EPR Signals Assigned to Fe/S Cluster N1c of the *Escherichia coli* NADH:Ubiquinone Oxidoreductase (Complex I) Derive from Cluster N1a†

Mareike Uhlmann and Thorsten Friedrich*

Albert-Ludwigs-Universität Freiburg, Institut für Organische Chemie und Biochemie, Albertstrasse 21, Chemiehochhaus, D-79104 Freiburg im Breisgau, Germany

Received August 30, 2004; Revised Manuscript Received November 12, 2004

ABSTRACT: The proton-pumping NADH:ubiquinone oxidoreductase, which is also called respiratory complex I, transfers electrons from NADH to ubiquinone via one flavin mononucleotide (FMN) and up to nine iron−sulfur clusters. A structural minimal form of complex I consisting of 14 different subunits called NuoA to NuoN (or Nqo1 to Nqo14) is found in bacteria. The isolated *Escherichia coli* complex I can be split into a NADH dehydrogenase fragment, a connecting fragment, and a membrane fragment. The soluble NADH dehydrogenase fragment represents the electron input part of the complex and consists of the subunits NuoE, F, and G. The FMN and four iron−sulfur clusters have been detected in this fragment by means of EPR spectroscopy. One of the EPR signals, called N1c, has spectral properties, which are not found in preparations of the complex from other organisms. Therefore, it is attributed to an additional binding motif on NuoG, which is present only in a few bacteria including *E. coli*. Here, we show by means of EPR spectroscopic analysis of the NADH dehydrogenase fragment containing site-directed mutations on NuoG that the EPR signals in question derived from cluster N1a on NuoE. The mutations in NuoG disturbed the assembly of the overproduced NADH dehydrogenase fragment indicating that a yet undetected cluster might be bound to the additional motif. Thus, there is no third binuclear iron−sulfur "N1c" in the *E. coli* complex I but an additional tetranuclear cluster that may be coined N7.

The proton-pumping NADH:ubiquinone oxidoreductase,[1] also called respiratory complex I, couples the transfer of two electrons from NADH to ubiquinone with the translocation of four protons across the membrane (*1*−*5*). In doing so, it contributes to the proton motive force, which is essential for energy consuming processes. The bacterial complex consists, in general, of 14 different subunits. Seven subunits are peripheral proteins including all subunits that bear the known redox groups of complex I, namely, one flavin mononucleotide (FMN) and up to nine iron−sulfur (Fe/S) clusters. The remaining seven subunits are very hydrophobic proteins predicted to fold into 54 α-helices across the membrane (*1*−*5*).

The genes of the *Escherichia coli* complex I are organized in the *nuo*-operon (from NADH:ubiquinone oxidoreductase) (*6*). As a particularity of *E. coli nuoC* and *D* are fused and code for one subunit NuoCD (*7*, *8*). The *E. coli* complex I has been isolated in the presence of dodecyl maltoside by means of chromatographic steps (*9*, *10*). The preparation is solely made up of the 13 different subunits being encoded by the *nuo* genes. One noncovalently bound FMN, two binuclear Fe/S clusters coined N1b and N1c, and five tetranuclear clusters called N2, N3, N4, N6a, and N6b have been detected in the preparation (Table 1; *9*−*11*). By means of salt treatment and raising the pH, the isolated complex was split into a NADH dehydrogenase fragment, a connecting fragment, and a membrane fragment (*9*). The soluble NADH dehydrogenase fragment is the electron input part of the complex and consists of the subunits NuoE, F, and G (*7*, *9*, *12*). Overexpression of *nuoB*−*G* in *E. coli* led to the overproduction of the fully assembled NADH dehydrogenase fragment (*7*). The *Strep*-tag II affinity peptide was fused to the C-terminus of NuoF enabeling a simple purification of the engineered fragment by means of chromatography on *Strep*-Tactin (*13*).

Table 1: Localization of the Fe/S Clusters Detected in the Preparation of the *E. coli* Complex I

Fe/S cluster	type	subunit	fragment of the complex
N1a	2Fe/2S	NuoE	NADH dehydrogenase fragment
N1b	2Fe/2S	NuoG	NADH dehydrogenase fragment
N1c	not present[a]	not present[a]	
N2	4Fe/4S	NuoB	connecting fragment
N3	4Fe/4S	NuoF	NADH dehydrogenase fragment
N4	4Fe/4S	NuoG	NADH dehydrogenase fragment
N5	4Fe/4S	NuoG	NADH dehydrogenase fragment
N6a/N6b	4Fe/4S	NuoI	connecting fragment
N7[b]	4Fe/4S[b]	NuoG[b]	NADH dehydrogenase fragment

[a] See this study. [b] Has not yet been detected in the entire complex but represents most likely the cluster ligated by the additional binding motif on NuoG so far only found in a few bacteria.

The NADH dehydrogenase fragment contains the binding sites for NADH and FMN and motifs for the binding of up to six Fe/S clusters. Four Fe/S clusters have been detected

† This work is supported by the Deutsche Forschungsgemeinschaft.

* Corresponding author. Phone +49-(0)761-203-6060. Fax +49-(0)-761-203-6096. E-mail: tfriedri@uni-freiburg.de.

[1] Abbreviations: complex I, proton-pumping NADH:ubiquinone oxidoreductase; EPR, electron paramagnetic resonance; FMN, flavin mononucleotide; Fe/S: iron sulfur; mT, milliTesla.

10.1021/bi048136n CCC: $30.25
Published on Web 01/12/2005

```
                221        *  *   *                              *    270
E.coli            WDMQFAPSICQQCSIGCNISPGERYGELRRIENRY-NGTVNHYFLCDRGRF
A.aeolicus        WLLEKGRTVCNLCPVGCEIQIEYGVGDWRSKRKVYRTKPTDELNICAKGFF
T.thermophilus    WEMEETPTTCALCPVGCGITADTRSGELLRIRARE-VPEVNEIWICDAGRF

P.denitrificans   WELTKTESIDVMDALGSSIRIDTKGREVMRILPRN-HDGVNEEWISDKTRF
R.capsulatus      WELVKTESIDVMDALGSNIRIDAKGREVKRIVPRN-HDGVNEEWLSDKGRF
N.crassa          WELKKTESIDVLDGLGSNIRVDTRGLEVMRILPRL-NDEVNEEWINDKTRF
H.sapiens         WETRKTESIDVMDAVGSNIVVSTRTGEVMRILPRM-HEDINEEWISDKTRF
```

FIGURE 1: Sequence comparison of the homologues of NuoG from *E. coli* [Acc.No.: P33602], *A. aeolicus* [Acc.No.: AAC06711], *T. thermophilus* [Acc.No.: Q56223], *P. denitrificans* [Acc.No.: P29915], *Rhodobacter capsulatus* [Acc.No.: AAC24995], *Neurospora crassa* [Acc.No.: P24918], and *Homo sapiens* [Acc.No.: XP_002669]. The numbering is according to the *E. coli* sequence. The cysteines that are not conserved throughout all species and presumably bind an additional Fe/S cluster are marked with asterisks.

Table 2: Oligonucleotides Used for Site-Directed Mutagenesis[a]

oligonucleotide	sequence
Cys^{230}Ala_for	5′-CAGTTTGCGCCGTCGATC**GCC**CAGCAATGTTCCATCGGC-3′
Cys^{230}Ala_rev	5′-GCCGATGGAACATTGCTGGGCGATCGACGGCGCAAACTG-3′
Cys^{233}Ala_for	5′-GCATCTGCCAGCAA**GCT**TCCATCGGCTGTAAC-3′
Cys^{233}Ala_rev	5′-GTTACAGCCGATGGAAGCTTGCTGGCAGATGC-3′
Cys^{237}Ala_for	5′-CAATGTTCCATCGGC**GCC**AACATCAGCC-3′
Cys^{237}Ala_rev	5′-GGCTGATGTTGGCGCCGATGGAACATT-3′
Cys^{265}Ala_for	5′-CACTACTTCCTC**GCG**GACCGTGGTCGTTTCG-3′
Cys^{265}Ala_rev	5′-CGAAACGACCACGGTCCGCGAGGAAGTAGTG-3′

[a] Underlined positions indicate new restriction sites; bold positions are responsible for the indicated mutation.

in the preparation by EPR spectroscopy and were attributed to the Fe/S clusters N1b, N1c, N3, and N4 (*7*−*9*, *13*, *14*). So far, no EPR signals have been assigned to the binuclear cluster N1a in the preparation of the *E. coli* complex I. However, this cluster is present in complex I preparations from other sources. N1a is expected to be located on NuoE due to the preservation of a conserved binding motif (*8*, *15*) and shows a midpoint potential in the range of −410 to −370 mV, which is pH dependent (*16*, *17*). It was assumed that N1a is not detected in the *E. coli* complex because of its proposed negative midpoint potential (*14*). Although EPR signals of a binuclear cluster at $g_{x,y,z} = 1.92$, 1.94, and 2.00 reminiscent of the signals of cluster N1a were detected in the preparation of the *E. coli* complex, they were attributed to a novel cluster called N1c due to its high midpoint potential of −250 mV, which showed no pH dependence (*9*, *14*). Cluster N1c has not been detected in complex I from any another species. It is a true component of the *E. coli* complex I as its EPR signals have been detected in *E. coli* cytoplasmic membranes (*9*, *14*), the isolated complex I (*9*, *10*), and in the isolated NADH dehydrogenase fragment (*7*, *13*). In addition, the EPR signals of N1c were increased in cytoplasmic membranes of the *E. coli* strain overproducing complex I (*10*). N1c was assumed to be located on NuoG, which contains an additional Fe/S cluster binding motif, that has so far been detected only in *E. coli*, *Salmonella thyphimurium*, *Thermus thermophilus*, *Aquifex aeolicus*, and a few other bacteria (Figure 1; *18*, *19*).

In this study, we generated mutants of the additional Fe/S cluster binding motif on NuoG. The four cysteines comprising the binding motif were changed individually to alanine. The mutations were introduced into the plasmid that was used to overproduce the NADH dehydrogenase fragment (*13*). Spectroscopic analysis of the isolated NADH dehydrogenase fragment from the various mutants showed that the EPR signals attributed to N1c derive from cluster N1a bound to NuoE. The additional binding motif on NuoG most likely binds another, not yet detected Fe/S cluster as the mutations in NuoG disturbed the assembly of the NADH dehydrogenase fragment.

MATERIALS AND METHODS

Materials and Strains. E. coli strains RR1ΔM15 (*20*) and BL21(DE3) (*21*), cloning vector pUC18 (Biolab, Schwalbach), and expression vector pET11-a (AGS, Heidelberg) were used. When required for maintenance of plasmids, ampicillin was added to 100 μg/mL. All enzymes used for recombinant DNA techniques were from MBI Fermentas, St. Leon-Rot, or Amersham Biosciences.

Site-Directed Mutagenesis. For expression of the NADH dehydrogenase fragment, a construct of pET11-a was used containing *nuoB-G* with a *Strep*-tag II coding sequence C-terminal on NuoF (*13*). This plasmid (pET11a/*nuoB-G*/$NuoF_c$) was used to transform competent *E. coli* cells BL21-(DE3). For site-directed mutagenesis *nuoG* was cloned in pUC18. Mutations in *nuoG* were created using the QuikChange site-directed mutagenesis kit (Stratagene). The sequences of the corresponding oligonucleotides are given in Table 2. Because of the mutations the cysteines 230, 233, 237, and 265 (*E. coli* numbering) giving rise to the additional Fe/S cluster binding motif on NuoG were changed to alanine (Table 2). All mutations were confirmed by DNA sequencing. The plasmid was digested with *Ade*I and the fragment of *nuoG* containing the mutation was cloned into pET11-a/*nuoB-G*/$NuoF_c$.

Expression of NuoB-G. Competent *E. coli* cells BL21-(DE3) were transformed with pET11-a/*nuoB-G*/$NuoF_c$ carrying either the wild type or mutated NuoG. The cells were grown, induced, harvested, and stored as described (*13*).

Purification of the NADH Dehydrogenase Fragment. All steps were carried out at 4 °C. A total of 20 g cells (wet weight) were resuspended in 60 mL of 50 mM MES/NaOH, 50 mM NaCl, pH 6.0, 20 μg of avidine, 10 μg/mL DNaseI (Boehringer, Mannheim), protease inhibitor (Complete, Boehringer, Mannheim), and disrupted by a single passage through a French pressure cell (SLM Amicon) at 110 MPa.

The cytoplasmic fraction was obtained by ultracentrifugation at 160000g for 60 min and applied to a 50 mL DEAE-Sepharose column (Pharmacia, 2.8 × 10 cm**)** equilibrated with 50 mM MES/NaOH, 50 mM NaCl, pH 6.0. Proteins were eluted with a 400 mL linear gradient from 50 to 400 mM NaCl in 50 mM MES/NaOH, pH 6.0. Fractions exhibiting NADH/ferricyanide activity were pooled and adjusted to pH 7.0 with either 0.1 or 1 N NaOH. The sample was applied to a 5 mL *Strep*-Tactin Sepharose column (IBA, 0.7 × 6.5 cm) equilibrated with 100 mM Tris/HCl, 1 mM EDTA, pH 7.0. The NADH dehydrogenase fragment was eluted with 2.5 mM D-desthiobiotine in the same buffer. The fractions with NADH/ferricyanide reductase activity were pooled, concentrated by ultrafiltration (Centricon 100, Amicon), and stored at −80 °C.

EPR Spectroscopy. Low-temperature EPR measurements were conducted with a Bruker EMX 1/6 spectrometer. The sample temperature was controlled with an Oxford Instruments ESR-9 helium flow cryostat. The purified NADH dehydrogenase fragment was concentrated 10 times and reduced with dithionite in the presence of redox mediators. Cytoplasmic fraction of either wild type or NuoG mutant cells were concentrated 50 times and reduced with 20 mM NADH (final concentration). For difference spectroscopy, an aliquot of the concentrated cytoplasmic fraction was diluted with the same volume of buffer. EPR tubes were frozen in 5:1 isopentane/methylcyclohexane (v/v) at 150 K. The magnetic field was calibrated using a strong pitch standard. Spin quantitation of the signals of cluster N1a in the spectrum of the NuoEF subcomplex (Figure 5) was conducted by double integration using 1 mM Cu^{2+} as a standard under nonpower saturated conditions and correction for differences in *g* values (*22*, *23*).

Other Analytical Procedures. Protein concentration was determined according to the biuret method. NADH/ferricyanide activity was measured as described (*24*).

RESULTS

EPR Spectroscopic Characterization of the NADH Dehydrogenase Fragment in the Cytoplasmic Fraction. By overexpressing *nuoB-G*, the NADH dehydrogenase fragment engineered with the *Strep*-tag II peptide is fully assembled in the cytoplasmic fraction (*13*). It is known that *nuoB* and *nuoCD* have to be expressed for a proper assembly of the fragment. However, these proteins are not contained within the preparation (*7*). Subtraction of the EPR spectrum of the air-oxidized cytoplasmic fraction from the spectrum of the cytoplasmic fraction reduced with NADH results in an EPR difference spectrum that contained all Fe/S clusters of the purified NADH dehydrogenase fragment (*7*; Figure 2a). At 40 K, the signals of cluster N1b ($g_{II,\perp}$ = 2.03, 1.94) and the signals attributed to N1c ($g_{x,y,z}$ = 1.92, 1.94, 2.00) were detected. EPR signals of the tetranuclear clusters N3 and N4 were detectable at 13 K, although these signals overlapped with signals from other Fe/S clusters (data not shown). In contrast, the EPR difference spectra of the cytoplasmic fraction prepared from the mutants Cys^{233}Ala and Cys^{237}Ala showed only the signals attributed to cluster N1c (Figure 2c,d). The signals of cluster N1b were nearly completely gone. The cytoplasmic fraction prepared from the mutants Cys^{230}Ala and Cys^{265}Ala revealed poor EPR difference spectra. Minor amounts of the signals attributed to N1c were detected in these spectra at less than 5% amplitude compared to wild type (Figure 2b,e). The signals of N1b, N3, and N4 were completely lost (Figure 2 and data not shown). These data indicate that the cysteines comprising the additional binding motif on subunit NuoG were not involved in the ligation of the cluster causing the signals at $g_{x,y,z}$ = 1.92, 1.94, and 2.00 attributed to N1c.

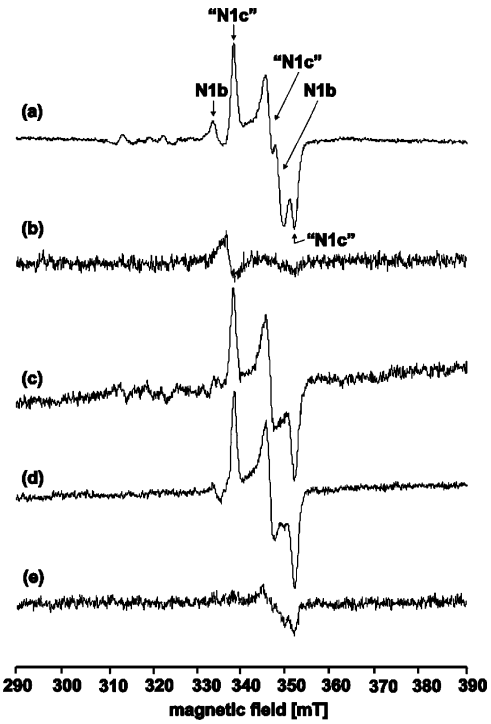

FIGURE 2: EPR (NADH-reduced minus air-oxidized) difference spectra of the cytoplasmic fraction from *E. coli* wild type (a) and from the NuoG mutant strains Cys^{230}Ala (b), Cys^{233}Ala (c), Cys^{237}-Ala (d), and Cys^{265}Ala (e) at 40 K and 10 mW microwave power. The absorptions of the individual Fe/S clusters are indicated. Other EPR conditions were microwave frequency: 9.44 GHz; modulation amplitude: 6 mT; time constant: 0.164 s; scan rate: 17.9 mT/min.

Purification of the NADH Dehydrogenase Fragment. To ensure that the EPR difference spectra obtained with the cytoplasmic fraction did not result from interference with other Fe/S proteins, the NADH dehydrogenase fragment was purified from the cytoplasmic fraction by means of anion-exchange and affinity chromatography. As an example, the preparation of the fragment from the mutant Cys^{233}Ala is shown in Figure 3. The enzyme eluted from the anion-exchange chromatography at 160 mM NaCl and in a single peak from affinity chromatography (Figure 3). The preparations of the NADH dehydrogenase fragment from the parental strain as well as from the mutants Cys^{237}Ala and Cys^{265}Ala showed similar elution profiles and were all made up of the subunits NuoE, F, and G constituting this fragment (Figure 4). From 10 g of cells (wet weight) of the parental strain 5 mg of NADH dehydrogenase fragment were obtained. The yield of the preparations from the mutant strains varied from 0.5 to 1.5 mg of starting from 50 g of cells.

Preparations from the mutant Cys^{237}Ala tend to lose NuoG when diluted and adjusted with 1 M NaOH to pH 7.0 prior to affinity chromatography (see Materials and Methods). Depending on this procedure, these preparations contained

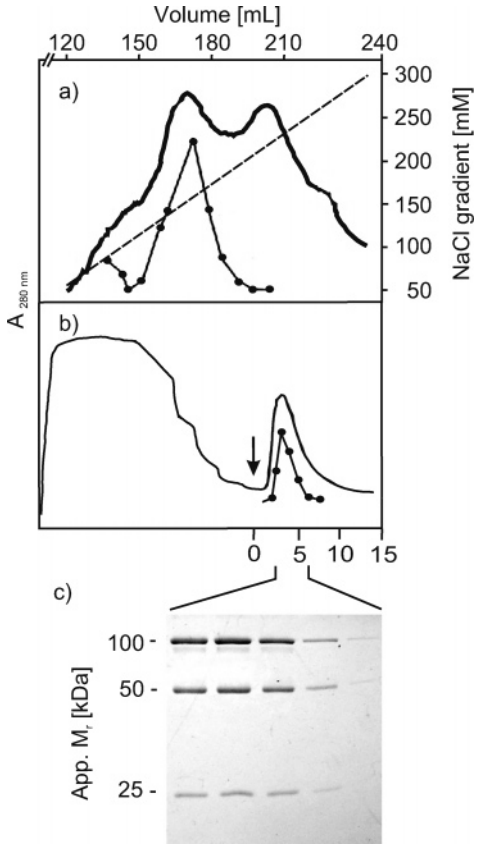

FIGURE 3: Isolation of the NADH dehydrogenase fragment of the *E. coli* complex I from the mutant strain pET11-a/*nuoB-G*/NuoF$_c$/NuoGCys^{233}Ala. Chromatography on DEAE-Sepharose (a); chromatography on *Strep*-Tactin Sepharose (b); absorbance at 280 nm (−); NADH/ferricyanide reductase activity (●); NaCl gradient (- - -). SDS/PAGE of the indicated fractions of the second anion exchange chromatography (c). The arrow indicates the start of the elution with D-desthiobiotin buffer.

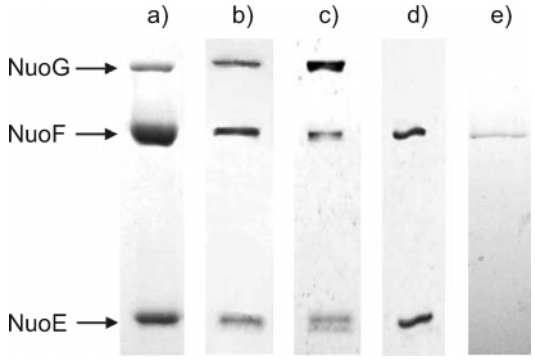

FIGURE 4: SDS-PAGE of the NADH dehydrogenase fragment purified from wild type (a) and from the NuoG mutants Cys^{265}Ala (b), Cys^{237}Ala (c) and (d), and Cys^{230}Ala (e). The fragment from the mutant Cys^{237}Ala was either isolated under mild conditions (c) or diluted before rechromatography on *Strep*-Tactin (d). The designation of the individual Nuo subunits is shown. The additional band with an apparent molecular mass of 18 kDa in (c) represents a proteolytic fragment of NuoG (*13*).

either the fully assembled NADH dehydrogenase fragment or a subcomplex made up of NuoE and F (Figure 4). We were not able to isolate the fragment from the mutant Cys^{230}-Ala most likely because it was not assembled. This is indicated by the absence of the EPR signal typical for the Fe/S clusters of the NADH dehydrogenase fragment in the cytoplasmic fraction of this mutant strain (Figure 2). Only

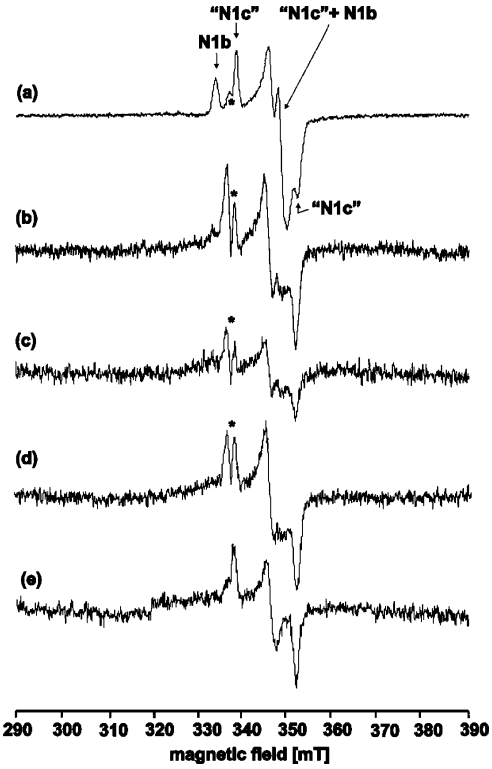

FIGURE 5: EPR spectra of the NADH dehydrogenase fragment purified from wild type (a) and from the NuoG mutants Cys^{233}Ala (b), Cys^{237}Ala (c), and Cys^{265}Ala (d). The EPR spectrum of the NuoEF subcomplex isolated from the mutant Cys^{237}Ala is shown in (e). Spectra were recorded at 40 K and 2 mW. The samples were reduced with an excess of dithionite in the presence of redox mediators. The signal marked with an asterisks corresponds to the radical signal caused by redox mediators. The absorptions of the individual Fe/S clusters are indicated. Other EPR conditions were microwave frequency: 9.44 GHz; modulation amplitude: 6 mT; time constant: 0.164 s; scan rate: 17.9 mT/min.

the single subunit NuoF containing the *Strep*-tag was bound to the *Strep*-Tactin column (Figure 4).

EPR Spectroscopic Characterization of the Purified NADH Dehydrogenase Fragment. The EPR spectral properties of the Fe/S clusters of the NADH dehydrogenase fragment in the cytoplasmic fraction and as purified did not show any significant differences, indicating that the purification protocol did not change the microenvironment of the clusters (Figures 2a and 5a). The EPR spectra of the NADH dehydrogenase fragment isolated from the mutants Cys^{233}-Ala, Cys^{237}Ala, and Cys^{265}Ala at 40 K showed the signals attributed to cluster N1c (Figure 5). Very minor signals deriving from cluster N1b were also detected in these spectra (Figure 5). Clusters N3 and N4 were not detected in these preparations (data not shown). In essence, mutations in the additional binding motif of NuoG had an influence on the EPR signals of every Fe/S cluster with the exception of the signals attributed to N1c. EPR spectra of the subcomplex made up of NuoEF isolated from the mutant Cys^{237}Ala showed the presence of the signals attributed to N1c; no other Fe/S clusters were detected (Figure 5). The content of the Fe/S cluster was determined by spin quantitation to approximately 0.7 mol per NuoEF subcomplex assuming a molecular mass of 68 kDa. This excludes the possibility that the signals derived from an impurity of the preparation. Therefore, the additional binding motif on NuoG, which is not present in this preparation, cannot supply the ligands of

the Fe/S cluster causing the signals attributed to N1c. The only binding motif for a binuclear Fe/S cluster in this subcomplex is present in NuoE and has been attributed to cluster N1a (*15*, *25*).

DISCUSSION

EPR signals at $g_{x,y,z} = 1.92$, 1.94, and 2.00 measured in *E. coli* cytoplasmic membranes and in preparations of the *E. coli* complex I and the NADH dehydrogenase fragment of the complex were attributed to a novel binuclear Fe/S cluster called N1c (*7*, *9*, *14*). The spectral properties of this cluster were reminiscent of the binuclear cluster N1a described in other organisms (*16*); however, it did not match the common features of this cluster, namely, its very low and pH-dependent midpoint potential (*9*, *14*). The novel cluster N1c was supposed to be located on NuoG, due to an additional Fe/S cluster binding motif on this subunit.

Here, we have shown that the EPR signals attributed to cluster N1c derive from cluster N1a and that there is no cluster N1c in the *E. coli* complex I. Mutagenesis of the four cysteines comprising the additional binding motif on NuoG led to a more or less complete loss of all Fe/S clusters located on this subunit, while the EPR signals attributed to N1c were still present (Figures 2 and 5). These EPR signals were also detected in a NuoEF subcomplex isolated from the mutant $Cys^{237}Ala$ (Figures 4 and 5) demonstrating that the Fe/S cluster causing these signals is not bound to NuoG. The presence of these EPR signals in the preparation of the subcomplex NuoEF clearly demonstrates that the EPR signals in question derived from cluster N1a on NuoE.

The overproduced and purified single subunit NuoE from *E. coli* contains a binuclear Fe/S cluster with g-values and a midpoint potential identical to the cluster named N1c (*17*). However, it was not clear whether the Fe/S cluster N1a giving rise to the EPR signals in the overproduced single subunit NuoE would have the same spectral and thermodynamic properties in the assembled NADH dehydrogenase fragment or complex I. It is known that EPR spectra of Fe/S cluster in single subunits may differ significantly from the spectrum of the same cluster being part of the fully assembled complex (*26*). In addition, it was shown that the midpoint potential of cluster N1a in *E. coli* is approximately 100 mV more positive than in other organisms and that its pH dependence is strongly influenced by the ionic strength of the buffer (*17*). At high salt concentrations, the midpoint potential of N1a does not show a pH dependence, while its potential varies between −348 and −246 mV in the pH range from 3.5 to 9.5 in a buffer containing 10 mM NaCl (*17*). Cluster N1a in complex I from *E. coli* showed no pH dependence in the pH range from 6.0 to 8.2 in a buffer containing 300 mM NaCl leading to its misnomer "N1c" (*9*, *14*).

Our studies indicate that the additional motif on NuoG indeed binds an Fe/S cluster because the mutations had a severe effect on the assembly of the NADH dehydrogenase fragment. EPR difference spectroscopy showed a strongly decreased amount of the fragment especially in the cytoplasmic fraction of the mutants $Cys^{230}Ala$ and $Cys^{265}Ala$ (Figure 2). Just the single subunit NuoF was isolated from the mutant $Cys^{230}Ala$, revealing a completely blocked assembly in this mutant. In addition, the fragment isolated from the mutant $Cys^{237}Ala$ showed a decreased stability as it easily disassembled to a NuoEF subcomplex (Figure 4). Such a subcomplex has been obtained by overexpression of NuoE and F in *Paracoccus denitrificans* (*27*). The EPR spectra of this preparation also reveal the presence of cluster N1a, which is consistent with our measurements. The cluster which is bona fide bound to the motif on NuoG awaits further characterization, especially concerning its function. It has been discussed that it might be a molecular switch between aerobic/anaerobic growth (*28*) or linked with the ability of complex I to react with menaquinone (*19*), which are related phenomena (*29*).

It has been proposed that the additional binding motif on NuoG ligates a tetranuclear Fe/S cluster, which might be converted to a binuclear cluster under oxidizing conditions (*28*). Another possibilty would be the presence of an EPR silent binuclear cluster bound to this motif. However, motifs with a similar cysteine spacing are found in other enzymes such as the *E. coli* formate dehydrogenase binding a tetranuclear Fe/S cluster (*28*). Overexpression of a short fragment of NuoG containing this motif under anaerobic conditions led to the production of a peptide containing a tetranuclear cluster. Thus, it is most likely that the additional motif of NuoG binds a tetranuclear cluster in complex I as well (*28*). However, the presence of an EPR silent binuclear cluster at this position cannot be completely excluded because the overexpressed fragment of NuoG might adopt a different fold compared to the entire NuoG within complex I. This in turn could lead to a change of the magnetic properties of the cluster. To avoid further confusion in the nomenclature of the Fe/S clusters of complex I, we would like to propose to name the additional Fe/S cluster on subunit NuoG, not present in all organisms, N7 because the expression "N1c" would imply that it is a binuclear Fe/S cluster and it is connected with the wrong assignment of the EPR signals of cluster N1a.

ACKNOWLEDGMENT

We are grateful to Julia Hinderberger, Michael Mittler, and Thomas Pohl for help in protein purification and to Franz Butz for excellent technical assistance.

REFERENCES

1. Walker, J. E. (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains, *Quart. Rev. Biophys. 25*, 253−324.
2. Friedrich, T. (2001) Complex I: a chimaera of a redox and conformation-driven proton pump? *J. Bioenerg. Biomembr. 33*, 169−177.
3. Magnitsky, S., Toulokhonova, L., Yano, T., Sled, V., Hagerhall, C., Grivennikova, V. G., Burbaev, D. S., Vinogradov, A. D., and Ohnishi, T. (2002) EPR characterization of ubisemiquinones and iron−sulfur cluster N2, central components of the energy coupling in the NADH-ubiquinone oxidoreductase (complex I) in situ, *J. Bioenerg. Biomembr. 34*, 193−208.
4. Brandt, U., Kerscher, S., Dröse, S., Zwicker, K., and Zickermann, V. (2003) Proton pumping by NADH:ubiquinone oxidoreductase. A redox driven conformational change mechanism? *FEBS Lett. 545*, 9−17.
5. Yagi, T., and Matsuno-Yagi, A. (2003) The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked, *Biochemistry 42*, 2266−2274.
6. Weidner, U., Geier, S., Ptock, A., Friedrich, T., Leif, H., and Weiss, H. (1993) The gene locus of the proton-translocating NADH: ubiquinone oxidoreductase in *Escherichia coli*. Organization of

the 14 genes and relationship between the derived proteins and subunits of mitochondrial complex I, *J. Mol. Biol. 233*, 109−122.
7. Braun, M., Bungert, S., and Friedrich, T. (1998) Characterization of the overproduced NADH dehydrogenase fragment of the NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli*, *Biochemistry 37*, 1861−1867.
8. Friedrich, T. (1998) The NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli*, *Biochim. Biophys. Acta, 1364*, 134−146.
9. Leif, H., Sled, V., Ohnishi, T., Weiss, H., and Friedrich, T. (1995) Isolation and characterization of the proton-translocating NADH: ubiquinone oxidoreductase from *Escherichia coli*, *Eur. J. Biochem. 230*, 538−548.
10. Spehr, V., Schlitt, A., Scheide, D., Guenebaut, V., and Friedrich, T. (1999) Overexpression of the *Escherichia coli nuo*-operon and isolation of the overproduced NADH:ubiquinone oxidoreductase (complex I), *Biochemistry 38*, 16261−16267.
11. Rasmussen, T., Scheide, D., Brors, B., Kintscher, L., Weiss, H., and Friedrich, T. (2001) Identification of two tetranuclear FeS clusters on the ferredoxin-type subunit of NADH:ubiquinone oxidoreductase (complex I), *Biochemistry 40*, 6124−6131.
12. Friedrich, T., and Scheide, D. (2000) The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases, *FEBS Lett. 479*, 1−5.
13. Bungert, S., Krafft, B., Schlesinger, R., and Friedrich, T. (1999) One-step purification of the NADH dehydrogenase fragment of the *Escherichia coli* complex I by means of *Strep*-tag affinity chromatography, *FEBS Lett. 460*, 207−211.
14. Sled, V., Friedrich, T., Leif, H., Weiss, H., Meinhardt, S., Fukumori, Y., Calhoun, M., Gennis, R., and Ohnishi, T. (1993) Bacterial NADH-quinone oxidoreductases: iron−sulfur clusters and related problems, *J. Bioenerg. Biomembr. 25*, 347−356.
15. Yano, T., Sled, V., Ohnishi, T., and Yagi, T. (1994) Identification of amino acid residues associated with the [2Fe-2S] cluster of the 25 kDa (NQO2) subunit of the proton-translocating NADH-quinone oxidoreductase of *Paracoccus denitrificans*, *FEBS Lett. 345*, 160−164.
16. Ohnishi, T. (1998) Iron−sulfur clusters/semiquinones in complex I, *Biochim. Biophys. Acta 1364*, 186−206.
17. Zu, Y., Di Bernardo, S., Yagi, T., and Hirst, J. (2002) Redox properties of the [2Fe-2S] center in the 24 kDa (NQO2) subunit of NADH:ubiquinone oxidoreductase (complex I), *Biochemistry 41*, 10056−10069.
18. Scheide, D., Huber, R., and Friedrich, T. (2002) The proton-pumping NADH:ubiquinone oxidoreductase (complex I) of *Aquifex aeolicus*, *FEBS Lett. 512*, 80−84.
19. Finel, M. (1998) Organization and evolution of structural elements within complex I, *Biochim. Biophys. Acta 1364*, 112−125.
20. Bolivar, F., Rodriquez, R., Bettlach, M., and Boyer, H. (1977) Construction and characterization of new cloning vehicles I. Ampicillin-resistant derivatives of the plasmid pMB9, *Gene 2*, 75−91.
21. Studier, F., and Moffatt, B. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, *J. Mol. Biol. 189*, 113−130.
22. Malmström, B., Reinhammar, B., and Vänngard, T. (1970) The state of copper in stellacyanin and laccase from the lacquer tree *Rhus vernicifera*, *Biochim. Biophys. Acta 205*, 48−57.
23. Aasa, R., and Vänngard, T. (1975) EPR signal intensity and powder shapes: a reexamination, *J. Magnetic Res. 19*, 308−315.
24. Friedrich, T., Hofhaus, G., Ise, W., Nehls, U., Schmitz, B., and Weiss, H. (1989) A small isoform of NADH:ubiquinone oxidoreductase (complex I) without mitochondrially encoded subunits is made in chloramphenicol-treated *Neurospora crassa*, *Eur. J. Biochem. 180*, 173−180.
25. Yano, T., Sled, V., Ohnishi, T., and Yagi, T. (1994) Expression of the 25-kilodalton iron−sulfur subunit of the energy-transducing NADH-ubiquinone oxidoreductase of *Paracoccus denitrificans*, *Biochemistry 33*, 494−499.
26. Yano, T., Magnitsky, S., Sled, V., Ohnishi, T., and Yagi T. (1999) Characterization of the putative 2x[4Fe-4S]-binding NQO9 subunit of the proton-translocating NADH-quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans*. Expression, reconstitution, and EPR characterization, *J. Biol. Chem. 274*, 28598−28605.
27. Yano, T., Sled, V., Ohnishi, T., and Yagi, T. (1996) Expression and characterization of the flavoprotein subcomplex composed of 50-kDa (NQO1) and 25-kDa (NQO2) subunits of the proton-translocating NADH-quinone oxidoreductase of *Paracoccus denitrificans*, *J. Biol. Chem. 271*, 5907−5913.
28. Nakamuru-Ogiso, E., Yano, T., Ohnishi, T., and Yagi, T. (2002) Characterization of the iron−sulfur cluster coordinated by a cysteine cluster motif (CXXCXXXCX27C) in the Nqo3 subunit in the proton-translocating NADH-quinone oxidoreductase (NDH-1) of *Thermus thermophilus* HB-8, *J. Biol. Chem. 277*, 1680−1688.
29. Unden, G., and Bongaerts, J. (1997) Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors, *Biochim. Biophys. Acta 1320*, 217−234.

BI048136N