Iron-Sulfur Cluster N2 of the Escherichia coli NADH:Ubiquinone Oxidoreductase (Complex I) Is Located on Subunit NuoB

The proton-pumping NADH:ubiquinone oxidoreductase, also called respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. One FMN and up to 9 iron-sulfur (Fe/S) clusters participate in the redox reaction. There is discussion that the EPR-detectable Fe/S cluster N2 is involved in proton pumping. However, the assignment of this cluster to a distinct subunit of the complex as well as the number of Fe/S clusters giving rise to the EPR signal are still under debate. Complex I from Escherichia coli consists of 13 polypeptides called NuoA to N. Either subunit NuoB or NuoI could harbor Fe/S cluster N2. Whereas NuoB contains a unique motif for the binding of one Fe/S cluster, NuoI contains a typical ferredoxin motif for the binding of two Fe/S clusters. Individual mutation of all four conserved cysteine residues in NuoB resulted in a loss of complex I activity and of the EPR signal of N2 in the cytoplasmic membrane as well as in the isolated complex. Individual mutations of all eight conserved cysteine residues of NuoI revealed a variable phenotype. Whereas cluster N2 was lost in most NuoI mutants, it was still present in the cytoplasmic membranes of the mutants NuoI C63A and NuoI C102A. N2 was also detected in the complex isolated from the mutant NuoI C102A. From this we conclude that the Fe/S cluster N2 is located on subunit NuoB.

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The proton-pumping NADH:ubiquinone oxidoreductase, also known as respiratory complex I, is the first of the respiratory chain complexes providing proton motive force required for energy consuming processes such as synthesis of ATP (1, 2). It links the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. In general, the bacterial complex I consists of 14 different subunits that add up to a molecular mass of ~530 kDa (3, 4). Seven subunits are peripheral proteins including the subunits that bear all known redox groups of complex I, namely one FMN and up to 9 iron-sulfur (Fe/S) clusters. The remaining 7 subunits are hydrophobic proteins. Little is known about their function, but they are mostly involved in ubiquinone reduction and proton translocation (3). In addition to the homologues of the 14 procaryotic complex I subunits the mitochondrial complex I of eucaryotes contains up to 32 extra proteins (5). They add up to a molecular mass of ~1 MDa. The homologues of the 7 hydrophobic subunits are mitochondrially encoded in all eucaryotes (2).

The electron input part of the complex is made up of the FMN and Fe/S clusters N1b, N3, N4, N5, N6a, and N6b. Because of their similar midpoint potential they are called isopotential Fe/S clusters (6–9). From the isopotential clusters the electrons are transferred to cluster N2, which exhibits a more positive midpoint potential. The respective value, however, depends strongly on the organism from which complex I has been isolated. Cluster N2 has also been considered playing an important role in energy conservation, because its midpoint potential is pH-dependent (10), which is a prerequisite for an energy coupling device. It is believed to transfer electrons directly to the substrate ubiquinone and was thus proposed to be located close to the membrane (11, 12), although recent data from electron microscopy of immunolabeled single particles suggest a location more than 50 Å from the membrane (13). Whereas most of the Fe/S clusters have been attributed to individual subunits, the location of cluster N2 is still under debate.

The Escherichia coli complex I is made up of 13 subunits called NuoA to NuoN (14). As a special case, the gene nucCD is fused to one gene giving rise to 13 nuc genes (15). The preparation of E. coli complex I contains one non-covalently bound FMN, and two binuclear (N1a and N1b, Nomenclature according to Ohnishi and co-workers (14)) and three tetranuclear (N2, N3, and N4) Fe/S clusters have been detected by means of EPR spectroscopy. Two Fe/S clusters, N6a and N6b, have been detected by means of UV-visible spectroscopy (8). Upon treatment with salt, E. coli complex I is cleaved yielding a soluble NADH dehydrogenase fragment with three subunits containing the FMN and the Fe/S clusters N1a, N1b, N3, and N4, an amphipathic connecting fragment with three subunits and cluster N2, N6a, and N6b, and a hydrophobic membrane fragment with 7 subunits containing a chromophore with a yet unknown chemical structure (16–19).

The connecting fragment contains subunits NuoB and NuoI (the homologues of the bovine PSST and TYKY, respectively), which contain binding motifs for three Fe/S clusters (Fig. 1) (20). NuoB contains a unique binding motif with two of the four cysteines comprising that motif being sequentially located (Fig. 1A). The C-terminal cysteine residue is followed by a proline
suggesting that NuoB harbors a [4Fe4S] cluster. NuoI contains a typical $2 \times 4 [4Fe4S]$ ferredoxin binding motif made up of eight conserved cysteine residues with regular spacing (Fig. 1B). It has been shown that the overproduced subunits NuoB and NuoI from Paracoccus denitrificans are indeed Fe/S proteins (21, 22).

Site-directed mutagenesis of subunit NuoI from Rhodobacter capsulatus complex I was characterized by means of EPR spectroscopy of cytoplasmic membranes (23, 24). Five of the eight conserved cysteines were replaced by other amino acids. Whereas mutations in the fourth cysteine of each binding motif in NuoI resulted in a disturbed assembly of the complex, mutagenesis of the residual cysteines did not affect the phenotype, but the amplitude of the N2 signals was diminished (24). From this data it was proposed that complex I contains two clusters N2, whereas mutations of two of the eight conserved cysteine residues on NuoI resulted in a complex, still equipped with cluster N2. From this we conclude that the Fe/S cluster N2 is located on NuoB.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains—** E. coli B wild type, the E. coli strains AN387 (26), JC7623 (27), DH5α (28), MK65umS (29) and the plasmids pBAD33 (30), pCHK20 (31), pMKA-705 (31), pTTT3-19U (32), pNUO-13 (30), pNUO-2.3 (30), and pUM-24 (33) were used. When required for maintenance a typical 2 g/ml kanamycin to 20 μg NuoI and NuoB were used. When required for maintenance, 100 μg ampicillin to 20 μg NuoI and NuoB were used. When required for maintenance, 100 μg ampicillin to 20 μg NuoI and NuoB were used.

**Construction of Transformation Vectors—** In vitro DNA manipulations were carried out according to the methods described in Ref. 34 or the manufacturer’s instructions. For construction of the transformation vector pVSSacRB, pTTT3-19U was restricted with KpnI and religated after the sticky ends were digested with Klenow fragment. This vector was linearized with BamHI/Clai and ligated with a 2.2-kb fragment from pNUO-13 containing the nuoI gene that had been digested with the same enzymes. The resulting vector was called pVS3 and cut with Kpn I. A 1.3-kb BamHI fragment containing nptI of plasmid pCHK20 was filled in using Klenow enzyme and cloned into Klenow-treated pVS3. NptI was inserted in the same direction as nuoI. This vector was called pVS3Kan and restricted with SphiI/XbaI. After treatment with Klenow and religation, a 3.7-kb SacI fragment containing the sacR, sacB, and nptI genes was excised from plasmid pUM24 and cloned into the SacI site of pVS3Kan. The resulting vector was called pVSS3SacRB. It contained an in-frame deletion of 510 bp of nuoI corresponding to 94% of the coding region. For the construction of plasmid pVSS5SacRB, a 2.3-kb FstI fragment containing the nuoI gene was excised from pVSS3SacRB, and cut into Smal-digested pVS5. The resulting vector was called pVSS5SacRB. In addition, pVS5 was cut with Avai and incubated with exonucleases according to the manufacturer (Heidelberg), or Sigma. All chemicals were from Merck (Darmstadt), Riedel de Haen (Hannover), Serva (Heidelberg), or Sigma.

**Deamination—** Deaminase (d-NADH/ferricyanide) reductase activity was combined, concentrated by precipitation with 9% (w/v) ethanol and dialyzed against 2 ml MES/NaOH, pH 6.0, and stored at −80 °C. Complex I was isolated from the mutants as described (35). In principal, the membrane proteins were extracted with 3% (w/v) dodecyl maltoside in 50 mM MES/NaOH, pH 6.0, and applied to a first anion-exchange chromatography on Source 15-Q material (Pharmacia). Fractions containing NADH/ferricyanide reductase activity were combined, concentrated by precipitation with 9% (w/v) ethanol and dialyzed against 2 ml MES/NaOH, 50 mM NaCl, and 0.1% dodecyl maltoside. The protein was subjected to size exclusion chromatography on Ultrogel AcA 34 (Serva) in the same buffer. Peak fractions were pooled and applied to Source 15-Q material (Pharmacia). Fractions with NADH/ferricyanide reductase activity were pooled and stored at −80 °C.

**EPR Spectroscopy—** EPR measurements were conducted with a Bruker EMX 1.6 spectrometer operating at X-band (9.2 GHz). The sample temperature was controlled with an Oxford instrument ESR-9 helium flow cryostat. The magnetic field was calibrated using a strong helium flow cryostat. The magnetic field was calibrated using a strong

**Localization of Fe/S Cluster N2**

**Figure 1** Multiple sequence alignment of complex I subunits NuoB (A) and NuoI (B). The numbering is according to the E. coli sequences and the cysteine residues comprising the binding motifs are marked in a gray shading. The contribution of each cysteine residue of the binding of two different Fe/S clusters in NuoI is indicated.
isolated subunits were used for detection.

Characterization of Strains Carrying a Point Mutation

The deletion strain was called ANN023/pBAD33-nuoB. Two of the four constructs used for the construction of strain ANN023 were obtained from plasmids pBAD33-B^{63A} and pBAD33-B^{63S}, the latter carrying an additional 5′-CGTTACGTTGAGATGG-3′ and 5′-CTGTGACTGGTGACGCGTCAACCAAGTC-3′, respectively. Western blotting was performed with a mixture of antisera raised individually against NuoB and NuoI, respectively. The upper band corresponds to NuoB (25 kDa) and the lower band to NuoI (20 kDa).

Characterization of the Deletion Strains—The E. coli nuoB gene is made up of 660 bp (20). Positions 201 to 654 were deleted from the chromosome of wild type strain AN387 without interrupting the reading frame. The resulting strain ANN023 (Table II) contained a nuoB fragment coding for amino acids 1–67 and 219–220. Subunit NuoB was not detectable in the membranes of strain ANN023 by a Western blot analysis using a polyclonal antibody raised specifically against NuoB (Fig. 2). It was shown that this strain did not contain an assembled or functional complex I. The amount of NuoB in strain ANN093 was reduced (Fig. 2). Instead, fractions in the first third of the gradient exhibited ferricyanide activity were because of the alternative NADH dehydrogenase. The expression of this alternative NADH dehydrogenase is increased in the absence of complex I.

The amount of complex I in the cytoplasmic membranes of the deletion strains was estimated from the (d-)NADH/ferricyanide oxidase activity (Table III). The physiological activity of complex I in the cytoplasmic membranes of the deletion strains was determined by the (d-)NADH oxidase activity (Table III). The artificial substrate d-NADH was used in both assays to distinguish between the two membrane-bound NADH dehydrogenases of E. coli (43, 46). Whereas NADH can be used by both NADH dehydrogenases, d-NADH preferentially reacts with complex I. The d-NADH/ferricyanide activity was strongly reduced in both strains and the d-NADH oxidase activity was completely abolished (Table III). The activity measured with NADH as substrate was because of the alternative NADH dehydrogenase. The residual NADH oxidase activity of both deletion strains was not sensitive to 10 μM piericidin A, a concentration that inhibited 69% of the NADH oxidase activity in the parental strain AN387. Thus, complex I was missing in strains ANN023 and ANN093, demonstrating that both NuoB as well as NuoI are essential for the assembly of complex I.

Characterization of Strains Carrying a Point Mutation—When strain ANN023 is complemented with wild type nuoB on plasmid pBAD33 under control of the inducible pBAD promoter, complex I is assembled and enzymatically active (35). This strain was called ANN023/pBAD33-B^{63A}. Two of the four conserved cysteine residues of NuoB were replaced by alanine or serine residues by complementation of strain ANN023 with plasmids pBAD-B^{63A}, pBAD-B^{63S}, and pBAD-B^{155A}. The
ubiquinone. In contrast, strain ANN095 showed a very low but that of wild type. However, most mutants did not exhibit any mutations, indicating an amount of complex I comparable with reductase activity was normal or just slightly reduced by the in NuoI led to a variable phenotype. The d-NADH/ferricyanide oxidase activity depended on the redox reaction of cluster N2 (49). The physiological (d-)NADH activity as complex I (48). In contrast, the physiological (d-)NADH reductase activity involves the FMN and most likely the isopotential Fe/S clusters and does not include the redox reaction of cluster N2 (47). The presence of the succinate:ubiquinone oxidoreductase only the region around g = 1.91, the g_s signal of the cluster N2, was shown. In the different spectrum of the parental strain, the g_s signal of N2 at g = 1.91 was seen. The g_s values of clusters N3 and N4 were clearly present as shoulders at the g = 1.88 and g = 1.89 field positions, respectively (Fig. 4). The spectra of the mutants showed increased levels of the Fe/S signals of the succinate:ubiquinone oxidoreductase (data not shown). The g_s signal of N2 was completely missing in the difference spectra of the membranes from the mutants carrying a point mutation in NuoB, regardless of whether the cysteine residue was changed to an alanine or serine residue. However, the g_s signals of clusters N3 and N4 were seen in the difference spectra of the NuoB point mutants although at reduced levels (Fig. 4). The g_s signal of cluster N2 was also missing in most strains carrying a point mutation in NuoI, whereas the signals of clusters N3 and N4 were detectable at a reduced level (Fig. 4). However, two NuoI mutants were distinguished from the others by the presence of the g_s signal of N2 (Fig. 4). The signal was decreased and slightly shifted to g = 1.90 in strains with the NuoI C63A and NuoI C102A mutations. The decreased g_s signals of clusters N3 and N4 were also detectable. These data showed that the Fe/S cluster N2 was lost in all mutant strains with the exception of strains ANN094 and ANN095.

Isolation of Complex I from the Mutant Strains—For an unambiguous assignment of the EPR signals detected in the membranes we tried to isolate complex I from the various mutants by a protocol developed for mutants carrying a point mutation in NuoB (35). All steps were performed in the presence of 0.1% dodecyl maltoside at pH 6.0. The preparations from the mutant strains investigated in this study were very similar to those described in Ref. 35. The enzyme eluted from both anion-exchange chromatographies on Source Q-15 at 280 mM NaCl and from the Ultrogel AcA 34 size-exclusion column at 70 ml, showing chromatographic properties comparable with the complex from wild type (42). Approximately 2–4 mg of complex I were obtained from 60 g of cells from strains with a point mutation in either NuoB or NuoI.

The complex was isolated from all mutants carrying a point mutation in NuoB. Despite the fact that the Fe/S cluster N2 was not detectable in the cytoplasmic membranes of
strains, routinely 4 mg of a stable complex I were obtained. As the only exception, the preparation from the NuoB C158A mutant was unstable and tended to aggregate even at elevated detergent concentrations. Because of its instability, the preparation of the NuoB C158A mutant contained significant amounts of succinate:ubiquinone oxidoreductase (see below).

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TABLE III

| Strain          | NADH/ferricyanide reductase activity | d-NADH/ferricyanide reductase activity | NADH oxidase activity | d-NADH oxidase activity | Inhibition of the NADH oxidase activity by piericidin A |
|----------------|-------------------------------------|----------------------------------------|----------------------|------------------------|--------------------------------------------------------|
|                | μmol min⁻¹ mg⁻¹                      |                                        |                      |                        | %                                                      |
| AN387          | 1.0                                 | 0.8                                    | 0.15                 | 0.06                   | 69                                                     |
| AN023          | 0.6                                 | 0.3                                    | 0.05                 | 0                      | <10                                                    |
| AN093          | 0.6                                 | 0.1                                    | 0.04                 | 0                      | <10                                                    |
| AN0023/pBAD-B^-C63A   | 0.7                                | 0.3                                    | 0.04                 | 0                      | <10                                                    |
| AN0023/pBAD-B^-C63S   | 0.6                                | 0.3                                    | 0.1                  | 0                      | <10                                                    |
| AN0023/pBAD-B^-C63A   | 0.6                                | 0.2                                    | 0.1                  | 0                      | <10                                                    |
| AN0024         | 0.7                                 | 0.2                                    | 0.07                 | 0                      | <10                                                    |
| AN0025         | 0.5                                 | 0.2                                    | 0.08                 | 0                      | <10                                                    |
| AN0094         | 0.9                                 | 0.8                                    | 0.04                 | 0                      | <10                                                    |
| AN0095         | 1.2                                 | 0.7                                    | 0.06                 | 0.01                   | <10                                                    |
| AN0096         | 1.1                                 | 1.0                                    | 0.04                 | 0                      | <10                                                    |
| AN0098         | 1.2                                 | 0.9                                    | 0.07                 | 0                      | <10                                                    |
| AN0099         | 1.2                                 | 0.8                                    | 0.08                 | 0                      | <10                                                    |
| AN00910        | 1.1                                 | 0.9                                    | 0.07                 | 0                      | <10                                                    |
| AN00911        | 0.8                                 | 0.6                                    | 0.07                 | 0                      | <10                                                    |
| AN00912        | 1.1                                 | 0.5                                    | 0.08                 | 0                      | <10                                                    |
| AN00913        | 1.2                                 | 0.6                                    | 0.07                 | 0                      | <10                                                    |

and 25 μM decylubiquinone, complex I isolated from all NuoB mutants showed no NADH:decylubiquinone activity.

In contrast, it was nearly impossible to obtain a pure complex I preparation from the strains carrying a point mutation in NuoI. Whereas the detergent extract still contained d-NADH/ferricyanide reductase activity, this activity was completely lost by applying ion-exchange or size-exclusion chromatography. The fractions from sucrose gradient centrifugation (Fig. 3) contained too many impurities and too little of complex I to be used for EPR spectroscopy. Concentration of the fractions with either ultrafiltration or precipitation led to a disintegration of the complex as judged by a repeated sucrose gradient centrifugation (data not shown). However, it was possible to obtain a preparation from mutant strains NuoI C63A and NuoI C102A as well as enriched complex I fractions from mutant strains NuoI C99A and NuoI C105S. These preparations were not as stable as the ones obtained from the NuoB mutants but three of them were used for EPR spectroscopy although they contained considerable amounts of succinate:ubiquinone oxidoreductase (see below). The preparation from the NuoI C63A mutant was unstable and aggregated upon concentration and could therefore not be used for EPR spectroscopy. The presence of subunits NuoB and NuoI was confirmed in the preparations by Western blot analysis (Fig. 2). In the presence of 50 μM NADH and 25 μM decylubiquinone, complex I isolated from the four NuoI mutants showed no NADH:decylubiquinone activity.

EPR Spectroscopy of Complex I Isolated from Mutants—Five preparations obtained from the NuoB mutant strains and three preparations from the NuoI mutant strains were characterized by EPR spectroscopy (Fig. 5). The samples were reduced by dithionite in the presence of redox mediators. The EPR spectra recorded at 40 K were nearly identical, indicating no change in the microenvironment of binuclear Fe/S clusters N1a and N1b (data not shown). The spectrum of the wild type enzyme recorded at 13 K and 10 mW microwave power revealed the presence of EPR-detectable clusters N1a (g∥ = 1.92, 1.95, 2.00), N1b (g∥ = 1.94, 1.94, 2.03), N2 (g∥ = 1.94, 2.01, 2.05), and N3 (g∥ = 1.88, 1.92, 2.04, and N4 (g∥ = 1.89, 1.93, 2.09; Fig. 5). In the preparations from all NuoI mutants, the signals of cluster N2 were completely gone. The g∥ signal of cluster N3 at 2.04, which was overlapped in the spectrum of the wild type by the g∥ signal of cluster N2, appeared in the spectra of the NuoB mutants. The spectra of the NuoB C63A and NuoB C63S mutants showed slight contributions from the binuclear cluster S1 from succinate:ubiquinone...
Localization of Fe/S Cluster N2

Little is known about the mechanism of the respiratory complex I that is caused by the lack of high-resolution structural data and the uncertainty of the number and nature of its cofactors. Many Fe/S clusters of complex I have been attributed to distinct subunits by EPR spectroscopic characterization of overproduced subunits (9, 22, 52–55). In some cases, the spectroscopic and thermodynamic features of the Fe/S clusters have changed, leading to an ambiguity of the Fe/S cluster investigated. Alternatively, mutations can be introduced in the nuo operon and the effect of the mutation on the fully assembled complex can be studied (35). However, mutagenesis of individual subunits turned out to be very laborious as they have to be introduced as unmarked mutations in the chromosome using the sacRB suicide vector system (33). Insertion of a resistance cartridge in the nuo operon coding the complex I subunits disturbed the assembly of the complex. All cysteine residues of NuoI that are putative ligands of Fe/S clusters have been replaced by alanine or serine residues on the chromosome. In addition, two of the four conserved cysteine residues of NuoB have been replaced by alanine residues. In the meantime, a plasmid-borne system has been established in our laboratory allowing mutagenesis in NuoB on a plasmid and subsequent transformation of the ΔnuoB strain with that plasmid (35). Using this system, the remaining two cysteine residues on NuoB were changed to alanine and serine residues.

Subunits NuoB and NuoI are part of the connecting fragment of the complex and are located at the interface of the hydrophilic and hydrophobic portion of the complex (14, 56). However, recently it was proposed that NuoB is located in the hydrophilic region of the complex more than 50 Å away from the membrane (13). The ΔnuoB and ΔnuoI strains were not able to assemble a functional complex I, although NuoI was detected in the membranes of strain ANN023 (Fig. 2). It is possible that these strains contain assembly intermediates of complex I. Similar results have been obtained with the bacterial complex I of R. capsulatus (23, 24) and the mitochondrial complex of N. crassa (19, 57).

By introducing point mutations in either NuoB or NuoI, the assembly of the complex was not disturbed (Figs. 2 and 3). However, there was a major difference concerning point mutations introduced in either NuoB or NuoI. Although mutagenesis in NuoB led to a complete loss of cluster N2 (Figs. 4 and 5), a stable complex missing N2 was isolated. This indicated that the three-dimensional structure of NuoB does not rely on the presence of the Fe/S cluster but on conserved protein/protein interactions. This agreed with the fact that NuoB shows the highest degree of sequence identity to its homologues compared with other complex I subunits (5). Mutagenesis in NuoI led to a dramatic decrease of the stability of complex I (Table III, Fig. 3). Although the amount of complex I in the mutant membranes was comparable with that in wild type, only one-third of the complex protein was extracted with detergent and remained stable during sucrose gradient centrifugation. The complex extracted from most of the NuoI mutants disassembled during column chromatography and could not be further purified. Preparations leading to a partly purified complex I were only possible from strains with mutations Nuo C63A, Nuo C66A, Nuo C102A, and Nuo C105S with the one from Nuo C63A being extremely unstable. This was also reported for mitochondrial complex I from N. crassa (19, 57). Whereas mutations of the conserved cysteine residues on NuoB led to

oxidoreductase at $g = 2.03$ overlapping with the $g_e$ of N3 and around $g = 1.93$ (50) overlapping with the major inflection point of the complex I spectrum. These contributions dominated the spectrum of complex I with the NuoC C158A mutation and thus they were omitted in Fig. 5. However, this caused no loss of information as the signals of S1 did not overlap with the signals of N2. Cluster N2 was also lost in the preparation of the NuoB C63S mutant, although it has been demonstrated that serine residues are possible ligands of Fe/S clusters (51).

The spectrum of the preparation from the mutant NuoI C102A contained the signals of cluster N2 at $g = 1.90$ and 2.045 (Fig. 5). Thus the signals of N2 were slightly shifted compared with the wild type. This was also seen in the EPR difference spectrum of the membranes from this strain (Fig. 4). The intensity of the signal at $g = 2.045$ was diminished by about 30% compared with the wild type spectrum as judged by its amplitude. Small contributions from cluster S1 of succinate:ubiquinone oxidoreductase at $g = 2.03$ and 1.93 were present in this spectrum. The spectra of the enriched complex I fractions from mutants NuoI C99A and NuoI C105S were strongly overlapped by signals of the Fe/S clusters of succinate:ubiquinone oxidoreductase. Nevertheless, the lack of cluster N2 in these enriched complex I fractions was detectable (data not shown).

### DISCUSSION

A. Berger, V. Spehr, and T. Friedrich, unpublished results.
the assembly of a stable complex I, mutations of five of the eight conserved cysteine residues on NuoI led to an instable complex or prevented assembly of the complex (57). Mutagenesis in *R. capsulatus* NuoI led either to an active enzyme (Cys-106; *E. coli* Cys-102), an enzyme with reduced activity (Cys-64, -67, and -70; *E. coli* Cys-60, -63, and -66), or to an inactive enzyme (Cys-74, *E. coli* Cys-70) (23, 24). This indicated that the two Fe/S clusters on NuoI are needed for a proper folding of the protein.

Our data clearly showed that the Fe/S cluster N2 is located on subunit NuoB, the bacterial homologue of subunit FSST of bovine complex I, and that there is only one Fe/S cluster giving rise to the corresponding EPR signals. All mutations in the conserved cysteine residues in NuoB led to a complete loss of N2 and an inactive enzyme (Fig. 5 and Table III). This was in agreement with data reported in the literature (19, 25). Mutagenesis of two conserved cysteine residues in the *N. crassa* NuoB led to the loss of cluster N2. Mutagenesis of conserved acidic amino acids in *Y. lipolytica* NuoB resulted in a change of its spectral properties. In addition, it has been shown that inhibition of complex I by a pyridaben derivative correlates with labeling of NuoB (58). It is suggested that this component inhibits the electron transfer between cluster N2 and ubiquinone (59). Thus, it was concluded that NuoB harbors N2 (58).

It has been proposed that cluster N2 is located on NuoI and that the EPR signals are because of the presence of two clusters N2a and N2b (23, 24, 60–63). In the study presented here, cluster N2 was detected in the membranes of the mutants NuoI C63A and NuoI C102A (Fig. 4). The mutations were confirmed by additional sequencing of genomic DNA. The same finding was reported for *R. capsulatus*, where the cysteine residues at the corresponding positions (Cys-67 and -106) were replaced by serine residues (24). From the EPR spectra recorded with *R. casulatus* mutant membranes it was calculated that the amount of N2 was reduced by 50%. From this, it was concluded that NuoI contains two clusters N2, one being lost because of the mutation while the other remained unchanged (24). However, our complex I preparation from the NuoI C102A mutant contained ~70% N2 and not 50% or less. Thus, the mutation on NuoI led to a destabilization of the complex that resulted in a partial loss of cluster N2 on subunit NuoB. This indicated a spatially close relationship between these two subunits. The interpretation that there are two clusters N2 is partly based on the assumption that the relative intensity of the signals of cluster N2 and that of binuclear clusters N1a and N1b are equal (24). This only holds true if it is assumed that both clusters N1a and N1b are completely reduced by NADH. However, this is in contrast to data obtained with bovine submitochondrial particles (64, 65) and, more important, with cytoplasmic membranes from *R. sphaeroides* (66). In *R. sphaeroides* the midpoint potential of cluster N1a was determined to ~380 mV and therefore, this cluster is not completely reducible with NADH (66). If only one of the binuclear clusters in *R. capsulatus* cytoplasmic membranes is reduced by NADH the calculated amount of cluster N2 in the membrane would be halved ending up with one cluster N2 per complex I. So far, no data concerning point mutations in NuoB are available for *R. capsulatus*.

The overproduced subunit NuoI of *P. denitrificans* has been characterized in detail (22). After in vitro reconstitution, NuoI contains nearly 8 mol each of Fe and S per molecule. Two axial-type tetranuclear Fe/S clusters have been detected in this preparation by EPR spectroscopy (22). However, their midpoint potential was shifted by more than 600 mV and the EPR signals could not be assigned to a signal of the intact complex (22). The UV-visible absorption spectra were similar to those of 2 × [4Fe/4S] ferredoxins. This is in line with results obtained with complex I from *N. crassa* and *E. coli* (8). It was shown by UV-visible spectroscopy that these preparations contain two additional Fe/S clusters that have not yet been identified by means of EPR spectroscopy (8). By comparison with a homologous [NiFe] hydrogenase (67, 68) and a 2× [4Fe/4S] ferredoxin it turned out that these two Fe/S clusters are of a typical ferredoxin type and are located on NuoI. As the midpoint potential of these Fe/S clusters (~280 mV) is not pH-dependent they cannot be attributed to cluster N2. They were named clusters N6a and N6b (8). Therefore, it is conclusive that all eight conserved cysteine residues of NuoI are needed for the ligation of clusters N6a and N6b (Fig. 1). Because of the lack of any structural data, it is not clear why the mutations of residues Cys-63, Cys-99, Cys-102, and Cys-105 led to a more stable complex than the corresponding mutation of the other four conserved cysteine residues on NuoI. In general, structural consequences of ligand mutations in Fe/S proteins are not predictable (51).

Localization of N2 on NuoB raises the question of the fourth ligand of this cluster. The arrangement of conserved cysteine residues in NuoB is rather uncommon. The first two conserved cysteine residues are located sequentially consecutively (Fig. 1). Because of sterical reasons it was assumed that only one of them may serve as a ligand for N2 (19, 25, 67). Because Cys-63 is not conserved in the homologous [NiFe] hydrogenase (68, 69), Cys-64 was considered to be the probable ligand. The suggestion that a conserved acidic amino acid on NuoB would be ligand for N2 has been ruled out by site-directed mutagenesis studies in *Y. lipolytica* (25). It was also proposed that the fourth ligand of N2 might be located on subunit NuoD (67). The soluble [NiFe] hydrogenases and complex I arose from a common ancestor (68), with subunits NuoB and NuoD being the homologues of the small and large subunit of hydrogenases, respectively (68, 69). As NuoB and NuoD seem to constitute a functional domain (67, 68), it could be possible that the fourth ligand of cluster N2 derives from NuoD (67). As all conserved cysteine residues of NuoI are involved in the binding of clusters N6a and N6b it can be excluded that one of these are involved in binding cluster N2. However, we have shown by molecular dynamics studies that in principal it is possible that two sequentially located cysteine residues are ligands of the same tetranuclear Fe/S cluster. Using the known structure of the small subunit of [NiFe] hydrogenases containing a regular cysteine motif in silico mutant with two sequentially consecutive cysteines was generated. Molecular dynamics simulation showed that a slight main chain conformational change in the structure of the hydrogenase small subunit would allow two sequentially consecutive cysteines to coordinate a [4Fe/4S] cluster. This would fit with the finding that individual removal of each of the two consecutively located cysteines on NuoB led to a loss of the Fe/S cluster. If only one of the cysteine residues were ligand of the cluster it would be reasonable to assume that the other undertook its function when the first cysteine residue was replaced by alanine or serine residues. This was experimentally verified by “scanning mutagenesis” of ferredoxins (71, 72). Thus, it is most likely that either all four conserved cysteine residues on NuoB are ligand to cluster N2, or that the fourth ligand derives from NuoD and has to be identified.

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