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Subunit analysis of bovine heart complex I by reversed-phase high-performance liquid chromatography, electrospray ionization–tandem mass spectrometry, and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry

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ABSTRACT

An effective method was developed for isolation and analysis of bovine heart complex I subunits. The method uses C18 reversed-phase high-performance liquid chromatography (HPLC) and a water/acetonitrile gradient containing 0.1% trifluoroacetic acid. Employing this system, 36 of the 45 complex I subunits elute in 28 distinct chromatographic peaks. The 9 subunits that do not elute are B14.7, MLRQ, and the 7 mitochondrial-encoded subunits. The method, with ultraviolet (UV) detection, is suitable for either analytical (<50 μg protein) or preparative (>250 μg protein) applications. Subunits eluting in each chromatographic peak were initially determined by matrix-assisted laser desorption/ionization–time-of-flight/mass spectrometry (MALDI–TOF/MS) with subsequent positive identification by reversed-phase HPLC–electrospray ionization (ESI)/tandem mass spectrometry (MS/MS) analysis of tryptic digests. In the latter case, subunits were identified with a 99% probability using Mascot for database searching and Scaffold for assessment of protein identification probabilities. The reversed-phase HPLC subunit analysis method represents a major improvement over previous separation methods with respect to resolution, simplicity, and ease of application.

Nicotinamide adenine dinucleotide (NADH)[2] dehydrogenase ubiquinol oxidoreductase (complex I, EC 1.6.5.3) is the largest, most complex, and least understood multisubunit enzyme of the electron transport chain system of bacteria and mitochondria. The enzyme catalyzes electron transfer from NADH to ubiquinone and couples this to the translocation of protons across the inner mitochondrial membrane [1,2]. The mammalian enzyme is composed of 45 nonidentical subunits [3], a noncovalently bound flavin mononucleotide (FMN), and eight iron–sulfur clusters, with a combined molecular mass of approximately 980 kDa [1,4]. Seven subunits are encoded by mitochondrial DNA, and the remaining subunits are nuclearly encoded [5,6].

Subunit analysis of complex I is difficult by conventional methodologies because of its high degree of complexity. Two approaches have been used to resolve the subunits: (i) two-dimensional resolution of subunits by isoelectric focusing combined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (high- and low-pH isoelectric focusing is required to resolve all subunits [7]) and (ii) fractionation of the enzyme into three major subcomplexes (Iα, Iβ, and Iγ) followed by reversed-phase high-performance liquid chromatography (RP–HPLC) and mass spectrometry (MS) [8]. In each case, subunit identifications were based on either N-terminal amino acid sequencing of the purified subunits or MS analysis of tryptic peptides [8–10].[3] However, each experimental approach involves several procedures and provides only a qualitative assessment of the subunit content.

Interest in the individual subunits of complex I has recently increased because chemical modification of individual subunits has been linked to important biological processes. Examples include

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[2] *Abbreviations used:* NADH, nicotinamide adenine dinucleotide; FMN, flavin mononucleotide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RP–HPLC, reversed-phase high-performance liquid chromatography; MS, mass spectrometry; ROS, reactive oxygen species; BCA, bicinchoninic acid; MWCO, molecular weight cutoff; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; TFA, trifluoroacetic acid; RT, room temperature; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; ATP, adenosine triphosphate.

[3] Subunit nomenclature for complex I is that described by Finel and coworkers [9].

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(i) pathological states initiated by reactive oxygen species (ROS), such as Parkinson's disease [11], cardiac ischemia and reperfusion injury [12], and Alzheimer's disease [13], and (ii) phosphorylation sites implicated in regulation of complex I function [14–19]. Because studies focus on individual complex I subunits, an analytical method for their separation and analysis is required. Our laboratory previously developed RP–HPLC methods for resolving all but the most hydrophobic, mitochondrial-encoded subunits of bovine heart cytochrome *c* oxidase [20,21] and cytochrome bc_1 [22]. Combined with MS analysis of the resolved subunits, we have used this methodology to detect and quantify ROS-induced damage at selective sites within cytochrome *c* oxidase [23,24]. Although the subunit content of the two complexes is much less complicated than complex I, the polarity, hydrophobicity, and mass range are similar, suggesting that subunit analysis of complex I by RP–HPLC is feasible.

Materials and methods

Materials

Reagents and their sources were as follows: dodecyl maltoside (Anatrace), bicinchoninic acid (BCA) assay reagents (Pierce Biotechnology), phenylmethanesulfonylfluoride (USB), NADH and ubiquinone-1 (Sigma–Aldrich), and sequencing-grade bovine pancreatic trypsin (Promega). Ultracell YM-10, regenerated cellulose centrifugal filters (10,000 molecular weight cutoff [MWCO]), were purchased from Millipore, the C18 high-performance RP–HPLC column (300 Å, 4.6 × 250 mm, 10 μm) was purchased from Vydac (cat. no. 218TP104), and the high-performance Q-Sepharose column (24.5 × 0.9 cm) was purchased from Amersham Biosciences. All other chemicals were of analytical grade.

Isolation of complex I from bovine heart

Complex I was isolated from bovine heart mitochondria according to the procedure of Sazanov and coworkers [8] with minor modifications. Mitochondrial membranes were suspended to a final protein concentration of 12 mg/ml (determined by the Biuret method) in 20 mM Tris–Cl buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 300 μM phenylmethylsulfonylfluoride (PMSF), and 9.5 mg/ml dodecyl maltoside. The suspension was placed on ice and stirred for 30 min, followed by centrifugation for 30 min at 30,000*g*. The resulting supernatant containing dodecyl maltoside-solubilized proteins was applied to a high-performance Q-Sepharose column (24.5 × 0.9 cm) that had been equilibrated with 20 mM Tris–Cl buffer (pH 7.4) containing 1 mM EDTA, 10% ethylene glycol, and 2 mM dodecyl maltoside at a flow rate of 0.5 ml/min. Bound proteins were eluted from the column at 0.5 ml/min by increasing the NaCl concentration of the buffer as follows: (i) linear gradient from 0 to 250 mM NaCl in 40 min, (ii) isocratic elution with buffer containing 250 mM NaCl for 140 min, and (iii) elution of complex I with a linear gradient from 250 to 350 mM NaCl in 80 min. Eluant containing the enzyme was collected, and the protein was concentrated by ultracentrifugation in a Beckman 50Ti rotor at 165,000*g* at 4 °C. The resulting pellets containing complex I were resuspended in 20 mM Tris–Cl buffer (pH 7.4) containing 1 mM EDTA, 10% ethylene glycol, 2 mM dodecyl maltoside, and 125 mM NaCl and were rechromatographed on the Q-Sepharose high-performance column using the same gradient elution conditions. Once again, complex I was concentrated by ultracentrifugation, and the pellets were resolubilized in 20 mM Tris–Cl buffer (pH 7.4) containing 1 mM EDTA, 10% ethylene glycol, 2 mM dodecyl maltoside, and 350 mM NaCl. The protein concentration was determined by BCA assay using bovine serum albumin as a standard. The enzyme was stored in 0.5-mg aliquots at −80 °C.

Molecular activity assay

The NADH/ubiquinone oxidoreductase activity of complex I was determined spectrophotometrically by following the oxidation of NADH at 340 nm using $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ [25]. The reaction was initiated by adding 12 μM NADH to 3.6 nM complex I (solubilized in 50 mM Tris–acetate buffer [pH 7.8] containing 2 mM dodecyl maltoside and 100 μM ubiquinone-1). The NADH/ubiquinone$_1$ activity of purified complex I was 9 to 11 (mol NADH) (mol complex I)$^{-1}$ (s)$^{-1}$.

Resolution of subunits by RP–HPLC

Isolated complex I (250 μg in 160 μl) was dialyzed against 50 mM Tris–acetate buffer (pH 7.8) containing 350 mM NaCl and 0.2 mM dodecyl maltoside) to remove ethylene glycol, and trifluoroacetic acid (TFA) was added to a final concentration of 0.1%. After 5 min at room temperature (RT) in 0.1% TFA, the sample was applied at 1.0 ml/min to a C18 RP–HPLC column (300 Å, 4.6 × 250 mm, 10 μm). The HPLC system (Waters), controlled by a Waters System Interface Module using Millennium^{32} software (version 3.20, Waters), consisted of two 501 HPLC pumps, a manual injector with a 2-ml loop, a Vydac C18 high-performance guard column, the Vydac C18 column, and a Waters photodiode array detector. The mobile phases consisted of water containing 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). The following gradient was applied at a flow rate of 1.0 ml/min: linear gradient from 0 to 25% B in 5 min, linear gradient from 25 to 30% B in 5 min, linear gradient from 30 to 55% B in 95 min, isocratic elution with 55% B for 5 min, linear gradient from 55 to 100% B in 36 min, isocratic elution with 100% B for 5 min, linear gradient from 100% B to 100% A in 6 min, and isocratic elution with 100% buffer A for 30 min to reequilibrate column for the next injection. Elution of the protein was monitored at 214 nm, and elution fractions were collected at 1-min intervals.

MALDI–TOF/MS of RP–HPLC-purified subunits

Each 1-ml RP–HPLC fraction was made 0.8 mM in dodecyl maltoside and reduced to approximately 50 μl by vacuum centrifugation at 65 °C (SpeedVac), and 1 μl of the concentrated sample was spotted on the matrix-assisted laser desorption/ionization (MALDI) target plate. After partial drying, 1 μl of a saturated solution of sinapinic acid in 50% acetonitrile containing 0.1% TFA was added to the applied complex I and dried at RT under a gentle stream of air. MALDI–time-of-flight (TOF) mass spectra for each fraction were acquired on an Applied Biosystems Voyager-DE STR mass spectrometer in reflectron mode by averaging the results of 100 laser shots. Mass assignments were made by the close external calibration method using insulin, thioredoxin, apomyoglobin, and enolase as references. Noise reduction and smoothing algorithms were applied to each spectrum.

HPLC–ESI-MS/MS analysis of tryptic digests of RP–HPLC fractions

Each 1-ml sample was concentrated to approximately 50 μl by vacuum centrifugation at 65 °C, adjusted to approximately 200 μl with 50 mM NH_4HCO_3 containing 80% acetonitrile, and digested with trypsin (0.5 μg) at 37 °C for 4 h. The resulting digests were dried completely by vacuum centrifugation, dissolved in 10–15 μl of 0.1% TFA, and 5 μl was analyzed by capillary HPLC–electrospray ionization (ESI)–tandem mass spectrometry (MS/MS). Spectra were acquired on a Thermo Fisher LTQ linear ion trap mass spec-