Overexpression of the *Escherichia coli nuo*-Operon and Isolation of the Overproduced NADH:Ubiquinone Oxidoreductase (Complex I)†

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ABSTRACT: The proton-pumping NADH:ubiquinone oxidoreductase (complex I) of *Escherichia coli* is composed of 13 different subunits. The corresponding genes are organized in the *nuo*-operon (from NADH: ubiquinone oxidoreductase) at min 51 of the *E. coli* chromosome. To study the structure and function of this complex enzyme, a suitable purification protocol yielding sufficient amount of a stable protein is needed. Here, we report the overproduction of complex I in *E. coli* and a novel isolation procedure of the complex. Overexpression of the *nuo*-operon on the chromosome was achieved by replacing its 5′-promotor region with the phage-T7 RNA polymerase promotor and by expressing the genes with the T7 RNA polymerase coded on an inducible plasmid. It is shown by means of enzymatic activity and EPR spectroscopy of cytoplasmic membranes that complex I is overproduced 4-fold after induction. Complex I was isolated by chromatographic steps performed in the presence of dodecyl maltoside. The preparation comprises all subunits and known cofactors and exhibits a high enzymatic activity and inhibitor sensitivity. Due to its stability over a wide pH range and at very high salt concentrations, this preparation is well suited for structural investigations.

The respiratory chains of most proteobacteria contain two primary NADH dehydrogenases, namely NDH−I and NDH−II (*1*). The NDH−II is also called alternative NADH dehydrogenase and is a single polypeptide enzyme with FAD as the only redox group (*2*, *3*). This enzyme transfers electrons from NADH to ubiquinone without contributing to the membrane potential (*4*). The NDH−I is a proton-pumping NADH:ubiquinone oxidoreductase, also called respiratory complex I. It couples the transfer of two electrons from NADH to ubiquinone with the translocation of four protons across the membrane (*5*−*7*). In general, the bacterial complex I consists of 14 different subunits that add up to a molecular mass of approximately 530 kDa (*8*−*12*). Seven subunits are peripheral proteins including all subunits that bear the known redox groups of complex I, namely one FMN and up to nine iron−sulfur (FeS)[1] clusters (*13*, *14*). The remaining seven subunits are very hydrophobic proteins and are predicted to fold into 54 α-helices across the membrane. Nothing is known about their function, but they are most likely to be involved in ubiquinone reduction and proton translocation (*14*).

The genes of the *Escherichia coli* complex I are organized in the so-called *nuo*-operon (from NADH:ubiquinone oxidoreductase), a 16 kb DNA region localized at min 51 of the chromosome (*8*). In *E. coli*, the genes *nuoC* and *D* are fused to form one gene *nuoCD* leading to a complex consisting of 13 subunits (*15*, *16*). This complex has been isolated in the presence of the technical alkylglucoside detergent APG (*10*). The preparation is solely made up of the subunits encoded by the *nuo*-operon and contains one noncovalently bound FMN, two binuclear (N1b and N1c), and three tetranuclear (N2, N3, and N4) EPR detectable FeS clusters (*10*, *17*). This preparation is only stable in the pH range from 6.0 to 6.5 and at salt concentrations below 250 mM (*10*).

Here, we report the overproduction of the *E. coli* complex I and a novel isolation procedure yielding sufficient quantities of a stable complex I, which is a prerequisite for further characterization of this enzyme by biochemical and biophysical techniques.

MATERIALS AND METHODS

Materials and Strains. The *E. coli* B wild-type, the *E. coli* strains AN387 (*18*) and JC7623 (*19*), and the plasmids pAR-1219 (*20*), pHP-45 Ω (*21*), pMAK-705 (*22*), pT7T3−18U (Pharmacia, Freiburg), pET-11a (*23*; ASG Heidelberg), pUM-24 (*24*), and pYB-3 (*25*) were used. When required for maintenance of plasmids, spectinomycin was added to 20 μg/mL, kanamycin to 50 μg/mL, chloramphenicol to 20 μg/mL, and ampicillin to 100 μg/mL. All enzymes used for recombinant DNA techniques were from Pharmacia (Freiburg) or Boehringer-Mannheim (Mannheim). [α-^{32}P]dATP (600 mCi/mmol) was obtained from Amersham Buchler (Braun-

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[1] Abbreviations: APG 225, a technical alkyl glucoside of the general formula $C_nH_{2n+1}O(C_6H_{10}O_5)_xH$ with $n = 8$, 10 and $x = 1$, 2; dodecyl maltoside, *n*-dodecyl-β-D-maltopyranoside; FeS cluster, iron−sulfur cluster; EPR, electron paramagnetic resonance; IPTG, isopropyl-β-D-thiogalactopyranoside; mT, millitesla.

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schweig). All other 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 (*26*). DNA-sequencing was performed according to ref *27* using the T7-sequencing kit (Pharmacia, Freiburg). The following oligonucleotides were used as PCR primers in which the mutated nucleotides are underlined: *nuoANde*I, 5′-TAACATATGAGTATGTCAA-CATCCAC-3′; (positions +91 to +110 of the *nuo*-promotor, numbered according to ref *8*, *nuoBBam*HI, 5′-GAAGGATC-CCTTTGCCGACAGGCGC-3′ (complementary to positions 1232−1213 according to ref *8*). The construction of the promotor replacement vector, pVS-5, is described in the legend of Figure 1. Positions −560 to −1 of the *nuo*-operon including all known promotor sites (*28*) were replaced using vector pVS-5.

Genomic Replacement. Strain JC7623 was used to construct gene replacement by the method of Oden et al. (*29*) with pVS-5. Selection was made for the spectinomycin-resistance. The resulting strain was designated JCN003 and tested for the absence of vector encoded chloramphenicol-resistance. Strain ANN003 was constructed from AN387 (*18*) by P1 transduction (*30*). P1 lysate was prepared from strain JCN003. Transductants were selected by spectinomycin-resistance. Genomic DNA from cells was isolated according to ref *31* and characterized by Southern analysis (*32*).

Expression of the nuo-Operon and Overproduction of Complex I. For expression of the *nuo*-operon under control of the inducible T7Φ10-promotor, the strain ANN003 was transformed with the plasmid pAR1219 (*20*). The plasmid pAR1219 provides the genes for expression of T7 RNA polymerase. It consists of the gene I protein of phage T7 under the control of the inducible *lac* UV5 promotor. The transformants were called ANN003/pAR1219. They were grown in a 10 L culture of LB medium with 10 mg/L ferric ammonium citrate, 2 mg/L sodium sulfide, and 10 mg/L riboflavin. Cells were grown at 37 °C and isopropyl-β-D-thiogalactopyranoside (IPTG) was added (final concentration 0.1 mM) at an approximate OD_{600} of 0.8. Three hours after induction, 40−60 g of cells was harvested by centrifugation for 10 min at 4000*g*. The cells were washed with 50 mM Mes/NaOH, pH 6.0, and stored at −70 °C. *E. coli* B-strain and the K-12 derivatives AN387 and ANN003 were grown accordingly in LB medium without addition of IPTG.

Isolation of Complex I. All steps were carried out at 4 °C. A total of 100 g of cells was resuspended in 200 mL of 50 mM Mes/NaOH, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), pH 6.0, with 10 μg/mL DNAse I and disrupted by a single pass through a French Pressure cell (SLM Aminco) at 110 MPa. Cell debris was removed by centrifugation for 25 min at 14 000*g*, and cytoplasmic membranes were obtained by centrifugation for 1 h at 250 000*g*. The membranes were resuspended in 50 mM Mes/NaOH, pH 6.0, and 1.8 M NaBr to a concentration of 80 mg/mL and sedimented as described above. They were washed free of NaBr by resuspension in 50 mM Mes/NaOH, pH 6.0, followed by centrifugation as above. Membrane pellets were resuspended in the same buffer at a concentration of 80 mg/mL. Dodecyl maltoside was added to a final concentration of 3%, and the solution was gently homogenized and centrifuged for 1 h at 250 000*g*. The supernatant was diluted 2-fold and applied

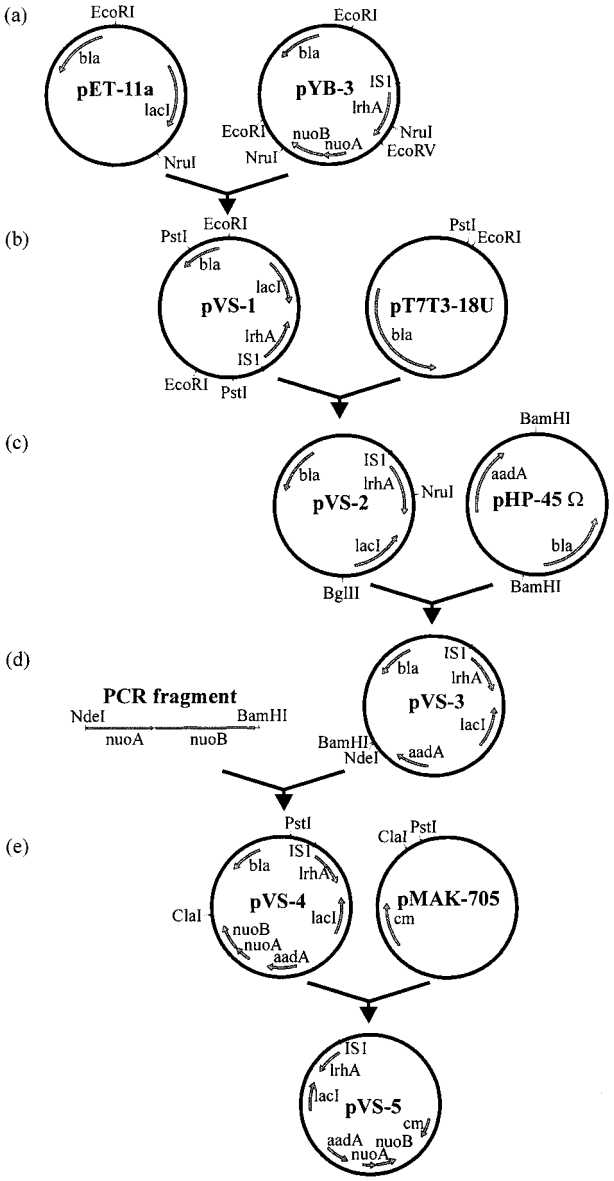

FIGURE 1: Scheme for the construction of the expression vector pVS-5. (a) A 2.4 kb *Eco*RI−*Eco*RV fragment containing the 5′ flanking region of the *nuo*-operon was excised from pYB-3 treated with Klenow fragment and was cloned into the *Nru*I site of pET-11a. The resulting vector was called pVS-1. (b) A 3.9 kb *Eco*RI−*Pst*I fragment containing the 5′ flanking region of the *nuo*-operon, the *lac*I gene and the T7Φ10 promotor was excised from pVS-1 and cloned into the *Eco*RI−*Pst*I sites of pT7T3−18U. The resulting vector was called pVS-2. (c) A 2.1 kb *Bam*HI fragment containing the spectinomycin-resistance mediating *aad*A gene was excised from pHP-45 Ω and cloned into the *Bgl*II site of pVS-2. The resulting vector was called pVS-3. (d) The genes *nuoA* and *nuoB* were amplified by PCR using the oligonucleotides *nuoANde*I and *nuoBBam*HI described in Material and Methods. In this way, a *Nde*I restriction site including the ATG of *nuoA* was created. The PCR-product was digested with *Bam*HI and *Nde*I, and the resulting 1.1 kb fragment was ligated with pVS-3 which had been digested with *Bam*HI and *Nde*I. The resulting vector was called pVS-4. (e) A 7.1 kb *Cla*I−*Pst*I fragment containing *aad*A, *lac*I, the T7Φ10-promotor, and the flanking regions of the *nuo*-operon of this plasmid was cloned into *Cla*I and *Pst*I digested pMAK-705. The resulting vector was called pVS-5. The T7Φ10-promotor and the flanking DNA of this final construct were verified by sequencing. The following abbreviations are used: bla, β-lactamase gene; lacI, lac repressor; IS1, insertion sequence IS1; lrhA, LysR homologue A gene; aadA, aminoglycoside-3′-adenyltransferase gene; cm, chloramphenicol acetyltransferase gene.

to a 30 mL Source 15Q (Pharmacia Biotech) column equilibrated in 50 mM Mes/NaOH, 50 mM NaCl, and 0.15% dodecyl maltoside, pH 6.0, at a flow rate of 90 mL/h. The column was washed with 30 mL of the same buffer and eluted with a 280 mL linear gradient of 50−300 mM NaCl in 50 mM Mes/NaOH, 0.15% dodecyl maltoside, and 0.1 mM PMSF, pH 6.0. Fractions containing NADH/ferricyanide reductase activity were combined, concentrated by precipitation with 9% (w/v; final concentration) poly(ethylene glycol) 4000 and dissolved in 3 mL of buffer. The concentrated protein was subjected to size-exclusion chromatography on a 180 mL Ultrogel AcA 34 (BioSepra, Serva) column in 50 mM Mes/NaOH, 50 mM NaCl, 0.15% dodecyl maltoside, and 0.1 mM PMSF, pH 6.0, at a flow rate of 7 mL/h. Peak fractions were combined and applied to a 1 mL MonoQ (Pharmacia) FPLC-column. The column was washed with 1 mL 50 mM Mes/NaOH and 0.15% dodecyl maltoside, pH 6.0, and eluted with a 20 mL linear gradient of 50 to 350 mM NaCl in the same buffer at a flow rate of 12 mL/h. The combined peak fractions with NADH/ferricyanide reductase activity were pooled and stored without any further treatment at −70 °C. Routinely, the protein was stored at 5 mg/mL. Under these conditions, the preparation was stable for at least 1 month.

Other Analytical Procedures. The molecular weight of the isolated complex I was estimated by gel filtration on a TSK Fractogel G 4000 column (TosoHaas) calibrated with conalbumin (81 kDa), γ-globulin (160 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) in 50 mM Mes/NaOH, 50 mM NaCl and 0.15% dodecyl maltoside, pH 6.0. The void volume was determined with Dextran Blue. Sucrose-gradient centrifugation in the presence of 0.2% dodecyl maltoside was performed as described (*10*). SDS−PAGE was performed in 16% gels according to ref *33*. Reconstitution of the complex into artificial membranes from soy bean lipids and measurement of kinetic parameters were performed according to ref *34*. The protein concentration was measured either by modified Biuret or Lowry methods. For routine assays, the protein concentration of complex I was spectroscopically determined using an extinction coefficient of $\epsilon_{280nm} = 764\ mM^{-1}\ cm^{-1}$ derived from the sequence. Nonheme iron content was determined according to ref *35*, and the flavin content was determined according to ref *36*. For sequencing, complex I was resolved by SDS−PAGE, and individual subunits were transferred onto a poly(vinylidene difluoride) membrane (*37*). Protein sequences were determined by Dr. E. Wachter (University of Munich). 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 following redox mediators were used: indigotetrasulfonate, indigodisulfonate, 2-hydroxy-1,4-naphthoquinone, Safranine T, Neutral Red (all 50 μM), methyl viologen and benzyl viologen (both 40 μM).

RESULTS

Overproduction of Complex I. To increase the amount of complex I in the cytoplasmic membrane, we overexpressed the *nuo*-operon on the chromosome by replacing its promotor region with a phage promotor. The *E. coli* strain ANN003 was derived from the K12-derivative strain AN387 and contains the phage T7Φ10-promotor instead of the *nuo*-operon promotor region but no phage T7-RNA polymerase. Strain ANN003/pAR1219 harbors the gene for the phage RNA polymerase on the inducible plasmid pAR1219. The amount of complex I in the cytoplasmic membranes of these strains as well as from the B-type strain was estimated from the specific NADH and deamino-NADH/ferricyanide reductase activity (Table 1). While the NADH/ferricyanide activity of the membranes reflects the amount of both NADH dehydrogenases, the major portion of the deamino-NADH/ferricyanide activity stems from complex I (*38*, *39*). In general, the B-strain shows a higher specific activity with both substrates than the K-strains. Compared to the parental strain AN387, the specific d-NADH/ferricyanide acitivity of strain ANN003 is significantly decreased. When the gene for the phage RNA polymerase is provided on the inducible plasmid pAR1219, the activity is increased four times compared to the parental strain AN387, but only after induction with IPTG (Table 1). Thus, the expression of the *nuo*-operon is under the control of the T7Φ10-promotor.

Table 1: Specific NADH/Ferricyanide and d-NADH/Ferricyanide Reductase Activity of Cytoplasmic Membranes of various *E. coli* Strains

strain	specific activity [μmol of NADH/min mg protein]	
	NADH/ferricyanide	d-NADH/ferricyanide
B-strain	2.1	1.7
AN387	1.5	1.2
ANN003	1.2	0.3
ANN003/pAR1219 (without IPTG)	1.3	0.5
ANN003/pAR1219 (with IPTG)	5.9	4.7

The sedimentation rate of the protein fraction showing NADH/ferricyanide activity in the sucrose gradient centrifugation corresponds to an approximately 550 000 M_r protein which is characteristic for the *E. coli* complex I (*10*). Dodecyl maltoside extracts of cytoplasmic membranes of strains AN387 and ANN003/pAR1219 grown in the presence of IPTG were subjected to sucrose gradient centrifugation (Figure 2). Roughly the same amount of protein was loaded on both gradients. The NADH/ferricyanide reductase activity sedimenting with complex I is approximately four times higher in the extract obtained from strain ANN003/pAR1219 than from strain AN387.

The increased complex I content in the cytoplasmic membranes of strain ANN003/pAR1219 was also detected by EPR-spectroscopy (Figure 3). Difference spectra of NADH plus succinate-reduced membranes minus succinate-reduced membranes show the known signals of the EPR-detectable binuclear FeS clusters N1b and N1c at 40 K (Figure 3A) and of the tetranuclear FeS clusters N2, N3, and N4 at 13 K (Figure 3B). The spectra obtained with the membranes from the strains AN387 and ANN003/pAR1219 are corrected for protein concentration, gain, and tube thickness. Due to the overlap with signals of the FeS clusters of other enzymes, quantification of the EPR signals by double-integration was not possible. Therefore, the relative amount of the individual FeS clusters in the wild-type and the strain overproducing complex I was estimated by their signal amplitudes. With this method, a 4-fold increase of

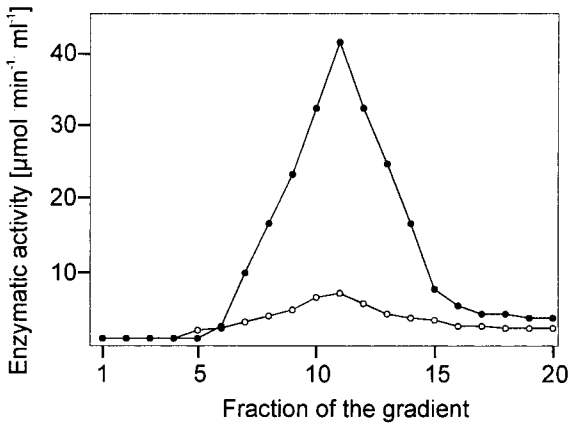

FIGURE 2: Sucrose gradient centrifugation of dodecyl maltoside-solubilized cytoplasmic membranes of *E. coli* strains AN387 (○) and ANN003/pAR1219 (●). Proteins were extracted with 3% dodecyl maltoside (w/v) and separated by means of gradients of 5−20% (w/v) sucrose in 50 mM Mes/NaOH, pH 6.0, 50 mM NaCl and 0.2% dodecyl maltoside. Each gradient was loaded with a total of 13 mg of protein. Fraction 1 refers to the top of the gradient and fraction 20 to the bottom of the gradient.

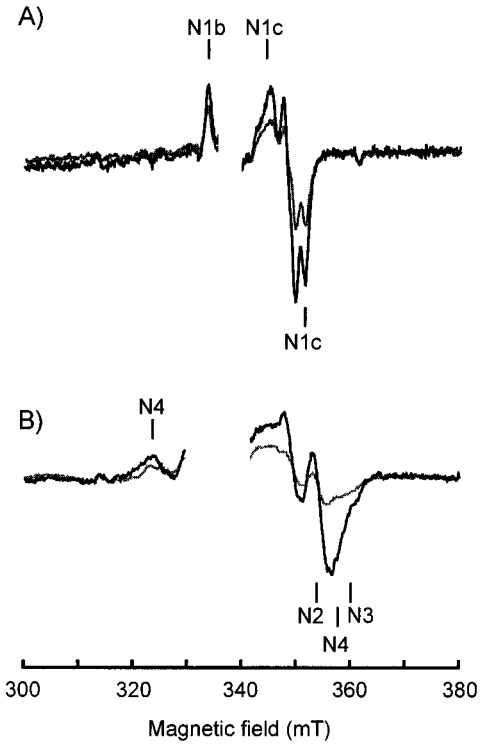

FIGURE 3: EPR spectra of iron−sulfur clusters of cytoplasmic membranes of strains AN387 (grey) and ANN003/pAR1219 (black). Difference spectra were obtained from samples reduced with 2 mM NADH plus 5 mM succinate minus samples reduced with 5 mM succinate both in the presence of redox mediators in order to completely reduce the succinate dehydrogenase in both samples. The signals of the FeS clusters N1b, N1c, N2, N3, and N4 are indicated. (A) sample temperature 40 K and microwave power 5 mW; (B) sample temperature 13 K and microwave power 20 mW. Other EPR conditions were microwave frequency, 9.44 GHz; modulation amplitude, 0.6 mT; time constant, 0.064 s; scan rate, 17.9 mT/min.

the amount of the complex I FeS clusters in the membranes of strain ANN003/pAR1219 after induction with IPTG was determined.

Stability of the Overproduced Complex I. The stability of the overproduced complex I in dodecyl maltoside was tested as described (*10*). Cytoplasmic membranes were adjusted to distinct salt and pH conditions and incubated for 15 min at 4 °C. The membrane suspension was mixed with dodecyl maltoside to a final concentration of 3%, and the extracted proteins were separated by sucrose gradient centrifugation in the corresponding buffer. The intact complex I sediments as an approximately 550 kDa protein as judged by the NADH/ferricyanide activity (Figure 2). Disintegration of the complex can be assayed by the formation of a so-called NADH dehydrogenase fragment which sediments as a 200 kDa protein (*10*, *15*). In contrast to the enzyme in APG, the overproduced complex I in dodecyl maltoside was stable at a pH range 5.0−7.5 and up to 1 M NaCl. Applying the same procedure to membranes of the B-strain, the complex is stable in the same pH range, but tends to disintegrate at NaCl concentrations higher than 250 mM.

Isolation of Complex I. Complex I was isolated from strain ANN003/pAR1219 grown in the presence of IPTG. The enzyme eluted in Source 15Q anion-exchange chromatography between 200 and 230 mM NaCl and from the MonoQ column between 190 and 210 mM NaCl (Figure 4). All steps were performed in the presence of 0.15% dodecyl maltoside at pH 6.0. A total of 8−12 mg of approximately 90% pure complex I (as judged from SDS−PAGE) was obtained from 100 g of cells (see Table 2). The same isolation procedure was performed with the B-strain yielding 1−3 mg of protein of comparable purity. The complex from both strains showed nearly identical chromatographic properties.

Subunit Composition, Cofactor Content, and Catalytic Properties. Native molecular weight was estimated by gel filtration using a TSK Fractogel G 4000 column. Although size-exclusion chromatography does not provide the accurate molecular mass of membrane proteins, it gives an indication whether the preparation is in a monomeric, dimeric, or higher aggregation state. The column was calibrated with six soluble proteins in a buffer containing dodecyl maltoside. The elution volume of the isolated complex I was determined in a separate experiment and corresponds to a particle with a molecular mass of 600 kDa. Using the micellar molecular mass of about 50 kDa (*40*), this mass can be corrected for the bound detergent. The calculated molecular mass of 550 kDa fits well with the molecular mass of 535 kDa as derived from the DNA sequence (see below). This value is incompatible with dimeric or oligomeric protein species.

By SDS−PAGE, the preparation was resolved into 13 bands (Figure 4D). The subunit being encoded by *nuoCD* has an apparent molecular mass in SDS of 70 kDa, which is in good agreement with the mass deduced from the sequence of *nuoCD*, namely 68 kDa (*15*). A protein with the same apparent molecular mass was also found in the preparation in APG and had been assigned as NuoD by an internal amino acid sequence (*10*). In *Buchnera aphidicola*, the endosymbiont of aphids, the homologous gene *nuoCD* has also been fused to form one gene (*41*). A proteolytic fragment of NuoG revealed the sequence "ISVHEPRQPQDID", which cannot be matched with the protein sequence derived from *nuoG* as reported by Weidner et al. (*8*). However, this sequence matches NuoG as derived from the sequence of the *E. coli* genome (*42*). The previously determined stop codon of *nuoG* (*8*) results from a frame-shift in DNA sequencing. The molecular mass deduced from the corrected DNA sequence is 100.5 kDa (Figure 4D).

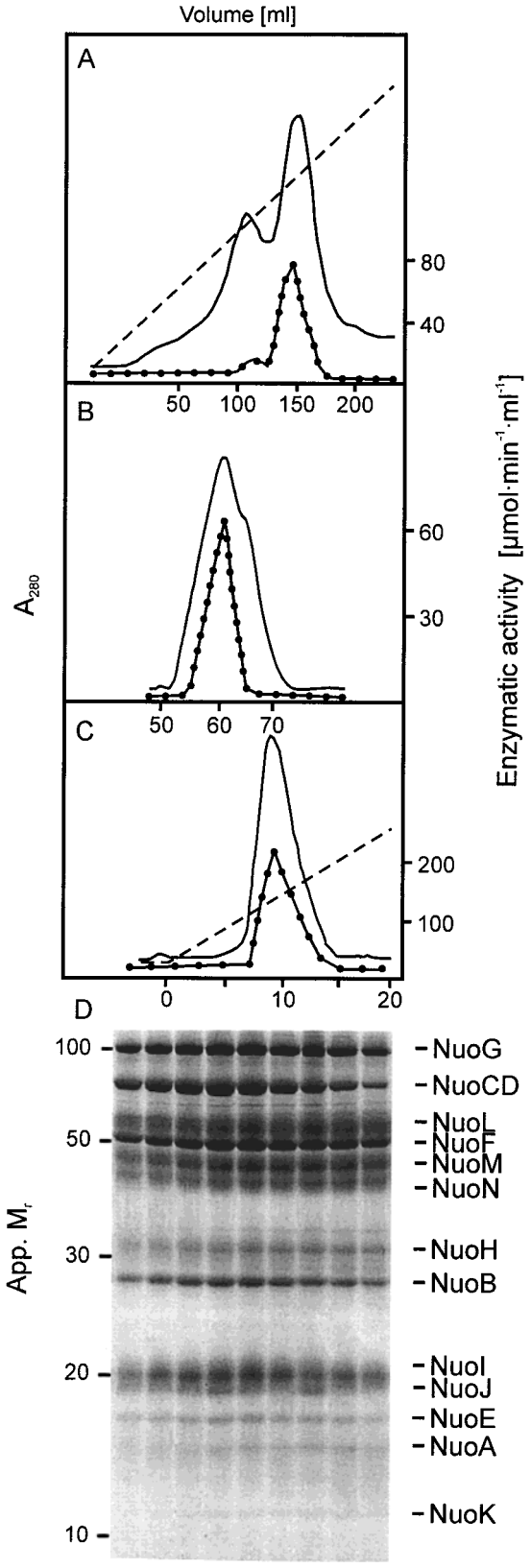

FIGURE 4: Isolation of the *E. coli* complex I from the strain ANN003/pAR1219. Prior to detergent extraction, the membranes have been washed with NaBr. (A) Chromatography on Source 15Q; (B) chromatography on Ultrogel AcA 34; (C) chromatography on Mono Q; (—) absorbance at 280 nm; (●) NADH/ferricyanide reductase activity; (- - -) NaCl gradient. (D) SDS/PAGE of the fractions of the Mono Q chromatography with NADH/ferricyanide activity (fractions 8−16). The numbers refer to the apparent (app.) molecular masses × 10^{-3} Da. The assignment of the individual subunits to the Nuo-proteins is given on the right.

Table 2: Isolation of *E. coli* Complex I from Strain ANN003/pAR1219[a]

	vol (mL)	protein (mg)	NADH/ferricyanide reductase activity: total (µmol min^{-1})	NADH/ferricyanide reductase activity: specific (µmol min^{-1} mg^{-1})	yield (%)
membranes	60	3710	12 240	3.3	100
membranes after NaBr-treatment	30	1320	4490	3.4	37
extract	24	430	3740	8.7	31
Source 15Q	21	60	1370	24	11
AcA 34	11	14	750	54	6
Mono Q	3	9	610	68	5

[a] The preparation was started from 100 g of wet cells.

The cofactor content of the complex I preparation in dodecyl maltoside is essentially the same as that of the preparation obtained from the B-strain in APG (*10*). The specific FMN contents of three independent preparations varied from 1.65 to 1.85 nmol/mg and specific iron contents from 38 to 45 nmol/mg. Use of the calculated protein molecular mass of 535 kDa gives 0.9−1.0 mol of FMN and 20−23 mol of iron/mol complex I. The EPR spectra of the isolated complex I and the thermodynamic properties of the FeS clusters are identical regardless of the detergent or strain used for isolation (*10* and data not shown).

The complex I preparation in dodecyl maltoside shows K_M values for NADH and ferricyanide of 5 and 130 µM, respectively, characterizing the NADH-binding site of complex I. After reconstitution into phospholipid membranes, the preparation catalyzes the piericidin A-sensitive electron transfer from NADH to decylubiquinone. The V_{max} of this reaction is about 50 s^{-1}, the K_M^{app} value of decylubiquinone is 10 µM, and the K_i of piericidin A is 45 nM. Thus, with the same K_M-values for the substrates, the preparation in dodecyl maltoside shows a 10-fold higher turnover and an 80-fold higher inhibitor sensitivity compared to the preparation in APG (*10*).

DISCUSSION

The progress in complex I research is hampered from the complexity of the enzyme in combination with the lack of structural data. Our previously described preparation of the *E. coli* complex I in the technical detergent APG, a mixture of various alkyl glycosides with a high content of free fatty alcohols (greater than 10%; w/v), readily resolves into three fragments (*10*) and was not well suited for structural investigations. Most likely, this instability stems from impurities in the technical detergent disintegrating the protein structure (*43*). We therefore tried to obtain a sufficient amount of a stable preparation by (i) overproducing complex I and (ii) using 99.8% pure dodecyl maltoside as detergent in the isolation procedure. The first step, aimed at amplifying the amount of complex I, was to replace the promotor region of the *nuo*-operon with the T7Φ10-promotor. The resulting mutant ANN003 is devoid of all known promotor sites of the *nuo*-operon (*28*) and shows a strongly decreased complex I content (Table 1). When the cells are supplied with the phage T7 polymerase on an inducible plasmid, the expression of the *nuo*-operon depends on the addition of IPTG (Table 1). After induction, the amount of complex I is increased

four times as judged by the specific d-NADH/ferricyanide reductase activity of cytoplasmic membranes (Table 1), the specific NADH/ferricyanide reductase activity of the membrane extract after sucrose gradient centrifugation (Figure 2), and the amount of the EPR detectable FeS clusters in the membrane (Figure 3). To our knowledge this is the first report of overproducing a multisubunit membrane complex by overexpression of the correspoding genes on the chromosome.

It has been reported that membraneous multienzyme complexes have been overexpressed more than 30-fold using recombinant plasmids (*44*, *45*). When synthesis of membrane proteins is amplified at least 10-fold, special lipid−protein organelles are induced. These membrane cisternae or vesicle-like structures harbor the overproduced membrane proteins (*44*, *45*). The 4-fold level of amplification of the *nuo*-operon is too low to induce the synthesis of these intracellular organells. The low amplification is most likely due to the features of the T7 promotor. It has been shown that this promotor is one of the strongest known, e.g., 15-fold stronger than the induced *lac* promotor (*46*). However, the transcripts are only poorly translated. The protein yield per transcript obtained by the T7 RNA polymerase is even lower than using the *E. coli* polymerase. The phage polymerase moves faster than the ribosomes, leading to a desynchronization of the translation process (*46*, *47*). The *nuo*-operon consists of 15 020 base pairs (*8*), making it most likely that desynchronization takes places before all 13 genes have been translated. However, all genes of the *nuo*-operon have to be translated for a proper assembly of complex I (*48*; Berger, A., Spehr, V., and Friedrich, T., manuscript in preparation).

Using the overproducing strain and dodecyl maltoside as detergent, the preparation contained ATP synthase, which persisted as a significant contaminant. To remove this enzyme complex, cytoplasmic membranes were washed with the chaotropic agent NaBr as described (*49*). The F_1 part of the ATPase is removed from the F_o part as a result of this treatment. Both parts of the ATPase exhibit different chromatographic properties than complex I. Treatment with NaBr had no effect on the EPR spectroscopic properties of complex I, although more than half of the amount of complex I was lost due to this procedure as judged from the NADH/ferricyanide reductase activity (Table 2). This problem was mostly compensated by the use of the strain ANN003/pAR1219 to overexpress the *nuo*-operon. The yield according to the new preparation protocol was roughly two-thirds of the preparation in APG (*10*). Nevertheless, the preparation in dodecyl maltoside shows a 10-fold higher turnover and an 80-fold higher affinity for the inhibitor piericidin A, which might reflect a larger amount of an active population (*17*). The enzyme activity and inhibitor sensitivity is comparable to preparations of the mitochondrial complex I (*50*, *51*). The complex was stable in a pH range 5.0−7.5 in dodecyl maltoside. It is noteworthy that the preparation in dodecyl maltoside from the B-strain is stable in the same pH range but is less stable at high salt concentrations. The tolerance toward high salt concentrations enabled us to obtain electron micrographs of the preparation of the overproduced complex I in dodecyl maltoside (*52*). The preparations in APG or from the B-strain broke into small pieces, most likely during the staining procedure using 1% (w/v) uranyl acetate (data not shown). Thus, the use of both the strain ANN003/pAR1219 and dodecyl maltoside leads to the isolation of a stable and highly active complex and is the first step toward a structural characterization of a bacterial complex I.

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