A Role for Native Lipids in the Stabilization and Two-dimensional Crystallization of the Escherichia coli NADH-Ubiquinone Oxidoreductase (Complex I)*

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NADH-ubiquinone oxidoreductase (complex I or NDH-1) was purified from the BL21 strain of Escherichia coli using an improved procedure. The complex was effectively stabilized by addition of divalent cations and lipids, making the preparation suitable for structural studies. The ubiquinone reductase activity of the enzyme was fully restored by addition of native E. coli lipids. Two different two-dimensional crystal forms, with p2 and p3 symmetry, were obtained using lipids containing native E. coli extracts. Analysis of the crystals showed that they are formed by fully intact complex I in an L-shaped conformation. Activity assays and single particle analysis indicated that complex I maintains this structure in detergent solution and does not adopt a different conformation in the active state. Thus, we provide the first experimental evidence that complex I from E. coli has an L-shape in a lipid bilayer and confirm that this is also the case for the active enzyme in solution. This suggests strongly that bacterial complex I exists in an L-shaped conformation in vivo. Our results also indicate that native lipids play an important role in the activation, stabilization and, as a consequence, crystallization of purified complex I from E. coli.

NADH-ubiquinone oxidoreductase (complex I or NDH-1, EC 1.6.5.3) is the first enzyme of the respiratory chains of most mitochondria and many bacteria. It catalyzes the transfer of two electrons from NADH to ubiquinone-10, coupled to the translocation of about 4 protons across the membrane, against the electrochemical concentration gradient (for reviews, see Refs. 1–3). Complex I is one of the largest known membrane protein complexes. The bovine enzyme has a mass of ~980 kDa and is composed of about 46 subunits, including seven hydrophobic ND subunits encoded in the mitochondrial genome (4). The simplest version of the complex in terms of protein content is the prokaryotic enzyme, which has 13–14 subunits and a combined molecular mass of about 550 kDa (5). Complex I is currently the least understood component of the respiratory chain. In contrast to other enzymes of the respiratory chain, the atomic structure of complex I is not known and as a consequence, the mechanisms of proton pumping and electron transfer are also not established.

Electron microscopy has shown that the mitochondrial as well as the bacterial enzyme have a characteristic L-shaped structure. One arm is embedded in the membrane and the other, the peripheral arm, protrudes into the mitochondrial matrix or bacterial cytoplasm. The first structural model of complex I derived from two-dimensional crystals of the Neurospora crassa enzyme in negative stain was at about 30 Å resolution (6, 7). The highest resolution three-dimensional model is currently the 22 Å structure of bovine complex I produced by single particle averaging of molecules embedded in vitrified ice (8). Complex I from Escherichia coli is similar in size and shape to mitochondrial enzymes, although it is thinner, as indicated by single particle analysis of negatively stained samples (9, 10). This established view has been challenged recently by a proposal that active complex I adopts a different, “horseshoe”-like conformation and that the accepted L-shape is an artifact because of solubilization by detergent. This alternative conformation would arise from folding of the peripheral arm toward the distant end of the membrane arm (11). All of the subunits of a minimal complex I proposed from known sequences (2) have analogues in the bacterial enzyme, which is considered to be the simplest version of the enzyme.

Dissociation of complex I by chaotropes and detergents indicated that all the redox centers of the enzyme (flavin mononucleotide and up to 8–9 iron-sulfur clusters) are in the peripheral hydrophobic arm (2, 12–14). The membrane arm is composed of highly hydrophobic subunits and some of these are likely to participate in proton pumping. Sequence comparisons suggested that the hydrophobic subunits ND2, ND4, and ND5 (bovine nomenclature) evolved from a common ancestor related to subunits of K+ or Na+/H+ antiporters (1, 15, 16). Further disruptions indicated that subunits ND4 and ND5 are likely to be situated in the part of the membrane arm distal from the peripheral arm (14). This was confirmed by our electron crystallography studies, which located subunit ND5 to the distal end of the membrane arm (17). These findings led us to suggest that the proton-pumping machinery of complex I might involve a combination of two mechanisms: direct (redox-driven) and indirect (conformation-driven). A more detailed discussion of such a two-mode combination is given in a recent review (1).

A high-resolution structure of the enzyme is necessary to establish a detailed mechanism of complex I function, and this is particularly important in view of the increasing number of human disorders associated with mutations in complex I subunits (18). As can be expected for a large membrane protein, three-dimensional crystals of complex I are extremely difficult to obtain, whereas two-dimensional crystallization is more feasible. Previously, the only reported two-dimensional crystals of intact complex I were obtained with the N. crassa enzyme and studied in negative stain, which limits the resolution to about 20–25 Å (6, 7). We have produced two-dimensional crystals of major subcomplexes of bovine complex I and reported a projec-
tion map of frozen-hydrated crystals at 13-Å resolution (17). However, it has proved difficult to obtain two-dimensional crystals of the intact bovine complex. Additionally, the medium-resolution projection maps of the mitochondrial enzyme are difficult to interpret because of the large number of subunits (more than 20 subunits in the membrane arm alone).

As the bacterial enzyme is much simpler than the mitochondrial one, but is expected to have a similar mechanism, we decided to utilize it for structural studies, using E. coli as a model system. This report contains the structural and biochemical characterization of E. coli complex I purified by an improved procedure from BL21 cells. The activity of the enzyme toward decyl-ubiquinone was fully restored by the addition of native E. coli lipids. Two different forms of two-dimensional crystals were obtained using lipids containing native E. coli extracts. The crystals were studied by electron microscopy in negative stain and found to contain intact, L-shaped complex I. Single particle analysis and activity assays have shown that active complex I maintains an L-shape in detergent solution. These results suggest that complex I is L-shaped in vivo, and indicate a role for native lipids in the activation, stabilization, and crystallization of purified complex I from E. coli.

EXPERIMENTAL PROCEDURES

Chemicals—Dodecylmaltoside (DDM) was purchased from Anatec (Maunee, OH) and dodecanedioylphosphatidylcholine (DOPC) from Fluka (Gillingham, Dorset, United Kingdom), other detergents from Calbiochem (Nottingham, UK), Complete protease inhibitors tablets were obtained from Roche Diagnostics (Lewes, UK), Bio-scale DEAE column and Bio-Beads from Bio-Rad (Hemel Hempstead, Herts., UK), other chromatography columns from Amersham Biosciences, and E. coli total and polar lipid extract from Avanti Polar Lipids (Alabaster, AL). All other chemicals were purchased from Sigma.

Bacterial Growth and Membrane Preparation—E. coli strain BL21 (strain for K-12, but is expected to have a similar mechanism, we decided to utilize it for structural studies, using E. coli as a model system. This report contains the structural and biochemical characterization of E. coli complex I purified by an improved procedure from BL21 cells. The activity of the enzyme toward decyl-ubiquinone was fully restored by the addition of native E. coli lipids. Two different forms of two-dimensional crystals were obtained using lipids containing native E. coli extracts. The crystals were studied by electron microscopy in negative stain and found to contain intact, L-shaped complex I. Single particle analysis and activity assays have shown that active complex I maintains an L-shape in detergent solution. These results suggest that complex I is L-shaped in vivo, and indicate a role for native lipids in the activation, stabilization, and crystallization of purified complex I from E. coli.

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Bacterial Growth and Membrane Preparation—E. coli strain BL21 was grown from a 0.3-liter overnight culture in a 3-liter fermenter at 37 °C and 2% dissolved oxygen in Luria-Bertani (LB) media for about 24 h until late exponential stage (A600 ~ 3.0). Cells (~ 180 g) were harvested by centrifugation at 3900 × g (average value here and throughout) for 20 min, re-suspended in RB buffer (50 mM MES, pH 6.0) and collected by centrifugation at 6800 × g for 15 min. When necessary, cells were kept frozen as a pellet at −20 °C. For a small scale preparation, the cells were grown in three-fourths full 2-liter flasks with slow (140 rpm) agitation. Cells (120 g wet weight) were re-suspended in ~500 ml of RB buffer (RB buffer: 0.002% phenylmethylsulfonyl fluoride, always added fresh from a 1% stock in absolute ethanol). The suspension was passed twice through a Z-plus 2.2 kilowatt cell disruptor (Constant Systems Ltd.) at 300,000 psi. Cell debris was removed by centrifugation at 9,600 × g for 15 min followed by 18,800 × g for 25 min. The supernatant was centrifuged for 4 h at 150,000 × g. The brown membrane pellets were re-suspended in an ~150 ml total volume of RB buffer (RB buffer with Complete EDTA-free protease inhibitors tablets added according to the manufacturer’s instructions) using a glass-Teflon homogenizer. When necessary, membranes were stored at −80 °C.

Purification of Complex I—Membranes from the equivalent of 120 g of cells were used for a single purification. DDM from 10% stock in water was added dropwise (under constant stirring and on ice) to the membrane suspension to a final concentration of 2%. After a 1-h incubation, non-solubilized material was removed by centrifugation for 1 h at 150,000 × g. The supernatant was passed through a 0.45-micron filter and adjusted to 150 m M NaCl by adding dropwise a solution of 1 M NaCl. This stage, all deamino-NADH-ferricyanide (FeCy) activity present in the membranes was found in solution (about 8000 μmol of dNADH oxidized per min).

The AKTA Explorer chromatography system (Amersham Biosciences) was used, with monitoring at 280/420/605 nm to follow absorbance changes because of co-factors present in various proteins. A HiLoad 26/10 Q-Sepharose column was equilibrated with buffer A (20 mM MES, pH 6.0, 0.1% DDM, 10% glycerol, 0.002% phenylmethylsulfonyl fluoride). Solubilized membranes were applied and eluted at 1 ml/min using 60 ml of 15–20% linear gradient of buffer B (A with 1 M NaCl) in buffer A, followed by 800 ml of 20–35% linear gradient of buffer B in buffer A. Complex I eluted at about 500 ml of the elution volume (~280 mM NaCl), as judged from the dNADH:FeCy activity of fractions. Active fractions (about 150 ml) were pooled, diluted with an equal volume of buffer A, and applied to a Bio-Scale DEAE 20 column equilibrated with buffer A. Retained material was eluted with 10 ml of 0–15% linear gradient of buffer B in buffer A, followed by 600 ml of 15–30% linear gradient of buffer B in buffer A. Complex I eluted as a first peak at about 280 ml (as judged from the dNADH:FeCy activity and SDS-PAGE profiles of the fractions), well separated from the sec- ond peak at about 450 ml, which is likely to be bacterial oxidases. Fractions containing complex I were pooled (about 180 ml) and concentrated on Vivacell 70 (100 kDa cut-off) concentrators to a volume of about 2 ml. A HiLoad Superdex 200 16/60 Prep Grade gel filtration column was equilibrated with 20 mM MES, pH 6.0, 200 mM NaCl, 3 mM CaCl2, 0.5% DDM, 10% glycerol, 0.002% phenylmethylsulfonyl fluoride. The concentrated sample was applied and eluted at a flow rate of 0.5 ml/min. Fractions of 1.5 ml were collected (at this stage, all dNADH:FeCy activity co-eluted with the main protein peak), analyzed by SDS-PAGE, and those containing pure complex I were pooled and diluted with buffer A to reduce the NaCl concentration to about 50 mM. Then the sample was concentrated to about 6–8 mg/ml using Vivacell 20 (100 kDa cut-off) concentrators. Purified complex I was stored in small aliquots under liquid nitrogen.

Identification of the Subunits of the Complex—Proteins resolved by SDS-PAGE and stained with 0.1% colloidial Coo massie G-250 (3% phosphoric acid, 6% ammonium sulfate) were identified by peptide mass fingerprinting and tandem MS peptide sequence data. Excised gel bands were digested "in-gel" (19) with trypsin (200 μg/ml Tris-2HCl, pH 8.5, 5 mM CaCl2, 37 °C) or CNBr (20), without prior reduction and alkylation. Portions of the digest were examined in positive ion mode by matrix-assisted laser desorption ionization time-of-flight mass spectrometry with a ToFSpec 2E instrument (Micromass, Altrincham, UK) in the presence of α-cyano-4-hydroxycinnamic acid as matrix. Spectra were calibrated with trypsin autolysis peptides (2103.4 m/z) and a matrix-related ion (1060.048). Peptide mass data were screened against databases of protein sequences using the ProteinProtein program (Micromass).

Peptide sequence analysis was performed on a Q-TOF mass spectrometer equipped with ESI (Micromass) and coupled on-line to a capillary high performance liquid chromatography (CapLC; Micromass). Peptide mixtures were separated using a PepMap C18 column (180 μm × 100 mm, LC Packings, Amsterdam, The Netherlands) with an acetonitrile gradient in 0.1% formic acid. Acquired tandem MS spectra were interpreted manually, assembled into Peptide Sequence Tags (21), and compared with protein sequence databases.

Two-dimensional Crystallization—Lipids in organic solvents were mixed to the desired proportion, washed in chloroform and diethyl ether, dried under a stream of nitrogen, and resuspended in a buffer containing 20 mM MES, pH 6.0, with 2% detergent. For crystallization, the enzyme (0.5 mg/ml final concentration) was mixed with lipid (lipid/protein ratio, 0.3–0.4) in an Eppendorf tube, with the addition of other stock solutions to achieve the desired conditions (0.1 mM MES, pH 6.5, 20–40 mM NaCl, 5–10 mM CaCl2). Bio-Beads SM-2 (15 mg/mg of total amount of detergent present in a set-up) were added to allow for slow (about 6–10 h) removal of detergent (22). The samples were flushed with nitrogen, sealed, and kept at the desired temperature.

Electron Microscopy and Image Processing—For single particle analysis, protein in buffer as mixed in in-gel figure legends and applied to carbon-coated copper grids (flow-discharged in air). After a 2-min incubation, excess buffer was removed by blotting, the grid was washed twice with the same buffer containing no protein or detergent and then stained with either 2% uranyl acetate or 1% gold thioglucose for 10 s. Crystals were stained with 2% uranyl acetate. Images were recorded with a Phillips Tecnai 12 electron microscope at 120 kV and magnification of ×42,000, on Kodak SO163 film. Electron micrographs were checked for astigmatism using an optical diffraction beam. Good crystalline areas (or whole films for single particle analysis) were digitized on a Zeiss-SCAI scanner at a 7-Å step size (corresponding to 1.67 Å at the specimen level) and demagnified by linear interpolation of the computer to obtain a pixel size corresponding to the specimen level for crystals and to 5 Å for single particles. Individual images of crystals were corrected for long-range disorder and projection maps calculated using MRC (23, 24) and CCP4 (25) software suites. The defocus of images was estimated using program CTFFIND2 (8). Some of
the MRC programs were modified to accommodate large unit cells.

Single particle analysis was performed with the IMAGIC 5 software package (26). In total, 500 particles were picked (without selection for appearance) and boxed off from the micrographs of complex I that had been incubated for 2 days at 4 °C with 50 mM NaCl in the buffer and then stained with uranyl acetate. The particle images were normalized and band-pass filtered using a low frequency cut-off of 1/500 Å⁻¹ and a high frequency cut-off of 1/15 Å⁻¹ and centered by translational alignment to a rotational averaged total sum. The centered particle images were grouped into 24 classes using multivariate statistical analysis. All 24 class-sum images were used as references for a multireference alignment and the process was iterated until stable class-sum images were obtained. After the final iteration, aligned particle images were grouped into 6, 12, or 24 classes using multivariate statistical analysis and class-sum images were calculated and compared.

Analytical Methods—Protein concentrations were determined by the Bradford (40) (Bio-Rad) method with bovine serum albumin as standard. Pre-prepared Tris glycine-polyacrylamide gels containing a 10–20% acrylamide gradient were used according to the manufacturer’s instructions (Novex). Enzyme activity assays were performed at 30 °C in a Shimadzu UV-1601 spectrophotometer with a magnetic stirrer attachment. Reduction of FeCy was followed at 420 nm, and oxidation of NADH in the presence of decyl-ubiquinone, at 340 nm. All reactions in the presence of lipids or quinones were observed under constant stirring. Reactions were started by addition of 1–2 μl of protein to a 2-ml assay mixture, except for assays in the presence of lipids or quinones, when all the ingredients, except for NADH, were incubated for 2 min at 30 °C with stirring, and the reaction was started by addition of NADH. The rates were estimated using the steady-state (linear) part of the time course of the reaction.

Analytical gel-filtration chromatography was performed using a Superose 6 column equilibrated with a buffer containing 20 mM MES, pH 6.0, 200 mM NaCl, 2 mM CaCl₂, 0.1% DDM, 10% glycerol, 0.002% phenylmethylsulfonyl fluoride at a flow rate of 0.3 ml/min. Fractions of 0.5 ml were collected and analyzed by SDS-PAGE. The column was calibrated using a high molecular weight gel filtration calibration kit (Amersham Biosciences).

RESULTS

Purification and Subunit Composition of Complex I—An increase in the levels of enzyme in the E. coli membranes is required to obtain sufficient amounts of purified complex I for structural studies. We opted for the induction of its expression by limited oxygen availability as an alternative to overexpression by promoter replacement (27). It is known that under aerobic conditions complex I expression decreases in exponential and stationary phases of E. coli growth (28), when the non-coupling, non-proton pumping dehydrogenase NDH-2 is expressed and used preferentially (29). An optimal level of complex I induction was achieved in Luria-Bertani medium at 2% dissolved oxygen. Under these conditions the activity of complex I in the cytoplasmic membranes (assayed with a specific complex I substrate deamino-NADH and FeCy) was about 1.5–2 μmol/min/mg of protein, 2–3 times higher than during any stage of aerobic growth. Deamino-NDH:FeCy activity was about 80% of NADH:FeCy activity, indicating that only low amounts of NDH-2 were present in our preparations.

The purification procedure (Fig. 1) was developed using elements of previously described methods (27, 30), but with different chromatography steps and avoiding NaBr treatment of the membranes and associated loss of protein. The final gel-filtration step produced a pure preparation with a molecular weight of about 600,000 (by comparison with molecular weight standards), indicating that the complex is monodisperse (Fig. 1C). Notably, a contaminant of about 150 kDa that was present throughout the purification could be removed only when the final gel-filtration step was conducted with 0.2 M NaCl in the buffer. This protein was identified as subunit NuoCD by peptide mass mapping and is likely to be a multimer of this hydrophobic subunit. The significance of this finding remains to be established; it is possible that NuoCD is transiently present as a multimer during assembly of complex I. Previously, co-

expression of NuoCD was found to be essential for overexpression and assembly of an NADH dehydrogenase fragment containing NuoE, -F, and -G in E. coli (31). A summary of the purification procedure is given in Table I.

All 13 subunits encoded by the nuo operon have been positively identified in the preparation by mass spectrometry using a combination of peptide mass mapping and tandem MS analyses (Fig. 1D, Table II). No significant contaminants were identified on Coomassie-stained gels (Fig. 1D). For some of the highly hydrophobic subunits (NuoL, -M, and -J) few tryptic peptides were recovered and reliable identifications were possible only after tandem MS analyses of the peptides. Table II contains data on sequences of tryptic peptides recovered for the hydrophobic subunits. These peptides are likely to originate from hydrophilic loops and might be useful in secondary structure predictions. Two subunits, NuoE and NuoJ, are not resolved by our gel system, whereas NuoM and NuoN run close to each other, but are partially separated. The relative positions of some subunits differ from those previously reported (27, 32), probably because of slight differences in the gel systems used. We have not observed two separate bands for the NuoL subunit as reported recently (32), although the NuoL band is broad and partially overlaps the NuoE/J band (Fig. 1D).

Activation of Complex I by Native Lipids and Stabilization by Divalent Cations—To assess whether complex I purified by our
procedure was active, we conducted kinetic measurements with decyl-ubiquinone, an analogue of the physiological acceptor, over a range of NaCl concentrations both in detergent solution and with added lipids. In solution the activity was optimal with the detergent DDM at a concentration (0.03%) just above the critical micelle concentration (CMC). The activity was reduced over a range of NaCl concentrations both in detergent solution and with added lipids. In solution the activity was optimal with ETL (Fig. 2). The activity depended on the method of solubilization of lipids; solubilization in CHAPS was more effective than in DDM or by sonication, probably because of better dispersion of lipids by CHAPS, which has a high CMC. Native lipids from *E. coli* were more effective in stimulating activity than plant (soybean) or synthetic (DOPC) lipids. If a mixture of DOPC with synthetic (DOPC) lipids. If a mixture of DOPC with

Membrane proteins are often more active in the presence of various lipids, the activity was stimulated up to 8-fold attaining −22 μmol/min/mg of protein (Fig. 2B). The activity depended on the method of solubilization of lipids; solubilization in CHAPS was more effective than in DDM or by sonication, probably because of better dispersion of lipids by CHAPS, which has a high CMC. Native lipids from *E. coli* were more effective in stimulating activity than plant (soybean) or synthetic (DOPC) lipids. If a mixture of DOPC with *E. coli* total lipids (ETL) in a 3:1 ratio (similar to the crystallization conditions, see below) was used, the activity was significantly higher than with DOPC alone, nearly approaching the levels observed with ETL (Fig. 2B). The activity was maximal at 20–50 mM NaCl, similar to assays containing no lipid. The enzyme was not sensitive to rotenone or piericidin A. Inhibitor sensitivity

The effects of divalent cations on activity were studied using

### Table I

**Purification of complex I from *E. coli* BL21**

| Purification step       | Vol  | Protein | Total activity | Specific activity |
|-------------------------|------|---------|----------------|------------------|
|                         | ml   | mg      | μmol min⁻¹     | μmol min⁻¹ mg⁻¹  |
| Membrane fraction       | 100  | 3480    | 7600           | 2.2              |
| Solubilized membranes   | 110  | 1540    | 7400           | 4.8              |
| Q-Sepharose             | 150  | 190     | 4900           | 25.8             |
| DEAE                    | 170  | 62      | 2800           | 45.2             |
| Superdex 200            | 9    | 38      | 2200           | 57.9             |

Data are for tryptic peptides.

### Table II

**Identification by mass-spectrometry of subunits present in *E. coli* complex I**

| Subunit (SWISS-PROT accession No.) | Peptide mass fingerprint data | Tandem MS data |
|------------------------------------|-------------------------------|----------------|
|                                    | No. of peptide masses matched | Sequence⁡ | MH⁻¹ (monoisotopic) | MH⁻¹ (sequence) |
| NuG (P33602)                       | 25                            | 39.0%      | 894.48            | 894.47          |
| NuGCD (P78089)                     | 25                            | 49.8%      | 1025.50           | 1025.51         |
| NuF (P31979)                       | 15                            | 38.0%      | 1237.63           | 1237.63         |
| NuF (P33608)                       | 3                             | 8.2%       | 1583.78           | 1583.86         |
| NuF (P33608)                       | 7                             | 16.0%      | 2278.20           | 2326.18         |
| NuG (P33598)                       | 8                             | 34.6%      | 960.58            | 960.56          |
| NuG (P3360)                        | No significant data           |             |                   |                 |
| NuG (P31978)                       | No significant data           |             |                   |                 |
| NuF (P33608)                       | 3                             | 8.2%       | 1234.66           | 1234.68         |
| NuG (P33603)                       | 7                             | 16.0%      | 1286.68           | 1286.71         |
| NuG (P33603)                       | (188–196, 293–303, 368–382)   | 145,148,294–303 | 2278.20           | 2326.18         |
| NuG (P33598)                       | 8                             | 34.6%      | 960.58            | 960.56          |
| NuG (P33604)                       | 7                             | 35.0%      | 1151.48           | 1151.52         |
| NuG (P33601)                       | 6                             | 28.3%      | 1218.60           | 1218.68         |
| NuE (P33601)                       | 6                             | 28.3%      | 1490.62           | 1490.74         |
| NuE (P3360)                        | 6                             | 28.3%      | 802.46            | 802.47          |
| NuE (P33601)                       | 6                             | 35.0%      | 1446.76           | 1446.77         |
| NuE (P33598)                       | 8                             | 34.6%      | 2215.93           | 2216.00         |
| NuE (P33597)                       | 6                             | 27.2%      | 845.44            | 845.45          |
| NuE (P3360)                        | 6                             | 27.2%      | 1099.58           | 1099.59         |
| NuE (P33601)                       | (45–61, 45–61, 120–129)       | 1405.62     | 1405.67           |
| NuE (P33598)                       | (87–99a, 87–99b, 88–100b)     | 1534.66     | 1534.75           |

⁡Identified sequences within peptides are underlined.

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References and footnotes are not included in the provided text.
Crystallization of E. coli Complex I

Fig. 2. Activity of complex I from E. coli. Assayed with NADH and decyl-ubiquinone in buffers containing 5 mM MES, pH 6.0, and the indicated NaCl (A and B) or CaCl₂ (C) concentrations. Points at 0 mM NaCl correspond to 0.1 mM NaCl carried over with the protein. A, activity in lipid-free buffers in the presence of detergents or without any added detergent, as indicated. B, activity measured in the presence of lipids, which were solubilized in the detergent shown or dispersed by sonication (in H₂O). The final concentration of the detergent in the assay mixture used for crystallization (DOPC:ETL). The activity in the presence of lipids compare well with previous values of ~1.5–4 μmol/min/mg of protein (11, 32).

The structural stability of complex I in detergent solutions was examined by electron microscopy. Samples were incubated for various time periods and then images of single particles were examined. The presence of small fragments indicated dissociation of the enzyme. When the purified enzyme was stored in 20 mM MES, pH 6.0, and 0.1% DDM at room temperature, it dissociated after a few hours, but the presence of 10 mM CaCl₂ maintained stability for about 24 h. At 4 °C complex I was intact for about 2 days, but this was increased to 3–5 days with the inclusion of 10 mM CaCl₂. Addition of MgCl₂ had a similar effect as CaCl₂. The presence of NaCl at 10–50 mM did not improve the stability, again indicating a specific effect of divalent cations. The complex was most stable when DDM was used as the detergent, but taurodeoxycholate and CHAPS could be tolerated for shorter periods (up to about 24 h). This was indicated by gel-filtration chromatography and electron microscopy (data not shown). Two-dimensional crystallization trials showed that the complex remains intact for about a week at 22–37 °C when reconstituted into a lipid bilayer by detergent removal (see below). Thus, complex I from E. coli can be stabilized by the procedures described and so is suitable for structural studies.

Single Particle Analysis of Complex I—Initial structural characterization of purified complex I was conducted by single particle analysis. Electron micrographs of isolated complex I have shown the familiar L-shape of this enzyme under a variety of conditions and importantly in buffers optimal for activity (50 mM NaCl, Fig. 3A, and 10 mM CaCl₂, not shown). When the enzyme was stored at 4 °C for extended periods (~2 days) at ≤50 mM NaCl, there was a tendency for dimerization (Fig. 3B). The dimers appear to be joined through the membrane arms; these hydrophobic domains are usually better stained because of stronger exclusion of negative stain. This interpretation was confirmed by gel-filtration chromatography; after several days of incubation at 4 °C, the main protein peak gradually shifted to a position corresponding to ~600 kDa to that corresponding to ~1100 kDa (Fig. 4). SDS-PAGE analysis of the fractions eluted from the column indicated that ~1100-kDa peak appears to contain all the subunits of the enzyme (not shown). Taking into account that the relative amount of protein-bound detergent is likely to decrease in a dimer conformation, the observed increase in molecular weight is consistent with a monomer to dimer transition.

The conformation of the complex appeared to be less well defined at low ionic strength (0.1 mM NaCl) conditions, as along with many L-shaped particles there were some dimers and larger aggregates (Fig. 3D). To avoid possible artifacts because of the high ionic strength of the uranyl acetate stain, we used the low ionic strength stain, gold thioglucose, as suggested previously (11). The observed aggregation may result from a “salt washing” effect, a decrease in solubility of proteins at very low ionic strength. A small proportion of the molecules looked like dimers with peripheral arms either missing or inadequately stained. Similar particles were also observed at 50 mM NaCl with protein incubated for several days at 4 °C and are indicated by black-and-white arrows in Fig. 3, B and D. The enzyme stained with gold thioglucose in high ionic strength buffer appeared L-shaped (Fig. 3C), as reported before (11).
Crystallization of E. coli Complex I

The nature of the dimers was investigated further by single particle analysis using the IMAGIC 5 software package (26). About 500 particles were picked from images similar to those in Fig. 3B, as under these conditions there were no larger aggregates, allowing for sufficient numbers of different types of particles for classification. Nearly all the particles represented side views of the enzyme because of a preferred orientation of complex I attachment to the carbon on the grid. The particle images were grouped into 6, 12, or 24 classes by multivariate statistical analysis and multireference alignment. We found that grouping into 6 classes, as shown in Fig. 3E, represented all the major types of particles, as 12 or 24 classes have shown only subpopulations of these six (data not shown).

Comparison of different class-sum images shown in Fig. 3E confirms that dimers in class 1 are joined through strongly stain-excluding membrane arms. The molecules in class 2 represent dimers with missing or inadequately stained peripheral arms, because they can be perfectly aligned (including a slight outside-facing curvature of the membrane arms) with the core of dimers in classes 1 and 3. Similarly, molecules in class 4 are in the opposite orientation on the grid to those in class 2 and can be aligned with the core of dimers in class 6 (in the opposite orientation and a “mirror” image of class 1). Most of the dimers in class 3 appear to have only one of the peripheral arms present. Such particles, with one of the peripheral arms missing, could also be seen in raw images. Class 5 represents a monomer with the membrane arm oriented horizontally, as indicated by comparison with the shape of the monomer in classes 1, 3, and 6.

Active Complex I Is L-shaped in Solution—It was suggested recently (11) that the familiar L-shape of complex I is an artifact because of its removal from the membrane environment by detergent solubilization. It was proposed that the enzyme adopts a horseshoe conformation in vivo, which could be observed in solution only at low ionic strength (0.1 mM NaCl), conditions associated with the activation of the complex (11). In contrast, we did not observe a strong stimulation of activity at 0.1 mM NaCl as compared with 50 mM (Fig. 2A), or complete inhibition by 200 mM NaCl. The optimal NaCl concentration was between 20 and 50 mM. A clear transition to a different conformation in low ionic strength was not observed by electron microscopy (Fig. 3). A small proportion of molecules appear to be similar to the reported horseshoe-like structure (11) and are represented by classes 2 and 4 in Fig. 3E. However, as discussed above, these classes are likely to represent dimers of complex I without peripheral arms.

Importantly, L-shaped molecules were by far the major form present in 50 mM NaCl (Fig. 3A and similar data in gold thioglucose, not shown), the salt concentration at which we observed optimal or close to optimal activity (Fig. 2). This is in contrast to the proposal that complex I adopts an alternative conformation in the active state.

Two-dimensional Crystallization of Complex I—For further structural analysis, two-dimensional crystallization trials were carried out initially with the commonly used lipid DOPC (C18 carbon chain, temperature of phase transition \( T_m = -20 \, ^\circ C \)) which remains fluid and can support crystal growth at 4 \(^\circ C \). For insertion of protein into the lipid bilayer (as observed by the appearance of proteoliposomes in EM) to occur, the salt concentration in the incubation buffer had to be increased to >0.4 M, which led to dissociation of the complex. Short chain lipid dilauroyl-PC (C12, \( T_m = -1 \, ^\circ C \)) supported protein insertion at NaCl >0.2 M, but no crystals were observed. Palmitoyloleoyl-PC (C16–18, \( T_m = -2 \, ^\circ C \)) supported insertion at any salt concentration, leading to the formation of tightly packed pro-
teoliposomes, but not crystals. If E. coli polar or total lipid extracts (EPL or ETL, ~60% phosphatidylethanolamine + other lipids, \( T_m > 25 \) °C) were used, efficient insertion of the protein was observed at any salt concentration. Tightly packed proteoliposomes had some ordered areas, but they were very small, presumably because of the limited fluidity of these lipids at 4 °C or room temperature. Therefore, a combination of DOPC with ETL/EPL was explored to combine DOPC fluidity with the affinity of complex I for native lipids.

Two-dimensional crystals were obtained at DOPC:ETL ratios of 3:1 to 4:1, in the pH range 6.0–6.5, with the addition of 20–50 mM NaCl and 5–10 mM CaCl\(_2\) or MgCl\(_2\). The presence of divalent cations was necessary for crystallization, possibly because of stability requirements. Crystals also formed if ETL was replaced with EPL or purified E. coli phosphatidylethanolamine (Fig. 5). The best crystals were obtained when both lipids and protein were solubilized in DDM, although taurodeoxycholate could also be used. Crystals formed after about 5 days of incubation at 22 °C, and were also observed at 37 °C; however, constant or cyclic exposure to elevated temperatures did not improve the quality of crystals. At 4 °C under similar conditions only very small ordered areas were observed. The formation of crystals after detergent removal by Bio-Beads progressed from small liposomes (Fig. 5A) to larger formations, either collapsed liposomes where two lipid bilayers interact to form a crystal (Fig. 5B) or single-layer sheets (Fig. 5, C and D).

The folded edge of a crystal in a collapsed liposome clearly shows peripheral arms of complex I protruding of the bilayer in rows (Fig. 5A). Single-layer crystals were better ordered than collapsed liposomes and were present in two crystal forms obtained under slightly different conditions (Fig. 5, C and D, and Fig. 6). With DOPC:E. coli phosphatidylethanolamine at a 4:1 ratio and a lipid:protein ratio of 0.4, the crystals obtained belonged to space group \( p2 \) (Fig. 5D and Fig. 6, A and B), as indicated by analysis of phase residuals using the ALLSPACE program (24). If DOPC:ETL in the 3:1 ratio and a lipid:protein ratio of 0.3 was used, the crystals had a hexagonal diffraction pattern (Fig. 5C and Fig. 6, C and D) and belonged to space group \( p3 \), as determined by ALLSPACE. In both cases, single-layer crystals with \( p2 \) or \( p3 \) symmetry were present in lower proportional amounts than collapsed liposomes. Crystals were usually of approximately circular form with diameters up to 1 \( \mu \)m. Coherent ordered areas were about 0.2–0.4 \( \mu \)m in size. Spots in diffraction patterns of ordered areas extend to about 25–30 Å in both crystal forms (Fig. 6, B and D), close to the limit imposed by the negative stain technique. The projection map of \( p2 \) crystals (Fig. 7A) shows prominent stain-excluding areas that are likely to be the peripheral arms of the complex protruding over the lipid bilayer (and a layer of negative stain). The size of these areas (about 70–80 Å in diameter) is consistent with each being a projection view of a peripheral arm (9). Each of two arms of bacterial complex I is expected to be about 180 Å in length with a thickness of up to about 70 Å (Ref. 9 and Fig. 3E). Both the observed symmetry and the size of the unit cell of \( p2 \) crystals (147 ± 1 Å × 199 ± 2 Å) are consistent with 2 molecules of intact complex I being present in one unit cell. The integrity of the complex in \( p2 \) and \( p3 \) crystals was confirmed by SDS-PAGE; the gel profile of crystals purified on sucrose gradients was identical to that of purified complex I. Thus, under these conditions complex I from E. coli is more stable than the bovine enzyme, because our repeated crystallization attempts with bovine complex I even at 4 °C have always lead to the loss of the ND5 subunit (17).

An axis of symmetry perpendicular to the lipid bilayer indicates that all molecules are facing the same side of the bilayer. Because negative stain does not penetrate significantly into lipids, membrane arms are not visible in projection maps of \( p2 \) crystals, but their position can be suggested on the basis of relative positions of the peripheral arms and symmetry axes. This is indicated by the contour in Fig. 7A, using an approximate outline of the molecule from previous studies (9).

The symmetry and unit cell size (309 ± 2 Å × 309 ± 2 Å) of \( p3 \) crystals are consistent with 3 molecules of intact complex I forming each unit cell. The projection map of \( p3 \) crystals (Fig. 7B) can be interpreted on the basis that 180-Å long membrane arms are arranged around a \( p3 \) symmetry axis, as indicated by the contour. The crystal used for the map in Fig. 7B was embedded in a relatively thin layer of negative stain (similar to Fig. 6C). Therefore, both the peripheral arm and the distant end of the membrane arm, which is likely to have some area extending over the lipid bilayer (Ref. 9 and Fig. 3E), appear to be visible at the ends of the membrane arm. When \( p3 \) crystals are embedded in a relatively thick layer of stain (similar to Fig. 5C), peripheral arms stand out much more in both the raw
crystal. Above the mean whereas negative contours are shown in broken lines. Continuous lines embedded in a thick layer of stain.

The membrane arm of complex I in the projection is indicated. Unit cells and symmetry elements are outlined.

Resolution with either p2(Å) or p3(B and C) symmetry imposed. A. p2 crystal. B. p3 crystal embedded in a thin layer of stain. C. p3 crystal embedded in a thick layer of stain. Continuous lines indicate density above the mean whereas negative contours are shown in broken lines. Unit cells and symmetry elements are outlined. A putative outline of the membrane arm of complex I in the projection is indicated.

Images (Fig. 5C) and in the projection map (Fig. 7C), indicating the orientation of the complex and position of the hydrophilic domain. The crystals appear to be packed so that at one point, 3 complexes contact close to a p3 axis through the peripheral arm and/or nearest end of the membrane domain and at the other p3 axis, 3 complexes contact through the distal end of the membrane domain. A different packing would allow different penetration of negative stain, this might explain why the distal end of the membrane arm is visible in p3 crystals, but not in p2. The exact positions of the membrane arms in crystals should be clear from future work with frozen-hydrated samples.

Complex I Is L-shaped in a Lipid Bilayer—The data in Figs. 5–7 present the first structural information on bacterial complex I in a native membrane environment, as previously the enzyme was only studied in detergent solution by single particle analysis. Our two-dimensional crystals are a good approximation of the in vivo situation because they were obtained with native E. coli lipids as a component of the membrane. The analysis of images similar to Fig. 5A, showing peripheral arms protruding from the lipid bilayer, indicates that the enzyme is likely to have an L-shape in the E. coli membrane. We did not see evidence for an expected “double arm” pattern of the alternative horseshoe shape (11) in any orientation of the crystal lattice folding.

Importantly, projection maps in Fig. 7 are consistent only with the L-shaped conformation of complex I in the bilayer. The peripheral arm at one end of the molecule is clearly protruding much further away from the bilayer than any other part of the molecule, which would not be the case for the horseshoe model with approximately equal arms at both ends of the molecule. Thus, our data suggest an L-shaped complex I conformation in a lipid membrane.

**DISCUSSION**

Structural characterization of complex I depends on the availability of large scale preparations of pure, monodisperse and stable enzyme. There has been substantial progress toward these goals with preparations of bovine complex I, a close homologue of the human enzyme (13, 14). To date, the only published purification procedures for bacterial complex I in an intact form were for the E. coli enzyme (27, 30, 32). Complex I from other prokaryotic sources, including Thermus thermophilus, appears to be unstable (1, 5). The E. coli enzyme was reported to be unstable in salt (˃0.2 m NaCl) and at alkaline pH (˃6.5) (30). This was apparently improved if the complex was purified from an overexpressing strain (27) or a NDH-2-lacking strain (32) using DDM as a detergent. The yield (9–20 mg) and purity of the protein were limited and preparations contained ATPase and several other contaminating proteins (27, 32).

The final protein yield (40 mg) in our procedure is at least twice that of previous methods (27, 32) and the purity is also improved, as the preparation contains no ATPase or other contaminating bands (Fig. 1D). The most effective additional step, as compared with published procedures, appears to be the chromatography on a high-resolution Bio-Scale DEAE column (Fig. 1B). The subunit composition of the purified complex was defined by mass spectrometry and shown to be complete (Fig. 1D, Table II).

Enzyme activity assays with quinones as electron acceptors can often be problematic because of the low solubility of quinones in water and possible interference of detergent with quinone-binding sites. In the case of the bovine enzyme, only the preparation made with a series of precipitation steps and still containing bound native lipids, shows high activity (8–9 μmol/min/mg of protein) with quinones (34), whereas complex I highly purified by a series of chromatography steps and devoid of most lipids shows rather low activity (1.5 μmol/min/mg of protein) even after addition of lipids (13, 14). Reported NADH-decyl-ubiquinone activities of purified complex I from E. coli range from about 4–5 μmol/min/mg of protein for the enzyme reconstituted into soybean lipids (27) or assayed in low ionic strength buffer (11) to about 1.5 μmol/min/mg of protein for the enzyme assayed in the presence of soybean lipids (32). We found that native E. coli lipids were more effective than other lipids in this respect and restored activity to a level significantly higher than previously reported (up to 22 μmol/min/mg of protein, Fig. 2B) and near to that expected in the cytoplasmic membrane (32).

The two-dimensional crystals described here are the first obtained with bacterial complex I and provide the first experimental structural information on the enzyme in a native membrane environment. Importantly, detailed analysis of crystals showed that intact complex I from E. coli is L-shaped in a lipid bilayer (Figs. 5 and 7). This strongly supports an L-shaped conformation of the enzyme in vivo.

This conclusion contrasts a recent proposal that complex I adopts a different, horseshoe-like conformation in vivo, based on the observations of this alternative shape and activation of
the enzyme in solutions of low ionic strength (11). We did not observe the same stimulation of activity (Fig. 2A). One reason for this discrepancy could be that activity measurements by Bottcher et al. (11) were performed mostly with sub-CMC amounts of detergent (carried over with the protein) in the assay buffer. The activity varies significantly depending on the DDM concentration in the buffer (Fig. 2A). With sub-CMC amounts of detergent in the assay and without added lipid, the access of the hydrophobic decyl-ubiquinone to the active sites on complex I may be problematic and in addition the enzyme is likely to aggregate. In this situation the rates observed might depend on the amount of remaining protein-bound native lipid. Under the same conditions as reported previously (11), we observe (Fig. 2A, no detergent) relatively low rates of NADH oxidation with the maximum at around 20–50 mM NaCl. In addition, it should be noted that our preparation was obtained from the wild-type BL21 strain of E. coli, whereas previous experiments were conducted with protein overexpressed in a derivative of the K12 strain (11, 27), which may account for some differences.

We have not observed a change from an L-shape to a horse-shoe-like shape in low ionic strength buffer (Fig. 3). Instead, single particle analysis showed (Fig. 3E) that dimers of complex I with missing or inadequately stained peripheral arms have an appearance similar to the horse-shoe-like shape. It should be mentioned that the lower proportion of L-shaped particles observed at low ionic strength (Fig. 3D), apart from protein aggregation, might also be because of less effective attachment of particles to the carbon layer on the microscope grid. Under these conditions we had to increase the protein concentration from the standard 10 to 50 μg/ml to achieve a similar spread of particles. This could lead to an under-representation of some classes of particles in images and ambiguities in interpretation.

With regard to alternative conformations of complex I in solution the most relevant result from our studies is that the enzyme clearly had an L-shape under conditions optimal for activity (50 mM NaCl; Figs. 2A and 3A). This confirms that intact, active bacterial complex I has indeed a classic L-shape in detergent solution, as well as in a biological membrane.

Native E. coli lipids were necessary for the stabilization and effective insertion of complex I into a lipid bilayer, which allowed us to produce crystals containing intact enzyme at room temperature. Because E. coli lipids are also needed for optimal activity, either in pure form or in combination with other lipids, such as DOPC (Fig. 3B), it appears that they play an important role in maintaining the integrity of the purified enzyme. Bound lipids have been identified in three-dimensional structures of several membrane proteins and were suggested to have a stabilizing effect through specific interactions via their lipid and head group moieties (35, 36). For example, cardiolipin cannot be removed from bovine cytochrome c oxidase and bc1 complexes without loss of activity (37, 38). The mechanism of stabilization and activation (Fig. 2C) of complex I by divalent cations remains to be established. A bound Mg2+ ion found in cytochrome c oxidases was suggested to have a structural role in the stabilization of the interface between two subunits (39).

Further structural characterization of the enzyme will require imaging of frozen-hydrated two-dimensional crystals. Additionally, dimers of intact complex I (Fig. 3, B and E) might be useful in structure analysis by single particle averaging in a frozen-hydrated state, as the alignment of the particles will be more effective because of the doubled molecular size, symmetry of the particle, and removal of uncertainty in assigning peripheral and membrane arms of the complex.

In conclusion, we have developed an improved procedure for the purification of complex I from E. coli BL21 cells. The enzyme activity with quinones was fully restored by addition of native E. coli lipids, and the complex was stabilized effectively in the presence of divalent cations and lipids. Activity measurements and single particle analysis confirmed that active bacterial complex I has an L-shape in solution. Two-dimensional crystals were obtained using native E. coli lipids. Analysis of these crystals showed for the first time that bacterial complex I is L-shaped in a lipid bilayer, and thus is likely to have the same conformation in vivo.

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A Role for Native Lipids in the Stabilization and Two-dimensional Crystallization of the *Escherichia coli* NADH-Ubiquinone Oxidoreductase (Complex I)
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