Resolution of the Membrane Domain of Bovine Complex I into Subcomplexes: Implications for the Structural Organization of the Enzyme

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ABSTRACT: Complex I (NADH:ubiquinone oxidoreductase) purified from bovine heart mitochondria was treated with the detergent *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO). The enzyme dissociated into two known subcomplexes, Iα and Iβ, containing mostly hydrophilic and hydrophobic subunits, and a previously undetected fragment referred to as Iγ. Subcomplex Iγ contains the hydrophobic subunits ND1, ND2, ND3, and ND4L which are encoded in the mitochondrial genome, and the nuclear-encoded subunit KFYI. During size-exclusion chromatography in the presence of LDAO, subcomplex Iα lost several subunits and formed another characterized subcomplex known as Iλ. Similarly, subcomplex Iβ dissociated into two smaller subcomplexes, one of which contains the hydrophobic subunits ND4 and ND5; subcomplex Iγ released a fragment containing ND1 and ND2. These results suggest that in the intact complex subunits ND1 and ND2 are likely to be in a different region of the membrane domain than subunits ND4 and ND5. The compositions of the various subcomplexes and fragments of complex I provide an organization of the subunits of the enzyme in the framework of the known low resolution structure of the enzyme.

In aerobic eubacteria and mitochondria, complex I (NADH: ubiquinone oxidoreductase, EC 1.6.5.3) is the first and largest enzyme of the respiratory chain. It catalyzes the transfer of two electrons from NADH to ubiqinone-10 which is coupled to the translocation of four to five protons from the matrix across the inner mitochondrial membrane (*1*−*4*). Bovine complex I is composed of 42 (possibly 43) subunits, including seven hydrophobic proteins encoded in the mitochondrial genome (*5*). Together they make a complex of ∼900 kDa. The simpler version is the prokaryotic enzyme which has 13 subunits in *Escherichia coli* and 14 subunits in other bacteria with a combined molecular mass of ∼550 kDa (*3*). Atomic resolution structures of the cytochrome bc_1 complex (*6*−*8*), cytochrome *c* oxidase (*9*−*10*), F_1-ATPase (*11*), and of the F_1:c_{10}-ring fragment of ATP synthase (*12*) are known. In contrast, only low resolution structures based on electron microscopic analysis of the bovine (*13*), *E. coli* (*14*), and *Neurospora crassa* complexes I (*14*−*17*) have been described. The complex is an L-shaped assembly with one arm in the membrane and the other peripheral.

By controlled disruption of the complex with chaotropes and with detergents, many hydrophilic subunits have been ascribed to the peripheral arm and hydrophobic ones to the membrane arm (*1*). Treatment of the bovine enzyme with perchlorate releases a water-soluble fragment known as the FP (flavoprotein) fraction which retains the ability to transfer electrons from NADH to ferricyanide. It consists of three subunits: the 51 kDa subunit (the site of NADH binding and of the primary electron acceptor FMN) and the 24 and 10 kDa subunits (*18*, *19*). Both the 51 and 24 kDa subunits contain Fe−S clusters, as does the 75 kDa subunit, which is probably intimately associated with the 51 and 24 kDa subunits in the intact complex (*20*). Treatment of the bovine complex I with the nondenaturing detergent *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO[1]) dissociates the enzyme into two subcomplexes termed Iα and Iβ (*21*). Subcomplex Iα is also an NADH:ferricyanide oxidoreductase. It consists of ∼23 mostly hydrophilic subunits and contains all the redox centers of the enzyme. It is likely to represent the peripheral arm and part of the membrane domain in the intact enzyme. Subcomplex Iβ contains ∼13 mainly hydrophobic subunits and has no known prosthetic groups or biochemical activity. It is derived from the membrane arm of the complex. Under slightly different conditions of dissociation another active hydrophilic subcomplex known as Iλ has been obtained. It contains 14 of the subunits in subcomplex Iα, and it represents most or all of the peripheral arm of complex I (*22*, *23*). Several highly conserved hydrophobic subunits of complex I, including ND3, ND4L, and ND6 subunits, were not found in any of these subcomplexes. In other cases, notably ND1 and ND2, they appeared to be present in substoichiometric amounts in subcomplex Iα (*21*, *24*).

As described below, bovine complex I has been purified by modification of earlier methods (*21*, *25*), yielding a highly pure and monodisperse enzyme. This preparation of complex I has been disrupted with the detergent LDAO, and in addition to subcomplexes Iα and Iβ, a third subcomplex

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[1] Abbreviations: LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; DM, dodecyl maltoside; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, MALDI-TOF, matrix assisted laser desorption ionization time-of-flight mass spectrometry.

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containing subunits ND1, ND2, ND3, ND4L, and KFYI has been isolated. During chromatography of the subcomplexes, subunits ND1 and ND2 were always associated together, as were subunits ND4 and ND5, but in different subcomplexes. Therefore, the pairs of subunits ND1/ND2 and ND4/ND5 are likely to be in different regions of the membrane domain of the intact enzyme. These findings have been related to structural models of complex I.

MATERIALS AND METHODS

Chemicals. LDAO and ethylene glycol were obtained from Fluka (Gillingham, Dorset, U.K.), chromatography columns from Pharmacia (St. Albans, Herts., U.K.). All other chemicals were purchased from Sigma (Poole, Dorset, U.K.).

Analytical Methods. Protein concentrations were determined by the BCA (Pierce, Chester, Cheshire, U.K.) and Bradford (Bio-Rad, Hemel Hempstead, Herts, U.K.) methods with bovine serum albumin as standard. Polyacrylamide gels containing a 10−20% acrylamide gradient were prepared and run in the buffer system of Laemmli (*26*). Enzyme activity assays were performed as described before (*21*).

Purification of Complex I from Bovine Heart Mitochondria. Mitochondrial membranes were suspended in 100 mL of buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% v/v glycerol) at a protein concentration of 12 mg/mL containing PMSF (final concentration of 0.005% w/v), 2 mM dithiothreitol, and dodecyl maltoside (DM, 1% w/v). The suspension was stirred for 30 min on ice and then centrifuged (30 min, 30000*g*). The supernatant was applied to a HiLoad 26/10 Q-Sepharose HP column equilibrated in buffer A (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1% DM, 10% ethylene glycol, 1 mM dithiothreitol) at a flow rate of 2 mL/min, with the AKTA Explorer chromatography system (Pharmacia). The column was washed with 50 mL of buffer A, then with 30 mL of a linear gradient of 0−25% buffer B (buffer A with 1 M NaCl) in buffer A, and finally with 150 mL of 25% buffer B. Complex I was then eluted with 200 mL of a 25−35% linear gradient of buffer B in buffer A. Fractions containing complex I (at 260−330 mM NaCl, as judged from absorbance traces at 280 and 420 nm) were combined (total volume ca. 80 mL) and diluted with the same amount of buffer A. At this stage, the enzyme was about 70% pure, as judged by SDS−PAGE. The sample was then reapplied to the same Q-Sepharose column equilibrated with buffer A. Complex I was eluted with the same gradient profile as done previously. Fractions containing complex I were pooled, and the enzyme was precipitated by addition of DM to 1%, sodium cholate to 1.8%, and ammonium sulfate to 50% saturation. The suspension was stirred for 10 min and centrifuged (30 min, 10000*g*). The pellet was dissolved in 1 mL of buffer A containing 1% DM. The sample was divided into two aliquots. Each was applied and chromatographed separately on a Superose 6 column equilibrated with buffer C (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1 M NaCl, 0.2% DM, 10% ethylene glycol, 2 mM dithiothreitol) at a flow rate of 0.3 mL/min. Complex I eluted in a symmetrical peak at about 11 mL, separate from any traces of contaminants. Peak fractions were pooled, and DM was added to a final concentration of 0.5%. The solution was concentrated on a Microsep 300K concentrator (Filtron, Northborough, MA) to a concentration of 10 mg/mL. The preparation was either used immediately or frozen in liquid nitrogen (after addition of glycerol to 20%) and kept at −80 °C. Each preparation yielded about 25−30 mg of pure complex I.

Isolation of Subcomplexes. Resolution of complex I into subcomplexes was performed by modifying the previous procedure (*21*) to improve separation of subcomplexes Iγ and Iα. Purified complex I (10 mg) was exchanged into buffer AP consisting of 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 10% ethylene glycol, 1% LDAO, and 2 mM dithiothreitol on a PD-10 column (Pharmacia). The sample was kept for 1 h on ice and then applied at a flow rate 0.5 mL/min to a Mono-Q HR 5/5 column (Pharmacia) equilibrated with AP buffer. The sample was eluted with 20 mL of AP buffer followed by a 0−60% linear gradient of buffer BP (buffer AP containing 0.5 M potassium phosphate) in 120 mL. Subcomplex Iγ did not bind to the column whereas subcomplexes Iα and Iβ eluted at 110 and 175 mM potassium phosphate, respectively. Fractions (1.5 mL) containing pure subcomplexes were pooled and concentrated on a Centricon 100K concentrator (Amicon, Danvers, MA). Alternatively, subcomplex Iα was precipitated with ammonium sulfate in the presence of DM and sodium cholate, similar to complex I. Comparable resolution of subcomplexes was obtained by scaling up the procedure 5-fold on a Mono-Q HR 10/10 column (data not shown).

Concentrated solutions of subcomplexes (0.2−0.4 mL, 2−4 mg/mL) were applied to a Superose 6 column equilibrated in AP buffer and eluted at at flow rate of 0.3 mL/min. The column was calibrated in the same buffer with molecular weight standards.

Characterization of the Subunit Compositions of the Subcomplexes. Proteins were separated by SDS−PAGE and transferred by electrophoresis for 15 h at 150 mA in a buffer containing 10 mM sodium bicarbonate, 3 mM sodium carbonate, and 0.025% sodium dodecyl sulfate to poly(vinylidene difluoride) membranes. Their N-α-formyl groups were removed by exposure of the membrane bound protein to 1.5 M methanolic HCl vapor for 2 h at room temperature. Then the proteins were examined by N-terminal sequence analysis with a model 494 Procise protein sequencer (PE Applied Biosystems, U.K.). Proteins resolved by SDS−PAGE and stained with Coomassie blue dye were identified by peptide mass mapping. The protein bands were excised from the gels and digested with trypsin (*27*). Portions of the digest were examined in positive ion mode by MALDI-TOF mass spectrometry with a Perseptive Biosystems DE-STR instrument in the presence of α-cyano-4-hydroxy-cinnamic acid as matrix. Spectra were calibrated with a mixture of synthetic peptides with monoisotopic molecular masses of 904.4681, 1296.6853, 1570.6774, 2093.0867, and 2465.1989, deposited in spots adjacent to each sample. Peptide mass data were screened against databases of protein sequences using either the MS-FIT program or using "Peptide Search" (*28*).

RESULTS

Purification of Complex I. The modified chromatographic procedure yielded a highly pure and monodisperse enzyme. The main differences from the earlier procedure (*21*, *25*) are that the Mono-Q HR 10/10 column has been replaced by Q-Sepharose and the anion exchange chromatography is

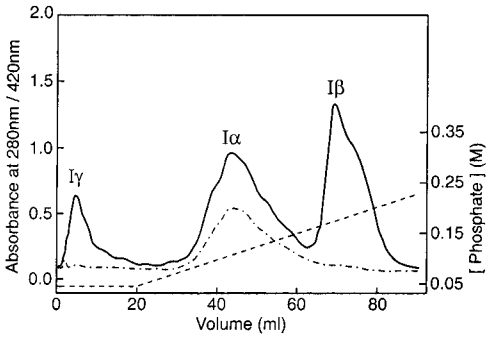

FIGURE 1: Resolution of the subcomplexes on the Mono-Q column. Purified complex I (~10 mg) was treated with LDAO and loaded onto the Mono-Q HR 5/5 column in the presence of LDAO. (—) Absorbance at 280 nm; (- · -) absorbance at 420 nm; (- - -) potassium phosphate gradient.

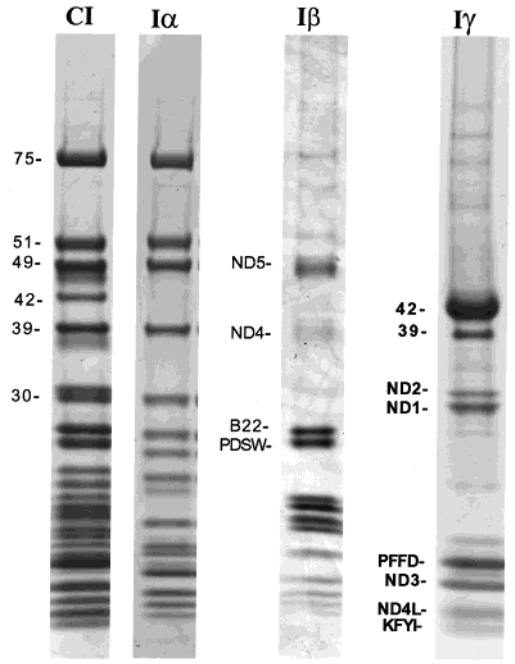

FIGURE 2: Subunit composition of the purified complex I and its subcomplexes. Samples were subjected to SDS−PAGE and stained with Coomassie blue. The lanes correspond to the pooled peak fractions of the following: CI − purified complex I; Iα, Iβ, and Iγ − fractions as indicated in Figure 1. Subunits in Iα and Iβ subcomplexes were identified by comparison with previous preparations (*21*). Subunits in the Iγ fraction were identified experimentally (Table 1) and are indicated in bold.

repeated to remove contaminating proteins. In the final step the enzyme eluted from a size-exclusion column in a single symmetric peak with a molecular weight of ~900 kDa (data not shown). This value is consistent with the enzyme being monomeric and therefore monodisperse. Analysis of the fractions from the size-exclusion column by SDS−PAGE shows that in the peak fractions the preparation is highly pure and devoid of other mitochondrial membrane proteins (Figure 2). Small amounts of contaminating proteins eluted after complex I.

The main advantage of the modified procedure is that it avoids the formation of aggregates and ensures the removal of traces of contaminating enzymes, notably transhydrogenase which hitherto has proved difficult to remove completely. The activity of the enzyme was about 100−130 units (micromoles of NADH oxidized per minute per milligrams of protein) with ferricyanide as an electron acceptor and about 1.0−1.4 units with ubiquinone-5 (Q-1), similar to previous chromatographic preparations (*21*, *25*). The enzyme was relatively insensitive to rotenone because of almost complete removal of phospholipids, as discussed before (*21*). It appears to contain a full complement of all the known subunits of complex I.

Resolution of Complex I into Subcomplexes. During chromatographic separation of complex I after treatment with LDAO, about 25% of the protein eluted in the flowthrough, and subcomplexes Iα and Iβ eluted subsequently at higher ionic strength. Although the breakthrough fraction contained small amounts of intact complex I, the major component had a protein composition different from that of subcomplexes Iα or Iβ; therefore, it appeared likely that it represents another fragment of complex I. The resolution of the subcomplexes was improved by replacing NaCl with potassium phosphate in the buffers and by increasing the LDAO concentration in column buffers from 0.1 to 1.0%. No intact complex I was observed in the breakthrough under these conditions (Figure 1). The absorbance trace at 420 nm indicates that the redox centers are present only in subcomplex Iα, as reported before (*21*).

Polypeptide subunit composition of subcomplexes Iα and Iβ obtained in this way, as well as of the flowthrough fraction we termed Iγ, is shown in Figure 2. Subcomplexes Iα (~21 subunits) and Iβ (13 subunits) are nearly identical in composition to previously reported preparations (*5*, *21*, *24*), as judged by comparison of bands on SDS−PAGE. The exception is that subunits ND1, ND2, and 42 kDa, which were previously found in substoichiometric amounts in Iα (*21*, *24*), are not found in Iα prepared by the current method. The reason for this difference is likely to be more complete disruption of complex I in the presence of phosphate and increased LDAO concentration.

The activity of subcomplex Iα (and subcomplex Iλ described below) was similar to that previously reported (*21*, *23*): ~150−200 μmol NADH min^{-1} mg^{-1} with ferricyanide as an acceptor and ~2.5−3.0 μmol NADH min^{-1} mg^{-1} with Q-1.

Subunit composition of Iγ fraction, as shown in Figure 2, was determined by N-terminal sequencing, in combination with peptide mass mapping (Table 1). The presence of hydrophobic ND1, ND2, and ND3 subunits was indicated by mass mapping experiments. The identity of these subunits, as well as the presence of the ND4L subunit, was confirmed by sequencing after transfer to poly(vinylidene difluoride) membranes using modified protocol (see Methods section). Additionally, nuclear-encoded 42 kDa, 39 kDa, PFFD, and KFYI subunits were identified in the Iγ fraction. Therefore, nearly all subunits previously not clearly assigned to any subcomplex are in fact present in Iγ, and only the ND6 subunit still remains to be identified.

It should be noted that in peptide mapping experiments the percent coverage is lower for ND subunits because hydrophobic segments of proteins are not recovered in this procedure. As can be seen from Table 1 (and Table 3), in ND subunits mainly C- or N-terminal domains of the subunits are digested by trypsin, as could be expected if these domains form extramembraneous loops.

Size-Exclusion Chromatography of Subcomplexes. To establish whether the Iγ fraction represents a subcomplex,

Table 1: Identification of the Subunits Present in the Iγ Fraction[a]

subunit NCBI accession no.	no. of peptide fragments consistent with the sequence, percent coverage, and start−end of peptide sequence	identification by N-terminal sequencing (residues determined)
ND1	6 (18%)	1−17
128632	27−34, 35−54, 36−54, 127−134, 263−274, 282−291	
ND2	3 (11%)	1−7
128668	264−272, 273−295, 315−321	
ND3	2 (18%)	1−12
128703	34−48, 49−54	
ND4L		1−12
42 kDa	7 (25%)	1−10
464254	111−118, 119−127, 128−149, 150−163, 164−168, 266−277, 269−277	
39 kDa		1−10
PFFD	5 (34%)	1−10
400587	10−16, 58−64, 75−80, 76−80, 92−105	
KFYI	2 (51%)	1−10
400579	29−43, 61−70	

[a] The subunits (in bold) were identified by peptide mass mapping and N-terminal sequencing. The number of peptides produced by trypsin digest, the masses of which were consistent with known sequences, and the percent coverage of the sequence by these peptides are shown. The residue numbers for the start and end of the sequence for each peptide are in accordance with the classification used by the National Center for Biotechnology Information (NCBI) and include transit peptide sequence where applicable.

as well as to characterize further subcomplexes Iα and Iβ, we performed size-exclusion chromatography in the presence of LDAO on all three preparations. As the column was calibrated with soluble molecular weight standards, in subsequent estimations of molecular weights of subcomplexes we ignore possible contribution from a detergent shell.

After application of subcomplex Iα to a Superose 6 column we observe a major peak of ~540 kDa (Figure 3A, peak 1) and two smaller peaks, containing the 39 kDa subunit (data not shown) and several low molecular weight subunits (Figure 3A, peak 2; Figure 4A). The major peak apparently contains all the redox centers (420 nm absorbance trace, Figure 3A) and, as can be judged from subunit composition (Figure 4A and Table 2), is identical to subcomplex Iλ, previously purified on sucrose gradients (*22, 23*). Our procedure is thus a viable alternative to previous Iλ preparations, as reproducibly large amounts of pure Iλ can be obtained. Subcomplex Iλ does not contain subunits MLRQ, B9, and MWFE, all of which are likely to have one hydrophobic segment and are present in Iα (*21*). Therefore subcomplex Iλ is likely to be more water-soluble than Iα. However, if Iα or Iλ are chromatographed on Superose 6 in the absence of any detergent in the column buffer, the subcomplexes elute as high molecular weight species in the void volume (data not shown). This indicates that, although both subcomplexes are largely hydrophilic and remain in solution in detergent-free buffers, they tend to form large aggregates without detergent.

Application of subcomplex Iβ results in two major peaks of about 320 and 100 kDa (Figure 3B). The first peak (or subcomplex IβL) contains clearly ND4 and ND5 subunits as well as low molecular weight subunits PDSW and B17 (Figure 4B, Table 3). The second peak (or subcomplex IβS) contains the rest of the Iβ subunits (Figure 4B, Table 3). Subunits PDSW and B17 appear to be present both in IβL and IβS, probably due to incomplete disruption of the Iβ subcomplex.

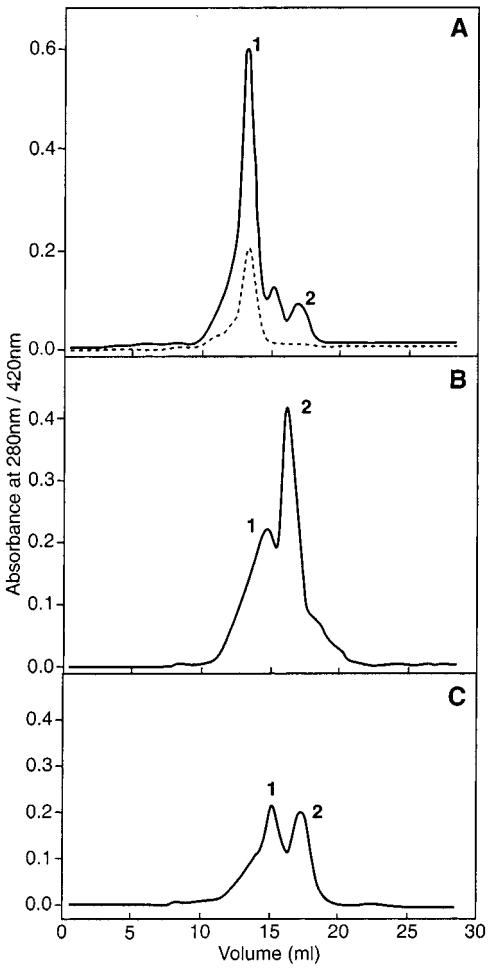

FIGURE 3: Size-exclusion chromatography of the subcomplexes of complex I. The following samples were applied to the Superose 6 column in the presence of LDAO: A − Iα subcomplex, B − Iβ subcomplex, and C − Iγ fraction. (—) Absorbance at 280 nm; (- · -) absorbance at 420 nm.

Since we observe only the fragments of Iβ during size-exclusion chromatography, a question arises as to whether it was indeed a subcomplex initially. To verify this, we took Iβ preparation as obtained from the Mono-Q column, dialyzed it against salt-free buffer, and reapplied it to the Mono-Q column under the same conditions as in the first run. This second time Iβ eluted not in the same position of the gradient (i.e.,~175 mM KP_i) but instead in the flowthrough (data not shown). This indicates that during the first run Iβ was indeed a subcomplex, that is, its components did not just coelute in the same position. It is therefore likely that complex I after initial exposure to LDAO (~1 h) dissociates into Iα, Iβ, and Iγ; then, after prolonged exposure (>4 h) Iα forms Iλ, while Iβ fragments into IβL and IβS.

Fraction Iγ, when applied to Superose 6, elutes as two peaks of about 300 and 100 kDa (Figure 3C); the first of which contains ND1 and ND2, as well as 42 and 39 kDa subunits (Figure 4C, lane 1). The second peak contains all the other low molecular weight subunits of the Iγ fraction (Figure 4C, lane 2). It should be noted that although 42 and 39 kDa subunits coelute with ND1 and ND2 at this chromatography step, they did not coelute exactly in the same position with the main bulk of protein during the first

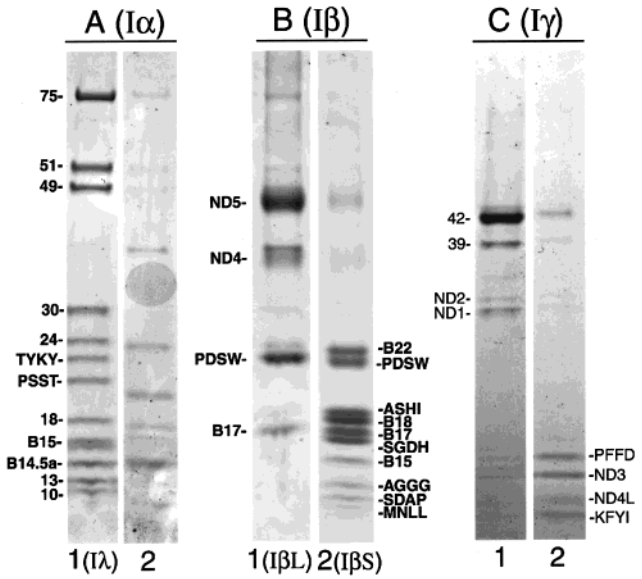

FIGURE 4: Subunit composition of the fragments of the complex obtained by size-exclusion chromatography of the subcomplexes. Samples obtained by chromatography of Iα (A), Iβ (B), and Iγ (C) were subjected to SDS−PAGE and stained with Coomassie blue. In A, B, and C lanes 1 and 2 correspond to the pooled peak fractions 1 and 2 as indicated in Figure 3A, B, and C, respectively. Subunits in C were identified by comparison with gels of the Iγ fraction; subunits in A and B were identified experimentally (Tables 2 and 3) and are indicated in bold.

Mono-Q step (data not shown). It is therefore likely that only a proportion, if any, of 42 and 39 kDa subunits is associated with ND1 and ND2. It is possible that initially most of the subunits found in Iγ formed a subcomplex, which dissociated into fragments only after prolonged exposure to LDAO and phosphate, similarly to the Iβ subcomplex. However, since ND1 and ND2 coeluted during both Mono-Q and Superose 6 steps, we conclude that at least ND1 and ND2 from the Iγ fraction form a subcomplex.

DISCUSSION

Bovine complex I was originally purified using a series of precipitation steps (*29*), which gives highly active and rotenone-sensitive preparation, presumably because some native lipids remain associated with the enzyme. However, this preparation still contains some impurities (mainly transhydrogenase and ATPase), which makes it unsuitable for structural studies. More recently developed chromatographic procedures (*21*, *25*) result in significantly purer preparations, although with lower activity and rotenone sensitivity. The procedure described in ref *21* is quick, but the preparation often still contains some traces of contaminants and is not monodisperse (gel-filtration analysis shows that a significant proportion of the enzyme is aggregated). A more recent procedure (*25*) addresses some of these problems, but since it was optimized for the simultaneous purification of F_1-F_0-ATPase and complex I, it is not suitable for routine complex I studies. The modified method of complex I purification described here is quick, easily reproducible, and can be scaled up. The preparation is monodisperse and free of any contaminants, which makes it suitable for structural and crystallization studies.

We show that treatment of the purified complex I with relatively "harsh" nondenaturing detergent LDAO produces not only subcomplexes Iα and Iβ, but also an additional fraction we term Iγ (Figures 1 and 2). It contains nearly all subunits previously not assigned to either Iα or Iβ, with the exception of only ND6. Size-exclusion chromatography (Figures 3C and 4C) shows that at least ND1 and ND2 subunits from the Iγ fraction form a subcomplex.

Half of the subunits in the Iγ fraction are the hydrophobic ND subunits (ND1, ND2, ND3, and ND4L). In addition, the KFYI subunit is likely to have one hydrophobic segment (*21*). Two hydrophilic subunits (42 and 39 kDa) are likely to be loosely, if at all, associated with other proteins in the Iγ fraction, as discussed above. The 42 kDa subunit is generally weakly associated with complex I (*21*, *25*). The 39 kDa subunit and PFFD (also known as a 15 kDa (IP) subunit) are present not only in Iγ but also in Iα subcomplex (*21*). Therefore, most of the Iγ fraction (ND1/ND2 "core" plus ND3, ND4L, and KFYI) is likely to represent a fragment of the membrane domain of complex I which, importantly, is separate from Iβ. Thus, the Iγ membrane fragment will represent ~12% (~105 kDa) and Iβ ~33% (282 kDa) of the entire complex, the rest being Iα.

Size-exclusion chromatography also shows that Iα can be converted into the slightly smaller subcomplex Iλ (Figures 3A and 4A) and that Iβ dissociates further into subcomplexes IβL (containing as major components ND4 and ND5) and IβS (containing the rest of the Iβ subunits) (Figures 3B and 4B). Thus, our results show that after exposure to LDAO complex I dissociates initially into three major fragments

Table 2: Identification of the Subunits Present in the Iλ Subcomplex[a]

subunit NCBI accession no.	no. of peptide fragments consistent with the sequence, percent coverage, and start−end of peptide sequence	identification by N-terminal sequencing (residues determined)
75 kDa		1−10
51 kDa		1−10
49 kDa		1−10
30 kDa		1−7
24 kDa		1−10
TYKY		1−10
PSST	11 (29%)	
1171865	38−49, 50−62, 70−78, 70−79, 139−148, 139−148,* 140−148, 140−148,* 209−215, 210−215, 212−216	
18 kDa	11 (33%)	1−10
400578	59−73, 79−84, 97−104, 97−104,* 99−104, 99−104,* 132−140, 132−150, 141−150, 159−168, 159−169	
B15	3 (35%)	
1346666	11−30, 45−56, 73−85	
B14.5a	6 (48%)	
547984	7−12, 28−34, 35−48, 49−57, 94−103, 103−113	
B14	3 (27%)	
400384	8−23, 70−77, 106−116	
13 kDa	5 (41%)	1−10
1709407	39−52, 39−53, 60−83, 62−83, 109−120	
B13	4 (33%)	
400650	7−23, 8−23, 72−92, 72−93	
10 kDa	3 (36%)	1−10
128905	57−73, 74−94, 74−96	
B8	3 (25%)	
400515	46−56, 47−56, 76−89	

[a] The subunits (in bold) were identified by peptide mass mapping and N-terminal sequencing (see notes for Table 1). Asterisk indicates that peptide contains oxidized methionine residue.

Table 3: Identification of the Subunits Present in IβL and IβS Subcomplexes[a]

subunit NCBI accession no.	no. of peptide fragments consistent with the sequence, percent coverage, and start−end of peptide sequence	identification by N-terminal sequencing (residues determined)
	IβL Subcomplex	
ND5	5 (8%)	
128778	426−436, 437−455, 437−456, 513−52, 536−547	
ND4	5 (9%)	
128741	4−19, 136−142, 417−432, 419−432, 453−459	
PDSW	10 (47%)	
400533	16−35, 17−35, 36−49, 59−66, 61−66, 95−109, 98−109 128−137, 138−153, 143−153	
B17	5 (23%)	
400396	10−16, 22−31, 32−39, 50−58, 122−128	
	IβS Subcomplex	
B22	7 (50%)	
400513	1−15, 60−65, 66−93, 131−135, 139−150, 151−158, 160−175	
PDSW	6 (36%)	
400533	16−35, 36−49, 61−66, 95−109, 98−109, 143−153	
ASHI	6 (35%)	1−10
400525	59−77, 59−81, 82−98, 87−98, 160−170, 171−186	
B18	6 (34%)	
400397	8−21, 9−21, 22−34, 22−35, 22−35,* 38−56	
B17	10 (48%)	
400396	1−9, 1−11, 22−31, 25−31, 32−39, 50−58, 104−121, 104−121,* 104−128, 122−128	
SGDH	6 (25%)	1−10
400588	54−65, 136−150, 136−150,* 151−157, 162−176, 164−176	
B15	4 (27%)	
1346666	45−56, 46−56, 58−67, 73−85	
AGGG	3 (26%)	1−10
400572	37−46, 47−55, 49−55	
SDAP		1−10
MNLL		1−10

[a] The subunits (in bold) were identified by peptide mass mapping and N-terminal sequencing (see notes for Table 1). Asterisk indicates that peptide contains oxidized methionine residue.

(Iα, Iβ, and Iγ), which then dissociate further into smaller subcomplexes. It is clear that ND4 and ND5, on one hand, and ND1 and ND2, on the other hand, form what can be called "core" subcomplexes, which originate from the different fragments of the membrane domain (Iβ and Iγ, respectively). This information can be interpreted in the context of known low resolution structures of complex I.

To date, the best model for bovine complex I is a 22 Å reconstruction obtained by single-particle averaging of the molecules embedded in ice (*13*). The model, in agreement with previous models (*14*−*17*), is L-shaped, with two arms of approximately equal lengths of ~200 Å. Both arms have a narrow constriction closer to the interconnecting point, which effectively divide each arm into two components, larger and smaller. The assignment of membrane and peripheral arms as in ref 13 may be uncertain at the moment due to limited resolution. However, for the purposes of our discussion here this is not critical since both arms have rather similar features (constrictions) at low resolution. We show in Figure 5 a general outline of the molecule, using features from the reconstruction in ref *13*.

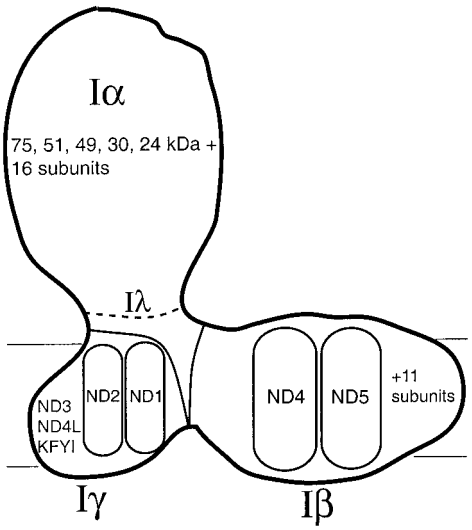

FIGURE 5: Proposed model for the subunit organization within the membrane domain of complex I. Data from the fragmentation of the membrane domain presented here and the low resolution outline of the molecule (*13*) are considered.

As the constriction divides the membrane arm at about a 1 to 2 ratio, it is reasonable to propose that smaller Iγ fraction (or most of it) contributes to the smaller part of the membrane arm while the Iβ subcomplex constitutes the bigger part (Figure 5). The bulk of the Iα subcomplex forms a peripheral arm. Such an arrangement places the ND1 subunit as part of Iγ in the proximity of the Iα subcomplex. This is in agreement with the proposed role of ND1 in the ubiquinone binding, which requires this subunit to be close to the redox centers of Iα (*1*, *30*−*32*). Our data suggest that ND2 is situated close to ND1, but the exact positions of other subunits from Iγ are not clear at the moment, and they are marked only as belonging to the Iγ fraction in Figure 5. Similarly, for Iβ we suggest that ND4 and ND5 will be adjacent to each other (Figure 5), but the positions of other subunits in Iβ are not clear.

Sequence comparisons suggest that ND2, ND4, and ND5 subunits evolved from a common ancestor and are probably involved in proton pumping (*33*, *34*). The ND4 and ND5 subunits are more closely related to each other than to ND2. The proposed hypotheses on the energy transducing mechanism of complex I can be roughly divided into two types: with direct (*35*, *36*) and indirect (*3*, *37*) coupling between the electron transport and proton translocation. Since proton/electron stoichiometry of compex I is very high (4−5 $H^+/2e^-$) it is possible that both mechanisms are at work. Our model in Figure 5 is compatible, for example, with the possibility that ND1/ND2 subunits are involved in a direct coupling site and ND4/ND5 subunits − in an indirect site, through long-range conformational changes. More detailed studies on this matter are required.

Previous studies on the fragmentation of complex I from different sources (reviewed in refs *1*, *4*, and *30*) contributed insight to its general structural organization, particularly in the peripheral arm. Our results on the fragmentation of the membrane arm allowed us to propose the first experiment-based model for the subunit organization in the membrane domain of complex I, which will be helpful for future structural studies.

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