Characterization of the Overproduced NADH Dehydrogenase Fragment of the NADH:Ubiquinone Oxidoreductase (Complex I) from *Escherichia coli*†

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ABSTRACT: The proton-pumping NADH:ubiquinone oxidoreductase of *Escherichia coli* is composed of 14 different subunits and contains one FMN and up to nine iron−sulfur clusters as prosthetic groups. By use of salt treatment, the complex can be split into an NADH dehydrogenase fragment, a connecting fragment and a membrane fragment. The water-soluble NADH dehydrogenase fragment has a molecular mass of approximately 170 000 Da and consists of the subunits NuoE, F, and G. The fragment harbors the FMN and probably six iron−sulfur clusters, four of them being observable by EPR spectroscopy. Here, we report that the fully assembled fragment can be overproduced in *E. coli* when the genes *nuoE*, *F*, and *G* were simultaneously overexpressed with the genes *nuoB*, *C*, and *D*. Furthermore, riboflavin, sodium sulfide, and ferric ammonium citrate have to be added to the culture medium. The fragment was purified from the cytoplasm by means of ammonium sulfate fractionation and chromatographic steps. The preparation contains one noncovalently bound FMN per molecule. Two binuclear (N1b and N1c) and two tetranuclear (N3 and N4) iron−sulfur clusters were detected by EPR in the NADH reduced preparation with spectral characteristics identical with those of the corresponding clusters in complex I. The preparation fulfills all prerequisites for crystallization of the fragment.

The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I,[1] is the first enzyme in the respiratory chains of mitochondria and several bacteria. It couples the transfer of two electrons from NADH to ubiquinone with the translocation of four protons across the membrane. One FMN and six to nine iron−sulfur (FeS) clusters serve as prosthetic groups in this reaction (Weiss et al., 1991; Weiss & Friedrich, 1991; Walker, 1992; Friedrich et al., 1995). The mitochondrial complex of the bovine is made up of 42 different subunits (Buchanan & Walker, 1996) and that of the fungus *Neurospora crassa* of at least 35 subunits (Schulte & Weiss, 1996). The seven most hydrophobic subunits are encoded by mitochondrial DNA. Purple bacteria contain a minimal form of complex I composed of 14 subunits (Friedrich et al., 1995; Friedrich & Weiss, 1996, 1997). Among these are all subunits presumed to bind substrates and prosthetic groups, as well as the homologues of the mitochondrially encoded subunits of the eucaryotic complex I.

The genes of the complex of *Escherichia coli*, *Paracoccus denitrificans*, *Rhodobacter capusulatus*, and *Thermus thermophilus* (Weidner et al., 1993; Xu et al., 1993; Dupuis et al., 1995; Yano et al., 1997) are clustered in a conserved order (Friedrich et al., 1995). In *E. coli*, they are organized in the so-called *nuo*-operon (from *N*ADH:*u*biquinone *o*xidoreductase), a 16 kb DNA region localized at minute 51 of the chromosome (Weidner et al., 1993). On the basis of sequence comparisons and biochemical approaches, it has been predicted that the subunit NuoF contains the binding sites for NADH and FMN as well as one FeS cluster. The subunits NuoB, E, G, and I are predicted to be iron−sulfur proteins, while NuoH has been proposed to contain the ubiquinone binding site (Weiss et al., 1991; Walker, 1992; Fearnley & Walker, 1992; Weidner et al., 1993).

The *E. coli* complex I has been isolated in the presence of an alkylglucoside detergent by chromatographic steps (Leif et al., 1995). The monodisperse preparation is solely made up of the 14 subunits encoded by the *nuo*-genes. One noncovalently bound FMN, two binuclear (N1b and N1c), and three tetranuclear (N2, N3, and N4) EPR detectable FeS clusters have been found in the preparation. The binuclear FeS cluster N1a found in other species was not detected. Since the sequence motif presumed to harbor this cluster is present on NuoE, we suggest this cluster to be present in the *E. coli* complex while not being reducible with NADH (Ohnishi, 1993; Sled' et al., 1993, Leif et al., 1995).

Upon treatment with salt, the isolated *E. coli* complex I falls into three parts which are called NADH dehydrogenase fragment, connecting fragment, and membrane fragment (Leif et al., 1995). The water-soluble NADH dehydrogenase fragment contains the subunits NuoE, F, and G and harbors the FMN and the EPR-detectable FeS clusters N1b, N1c, N3, and N4. The connecting fragment consists of NuoB, C, D, and I and contains the EPR detectable FeS cluster N2. This fragment is only soluble in the presence of detergent although none of its subunits is predicted to form membrane-spanning helixes. The fragment is assumed to connect the

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[1] Abbreviations: complex I, proton-pumping NADH:ubiquinone oxidoreductase; FeS cluster, iron−sulfur cluster; EPR, electron paramagnetic resonance; UQ-0, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone; UQ-2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; IPTG, isopropyl β-D-thiogalactopyranoside; mT, millitesla.

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NADH dehydrogenase fragment with the membrane fragment. The membrane fragment is composed of NuoA, H, and J−N, which are the homologues of the mitochondrially encoded subunits of the eucaryotic complex. These most hydrophobic subunits are predicted to fold into 55 membrane spanning helixes (Weidner et al., 1993).

We report here the homologous overexpression, isolation, and characterization of the NADH dehydrogenase fragment. We show that subunits of the connecting fragment and addition of riboflavin, iron ions, and sulfide to the culture medium are needed for proper assembly of the fragment. The isolated NADH dehydrogenase fragment contains the expected cofactors in equimolar amounts with identical spectroscopic features to those of the entire complex.

MATERIAL AND METHODS

Materials and Strains. The *E. coli* K-12 strains AN387 (Stroobant et al., 1972) and BL21(DE3) (AGS, Heidelberg) and the plasmids pT7T3 19U (Pharmacia, Freiburg) and pET-24a (AGS, Heidelberg) were used. When required for maintenance of plasmids, ampicillin was added to 100 μg/mL and kanamycin to 30 μg/mL. All enzymes used for recombinant DNA techniques were from Pharmacia (Freiburg) or Boehringer Mannheim (Mannheim). [α-^{35}S]dATP (600 mCi/mmol) was obtained from Amersham Buchler (Braunschweig). All others chemicals were from Merck (Darmstadt), Riedel de Haen (Hannover), Serva (Heidelberg), or Sigma (München).

Construction of Expression Vectors. Standard methods for molecular biology were used according to Sambrook et al. (1989). DNA sequencing was performed according to Sanger et al. (1977) using the T7-sequencing kit (Pharmacia, Freiburg). For construction of the expression plasmid pET-24/*nuoE-G*, a 5913-bp *Bam*HI DNA fragment was excised from pNUO-13 (Weidner et al., 1993) and ligated in *Bam*HI/pET-24a. The resulting plasmid is designated pET-24/*nuoD-G*. Two primers were synthesized to amplify the 5′-^{3389}*Nde*I/*Xho*I^{3713}-3′ DNA *nuo* fragment [the numbering refers to Weidner et al. (1993)]: 5′-CTACATATGCACGAGAATCAACAACCAC-3′, which is designed to introduce a *Nde*I recognition site at the 5′ end of *nuoE* (the underlined residues are modified from the *E. coli* DNA), and 5′-GCTTTTTCTCGAGCGCCGCCTG-3′ (the underlined residues mark the *Xho*I recognition site). The resulting *Nde*I/*Xho*I DNA *nuo* fragment was ligated into *Nde*I/*Xho*I cut pET-24/*nuoD-G*.

For construction of the expression plasmid pET-24/*nuoB-G*, two primers were synthesized to amplify the 5′-^{824}*Nde*I/*Sal*I^{2310}-3′ DNA *nuo* fragment (Weidner et al., 1993). 5′-ATTCATATGGATTATACGCTCACCCGC-3′ was designed to introduce a *Nde*I recognition site at the 5′ end of *nuoB* (the underlined residues are modified from the *E. coli* DNA) and 5′-ACGCAGTCGACAATCTCTTCGCCATCG-3′ with the *Sal*I recognition site (underlined residues). The *Nde*I/*Sal*I fragment was ligated into the *Nde*I/*Sal*I site of pET-24/*nuoD-G*. Both constructs were verified by sequencing.

Expression of NuoE-G and NuoB-G Subunits. Competent *E. coli* cells BL21(DE3) were transformed with pET-24/*nuoE-G* or pET-24/*nuoB-G*, respectively. The resulting transformants were grown in a 10 L culture of LB medium with 100 μg/mL ferric ammonium citrate added in aliquots of 20 μg/mL every hour, 2 μg/mL sodium sulfide, and 20 μg/mL riboflavin. Cells were grown at 37 °C and isopropyl β-D-thiogalactopyranoside (IPTG) was added (final concentration 0.1 mM) at an absorbance of approximately 0.8 at 600 nm. Three hours later, the cells were harvested after entering the stationary growth phase.

Purification of the NADH Dehydrogenase Fragment. Approximately 50 g cells (wet mass) were harvested by centrifugation for 10 min at 4000*g*. Cells were washed with 50 mM MES/NaOH, pH 6.0, and stored at −70 °C. All further steps were carried out at 4 °C. The cells were resuspended in 200 mL 50 mM MES/NaOH, pH 6.0, 50 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride (PMSF) with 10 μg/mL deoxyribunuclease I and disrupted by a single pass through a French pressure cell (SLM Aminco) at 110 MPa. Cell debris and the cytoplasmatic membranes were removed by centrifugation for 1 h at 250000*g*. The supernatant was applied to a 250 mL DEAE-Sepharose (Pharmacia) column equilibrated in isolation buffer using a 2500 mL linear gradient of 50 to 400 mM NaCl in isolation buffer at a flow rate of 240 mL/h. The NADH dehydrogenase fragment eluted between 150 and180 mM NaCl as tested by determination of the NADH/ferricyanide reductase activity (Friedrich et al., 1989). The peak fractions were pooled and subjected to ammonium sulfate fractionation. The precipitate between 40 and 60% ammonium sulfate saturation was dissolved in 50 mM MES/NaOH, pH 6.0, 1 mM PMSF, and 800 mM ammonium sulfate and pumped onto a 15 mL Phenyl-Sepharose High Performance column (Pharmacia) equilibrated in the same buffer. In a 150 mL linear gradient of 800 to 0 mM ammonium sulfate in the above buffer at a flow rate of 180 mL/h, fractions containing NADH/ferricyanide reductase activity eluted between 300 and 250 mM ammonium sulfate. They were combined, concentrated to 500 μL by ultrafiltration (Centripep 100, Amicon), and applied to size-exclusion chromatography on a 25 mL Superdex 200 HR column (Pharmacia) in 50 mM MES/NaOH, pH 6.0, 50 mM NaCl, and 1 mM PMSF at a flow rate of 18 mL/h. For Stokes radius determination, the elution volume of the NADH/ferricyanide reductase activity was compared with that of thyroglobin, ferritin, catalase, aldolase, and γ-globulin as calibration standards.

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. To quantitate the concentration of individual FeS clusters, spectra were recorded under nonpower saturated conditions. These spectra were fitted to a linear combination of the simulated spectra of individual FeS clusters. The relative contribution of an individual cluster to the spectrum was used as its relative spin concentration (Wang et al., 1991; Leif et al., 1995). The spin concentration of all FeS clusters were determined under nonpower saturated conditions using 1 mM Cu-EDTA as standard. The transition-probability corrections were carried out according to Aasa and Vänngård (1975).

Other Analytical Procedures. UV−visible absorption spectra were recorded on a Lambda-2 spectrometer (Perkin-Elmer) at room temperature. Measurement of kinetic parameters were performed as described elsewhere (Friedrich et al., 1989). The protein concentration was measured by

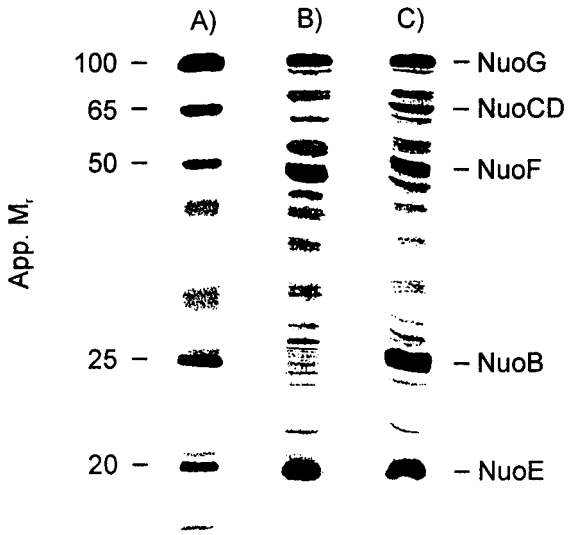

FIGURE 1: Coomassie stained SDS−PAGE of (A) isolated complex I; (B) cytoplasm of cells overproducing NuoE, F and G; (C) cell lysates of cells overproducing NuoB, C, D, E, F, and G. The apparent molecular masses of the Nuo subunits are shown on the left and their designation on the right.

either modified Biuret or Lowry methods. For routine assays, the protein concentration of the NADH dehydrogenase fragment was spectroscopically determined using an extinction coefficient of $\epsilon_{280nm} = 190\ mM^{-1}\ cm^{-1}$ derived from the sequence. Non-heme iron content was determined according to Fish (1988), and the flavin content was determined according to Koziol (1971).

RESULTS

Overproduction of the Subunits NuoE, F, and G and NuoB, C, D, E, F, and G. The growth rates of *E. coli* cultures containing the expression plasmids pET-24/*E*−*G* and pET-24/*B*−*G* decreased significantly after induction by IPTG. SDS−PAGE of total cell lysates from IPTG-treated *E. coli* strain hosting plasmid pET-24/*E*−*G* that harbors the genes *nuoE*, *F*, and *G* showed the overproduced subunits NuoE, F, and G (Figure 1). All three subunits are located in the cytoplasm. However, the cytoplasm did not exhibit enhanced NADH/ferricyanide reductase activity compared to wild-type cells. EPR signals typical for the FeS clusters of complex I could not be detected in the cytoplasm (data not shown). From these results we conclude that NuoE, F, and G are produced, but neither equipped with their cofactors nor properly assembled.

SDS−PAGE of cell lysates from the strain hosting the plasmid pET-24/*B*−*G* that harbors the genes *nuoB*, *C*, *D*, *E*, *F*, and *G* reveals the production of five proteins (Figure 1). NuoB was found in inclusion bodies in the sediment of a centrifugation at 14000*g*. NuoC and D were not detected but a overproduced polypeptide with an apparent molecular mass in SDS of 65 kDa was present in the cytoplasm as well as in inclusion bodies. The apparent molecular mass of this protein matches the sum of the masses of NuoC and D as derived from their DNA sequences. A protein with the same apparent molecular mass was also found in preparations of the entire complex I and had been assigned to subunit NuoD by an internal amino acid sequence (Leif et al., 1995). We will call this subunit NuoCD. The EPR signals of the FeS clusters expected to be present in the

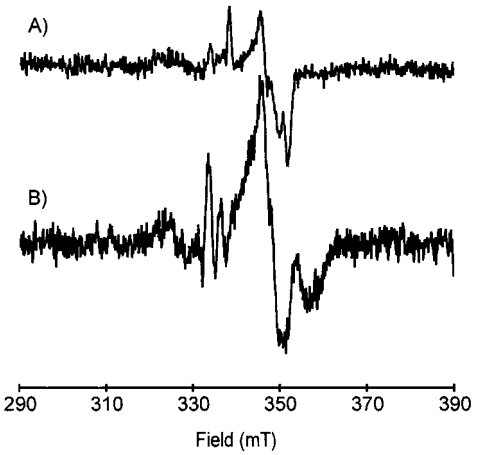

FIGURE 2: EPR spectra of the cytoplasm of *E. coli* cells overproducing NuoB-G. The spectra are difference spectra obtained from samples reduced with 2 mM NADH minus air-oxidized samples. (A) Sample temperature 40 K and microwave power 2 mW; (B) sample temperature 13 K and microwave power 10 mW. Other EPR conditions were: microwave frequency, 9.44 GHz; modulation amplitude, 6 mT; time constant, 0.064 s; scan rate, 17.9 mT/min.

NADH dehydrogenase fragment, namely the binuclear FeS clusters N1b and N1c and the tetranuclear clusters N3 and N4, were detected in the cytoplasm when reduced by NADH (Figure 2). This indicates the functional assembly of the three FeS polypeptides.

The production of the NADH dehydrogenase fragment was increased by 60% by addition of riboflavin, ferric ammonium citrate, and sodium sulfide to the medium. The addition of either riboflavin or ferric ammonium citrate and sodium sulfide enhanced the production of the fragment only about 30%. This suggests that the incorporation of flavin and the FeS clusters into the protein is a highly cooperative process which promotes the correct folding of the protein. The same observation has been made in the overexpression of the homologues of NuoE and F of *P. denitrificans* (Yano et al., 1996).

Isolation of the NADH Dehydrogenase Fragment. Isolation to homogeneity of the NADH dehydrogenase fragment was achieved by ion-exchange chromatography on DEAE-Sepharose, ammonium sulfate fractionation, hydrophobic-interaction chromatography on Phenyl-Sepharose, and size-exclusion chromatography on Superdex 200. The fragment eluted from the DEAE-Sepharose at 150 mM NaCl, precipitated between 40 and 60% ammonium sulfate concentration, and eluted from the Phenyl-Sepharose at 300 mM ammonium sulfate (Figure 3). Chromatography on Superdex 200 resulted in two peaks of NADH/ferricyanide reductase activity corresponding to proteins with a molecular mass of 320 000 and 160 000 Da, respectively. These values match the molecular masses derived from the DNA sequence for a dimeric and a monomeric form of the NADH dehydrogenase fragment. Both peaks show the same protein pattern in SDS−PAGE and have the same specific NADH/ferricyanide reductase activity of 120 units/mg. The monomer and dimer could not be converted to each other by size-exclusion rechromatography, indicating that both forms are produced separately by the cell. Routinely, 40 mg fragments, 95% pure as judged by SDS−PAGE, were obtained from 40 g cells (Table 1).

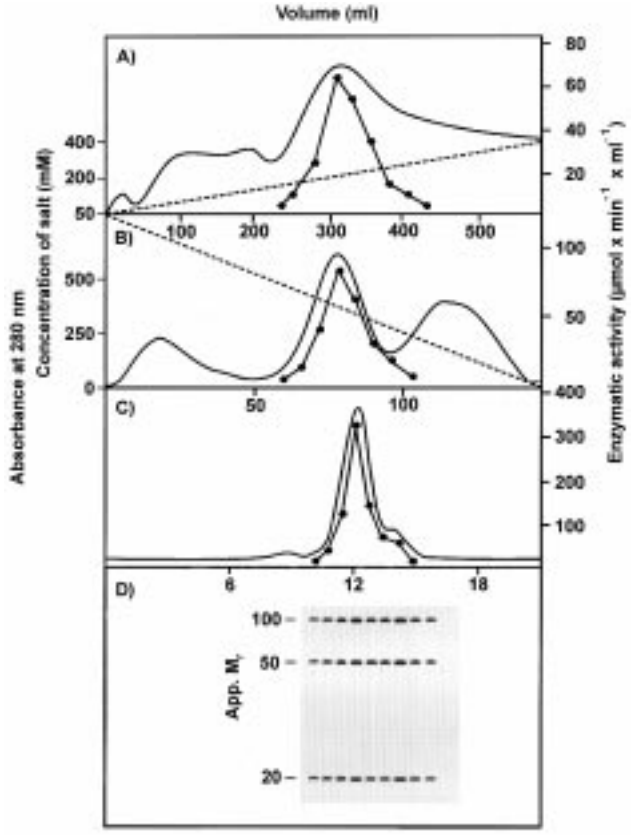

FIGURE 3: Isolation of the overproduced NADH dehydrogenase fragment of *E. coli*. (A) Chromatography on DEAE-Sepharose; (B) chromatography on Phenyl-Sepharose; (C) size-exclusion chromatography on Superdex-200; (D) SDS−PAGE of the fractions with NADH/ferricyanide reductase activity; (—) absorbance at 280 nm; (●) NADH/ferricyanide reductase activity; (--) used gradients. The numbers in panel D refer to apparent molecular masses × 10^{-3} Da.

Internal Redox Groups. The specific FMN contents of four independent preparations varied from 5.8 to 6.9 nmol/mg, and specific iron contents from 100 to 115 nmol/mg. Use of the calculated protein molecular mass of 160 kDa gives 0.9−1.1 mol of FMN and 16−18 mol of iron/mol of NADH dehydrogenase fragment.

The UV−visible spectrum of the air oxidized NADH dehydrogenase fragment shows the broad absorption band of the FMN at 450 nm and that of the FeS clusters at 330 and 410 nm (Figure 4). These absorbances are bleached by reduction with dithionite (Figure 4A). The UV−visible spectrum of the isolated complex I at the same molarity shows a more intense absorption at 410 and 375 nm while the absorbances at 450 and 550 nm are roughly comparable (Figure 4B). The absorbances at 410 and 375 nm are predominantly caused by the tetranuclear FeS clusters (Palmer, 1973; Ragan et al., 1982). This indicates a higher amount of tetranuclear FeS clusters in complex I than in the NADH dehydrogenase fragment, while both contain comparable amounts of binuclear FeS clusters and FMN.

The FeS clusters of the isolated NADH dehydrogenase fragment were studied by EPR spectroscopy. In the NADH-reduced preparation, EPR signals of four clusters (N1b, N1c, N3, and N4) were resolved as intrinsic components (Figure 5), indicating an intact electron pathway in the fragment. The properties of the ESR signals were compared to those of the isolated complex I. The *g* positions of all signals were identical with those obtained from complex I (Figure 5, Leif et al., 1995). While the *g* values and the corresponding line widths of the clusters N1b, N1c, and N3 are identical in both preparations, the g_z signal of cluster N4 is broadend in the NADH dehydrogenase fragment (Figures 5 and 6). Since this is already the case in the spectrum obtained from the NADH-reduced cytoplasm (Figure 2), the isolation process does not alter the spectral properties of the FeS clusters. The spin concentration of the FeS clusters relative to each other were calculated by fitting a linear combination of the simulated spectra of individual FeS clusters to a spectrum recorded under nonsaturating conditions (Figure 6). The relative spin concentration of all FeS clusters is approximately equivalent to that of the flavin, namely FMN:N-1b:N-1c:N-3:N-4 = 1:1.1:1.2:0.9:0.8 (Figure 6).

Electron-Transfer Properties. The K_M values of the NADH dehydrogenase fragment and complex I for several substrates are shown in Table 2. The K_M values for NADH and ferricyanide, characterizing the NADH-reducing site of complex I, are identical for both preparations. Therefore, we conclude that the electron input site of the fragment is identical with that of complex I. On the contrary, the fragment has a substantial lower affinity to UQ-2 and UQ-0 than complex I. The electron transfer from NADH to ubiquinone-2 is insensitive to piericidin A and annonine VI. This shows that the NADH/ubiquinone reductase activity is artificial, and the electron output site of the fragment is different from that of complex I.

DISCUSSION

In this paper, we report the successful overproduction and isolation of the NADH dehydrogenase fragment of the respiratory complex I of *E. coli*. Although the fragment is only made up of the subunits NuoE, F, and G, overexpression of the corresponding genes leads to overproduction of three apoproteins that are not assembled. With simultaneous overexpression of *nuoB*, *C*, and *D* coding for subunits of the connecting fragment, the cofactors are incorporated and the fragment is properly assembled. Since NuoB is found in inclusion bodies and is therefore not available, it is most likely that the soluble fraction of NuoCD acts as a base on which the NADH dehydrogenase fragment is built up.

Unexpectedly, overexpression of the genes *nuoC* and *D* led to the formation of only one protein, NuoCD. The apparent molecular mass of this protein is 68 kDa as determined by SDS−PAGE. This mass corresponds to the sum of the masses of NuoC and D as deduced from their DNA sequence. The TGA stopcodon of *nuoC* is present in the sequence of the *nuo*-operon of Weidner et al. (1993) as well as in the sequence of the *E. coli* genome published by Blattner et al. (1997). Due to a mismatch between both sequences upstream of that stop codon, the TGA is in frame in the *nuo*-sequence but not in Blattner's sequence. If the latter is correct, *nuoC* and *D* are fused and would code for one protein. The genes *nuoC* and *D* are fused to one gene *nuoCD* in *Buchnera aphidicola* (Clark et al., 1997). The homologues of *nuoC* and *D* are also fused to the gene *hycE* in the formate hydrogenlyase of *E. coli* (Videira & Azevedo, 1994; Friedrich & Weiss, 1997). The N-terminal part of HycE [the large subunit of the hydrogenase (Sauter et al., 1992)] is homologous to NuoC, the C-terminal part to NuoD.

Table 1: Isolation of the NADH Dehydrogenase Fragment from 40 g *E. coli* Cells Yielding 100 mL of Cytosol with 4 g of Total Protein

			NADH/ferricyanide reductase activity		
preparation	vol (mL)	protein (mg)	total [μmol min^{-1}]	specific [μmol min^{-1} mg^{-1}]	yield (%)[a]
eluate from DEAE-Sepharose	90	580	9860	17	100
resuspended sediment from ammoniumsulfate precipitation	10	400	8400	21	85
eluate from Phenyl-Sepharose	20	100	6600	66	67
eluate from Superdex 200	3	40	4800	120	49

[a] Due to the presence of other cytoplasmic NADH dependend dehydrogenases, the yield is referred to activity after the first preparation step.

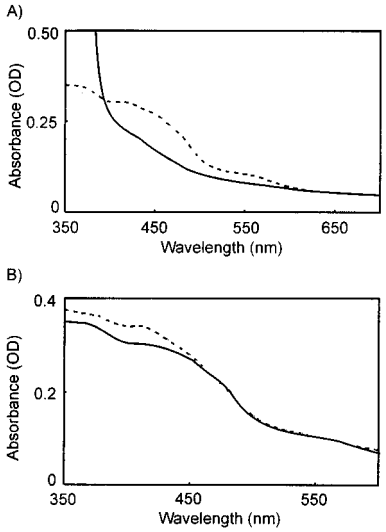

FIGURE 4: UV−visible absorption spectra of complex I and the NADH dehydrogenase fragment of *E. coli*. The fragment was dissolved in 50 mM MES, pH 6.0; 50 mM NaCl at a concentration of 0.1 mg/mL. Complex I was dissolved in the same buffer with the addition of 0.1% laurylmaltoside at 0.35 mg/mL. (A) Spectra of the oxidized (dashed line) and with 10 mM dithionite reduced (solid line) NADH dehydrogenase fragment; (B) Spectra of oxidized NADH dehydrogenase fragment (solid line) and complex I (dashed line) at the same molarity.

Nevertheless, if the *nuo*-sequence of Weidner et al. (1993) was correct, it would be also possible that the TGA codon codes for the natural amino acid selenocysteine (Böck et al., 1991) because the region around this codon shares 48% sequence identity with the conserved DNA region containing the codon for selenocysteine in formate dehydrogenases H and N of *E. coli*. Translation of TGA as selenocysteine would not change the reading frame downstream of *nuoD* but lead to the translation of three more amino acids in NuoCD.

The preparation contains all cofactors that have been detected in the NADH dehydrogenase fragment obtained by splitting of the isolated complex I (Leif et al., 1995). With the exception of the g_z value of cluster N4, the isolated NADH dehydrogenase fragment and complex I show identical EPR-spectroscopic features, indicating a similar local environment of the FeS clusters in both preparations. The broadening of the g_z signal of cluster N4 has also been observed in the NADH dehydrogenase fragment obtained by splitting of the isolated complex I and in preparations of parts of complex I from *N. crassa* lacking cluster N2 (Leif et al., 1995; Wang et al., 1991; Nehls et al., 1992). This points to a close spatial arrangement of the clusters N2 and N4 (Ohnishi, 1993). The cofactors of the preparation are present in the same stoichiometric amounts as in complex I. The high amount of iron in the preparation would allow the

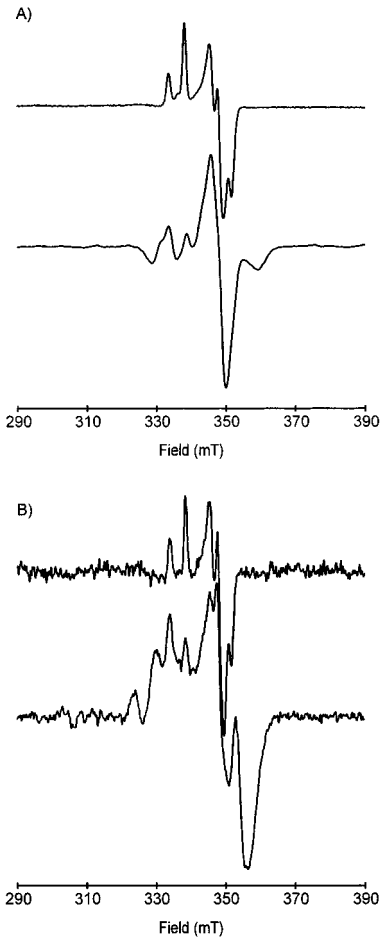

FIGURE 5: EPR spectra of (A) the isolated NADH dehydrogenase fragment and (B) the isolated complex I. The samples were reduced with 2 mM NADH and spectra recorded at 40 K and 2 mW (upper curves) or 13 K and 10 mW (lower curves). Other EPR conditions were the same as in Figure 2.

presence of three binuclear and three tetranuclear FeS clusters in the NADH dehydrogenase fragment. The subunit NuoG contains an atypical but conserved motif for the ligation of an additional tetranuclear FeS cluster (Fearnley & Walker, 1992; Weidner et al., 1993). The binuclear cluster N1c has not been detected in preparations from any other organism. We expect it to be located on NuoG, which contains a typical cysteine motif for binding such a cluster. This motif is also found in the homologous subunits of *Salmonella thyphimurium* and *Th. thermophilus* (Archer et al., 1993; Yano et al., 1997) from which the complex has not been isolated yet.

The groups of Yagi and Ohnishi detected a binuclear FeS cluster on the homologue of NuoE and one binuclear and one tetranuclear cluster on the homologue of NuoG of the *P. denitrificans* complex I by EPR spectroscopic analysis of overexpressed, individual subunits (Yano et al., 1994,

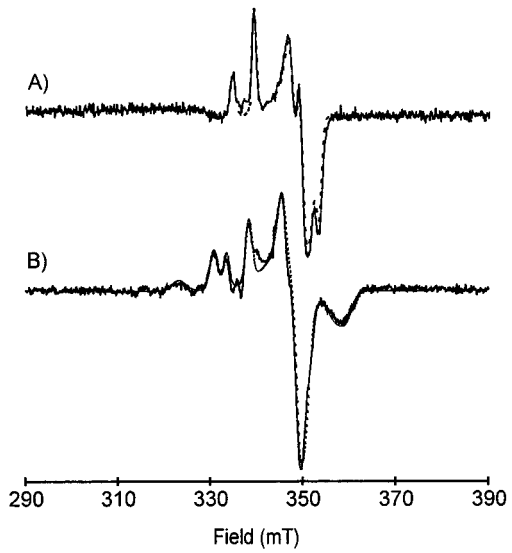

FIGURE 6: EPR spectra of the isolated NADH dehydrogenase fragment reduced with NADH and simulated spectra. EPR spectra were recorded at (A) 40 K and 1 mW microwave power and (B) 13 K and 1 mW microwave power. Other EPR conditions were the same as in Figure 2. The theoretical spectra (dashed line) were calculated using the following simulation parameters: N1b, $g_{x,y,z}$ = 1.93, 1.94, 2.02; $L_{x,y,z}$ = 1.7, 1.2, 1.1 mT; N1c, $g_{x,y,z}$ = 1.92, 1.95, 1.99; $L_{x,y,z}$ = 1.2, 1.55, 0.95 mT; N3, $g_{x,y,z}$ = 1.88, 1.94, 2.044; $L_{x,y,z}$ = 2.8, 1.6, 1.8 mT; N4, $g_{x,y,z}$ = 1.89, 1.94, 2.09; $L_{x,y,z}$ = 2.6, 1.1, 3.0 mT. The relative spin contributions of individual clusters for simulation of the spectrum were (A) N1b/N1c = 0.92:1 and (B) N1b/N1c/N3/N4 = 0.92:1:0.75:0.66.

Table 2: Enzymatic Properties of the Isolated NADH Dehydrogenase Fragment and Complex I of *E. coli*

preparation	K_M^{NADH}	$K_M^{Fe(CN)_6^{3-}}$	K_M^{Q-2}	K_M^{Q-0} (μM)
complex I	5	130	2	250
NADH dehydrogenase fragment	5	130	115	125

1995, 1996). The assignment of these FeS clusters to signals obtained from the entire complex is very difficult, since the EPR spectroscopic properties of the overexpressed FeS-proteins differed considerably from the ones of the entire complex.

The NADH dehydrogenase fragment represents a functional protein module as became clear by revealing its evolutionary relationship to the diaphorase part of a bacterial NAD^+-reducing hydrogenase (Pilkington et al., 1991; Walker, 1992; Friedrich & Weiss, 1996; Friedrich & Weiss, 1997). The NAD^+-reducing hydrogenase from *Alcaligenes eutrophus* is made up of the α, β, γ, and δ subunits. The β/γ dimer is working as a hydrogenase, the α/γ dimer as a diaphorase (Friedrich & Schwartz, 1993). The diaphorase part contains one FMN and several FeS clusters (Tran-Betcke et al., 1990). The N-terminus of the α subunit is homologous to NuoE, its C-terminus to NuoF. The γ subunit is homologous to the N-terminus of NuoG. Homologous hydrogenase genes have been described in *Anabaena variabilis, Desulfovibrio fructosovorans*, and *Synechocystis* sp. PCC6803 (Schmitz et al., 1995; Malki et al., 1995; Appel & Schulz, 1996). However, this homology does not reveal the number and type of the FeS clusters, since the cofactor composition of these enzymes is still under discussion (Schneider et al., 1979, 1984; Erkens et al., 1996). Furthermore, the different EPR spectra and different number of conserved cysteines (Finel, 1993) of the NADH dehydrogenase fragment and the diaphorase part of the hydrogenases points to a divergent evolution after phylogenetic separation (Friedrich & Weiss, 1997).

The NADH-reducing site has not been changed during preparation, and the FeS clusters detectable by EPR spectroscopic are reducible with NADH. This confirms the preservation of the physiological electron pathway in the fragment. Therefore, the water soluble and monodisperse preparation fulfills all prerequisites for crystallization.

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