Iron-Sulfur Cluster N5 Is Coordinated by an HXXXXCXXXHXXXX Motif in the NuoG Subunit of Escherichia coli NADH:Quinone Oxidoreductase (Complex I)*

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NADH:quinone oxidoreductase (complex I) plays a central role in cellular energy metabolism, and its dysfunction is found in numerous human mitochondrial diseases. Although the understanding of its structure and function has been limited, the x-ray crystal structure of the hydrophilic part of Thermus thermophilus complex I recently became available. It revealed the localization of all redox centers, including 9 iron-sulfur clusters and their coordinating ligands, and confirmed the predictions mostly made by Ohnishi et al. (Ohnishi, T., and Nakamaru-Ogiso, E. (2008) Biochim. Biophys. Acta 1777, 703–710) based on various EPR studies. Recently, Yakovlev et al. (Yakovlev, G., Reda, T., and Hirst, J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 12720–12725) claimed that the EPR signals from clusters N4, N5, and N6b were misassigned. Here we identified and characterized cluster N5 in the Escherichia coli complex I whose EPR signals had never been detected by any group. Using homologous recombination, we constructed mutant strains of H101A, H101C, H101A/C114A, and cluster N5 knock-out. Although mutant NuoEFG subcomplexes were dissociated from complex I, we successfully recovered these mutant NuoCD subunits by expressing the His-tagged NuoCDEFG subunits, which had high affinity to NuoG. The W221A mutant was used as a control subcomplex carrying wild-type clusters. By lowering temperatures to around 3 K, we finally succeeded in detecting cluster N5 signals in the control for the first time. However, no cluster N5 signals were found in any of the N5 mutants, whereas EPR signals from all other clusters were detected. These data confirmed that, contrary to the misassignment claim, cluster N5 has a unique coordination with His(Cys)3 ligands in NuoG.

The proton-translocating NADH:ubiquinone oxidoreductase (EC 1.6.5.3) (complex I) is the largest energy-transducing complex in the aerobic respiratory chains of many prokaryotes and eukaryotes (1–3). Complex I is one of the most complicated and elaborate iron-sulfur (Fe/S)3 proteins yet known (4). Recently, the three-dimensional structure of the hydrophilic domain of Thermus thermophilus HB-8 complex I has been determined at 3.3 Å resolution (5). It revealed the spatial localization of all of the redox centers and their coordinating amino acid residues. For the sake of simplicity, we will use the Escherichia coli nomenclature for each subunit. The primary electron acceptor of complex I is a noncovalently bound flavin mononucleotide (FMN) located in the NuoF subunit. Electrons are believed to flow through seven Fe/S clusters. They are as follows: a tetranuclear [4Fe-4S] cluster N3 in NuoF, a binuclear [2Fe-2S] cluster N1b and two [4Fe-4S] clusters N4 and N5 in NuoG, two [4Fe-4S] clusters N6a and N6b in NuoI, and a [4Fe-4S] cluster N2 in NuoB (Fig. 1A) (5, 6). These seven Fe/S clusters are lined up to form the main electron transfer pathway, whereas a [2Fe-2S] cluster N1a in NuoE and a [4Fe-4S] cluster N7 in NuoG stick out from the main pathway. It has been suggested that clusters N1a and N7 may have different roles (5, 7). The sequence analysis has confirmed that all of the Fe/S clusters except cluster N7 are conserved (8).

EPR spectroscopy has been most informative for the analysis of Fe/S clusters. However, because the sensitivity and resolution of EPR spectroscopy are much lower than those of spectrophotometry, considerable spectral overlaps exist. Therefore, to make a definitive assignment of the spectra to each of the specific Fe/S clusters is sometimes very difficult. In addition, some Fe/S clusters may not be detectable by EPR when the spin relaxation time is too short, or when the Fe/S cluster is not paramagnetic under certain chemical or electronic conditions. In fact, E. coli complex I contains at least six EPR-detectable Fe/S clusters as follows: N1a, N1b, N2, N3, N4, and N7. However, N5 signals have not been detected so far. Cluster N6a and N6b signals have not been characterized sufficiently. Another problem is that EPR identification of an Fe/S cluster residing in the overexpressed single subunit could be misleading, because its EPR signals may be altered from those in the intact complex I system (9). EPR spectral properties of Fe/S clusters, such as the principal g values, line widths, and the spin-relaxation rates can be very sensitive to the micro-environment around the Fe/S cluster, especially in a delicate multicomponent membrane.
protein like complex I (9). Therefore, assigning the observed EPR signals to the structurally defined clusters has been an extremely difficult task.

Cluster N5 has a very fast spin relaxation. Therefore, its EPR spectra are detectable only when an extremely low temperature and high microwave power are used (6). For these reasons, it has been most difficult to study cluster N5 among all Fe/S clusters in complex I. In fact, cluster N5 was detected only in several species such as pigeon (10), bovine heart (6), Yarrowia lipolytica (11), Rhodobacter sphaeroides (12), and Paracoccus denitrificans (13). EPR characterization of cluster N5 was limited mostly to complex I from bovine heart mitochondria and Y. lipolytica. Cluster N5 has been assigned to the second binding motif in the N-terminal part of the 75-kDa subunit of mitochondrial complex I or the NuoG subunit in E. coli complex I (Fig. 1B). The unusual Fe/S binding motif “HXXXXCX-CXX” that includes one His and three Cys residues is conserved in complex I in all species so far studied, and is also seen in other enzymes such as iron-only hydrogenases (14). Ohnishi (15) already predicted as early as in 1993 that this mixed-ligand cluster N5 might have mixed ground states of $S = 1/2$ and $3/2$. This could explain why its detected spin concentration at the $S = 1/2$ ground state was only 0.25 per complex I (16).

Recently, Hirst and co-workers (17) raised controversial arguments that there were EPR “misassignments” among clusters N4, N5, and N6b by simply comparing EPR spectra observed in the overexpressed single NuoG subunit and in intact complex I. Here we challenge their misassignment claim on cluster N5. First, we identified the spectroscopic property of cluster N5 in Escherichia coli complex I, which had not been previously detected by EPR. Second, we designed experiments to determine whether cluster N5 has His(Cys)$_3$ mixed ligands or regular four Cys ligands. By introducing mutations into the genomic DNA with homologous recombination, we constructed four different cluster N5 mutant strains that include single mutants H101A and H101C, a double mutant H101A/C114A, and a cluster N5 knock-out (ΔN5) mutant. As a result, without any chemical reconstitution, we were able to detect cluster N5 signals for the first time in the control but not in any cluster N5 mutant samples. These data suggest that the misassignment claim to clusters N4 and N5 and their proposal of a four-cysteine coordination to cluster N5 by Hirst and co-workers (17) are incorrect.

EXPERIMENTAL PROCEDURES

Materials—The pCRScript cloning kit was from Stratagene (La Jolla, CA), and the PCR product purification and gel extraction kits were from Qiagen (Valencia, CA). The gene replacement vector, pKO3, was a generous gift from Dr. George M. Church (Harvard Medical School, Boston). All chemicals used were of the highest grade available from Sigma. Bovine complex I was purified as described previously (18).

Preparation of the Cluster N5 Mutant Strains and Expression Vector for the NuoCD Subunit—Four mutants for cluster N5 in the E. coli NuoG subunit were constructed as described previously (19). The oligonucleotides used for the site-directed mutagenesis are listed in Table 1. Site-directed mutagenesis was performed mainly by using the GeneEditor™ (Promega, Madison, WI) except for the H101C mutation, which was carried out by QuickChange™ II XL (Stratagene, La Jolla, CA). All of the mutations were verified by DNA sequencing. Each mutated
fragment was inserted into pKO3/nuoG (19). The *E. coli* NuoG-deficient strain [MC4100 (*nuoG::Spcl*)] (19) was transformed with those pKO3/nuoG-mutant vectors, and homologous recombination was carried out as described previously (19, 20). The *nuoCD* gene was obtained from genomic DNA from *E. coli* DH5α by PCR (Table 2). After the DNA sequence was verified by sequencing, the Ndel/Xhol fragment harboring the entire *nuoCD* gene was ligated into pET16b, and we designated it as pET16b/*nuoCD*.

**Bacterial Growth and Membrane Preparation**—The mutant *E. coli* strains (Table 2) were grown in 250 ml of the Terrific Broth medium for 4–5 h at 37 °C until *A*<sub>600 nm</sub> reached ~1.5. The cells harvested were resuspended to 10% (w/v) in an ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 10% (w/v) glycerol. A lysozyme from chicken egg white was added to a final concentration of 0.5 mg/ml and incubated for 30 min. The cell suspension was briefly sonicated and then passed once through a French press (Spectronic Instruments, Rochester, NY) at 15,000 p.s.i. and centrifuged again at 23,000 rpm. The cell pellets were broken by two passages through a French press at 15,000 p.s.i. After unbroken cells and inclusion bodies were removed by centrifugation, the supernatant was ultracentrifuged at 250,000 × *g* for 60 min. The NuoCDEFG subcomplex was purified from the supernatant under anaerobic conditions (<1 ppm) at room temperature. All solutions were degassed and purged with oxygen-free argon and equilibrated in the chamber overnight before use. The sample was loaded onto a nickel-nitritolriacetic acid column (1.5 × 5.0 cm) equilibrated with buffer A. The column was washed with 50 ml of the same buffer, and then the subcomplex NuoCDEFG protein was eluted with the same buffer containing 200 mM L-histidine and Complete<sup>TM</sup> protease inhibitor mixture (Roche Applied Science). After removing histidine with a desalting column (10-DG, Bio-Rad), the protein was concentrated using an Amicon Ultra filter (molecular mass cutoff of 100 kDa; Millipore, Billerica, MA).

**EPR Spectroscopy**—EPR samples were immediately prepared anaerobically after their purification. Redox mediators, methyl viologen and benzyl viologen, were added at 5 μM each. After the addition of 10 mM neutralized sodium dithionite solution, each sample was incubated for 5–10 min, transferred to an EPR tube, and then frozen immediately. EPR spectra were recorded by a Bruker ESP 300E spectrometer at X-band (9.4 GHz) using an Oxford Instrument ESR900 helium flow cryostat. EPR spectra of the Fe/S clusters were simulated using SimFonia software (Bruker, Germany). Spin quantitations were carried out under nonsaturating conditions using 0.5 mM Cu(II)EDTA or 0.5 mM Cu(II) perchlorate as standards according to Ref. 22. Power saturation data were analyzed with a computer fitting method (23, 24).

**Other Analytical Procedures**—UV-visible absorption spectra were recorded on a Beckman DU640 spectrophotometer at room temperature. Protein assay was routinely done by the methods of Lowry et al. (25) and Bradford (26). SDS-PAGE and blue native PAGE were carried out according to Laemmli (27) and Schägger (28), respectively. Immunoblotting was conducted as described previously with affinity-purified antibodies (29). Non-heme iron and acid-labile sulfide were determined according to Refs. 30, 31, respectively.

### Table 1

| Oligonucleotides | Sequences |
|------------------|-----------|
| W221A            | 5′-GCCATACACCATTGAGTACCTGAGTCCG-3′ |
| H101A            | 5′-CTGTGGATACCAAGCAGGCCTCAGCTGCGCGCCG-3′ |
| C114A            | 5′-GAGAAAGGCGTTAGACCCATCTTCAAGATACG-3′ |
| C105A/C108A/C114A| 5′-AACGCCGCCAGCAGGCTCGAGTGAGAGGCCGTTAACGCCCATCTTCAAGATACG-3′ |
| nuoCD-forward(NdeI)| 5′-CACCACGACCATTTTCAATGTGTTGACCAACAT-3′ |
| nuoCD-reverse(XhoI)| 5′-GTGGTGAATCACCAGTCGCCACACTGCCG-3′ |
| nuoCD-forward(NdeI)| 5′-CACCACGACCATTTTCAATGTGTTGACCAACAT-3′ |
| nuoCD-reverse(XhoI)| 5′-GTGGTGAATCACCAGTCGCCACACTGCCG-3′ |

### Table 2

**E. coli** strains used in this study

| Strain         | Genotype          | Source          |
|----------------|-------------------|-----------------|
| MC4100         | F<sup>−</sup>, araD139, (arg F-lac)U169, ptsF25, relA1, flb5301, rplS 150<sup>−</sup> | Ref. 44        |
| ΔNuoG          | MC4100, nuoG<sup>−</sup>:spcl | Ref. 19        |
| W221A          | MC4100, nuoG W221A  | This study      |
| ΔN5            | nuoG<sup>−</sup>MC4100/C105A/C108A/C114A | This study      |
| H101A          | MC4100, nuoG H101A | This study      |
| H101C          | MC4100, nuoG H101C | This study      |
| H101A/C114A    | MC4100, nuoG H101A/C114A | This study      |
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FIGURE 2. A, localization of the NuoG subunit from the wild type, W221A, and four cluster N5 mutants (ΔN5, H101C, H101A, and H101A/C114A). Western blot analysis of membrane (M) fractions and soluble (S) fractions from each E. coli strain were carried out with affinity-purified anti-NuoG antibody. 10 μg of samples (except 1 μg for wild type) were applied to each lane of a Laemmli 10% SDS-polyacrylamide gel. B, partial purification of the NuoCDEFG subcomplex from the soluble fractions of the W221A cells overexpressed with the His-tagged NuoCD subunit. Immunoblotting analysis of the samples from different purification stages were shown. Lane 1, soluble fraction; lane 2–5, flow-through fraction, front elution, main elution, and tail elution from the nickel affinity column chromatography, respectively; lane 6, after gel filtration; lane 7, after concentration with the 100-kDa cutoff centrifugation filter. Protein samples (10 μg each) were applied to each lane of a Laemmli 10% SDS-polyacrylamide gel. Affinity-purified anti-NuoG, anti-NuoCD, and anti-NuoF antibodies were used. The molecular sizes in kilodaltons are shown on the left. C, Western blot analysis of the partially purified mutant NuoCDEFG subcomplexes with anti-NuoG, anti-NuoF, and anti-NuoE antibodies (left panel) and anti-NuoCD antibodies (right panel). Lane 1, W221A mutant; lane 2, H101A; lane 3, ΔN5; lane 4, H101A/C114A. 15 μg of samples were applied to each lane of a Laemmli 12.5% SDS-polyacrylamide gel. The molecular sizes in kilodaltons are shown in the middle.

Any variations from the procedures and other details are described in the figure legends.

RESULTS

Effects of Mutations in E. coli Cluster N5 and Partial Purification of the NuoCDEFG Subcomplex by Expressing His-tagged NuoCD Subunit—We constructed single mutant strains H101A and H101C, a double mutant H101A/C114A, and a cluster N5 knock-out (ΔN5) mutant (Table 1). The mutations in the predicted binding motif for cluster N5 drastically affected the stability of complex I. We did not find any deamino-NADH dehydrogenase activity in the membrane preparations from any of those mutant strains. As shown in Fig. 2A, the NuoG subunit was mainly detected in soluble fractions from the H101A and H101C mutants. It was weakly detected in soluble fractions and not in the membrane fractions from the double mutant H101A/C114A and the ΔN5 mutant. Western blot analyses with anti-NuoE and anti-NuoF antibodies gave similar results (data not shown). These data suggest that the NADH dehydrogenase subcomplex (NuoEFG subcomplex) in those cluster N5 mutants was unstable and dissociated from the membrane. A similar destabilizing effect on the complex I assembly has been reported for the H129A mutant (corresponding to E. coli H101A) in the 75-kDa subunit of mitochondrial complex I from Y. lipolytica (32).

To recover the mutated NuoEFG subcomplex from these soluble fractions, we overexpressed the His-tagged NuoCD subunit in the mutant strains. We used the W221A mutant (Fig. 2B) to obtain the wild-type Fe/S clusters in the NuoG subunit for the following reasons. (i) The NuoEFG subcomplex was dissociated from the membrane in W221A as the cluster N5 mutants (Fig. 2A). (ii) Based on the crystal structure, Trp-221 is located away from all Fe/S clusters including cluster N5. (iii) A low level of complex I activity was detected in the membrane fraction of the W221A strain, where a trace amount of NuoG remained. (iv) Another Trp-221 mutation (W221F) had no effect at all on either complex I activity or its integrity.

We were able to partially purify the W221A and cluster N5 mutant subcomplexes from these cytoplasmic fractions with nickel chelation affinity column chromatography (Fig. 2B). In these preparations, the levels of NuoCD, NuoE, NuoF, and NuoG subunits were basically similar according to the Western blot analysis (Fig. 2C), but neither NuoB nor NuoI was detected (data not shown). All samples exhibited the typical absorption spectra containing [4Fe-4S] clusters (data not shown). The absorption peaks around 420 nm were roughly proportional to non-heme iron contents in the preparations (79–420 μM).

Cluster N5 Mutants Have EPR Signals from Other Fe/S Clusters Except N5—To characterize bound iron-sulfur clusters in the cluster N5 mutants, we performed EPR analysis. First, we examined EPR spectra of the [2Fe-2S] clusters in the dithionite-reduced samples in the g = 2 region at 40 K and 5 milliwatts (Fig. 3, left). We detected two sets of EPR signals, one with
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At 6 K and 5 milliwatts, EPR signals arising from multiple [4Fe-4S]+ clusters were detected (Fig. 3, right). The EPR spectra from W221A displayed signals with g values of 2.07, 2.045, 2.02, 1.93, 1.89, and 1.88. Because our samples did not contain the NuoB and NuoI subunits, EPR signals from four different [4Fe-4S] clusters were expected. They are cluster N3 in NuoF and clusters N4, N5, and N7 in NuoG. By comparing published g values in E. coli complex I (7, 35), it was concluded that the EPR signals with g\textsubscript{\text{Z,y,x}} = ~2.04, ~1.94, ~1.88; g\textsubscript{\text{X,x}} = ~2.07, ~1.93, ~1.88; and g\textsubscript{\text{Z,y,x}} = 2.045, ~1.94, ~1.89 arose from three [4Fe-4S] clusters N3, N4, and N7, respectively. All of the mutant subcomplexes displayed EPR signals for these [4Fe-4S] clusters. This suggests that the mutations in the cluster N5-binding site did not lead to a drastic change in the characteristics of neighboring Fe/S clusters. However, there were some variations in signal intensities. For direct comparison among mutants, EPR data at 6 K (Fig. 3, right) were normalized based on the signal amplitude at g = 2.045 of the EPR signal from cluster N7. Cluster N7 is distant from the main electron transfer pathway and is unlikely to have significant paramagnetic interactions with other Fe/S clusters. We observed that the cluster N4 signals were increased in ΔN5 but decreased in H101C compared with the control. These changes of N4 signals seem to be caused by a loss of interactions between clusters N4 and N5 or by an altered interaction in these mutants. Almost no [3Fe-4S] signals were detected in the mutants.

Cluster N5 Signals in Bovine Heart Complex I—Because it is extremely difficult to detect signals from cluster N5 in E. coli samples, we first reexamined EPR spectra in the bovine heart complex I where the cluster N5 signals were well characterized. As shown in Fig. 4, we measured EPR signals from the iron-sulfur clusters in the purified bovine heart complex I in a wide range of temperatures (from 45 to ~3 K) at 1 milliwatt microwave power. The complex I sample (prepared at the Yoshikawa laboratory) was reduced with 1 mM NADH. At higher temperatures (~40 K), only the cluster N1b spectrum was observed as expected because this is a binuclear Fe/S cluster. By decreasing the sample temperature, signals from cluster N2 were first observed in the range of 20 to 10 K. Signals from cluster N3 were optimally observed at 10 to 8 K, whereas those from N4 were at <10 K. When the temperature was further lowered to around 3 K and the microwave power was increased >5 milliwatts, signals from cluster N5 finally became detectable. The N5 spectrum exhibited a rhombic symmetry (g\textsubscript{x,y,z} = 2.06, ~1.93, 1.90). It should be pointed out that the N5 spin relaxation differs considerably among complex I samples prepared with different methods. For example, complex I from the Hatfield laboratory was detectable even at 7 K with 5 milliwatts (6) suggesting that it had a slower spin relaxation. Another interesting observation is that when microwave power was increased from 1 to 20 milliwatts around 3 K, the N5 signals at g\textsubscript{x} = 2.06 and g\textsubscript{y} = 1.90 were significantly enhanced. The cluster N4 signals also increased at higher microwave power around 3 K. This suggests a possible spin-spin interaction between N4 and N5 (their edge-to-edge distance is only 8.5 Å; see Fig. 1A). This is consistent with the results for cluster N4 in Y. lipolytica complex I (11).

The EPR Signal Arising from Cluster N5 in E. coli—We detected cluster N5 signals in our W221A mutant NuoCDEFG subcomplex around 3 K and with 5 milliwatts. We analyzed the g\textsubscript{x} region of the EPR spectra for the comparison among mutants, because there was an interfering broad negative trough in the magnetic field below 3500 G at temperatures <4 K. We also measured bovine heart complex I as a biological temperature “standard.” As seen in Fig. 5, the g = 1.90 signal was successfully observed in W221A, but not in H101A, H101C, ΔN5. Also, there was no discernible N5 signal in H101A/C114A (data not shown).

We compared the EPR spectra of W221A, H101C, and ΔN5 around 3 K and at both 5 and 0.5 milliwatts (Fig. 6). It is apparent that the g = ~1.89 signal arising from cluster N7 was completely saturated at this low temperature. In W221A, the newly appeared g = 1.90 signal and the g = 1.88 arising from cluster N4 became noticeable at 5 milliwatts as compared with those obtained at 0.5 milliwatt. In contrast, in H101C and ΔN5, a signal increase was only observed at g = 1.88 (Fig. 5). Around 3 K, the g = 1.90 signal in W221A became more pronounced with an increase of microwave power from 0.5 milliwatt to 20 milliwatts, indicating an extremely fast spin relaxation (data not shown). All these data suggest that the newly appearing g = 1.90 signal around 3 K in W221A is the g\textsubscript{x} of cluster N5.

DISCUSSION

Because EPR identification of cluster N5 in the reconstituted overexpressed NuoG subunit was unsuccessful (19), we introduced a different mutagenesis strategy to test the possibility of a histidine ligation to cluster N5 in E. coli complex I. We constructed four different cluster N5 mutant strains by introducing mutations into the genomic DNA. The NuoEFG subcomplex was dissociated from the complex I assembly in these mutants. We expressed the His-tagged NuoCD subunit in the cytosol and purified the mutant NuoG subunit as NuoCDEFG subcomplexes with nickel affinity column chromatography. Without any chemical reconstitution, we successfully identified the EPR signals arising from cluster N5 for the first time as well as those from all other existing clusters. Our site-directed mutagenesis experiments strongly suggested that cluster N5 is indeed coordinated with an unusual binding motif HXXXXCXXCXXXXX.
The Mutation of Fe/S Clusters Often Causes Destabilization—
We often found that the mutation of ligand(s) of a [4Fe-4S] cluster destabilized the cluster so much that the cluster assembly or even protein folding itself was impaired. In fact, when a H106A mutant (which corresponds to H101A in this study) in the *P. denitrificans* NuoG homologue (Nqo3) was overexpressed, almost no iron-sulfur clusters were incorporated into the subunit, making further investigations at the subunit level impossible (13). There is another report that the His to Cys variant of *Clostridium acetobutylicum* iron-only hydrogenase, which has the same binding motif as cluster N5, was not produced in quantities large enough for structural and thermodynamic studies (36). In this respect, it was fortunate for us that the Fe/S clusters remained intact (Fig. 3), even though the mutations in the cluster N5-binding site caused dissociation of the NuoEFG subcomplex from the membrane.

About Histidinyl Ligation—The HXXXXCXXXXXC coordination motif only exists in membrane-bound nitrate reductases, iron-only hydrogenases, and complex I. The HoxU subunit of the soluble [NiFe]-hydrogenase from *Ralstonia eutropha*, which is homologous to NuoG, contains the same binding motifs for cluster N1b, N4, and N5 (37). However, its motif for cluster N5 is GXXHCXXX, which is slightly different from that for cluster N5 in complex I. But it was conserved in this enzyme group.

It has been speculated that the histidine ligand may tune the midpoint potential. Rieske [2Fe-2S] proteins that are coordinated by two cysteines and two histidines have a high redox midpoint potential (*E_m*) of about +300 mV. In the case of cluster N5, histidine is probably ligated to the iron atom that is one of the iron pairs, which does not receive electrons (38). That would explain why cluster N5 has a similar *E_m* to other isopotential groups of Fe/S clusters (*E_m* = approximately −250 mV), which have regular (Cys)_4 ligands (6).

Non-cysteiny1 coordinations such as histidine are known to produce a [4Fe-4S] cluster having mixed ground states of $S = 1/2$ and 3/2. A good example is Pyrococcus furiosus ferredoxin, which contains aspartate coordination, CXXDXXCX (38). The EPR spectra from the mixed ligand [4Fe-4S] cluster showed two different EPR signals from the ground spin states, $S = 1/2$ and 3/2. In the second ligand mutant (D14C) of this ferredoxin, there were only EPR signals from the $S = 1/2$ state (39). In light of all this, it is highly possible that cluster N5 is in a mixed ground spin state of $S = 1/2$ and 3/2. In fact, the over-
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The properties of the [4Fe-4S] cluster with a His ligand have not yet been clearly determined. In the study of Y. lipolytica complex I, Waletko et al. (32) reported that the H129A mutation in the binding motif HXXCXXXCCXXX of cluster N5 did not affect the EPR signals from cluster N5. This is contrary to our present data that the cluster N5 signal was abolished in our corresponding H101A mutant. However, there may be problems in their experimental results. First, it is not clear whether the loss of complex I activity in the H129A membrane (to $< 3\%$ of that in the wild-type membrane) is a direct consequence of the mutation. They showed that the intensities of all subunit bands of complex I in H129A were greatly reduced (probably $\sim 10\%$) by two-dimensional gel analysis as compared with those in the wild type. Therefore, the loss of activity in the mutant could have resulted from the loss of assembled complex I in the His-129 mutant membrane. Second, it is difficult to understand why the cluster N2 signals were still observed in their H129A mutant complex I, which was reduced with NADH. According to the crystal structure, cluster N2 is located downstream of the Fe/S cluster with His-129 in the electron transfer pathway. If this H129A mutation indeed affected this Fe/S cluster, the electron flow from NADH should be blocked at this site, and cluster N2 in H129A could not be reduced with NADH. Then the cluster N2 signals should not appear in their EPR spectra. Apparently, their biochemical data on the H129A mutation contradict their EPR data.

In the periplasmic NiFe hydrogenases, when the histidine (His-184 in Desulfovibrio fructosovorans) in the binding motif “HXXCXXXCCXX” was changed to a cysteine or glycine, the iron-sulfur cluster was still assembled, but the electron transfer activity of the mutants was almost completely lost (36). For the H184G variant, the activity was partially restored by adding exogenous ligands such as imidazole (36).

We Have Not Misassigned Cluster N5—Recently Hirst and co-workers (17) claimed that the EPR signals of cluster N4, N5, and N6b were misassigned to their structurally defined clusters. They proposed the following: (i) the EPR signals of N5 were misassigned to N4; (ii) the signals from N6b, which resides in NuoI but not in NuoG, were misassigned to N4; and (iii) cluster N5 is a four cysteine-ligated [4Fe-4S] cluster in NuoG (not the cluster with His(Cys)$_3$ ligands). We will discuss points i and iii because they are relevant to our present results.

In E. coli complex I, only five reduced iron-sulfur clusters were typically observed, and their signals are referred to as N1a, N1b, N2, N3, and N4. As Hirst and co-workers (17) pointed out, the reported g values of cluster N4 in whole E. coli complex I are 2.09, 1.93, 1.89 (33, 41), and they are not quite the same as those from the overexpressed, His-tagged NuoG subunit ($g_{x,y,z}$ = 2.07, 1.95, 1.89) (17) and those from the maltose-binding protein-fused NuoG ($g_{x,y,z}$ = 2.06 to 2.07, 1.94, 1.89) (19). Similarly, the g values of cluster N4 in the NuoEFG subcomplex varied. Two papers reported $g_{x,y,z}$ = 2.09, 1.93, 1.89 for cluster N4 (33, 41), which are similar to the g values of cluster N4 assigned in the whole complex I, whereas another report showed that no
signals from cluster N4 were detected (42). In our study, the \(g_{z,y,x} = 2.07, 1.93, 1.88\) signals were assigned as cluster N4 in the NuoCDEFG subcomplex. Cluster N4 is known to be very sensitive to alteration of its microenvironment, and the \(g\) values, especially \(g_z\), can be easily shifted and broadened in bovine complex I (9, 10, 16, 43). Therefore, it is risky to depend on \(g\) values alone for EPR signal assignments. Hirst and co-workers (17) seem to have overlooked this point when they raised the misassignment issue.

Because we have already shown that EPR signals of \(g_{z,y,x} = 2.07, 1.93, 1.88\) arose from cluster N4 in our previous cluster knock-out experiment, there is no doubt that our cluster N4 assignment was correct (19). In the present study, the \(g = 1.90\) signal was detected only in the W221A variant harboring cluster N5, along with other existing clusters, including cluster N4. There is no way to assign the \(g = 1.90\) signal to cluster N4. The characteristics of the \(g = 1.90\) signal in the W221A variant resemble those of cluster N5 from the bovine complex I that has been investigated in detail (Fig. 4) (6). We would also like to point out that cluster N5 signals are not detectable under the EPR conditions that Hirst and co-worker employed (5–40 K, 0.1 milliwatt) (17). The signals can only be detected in \(E. coli\) at lower temperature and with higher power (\(\sim 3\) K, 5 milliwatts) as we demonstrated. Therefore, the \(g_z = 2.07\) signals from the overexpressed subunit shown in Fig. 5 of Ref. 17 cannot be assigned to N5 in their system. Again, our mutation data clearly showed that the histidine in the binding motif HXXXCXXXCXXXXX is a ligand for cluster N5. For these reasons, we believe that the misassignment claim and their proposal of four-cysteine coordination for cluster N5 are incorrect.

In conclusion, cluster N5 in \(E. coli\) complex I is hardly detectable by EPR above 4 K, because it has extremely fast spin relaxation. It is highly likely that this particular behavior of cluster N5 is caused by the unusual His(Cys)\(_3\) mixed coordination. Our current mutation study strongly supported this working hypothesis. Considering that this histidine in the motif is evolutionarily conserved in complex I, there must be some functional importance as to why histidine was selected for use as a cluster N5 ligand in complex I. Further comprehensive study of this unique Fe/S cluster is eagerly awaited.

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**Note Added in Proof**—After submission of this manuscript, Hirst and co-workers (Reda, T., Barker, C. D., and Hirst, J. (July 4, 2008) *Biochemistry* 10.1021/bi800437g) described, in the Abstract, that “Spectrum N1a, from the 24 kDa subunit [2Fe-2S] cluster, is not observed in bovine heart complex I at any potential.” This statement seems to be misleading because N1a signals defined in their Table 1 as \(g_{z,y,x} = 2.004, 1.945, 1.917\) are values for a “modified form” of N1a. The actual \(g\) values for N1a in the intact membrane are \(g_{z,y,x} = 2.02, 1.94, 1.92\). We always found N1a signals with these latter \(g\) values in the isolated bovine heart complex I. Therefore, one cannot find any sig-
nals with $g_{xx} = 2.004, 1.945, 1.917$ in complex I at any potential. This was discussed more in detail in Ref. 9.

Proposal of the isopotential group was originally based on the redox titrations made on iron-sulfur clusters present within mitochondria (in situ). To date, the truly intact form of complex I has not been established. There are possible redox and magnetic interactions among iron-sulfur clusters in addition to the modification that occurred during the lowering to the extremely low potential ($E_p$) as well as in the isolation process. Ohnishi et al. reported an example of modification of the line shape of N1a by going down to below $-400$ mV in the complex I isolated by Hatefi and co-workers (Ohnishi, T., Blum, H., Galante, Y. M., and Hatefi, Y. (1981) J. Biol. Chem. 256, 9216–9220).

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