Catalytic Importance of Acidic Amino Acids on Subunit NuoB of the Escherichia coli NADH:Ubiquinone Oxidoreductase (Complex I)*§

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The NADH:ubiquinone oxidoreductase (complex I) from Escherichia coli is composed of 13 subunits called NuoA through NuoN and contains one FMN and 9 iron-sulfur clusters as redox groups. Electron transfer from NADH to ubiquinone is coupled with the translocation of protons across the membrane by a yet unknown mechanism. Redox-induced Fourier transform infrared difference spectroscopy showed that the oxidation of iron-sulfur cluster N2 located on NuoB is accompanied by the protonation of acidic amino acid(s). Here, we describe the effect of mutating the conserved acidic amino acids on NuoB. The complex was assembled in all mutants but the electron transfer activity was completely abolished in the mutants E67Q, D77N, and D94N. The complex isolated from these mutants contained N2 although in diminished amounts. The protonation of acidic amino acid(s) coupled with the oxidation of N2 was not detectable in the complex from mutant E67Q. However, the conservative mutations E67D and D77E did not disturb the enzymatic activity, and the signals because of the protonation of acidic amino acid(s) were detectable in the E67D mutant. We discuss the possible participation of Glu67 in a proton pathway coupled with the redox reaction of N2.

The proton-translocating NADH:ubiquinone oxidoreductase (EC 1.6.99.3), also called respiratory complex I, is the largest and least understood of the complexes of the respiratory chains (1–5). Its redox reaction is coupled with a translocation of protons across the membrane (6, 7). The mechanism of the complex is poorly understood because of its complex composition. The bacterial complex generally consists of 14 different subunits with seven hydrophilic proteins building the peripheral arm of the complex. Among these are the subunits that bear all known redox groups. The remaining 7 subunits are very hydrophobic proteins predicted to fold into 54 transmembrane α-helices. One FMN and, depending on the species, up to 9 iron-sulfur (Fe/S) clusters take part in electron transfer (1–5). The structure of the peripheral arm of the Thermus thermophilus complex has been determined at 3.3-Å resolution (8).

The Escherichia coli complex I genes are named nuoA through nuoN including the fused gene nuoCD (9). The corresponding 13 subunits are called NuoA through NuoN (9, 10). The presence of one noncovalently-bound FMN, two binuclear (N1a and N1b), and five tetranuclear (N2, N3, N4, N6a, and N6b) Fe/S clusters has been proven in the preparation of the E. coli complex I (11–13). Two additional Fe/S clusters detected in complex I from other species (N5 and N7) should be present in E. coli due to the preservation of the corresponding binding motifs (8, 14–16).

The Fe/S cluster N2 is located on subunit NuoB, the bacterial homologue of the mitochondrial PSST subunit (8, 17–20). The midpoint potential of N2 is, in contrast to most other clusters, pH-dependent (21). Therefore, it has been proposed that N2 is involved in proton translocation and functions as the direct electron donor of quinone (21, 22). This idea is supported by the fact that N2 interacts with ubisemiquinone radicals identified in submitochondrial particles (22–26). Two semiquinone radicals coined SQN5 and SQN6 have been identified by the group of T. Ohnishi. Distances of 12 Å and more than 30 Å between N2/SQN5 and N2/SQN6, respectively, were calculated (26).

If N2 participates in proton pumping, its redox reaction is coupled to a protonation/deprotonation of the cluster itself and/or of surrounding amino acids. We demonstrated by means of electrochemically induced FT-IR difference spectroscopy of complex I from site-directed mutants that the reduction of N2 is accompanied by the protonation of Tyr114 and Tyr190 on NuoB (numbering according to the E. coli sequence; Ref. 27). Other important features of the infrared difference spectra are a positive absorption around 1,712 cm−1, which has been attributed to protonated aspartic or glutamic side chains, and negative absorptions at 1,556 and 1,410 cm−1 attributed to the respective signals of the deprotonated forms (28). These data indicated that the oxidation of N2 is coupled with the
protonation of acidic amino acid(s). In this study, we mutated the conserved acidic amino acids on NuoB and characterized the mutants by their enzymatic activity and by means of EPR and FT-IR-spectroscopy. Our data showed that Glu67, Asp77, and Asp94 are essential for electron transfer. We are discussing the role of Glu67, the acidic amino acid most likely protonated by the oxidation of N₂, for proton translocation.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains—**E. coli strain AN387 (29) and was generated using a genomic replacement method as described (27). It contains a chromosomal in-frame deletion of *nuoB*. E. coli strains DH5α (30), XL1-Blue Supercompetent cells (Stratagene) and the plasmids pSTBlue-1 (Novagen) and pBAD33 (31) were used. When required for maintenance of plasmids, chloramphenicol was added to 20 mg/liter and ampicillin to 100 mg/liter. All enzymes used for recombinant DNA techniques were from MBI Fermentas (St. Leon-Roth). All other chemicals were from Merck and Sigma.

**Site-directed Mutagenesis and Expression of nuoB—**Point mutations were created using the QuikChange site-directed mutagenesis kit (Stratagene) and the corresponding primers were used for DNA sequencing. The resulting plasmids containing the desired mutation were used for transformation of the deletion strain ANN023. In this strain, expression of NuoB and complementation of the chromosomal deletion was carried out by adding arabinose to a final concentration of 0.2% (w/v) as described (19).

**Growth of Cells and Isolation of Complex I—**Parental strain and mutant *E. coli* strains were grown aerobically in 10 liters of LB medium at 37 °C to the late exponential growth phase. Cells were harvested by centrifugation for 10 min at 4,000 × g, washed with 50 mM MES/NaOH, pH 6.0 and stored at −80 °C. Complex I was isolated from the mutants as described (27). In principle, the proteins were extracted from the cytoplasmic membrane with 3% (w/v) dodecyl maltoside (Glycon) in 50 mM MES/NaOH, 50 mM NaCl, pH 6.0 and applied to a first anion-exchange chromatography on Source 15Q (Amersham Biosciences). Fractions containing NADH/ferricyanide reductase activity were combined, concentrated by precipitation with 9% (w/v) final concentration poly(ethylene glycol) 4,000 and dissolved in 2 ml of 50 mM MES/NaOH, 50 mM NaCl, and 0.1% dodecyl maltoside, pH 6.0. The protein was subjected to size-exclusion chromatography on Sephacryl S-300 (Amersham Biosciences) in the same buffer. Peak fractions were pooled and applied to a 10 ml Source 15Q column (Amersham Biosciences) equilibrated as described above. Fractions with NADH/ferricyanide reductase activity were pooled and stored at −80 °C.

**Electrochemistry—**The ultra-thin layer spectroelectrochemical cell for the visible (vis) and infrared (IR) was used as previously described (32). Sufficient transmission in the 1,800 cm⁻¹ to 1,000 cm⁻¹ range, even in the region of strong water absorbance around 1,645 cm⁻¹, was achieved with the cell pathlength set to 6–8 μm. To avoid protein denaturation, the gold grid working electrode was chemically modified by a 2 mM cysteamine solution as reported (32). In order to accelerate the redox reaction, mediators were used at a final concentration of 45 mM each as described (28). At the given concentrations and with the pathlength below 10 mm, no spectral contributions from the mediators in the vis and IR range were used were detected in control experiments with samples lacking the protein. Approximately 6–7 μl of the protein solution were sufficient to fill the spectro-electrochemical cell.

**FT-IR Spectroscopy—**FT-IR and vis difference spectra as a function of the applied potential were obtained simultaneously from the same sample with a setup combining an IR beam from the interferometer (modified IFS 25, Bruker, Germany) for the 4,000 cm⁻¹ to 1,000 cm⁻¹ range and a dispersive spectrometer for the 400–900 nm range as reported previously (33). First, the protein was equilibrated with an initial potential at the electrode, and single beam spectra in the vis and IR range were recorded. Then, a potential step toward the final potential was applied, and single beam spectra of this state were recorded after equilibration. Difference spectra were calculated from the two single beam spectra with the initial single beam spectrum taken as reference. IR-double difference spectra were obtained by a subtraction of the samples normalized at 460 nm. No smoothing or deconvolution procedures were applied. The equilibration process for each applied potential was followed by monitoring the electrode current and by successively recording spectra in the vis range until no further changes were observed. Generally, the equilibration took less than 8 min for the full potential step from −0.7 V to 0.2 V. Typically, 128 interferograms at 4 cm⁻¹ resolution were coadded for each single beam IR spectrum and Fourier-transformed using triangular apodization. 20–25 difference spectra have been averaged. The presence of atmospheric water vapor was avoided as described (28).

**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 or a weak pitch standard. The isolated complex I (2–4 mg/ml) was reduced with a few grains of dithionite in the presence of 15 mM NADH (12). The relative amount of the individual clusters was determined by simulation of the spectra as described (11). Potentiometric redox titrations were performed as described (11).

**Other Analytical Procedures—**The (deamino-) NADH/ferricyanide reductase activity and the (deamino-) NADH oxidase activity were measured as described (34, 35). Protein concentration was measured with the biuret method using bovine serum albumin as standard. Sucrose gradient centrifugation in the presence of 0.2% dodecyl maltoside was performed as described (11, 12). Following SDS-PAGE protein bands were either stained with Coomassie Blue or silver (36). Alternatively, the separated proteins were electroblotted onto 0.45-μm pore size nitrocellulose membrane (Schleicher und Schüll) for Western blot analysis (37). A rabbit polyclonal antibody raised against NuoB was used for detection. Analytical gel filtration was performed on a 14 ml of PolySep-GFC-P4,000 column (Phenomenex) in 50 mM MES/NaOH, 50 mM NaCl, and 0.1%
RESULTS

Conserved Acidic Amino Acids on NuoB—The homologues of NuoB from bacterial and mitochondrial complex I from 35 species were aligned. As an example the alignment of five different sequences is shown in Fig. 1. NuoB contains eight conserved acidic amino acids including two glutamates and five aspartates, respectively. Either a glutamate or an aspartate is present at position 119 (E. coli numbering). In this study, these acidic amino acids were changed individually to the corresponding amide. The assembly and the enzymatic activity of complex I in these mutants was investigated. The enzyme was isolated from the inactive mutants and characterized by means of EPR and FT-IR spectroscopy. Aspartic acids that were identified to be of functional importance were subsequently changed to glutamic acids and vice versa.

Catalytic Activity of the Mutant Strains—The E. coli strain ANN023 lacks subunit NuoB and is therefore not able to assemble an intact complex I (27). By complementing the chromosomal in-frame deletion strain with wild-type complex I in membranes was restored to 90% of the wild-type level by complementing the chromosomal in-frame deletion strain with wild-type complex I in these mutants was investigated. The amount of complex I in the mutant membranes after induction with arabinose was confirmed by Western blot analysis (supplemental data, Fig. S3). Sucrose gradient centrifugation showed the presence of an assembled complex I in the cytoplasmic membrane of all mutant strains (supplemental data, Fig. S4).

The NADH dehydrogenase activity of bacterial membranes was measured as NADH/ferricyanide and deamino (d)-NADH/ferricyanide reductase activity, respectively, to discriminate the two membrane-bound NADH dehydrogenases of E. coli (35, 38). Whereas the non-energy converting, alternative NADH dehydrogenase has a very low affinity to d-NADH, complex I has the same affinity toward NADH and d-NADH (35, 38). Because NuoB is not involved in this reaction, the amount of complex I in the cytoplasmic membranes of the mutants can be estimated from the d-NADH/ferricyanide reductase assay (Table 1). The control strain ANN023/pBAD did not show any complex I activity. Complex I content was reduced by approximately one third in mutants D152N and E163Q and by one-fourth in mutants E67Q, D77N, D94N, and D146N. The amount of complex I in the cytoplasmic membranes of the D115N and E119Q mutant strains was comparable to the one of the parental strain (Table 1). The amount of complex I in the mutant membranes as determined by the d-NADH/ferricyanide reductase activity correlates well with the

dodecyl maltoside, pH 6.0 at a flow rate of 0.5 ml/min. Each run was loaded with 0.2 mg of complex I.

TABLE 1
Enzyme activities of the membrane-bound NADH dehydrogenases of E. coli parental strain and various NuoB mutants

| Strain       | NADH/ferricyanide reductase activity | d-NADH/ferricyanide reductase activity | NADH oxidase activity | d-NADH oxidase activity | Normalized d-NADH oxidase activity | Sensitivity to piericidin A |
|--------------|-------------------------------------|----------------------------------------|-----------------------|------------------------|-----------------------------------|--------------------------|
| Parental     | 0.9                                 | 0.8                                    | 0.08                  | 0.052                  | 100                               | +                        |
| Control      | 0.6                                 | 0.1                                    | 0.05                  | 0                      | 0                                 | 0                        |
| E67Q         | 0.8                                 | 0.6                                    | 0.04                  | 0.004                  | 10                                | 0                        |
| D77N         | 0.9                                 | 0.6                                    | 0.12                  | 0.005                  | 12                                | 0                        |
| D94N         | 0.7                                 | 0.6                                    | 0.03                  | 0.005                  | 12                                | 0                        |
| D115N        | 0.7                                 | 0.7                                    | 0.08                  | 0.021                  | 45                                | 0                        |
| E119Q        | 0.7                                 | 0.7                                    | 0.09                  | 0.023                  | 88                                | 0                        |
| D146N        | 0.8                                 | 0.6                                    | 0.07                  | 0.023                  | 59                                | 0                        |
| D152N        | 0.7                                 | 0.5                                    | 0.09                  | 0.019                  | 59                                | 0                        |
| E163Q        | 0.7                                 | 0.5                                    | 0.09                  | 0.025                  | 76                                | 0                        |
| E67D         | 0.9                                 | 0.7                                    | 0.08                  | 0.046                  | 78                                | 0                        |
| D77E         | 0.7                                 | 0.6                                    | 0.07                  | 0.021                  | 54                                | 0                        |
| D94E         | 0.8                                 | 0.5                                    | 0.10                  | 0.027                  | 83                                | 0                        |

* d-NADH oxidase activity referring to the complex I content as deduced from the d-NADH/ferricyanide reductase activity of the respective membranes.
amount of subunit NuoB determined by Western blotting (supplemental data, Fig. S3). The NADH/ferricyanide reductase activity was only slightly reduced in all mutant strains indicating that the production of the alternative NADH dehydrogenase was increased in E. coli strains with a reduced amount of complex I as reported earlier (12).

The physiological activity of complex I was measured as d-NADH oxidase activity (Table 1). This reaction rate normalized to the amount of the complex in the membranes as deduced from the d-NADH/ferricyanide reductase activity reveals the catalytic rate of the mutant complex. The rate was 45–90% of the activity of the parental strain in the D115N, E119Q, D146N, D152N, and E163Q mutants (Table 1). This activity was sensitive to piericidin A, a specific complex I inhibitor, indicating that complex I was functional in these mutants although at a slower rate. The mutants E67Q, D77N, and D94N showed about 10% of the activity of the parental strain. This residual activity was not inhibited by 10 μM piericidin A and therefore was not derived from the physiological activity of the complex. Thus, complex I is inactive in these mutants. From this we concluded that Glu67, Asp77, and Asp94 are essential for the physiological electron transfer in the E. coli complex I and focused our further studies on these mutants.

Isolation of Complex I from the Mutant Strains—To determine whether the reduced activity of complex I in the distinct mutants was due to the loss of a cofactor or the replacement of a functionally important amino acid, we attempted to isolate the enzyme from the mutants and to characterize them by EPR and FT-IR spectroscopy. The complex from the E67Q and the D77N mutants showed comparable chromatographic behavior. It eluted from both anion-exchange chromatographies on Source 15Q at 280 mM NaCl and from the Sephacryl S-300 size-exclusion column at 70 ml (supplemental data, Fig. S5). On an average 4 mg of complex I were obtained from 80 g of cells (supplemental data, Table S2). Upon SDS/PAGE the preparation was resolved into 12 subunits corresponding to the proteins encoded by the nuo-operon (supplemental data, Fig. S5). The subunits NuoE and J were not separated by SDS-PAGE as reported (11, 12, 39). Because of the presence of protein impurities additional bands were detectable in the early fractions of the gradient. These fractions were discarded. It was not possible to obtain a stable preparation from the D94N mutant. Thus, the preparations from the E67Q and D77N mutants were further investigated. They did not show any piericidin-sensitive NADH:decyl-ubiquinone reductase activity, even after activation with phospholipids (7).

The structural integrity of the preparations were examined by analytical gel filtration (supplemental data, Fig. S6). The elution profile of the preparations from parental strain and the E67Q and D77N mutants showed a similar profile. The protein eluted at the same volume with the same peak width at one-half height. These data demonstrate the structural integrity of the preparations of the complex from the mutants.

EPR Spectroscopic Analysis of the NuoB Mutants—The content of the Fe/S clusters in the preparations was determined by means of EPR-spectroscopy. Samples containing 2–4 mg of each preparation were reduced by adding an excess of NADH and dithionite. The EPR spectra of the complex from the NuoB mutants E67Q and D77N recorded at 40 K revealed the presence of the binuclear clusters N1a and N1b in stoichiometric amounts and with the same spectral characteristics as in the parental strain (Fig. 2). In addition to these clusters, the tetranuclear clusters N2, N3, and N4 were detected in the spectra recorded at 13 K (Fig. 3). The preparation of the complex from the mutant D77N contains very minor contamination from succinate dehydrogenase as seen in the positive signal at g = 2.027 and the larger trough at g = 1.94. The g value of N2 (2.052) overlaps with the g values of N3 (2.048) and the g value of N1a (2.049) with the g values of N3 and N4 (2.08) and 1.89, respectively; Ref. 11). Thus, the amount of cluster N2 in the preparation of the mutant complex was obtained by simulation of the individual Fe/S clusters as described (11). The amount of N2 in the E67Q mutant enzyme was reduced to ~30% compared with the parental strain and to 20% in the D77N mutant enzyme. However, the spectral position and the line width of the signals of cluster N2 remained unchanged. The clusters N1a, N1b, N3, and N4 were present at parental strain levels.
FT-IR Spectroscopic Analysis of the NuoB Mutants—The difference spectra calculated from the completely oxidized and the completely reduced complex from the parental strain and from the mutant strains E67Q and D77N are shown in Figs. 5 and 6. Three main regions in the spectra are discussed: (i) the amide I range, that includes the \( v(C=O) \) signals from the backbone, (ii) signals previously attributed to tyrosine 114 and 139 and discussed to be part of the proton pathway around cluster N2 (27), and (iii) the signals characteristic for acidic groups, which are possibly perturbed upon mutation.

The amide I range between 1,700 and 1,620 cm\(^{-1}\) included a prominent differential feature. Peaks that reflect structural reorganizations of \( \beta \)-sheets, loops, unordered elements, and helices were distinguished at 1,696, 1,670, 1,648, 1,634, and 1,624 cm\(^{-1}\) (Figs. 5 and 6). Small contributions from FMN and individual amino acid side chains, in particular Asn, Gln, or Arg cannot be excluded in this spectral range (40). The large conformational rearrangement that was detected in the parental strain complex was partially decreased in the spectra of the complex isolated from the mutants.

The characteristic tyrosine signals at 1,515 and 1,498 cm\(^{-1}\) that were proposed to be protonated upon reduction of cluster N2 (27) were shifted by some wavenumbers by the E67Q mutation and slightly shifted by the D77N mutation (Figs. 5 and 6). The influence of the mutations of the acidic amino acids on the modes of the tyrosine side chains corroborates the idea that there could be a direct interaction between these two types of amino acids.

Signals attributed to the protonation of aspartic or glutamic acid side chain(s) were detected in the redox-induced FT-IR difference spectra of complex I (28). The \( v(C=O) \) mode of protonated aspartic or glutamic acids contributes as positive absorption at 1,716–1,712 cm\(^{-1}\) and the respective signals for the deprotonated forms are observed as negative absorptions at 1,555–1,580 cm\(^{-1}\) \( (v(COO^–)_{\text{w}}) \) and at 1,402–1,404 cm\(^{-1}\) \( (v(COO^–)_{\text{a}}) \).

The small shoulder at about 1,712 cm\(^{-1}\) reflecting the protonation of aspartic or glutamic acid(s) coupled with the oxidation of cluster N2 (28) was not detectable in the spectra of the complex from the mutant E67Q and slightly reduced for the complex from the mutant D77N (Figs. 5 and 6). The loss of the signal at 1,712 cm\(^{-1}\) is seen in the double difference spectrum from the spectra of
the complex from the parental strain minus the spectra of the complex from the mutant E67Q (Fig. 5, inset). This provides evidence that Glu67 is affected by the electron transfer induced by the electrochemical approach. In contrast, the shoulder at about 1,712 cm\(^{-1}\) was detectable in the preparations from the mutants E119Q and D115N (data not shown).

Properties of the E67D, D77E, and D94E Mutants—Positions Glu67, Asp77, and Asp94 were classified as important for the electron transfer activity of complex I (Table 1). Therefore, we introduced conservative changes from aspartic acid to glutamic acid and vice versa at these positions. The d-NADH oxidase activity was only slightly reduced by \(\approx 20\%\) in the E67D and D94E mutant enzymes, whereas half of the parental strain activity remained in the D77E mutant enzyme (Table 1). These activities were sensitive to 10 \(\mu M\) piericidin A. The preparation from the D77E mutant showed an inhibitor-sensitive activity of \(1.0 \pm 0.2 \mu\)mol of NADH/min/mg. The complex from the D94E mutant showed no NADH:decyl-ubiquinone activity, most likely because of its instability as judged from multiple peaks after analytical gel filtration (data not shown). Under the same conditions, the complex from the parental strain exhibited an activity of \(2.4 \pm 0.1 \mu\)mol of NADH/min/mg.

EPR spectroscopy at 40 K revealed that the spectral positions and the relative amount of the signals of the binuclear clusters of the preparation from the E67D and D77E mutants did not change compared with the parental strain. The spectra were virtually identical to the ones shown in Fig. 2 (supplemental data, Fig. S7). The spectra recorded at 13 K showed that both preparations contained minor contaminating amounts of succinate dehydrogenase as seen in signals at \(g = 2.027\) and 1.94. The preparation of the complex from the E67Q mutant contained 45\% of the amount of cluster N2 in the parental strain, the preparation from the mutant D77E \(\approx 35\%\), while the amount of the remaining Fe/S clusters was similar to the preparation from the parental strain (Fig. 3). Thus, these mutants contained an increased amount of N2 compared with the corresponding amide mutants. The fact that the content of N2 in the complex isolated from the E67D and D77E mutants was one-half and one-third of the content of the parental strain, respectively, whereas the d-NADH oxidase activity was 78 and 54\% of the activity of the parental strain (Table 1) could be due either to a loss cluster N2 during the purification or possibly to a higher activity of the complex in the mutant strains.

FIGURE 5. Electrochemically induced FT-IR difference spectra of the parental strain (trace a) and the preparations from the strains E67Q (trace b) and E67D (trace c) at a potential step from \(-0.5\) to 0.2 V. The signal assigned to a protonated acidic amino acid at about 1,712 cm\(^{-1}\) and the signals of the protonated/deprotonated tyrosines at 1,515 and 1,498 cm\(^{-1}\) are marked with lines. The inset shows the double difference spectrum from the spectra of the parental strain complex minus the one of the E67Q mutant enzyme for the spectral region characteristic for protonated acidic residues.

FIGURE 6. Electrochemically induced FT-IR difference spectra of the parental strain (trace a) and the preparations from the strains D77N (trace b) and D77E (trace c) at a potential step from \(-0.5\) to 0.2 V. The signal at about 1,712 cm\(^{-1}\) discussed in the text is marked with a line.
FT-IR spectroscopy with the complex from the E67D and the D77E mutants revealed the presence of a broad signal at 1,718 cm\(^{-1}\) (Figs. 5 and 6). A possible explanation for the appearance of this signal is the different side chain length. This change could induce the shift of a \(\nu(C=O)\) vibrational mode, as the acidic group may now face a less hydrophilic environment or experience weaker hydrogen bonding. In addition, the local \(pK_a\) of another acidic residue in the direct vicinity may have changed, leading to an altered protonation state. Noteworthy, the amplitude of the amide \(I\) region became similar to the amplitude in the spectrum of the complex from the parental strain and the characteristic tyrosine signals at 1,515 and 1,498 cm\(^{-1}\) were similar to parental strain.

**DISCUSSION**

Two scenarios for the mechanism of complex I are discussed, in which proton-translocation is either directly driven by the electron transfer or indirectly by conformational changes (3–5). Because of the modular evolution of the complex, we have proposed that both types of coupling are combined in complex I (3). In our mixed model the conformational changes of the subunits NuoL, M, and N, which are homologous to cation/H\(^+\) antiporters and which do not contain a redox group (2, 41–43). The redox-driven coupling should be provided by the interaction of the Fe/S cluster N2 with the semiquinone species detected under steady state conditions (22–26, 44). The pH dependence of the midpoint potential of N2 (21), its close proximity to the membrane (8, 45, 46), and its interaction with semiquinone radicals (23–26) make it an ideal candidate for being the direct electron donor to quinone and involved in proton translocation (21, 22). The location of N2 close to the membrane has been questioned recently by work performed with *Yarrowia lipolytica*. From the analysis of immunolabeled single particles it was concluded that NuoB is distant from the membrane (47) and first evidence was reported from *Y. lipolytica* mutants that cluster N2 is not needed for the physiological electron transfer within the complex (48). However, the structure of the peripheral arm of complex I shows a near linear arrangement of seven Fe/S clusters from the NADH binding site to the putative quinone binding site (8). Cluster N2 is located just 15 Å away from the membrane contradicting the data derived by electron microscopy (8, 47). In addition, it was suggested that the *Y. lipolytica* mutants mentioned above still contain N2, but that its midpoint potential was dramatically reduced because of introduced mutations (8).

We have investigated the role of individual acidic amino acids on NuoB in close vicinity of N2 for the mechanism of complex I. Remarkably, the residues Glu\(^67\), Asp\(^77\), and Asp\(^94\) are not involved in substrate and cofactor binding although their mutation to the corresponding amide completely abolished electron transfer of the complex (Table 1). The mutations did not prevent the assembly of the complex. For spectroscopic characterization, the complex was isolated from the mutants. The homogeneity and stability of the preparations from the Glu\(^67\) and Asp\(^77\) mutants was confirmed by analytical gel filtration (supplemental Fig. S6) and by EPR spectroscopy (Figs. 2 and 3). We are aware that the mutations leading to a partial loss of N2 might cause local structural changes, which in turn could lead to perturbations of the FT-IR spectra. However, it is reasonable to assume that the spectral changes observed in FT-IR spectroscopy are mostly because of the mutations introduced on NuoB, because of the spectroscopic changes do not occur in preparations from NuoB and NuoCD mutants, which have partially lost N2.\(^4\)

From studies with the mitochondrial complex from *Y. lipolytica* (18, 50) it has been proposed that Asp\(^115\) and Glu\(^119\) (corresponding to Asp\(^136\) and Glu\(^140\) in *Y. lipolytica*) play a central role in the energy coupling mechanism of the complex while contributing to a channel that transfers protons to or from N2 (18). In *E. coli* these mutations had only a minor effect on enzymatic activity (Table 1) and had neither an effect on the EPR spectroscopic properties of the Fe/S clusters nor on the FT-IR signals of the protonation/deprotonation of acidic amino acid(s) (data not shown). Thus, these positions are not important for electron transfer and proton translocation, at least in the bacterial complex I.

The most interesting acidic amino acids on NuoB are Glu\(^67\), Asp\(^77\), and Asp\(^94\) (corresponding to Glu\(^69\), Asp\(^99\), and Asp\(^115\) in *Y. lipolytica*). In both organisms, the change of Asp\(^77\) and Asp\(^94\) to the corresponding amide led to a complete loss of enzymatic activity (Table 1 and Refs. 18 and 50). The amount of cluster N2 is strongly reduced without a change in its spectral properties (Fig. 3 and Ref. 18). Mutation of the aspartic acid to glutamic acid did not rescue the *Y. lipolytica* complex (50). However, the *E. coli* mutants D94E and D77E clearly exhibited enzymatic activity (Table 1), contained a less decreased amount of cluster N2 (Fig. 3), and showed an influence on the \(\nu(C=O)\) modes of protonated aspartic and glutamic acid side chains (Fig. 6). A clear disagreement between the mitochondrial and the bacterial complexes remains concerning the role of Glu\(^67\) (Glu\(^99\) in *Y. lipolytica*). Mutation of this position to the amide had no effect on the enzymatic activity of the *Y. lipolytica* enzyme and led to a shift of the \(g_z\) value of N2 but not to a loss of the cluster (18). In *E. coli* this mutation terminated activity and led to a loss of more than two-thirds of N2 (Table 1 and Fig. 3). In addition, the FT-IR signature for the redox-induced protonation of an acidic amino acid including the signal at 1,712 cm\(^{-1}\) was lost, suggesting that this acidic residue is most likely protonated coupled with the oxidation of N2 (Fig. 5). This FT-IR signature was present in the E67D mutant (Fig. 5). Noteworthy, the E67Q mutation led to a shift of the FT-IR signal for a protonated acidic amino acid side chain concomitant with oxidation of the enzyme. The fact that acidic amino acids substitute each other in the bacterial but not in the mitochondrial complex possibly points to a more flexible structure of the bacterial complex.

We have shown that the oxidation of N2 is coupled with the protonation of an acidic amino acid in the *E. coli* complex I (28). This finding could not be reproduced by means of redox induced ATR-FT-IR difference spectroscopy using the mitochondrial complex I from bovine heart and *Y. lipolytica* (51). However, our data indicate that this acidic amino acid most likely corresponds to Glu\(^67\) on NuoB because Glu\(^67\) is proto-

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\(^4\) P. Hellwig and T. Friedrich, unpublished results.
Function of Acidic Amino Acids of Complex I Subunit NuoB

![Diagram of Complex I Subunit NuoB](image)

**FIGURE 7. Environment of cluster N2 in the crystal structure of *T. thermophilus* complex I (8).** The subunits Nqo6 (corresponding to NuoB) and Nqo4 (corresponding to NuoD) are drawn in blue and green, respectively. A different conformer, drawn in transparent pink, brings the carboxylate group within hydrogen bonding distance to the cluster ligand C46. The space for this rotation is provided by the prolines 141 and 142. b, side view.

Glu67 is not the redox-Bohr group, as its midpotential of N2 did not change due to the E67Q mutation. The pH dependence of the midpotential of N2 was significantly decreased in the spectra of the mutant E67D (Fig. 6). The oxidation of N2 could induce conformational changes that alter the environment of Glu67 as described above leading to a decrease of its pK value and its subsequent protonation.

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aspartic acid is responsible for the signal at 1,712 cm\(^{-1}\) because this signal is only slightly reduced and not lost in the D77N mutant (Fig. 6). We keep in mind that interactions with another acidic side chain would induce a secondary effect and the D77N mutation may lead to a perturbation of residue Glu67.

The pH dependence of the midpotential of N2 did not change due to the E67Q mutation. Thus, Glu67 is not the redox-Bohr group associated with N2. In addition, the midpotential of N2 exhibits a slope of \(-60 \text{ mV/pH.unit (11, 21).}

The direct coupling of the oxidation of N2 with the protonation of Glu67 would lead to an inverted slope, which is not observed. Therefore, we propose that the protonation of Glu67 is coupled to a conformational change.

It is noteworthy that the prominent differential feature in the FT-IR difference spectra between 1,700 and 1,620 cm\(^{-1}\) that include structural reorganizations like the direct reorganization around the Fe/S clusters as well as coupled conformational changes, is significantly decreased in the spectra of the mutant complex (Figs. 5 and 6). The oxidation of N2 could induce conformational changes that alter the environment of Glu67 as described above leading to a decrease of its pK value and its subsequent protonation.

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