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Delipidation of Cytochrome *c* Oxidase from *Rhodobacter sphaeroides* Destabilizes its Quaternary Structure

Andrej Musatov^{a,b}, Rastislav Varha^{a,c}, Jonathan P. Hosler^d, and Erik Sedlák^{a,c,e,*}

^aDepartment of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78229-3900, U.S.A. ^bDepartment of Biophysics, Institute of Experimental Physics, Slovak

Academy of Sciences, Watsonova 47, 04001 Košice, Slovakia; andrej.musatov@gmail.com

^cDepartment of Biochemistry, P.J. Šafárik University, Moyzesova 11, 04001 Košice, Slovakia;

rastislav.varhac@upjs.sk ^dDepartment of Biochemistry, The University of Mississippi Medical

Center, Jackson, Mississippi 39216, U.S.A.; jhosler@umc.edu ^eCentre for Interdisciplinary

Biosciences, P.J. Šafárik University, Jesenná 5, 04001 Košice, Slovakia, P.J. Šafárik University, Jesenná 5, 04001 Košice, Slovakia

Abstract

Delipidation of detergent-solubilized cytochrome *c* oxidase isolated from *Rhodobacter sphaeroides* (*Rbs*-CcO) has no apparent structural and/or functional effect on the protein, however affects its resistance against thermal or chemical denaturation. Phospholipase A₂ (PLA₂) hydrolysis of phospholipids that are co-purified with the enzyme removes all but two tightly bound phosphatidylethanolamines. Replacement of the removed phospholipids with nonionic detergent decreases both thermal stability of the enzyme and its resilience against the effect of chemical denaturants such as urea. In contrast to nondelipidated *Rbs*-CcO, the enzymatic activity of PLA₂-treated *Rbs*-CcO is substantially diminished after exposure to high (>4M) urea concentration at room temperature without an alteration of its secondary structure. Absorbance spectroscopy and sedimentation velocity experiments revealed a strong correlation between intact tertiary structure of heme regions and quaternary structure, respectively, and the enzymatic activity of the protein. We concluded that phospholipid environment of *Rbs*-CcO has the protective role for stability of its tertiary and quaternary structures.

Keywords

membrane protein stability; protein-lipid interaction; delipidation; thermal denaturation; chemical denaturation

*To whom correspondence should be addressed: Erik Sedlák, erik.sedlak@upjs.sk.

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1 Introduction

The *aa₃*-type cytochrome c oxidase (CcO) is a multisubunit integral membrane protein with a crucial role in energy metabolism in many aerobic organisms. It is localized within the inner membrane of all mitochondria and in the cytoplasmic membrane of many bacteria. CcO catalyzes the reduction of oxygen to water by cytochrome *c* and couples this exergonic redox reaction to the translocation of protons across the membrane. Proton translocation by CcO contributes to the proton-motive force that powers ATP synthesis. The number of CcO subunits varies among different organisms, from four in prokaryotes [1] to thirteen in mammalian mitochondrial CcO [2–4]. Regardless of the origin, the catalytic core of CcO is composed of three highly hydrophobic subunits. Subunits I and II contain all of the redox-active centers. Subunit III is necessary for optimizing the proton pumping capability and the catalytic lifetime of the enzyme [5,6].

In the membrane, the transmembrane regions of integral membrane proteins are, of course, surrounded by annular phospholipid (PL). Isolation and purification of these proteins in detergent results in the removal of much PL, but some PL remains bound at specific sites [7]. Bound PL was seen in crystal structures of bovine heart CcO [8], bacterial CcO [1,9], other electron transfer complexes [10–12] and the photosynthetic reaction center of *Rhodobacter sphaeroides* [13]. Studies have revealed that bound PLs affect both structural and functional integrity of membrane protein complexes, i.e. the bound PLs are essential components of the individual enzymes or supercomplexes [10,12,14–18].

In many cases the bound PLs argued to have structural and/or functional roles are identified as cardiolipin (CL). For example, experimental data and molecular dynamic simulations suggest the direct or indirect participation of bound CL in proton uptake and proton pumping by CcO [19–21]. Other findings indicate a role for bound CL in stabilizing the quaternary structures of CcO and the photosynthetic reaction center [22–24]. Studies also indicate a functional and/or stabilization role for phospholipids other than CL. For example, CcO contains evolutionarily conserved lipid binding sites within subunit III that contain PE molecules in bacterial CcO [1], although CL can be modeled into bacterial subunit III [21], and PG in a structure of mammalian CcO [8]. Modification of these lipid binding sites within subunit III inhibits 1) the assembly of subunit III into the CcO complex, and 2) the protective function of subunit III on CcO activity even when all of the subunit is present [18]. Negatively charged PG stabilizes a dimer of photosystem II [25] similar to the role of negatively charged CL in mammalian CcO [20]. Bound PE has been proposed to stabilize a bacterial photosynthetic reaction center [26] but to destabilize respiratory supercomplexes in mitochondria [16].

In the present work, a mild delipidation method utilizing phospholipase A₂ (PLA₂), developed previously to delipidate bovine heart CcO and the bc₁ complex, was applied to the purified *aa₃*-type CcO of *Rb. sphaeroides* (*Rbs*-CcO) [11,22]. *Rbs*-CcO has been extensively studied (along with the similar enzyme from *Paracoccus denitrificans*) due to its functional similarity to mitochondrial CcO and its high structural similarity to the catalytic core of eukaryotic CcO. Our goal was to examine the effect of annular and protein-bound PL on function and structure of CcO using the structurally simpler bacterial CcO.

2 Material and methods

2.1 Preparation of CcO, reduced cytochrome c and phospholipase A₂

Purification of the *aa3*-type CcO of *Rbs*-CcO was achieved using a six-histidine tag on the C-terminus of subunit I performed as described by Zhen et al. [27]. CcO concentrations were calculated for the oxidized enzyme using $\epsilon_{422} = 1.54 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ [28]. Horse heart cytochrome (Type III) (Sigma) was reduced with freshly prepared sodium dithionite and the excess dithionite was removed by gel filtration on PD-10 column containing G-25 Sephadex. The concentration of ferrocytochrome *c* was determined using $\epsilon_{550} = 29.5 \text{ mM}^{-1}\text{cm}^{-1}$ [29]. Phospholipase A₂ was isolated from *Crotalus atrox* venom as described by Wells and Hanahan [30]. Coomassie stained protein gels showed it to be >95% pure. The concentrations of phospholipase A₂ was determined using $\epsilon_{280}^{1\%} = 27.2 \text{ M}^{-1}\text{cm}^{-1}$ [30].

2.2 Phospholipase A₂ Digestion of Phospholipids Bound to CcO

Delipidation of *Rbs*-CcO was achieved by incubation of detergent-solubilized enzyme with a five-fold molar excess of PLA₂ for 2 hours at 25°C, following a protocol developed for bovine CcO [22]. Briefly, CcO (30 μM , ~6 mg of protein) was treated with PLA₂ (150 μM) at 25°C in 50 mM MOPS, pH 7.2 containing 10 mM CaCl₂, and 1 mg of detergent (Triton X-100 or dodecyl maltoside) per mg of CcO. The cleavage activity of PLA₂ was quenched after the indicated time by adding EDTA to 50 mM EDTA. CcO was re-purified by anion-exchange chromatography as follows.

2.3 HiTrapQ FPLC Ion Exchange Chromatography

Chromatography of CcO was performed using a one mL HiTrapQ anion-exchange column as described by Sedláč and Robinson [22], on a Laboratory Data Control HPLC system. CcO was loaded onto the column in 20 mM MOPS, pH 7.2, 0.5 mg/mL dodecyl maltoside. CcO was eluted from the column in the same buffer plus 0.4 M Na₂SO₄, by (1) a two mL linear gradient from 0 to 25% elution buffer, (2) isocratic elution with ten mL of 25% elution buffer, and (3) a 5 mL linear gradient from 25 to 100% elution buffer. Delipidated CcO eluted from the column in one peak ~0.3 M Na₂SO₄. The concentration of dodecyl maltoside in eluted sample was increased to 1 mg/mL and the sample was dialyzed against 20 mM MOPS, pH 7.2 for 12 hours at 4 °C to remove Na₂SO₄.

2.4 Re-purification of urea-treated *Rbs*-CcO

Urea-dissociated subunits were removed from CcO by chromatography on a one mL HiTrapQ Ni²⁺ column (GE HealthSciences). The column was equilibrated with 10 mL of 20 mM, pH 7.4, 10 mM imidazole and 0.5 M NaCl. After the sample was applied and washed with the same buffer, *Rbs*-CcO was eluted with the same buffer plus 0.5 M imidazole. Excess imidazole was removed by dialysis against

2.5 MALDI-TOF/MS

Mass spectrometry analyses were performed in the Mass Spectrometry Laboratory of the University of Texas Health Science Center at San Antonio using sample preparation and analysis conditions described previously [31].

2.6 SDS-PAGE

was used to determine the relative content of *Rbs*-CcO subunits after exposure to high temperature [32]. Gels were scanned and quantitatively analyzed with the software Scanner Control, Molecular Dynamics Version 4.0. The signals from subunits II and III were normalized to subunit I.

2.7 Analysis of Phospholipids

Phospholipids were extracted from *Rbs*-CcO as previously described [11] and quantified by a phospho-molybdate colorimetric method after wet ashing in perchloric acid [33]. The precision of this assay is ± 2 nmol. Phospholipids were also qualitatively analyzed by thin-layer chromatography (TLC) using Whatman K5 silica gels, 80 Å. The plates were developed with $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{NH}_4\text{OH}$ using a volume ratio of 65:35:4:0.25. Spots containing phospholipid were visualized by spraying the plates with phosphor-molybdate solution. Subsequently, all of the spots were visualized with a charring spray (6N H_2SO_4).

2.8 Determination of CcO Activity

Rbs-CcO activity was measured spectrophotometrically by following the pseudo-first-order rate of oxidation of 30–32 μM ferrocytochrome *c* by 0.9 nM CcO in 25 mM buffer containing, 1.0 mg/mL dodecyl maltoside [34]. The activity of purified, lipidated CcO was $1450 \pm 100 \text{ s}^{-1}$. The effect of different concentrations of urea on the activity of lipidated and delipidated CcO was determined as follows. CcO (2.5 μM) was incubated in 40 mM MOPS, pH 7.2, 1.0 mg/mL Triton X-100, with different concentrations of urea at 25°C for 10 minutes. A 10 μL aliquot of urea-treated CcO was transferred into 190 μL of the same buffer and incubated at 4 °C for 30 minutes. 3) A 5 μL aliquot was added to 700 μL of buffer, pH 7.0, 1.0 mg/mL dodecyl maltoside and assayed for activity as described above [34].

2.9 Circular Dichroism (CD) Measurements

CD measurements were performed on an OLIS Cary-16 spectropolarimeter using 1.1 μM CcO in either 20 mM phosphate buffer or 40 mM MOPS, pH 7.2, 1.0 mg/mL dodecyl maltoside. The appropriate dilution of CcO was made after 10 minute of incubation in the presence of 6.0 M urea at 25°C. The presented CD spectra of CcO represent the average of 15 consecutive scans collected with a 1 mm pathlength cuvette, a time constant of 1s, and a bandwidth of 2.0 nm at a setting of 200 points/100 nm. Thermal transitions were monitored by following the decrease in the negative ellipticity at 222 nm. Changes in temperature were controlled by water circulation at a rate of 1.5°C/min.

2.10 Differential Scanning Calorimetry (DSC)

DSC measurements of CcO were performed on VP DSC microcalorimeter. The presented DSC traces were corrected for a baseline represented by the re-scan trace. Visualization of thermal transitions was accomplished using Microcal analysis package for equilibrium thermal transitions provided by microcalorimeter manufacturer. The protein concentration for DCS assay was always $\sim 5 \mu\text{M}$ of protein in 20 mM sodium phosphate buffer containing 1 mg/mL dodecyl maltoside, pH 7.2.

2.11 Absorbance Spectroscopy

Optical spectra were recorded with AVIV spectrophotometer Model 14DS UV-VIS at 25°C. Spectra were recorded in 1-nm steps with a spectral bandwidth of 2.0 nm and averaged after 3–5 consecutive scans. Second derivatives spectra were generated from the collected data using the software available in spectrophotometer. Briefly, spectra after being fitted with polynomial of 7th degree were smoothed by 10 point moving window by Savitzky and Golay method and subsequently derived.

2.12 Analytical Ultracentrifugation

Sedimentation velocity experiments were performed using a Beckman XL-A ultracentrifuge equipped with absorbance optics. All analytical centrifugation experiments and data analysis were as previously described [35,36]. Before some of the sedimentation analyses, a 10 min incubation of CcO with 4 or 6 M urea was stopped by 5 times dilution with 20 mM phosphate buffer, 1.0 mg/mL DDM. Urea was removed by overnight dialysis against 20 mM at 4°C.

3 Results

3.1 Subunit Analysis of *Rbs*-CcO

MALDI-TOF mass spectrometry was applied to evaluate the subunit composition of *Rbs*-CcO. Four *Rbs*-CcO subunits were detected (Fig.1). Two peaks with m/z of 64,050 and 5,335 were assigned to subunits I and IV since subunits I and IV have the highest and lowest molecular masses, respectively. A singly-charged protonated molecular ion of subunit I is 903 higher than the value calculated from the amino acid sequence (63,147 Da). This difference is almost certainly due to six histidines attached to subunit I and a possible modification at the N-terminus [37]. For subunit IV, an observed m/z value of 5,335 is close to predicted mass (5,403 Da) of the ‘short’ form of subunit IV, which is predominant in CcO isolated from wild-type *Rb. sphaeroides* [37]. The sequence-predicted molecular masses of subunits II and III are 32,931 and 30,172 Da, respectively. Therefore, the peak with an m/z of 32,070 was assigned to subunit II and the peak with an m/z of 30,028 to subunit III. The observed mass for subunit II was 861 Da lower than its calculated mass (32,931 Da) while the observed mass for subunit III was close (only 144 Da lower) to its calculated mass (30,172 Da). These differences are likely due to the post-translational processing removal of amino-acids [37].

3.2 Effect of Delipidation on Tertiary and Quaternary Structures of CcO

The phosphorus content and phospholipid analysis by TLC indicated that 50–70 phospholipids co-isolated with CcO, mostly PE and PC (Table 1). Analyses, performed on CcO after delipidation with PLA₂ and re-purification indicated the presence of only ~2 phospholipids per protein monomer, identified as PEs (Fig.3, Table 1). These two PEs remained present even if the delipidation time was extended to 4 hours and/or PLA₂ concentration was increased two fold, indicating that two tightly bound molecules of PE are inaccessible to PLA₂.

Delipidation only slightly altered the CD spectrum of CcO indicating the absence of any significant change in secondary structure (Fig.4). To look for possible alterations in the environments of the two heme centers, the second derivative visible spectrum was obtained (Fig. 4, inset). Both delipidated and PL-containing *Rbs*-CcO exhibited signals at 432 nm and 422 nm corresponding to the heme *a* and heme *a*₃, respectively [32,38]. The slight differences between the spectra indicated that delipidation has little effect on heme A centers of subunit I.

3.3 Thermal Denaturation of Lipidated and Delipidated *Rbs*-CcO

Thermal denaturation of lipidated *Rbs*-CcO monitored by DSC revealed four distinct transitions with the apparent melting temperatures of ~38°C, ~45°C, ~53°C, and ~73°C (Fig. 5, upper panel). These results are in agreement to those reported for bovine heart CcO [23,39]. The thermal denaturation was irreversible because the CcO complex does not spontaneously reassemble after unfolding and scan-rate dependent (data not shown). Therefore, the transitions fitted to the DSC scan may be assigned to individual subunits, but thermodynamic analysis of unfolding is problematic. In a study of the structurally similar CcO from *Paracoccus denitrificans*, Haltia et al. [40] assigned the highest peak (here 73°C) to the cooperative unfolding of subunits I and II, and a lower peak (here 53°C) to the unfolding of subunit III. In addition to normal, lipidated CcO, we have also examined *Rbs*-CcO forms containing mutations in the lipid binding sites of subunit III (data not shown). In the DSC scans of these mutants, the 38°C, 45°C and 53°C peaks are all altered, but the 73°C peak is not. Hence we assign all three of the lower peaks to subunit III and the 73°C peak to subunits I and II. Thermal denaturation of subunits with molecular weight corresponding to subunit IV is usually not accompanied with calorimetric enthalpy sufficient for its detection by DSC [41,42].

The thermal transitions of *Rbs*-CcO were also monitored by ellipticity changes at 222 nm, i.e. at the dichroic band predominantly associated with α -helical $n \rightarrow \pi^*$ amide transitions [43] which reflects the amount of helical secondary structure. The normalized thermal transitions corresponded to unfolding of 40–50% of the α -helical CcO secondary structure. In contrast to the DSC data, only three transitions could be detected. The main thermal-induced transition in ellipticity at 222 nm corresponds to the DSC-revealed transition with a melting temperature ~53°C. Two shoulders in the main transition are assigned as transitions with melting temperatures ~38°C and ~45°. The DSC transition with a melting temperature ~73°C was not observed by CD analysis. Therefore, CD spectroscopy monitors the transitions assigned to subunit II and III, the subunit most intimately associated with phospholipid in a structure of *R. sphaeroides* CcO [1].

The effect of delipidation on the thermal stability of *Rbs*-CcO was analyzed by CD spectroscopy. Delipidation of *Rbs*-CcO altered the thermal stability of the secondary structure of subunit III as indicated by decrease of the main transition from 52.3 °C to 45.8 °C (Fig. 5, Table 1). For both lipidated and delipidated *Rbs*-CcO, heating to 80°C causes extensive aggregation of subunit III (Fig.5, inset in lower panel). These data are in good agreement with previously observed temperature-induced precipitation of subunit III of bovine heart CcO [23,39] and of *Paracoccus denitrificans* CcO [40].

3.4 Activity of Lipidated and Delipidated *Rbs*-CcO

Delipidation of *Rbs*-CcO by itself does not strongly affect its steady-state electron transfer activity. The activity of the enzyme declined by only 10% after incubation with a 5 times molar excess of PLA₂ for 4 hours at room temperature (data not shown). However, delipidation did make *Rbs*-CcO much more sensitive to chemical denaturation. While lipidated *Rbs*-CcO retained ~80% of its original activity after 10 minutes incubation at 8 M urea at room temperature, the delipidated enzyme retained only 10–20% of the original activity (Fig.6). The loss of stability was also observed at lower urea concentrations if the time of incubation was extended. Lipidated *Rbs*-CcO lost 20% of its activity after a four hour incubation in a 4 M urea at room temperature, while the delipidated enzyme lost 50% (inset, Fig.6). In a control experiment, delipidated CcO lost 10% of its activity within 4 hours in the absence of urea. Second derivative absorbance spectra (not shown) indicated that the loss of activity for delipidated CcO in the presence of urea was accompanied by changes in the environment of the active site heme. The second derivative spectra of both lipidated and delipidated CcO's showed smooth minimum at ~424 nm indicating merging of both minima at 421 and 430 nm (and thus similar environment at hemes *a* and *a*₃) after 1 hr incubation at room temperature (data not shown). Interestingly, even such prolonged incubation of lipidated CcO in 6 M urea has no apparent effect on the CcO secondary structure.

3.5 Stability of Quaternary Structure of Delipidated *Rbs*-CcO

Both lipidated and delipidated CcO solubilized in phosphate buffer containing 1 mg/mL DDM sedimented as homogeneous species, each with a sedimentation coefficient (*S*_{20,w}) of 8.5±0.2 (Fig.7). The heterogeneity of delipidated CcO was significantly increased by treatment with 4 or 6 M urea for 10 min at room temperature, as seen by the expanded range of *S*_{20,w} (Fig.7). The range of *S*_{20,w} for lipidated CcO was not affected by treatment with urea (data not shown). The heterogeneity of delipidated CcO is probably a result of partial subunit dissociation since protein gels (not shown) indicated some loss of subunits II and III following treatment of delipidated CcO with urea. Correlation between the activity of delipidated CcO treated with urea and the percentage of the complex containing the full complement of subunits was nearly ideal (inset, Fig.7).

4 Discussion

4.1 Phospholipids-bound to *Rbs*-CcO

A high resolution structure of the four-subunit CcO complex of *Rb. sphaeroides* includes six PL molecules, assigned as PEs [1]. Two of the PE are bound within a V-shaped cleft of subunit III (dark blue spheres, Fig.2). The walls of this cleft are formed by two regions of subunit III, the back wall is formed by helices of subunit I, while the front of the cleft is open to the lipid bilayer. The protein residues that interact with the lipids in the cleft are highly conserved [18]. Hence, subunit III of bovine CcO contains two lipids in the same location, assigned as PGs [44]. A PE is also located in the cleft of subunit III in bovine CcO, above the two PGs [8]. The other four PEs seen in the *Rbs*-CcO structure [1] are clustered at another interface between subunits I and III, outside of the cleft (light blue spheres, Fig.2). The single helix subunit IV of *Rbs*-CcO is bound to the CcO complex by these four lipids. In

a high-resolution structure of *Rbs*-CcO lacking subunits III and IV, all six of the PE discussed above are missing [9]. This emphasizes the importance of subunits III and IV for the binding of these PL. The presence of acyl chains and detergent molecules in the structure of the subunit I-II *Rbs*-CcO [9] showed the position of several potential lipid binding sites on subunits I and II. At least six of the lipid binding sites identified on the subunit I-II *Rbs*-CcO structure appear to also bind lipid in bovine CcO [9], i.e. these lipid binding sites are conserved, like those within the cleft of subunit III [18].

The presence of specifically-bound PL in crystal structures of integral membrane proteins is not unusual. Most high resolution structures of electron transfer complexes include specifically-bound lipid, often termed tightly-bound lipid since these PL remain bound in the same position on all molecules of the protein throughout the purification and the crystallization process. Depending upon the isolation procedure, various amounts of PL co-purify with integral membrane proteins. This PL includes the tightly-bound PL discussed above, plus more loosely bound lipid, often termed annular lipid, that is more easily removed upon further purification of the protein. For example, CcO isolated from bovine heart commonly contains 20–60 mol of PL/mol of protein [14]. Most of this PL can be removed from bovine CcO without affecting its structure and/or function, but a small number of PL are essential for CcO stability and function. For bovine CcO, the essential lipids include two cardiolipins [22]. In this study we have sought to determine the type and number of PL that are essential for the function and structural integrity of *Rbs*-CcO.

According to phosphate and TLC assays, up to 70 PL, mostly PE and PC, co-purify with *Rbs*-CcO as the enzyme elutes from a Ni^{2+} affinity column. A similar amount of co-purifying PL was reported by Diestler et al. [37]. The number of PL associated with *Rbs*-CcO dropped to 6 following anion exchange chromatography in the presence of DDM, also as reported by Diestler et al. [37]. Delipidation with PLA_2 removed all but two PE. We hypothesize that these two remaining PL are those located in the evolutionary-conserved lipid binding sites in the V-shaped cleft formed by subunits III and I (Fig. 2). The structures of the four-subunit *Rbs*-CcO [1] and the two subunit *Rbs*-CcO [9] indicate that the PE within subunit III should be less accessible to hydrolysis by PLA_2 than all of the other PL. Moreover, the lipids within the cleft of subunit III show the greatest number of lipid-protein contacts, suggesting that they should be more tightly bound than the others [18].

4.2 Effect of Delipidation of *Rhodobacter sphaeroides* CcO on its Structure and Activity

The temperature dependence of the 222 nm CD signal demonstrated that removal of PL predominantly affected the secondary structure of subunit III. It is well known that the tertiary structure of helical membrane proteins, within the transmembrane region, is primarily stabilized by helix-helix interactions. Thus, the loss of helical structure in subunit III should perturb helix-helix interactions to diminish the tertiary structure of this subunit (for review see [45–48]). A similar slight decrease in the CD transition temperature, $\sim 7^\circ\text{C}$, was observed for membrane reconstituted bovine heart CcO after its solubilization, and potential delipidation, in Tween 80 [49]. More recently, we have demonstrated that delipidation of bovine heart CcO decreased the temperature of the transition corresponding to the denaturation of the subunit III by $\sim 6\text{--}7^\circ\text{C}$ [23]. Interestingly, destabilization by $\sim 7^\circ\text{C}$

due to delipidation was also observed for the *Rb. sphaeroides* reaction center [13,24], an integral membrane protein complex unrelated to CcO.

The activity of PLA₂ delipidated *Rbs*-CcO, containing two PL, was 10% lower than that of lipidated CcO. Recently, Alnajjar et al. [50] utilized PLA₂ to delipidate *Rbs*-CcO and obtained an enzyme containing less than one PL per monomer. While we do not yet know the reason for the difference in PL content, given a similar method of delipidation, we note that the activity of delipidated *Rbs*-CcO of Alnajjar et al. [50] was ~35% less than normal. The combined results suggest that the two PE remaining in our delipidated *Rbs*-CcO are important but not essential for the activity of the enzyme. Alnajjar et al. [50] observed that the addition of CL restored activity, similar to the ability of CL to restore activity to delipidated bovine CcO [22]. Alnajjar et al. [50] proposed that delipidated *Rbs*-CcO exists in a more open configuration than the lipidated enzyme. In this study, urea treatment of *Rbs*-CcO had an insignificant effect the lipidated enzyme while up to 50% activity was lost upon urea treatment of the delipidated enzyme. It seems possible that treatment of PL-depleted CcO with urea resulted in the generation of an open conformation of *Rbs*-CcO similar to that postulated by Alnajjar et al. [50]. Therefore, the remaining two PEs that could not be hydrolyzed by PLA₂ in our experiments appear capable of keeping *Rbs*-CcO in a more stable, enzymatically active conformation.

The role of CL for the function of the mitochondrial-like CcOs of bacteria has been debated [33,51,52]. In this study, we did not detect CL in the lipid extracted from isolated *Rbs*-CcO before delipidation with PLA₂. Neither the crystal structure of the four-subunit *Rbs*-CcO [1] nor the two-subunit *Rbs*-CcO [9] showed CL, although CL can be modeled into the cleft of subunit III [6]. About 5.3 μmol of CL/g of CcO protein was detected by MALDI MS analysis [52]. While Alnajjar et al. [50] found that CL could restore the activity of delipidated *Rbs*-CcO, we find that CL is not necessary for near normal activity of *Rbs*-CcO.

4.3 Comparison of *Rbs*-CcO and Bovine Heart CcO properties

Rb. sphaeroides CcO exhibits high structural and functional similarity with the catalytic core of bovine heart CcO [1,8,53], but there are several important functional and structural differences between these two enzymes, including the following:

- i. isolated and detergent-solubilized CcO from *Rb. sphaeroides* is more than 3-times active comparing with bovine CcO when analyzed at the same assay conditions [54,55; this work];
- ii. dodecyl maltoside-solubilized CcO from *Rb. sphaeroides* is most likely monomeric as judged by sedimentation velocity analysis. Self-association of *Rbs*-CcO is not affected if the enzyme was analyzed in conditions known to induce dimerization of bovine CcO (data not shown), i.e., in a mixture of dodecyl maltoside and sodium cholate [56]. Similarly, bacterial CcO's from *Escherichia coli*, *Paracoccus denitrificans*, *Thiobacillus novellus* solubilized in Triton X-100, octyl-pentaerythylene ether, *n*-octyl- β -D-thioglucoside, respectively were also found in monomeric form [35,57,58].

- iii. the dependence of molecular activity of CcO from *Rb. sphaeroides* on specific phospholipid environment is not decisively established [50,52]. It is very likely that 2 tightly bound PEs are required for full electron transfer activity, proton pumping activity and enzyme stability [21] and play a similar role as 2 tightly-bound CLs within monomer of CcO [20,22,23].

4.4 Conclusion

1. A large number of PL co-purify with *Rbs*-CcO upon its isolation via Ni²⁺ affinity chromatography. Further purification using anion exchange chromatography leaves approximately 6 PL per monomer. This is the same number of PL seen in a crystal structure of the four-subunit *Rbs*-CcO [1] although additional lipid binding sites have been identified on subunits I and II [9].
2. Delipidation of *Rbs*-CcO using PLA₂ leaves only two PE per monomer. Examination of the four-subunit structure of *Rbs*-CcO [1] leads us to propose that these two PE are likely located within a cleft formed by subunit III.
3. The activity of *Rbs*-CcO containing only two PE per monomer (within subunit III) is near normal, in contrast to a fully delipidated form reported by Alnajjar et al. [50]. This suggests that retention of the lipids within the cleft of subunit III is sufficient for near normal activity, in agreement with Alnajjar et al. [50].
4. The *Rbs*-CcO containing two PE per monomer (within subunit III) is more sensitive to thermal and chemical denaturation than the fully lipidated enzyme. This suggests that lipids bound outside of the cleft of subunit III contribute to the structural stability of *Rbs*-CcO.

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Highlights

- Detection of all four subunits of Rbs-CcO by mass-spectrometry.
- PL environment is essential for structural stability of Rbs-CcO
- Two tightly-bound PE are likely crucial for proper functional state of Rbs-CcO

Figure 1. MALDI-TOF/MS analysis of cytochrome c oxidase from *Rhodobacter sphaeroides* (upper panel) and single subunit I (lower panel)

One microliter aliquots were spotted on the MALDI target and overlaid with 1 μ l of 10 mg/ml sinapinic acid in 50% acetonitrile/0.1% TFA. Spectra were acquired in linear mode and represent the average of 100 laser shots.

Figure 2. Structure of cytochrome c oxidase from *Rhodobacter sphaeroides*

Left – a side view showing all subunits, right – a top view showing helix arrangement and localization of molecules of phosphatidylethanolamines co-crystallized with the enzyme. Subunit I, red; subunit II, green; subunit III, yellow; subunit IV, grey. Four molecules of phosphatidylethanolamine localized between subunits III and IV in proximity of the subunit I are colored in cyan; two molecules of phosphatidylethanolamine localized in cleft of helices of subunits III next to the subunit I are colored in dark blue. The structure is visualized with PyMol with Protein Data Bank entry 1M56 (Svennson-Ek et al. 2002).

Figure 3. Thin layer chromatography analysis

TLC analysis of standard phosphatidylcholine (PC) (left line) and phosphatidylethanolamine (PE) (right line) and extracted phospholipids from 2.5 nmoles delipidated CcO (middle line). TLC plate was sprayed with phosphor-molybdate solution to visualize spots containing phosphate (left plate) and then charred with the spray containing sulphuric acid to visualize carbon containing spots (right plate). On the plates, it is possible to detect also heme and used detergent dodecyl maltoside (DDM).

Figure 4. Spectral analysis of cytochrome c oxidase from *Rhodobacter sphaeroides*

Main panel: circular dichroism spectra of lipidated (thick lines) and delipidated (thin lines) CcO in the far-UV region indicate that delipidation does not affect secondary structure of the enzyme. Inset: nearly identical second derivative absorbance spectra of delipidated and lipidated CcO in the Soret region strongly suggest that delipidation does not affect heme region of CcO. The spectra of both lipidated and delipidated CcO were taken in 40 mM MOPS, pH 7.2, buffer containing 1 mg/mL dodecyl maltoside at room temperature. The

protein concentrations were 0.8 μM and 5 μM at circular dichroism and absorbance spectroscopy measurements, respectively.

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Figure 5. Temperature-induced denaturation of nondelipidated and delipidated *Rhodobacter sphaeroides* cytochrome c oxidase

Upper panel: Thermal denaturation of lipidated CcO followed by DSC (solid lines) and by ellipticity change at 222 nm (filled circles). Lower panel: Temperature-induced changes in ellipticity of lipidated (solid circles) and delipidated (empty circles) CcO followed by CD at 222 nm. Ellipticity changes were normalized to maximal differences. Inset: SDS-PAGE subunit separation. Line 1: lipidated CcO; Line 2 : lipidated CcO after heating to 80 °C; Line 3: delipidated CcO; Line 4: delipidated CcO after heating to 80 °C. After exposure of lipidated and delipidated CcO to high temperature the subunit III precipitated in both

samples – line 2 and 4, respectively. The protein concentrations were 5 μM and 0.6–0.9 μM in DSC and CD, respectively. The enzyme was solubilized in 20 mM sodium phosphate buffer, pH 7.2, containing 2 mM of dodecyl maltoside.

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Figure 6. Concentration-dependent effect of urea on the steady-state electron transfer activity of lipidated (solid circles) and delipidated (empty circles) *Rhodobacter sphaeroides* cytochrome c oxidase

Rbs-CcO was incubated at a concentration of 5 μ M in 40 mM MOPS, pH 7.2, with 1 mg/ml DDM and varying concentration of urea. After 10 minutes incubation at room temperature, the effect of urea was stopped by dilution of the sample with 40 mM MOPS buffer following by incubation on ice. The enzyme activity was normalized towards the activity at 0M urea.

Inset: Time dependence of normalized molecular activity of both lipidated (solid triangles) and delipidated *Rbs*-CcO (empty triangles) in 4M urea at room temperature.

Delipidation of the protein did not affect the enzyme's activity in the absence of urea.

Activities of both lipidated and delipidated enzymes were $1450 \pm 150 \text{ s}^{-1}$ in the absence of urea.

Figure 7. Sedimentation velocity analysis

Lipitated (asterisks) and delipitated (empty circles) CcO in the absence of urea, and delipitated CcO after 10 minutes incubation in 4M (solid squares) and 6M urea (empty squares) at room temperature. Urea-induced heterogeneity of the protein indicates disintegration of CcO. *Inset:* Correlation between molecular activity and the amount of subunit II (empty triangles) and subunit III (solid triangles). The quantity of the subunits II and III were normalized to amount of the subunit I.

Table 1Properties of nondelipidated and delipidated *Rbs*-CcO.

<i>Rbs</i> -CcO forms	[P]/ CcO [*]	Identified PLs ^{**}	Electron transfer activity [s ⁻¹] ^{***}	T _{trs} [°C] ^{****}
Lipidated <i>Rbs</i> -CcO	60±8	PE, PC	1450±150	52.3±0.2
Delipidated <i>Rbs</i> -CcO	1.9±0.2	PE	1390±110	45.8±0.3

^{*} Phosphorus content per monomer *Rbs*-CcO, average of 2 different preparations.

^{**} PLs identified on TLC plates.

^{***} Average of 3 different measurements.

^{****} Transition temperatures of the major conformational change obtained from CD melting experiments.