Lambda Red-Mediated Mutagenesis and Efficient Large Scale Affinity Purification of the *Escherichia coli* NADH:Ubiquinone Oxidoreductase (Complex I)†

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ABSTRACT: The proton-pumping NADH:ubiquinone oxidoreductase, the respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. The *Escherichia coli* complex I consists of 13 different subunits named NuoA-N (from *N*ADH:*u*biquinone *o*xidoreductase), that are coded by the genes of the *nuo*-operon. Genetic manipulation of the operon is difficult due to its enormous size. The enzymatic activity of variants is obscured by an alternative NADH dehydrogenase, and purification of the variants is hampered by their instability. To overcome these problems the entire *E. coli nuo*-operon was cloned and placed under control of the L-arabinose inducible promoter P_{araBAD}. The exposed N-terminus of subunit NuoF was chosen for engineering the complex with a hexahistidine-tag by λ-Red-mediated recombineering. Overproduction of the complex from this construct in a strain which is devoid of any membrane-bound NADH dehydrogenase led to the assembly of a catalytically active complex causing the entire NADH oxidase activity of the cytoplasmic membranes. After solubilization with dodecyl maltoside the engineered complex binds to a Ni^{2+}-iminodiacetic acid matrix allowing the purification of approximately 11 mg of complex I from 25 g of cells. The preparation is pure and monodisperse and comprises all known subunits and cofactors. It contains more lipids than earlier preparations due to the gentle and fast purification procedure. After reconstitution in proteoliposomes it couples the electron transfer with proton translocation in an inhibitor sensitive manner, thus meeting all prerequisites for structural and functional studies.

The proton-translocating NADH:ubiquinone oxidoreductase (EC 1.6.5.3), also called respiratory complex I,[1] is the main entry point for electrons into the respiratory chains of many bacteria and of most eukaryotes (*1*−*6*). It couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane, thus providing the proton motive force essential for energy-consuming processes (*7*−*9*). Due to its central metabolic function, complex I is of industrial interest as a possible target for insecticides or arcaricides (*10*, *11*). In humans, complex I deficiencies are associated with neurodegenerative diseases such as Parkinson's disease (*12*). Complex I is considered to be one of the largest membrane bound protein complexes; the enzyme in bovine heart mitochondria consists of 45 different subunits adding up to a molecular mass of 1 MDa (*13*). Electron microscopic studies of the eukaryotic and prokaryotic complex revealed its two-part structure with a peripheral arm protruding in the aqueous space and a membrane arm buried in the lipid bilayer (*14*, *15*). The prokaryotic enzyme catalyzes the same reaction as its eucaryotic homologue and harbors the same set of cofactors, but it consists of only 14 different subunits (*2*, *3*, *5*, *16*). The bacterial complex I is therefore regarded to be a structural minimal form of a proton pumping NADH:ubiquinone oxidoreductase and is used as a model for human diseases (*17*). Recently, the structure of the peripheral arm of complex I from *Thermus thermophilus* was solved at 3.3 Å resolution, revealing its subunit arrangement and the putative electron transfer pathway (*18*).

In *Escherichia coli* the complex I genes are organized in the 15 kb *nuo*-operon (from *N*ADH:*u*biquinone *o*xidoreductase) (*19*). As a peculiarity, the genes *nuoC* and *nuoD* are fused, giving rise to a complex I consisting of 13 subunits NuoA-N that add up to a molecular mass of 535 kDa. It harbors a noncovalently bound flavin mononucleotide (FMN) and nine iron−sulfur (Fe/S) clusters as redox cofactors (*6*). The first preparation of a bacterial complex I was obtained from an *E. coli* B wild type strain in the presence of an alkyl glucoside detergent allowing its biochemical characterization (*20*). The complex I production was enhanced approximately 4-fold by replacing the 5′-promoter region of the *nuo*-operon with the phage-T7 RNA polymerase promoter and by expressing the genes with the T7 RNA polymerase coded on an inducible plasmid (*21*). The purification with β-D-dodecyl maltopyranoside (DDM) as detergent was optimized by applying different chromatographic steps (*8*). Other groups

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[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; complex I, proton-pumping NADH:ubiquinone oxidoreductase; DDM, *n*-dodecyl-β-D-maltopyranoside; decyl-ubiquinone, 2,3-dimethoxy-5-methyl-6-decyl-benzoquinone; EPR, electron paramagnetic resonance; FMN, flavin mononucleotide; Fe/S, iron−sulfur; MES, 2-(*N*-morpholino)ethanesulfonic acid; Ni^{2+}-IDA, nickel iminodiacetic acid; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonylfluoride

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have reported similar protocols that yield stable and pure complex I in milligram quantities using *E. coli* grown under oxygen limiting conditions or a strain with a chromosomal inactivation of the alternative NADH dehydrogenase (*22*, *23*).

Here, we present a complex I overproduction system in a strain devoid of any membrane bound NADH dehydrogenase enabling easy site-directed mutagenesis of the operon, the measurement of complex I activities in the membrane, and the purification of the complex by affinity chromatography. This was achieved by the construction of an expression vector carrying the entire *nuo*-operon under the control of the inducible P_{araBAD} promoter. By applying λ-Red (*re*combination *d*efective)-mediated recombineering it is possible to introduce any desired mutation in the episomal encoded *nuo*-genes. With this technique, a hexahistidine coding sequence upstream of *nuoF* was inserted, leading to the production of a stable complex I engineered with a histidine-tag N-terminal on NuoF. This allowed the rapid and efficient purification of *E. coli* complex I. The preparation is pure and has a higher lipid content than other preparations due to the mild and fast purification procedure. It catalyzes the electron transfer coupled with proton translocation in an inhibitor sensitive manner.

MATERIALS AND METHODS

Materials and Strains. E. coli strain ANN0221 is a *nuoB::nptI, ndh::tet* derivative of strain AN387 (*24*) and was generated using a genomic replacement method as described (*21*). In addition, *E. coli* strains ANN003 (*21*) and DH5α Δ*nuo* (*25*) and the plasmids pBAD33 (*26*), pCA24N*nuoF* (*27*), pKD46 (*28*), pUC28 (*29*), and pUM24 (*21*) were used. Ampicillin (100 μg/mL), chloramphenicol (170 μg/mL), kanamycin (50 μg/mL), and tetracycline (12 μg/mL) were supplemented when necessary. All enzymes used for recombinant DNA techniques were from Fermentas (St. Leon-Roth). DNA oligonucleotides were from Operon Biotechnologies (Cologne; Supporting Information, Table S1).

Cloning of the nuo-Operon. Chromosomal DNA of strain ANN003 was isolated (*30*) and digested with *Sca*I. A 27′866 bp fragment consisting of the T7 promoter region and the complete *nuo*-operon was gel purified and cloned into *Sma*I linearized Cosmid III (Roche Diagnostics, Mannheim) giving a construct named ScaCos. To replace the T7 promoter region by P_{araBAD} from pBAD33, *nuoA* and the 5′-region of *nuoB* were PCR amplified from ScaCos using primers *Nsi*I-*nuoA* and *nuoB*rev (Supporting Information, Table S1). The PCR product was cut with *Nsi*I and *Sma*I and subcloned into pUC28 linearized with *Nsi*I and *Sma*I. The construct was named pUC*nuoA-B′*. ScaCos was cut with *Sma*I and the resulting 3′768 bp fragment comprising *nuoB′-F′* was ligated into the *Sma*I site of pUC*nuoA-B′* resulting in pUC*nuoA-F′*. The P_{araBAD} promoter of the arabinose operon and its regulatory gene *araC* were cut from pBAD33 with *Nsi*I and *Pst*I and ligated into pUC*nuoA-F′* linearized with *Nsi*I. The pUC28 vector backbone of the resulting construct pUCPROM-*nuoA-F′* was excised with *Bgl*II and *Nsi*I and replaced with a *Bam*HI/*Nsi*I fragment of pBAD33 comprising the resistance gene and the origin of replication, giving a construct named pBAD*nuoA-E′*. The remaining part of the *nuo*-operon comprising the genes *nuoE′-N* was obtained by cutting ScaCos with *Eco*0109I. The resulting 21′216 bp fragment was blunted by Klenow fill-in and cut with *Sgf*I. This 12′706 bp fragment was inserted into pBAD*nuoA-E′* to obtain pBAD*nuo*. For this purpose pBAD*nuoA-E′* was cut with *Pst*I, and the resulting 3′-overhang was digested with Klenow fragment and the blunt end DNA cut with *Sgf*I. A scheme illustrating the construction of pBAD*nuo* is presented in the Supporting Information (Figure S1).

λ-Red-Mediated Recombineering. Electrocompetent DH5αΔ*nuo*/pKD46 cells were prepared and electroporated as described (*25*). The *nptI-sacRB* cartridge was amplified from pUM24 by PCR with the primer pair *nuoF::nptI-sacRB* (Supporting Information, Table S1). To integrate the cartridge into pBAD*nuo* by λ-Red-mediated recombineering, electrocompetent DH5αΔ*nuo*/pKD46 was mixed with 50 ng of pBAD*nuo* and 500 ng of PCR product. After electroporation recombinants were selected on LB-agar supplemented with kanamycin. Plasmids were isolated from Km^R clones (*31*) and purified by transformation of DH5α and growth on LB-agar supplemented with kanamycin. To exchange *nuoF* with the modified version containing a hexahistidine coding sequence, the *nptI-sacRB* cartridge on pBAD*nuo* was replaced with a PCR product by recombineering. The respective linear dsDNA was amplified from a 1′337 bp *Ava*I fragment of pCA24N*nuoF* by PCR with primer pair His *nuoF*fwd and *nuoF*rev (Supporting Information, Table S1). Electrocompetent DH5αΔ*nuo*/pKD46 was co-transformed with 50 ng of pBAD*nuo*/*nuoF::nptI-sacRB* and 300 ng of PCR product. Recombinants were selected on YP-agar supplemented with chloramphenicol and 10% (w/v) sucrose at 30 °C. Plasmids from Cam^R and Suc^R clones were isolated and screened for the correct integration of the histidine-tag coding sequence by restriction analysis. The mutation was confirmed by DNA sequencing.

Growth of Cells. E. coli ANN0221 cells were transformed with pBAD*nuo* or pBAD*nuo*/His-*nuoF* and grown aerobically as 250 mL cultures in 1 L baffled conical flasks or in a 200 L fermenter. The medium was composed of 0.5% yeast extract, 1.0% peptone, 15 mM Na_3PO_4, 10 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 50 mg/L riboflavin, 30 mg/L ferric ammonium citrate, and 0.5 mM L-cysteine. Cells were grown at 37 °C, and the expression of the *nuo*-operon was induced by addition of 0.2% (w/v) L-arabinose after 1 h. Cells were harvested after entering the stationary growth phase by continuous flow centrifugation at 16000*g*, 4 °C and stored at −80 °C until use.

Preparation of Cytoplasmic Membranes. All steps were carried out at 4 °C. Cells were resuspended in a 5-fold volume of 50 mM MES/NaOH, 0.1 mM PMSF, pH 6.0, with 10 μg/mL DNAseI and 50 μg/mL lysozyme and disrupted by a single pass through a French pressure cell (SLM Aminco) at 110 MPa. Cell debris was removed by centrifugation at 36000*g* for 20 min, and cytoplasmic membranes were obtained by centrifugation at 250000*g* for 1 h. The membranes were resuspended in 50 mM MES/NaOH, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, pH 6.0 at a protein concentration of 60 mg/mL.

Isolation of Complex I. All steps were carried out at 4 °C. DDM (AppliChem) from 20% stock in water was added to the membrane suspension to a final concentration of 3% and the solution was gently homogenized using a glass−Teflon homogenizer. After 15 min incubation on ice, unsolubilized material was removed by centrifugation at 250000*g* for 20

min. The supernatant was applied to a 60 mL Fractogel EMD TMAE Hicap M (Merck) column equilibrated in 50 mM MES/NaOH, 50 mM NaCl and 0.1% DDM, pH 6.0. The column was washed with 35 mL of 150 mM NaCl in 50 mM MES/NaOH, 0.1% DDM, pH 6.0, and bound proteins were eluted with a 75 mL linear gradient of 150−350 mM NaCl in 50 mM MES/NaOH, 0.1% DDM, pH 6.0 at a flow rate of 6 mL/min. Fractions containing NADH/ferricyanide oxidoreductase activity were pooled and adjusted to 20 mM imidazole by adding 500 mM imidazole in 200 mM NaCl, 50 mM MES/NaOH, 0.1% DDM, pH 6.0. The combined fractions were loaded onto a 15 mL ProBond Ni^{2+}-IDA column (Invitrogen) equilibrated in 20 mM imidazole, 200 mM NaCl, 50 mM MES/NaOH, 0.1% DDM, pH 6.0 at a flow rate of 1 mL/min. The column was washed with the same buffer until the absorbance at 280 nm dropped to the initial value. Proteins were eluted with a step gradient from 20 mM to 470 mM imidazole in 200 mM NaCl, 50 mM MES/NaOH, 0.1% DDM, pH 6.0 at a flow rate of 1 mL/min and a step size of 48 mM imidazole per 10 mL. Fractions containing NADH/ferricyanide oxidoreductase activity were combined and washed three times by a 10-fold dilution with 50 mM NaCl, 50 mM MES/NaOH, 0.1% DDM, pH 6.0 and ultrafiltration in a 100 kDa MWCO Amicon Ultra-15 centrifugal filter (Millipore). The protein was stored until use at −80 °C in a concentration of 10 mg/mL.

The homogeneity of the preparation was verified by analytical size exclusion chromatography on a Superose 6 10/300 GL column (GE Healthcare) equilibrated in 50 mM NaCl, 50 mM MES/NaOH, 0.1% DDM, pH 6.0. The column was calibrated with Thyroglobulin (669 kDa), Ferritin (440 kDa), BSA (67 kDa), and RNAse A (13.7 kDa) for the determination of the molecular mass of the preparation.

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 (3 mg/mL) was reconstituted in phospholipids as described (*8*) and reduced by addition of a few grains of dithionite (*20*).

Enzyme Activity. Complex I activity in cytoplasmic membranes was measured either as NADH/ferricyanide oxidoreductase activity or as NADH oxidase activity as described (*32*, *33*). The NADH oxidase activity was blocked by addition of 20 μM piericidin A, a specific complex I inhibitor (*32*). The NADH:decyl-ubiquinone oxidoreductase activity of isolated and reconstituted complex I was measured at 25 °C with a TIDAS-II spectrophotometer (J&M) in 1 mL of 50 mM MES/NaOH, 50 mM NaCl, pH 6.0 in a stirred cuvette. 1 to 100 μM decyl-ubiquinone and 5 μg of complex I were added to the buffer, and the reaction was started after 1 min incubation by the addition of 1 to 200 μM NADH ($\epsilon_{340} = 6.2$ mM^{-1} cm^{-1}). The activity of the reconstituted complex was measured with the preparation (10 mg/mL) mixed 1:1 (w/w) with *E. coli* polar lipid extract (10 mg/mL; Avanti polar lipids) in 50 mM MES/NaOH, 50 mM NaCl, 2% DDM, pH 6.0 after incubation for 2 min on ice. Reconstitution of complex I into proteoliposomes and fluorescence spectroscopic measurement of proton translocation activity were performed as described (*8*).

Table 1: NADH Oxidase and NADH/Ferricyanide Activity of Cytoplasmic Membranes of Various *E. coli* Strains Used in This Study

strain	NADH/ferricyanide oxidoreductase activity [μmol·min^{-1}·mg^{-1}]	NADH oxidase activity [μmol·min^{-1}·mg^{-1}]	inhibition by piericidin A [%]
AN387	1.9 ± 0.2	0.70 ± 0.06	53
ANN0221	0.1 ± 0.1	0.00	0
ANN0221/pBAD*nuo*	5.7 ± 0.3	0.86 ± 0.04	98
ANN0221/pBAD*nuo*/His-*nuoF*	5.0 ± 0.2	0.96 ± 0.01	97

Other Analytical Procedures. Protein concentration was measured by the biuret method using BSA as standard. SDS−PAGE was performed according to Schägger and von Jagow (*34*) using a 10% T, 3% C separating gel. The concentration of isolated complex I was determined by the absorbance at 280 nm using an extinction coefficient of 764 mM^{-1} cm^{-1} for complex I as derived from the DNA sequence. Iron content was determined by a colorimetric assay using ferrozine as indicator according to ref *35*, and the flavin content was determined according to ref *36*. For the quantification of protein bound lipids, 300 μg of complex I was extracted with methanol:chloroform:water (4:2:1) according to ref *37*. The ether phase was washed in the presence of magnesium nitrate, and the amount of phosphorus was determined based on the color formed by the reduction of a phosphomolybdate complex with ascorbic acid (*38*).

RESULTS

Characterization of the Complex I Mutagenesis and Overproduction System. The enzymatic and biochemical characterization of mutations in the *nuo*-operon is hampered by the presence of the alternative NADH dehydrogenase in the *E. coli* cytoplasmic membranes and the instability of most mutants prohibiting their purification in multiple chromatographic steps. To circumvent the problem we created *E. coli* strain ANN0221 lacking complex I and the alternative NADH dehydrogenase by inactivating the *ndh* gene coding for the alternative NADH dehydrogenase by the insertion of a tetracycline resistance cartridge and by inactivating complex I by the insertion of a kanamycin resistance cartridge in *nuoB* (*33*). Strain ANN0221 is a derivative of strain AN387 (*24*). Cytoplasmic membranes of strain ANN0221 were devoid of any NADH dehydrogenase and NADH oxidase activity as expected due to the mutations introduced (Table 1). To obtain an overproduction of complex I and to enable site-directed mutagenesis of the *nuo*-operon, the *E. coli nuo*-genes lacking the promoter region were cloned in a pBAD33 vector (*26*) under control of the L-arabinose inducible promoter of the *araBAD*-operon, yielding a 21.3 kb construct named pBAD*nuo* (Supporting Information, Figure S1). *E. coli* strain ANN0221 was transformed with pBAD*nuo* resulting in an approximate 2−3-fold increase of the NADH/ferricyanide oxidoreductase after induction with arabinose compared to strain AN387 (Table 1). This activity derived exclusively from complex I because the NADH oxidase activity of this strain was fully sensitive to piericidin A (Table 1). This demonstrates that the episomal encoded complex I is overproduced and active in strain ANN0221/pBAD*nuo*. In strain AN387 approximately half

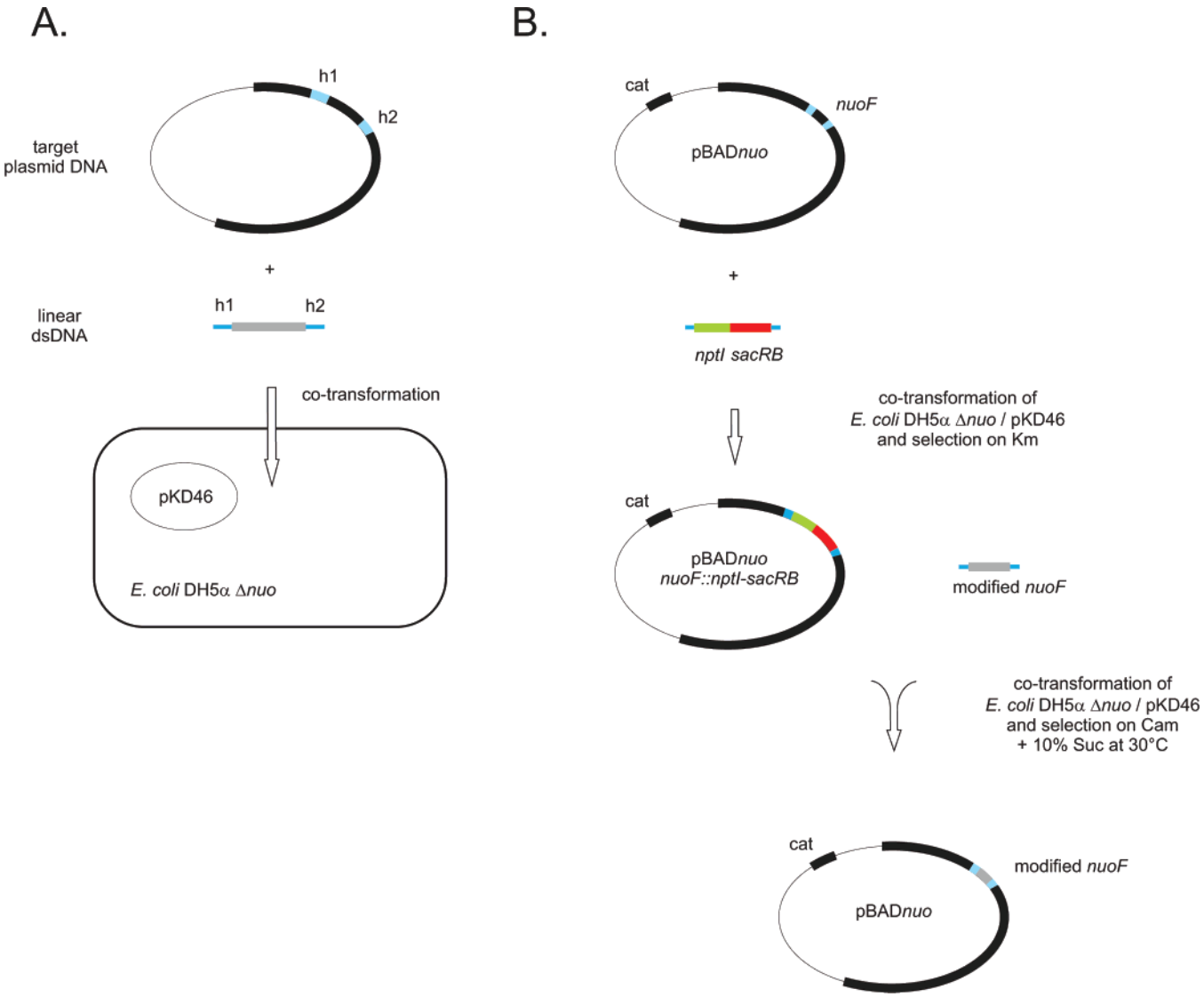

FIGURE 1: Scheme of the method used to introduce mutations in the cloned *nuo*-operon. (A) General approach for Lambda Red-mediated recombineering. *E. coli* DH5α*Δnuo* carrying the plasmid pKD46, which encodes the recombination functions of phage lambda, was co-transformed with plasmid DNA and linear dsDNA, typically a PCR product. "h1" and "h2" denote regions of homology between the targeted plasmid DNA and the linear dsDNA. In DH5α*Δnuo* recombination occurs between the linear dsDNA and the target plasmid DNA mediated by the recombination functions of phage lambda. (B) The *nuo*-operon coding for the subunits of the NADH:ubiquinone oxidoreductase was cloned in a pBAD vector which confers chloramphenicol (Cam) resistance due to the presence of the gene coding for the choramphenicol acetyltransferase (cat). This construct was named pBAD*nuo* and used for mutagenesis and expression of the *nuo*-operon. In a first recombineering event the targeted region of *nuoF* was replaced by the *nptI-sacRB* cartridge. The cartridge was obtained by PCR amplification with primers containing 5′-extensions homologous to *nuoE* and *nuoF*. The gene *nptI* encodes neomycin phosphotransferase which confers kanamycin (Km) resistance and *sacRB* encodes levansucrase leading to a sucrose (Suc) sensitive phenotype. The introduction of the cartridge was verified by selection in the presence of kanamycin. The plasmid pBAD*nuo/nuoF::nptI-sacRB* was isolated and *E. coli* DH5α*Δnuo*/pKD46 was co-transformed with this plasmid and a linear dsDNA containing the mutated version of *nuoF*. The exchange of the *nptI-sacRB* cartridge against the mutated version of *nuoF* was selected in the presence of 10% sucrose. The mutated *nuoF* fragment used for the second recombination event was amplified from a subclone carrying the mutation.

of the activity stems from complex I as judged by the inhibitor sensitivity (Table 1).

Because standard cloning techniques are not suited for the manipulation of large vector constructs like pBAD*nuo*, the λ-Red system was applied to introduce site-directed mutations (Figure 1). The bacteriophage λ encoded recombination functions recombine sequences efficiently with homologies as short as 35 to 50 base pairs enabling the use of PCR products with homology extensions to target pBAD*nuo* (*28*, *39*, *40*). By using a λ-Red system synthesized on an easily curable low copy plasmid the λ-Red functions were transferred to strain DH5α*Δnuo* to abolish unwanted recombination events between the episomal and chromosomal encoded *nuo*-operon (*25*). To enhance the efficiency of recombination a two-step approach employing the *nptI-sacRB* cartridge, which confers kanamycin resistance and sucrose sensitivity, was chosen (*41*, *42*). A first recombineering event with a 50-fold molar excess of linear DNA over targeted plasmid DNA typically resulted in 100−500 Kan^R Suc^S clones. Restriction analysis of the isolated plasmid DNA of these clones revealed that a mixture of modified and unmodified plasmids was present, possibly due to the formation of plasmid multimers (*43*). The modified plasmid was isolated by transformation of *E. coli* DH5α with low amounts (<1

Table 2: Isolation of *E. coli* Complex I from Strain ANN0221/pBAD*nuo*/His-*nuoF* Starting from 25 g of Cells (Wet Weight)

preparation	volume [mL]	protein [mg]	NADH/ferricyanide oxidoreductase activity [μmol min^{-1}] total	specific	yield [%]
membranes	9.5	530	2423	4.6	100
extract	31.0	412	1767	4.3	73
Fractogel EMD	49.0	86	1284	14.9	53
ProBond Ni^{2+}-IDA	23.1	11	1144	104	47

ng DNA/10^8 cells) of this plasmid mixture and selection in the presence of kanamycin. The plasmid DNA of these clones showed solely the restriction pattern of pBAD*nuo* which had integrated the cartridge. The clones were selected and the *nptI-sacRB* cartridge was exchanged with a PCR fragment carrying the mutation by a second recombineering event in the presence of 10% sucrose at 30 °C. About one-third of 20−100 Kan^S Suc^R clones aquired the desired mutation as judged from restriction analysis. This technique was already successfully used to mutate the cysteine residues ligating Fe/S cluster N7 on NuoG to alanine residues (*25*). Here, the cartridge was introduced in *nuoF* and replaced in the second step by the PCR product containing the sequence for a hexahistidine-tag N-terminal on NuoF. The correct introduction of the hexahistidine coding sequence upstream of *nuoF* was confirmed by DNA sequencing. The resulting strain was coined ANN0221/pBAD*nuo*/His-*nuoF*.

Cytoplasmic membranes from strain ANN0221/pBAD*nuo*/His-*nuoF* showed a NADH/ferricyanide oxidoreductase and NADH oxidase activity similar to strain ANN0221/pBAD*nuo* (Table 1). The NADH oxidase activity was fully sensitive to piericidin A, indicating that strain ANN0221/pBAD*nuo*/His-*nuoF* contains an overproduced and functional complex I.

Affinity Purification of E. coli Complex I. Cytoplasmic membranes from 25 g of cells of *E. coli* strain ANN0221/pBAD*nuo*/His-*nuoF* were prepared as described (*8*). Proteins were solubilized from the cytoplasmic membranes (50−60 mg/mL protein) by incubation with 3% (w/v) DDM for 15 min on ice. About three-quarters of the NADH/ferricyanide oxidoreductase activity of the membrane suspension was recovered (Table 2). To remove the excess detergent the clarified extract was applied to a fast anion exchange chromatography on Fractogel EMD. Fractions exhibiting NADH/ferricyanide oxidoreductase activity eluted between 170 and 230 mM NaCl (Figure 2). They were pooled, adjusted to 20 mM imidazole, and loaded onto a Ni^{2+}-IDA column. Unbound material was washed from the column, and minor amounts of unspecifically bound proteins were eluted in the presence of 20−200 mM imidazole. Fractions with NADH/ferricyanide oxidoreductase activity eluted at imidazole concentrations above 350 mM (Figure 2). This method yielded approximately 11 mg of complex I with a specific NADH/ferricyanide oxidoreductase activity of 100 μmol min^{-1} mg^{-1} from 25 g of wet weight of cells. Nearly 50% of the complex in cytoplasmic membranes was recovered by this protocol as judged from the NADH/ferricyanide oxidoreductase activity (Table 2).

SDS−PAGE of the preparation indicated the presence of all complex I subunits (Figure 3). The subunits NuoE and J were not separated as previously reported (*23*). The band with an apparent molecular mass of about 85 kDa corresponds to an proteolytically cleaved fragment of NuoG (*44*). Thus, the engineered complex is fully assembled. No impurities were detected in the Coomassie stained gel loaded with 75 μg of protein (Figure 2). Aliquots of the preparation were subjected to analytical size-exclusion chromatography on a 24 mL Superose 6 column (Figure 2). Protein with NADH/ferricyanide oxidoreductase activity eluted as a single symmetric peak after 11.1 mL corresponding to a molecular

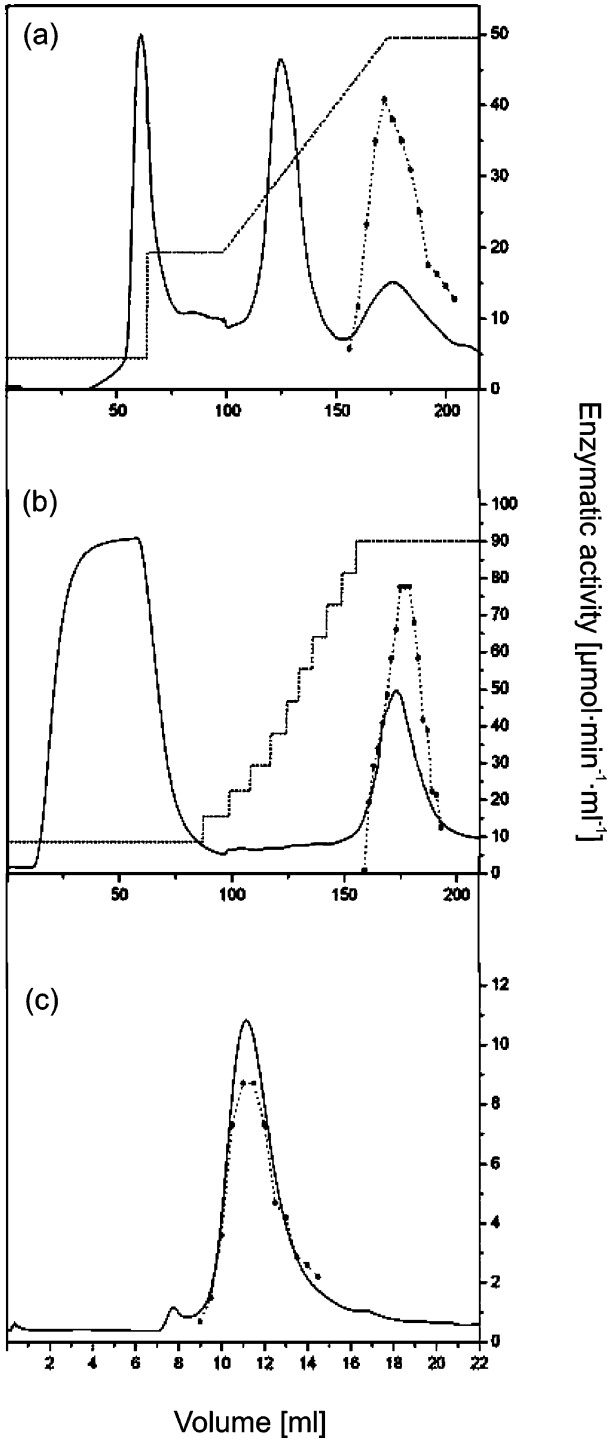

FIGURE 2: Isolation of *E. coli* complex I from strain ANN0221/pBAD*nuo*/His-*nuoF*. Chromatography on Fractogel EMD (a); chromatography on Pro Bond Ni^{2+}-IDA (b); chromatography on Superose 6 (c); absorbance at 280 nm (—); NADH/ferricyanide oxidoreductase activity (●); NaCl or imidazole gradient (- - -).

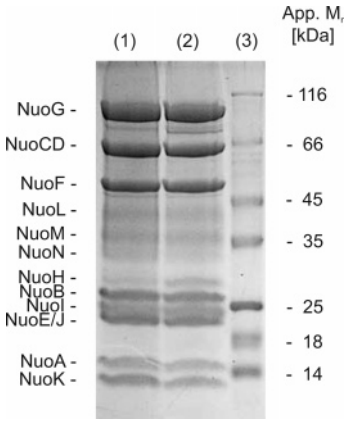

FIGURE 3: SDS−PAGE of the preparation of complex I after chromatography on Pro Bond Ni^{2+}-IDA. The gel was loaded with 75 μg of complex I of the combined fractions corresponding to elution volume 160−175 mL (lane 1) and 175−190 mL (lane 2). Lane 3 shows the position of marker proteins. The bands were assigned to the individual subunits due to their apparent molecular mass.

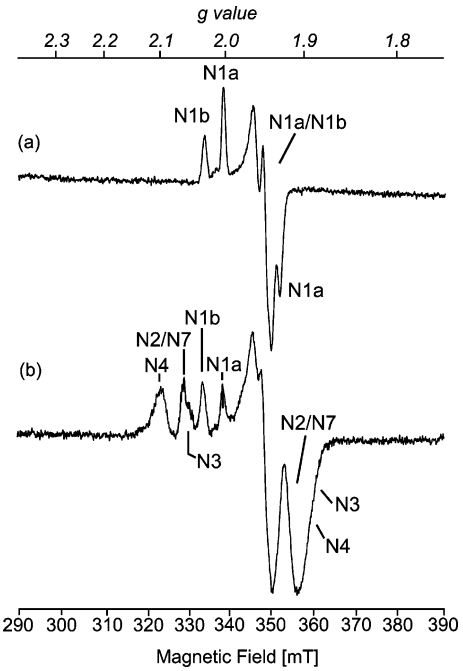

FIGURE 4: EPR spectra of complex I isolated from strain ANN0221/pBAD*nuo*/His-*nuoF*. The spectra were recorded at 40 K and 2 mW (a) and at 13 K and 5 mW (b). The sample was reduced by an addition of dithionite to reduce cluster N7 (*25*). The position of the signals of the individual clusters is indicated. Other EPR conditions were microwave frequency, 9.44 GHz; modulation amplitude, 0.6 mT; time constant, 0.164 s; scan rate, 17.9 mT/min.

mass of 780 kDa, which is in good agreement with the molecular mass of complex I with bound detergent (*45*). Approximately 2% of the protein eluted as aggregates in the void volume of the column as judged by the peak area. A small peak at 17 mL elution volume with less than 0.2% of the main peak area most likely stems from imidazole carried over from the affinity chromatography. An aliquot of the preparation was stored at −80 °C for 6 months, thawed, and applied to analytical size-exclusion chromatography, resulting in a virtually identical elution profile.

Cofactor Content. The content of noncovalently bound FMN was determined by fluorescence spectroscopy to 1.43−1.66 nmol/mg protein, corresponding to a ratio of 0.76−0.89 mol of flavin per mol of complex I. The iron content of the preparation was determined using the ferrozine method to 32 ± 3 mol of iron per mol of complex I, which is the expected value for seven tetranuclear and two binuclear iron−sulfur clusters. The Fe/S clusters of the preparation reconstituted in lipids were characterized by X-band EPR spectroscopy (Figure 4). The spectra of the dithionite reduced preparation obtained at 40 K and 2 mW microwave power showed the signals of the binuclear clusters N1a ($g_{x,y,z}$ = 1.92, 1.94, and 2.00) and N1b ($g_{\|,\perp}$ = 2.03 and 1.94) at the expected positions. The signals of the tetranuclear Fe/S clusters N2 ($g_{\|,\perp}$ = 1.91 and 2.05), N3 ($g_{x,y,z}$ = 1.88, 1.92, and 2.04), N4 ($g_{x,y,z}$ = 1.89, 1.93, and 2.09) and N7 ($g_{x,y,z}$ = 1.894, 1.953, and 2.047) were present in the spectrum recorded at 13 K and 5 mW microwave power in addition to the signals of the binuclear clusters. The stoichiometry of the individual clusters remained unchanged compared to the EPR spectroscopic characterization of previous preparations (*8*, *20*, *21*, *25*).

Enzymatic Activity. To assess whether the introduction of the histidine-tag had any influence on the physiological electron transfer reaction, the affinity purified complex was reconstituted in *E. coli* polar lipids and the kinetic parameters of the NADH:decyl-ubiquinone oxidoreductase activity were measured at various substrate concentrations. The apparent $K_M^{(NADH)}$ and $K_M^{(Dec-Q)}$ were determined to be 7.6 μM and 3.4 μM, respectively. Varying the NADH concentration a V_{max} of 3.1 μmol min^{-1} mg^{-1} was determined and variation of the decyl-ubiquinone concentration led to a V_{max} of 3.3 μmol min^{-1} mg^{-1}. The reaction was completely sensitive to the addition of 20 μM piericidin A, indicating that the physiological electron transfer reaction to the inhibitor sensitive ubiquinone binding site was measured. The IC_{50} value of the NADH:decyl-ubiquinone oxidoreductase activity of the preparation reconstituted in phospholipids for piericidin A was determined to 2.7 μM. An IC_{50} value of 0.4 μM was measured for the NADH oxidase activity of the membrane preparation. Both values are in agreement with data reported for the *E. coli* complex I (*20*, *21*, *32*).

Compared to complex I isolated by standard chromatographic procedures, the affinity purified enzyme exhibited a 3−4 times higher specific activity of 1.2 μmol min^{-1} mg^{-1} in detergent solution (*8*). The activity of the affinity purified complex in detergent solution was stimulated 2−3-fold upon addition of 1:1 (w/w) *E. coli* polar lipids, while the complex purified by standard chromatographic procedures was activated 8−9-fold (*8*). This could be due to an increased phospholipid content of the preparation owing to a decreased delipidation during the gentle purification conditions. This possibility was examined by determinating the phosphate content. The preparation obtained by affinity chromatography contained 53 mol of phosphorus per mol of complex I, while that obtained by standard chromatographic steps contained 22 mol of phosphorus per mol of complex I. Thus, the gentle purification of the complex resulted in a 2.5-fold higher lipid content.

To address the question whether the affinity purified preparation is capable of coupling the electron transfer reaction with proton translocation, the preparation was reconstituted in proteoliposomes made up of *E. coli* polar

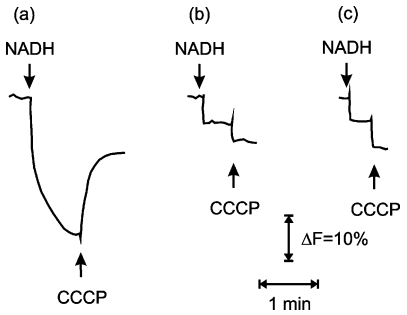

FIGURE 5: Generation of a pH gradient by the *E. coli* complex I isolated from strain ANN0221/pBAD*nuo*/His-*nuoF* reconstituted in proteoliposomes. The gradient was monitored by ACMA fluorescence. The assay contained 20 μg of complex I in 5 mM MES/NaOH, 50 mM NaCl, 65 μM decyl-ubiquinone, 2 μM ACMA pH 6.0 (a). The same reaction in the presence of 20 μM piericidin A (b) and in the presence of liposomes without complex I (c). The reaction was started by the addition of 100 μM NADH, and the gradient was dissipated by the addition of 20 μM CCCP as indicated by an arrow.

lipids as described (*8*). Approximately two-thirds of the complex were inserted in such an orientation in the phospholipid membrane that the NADH binding site was accessible. The generation of a proton gradient coupled with the redox reaction of complex I was followed by measuring the change of ACMA fluorescence (*8*). Addition of NADH to the proteoliposomes containing complex I incubated with decyl-ubiquinone and ACMA led to a quench of the fluorescence signal similar to that obtained in standard preparation indicating the proton-pumping of the complex (Figure 5). The quench of the fluorescence signal was fully sensitive to the protonophore CCCP and piericidin A, demonstrating the presence of a proton gradient established by the redox reaction of the complex (Figure 5). The offset of the ACMA signal in Figure 5b was due to the addition of NADH and CCCP, respectively (Figure 5c).

DISCUSSION

The mechanism of the respiratory complex I is still largely unknown, although the structure of the peripheral arm of the complex from *T. thermophilus* was recently determined at molecular resolution in a pioneering study (*18*). Site-directed mutagenesis is a valuable tool for exploring structure/function relationships. In the investigation of the *E. coli* complex I this has been hampered by three obstacles: (i) the huge size of the *nuo*-operon prohibiting its easy cloning on an expression plasmid, (ii) the presence of the alternative NADH dehydrogenase obscuring measurements of the physiological activity of the complex in the membrane, and (iii) the instability of most enzyme variants hindering their isolation by standard chromatographic methods for structural and spectroscopic characterization. Site-directed mutants were obtained either by the time-consuming introduction of unmarked mutations on the chromosome (*46*) or by inactivating the chromosomal *nuo*-genes by the insertion of a resistance cartridge and *in-trans* complementation with the corresponding gene on a plasmid (*33*, *47*−*50*). With the latter approach, a possible polar effect of the cassette on the expression of the downstream genes cannot be excluded. In addition, *in-trans* complementation with genes coding for the highly hydrophobic subunits is difficult because these proteins are only produced in low amounts (*17*, *50*). Finally, only single gene manipulation is possible with this method. To overcome the problem, the entire *nuo*-operon including the promoter region was cloned into a vector (*51*). Expression of the *nuo*-genes from this vector in a strain lacking complex I led to the production of a functional complex I. The major drawbacks of this system are the low protein production and the presence of the alternative NADH dehydrogenase in the expression strain (*51*). To circumvent the influence of the alternative NADH dehydrogenase, *E. coli* strains with an inactivated *ndh* gene were used (*22*, *52*). So far, no easy and efficient approach for the purification of complex I variants from *E. coli* has been found.

The three obstacles mentioned above can be solved with the system described here. The cloning of the *nuo*-operon coding region in the pBAD plasmid permits the straightforward manipulation of *nuo*-genes by λ-Red-mediated recombineering and verification of the mutation by restriction analysis and DNA sequencing. To enhance the efficiency of recombineering, the *nptI-sacRB* cartridge representing a positive and negative selection marker was employed. In a first step, the region of the *nuo*-operon, which should be mutagenesized, was replaced with the cartridge and subsequently exchanged with the mutated *nuo*-genes (Figure 1). Here, the versatility of this approach was verified by the insertion of a hexahistidine coding sequence upstream of *nuoF*. Compared to mutagenesis by *in-trans* complementation, it is possible to incorporate any desired mutation even on multiple genes. Compared to the unmarked chromosomal mutagenesis there is no need for the time-consuming construction of knock-out vectors, since any position can be targeted by the use of synthetic oligonucleotides.

As an expression host for pBAD*nuo* an *E. coli* strain devoid of any membrane-bound NADH dehydrogenases was constructed. This allowed the direct measurements of complex I activities using the mutant membranes (Table 1). The use of the expensive substrate d-NADH to discriminate between complex I and the alternative NADH dehydrogenase is no longer necessary, since the NADH oxidase activity of the strain is solely comprised by complex I encoded on pBAD*nuo* as demonstrated by the piericidin A sensitivity of the NADH oxidase activity (Table 1). The ratio of the physiological NADH oxidase activity to the artificial NADH/ferricyanide oxidoreductase activity was similar in the strains containing either pBAD*nuo* or pBAD*nuo*/His-*nuoF*, indicating that the modified enzyme is fully assembled and expresses its full catalytic activity. The complex and its variants, if assembled and resistant to detergent extraction, can be rapidly isolated under gentle conditions. The method was applied to purify complex I variants with mutations on NuoG (*25*). The introduction of a histidine-tag at the N-terminus of the NADH binding subunit NuoF allowed the purification of complex I to homogeneity within 1 day. The tight binding of the histidine-tagged complex I to the Ni^{2+}-IDA matrix yielded the recovery of approximately 90% of the activity loaded on the affinity column accompanied by a 7-fold increase of the specific activity (Table 2). Routinely more than 10 mg of complex I was obtained from 25 g of cells (Table 2). The preparation was homogeneous as judged from analytical size exclusion chromatography and contained a fully assembled complex with all subunits and cofactors present in stoichiometric amounts. The EPR spectroscopic

properties of the Fe/S clusters were identical with those of previous preparations (*20*, *21*). After reconstitution in *E. coli* polar lipids the preparation couples the inhibitor sensitive electron transfer from NADH to decyl-ubiquinone with the translocation of protons across the membrane (Figure 5). The structure of *T. thermophilus* complex I implies that the histidine-tag fused to NuoF could possibly interfere with substrate binding, because it is in an approximate 25−30 Å distance to the NADH binding site (*18*). However, the kinetic parameters of the NADH:decyl-ubiquinone oxidoreductase activity were virtually identical to those of the unmodified enzyme preparations (*8*, *21*, *52*), indicating that the tag does not influence substrate binding. In contrast to preparations obtained by standard chromatographic procedures, the affinity purified enzyme exhibits a 3−4 times greater specific NADH:decyl-ubiquinone oxidoreductase activity, which was stimulated 2−3-fold by the addition of lipids. This is most likely due to a decreased delipidation of the complex caused by omitting two ion exchange chromatographic steps, which has been reported to lead to a loss of lipids (*53*). Thus, the overproduction of a histidine-tagged complex I in an *E. coli* strain devoid of other NADH dehydrogenases enables the preparation of a pure and monodisperse complex I suitable for structural and functional studies even with enzyme variants.

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SUPPORTING INFORMATION AVAILABLE

Description of oligonucleotides used for site-directed mutagenesis (Table S1) and scheme illustrating the strategy used to clone the *nuo*-genes (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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