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Research paper

Functional and structural evaluation of bovine heart cytochrome c oxidase incorporated into bicelles☆

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ABSTRACT

Bilayered long- and short-chain phospholipid assemblies, known as bicelles, have been widely used as model membranes in biological studies. However, to date, there has been no demonstration of structural or functional viability for the fundamental mitochondrial electron transport complexes reconstituted into or interacting with bicelles. In the present work, bicelles were formed from the mixture of long- and short-chain phospholipids, specifically 14:0 and 6:0 phosphatidylcholines (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, (DHPC)). Isolated from bovine heart, cytochrome *c* oxidase was successfully incorporated into bicelles. Bicelles and cytochrome *c* oxidase incorporated into bicelles ("proteobicelles") were characterized by absorption spectroscopy, dynamic light scattering, atomic force microscopy, sedimentation velocity and differential scanning calorimetry. It was demonstrated that at total concentration of phospholipids $C_L = 24$ mM and the molar ratio (q) of long-chain DMPC over short-chain DHPC equal to 0.4, the diameter of bicelles formed at neutral pH is in the range of 30–60 nm with the thickness of bicelles of about 4 nm. Adding cytochrome *c* oxidase to bicelles unified the size of the resulting proteobicelles to about 160 nm. Cytochrome *c* oxidase in bicelles was fully reducible by artificial donors of electrons, exhibited "normal" reaction with external ligands, and was fully active. Both, sedimentation velocity analysis and temperature-induced denaturation indicated that enzyme in bicelles is monomeric. We concluded that cytochrome *c* oxidase in bicelles maintains its structural and functional integrity, and that bicelles can be used for more comprehensive investigation of cytochrome *c* oxidase and most likely other mitochondrial electron transfer complexes.

1. Introduction

Cytochrome *c* oxidase (EC 1.9.3.1; Complex IV, CcO) is a large transmembrane protein-phospholipid complex found in bacteria and mitochondria of eukaryotes. This multi-subunit enzyme catalyzes the four-electron reduction of dioxygen to water, a reaction coupled to proton translocation across the inner mitochondrial membrane [1]. Mammalian CcO is consisting of 13 dissimilar subunits, three or four tightly bound phospholipids, and four metal centers (Cu_A, heme *a*, heme a_3, and Cu_B) with a combined molecular weight of ~205 000 for the monomeric enzyme [2]. Mitochondrial proteins represent an important class of membrane proteins that remain especially difficult to study. One feature that most directly impacts structural and functional studies of this multi-subunit protein-lipid complex *in vitro* is its high hydrophobicity. For example, mitochondrially encoded CcO subunits I, II and III are very hydrophobic and have Gravy hydropathy scores of 0.685, 0.247 and 0.413, respectively [3,4]. To overcome issues due to the amphiphillic character of CcO, different strategies have been used, such as

Abbreviations: CcO, cytochrome *c* oxidase; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; PL, phospholipids; C_L, total molar concentration of phospholipids in bicelles; q, molar ratio of long-chain DMPC over short-chain DHPC; EDTA, ethylenediaminetetraacetic acid; DM, n-Dodecyl β-D-maltoside; TX-100, Triton X-100; AFM, atomic force microscopy; DSC, differential scanning calorimetry; DLS, dynamic light scattering.

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solubilization with different single detergents [5,6], mixture of detergents [7], or incorporation of enzyme into phospholipids multilamellar or unilamellar vesicles [8,9]. Although detergents are capable of effectively solubilizing such hydrophobic proteins, they cannot mimic the interactions between proteins and lipid membranes, one of the essential features of cell operation. Indeed, numbers of biochemical and biophysical studies have demonstrated that the lipid bilayer is important for structural and functional integrity of membrane proteins. Therefore, reconstitution of membrane proteins into phospholipid-based bilayer (liposomes) is one of the most useful techniques to study the structure and function of these proteins. In fact, incorporation of CcO into liposomes was successfully used for the examination of proton pumping mechanism, ligand binding, etc. [8–10]. However, the liposomal turbidity does not permit the use of some of the experimental techniques, such as absorbance spectroscopy or circular dichroism. This issue can potentially be overcome by finding new methodology for biophysical, enzymatic or structural investigations *in vitro*. Such approach was recently applied by Öjemyr et al. [11]. In this work two different terminal oxidases, aa_3 from *Rhodobacter sphaeroides* and ba_3 from *Thermus thermophilus* were reconstituted into nanodiscs composed of soybean lipids using the scaffold protein.

To probe the structure of membrane proteins in their native environment (i.e., bilayer), a new phospholipid mixture known as "bicelle" has been developed [12,13]. Bicelles are nanostructures formed by a long- and a short-chain phospholipids or detergent dispersed in aqueous solution. In contrast to liposomes, bicelles do not have an aqueous inside and there properties are close to a liquid crystal phase with one or two-dimensional ordering. Moreover, bicelles are transparent and, thus, amenable to optical spectroscopy. To date, the combination of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) as long-chain, bilayer-forming component with 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) as short-chain component (or combination with 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)) has remained the most popular choice for bicelle formation. The morphology of bilayered lipid mixtures highly depends on total concentration of lipids, ratios between long- and short-chain lipids, lipids composition, presence or absence of monovalent or divalent cations, time of hydration and/or temperature [14–16]. Several structural models for bicelles have been reported, such as disc-shaped, cylindrical micelles or perforated lamellae. However, the most recognized structural feature is a disc-shaped structure with the long-chain lipids organizing the disc plane and the short-chain lipids stabilizing the edges (or the rim) of the bicelles. This system is suitable for a number of various applications in different research areas, e.g., solution and solid-state NMR study on membrane proteins [17], crystallization of membrane proteins [18] circular dichroism (CD), and small-angle neutron scattering (SANS) [19,20]. Surprisingly, one very important group of proteins – mitochondrial electron transfer complexes, was completely ignored.

Our present work was designed to investigate the interaction of bicelles formed from the mixture of DMPC and DHPC with isolated mitochondrial electron transfer Complex IV or cytochrome *c* oxidase.

2. Materials and Methods

2.1. Materials

Both, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) in chloroform were obtained from Avanti Polar Lipids, Inc. and used without further purification. Triton X-100, sodium cholate, horse heart cytochrome *c* (≥95% purity), hydrogen peroxide and sodium dithionite were purchased from Sigma–Aldrich. *n*-Dodecyl β-D-maltoside was obtained from Anatrace. All other chemicals were reagent grade.

2.2. Methods

2.2.1. Bovine cytochrome c oxidase

Two forms of isolated from bovine heart CcO, designated as A and B forms, were used in this work. Preparation A (slow reacting with the external ligands) was isolated and purified from mitochondrial Keilin-Hartree particles by the method of Fowler et al. [21] as modified by Capaldi and Hayashi [22]. The final enzyme precipitate was solubilized at ~150 μM heme aa_3 in 0.1 M NaH_2PO_4 buffer (pH 7.4) containing 25 mM sodium cholate and then frozen in liquid nitrogen. CcO subunit composition was verified by reversed-phase HPLC as described previously [23]. The cytochrome *c* oxidase concentration was determined from the dithionite reduced minus fully oxidized spectrum and quantified using molar extinction coefficient $\Delta\varepsilon_{605\text{-}630} = 27\ mM^{-1}\ cm^{-1}$. The phosphorus content (15–20 P/aa_3) was determined as described previously [24]. Cytochrome *c* oxidase activity was measured spectrophotometrically following the pseudo-first-order rate of oxidation of 25–30 μM ferrocytochrome *c* by 2 nM cytochrome *c* oxidase at pH 7.0 in 25 mM phosphate buffer containing either 2 mM dodecyl maltoside or DMPC/DHPC mixture. Temperature effect on CcO activity was analyzed after solubilized enzyme was heated from 25 °C to 75 °C in water bath at rate of 1.5 °C/min. At 5 °C intervals, a 10 μL aliquot was removed and diluted with 190 mL ice-cold assay buffer, containing 2 mM DM. The activity was analyzed at 25 °C. Preparation B (reacting fast with the external ligands) involves Triton X-100 solubilization of enzyme from mitochondria at neutral pH followed by purification using ion-exchange chromatography in the presence of Triton X-100. The resulting CcO was concentrated to ~200 μM and frozen in liquid nitrogen. The phosphorus content (5–10 P/aa_3) was determined after extraction of phospholipids by the method of Bligh and Dyer [25]. Enzyme isolated in TX-100 according to modified protocol of Soulimane and Buse [26,27] was a gift from Dr. M. Fabian from The Center for Interdisciplinary Bioscience P.J. Safarik University in Kosice, Slovakia.

2.2.2. Formation of micelles, bicelles and proteobicelles

The different amount of single phospholipid (DHPC) or mixture of DMPC and DHPC was dried out under stream of nitrogen. The resulting PL film was suspended by brief and gentle vortexing in appropriate buffer for 10–20 s at room temperature. The hydration of phospholipids and the formation of bicelles was achieved by incubation of PLs for about 3 h at room temperature with an occasional (every 20–30 min) vortexing. The resulting clear bicellular solution was obtained. CcO incorporation to bicelles was accomplished by addition of a few microliters of concentrated detergent-solubilized enzyme (final CcO concentration was ~5 μM) to bicelles. All measurements with CcO in bicelles were performed after incubation of protein-phospholipid mixture for 30–60 min at room temperature.

2.2.3. Absorption spectroscopy

All spectra and kinetic measurements were recorded digitally using either a SLM Aminco 3000 or Jasco V-630 diode array spectrophotometer in 1 ml semimicro cuvettes with blackened walls and optical path of 1 cm. Difference spectra of the H_2O_2 or cyanide-induced spectral shifts were calculated by subtracting the oxidized absolute spectra of CcO from the ligand-induced absolute spectra.