Bovine Complex I Is a Complex of 45 Different Subunits*

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Mammalian mitochondrial complex I is a multisubunit membrane-bound assembly with a molecular mass approaching 1 MDa. By comprehensive analyses of the bovine complex and its constituent subcomplexes, 45 different subunits have been characterized previously. The presence of a 46th subunit was suspected from the consistent detection of a molecular mass of 10,566 by electrospray ionization mass spectrometry of subunits fractionated by reverse-phase high pressure liquid chromatography. The component was found associated with both the membrane arm of the complex, and it could not be resolved chromatographically from subunit SGDH (the subunit of bovine complex I with the N-terminal sequence Ser-Gly-Asp-His). It has now been characterized by tandem mass spectrometry of intact protein ions and shown to be a C-terminal fragment of subunit SGDH arising from a specific peptide bond cleavage between Ile-55 and Pro-56 during the electrospray ionization process. Thus, the subunit composition of bovine complex I has been established. It is a complex of 45 different proteins plus non-covalently bound FMN and eight iron-sulfur clusters.

Complex I (NADH ubiquinone oxidoreductase) catalyzes the first stages of oxidative phosphorylation in mitochondria by oxidizing NADH in the matrix and transferring the electrons via flavin mononucleotide and a series of iron-sulfur centers to ubiquinone in the inner membrane (1, 2). The passage of two electrons, one at a time, from NADH to ubiquinone is coupled to the translocation of four protons from the mitochondrial matrix to the intermembrane space (2, 3). The bovine enzyme is a membrane-bound assembly of ~45 polypeptides with a combined molecular mass approaching 1 MDa together with non-covalently bound FMN and eight iron-sulfur clusters (4). It has an L shape with one arm embedded in the membrane and another, the peripheral arm, orthogonal to it protruding into the mitochondrial matrix (5, 6). The complex can be dissociated under mild conditions into subcomplexes. Subcomplex Iλ, which corresponds to the peripheral arm, contains 15 subunits and all the known redox cofactors and the NADH binding site. Subcomplex Ia is subcomplex Iλ plus nine additional subunits (4, 7–11). Subcomplexes Iβ (13 subunits) and Iγ (6 subunits) provide most of the rest of the membrane arm (4, 8–12). On the basis of the presence of motifs for binding redox centers in various subunits and from the conservation of seven subunits of the complex in mitochondrial DNA sequences, 14 of the subunits were defined as providing the catalytic “core” of the enzyme, and the remaining subunits were defined as being “supernumerary” (2). Subsequently, it has been found that the simpler bacterial enzymes are composed of homologues of the core subunits and that the supernumerary subunits have no bacterial counterparts (13–15). Some of them appear to have functions that are unrelated directly to the electron transfer and proton pumping activities of the enzyme, but most of them have no known function (4, 9, 10, 16–19). The bovine enzyme has also provided a model for characterizing the much less readily available human enzyme (20). Mutations in subunits of human complex I have been associated with neurological and neuromuscular diseases (21).

In previous work, the protein subunit compositions of bovine complex I and subcomplexes Iλ, Iα, Iβ, and Iγ (2, 4, 8–12) have been investigated exhaustively by separating their subunits by a variety of different methods. They include one-dimensional gel electrophoresis, two-dimensional gel analysis involving equilibrium or non-equilibrium isoelectric focusing combined with SDS-PAGE, reverse-phase HPLC (11), and extraction of hydrophobic subunits with organic solvents coupled with hydrophilic interaction chromatography (3). Thereafter, the purified subunits were analyzed by Edman degradation and by mass spectrometric methods for measurements of intact protein masses by peptide mass fingerprinting and by amino acid sequencing of peptides by tandem MS (9, 10, 22–26). By these means, 45 different polypeptides have been identified in the complex and allocated to its peripheral and membrane arms. Stable post-translational modifications of all subunits except ND6 have been defined also (10, 11, 24, 26–28).

In numerous experiments, a component with a molecular mass of 10,566 Da has been detected by ESI-MS analysis of fractions resulting from reverse-phase HPLC separation of subunits from intact complex I and from subcomplex Iβ. This component co-eluted consistently with subunit SGDH, a known subunit of subcomplex Iβ (4, 11, 24). This mass was assumed to arise from an uncharacterized subunit of complex I (11, 25, 26), and until now, it has resisted characterization. No novel sequences were found in digests of appropriate fractions with trypsin, chymotrypsin, Asp-N, and cyanogen bromide or in combined digests with trypsin and cyanogen bromide (11), and the only components that were

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found were those arising from subunit SGDH. The 10,566 mass does not correspond to an N-terminal fragment of subunit SGDH. These results indicated the presence of an unknown protein with a modified N terminus and an unusual amino acid composition. Recently, the nature of the unattributed mass has been investigated by analysis of multiply charged protein ions by tandem MS in a quadrupole time-of-flight mass spectrometer. Partial amino acid sequences derived from these experiments have demonstrated that the 10,566 Da component is a fragment of subunit SGDH that arises by specific cleavage of a peptide bond during electrospray ionization of the protein.

**EXPERIMENTAL PROCEDURES**

Purification and Analysis of Complex I and Subcomplex Iβ from Bovine Heart Mitochondria—Complex I was purified from extracts of mitochondrial membranes made in the presence of n-dodecyl-β-D-maltoside (Anatrace; Maumee, OH) (9, 11, 12), and subcomplex Iβ was prepared from it as described previously.

The subunits of complex I and subcomplex Iβ were fractionated by reverse-phase HPLC, as described previously (11). Samples were introduced into mass spectrometers by flow injection (9, 25) or from a nanoelectrospray needle (29). Molecular masses of subunits were measured by positive ion ESI-MS. Mass measurements were made with a PE Sciex API III + triple quadrupole mass spectrometer (MDS Sciex; Concord, Ontario, Canada) using an orifice potential of between 70–80 V and potentials of ~4000 and 650 V on the capillary needle and interface plate, respectively, or 600–700 V in nanospray experiments (29). A Bio-Q quadrupole mass spectrometer (VG Instruments; Altrincham, UK) was also used with a cone voltage of 65 V and a capillary voltage of ~3000 V. The voltages applied to sample cones or orifices were chosen for the optimal ionization and transmission of myoglobin ions. Alternatively, both MS and tandem MS experiments were carried out with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF1) equipped with a Z-spray ion source and a nanoflow electrospray interface (Waters-Micromass; Manchester, UK) using a spray capillary of 20 μm inner diameter, a capillary voltage of 2500–2800 V, and sample and extraction cone voltages of between 50 and 60 and 4 V, respectively. Argon was used for collision-induced dissociation.

The Sciex API III+ triple quadrupole mass spectrometer was calibrated for measurements of protein mass as described previously (9), and the Q-TOF instrument was calibrated with horse heart myoglobin and bovine trypsinogen. Tandem mass spectra were interpreted manually. Leucine and isoleucine residues were not distinguished in any of these experiments, and they were assigned according to known sequences.

**RESULTS AND DISCUSSION**

Mass Spectral Analysis of the 10,566-Da Component—The ESI mass spectrum of the fraction from subcomplex Iβ that gave rise to the 10,566-Da component contained two overlapping series of molecular ions corresponding to the 10,566-Da component and to subunit SGDH (11, 24). Ions from subunit SGDH were more intense than those from the 10,566-Da component (Fig. 1). Their relative proportions differed in some spectra, but ions from subunit SGDH were usually predominant. The spectrum also contained minor ions that were not part of either series and are discussed later.

The multiply charged protein molecular ions produced by electrospray ionization were analyzed directly by tandem MS. A molecular ion (1057.5 m/z) containing ten protons in the ESI-MS spectrum of the 10,566 component was fragmented by collision-induced dissociation in a quadrupole time-of-flight mass spectrometer at low collision energy, similar to the conditions used for fragmenting peptide ions. The resultant fragment ion spectrum (Fig. 2) contained several singly charged ions from m/z 100–600. A pattern of ions was interpreted as the partial amino acid sequence Gly-Tyr-Val with a residual mass of 227 Da attached to the glycine residue. The presence of additional ions with masses of 28 Da less than this pattern, characteristic of a- and b-type ion pairs, suggested that the pattern derives from a-type ions, and therefore the N-terminal...
sequence of the component contains the sequence Gly-Tyr-Val, and the residual mass of 227 Da is N-terminal to the glycine (Fig. 2). The analysis of another protein ion (961.6 m/z) containing eleven positive charges produced a similar pattern of fragment ions and a partial amino acid sequence (data not shown). This partial sequence corresponds to residues 58–60 of the SGDH subunit, and the 227-Da residual mass is accounted for by Pro-56 and Glu-57. Hence, the 10,566 fragment has arisen by cleavage of the peptide bond between Ile-55 and Pro-56. The calculated molecular mass of residues 56–143 of SGDH is 10,566.0, which corresponds precisely with that of the unknown component.

Analysis of the same sample that was analyzed by mass spectrometry by Edman degradation gave only the N-terminal sequence of subunit SGDH, and the sequence from residue 56 onward was not detected. Moreover, the only band detected by SDS-PAGE analysis of the same sample was subunit SGDH. Therefore, the 10,566-Da component is not a proteolysis product of subunit SGDH, but it appears to have arisen by scission of an Ile–Pro bond during electrospray ionization. This proposal was confirmed by tandem MS by collision-induced dissociation of a protein ion (m/z 1116) containing 15 protons from the spectrum of the intact SGDH subunit (Fig. 3). This tandem mass spectrum contained ions that had arisen from the N-terminal region of subunit SGDH and also other ions that were common to both the ESI-MS spectrum of the two components (Fig. 1) and the tandem MS fragmentation of a protein ion from the 10,566-Da component (Fig. 2). A series of quintuply charged b-type fragments, b33–b42 (Fig. 3), defined the amino acid sequence IPVAIGITL correspond-
ing to residues 32–42 of subunit SGDH. The dipeptide Ile-Lys (residues 10–11 of subunit SGDH) was defined by the doubly charged fragment ions b10–b12. Several peaks (Fig. 3, denoted as A) corresponded to multiply charged molecular ions that defined the 10,566-Da component. Other fragment ions, labeled b2–b5 (Fig. 3, asterisks) defined the sequence PE)GYV. These ions were common to the tandem mass spectrum of 10,566 protein ions shown in Fig. 2. Other prominent ions with masses of 971.2 and 999.5 corresponded to b33 (4+) and b34 (4+) ions produced by cleavage of SGDH in a second Ile-Pro sequence at residues 34–35. These ions were observed also at lower abundance in the EI mass spectra (Fig. 1). A molecular mass corresponding to residues 1–55 of subunit SGDH with a predicted molecular mass of 6178.4 Da was not observed. Presumably, it had broken down into smaller fragments.

Protein Cleavage during Electrospray Ionization—The absence of fragmentation of biological macromolecules is a feature of soft ionization methods that has contributed to their success (30, 31), and therefore the cleavage of a protein during electrospray ionization is extremely unusual. The cleavage has been observed in three different mass spectrometers with different electrospray interfaces. They are 1) a triple quadrupole mass spectrometer with an electrospray interface, 2) a hybrid quadrupole time-of-flight instrument with a Z-spray electrospray source and a nanoflow interface, and 3) a second triple quadrupole time-of-flight instrument with a conventional ion-spray interface or with sample introduced from nanoelectrospray needles with single stage transfer of ions to high vacuum through a single orifice. Many thousands of proteins have been analyzed without fragmentation in the same instruments with the same operating parameters. However, it is known from collision-induced dissociation of peptides and from experiments, where the cone voltage is used to induce fragmentation, that bonds involving proline rupture most readily (32–34).

The Subunit Composition of Bovine Complex I—The experiments described here resolve a long-standing uncertainty about the subunit composition of bovine complex I. Recently, six of the seven hydrophobic subunits of the enzyme that are encoded in mitochondrial DNA have been characterized by mass spectrometry. Only subunit ND6 remains to be analyzed to complete the protein chemical characterization of the 45 subunits of the enzyme.

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