Biochimie 105 (2014) 159–164

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Bound cardiolipin is essential for cytochrome *c* oxidase proton translocation

Andrej Musatov*, Neal C. Robinson**

Department of Biochemistry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, TX 78229-3900, USA

ARTICLE INFO

Article history:
Received 19 May 2014
Accepted 7 July 2014
Available online 16 July 2014

Keywords:
Cytochrome *c* oxidase
Proton pumping
Cardiolipin
Liposomes

ABSTRACT

The proton pumping activity of bovine heart cytochrome *c* oxidase (CcO) is completely inhibited when all of the cardiolipin (CL) is removed from the enzyme to produce monomeric CcO containing only 11 subunits. Only dimeric enzyme containing all 13 subunits and 2–4 cardiolipin per CcO monomer exhibits a "normal" proton translocating stoichiometry of ~1.0 H^+ per/e^- when reconstituted into phospholipid vesicles. These fully active proteoliposomes have high respiratory control ratios (RCR = 7–15) with 75–85% of the CcO oriented with the cytochrome *c* binding sites exposed to the external medium. In contrast, reconstitution of CL-free CcO results in low respiratory control ratios (RCR < 5) with the enzyme randomly oriented in the vesicles, i.e., ~50 percent oriented with the cytochrome *c* binding site exposed on the outside of the vesicle. Addition of exogenous CL to the CL-free enzyme completely restores electron transport activity, but restoration of proton pumping activity does not occur. This is true whether CL is added to CL-free CcO prior to reconstitution into phospholipid vesicles, or whether CL is included in the phospholipid mixture that is used to form the vesicles. Another consequence of CL removal is the inability of the 11-subunit, CL-free enzyme to dimerize upon exposure to either cholate or the cholate/PC/PE/CL mixture used during proteoliposome formation (monomeric, 13-subunit, CL-containing CcO completely dimerizes under these conditions). Therefore, a major difference between reconstitution of CL-free and CL-containing CcO is the incorporation of monomeric, rather than dimeric CcO into the vesicles. We conclude that bound CL is necessary for proper insertion of CcO into phospholipid vesicles and normal proton translocation.

1. Introduction

Cardiolipin (CL) is a major mitochondrial glycerophospholipid that is localized exclusively within the mitochondrial inner membrane [1–3]. CL is essential for the functional and/or structural integrity of a number of mitochondrial inner membrane protein complexes including cytochrome *c* oxidase (CcO) [4–7], cytochrome bc_1 [8–11], succinate dehydrogenase (complex II) [12], NADH:CoQ oxidoreductase (complex I) [13,14], ADP/ATP carrier [15], and ATP synthase [16,17]. Among these proteins, CcO is the best characterized mitochondrial complex that depends upon CL.

Bovine heart cytochrome *c* oxidase is a member of the heme-copper oxidase super-family. It catalyzes the reduction of oxygen to water by ferrocytochrome *c* with coincident translocation of protons across the mitochondrial inner membrane [18–20]. The structural organization of bovine heart CcO is quite complicated. Within three dimensional crystals, bovine CcO is a dimer of 13-subunit monomers [21,22]. This organization is believed to reflect its structure within the inner mitochondrial membrane. Three of the thirteen subunits are mitochondrially encoded, ten are nuclearly encoded [23]. Mitochondrially encoded subunits I and II contain all four of the redox centers, (Cu_A, heme *a*, heme a_3 and Cu_B), that participate in individual electron transfer steps involved in the reduction of oxygen by ferrocytochrome *c* [22]. The functional importance of the third mitochondrially encoded subunit, subunit III, is currently not completely understood, although

Abbreviations: CcO, bovine heart cytochrome *c* oxidase; CL, cardiolipin; RP-HPLC, reversed phase high-performance liquid chromatography; RCR, respiratory control ratio; PLA2, phospholipase A2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; TMPD, *N,N,N′,N′*-tetramethyl-*ρ*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

* Corresponding author. Present address: Institute of Experimental Physics Slovak Academy of Sciences, Watsonova 47, 04001 Kosice, Slovakia. Tel.: +421 55 720 4132; fax: +421 55 633 6292.

** Corresponding author. Tel.: +1 210 567 3754; fax: +1 210 567 6595.

E-mail addresses: musatov@saske.sk (A. Musatov), robinson@uthscsa.edu (N.C. Robinson).

http://dx.doi.org/10.1016/j.biochi.2014.07.005
0300-9084/

thought to be involved in the proton pumping activity of CcO [24–26]. It also may function to stabilize the enzyme. For example, within the *Rhodobacter sphaeroides* enzyme, subunit III stabilizes the enzyme's catalytic lifespan and thus prevents its suicide inactivation [27,28].

Purified, detergent-solubilized bovine heart CcO has a structural and functional requirement for 3-4 CL that are tightly bound to the proteins within each monomer [5,6,29,30]. Two of these CL are clearly visible in the crystal structure and are located between transmembrane helices of adjacent protein subunits [21]. If CL is completely removed and replaced by detergent or other phospholipids, electron transport activity decreases by 50–60 percent of normal [5,6]. Full electron-transport activity is restored by exogenous CL, but not by other phospholipids. Coincident with CL removal is the irreversible dissociation of subunits VIa and VIb [5,6]. Removal of CL also destabilizes the association of subunits III and VIIa making them more susceptible to dissociation by structural perturbants [6]. These effects are consistent with the fact that CL is bound adjacent to subunits III, VIb, and VIIa, while subunit VIIa rests on top of subunit VIIb [21]. Cardiolipin stabilizes all of these interactions by forming strong ionic interactions between the negatively charged polar head group of CL and the positively charged side chains of the protein while the fatty acid tails contact apolar amino acids. A direct consequence of CL removal and dissociation of subunits VIa and VIb is the inability of the CcO monomers to dimerize since subunits VIa and VIb participate in major protein contacts at the dimer interface. CL, therefore, acts as a type of "glue" that stabilizes the quaternary structure of CcO. CL has been suggested to serve a similar role in the formation of supercomplexes within the mitochondrial inner membrane [31,32].

The importance of dimeric CcO to its function has been the subject of debate for several decades and is still not completely understood. Dimerization clearly is not essential for normal electron transfer activity since monomeric CcO has the same activity as the dimer. However, the possibility that dimerization plays a critical role in proton-translocation remains a topic of discussion. To address this problem, we have analyzed the effect of CL removal on proton translocation activity of CcO. Because CL removal prevents CcO self-association, these studies should help address the question whether such structural changes restrain alter, or inhibit the proton pumping activity of the enzyme.

2. Materials and methods

2.1. Materials

Bovine cardiolipin (1,3-diphosphatidyl-*sn*-glycerol); dioleoylphosphatidylcholine (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC); and dioleoylphosphatidylethanolamine (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE), each in chloroform, were obtained from Avanti Polar Lipids, Inc. Dodecyl maltoside (DM) was obtained from Anatrace. Horse heart cytochrome *c* (type III); *Crotalus atrox* venom; *N*,*N*,*N*′,*N*′-tetramethyl-1,4-benzenediamine dihydrochloride (TMPD); carbonyl cyanide 3-chlorophenylhydrazone (CCCP); valinomycin; phenol red; sodium ascorbate; and sodium cholate were obtained from Sigma Chemical Co. Bio-Beads SM-2 were purchased from Bio-Rad Laboratories. All other chemicals were reagent grade.

2.2. Methods

Bovine cytochrome c oxidase was isolated from Keilin-Hartree particles [33] by the method of Fowler et al. [34] with modifications described previously [35]. HiTrapQ anion-exchange column chromatography was subsequently used to remove small amounts of contaminating cytochrome bc_1 and all phospholipids except for 2-4 CL that remain tightly bound to the enzyme [5]. The purified complex had a molecular activity of 340–370 s^{-1} when assayed spectrophotometrically by following the pseudo-first order rate of ferrocytochrome *c* (30 μM) oxidation by 1.8 nM CcO in 50 mM phosphate buffer pH 7.2 containing 2 mM DM as described by Dale and Robinson [36]. Cardiolipin free detergent-solubilized CcO was obtained by treatment of CL-containing CcO with phospholipase A2 (PLA2) [6].

2.2.1. Incorporation of cytochrome c oxidase into liposomes

CcO-containing vesicles were prepared by a minor modification of the method of Casey [37]. The procedure was as follows: a suspension of 50 mg of phospholipids (defined below) in 1 mL of 0.1 M HEPES buffer, pH 7.4, containing 2% sodium cholate was sonicated on ice under nitrogen using a Branson model 250 Sonifier, until the solution became clear (~5 min). Unless otherwise specified, the suspension of phospholipids contained 25 mg DOPC and 25 mg DOPE (1:1 w/w). The purified CcO was exposed to Bio-Beads SM-2 to remove excess dodecyl maltoside before it was mixed with the phospholipid mixture. The procedure was to add 150 mg of Bio-Beads SM-2 per mL of CcO (2 mg/mL), stir the mixture for 15 min at 25 °C, and then remove the Bio-Beads by brief centrifugation [38]. The resulting detergent-depleted CcO was subsequently added to the phospholipid-cholate mixture described above at a final protein concentration of 1 mg/mL and a final protein to phospholipid ratio 1:40 (mg/mg). At the high molar ratio of phospholipid to protein that were used to prepare either CL-rich, or CL-free proteoliposomes, all of the enzyme was incorporated into the vesicles as indicated by the elution of a single peak when the products were analyzed by gel filtration chromatography (data not shown). The CcO-containing proteoliposomes were then formed by extensive dialysis of the CcO-phospholipid-cholate solution against 0.1 M HEPES, pH 7.4 for 8 h; 10 mM HEPES, 40.0 mM KCl, 50.0 mM sucrose, pH 7.4 for 12 h; and finally against 1 mM HEPES, 44.0 mM KCl, 55.0 mM sucrose, pH 7.4 for 8 h.

2.2.2. Characterization of proteoliposomes

The orientation of CcO that had been incorporated into vesicles was determined using visible difference spectroscopy ($\Delta A_{606-630}$) after reduction of cytochrome *a* within a CcO–CN complex [39]. Two impermeant electron donors (5 mM sodium ascorbate and 3.5 μM cytochrome *c*), were added followed by the addition of a permeant electron donor (2.5 mM TMPD). The ratio of $\Delta A_{606-630}$ before and after addition of TMPD yields the fraction of CcO with its cytochrome *c* binding site exposed on the external surface of the proteoliposome. The RCR of reconstituted CcO was calculated from the ratio of the rate of oxidation of ferrocytochrome *c* before and after the addition of 3 μM CCCP plus 30 μM valinomycin recorded at 550 minus 540 nm [18].

2.2.3. Measurement of proton translocation

Proton pumping activity of CcO incorporated into vesicles was measured spectrophotometrically using phenol red as the absorption pH indicator. Extra-vesicular pH changes were detected at 600 nm minus 564 nm [40]. Absorbance changes were calibrated using a standard solution of HCl. The H^+/e^- ratio was calculated by dividing the moles of protons extruded by the moles of cytochrome *c* added.

2.2.4. Cytochrome c oxidase subunit composition analysis

The content of the three mitochondrially encoded subunits was determined by quantitative scanning of Coomassie brilliant blue stained SDS-PAGE gels. The content of the 10 nuclearly encoded CcO subunits was determined using C18 reversed phase HPLC as described by Sedlák and Robinson [6] (Fig. 1).