyme was partially purified (19), and the gene cluster encoding the *T. thermophilus* NDH-1 was sequenced (22). The gene cluster is composed of 14 structural genes (designated *nqo1–14*) similar to those of other bacteria (3, 23). On the basis of its deduced primary structure, the *T. thermophilus* NDH-1 is predicted to harbor nine iron-sulfur clusters (22). In contrast, *P. denitrificans* NDH-1, which contains only UQ-10 (24), is believed to bear eight iron-sulfur clusters (3, 23, 25).

Interestingly, the *T. thermophilus* Nqo3 and *Escherichia coli* NuoG (a Nqo3 homologue) subunits are predicted to ligate an additional iron-sulfur cluster with a unique cysteine cluster motif (C<sup>27C</sup>XCCXXXCC<sub>27</sub>C<sup>27C</sup>C, *T. thermophilus* numbering), which is not found in *P. denitrificans* and higher organisms (Fig. 1A). On the other hand, *E. coli* NDH-1 shows slowly relaxing binuclear-type EPR signals (g<sub>⊥,⊥</sub> = 2.00, 1.95, 1.92), which have not been found in the NDH-1 from other bacteria. Therefore, it was proposed that the additional unique cysteine sequence motif in the NuoG subunit was responsible for the signals from the binuclear cluster N1c (26–28). However, no further detailed experiments have been conducted to date. The

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<sup>†</sup> The abbreviations used are: NDH-1, bacterial proton-translocating NADH-quione oxidoreductase; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; GST, glutathione S-transferase; MBP, maltose binding protein; Q, quinone; MQ, menaquinone; UQ, ubiquinone; N1c fragment, truncated form (85AA) of the *T. thermophilus* NuoG subunit; FNR, transcription factor for fumarate nitrate reductase; BS, biotin synthase; CBB, Coomassie Brilliant Blue; DTT, dithiothreitol; E<sub>m</sub>, mid-point redox potential; IPTG, isopropyl-β-d-thiogalactopyranoside.
same type of putative Fe/S binding sequence motif has been reported so far in ten other bacterial NDH-1 gene clusters (including Salmonella typhimurium) (see Fig. 1B) (29). It is, therefore, of interest to identify the iron-sulfur cluster coordinated by this motif and to characterize it to clarify the functional role(s) of this additional iron-sulfur cluster found in limited members of bacterial NDH-1.

The present paper describes the expression, reconstitution, and UV-visible and EPR spectroscopic characterization of the iron-sulfur cluster ligated by the additional cysteine cluster motif in the Nqo3 subunit. Our results indicate that this cysteine motif most likely coordinates a [4Fe-4S] cluster rather than a [2Fe-2S] cluster. The site-directed mutation experiments suggest that the four cysteine residues are involved in the ligation of this [4Fe-4S] cluster. This iron-sulfur cluster has a unique feature, very high sensitivity to oxygen exposure, which has not been observed on other EPR-detectable iron-sulfur clusters in NDH-1/complex I.

**EXPERIMENTAL PROCEDURES**

**MATERIALS—**pGEX4T-3 vector, glutathione-Septahorpe 4B gel, thrombin, and baculovirus expression system. Antibody were obtained from Novocea Inc. (Arlington Heights, IL). pMALc2g, amylase resin, genenase I, and anti-MBP antiseraum were from New England Biolabs (Beverly, MA). The SuperSignal was from Pierce (Rockford, IL). pGEX2TKCS was a generous gift from Dr. Masahiko Aoki (The Scripps Research Institute).

**Construction of Expression Vectors for the T. thermophilus N1c Fragments and Full-length Nqo3 Subunit—**The DNA fragment encoding the N1c region (85AA, see Fig. 1A) was obtained from pTHH-1 plasmid (22) by PCR using a sense primer to introduce Ndel restriction site at the translation initiation site and an antisense primer to construct XhoI site near the stop codon, respectively: NcF (sense), 5'-GACCATATGC-CCGCGTTCGCGCCGAAACTGG-3'; NcR2 (antisense), 5'-CTCGAGTCACCTCTCCAGCGCTCAGG-3'. The italized bases were altered from T. thermophilus DNA; the underlined bases indicate the newly introduced restriction sites, Ndel site for NcF and XhoI for NcR2; the boldface bases illustrate a constructed stop codon.

The construct was ligated into NdeI/XhoI sites of pET16b and pGEX2TKCS expression vectors. The final constructs were designated as pET16b/Nc1 and pGEX2TKCS/Nc1, respectively.

For overexpression of the full-length Nqo3 subunit, three different constructs were prepared. The nqo3 genes were obtained from the pTHH-1 plasmid (22) by PCR. The sense primers were designed to generate NdeI site for a pET21a expression vector, or SnaBI site for pMALc2g, or EcoRI site for pGEX4T-3 at the nqo3 initiation codon: nqo3F(NdeI), 5'-GGAGGTGTAATGTTCTCCGGGTTACAGG-3'; nqo3S(SnaB1), 5'-GAGAACGTAAATGTTCTCCGGGTCAAGTGTAACACCGCC-3'; nqo3S(EcoRII), 5'-GGAGATCTATGTTCTCCGGGTCAAGTGTAACACCGCC-3'; nqo3S(A); 5'-GCCGCTTC-3; C256A, 5'-GAGACCCCCACCCGGACCC-3'; C259A, 5'-GAGACCCCCACCCGGACCC-3' (mutated bases are underlined). Expression, purification, and subcloning of these DNA fragments (~800 bp) were carried out as described previously (22). Each DNA fragment was verified by sequencing. The NdeI/HindIII, SnaB1/HindIII, and EcoRI/Ndel fragments harboring the entire nqo3 gene were ligated into pET21a, pMALc2g, and pGEX4T-3, respectively. The proper constructs were confirmed by cleavage with several restriction enzymes. The final constructs were designated pET21a/nqo3, pMALc2g/nqo3, and pGEX4T-3/nqo3.

**Expression and Purification of the T. thermophilus N1c Fragments and Full-length Nqo3 Subunits—**Expression of the N1c fragment was conducted basically according to Yano et al. (10). Competent E. coli strain BL21(DE3) lysS was transformed with pET16b/Nc1 or pGEX2TKCS/Nc1. The cells were cultured into 50 mL of TB medium containing 200 μg/mL ampicillin, 100 μg/mL carbenicillin, 50 μg/mL ferric ammonium citrate, and 50 μg Na2S at 37 °C until the A600 nm reached 0.5. After addition of 0.1 mM IPTG, cells were grown for 16–18 h at 25 °C. The cells harvested were suspended in anaerobic 50 mM HEPES-NaOH buffer (pH 7.5) containing 250 mM NaCl, 2 mM DT, 1 mM phenylmethysulfonyl fluoride, 20 μg leupeptin and 1 mM EDTA. The cell suspensions were freeze-thawed twice and sonicated. After broken cells and inclusion bodies were removed by repeated centrifugations, the supernatant was spun in an Ultracentrifuge at 50,000 rpm for 45 min at 4 °C in a 60Ti rotor. The inclusion body fraction was prepared as described previously (9). The supernatant was degassed in vacuo and purged with argon and brought into an anaerobic chamber (Coy Laboratory Products). The following procedures were performed under anaerobic conditions (oxygen level ~1 ppb) at room temperature. The His-tagged N1c fragment was purified according to the protocol of the GST-fused N1c fragment was carried out as follows. The supernatant was applied on a glutathione-Sepharose 4B affinity column (1.5 × 3.0 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (TD buffer). The adsorbed protein was eluted with 10 mM reduced glutathione with 0.2 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride or 1% phenylmethylsulfonyl fluoride in vacuo

Expression of the full-length Nqo3 subunits was conducted by using pET21a/nqo3, pMALc2g/nqo3, and pGEX4T-3/nqo3 vectors according to the procedure of the N1c fragments (see above), except for E. coli strain BL21(DE3) was used as competent cells. The transformed cells were grown at 30–33 °C until the A600 nm reached ~2.0. After addition of 0.5 mM IPTG, the cells were further incubated for 6–8 h at 15 °C. The cells were suspended to 7.5% (w/v) in anaerobic 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and 200 mM NaCl on ice before use. The cells were broken up by two passages through a French Pressure cell at 2000 p.s.i. After unbroken cells and inclusion body were removed, the supernatant was spun in an Ultracentrifuge at 50,000 rpm for 60 min.

Expression in the soluble fraction of nqo3 subunit by pET21a/nqo3 was insufficient for conventional purification. GST-fused Nqo3 subunit by pGEX4T-3/nqo3 could not be detected in the soluble fraction. In contrast, MBP-fused Nqo3 subunit was purified under anaerobic conditions basically according to the manufacturer’s protocols.

**In Vitro Reconstitution of the Iron-Sulfur Clusters into the GST-fused N1c Fragment and the Nqo3 Subunit—**The reconstitution procedures were carried out in the anaerobic chamber by a modified method of Yano et al. (8, 11). The purified GST-fused N1c fragment was diluted to 10–20 μM with TB buffer containing 10% glycerol (TDG buffer). 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)2 and Na2S were added to the protein solution at a final concentration of 240–480 μM. The reconstitution proceeded for 2–3 h. Unbound Fe2+ and S2- were removed by the desalting 10DG column. The reconstituted GST-fused N1c fragments were applied on a DEA (Toyopearl 650M) column (0.8 × 10.0 cm) equilibrated with 0.8 mM Tris-HCl buffer. The non-fused N1c fragment was purified by treatment with thrombin for 3 h at room temperature after reconstitution, subsequent passing through a glutathione affinity column, and DEAE column chromatography. If required, the proteins were desalted by the 10DG column, and concentrated with Microcon-10 (Millipore). 10–20% glycerol was used to keep the isolated N1c fragments from precipitating during purification. The similar strategy was used for in vitro reconstitution of iron-sulfur clusters into the MBP-fused Nqo3 subunit. The non-fused Nqo3 subunit was isolated after reconstitution by overnight incubation with genenase 1 at room temperature and subsequent DEAE column chromatography.

**Site-directed Mutagenesis of the N1c Fragments—**Site-directed mutagenesis was performed by using the GeneEdior™ in vitro site-directed mutagenesis system kit (Promega). Each cysteine in the N1c fragment was to alanine to eliminate disulfide bonds. The following oligonucleotides were designed as target primers; C256A, 5’-GAGACCCCCACCCGGACCC-3’, C259A, 5’-ACACCCGTCGCGGTGTCG-3’, C263A, 5’-CTCAGCGCCTGTTTGGGCGC-3’, C265A, 5’-GGAGATCTATGTTCTCCGGGTTACAGG-3’. The purified GST-fused N1c fragment was digested with SnaBI and EcoRI, and the linearized GST-fused N1c fragment was ligated into the NdeI/XhoI site of pGEX4T-3, respectively. The reconstituted fractions were applied on the desalting 10DG column. The resulting fractions were used for in vitro reconstitution of iron-sulfur clusters into the GST-fused N1c fragment. The non-fused Nqo3 subunit was mutated after reconstitution by overnight incubation with genenase 1 at room temperature and subsequent DEAE column chromatography.

**Oxygen-induced Conversion of the Reconstituted GST-fused N1c Fragment—**Typically, a 500- to 1000-μl sample of anaerobically prepared GST-fused N1c (~4–5 mg/mL) in TDG buffer was taken out of the glove box and left in air at room temperature. Samples stirred slowly were monitored by UV-visible and EPR spectroscopy. UV-visible absorption was monitored by recording protein samples in a cuvette sealed with a Teflon cap after each sample was mixed with air by gently inverting the cuvette two or three times at periodic intervals during exposure to air.

**Reconstitution of the Air-oxidized Iron-Sulfur Cluster in the GST-fused N1c Fragment—**After the reconstituted GST-fused N1c fragment was exposed to air for 30 min, the sample was brought into the
glovebox and diluted to 20 μm with the anaerobic 50 mM Tris-HCl (pH 8.0) buffer containing 10% glycerol and 10 mM DTT. Fe(NH₄)₂(SO₄)₂ and Na₂S were slowly titrated into the protein solution to final concentrations of 480 μM. Excess iron, sulfide, and DTT were removed by a 10DG column.

Other Analytical Procedures—The molecular size of the purified GST-fused N1c fragment was determined by a gel filtration column TSK-G3000SW (Tosohaa) (2.5 x 30 cm). EPR spectra were measured by a Bruker ESP300E spectrometer operating at X band frequency (9.4 GHz). The sample temperature was varied using an Oxford ESR model 900 helium flow cryostat. Microwave frequency was monitored by a Hewlett-Packard 5350 frequency counter. Spin quantitation was performed under non-power-saturated conditions using 500 μM Cu-EDTA as a standard. UV-visible absorption spectra were recorded on an SLM-Amico DW-2000 and Beckman DU640 spectrophotometers at room temperature. For sequence analysis, GCG software programs were used to analyze the amino acid sequence and sequence comparison of polypeptides (30). A homology search was carried out using the BLAST program running at the NCBI (31). Non-heme iron and acid-labile sulfide were determined according to Doeg and Ziegler (32), and Fogo and Popowski (33) and Beiner (34), respectively. Protein concentration was routinely estimated by the method of Lowry et al. (35) and Bradford (36). Amino acid composition (37), amino acid sequences (38), DNA sequences (39), and immunoblotting (40) were done according to the references cited. SDS-PAGE was performed according to Laemmli (41) as a standard. UV-visible absorption spectra were recorded on an SLM-Aviove 1682 and Hewlett-Packard 5350 spectrophotometers.

RESULTS

Expression of the N1c Fragment of T. thermophilus Nqo3 Subunit—As shown in Fig. 1A, the P. denitrificans and T. thermophilus Nqo3 subunits contain at least three iron-sulfur cluster binding segments. From the N terminus, they are CXX₃CX₆CX₇C, HX₅CX₆CX₇C, and CX₆CX₇CX₈₋₉C, which have been suggested to ligate a binuclear cluster (N1b), a tetranuclear cluster (possibly N5), and a tetranuclear cluster (N4), respectively (10). Following these iron-sulfur cluster binding motifs, the T. thermophilus Nqo3 and E. coli NuoG subunits contain an extra cysteine cluster. 250(CXXCXXCX₁₇C₁₉²⁹₁ (T. thermophilus numbering), which is not found in P. denitrificans and higher organisms (Fig. 1A). Although this cysteine cluster has been considered to coordinate an iron-sulfur cluster, its properties have not been further investigated. Because the presence of these three iron-sulfur clusters may interfere with characterization of the fourth iron-sulfur cluster in the T. thermophilus Nqo3 subunit when a full-length form of subunit is used, we decided to express an internal polypeptide fragment (85AA), which encompasses the extra four-cysteine cluster (Cys²⁶⁵, Cys²⁶⁶, Cys²⁶⁷, and Cys²⁶⁸). The fragment (called the N1c fragment hereafter) starts just after the preceding run of cysteines (end at residue 230), which are the presumptive ligands of cluster N4, and contain the C-terminal stretch (see Fig. 1A and “Experimental Procedures”). When His-tagged forms of the N1c fragment were expressed in E. coli using pET16b/N1c/85AA), the product was predominantly expressed as dark red brown inclusion bodies under all conditions tested. Once the C-terminal region of the N1c fragment was truncated by 10 residues, the inclusion body appeared whitish. In contrast, the GST-fused N1c fragment was expressed in a soluble fraction and much less inclusion bodies were formed. The expressed GST-fused N1c fragment was purified from the cytoplasmic fraction by glutathione-affinity and DEAE column chromatography to nearly homogeneous form as shown in Fig. 2A (lanes 1, 2, and 4). The expressed GST-fused N1c fragment was readily recognized by affinity-purified anti-N1c antibody and anti-GST antibody (Fig. 2B, lane 1). We successfully isolated non-fused N1c fragment by treatment with thrombin (Fig. 2A, lanes 3 and 6). The apparent molecular sizes of the expressed GST-fused, His-tagged, and non-fused N1c fragments on the SDS gel were in good agreement with their deduced molecular sizes, 38.8, 12.3, and 9.8 kDa, respectively (Fig. 2A). A trace amount of cleaved GST domain was detected in the purified non-fused N1c fragment by Western blotting (Fig. 2B, right). However, this contaminated GST protein could be easily removed by incubation of the purified non-fused N1c fragment fractions at 65 °C for 2 min followed by centrifugation. These results indicate that the non-fused N1c fragment of T. thermophilus Nqo3 subunit is heat-stable.

In Vitro Reconstitution for the Iron-Sulfur Cluster into the N1c Fragment—The purified GST-fused N1c fragment was light brown, indicating that it contained iron-sulfur clusters. As shown in Fig. 3A, its UV-visible absorption spectrum exhibited very small but characteristic absorbance of iron-sulfur clusters. The contents of the non-heme Fe and acid-labile S²⁻ in the preparation were 0.1–0.2 atoms/mol. We attempted to reconstitute iron-sulfur clusters in vitro by following our previous reports (7, 11) by applying several different conditions regarding pH, protein concentration, Fe and S²⁻ concentrations, incubation time, and so forth. Because higher concentrations of proteins, Fe, and S²⁻ in the reaction mixture readily cause aggregation of the fragments, it was necessary to maintain lower concentrations of these factors (10–15 μM of protein; five to six times excess of Fe²⁺ and S²⁻) to ensure a high efficiency of reconstitution. The Fe²⁺ form in ferrous ammonium sulfate was preferred to Fe³⁺ form in ferric chloride for our reconstitution, and the reconstitution reaction was allowed to proceed at pH 8.0 for at least 2–3 h. Once the iron-sulfur cluster was reconstituted, the protein became stable at room temperature and anaerobic conditions for 1–2 weeks without any observable change. The reconstituted GST-fused N1c protein contained 1.8–3.1 atoms of Fe and 1.5–3.6 atoms of S²⁻/mol. The relative molecular mass of the reconstituted GST-fused N1c fragment was determined to be ~40 kDa by the molecular exclusion column chromatography, indicating that the reconstituted GST-fused N1c fragment is a monomer. This excludes a possibility of inter-subunit ligation of the iron-sulfur cluster. The GST segment was removed by thrombin digestion, and the non-fused N1c fragments were purified by a glutathione-affinity column and DEAE column chromatography. Although this digestion and the following isolation procedures appeared to result in a slight loss of the iron-sulfur clusters, the isolated non-fused N1c fragment still contained 0.7–1.5 atoms of Fe and 0.7–1.2 atoms of S²⁻/mol.

The absorption spectra of the reconstituted GST-fused N1c fragment (as prepared) exhibited a broad absorption peak around 410 nm (Fig. 3B). This spectrum is characteristic of a [4Fe-4S]²⁺ cluster but not of a [2Fe-2S]²⁺ cluster. The latter usually shows distinct absorption peaks/shoulders around 330, 420, 460, and 560 nm (43). Addition of 10 mM sodium dithionite resulted in a quench of the absorbance in the entire visible region, demonstrating that the reconstituted iron-sulfur cluster was redox-active. Similar UV-visible spectra were observed for the oxidized and the reduced forms of the non-fused N1c fragment isolated after thrombin digestion (Fig. 3C). These results indicated that the iron-sulfur cluster was coordinately in the N1c fragment by reconstitution. We determined a molar extinction coefficient (ɛ) at 410 nm for the iron-sulfur cluster chromophore housed in the GST-fused N1c fragment monomer (oxidized) based on the protein concentration and the non-heme Fe and acid-labile sulfide contents. The values obtained were ɛ₄₁₀ nm = 13,300–15,800 m⁻¹ cm⁻¹, which is in good agreement with the molar extinction, ɛ₄₁₀ nm = 15,000 m⁻¹ cm⁻¹ for a
EPR Analyses of the Reconstituted GST-fused and Non-fused N1c Fragments

We conducted EPR spectroscopic analyses of the reconstituted GST-fused and non-fused N1c fragments to confirm the presence of the iron-sulfur cluster and to determine the type of the iron-sulfur cluster. Neither the GST-fused nor non-fused N1c fragments as prepared showed detectable EPR signals in the g region at 10K (data not shown). Upon the reduction with dithionite, both the GST-fused and non-fused N1c fragments exhibited almost identical EPR signals with nearly axial symmetry (Fig. 4). The EPR signals exhibited rather broader line width and g values of g₁ = 2.045, ~1.94. In contrast to the non-fused N1c fragment, the reconstituted GST-fused N1c fragment could readily be concentrated at much higher concentrations (0.4 mg/ml) without any aggregation. Therefore, we decided to study the reconstituted GST-fused N1c fragment for further characterization. As shown in Fig. 4, the iron-sulfur cluster EPR signals could be observed at an optimum temperature range of 12–14 K with a fixed microwave power of 10 milliwatts (mW). The half-saturation parameter (P₁/₂) of cluster N1c is 1.97 mW at 12 K. No other EPR signals were detected at temperatures higher than 40 K. With three independent GST-fused N1c preparations, the ratio of the spin

FIG. 1. A, sequence alignments of the N-terminal segment (1–359 residues) of the Nqo3 subunit (total 783 AA) of T. thermophilus NDH1 with its homologues from various organisms; T.ther, T. thermophilus (GenBank accession number Q56223); D.mela, D. melanogaster mitochondria (AAAP46556); N.cras, Neurospora crassa mitochondria (CA91229); B.taur, Bos taurus mitochondria (AAAP39652); P.deni, Paracoccus denitrificans (AAAP46555); E.coli, E. coli (AACM30343). Conserved cysteine residues are highlighted by shadowed boxes and marked by asterisks. Cysteine residues shown by # were predicted to ligate an additional iron-sulfur cluster. The putative iron-sulfur binding sites are shown by broken lines. The arrows (s, and /c141) indicate the N-terminal and C-terminal of the N1c fragment (85AA) used in this study, respectively. B, comparison of the deduced amino acid sequence of the T. thermophilus N1c fragment (85AA) with its homologues (Nqo3/NuoG) containing the additional cysteine cluster from various organism. T.ther, T. thermophilus (Q56223); D.radi, Deinococcus radiodurans (AACM30335); S.coel, Streptomyces coelicolor (CA81229); S.meli, Sinorhizobium meliloti (CA81229); M.tube, Mycobacterium tuberculosis (CACM30343); E.coli, E. coli (AACM30343); S.typh, Salmonella typhimurium (AACM30343); P.fluo, Pseudomonas fluorescens (AACM30343); P.aeru, Pseudomonas aeruginosa (AACM30343); A.aeol, Aquifex aeolicus (AACM30343). Conserved cysteine residues are illustrated by shadowed boxes and # symbols.
Based on these observations and the fact that no EPR signal was detected in the reconstituted GST protein, it could be concluded that the iron-sulfur cluster coordinated in the N1c fragment is a [4Fe-4S] cluster.

Are the Four Conserved Cysteine Residues Involved in Coordination of the [4Fe-4S] Cluster?—To verify the cysteine coordination of the [4Fe-4S] cluster, each of the four conserved cysteine residues (Cys256, Cys259, Cys263, and Cys291) in the N1c fragment was individually mutated to alanine. The mutated GST-fused N1c fragments were expressed in E. coli, purified, reconstituted, and then subjected to UV-visible and EPR analyses. None of these mutations affected their expression level in E. coli, and the products could be purified from the cytoplasmic fraction. We noticed that the mutated GST-fused N1c fragments were much more susceptible to proteolysis during the purification. Smaller degradation products, which were recognized by anti-N1c-antibody, could be seen on a SDS gel (data not shown). The purified GST-fused N1c mutated fragments contained much lower amounts of non-heme Fe than the wild-type GST-fused N1c fragment before reconstitution. After reconstitution of iron-sulfur cluster, all mutants exhibited smaller absorbance of iron-sulfur clusters (around 410 nm) in UV-visible spectra, suggesting that iron-sulfur clusters could be formed in these mutants (data not shown). Nevertheless, these reconstituted mutants exhibited very small EPR signals in their reduced forms compared with that of the wild-type N1c fragments (data not shown). We could not detect any EPR signals in their oxidized forms, excluding a possibility that a [3Fe-4S] cluster was formed instead of a [4Fe-4S] cluster due to the removal of one cysteine residue. Unlike the wild-type, these mutants tended to aggregate and lost their yellow-brownish color within a few days after reconstitution even in strictly

**FIG. 2.** Overexpression and purification of the GST-fused N1c fragments of the Nqo3 subunit. A, CBB-stained SDS-polyacrylamide gel analyses of samples from different purification stages of the GST-fused and non-fused N1c fragments. Lane 1, soluble fraction (12.5 μg); lane 2, the GST-fused N1c fragment isolated by glutathione affinity column chromatography (6 μg); lane 3, the thrombin-treated (for 2 h) GST-fused N1c fragment (6 μg); lane 4, the purified GST-fused N1c fragment after DEAE chromatography (3 μg); lane 5, the purified GST (2 μg); lane 6, the purified N1c fragment (1.5 μg); lane 7, the His-tagged N1c fragment (3 μg). Protein samples were applied to each lane of a Schägger SDS-16.5% polyacrylamide gel. The molecular sizes in kilodaltons were shown on the left. B, immunoblotting of the purified GST-fused and non-fused N1c fragments with anti-N1c fragment (left panel) and anti-GST antibodies (right panel). Lane 1, the GST-fused N1c fragment (0.6 μg); lane 2, the isolated GST (0.4 μg); lane 3, the isolated N1c fragment (0.5 μg). The affinity-purified antibody for the N1c fragment was isolated from the antiserum against the Thermus Nqo3 subunit. Immunoblotting was carried out as described in Ref. 40, except that SuperSignal kit (Pierce) was used for the detection.

**FIG. 3.** UV-visible absorption spectra of the GST-fused N1c fragment (A), the reconstituted GST-fused N1c fragment (B), and the reconstituted non-fused N1c fragment (C). The proteins were dissolved in TD buffer containing 20% glycerol under anaerobic conditions. The oxidized forms, thick solid lines; the reduced forms by addition of 10 mM sodium dithionite, thin solid lines.

concentrations of the iron-sulfur cluster to those of the acid-labile sulfide was determined to be [spin]:[S²⁻] = 1.42 ± 0.28. Based on these observations and the fact that no EPR signal
an anaerobic conditions. This instability implies that the mutant N1c fragments could not stably fold mainly due to the substitution of Cys residues. Therefore, it may be that the iron-sulfur clusters could barely be reconstituted in the mutated N1c fragments probably using an external thiolate like β-mercaptoethanol or DTT, or water as a ligand (44–46) but they were extremely unstable. Taken together, it is conceivable that all four cysteine residues are involved in ligation of the [4Fe-4S] cluster.

**Expression of the Full-length Nqo3 Subunits**—To examine if the N1c iron-sulfur cluster can also be assembled as a [4Fe-4S]2+ cluster in the full-length Nqo3 subunit, we attempted to express the non-fused, GST-fused, and MBP-fused Nqo3 subunit in E. coli. The MBP-fused proteins were successfully expressed in the cytoplasmic phase in the E. coli body fraction of non-fused Nqo3 in lactosidase/H9251 T. thermophilus and expressed the non-fused, GST-fused, and MBP-fused Nqo3 subunit in the E. coli.

**EPR Analyses of the Expressed MBP-fused Nqo3 Subunit**—The purified MBP-fused Nqo3 subunit was considerably reddish-brown at about 4–5 mg/protein and showed significant optical absorption spectra of characteristic of iron-sulfur clusters (Fig. 6A). The purified MBP-fused Thermus Nqo3 subunit contained 2.6 ± 0.7 atoms of Fe and 1.7 ± 0.4 atoms of S2−/mol of protein.

If the T. thermophilus Nqo3 contains one binuclear and three tetranuclear clusters as described above, then the contents of Fe and S2− would be expected to be as high as 14 atoms of Fe and 6.5 ± 0.7 mol of S2−/mol of protein. The absorption spectra of the reconstituted MBP-fused Nqo3 subunit were shown in Fig. 6B, which exhibited the broad absorption peak at 417 nm with a tailing reaching around 700 nm and small shoulders at 320, 460, and 560 nm. This feature highly resembles that of the P. denitrificans Nqo3 subunit harboring one binuclear and two tetranuclear clusters (10). Although we attempted to isolate the Nqo3 subunit from the reconstituted MBP-fused Nqo3 with genenase I treatment, the isolated subunit tended to aggregate easily. Therefore, the reconstituted MBP-fused Nqo3 subunit was used for further characterization.
tetraneuronal on the basis of the temperature dependence of their signals. These iron-sulfur clusters display g values of 2.072, 2.046, 1.940, and −1.87 (Fig. 7B), apparently arising from multiple tetraneuronal clusters. An attempt to resolve these [4Fe-4S] cluster EPR signals by the potentiometric redox titration was not successful. These [4Fe-4S] species exhibited almost the same midpoint redox potentials (Em,n = −600 mV) and appeared to be cooperatively interacting each other, seen as the redox titration curve approaching to n = 2 (data not shown). In addition, they showed very similar spin relaxation behaviors. Therefore, it is difficult to identify these [4Fe-4S] clusters based on their spectral line shape.

Unique Feature of the [4Fe-4S] Cluster in the N1c Fragment—It has been reported that [4Fe-4S] clusters in certain enzymes can be inter-converted to other cluster forms upon air oxidation (47, 48). We observed that the color of the reconstituted N1c proteins changed from green-yellowish to reddish brown when they were exposed to air. We, therefore, attempted to study effects of oxygen exposure on the iron-sulfur cluster. As shown in Fig. 8A, just after 1 min of reaction of the GST-fused reconstituted N1c fragment with air, the absorbance at 340 nm and in the broad region of 420–600 nm immediately increased by −20–30% of the original intensity. Three new shoulders appeared at around 420, 460, and 560 nm, imparting a distinct red color characteristic of the [2Fe-2S]2+ cluster. By 10 min, it reached the maximum absorption and did not show any further change for up to at least 30–60 min. Then, further exposure to air resulted in progressive bleaching of the absorption in the entire visible region over a period of 10–12 h with t1/2 = 3–4 h, indicating the iron-sulfur cluster is being degraded due to oxygen. No precipitation was seen in the entire time course. These observations clearly suggested formation of a new, different cluster species from the [4Fe-4S]2+ cluster in the N1c center upon oxygen exposure. We also examined the reversibility of the newly appeared, air-oxidized N1c cluster. After the GST-fused N1c fragment was oxidized by air for 30 min, the reconstitution was carried out by addition of Fe2++, S2−, and DTT in the anaerobic chamber. The yellowish-red color of the iron-sulfur cluster in the N1c fragment reappeared, and it restored its absorption spectrum (Fig. 8B), indicating that the cluster conversion is at least partially reversible in vitro.

The inter-conversion between [4Fe-4S]2+ and [2Fe-2S]2+ in transcription factor for fumarate nitrate reduction (FNR) and biotin synthase (BS) was reported previously (49–55). E. coli FNR controls the switch from anaerobic to aerobic metabolism by the O2-induced conversion of the active [4Fe-4S] form of FNR into an inactive [2Fe-2S] form. Therefore, we compared the difference spectra of the air-exposed GST-fused N1c fragment (90 min) minus anaerobic GST-fused N1c fragment before exposure with the difference spectrum of FNR ([60 min] − [0 min]) and that of BS ([2 h] − [0 min]) re-plotted from Refs. 51 and 53, respectively. D, effect of air exposure on EPR spectrum of the reconstituted GST-fused N1c cluster. The samples of line 1 and line 2 in B were reduced by dithionite and then subjected to EPR measurements. EPR conditions are the same as in Fig. 4A.
[Fe2S-25]⁺ signal was detected. In the air-oxidized sample, very small EPR signals arising from [3Fe-4S]⁺ cluster was observed, which could account for less than 5% of the original [4Fe-4S] cluster signals (data not shown), suggesting that this [3Fe-4S] species was not a major product by air exposure in our case. Currently, we are not able to identify the iron-sulfur cluster generated by air-exposure. However, our results strongly suggest a possibility that a [2Fe-2S] type cluster is formed by air oxidation in the N1c fragment, which can spontaneously be converted to [4Fe-4S] cluster in the presence of strong reducing such as dithionite. This phenomenon (iron-sulfur cluster conversion) is akin to the cases of FNR and BS (51, 53). To confirm the formation of [2Fe-2S]²⁺ in the N1c cluster after air exposure, further experiment by Mössbauer or Resonance Raman spectroscopy would be needed.

Discussion

The Nqo3 subunit and its homologues contain multiple iron-sulfur clusters and are considered to play an important role in the electron transfer from the NADH binding site to the quinone reduction site (3, 4). We have shown previously that the expressed P. denitrificans Nqo3 subunit bears two EPR detectable iron-sulfur clusters, one binuclear cluster with axial symmetry (N1b) and one tetranuclear cluster with rhombic symmetry (N4) (10). The presence of another tetranuclear cluster (most likely cluster N5) was also proposed in the same subunit. In the present study, we have attempted to identify an additional iron-sulfur cluster coordinated by the cysteine motif (CXXCXXXCaC) in the Nqo3 subunits, which is found only in certain bacterial NDH-1 (Fig. 1). We expressed and reconstituted the polypeptide fragment containing the cysteine motif. Our present studies on the N1c fragment strongly indicated that this fragment is able to coordinate a [4Fe-4S] cluster based on the following experimental evidence: (i) This iron-sulfur cluster showed a broad axial type EPR spectrum with the maximal signal amplitude in the temperature range of 12–15 K. This is a much faster spin relaxation behavior than that of the usual binuclear iron-sulfur clusters. (ii) The ratio of spin concentration of the EPR signals of the reconstituted GST-fused protein and its chemical analysis of acid-jabile S²⁻ is about 1:4. (iii) The UV-visible absorption spectrum is characteristic of a [4Fe-4S] cluster but not of a [2Fe-2S] cluster. Mutagenesis studies confirmed that all four cysteine residues are needed for the ligation of this [4Fe-4S] cluster. This type of EPR signal, however, has not been observed in either T. thermophilus or E. coli NDH-1 enzyme complexes (27). This is an unexpected finding because the cysteine motif has previously been proposed to ligate a [2Fe-2S] cluster, which was thus designated N1c. In agreement with our observations, crystal structural analyses revealed that the same sequence motif ligates a [4Fe-4S] cluster in some enzymes, which include the formate dehydrogenase H in E. coli (CXXXXXXXXaC) (56), the periplasmic nitrate reductase in the sulfate reducing bacterium, Desulfovibrio desulfuricans ATCC27774 (133XXCXXXCaC) (57), the formate ferredoxin oxidoreductase and the aldehyde ferredoxin oxidoreductase from Pyrococcus furiosus (286XXCXXXCaC) (493), ferredoxin oxidoreductase numbering (58, 59). Therefore, these observations have raised a question regarding type of the iron-sulfur cluster coordinated in this cysteine motif in situ and assignment of the [2Fe-2S] clusters of the E. coli NDH-1. If we examine the “binuclear N1c” signal in E. coli NDH-1 carefully again, the line shape is quite similar to that of the N1a in the overexpressed Nqo2 subunits of P. denitrificans and T. thermophilus NDH-1 with the same g values (gₓᵧz = 2.00, 1.95, 1.92) (22) and resembles that of the binuclear cluster in the bovine FP fraction by Ragan et al. (60). These spectral similarities point to a possibility that cluster N1c signals detected in the E. coli NDH-1 may arise from the cluster N1a, if we assume that the midpoint redox potential (Eₚₒ) value of the cluster N1a was shifted up to −250 mV during aerobic membrane preparation. Nevertheless, at this moment we still cannot discriminate definitively the following possibilities one from the other: (i) the binuclear N1c signal detected in the E. coli NDH-1 corresponds to cluster N1a; (ii) The N1c cysteine motif in the E. coli NuoQ exceptionally ligates a [2Fe-2S] cluster in situ despite the fact that the motif is used to ligate a [4Fe-4S] cluster in other enzymes mentioned above; (iii) The binuclear N1c EPR signal is a direct consequence of aerobic conversion of the [4Fe-4S] center described as below. The last possibility (iii) would provide an alternative explanation for the discrepancy between our result and previous observation in E. coli system. Apparently, more work is needed to rigorously test these possibilities.

This report demonstrates that the T. thermophilus N1c fragment ligates the [4Fe-4S] cluster with a unique feature. At the present time, there are at least two speculations regarding function of this cluster. First, Finel (61) hypothesized that this cluster is related with electron transfer by menaquinone (MQ) whose Eₚₒ values are much lower than those of UQ. That is because many bacteria bearing this cysteine motif in the Nqo3 subunits catalyze electron transfer to MQ (e.g. T. thermophilus HB-8 contains only MQ-8 as Q). The second speculation was advocated by Steuber/Dimroth’s group (62). It has been reported that the membranes aerobically isolated from certain bacteria resulted in significantly loss of NDH-1 activity (e.g. Klebsiella pneumoniae (62), E. coli (62), Helicobacter pylori (63)). Steuber speculated that the inactivation of NDH-1 might be due to oxidative damage of some redox cofactors (62). It is known that these bacterial NDH-1 house the N1c-binding cysteine motif. The unique feature of the T. thermophilus N1c cluster may suggest that this cluster is a candidate of the redox cofactors. For this purpose, construction of center N1c-deficient mutants seems to be useful.

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