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Destabilization of the Quaternary Structure of Bovine Heart Cytochrome *c* Oxidase upon Removal of Tightly Bound Cardiolipin

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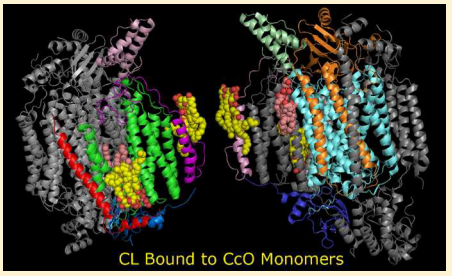
ABSTRACT: The quaternary structural stability of cardiolipin-containing (CcO^{CL+}) versus CL-free cytochrome *c* oxidase (CcO^{CL-}) was compared using structural perturbants as probes. Exposure to increasing concentrations of urea or guanidinium chloride causes sequential dissociation of five subunits from CcO^{CL+} in the order VIa and VIb, followed by III and VIIa, and ultimately Vb. Removal of CL from CcO destabilizes the association of each of these five subunits with the core of CcO. Subunits VIa and VIb spontaneously dissociate from CcO^{CL-} even in the absence of denaturant and are no longer present after purification of the CL-free 11-subunit complex by ion exchange chromatography. The other 11 subunits remain associated in a partially active complex, but the association of subunits III, VIIa, and Vb is weakened; i.e., the midpoints for the subunit dissociation curves are each shifted to a lower perturbant concentration (lower by 1.1−1.7 M urea; lower by 0.3−0.4 M GdmCl). This corresponds to a decrease of ∼9 kJ in the Gibbs free association energy for each of these subunits when CL is removed from CcO. With either CcO^{CL+} or CcO^{CL-}, loss of enzymatic activity occurs coincident with dissociation of subunits III and VIIa. The loss of activity is irreversible, and reactivation of CcO^{CL-} by exogenous CL occurs only if both subunits remain associated with the core of CcO. Inclusion of sulfate anions stabilizes the association of VIIa more than III, resulting in a slight separation of the urea-induced dissociation curves. In this case, activity loss correlates much better with dissociation of subunit VIIa than III. We conclude that (1) bound cardiolipin is an important stabilizing factor in the quaternary structure of CcO and (2) association of subunit VIIa (possibly together with subunit III) is critical for functional CL binding and full electron-transfer activity of CcO.



Bovine cytochrome *c* oxidase (CcO) (ferrocytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) is the terminal enzyme (complex IV) of the inner mitochondrial electron transport chain, catalyzing electron transfer from reduced cytochrome *c* to molecular oxygen. The complex is composed of 13 nonidentical protein subunits and two redox centers, one containing a copper atom and two heme A moieties; the other two copper atoms.[1,2] The multisubunit enzyme, an integral membrane complex, spans the inner mitochondrial membrane and is in contact with an annulus of membrane phospholipids.

CcO isolated from the mitochondrial membrane is a protein−phospholipid complex solubilized by detergent. The purified, detergent-solubilized complex typically contains 15−20 phospholipids,[3] 13 of which have been resolved within the three-dimensional crystal structure.[4] The 13 PLs are bound primarily at the interface between adjacent subunits, suggesting that they may function as a stabilizing factor in the quaternary structure. Most of these phospholipids can be removed without altering the functional activity of CcO or the structural stability of the detergent-solubilized complex. However, three or four cardiolipins (CLs) are tightly bound to each monomer and have an especially important functional and structural role.[3,5] Sequential removal of these CLs strongly correlates with a reversible loss of electron transport activity together with the dissociation of subunits VIa and VIb but does not appear to perturb either the quaternary or tertiary structure of the remaining 11-subunit enzyme.[5]

The functional importance of tightly bound CL is not unique to CcO because removal of CL from other purified, detergent-solubilized complexes, e.g., complex II or cytochrome bc_1, destabilizes their quaternary structures with concomitant loss of enzyme activity.[6,7] At least one role for cardiolipin tightly bound to mitochondrial multisubunit complexes appears to be the stabilization of subunit interactions within the structure. This hypothesis is analogous to CL functioning as a type of "glue" for the formation and stabilization of the large respiratory chain supercomplexes.[8,9]

Lipid-free CcO has approximately 50% of the original electron transport activity, but full activity is restored by reassociation of exogenous CL.[5] Any structural perturbation of the structure upon CL removal must, therefore, be relatively small and fully reversible. However, removal of the cardiolipin, together with subunits VIa and VIb, does increase its sensitivity to elevated hydrostatic pressure[10] and decrease its kinetic stability as probed by differential scanning calorimetry[11] (i.e., the rate of irreversible thermal protein denaturation increases). Thus, it appears that the structural stability of CcO is weakened upon removal of CL together with subunits VIa and VIb.

Structural perturbants such as urea or guanidium chloride (GdmCl) selectively induce the dissociation of subunits from CcO, with subunits VIa and VIb dissociating at the lowest

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perturbant concentration followed by VIIa, III, and Vb.[12] This occurs without perturbation of the tertiary structure of the other subunits in the enzyme. If the major role of bound CL is to stabilize the quaternary structure of CcO, as suggested by their location within the three-dimensional structure of CcO, it is predicted that the association of subunits adjacent to bound CL would be destabilized when CL is removed. To test this hypothesis, the urea- and GdmCl-induced dissociation of subunits from CL-free CcO was determined and compared with that of the CL-containing enzyme, which is the subject of this investigation.

EXPERIMENTAL PROCEDURES

Materials. Acetonitrile and phosphoric acid were of high-performance liquid chromatography (HPLC) grade and were obtained from Fisher Scientific. Chloroform and methanol were also HPLC grade and purchased from EM Science. Ultrapure Triton X-100, dodecyl maltoside, and cardiolipin were obtained from Boehringer Mannheim, Antrace, Inc., and Avanti Polar Lipids, respectively. Horse heart cytochrome *c* (type III) was obtained from Sigma Chemical Co. Phospholipase A_2 was isolated from *Crotalus atrox* venom as described by Wells and Hanahan.[13] All other chemicals were reagent grade. The C18 reversed phase column (10 μm, 4.6 mm × 250 mm, catalog no. 218TP104) was purchased from Vydac, and the 1 mL HiTrapQ fast protein liquid chromatography (FPLC) column was from Pharmacia.

Preparation of Bovine Heart Cytochrome *c* Oxidase (CcO^{CL+}). The enzyme was isolated from Keilin-Hartree heart particles by the method of Fowler et al.[14] with modifications described by Mahapatro and Robinson.[15] The resulting purified enzyme had a heme content of 9.4−9.9 nmol/mg, a phospholipid content of 15−30 P/aa_3, and an electron transport activity of 350−390 s^{-1}. Individual drops of purified enzyme [~25 μL drops of solution containing 20−25 mg/mL protein, 10 mg/mL sodium cholate, and 100 mM phosphate buffer (pH 7.4)] were quickly frozen by dripping the solution into liquid nitrogen. These individual aliquots of frozen enzyme were stored at −80 °C. Prior to any experiment, sodium cholate present in the enzyme preparation was exchanged for Triton X-100 or dodecyl maltoside by diluting the enzyme preparation to 1 mg/mL protein with buffer containing 1 mg/mL detergent followed by extensive dialysis to remove sodium cholate. The resulting Triton X-100- or dodecyl maltoside-solubilized enzyme was monomeric as judged by sedimentation velocity analysis.[16−18] CcO concentrations were calculated on the basis of an ε_{422} of 1.54 × 105 M^{-1} cm^{-1} for the fully oxidized enzyme.[19]

Three preparations of CcO, each with a different PL content, were utilized for the urea-induced dissociation experiments. Two preparations of CcO isolated from Keilin-Hartree heart particles contained 15 and 30 P_i/mol of oxidase (3 or 4 and ~6 CLs/mol of oxidase, respectively). Despite the different PL contents of these preparations, the subunit dissociation curves are identical, within experimental error. Subunit dissociation curves for a third preparation containing only 8 P_i/mol of oxidase (3−4 CLs), prepared by HiTrap Q ion exchange chromatography,[5] were only slightly shifted to lower denaturant concentrations (<0.2 M urea for each subunit).

Preparation of CL-Free CcO (CcO^{CL-}). All phospholipids, including CL, were removed from CcO using the phospholipase A_2 digestion method described previously.[5] This involves exposure of CcO (40 μM) to PLA_2 (40 μM) for 3 h at room temperature in 20 mM MOPS (pH 7.2) containing 10 mM $CaCl_2$ and 1 mg of detergent (Triton X-100 or dodecyl maltoside) per milligram of cytochrome *c* oxidase. The phospholipid-free enzyme (CcO^{CL-}) was purified and separated from PLA_2 lysophospholipids and free fatty acids by HiTrapQ FPLC[5] after inhibition of PLA_2 with 50 mM EDTA. Sodium sulfate present in the eluted CcO^{CL-} was removed by dialysis before subsequent denaturant-induced subunit dissociation experiments because the sulfate anions stabilize proteins[20,21] and would interfere with these experiments.

Quantitation of Phospholipids. The phospholipids were extracted from either 1 nmol of CcO^{CL+} or 5 nmol of CcO^{CL-} as previously described[22] and quantified by the phosphomolybdate colorometric method after wet ashing in perchloric acid.[23] The precision of this assay is ±2 nmol. Preparations of CcO^{CL+} typically contain 15−20 PLs/aa_3, 4 or 5 of which are CLs. Preparations of CcO^{CL-} contain <0.4 CL/aa_3 with no other PLs. The composition of the extracted PLs was determined using either isochratic or gradient normal phase HPLC on silicic acid.[24]

Determination of CcO Activity. CcO activity was measured spectrophotometrically at pH 7.0 in 25 mM phosphate buffer containing 2 mM dodecyl maltoside to an accuracy of ±5% by computer fitting the pseudo-first-order rate of ferrocytochrome *c* oxidation (25−30 μM) by cytochrome *c* oxidase (1.75 nM).[25] Ferrocytochrome *c* was freshly prepared by dithionite reduction, and excess dithionite was removed by G-25 Sephadex gel filtration. Initial concentrations of ferrocytochrome *c* were determined by use of ε_{550}(reduced-oxidized) of 21.4 mM^{-1} cm^{-1}. The activity of purified CcO was 360 ± 30 s^{-1}, i.e., moles of cytochrome *c* oxidized per mole of cytochrome *c* oxidase per second. The effect of different concentrations of urea or GdmCl on the activity of CcO^{CL+} and CcO^{CL-} enzymes, with or without addition of exogenous CL, was determined as follows. (1) CcO (5 μM) was incubated for 10 min at room temperature in 40 mM MOPS buffer (pH 7.2) containing either 0−7 M urea, or 0−2.5 M GdmCl, and 2.0 mM dodecyl maltoside. (2) The denaturant effect was quenched by dilution of a 10 μL aliquot of the solution into 190 μL of ice-cold buffer containing 50 mM $TrisSO_4$ (pH 7.4) and 1.5 mM Triton X-100 with or without 20 μM CL. (3) After incubation in the CL-containing solution for an additional 10 min, 5 μL of the aliquot was transferred into 700 μL of assay buffer at 25.0 °C for the determination of enzyme activity.[25]

Denaturant-Induced Dissociation of CcO Subunits. Dodecyl maltoside-solubilized CcO [0.5−1.0 mg of the protein in 200 μL of 40 mM MOPS (pH 7.2) containing 2.0 mM dodecyl maltoside] was mixed with the appropriate amount of 9 M urea or 5 M GdmCl in 200 mM MOPS (pH 7.2) containing 2.0 mM dodecyl maltoside and an appropriate amount of the same buffer without denaturant to achieve the desired denaturant concentration (0.0−7.0 M for urea and 0−2.5 M for GdmCl) in a final volume of 1 mL. After incubation for either 10 or 120 min at room temperature, the solution was diluted 5-fold with 20 mM MOPS (pH 7.2) and stored at 4 °C until dissociated subunits were removed by HiTrapQ FPLC as described previously.[5]

Analysis of Subunits. Quantitative determination of the 10 nuclearly encoded subunits (subunits IV−VIII, nomenclature according to Kadenbach et al.[26]) was determined by C18 reversed phase HPLC.[27] Subunits IV and Va were used as internal standards for normalization of the subunit content. The content of the three nuclear-encoded subunits (I−III) was

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determined by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis on 15% acrylamide gels that contained 2 M urea in addition to 0.1% SDS.[28] The Coomassie blue-stained gel was scanned and the subunit content quantified using Scanner Control, Molecular Dynamics version 4.0. The quantity of subunit III was normalized to the amount of subunit I.

Fitting of Sigmoidal Subunit Dissociation Data. Denaturant-induced dissociation of a subunit from CcO is adequately described by a two-stage process: reversible dissociation of the subunit from the enzyme core, followed by a slower, irreversible unfolding of the dissociated subunit.[12] Data acquired after exposure to denaturant for 10 min reflects the more rapid reversible subunit dissociation, while data acquired after exposure for 2 h at least partially involves the slower irreversible unfolding step. Therefore, data acquired after 10 min can be analyzed using the linear extrapolation method that has been used to evaluate the free energy change associated with reversible protein unfolding.[29] This model assumes a linear relationship between the denaturant concentration and the free energy change at that concentration, i.e., $\Delta G[\text{denaturant}] = \Delta G_{H_2O} + m[\text{denaturant}]$, where $\Delta G[\text{denaturant}]$ is the experimentally determined Gibbs free energy change at a particular denaturant concentration, ΔG_{H_2O} is the theoretical Gibbs free energy change that would be observed in the absence of denaturant, and m is the slope of the linear correlation between ΔG and urea concentration. Using this approach, the fraction of dissociated subunit, f_D, is a function of ΔG_{H_2O}, m, and [urea], according to the following equation:[12]

$$f_D = \frac{e^{-(\Delta G_{H_2O} + m[\text{urea}])/RT}}{1 + e^{-(\Delta G_{H_2O} + m[\text{urea}])/RT}} \quad (1)$$

To evaluate the fitting parameters, ΔG_{H_2O} and m, nonlinear least-squares fitting algorithms within SigmaPlot version 8.0 or Microsoft Office Excel 2003 are used to fit eq 1 to each set of dissociation data. The value of $[\text{urea}]_{1/2}$, the midpoint of the sigmoidal dissociation curve, is then evaluated from the equality $[\text{urea}]_{1/2} = \Delta G_{H_2O}/m$.

RESULTS

Bound CL Alters Urea-Induced Dissociation of Subunits from CcO. Urea-induced dissociation of subunits from CcO^{CL-} and CcO^{CL+} was compared after each was exposed to 0−7 M urea for 10 min at room temperature (Figure 1A). The only subunits within CcO^{CL-} that dissociate during exposure to these concentrations of urea are III, Vb, and VIIa (subunits VIa and VIb are absent because they dissociate during removal of CL and repurification of CcO^{CL-} by HiTrapQ FPLC). All other subunits remain associated with the stable core of CcO. Subunit dissociation data were acquired using three preparations of CcO^{CL+} that contained 30, 15, or 8 P_i/CcO monomer. Each preparation contained three or four molecules of tightly bound CL with 2−20 additional molecules of PC, PE, and CL. The midpoints for the dissociation of subunits III, VIIa, and Vb were within ±0.2 M urea for each of these preparations. Apparently, additional bound PL has very little influence on the individual urea-induced dissociation curves. Forms of CcO^{CL-} obtained from each of these preparations by PLA_2 hydrolysis and purification by HiTrap Q ion exchange chromatography were identical because each

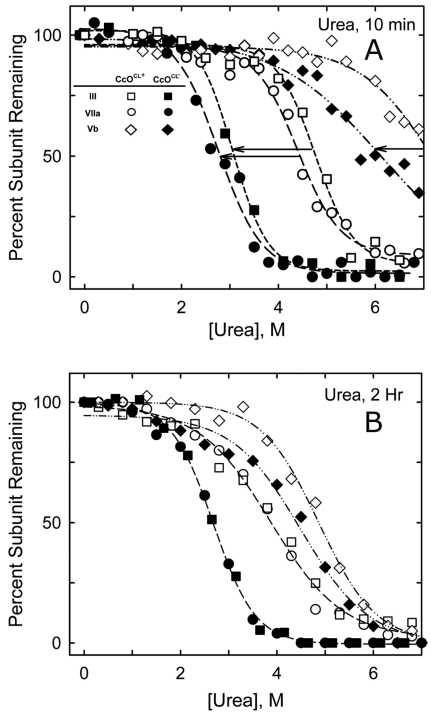

Figure 1. Urea-induced dissociation of subunits from CcO^{CL+} and CcO^{CL-}. (A) Percent of subunits III, Vb, and VIIa that remain associated with CcO after exposure to urea for 10 min at room temperature. (B) Percent of subunits III, Vb, and VIIa that remain associated with CcO after exposure to urea for 2 h at room temperature. In each panel, empty symbols are data acquired with CcO^{CL+} and filled symbols are data acquired with CcO^{CL-}. For each type of CcO, the dissociation of subunit VIIa (circles), III (squares), and Vb (diamonds) was fitted to eq 1, using nonlinear regression analysis. Arrows indicate the shift in the dissociation midpoints for each subunit upon removal of CL from CcO. For each set of data, 2.5−5 μmol of CcO was incubated in MOPS buffer (pH 7.2) containing 2 mM dodecyl maltoside and 0−7 M urea. After either 10 or 120 min, aliquots were taken for subsequent RP-HPLC subunit analysis after removal of dissociated subunits by HiTrap Q ion exchange chromatography (see Experimental Procedures for details). Dissociation of subunits VIa and VIb from CcO^{CL+} is not shown because these subunits are not present in either type of CcO. Fitting parameters for the nonlinear regression fits are summarized in Table 1.

was devoid of PL. The order of dissociation of the three subunits from CcO^{CL-} is the same as that from CcO^{CL+}, i.e., a nearly coincident dissociation of subunits VIIa and III followed by the dissociation of subunit Vb. However, with the CL-free enzyme, the midpoint of each urea dissociation curve is shifted by ∼1.5 M to a lower urea concentration, significantly more than the small shifts observed for CcO^{CL+} containing different amounts of other PLs. For example, the midpoints for the dissociation of subunits VIIa and III from CcO^{CL-} occur at ∼3 M urea, rather than at ∼4.5 M urea for CcO^{CL+}. Incubation of either CcO^{CL-} or CcO^{CL+} with urea for a longer period of time, e.g., 2 h, shifts each subunit dissociation curve to a lower urea concentration, but qualitatively, the result is similar to that observed after 10 min; i.e., the order of subunit dissociation is unchanged, and removal of CL shifts the dissociation curves to lower urea concentrations (Figure 1B).

Urea-induced dissociation of individual subunits from CcO behaves at least as a two-stage process: (1) rapid reversible dissociation of one or more subunits followed by (2) slower urea-induced unfolding.[5] Data acquired after 10 min reflect the

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faster, reversible stage, while data acquired after 2 h partially include some irreversible unfolding. Therefore, only the dissociation data acquired after exposure of CcO to urea for 10 min were analyzed by the linear extrapolation model for denaturant-induced reversible protein unfolding;[12,29] (see Experimental Procedures and eq 1). Using this procedure, values of ΔG_{H_2O}, m, and $[\text{urea}]_{1/2}$ were obtained for the dissociation of subunits from CcO^{CL+} and CcO^{CL-} (Table 1).

Table 1. Thermodynamic Parameters[a] for Urea-Induced Dissociation of Subunits from CcO

	ΔG_{H_2O}		$\Delta\Delta G_{H_2O}$	$[\text{urea}]_{1/2}$		$\Delta[\text{urea}]_{1/2}$
	CcO^{CL+}	CcO^{CL-}	$CcO^{CL+}-CcO^{CL+}$	CcO^{CL+}	CcO^{CL-}	$CcO^{CL+}-CcO^{CL+}$
VIIa	24.8	15.1	9.6	4.40	2.76	1.64
III	29.7	20.9	8.8	4.76	3.06	1.70
Vb	23.4	13.9	9.5	7.24	6.14	1.10
activity[b]		12.7			2.64	

[a]Average errors: ±2.2 kJ = ±11% for ΔG_{H_2O}, ±3.1 kJ = ±33% for $\Delta\Delta G_{H_2O}$; ±0.07 M = ±1.7% for $[\text{urea}]_{1/2}$, and ±0.10 M = ±6.4% for $\Delta[\text{urea}]_{1/2}$. [b]Calculated from the loss of molecular activity (s^{-1}).

The removal of CL from CcO decreases the Gibbs free energy for dissociation of each subunit, i.e., VIIa, III, and Vb, by 9−10 kJ, with the transition for each subunit dissociation occurring at a lower urea concentration, i.e., smaller $[\text{urea}]_{1/2}$ value.

Urea-Induced Loss of CcO Activity Correlates with Dissociation of Subunits VIIa and/or III. The molecular activity of CcO^{CL-} and CcO^{CL+} was measured after their exposure to a series of different urea concentrations. For either type of enzyme, the activity decreases coincident with the dissociation of subunits VIIa and III (Figure 2). The initial activity of CcO^{CL-} is approximately 50% of that of CcO^{CL+}, but nearly full activity is restored if the CL-free enzyme is incubated with exogenous CL prior to the assay.[5] The ability of exogenous CL to restore full activity to CcO^{CL-} is lost coincident with the urea-induced loss of activity and the dissociation of VIIa and III. The transition midpoints for the loss of activity before and after incubation with exogenous CL occur at nearly the same urea concentration (Figure 2B). This result implies that restoration of full activity by exogenous CL requires the presence of subunit VIIa and/or III because little or no activity is recovered when CcO missing these two subunits is incubated with exogenous CL. In contrast to the results obtained with CcO^{CL-}, addition of CL to CcO^{CL+} has almost no effect on electron-transfer activity regardless of whether it has been exposed to urea (Figure 2A). For example, enzyme that is 70% active after exposure to urea remains 70% active after addition of CL; enzyme that is 25% active after exposure to urea remains 25% active. Lastly, removal of subunits VIa and VIb during the preparation of CcO^{CL-} has little or no effect upon its electron transport activity; the molecular activities of CcO^{CL-} and CcO^{CL+} are nearly identical (~400 s^{-1}) if CL is reassociated with CcO^{CL-} prior to measurement of activity (Figure 2).

Time Dependence of Urea-Induced CcO Activity Loss Correlates with Dissociation of Subunits III and VIIa. Exposure of CcO^{CL-} to 2 M urea at room temperature results in a time-dependent exponential loss of subunits III and VIIa together with a similar exponential loss of electron-transfer activity (Figure 3). As described previously, the molecular activity of CcO^{CL-} (~220 s^{-1}) is ~50% of the activity of

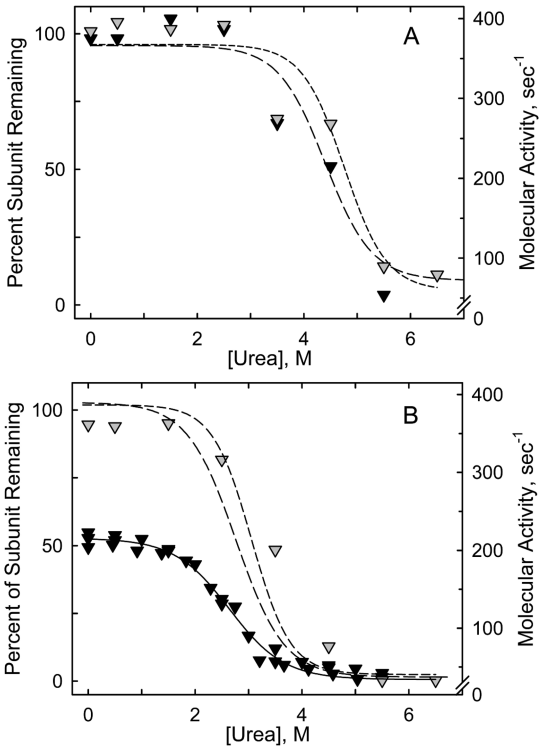

Figure 2. Comparison of urea-induced loss of electron transport activity with dissociation of subunits III and VIIa from CcO^{CL+}, and from CcO^{CL-}. (A) Data acquired using CcO^{CL+}. (B) Data acquired using CcO^{CL-}. With each type of enzyme, molecular activities were measured before (black-filled triangles) and after (gray-filled triangles) incubation with 20 μM exogenous CL (see Experimental Procedures for details). In each panel, the dissociation of subunit VIIa (long dashes) and subunit III (short dashes) corresponds to the nonlinear regression fits to the data of Figure 1.

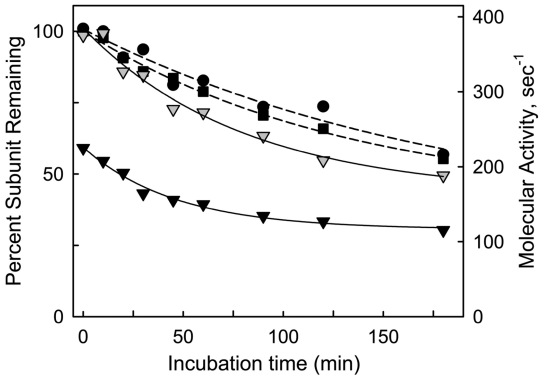

Figure 3. Time dependence of urea-induced activity loss and dissociation of subunits from CcO^{CL-}. The content of subunit III (filled squares) and subunit VIIa (filled circles) and the activity of CcO^{CL-} before (black-filled triangles) and after (gray-filled triangles) incubation with 20 mM exogenous CL (see Experimental Procedures for details). All data were collected after exposure of CcO^{CL-} to 2 M urea in 200 mM MOPS buffer (pH 7.2) containing 2 mM dodecyl maltoside.

CcO^{CL+} (~400 s^{-1}), but nearly full activity is restored by incubation with a CL in the presence of dodecyl maltoside micelles. However, during the exponential decay of CcO activity, exogenous CL loses its ability to restore activity to the urea-treated enzyme (gray triangles, Figure 3). Presumably, the slow time-dependent loss of activity, which is unresponsive to

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the addition of exogenous CL, reflects either the loss of the CL binding site, possibly on VIIa, or an irreversible structural change within CcO when it is exposed to urea for >10 min.

GdmCl-Induced Dissociation of Subunits III and VIIa. Exposure of CcO^{CL+} to a series of increasing GdmCl concentrations for 10 min at room temperature induces sequential dissociation of subunits similar to that observed with urea, i.e., dissociation of VIa and VIb, then III and VIIa, and finally Vb.[12] As was the case with urea-induced dissociation of subunits, removal of bound CL destabilizes the association of subunits VIIa and III so that they dissociate from CcO^{Cl-} at a lower GdmCl concentration (Figure 4). The major difference

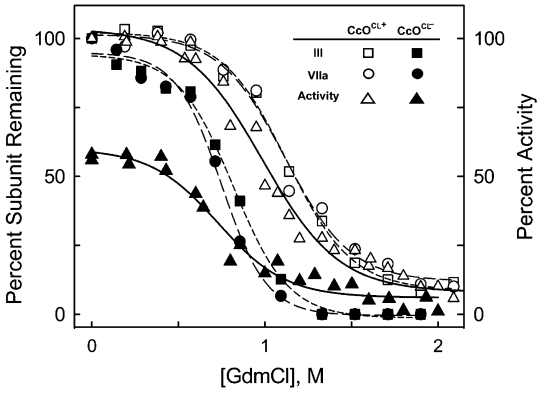

Figure 4. GdmCl-induced activity loss and dissociation of subunits III and VIIa from CcO^{CL+} and CcO^{CL-}. The percent activity (triangles) and the percent of subunits III (squares) and VIIa (circles) that remain associated with CcO^{CL+} (empty symbols) and CcO^{CL-} (filled symbols) were determined after exposure of each type of enzyme to 0–2 M GdmCl for 10 min at room temperature. Solid lines, short dashed lines, and long dashed lines represent nonlinear regression fits of eq 1 to the loss of activity data and to the dissociation of subunits III and VIIa, respectively.

in the GdmCl data is that the sigmoidal dissociation curves occur at denaturant concentrations lower than those that occur with urea, presumably because GdmCl is a stronger structural perturbant. Once again, dissociation of subunits III and VIIa from either CcO^{CL+} or CcO^{CL-} occurs nearly together and coincident with loss of activity (Figure 5). As with the urea-induced data, the GdmCl dissociation midpoints, $[GdmCl]_{1/2}$, for the activity loss data and the subunit dissociation data can be evaluated for each type of CcO by fitting sigmoidal curves to eq 1. Without bound CL, the midpoints are each shifted to lower GdmCl concentrations by 0.3–0.4 M.

Effect of Stabilization of Sulfate Anions on Urea-Induced Subunit Dissociation from CcO and Activity Loss. The stabilizing anion, sulfate, was included during urea-induced subunit dissociation in an attempt to resolve the dissociation of subunits III and VIIa and, thereby, possibly determine which dissociation is responsible for coincident CcO activity loss. High concentrations of sulfate anions are known to stabilize the protein tertiary structure from denaturant-induced unfolding.[20,21] Therefore, sulfate anions would be expected to stabilize the CcO quaternary structure against urea-induced subunit dissociation. Inclusion of 0.5 M sodium sulfate did stabilize the quaternary structures of both CcO^{CL+} and CcO^{CL-}, which is evident from the shift of the urea-induced subunit dissociation curves to higher urea concentrations (Figure 6). The stabilizing effect of sulfate anions in terms of an increase in

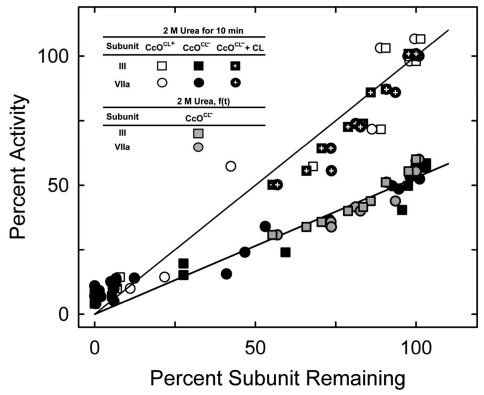

Figure 5. Correlation of the removal of subunits III and VIIa with percent activity remaining. Correlations of all of the subunit III data with activity are represented by squares; correlations of all of the subunit VIIa data are represented by circles. Data acquired after exposure of CcO^{CL+} to urea for 10 min are represented by empty circles and squares. Data acquired after exposure of CcO^{CL-} to urea (activities measured without addition of exogenous CL) are represented by solid filled circles and squares. Data acquired after exposure of CcO^{CL-} to urea (activities measured with the addition of exogenous CL) are represented by gray filled circles and squares with plus signs in the middle of the symbols. Data acquired by exposure of CcO^{CL-} to 2 M urea for various lengths of time (Figure 3 data) are represented by gray-filled circles and squares. Solid lines represent the perfect correlations between subunit removal and loss of activity.

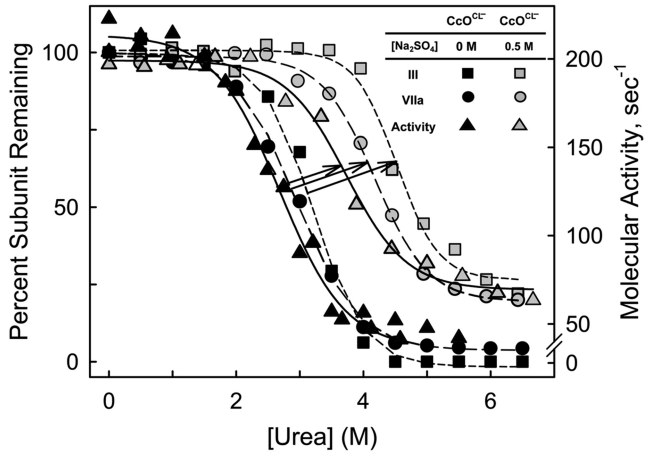

Figure 6. Stabilization of the quaternary structure and activity of CcO^{CL-} by sulfate anions. The percent of subunit III (squares) and VIIa (circles) remaining associated with CcO^{CL-} after exposure to 0–7 M urea was determined in the presence (gray-filled symbols) and absence (black-filled symbols) of 0.5 M Na_2SO_4. Electron transport activity was also determined after exposure to 0–7 M urea in the presence (gray-filled triangles) or absence (black-filled triangles) of 0.5 M Na_2SO_4. Dashed lines are nonlinear regression fits to the subunit dissociation data; solid lines are nonlinear regression fits to the activity data. Fitting parameters of all the nonlinear regression fits are summarized in Table 2. Arrows indicate the shift in the dissociation midpoints to a higher urea concentration in the presence of sulfate anions.

the Gibbs free energy for each subunit dissociation, ΔG_{H_2O}, and the urea concentration at the midpoint of dissociation, $[urea]_{1/2}$, were quantified by fitting each subunit dissociation curve to eq 1 (Table 2). Inclusion of 0.5 M sodium sulfate stabilized the association of each subunit by ~10 kJ and shifted the corresponding values of $[urea]_{1/2}$ for subunit dissociation to

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Table 2. Sulfate-Induced Changes in Thermodynamic Parameters[a] for Urea-Induced Dissociation of Subunits from CcO^{CL-}

	ΔG_{H_2O}			$[urea]_{1/2}$		
	0 M Na_2SO_4	0.5 M Na_2SO_4	$\Delta\Delta G_{H_2O}$	0 M Na_2SO_4	0.5 M Na_2SO_4	$\Delta[urea]_{1/2}$
VIIa	15.4	24.8	9.4	2.95	4.18	1.23
III	23.2	34.3	11.1	3.23	4.53	1.30
activity (s^{-1})	12.8	19.4	6.6	2.69	3.73	1.04

[a]Average errors: ±2.2 kJ = ±11% for ΔG_{H_2O}, ±3.1 kJ = ±33% for $\Delta\Delta G_{H_2O}$, ±0.07 M = ±1.7% for $[urea]_{1/2}$; ±0.10 M = ±6.4% for $\Delta[urea]_{1/2}$.

a higher urea concentration by ~1.3 M. Accordingly, the values of ΔG_{H_2O} and $[urea]_{1/2}$ for the decrease in electron-transfer activity are each shifted to higher values, i.e., by ~7 kJ and 1.0 M, respectively. The dissociation of subunits VIIa and III is better resolved in the presence of sulfate anions and clearly demonstrates that dissociation of subunit VIIa precedes dissociation of subunit III. Sulfate anions also stabilize the structural changes responsible for urea-induced activity loss. In the presence of sulfate anions, activity loss is nearly complete before subunit III dissociation begins, which apparently rules out the possibility that activity loss is coupled to dissociation of this subunit. Activity loss also seems to precede dissociation of subunit VIIa, although the difference of ~0.4 M urea between the subunit loss curve and the activity loss curve is only marginally larger than the experimental error (±0.1 M urea).

DISCUSSION

To test the hypothesis that PL removal, or more specifically CL removal, destabilizes the quaternary structure of CcO, the subunit content of CL-containing (CcO^{CL+}) and CL-free (CcO^{CL-}) was determined after each had been exposed to a series of different urea or GdmCl concentrations. Three preparations of CcO^{CL+} were analyzed that contained 3 or 4 CLs/CcO monomer together with ~2, ~8, or ~20 other PLs (primarily PC and PE). The additional amounts of PL had a minimal effect upon the denaturant-induced subunit dissociation curves (the largest amount of PL stabilized each dissociation by <0.2 M urea). With each preparation of CcO^{CL+}, subunits with less affinity (VIa and VIb) dissociate at low perturbant concentrations, those with higher affinity (III, Vb, and VIIa) dissociate at higher perturbant concentrations, and the most tightly bound remain associated in an eight-subunit core containing all of the redox centers.[12] Removal of CL decreases the affinity of each of these subunits for the core of CcO. Association of subunits VIa and VIb is so weakened that structural perturbants are no longer necessary for their removal and both dissociate during the CL removal procedure. Spontaneous dissociation of subunits other than subunits VIa and VIb is not observed, and CcO^{CL-} contains a full complement of the 11 remaining subunits.[5] However, the association of subunits III, Vb, and VIIa is weakened, and each dissociates from CcO^{CL-} at a urea or GdmCl concentration lower than that for dissociation from CcO^{CL+} (lower by 1.1−1.7 M urea; lower by 0.3−0.4 M GdmCl). Analysis of the urea-induced sigmoidal subunit dissociation curves using a denaturant-induced reversible protein unfolding model[12,29] allowed us to quantify the stabilization effect of CL. Association of subunits III, Vb, and VIIa is each stabilized by 9−10 kJ when CL is bound to CcO. Presumably, subunits VIa and VIb are stabilized by a similar amount.

Stabilization of only 10 kJ/mol seems to be quite small, but it must be emphasized that these values are relative rather than absolute and do not necessarily measure the absolute stabilization effect *in vivo*. All experiments were conducted in the presence of the solubilizing detergent dodecyl maltoside so that all of the hydrophobic surfaces that become exposed during subunit dissociation are immediately coated with detergent, thereby making the values underestimates of the true ΔG°_{diss}. It is more accurate to consider each experimental value as the free energy of transfer of a subunit from CcO to the interior of a micelle.

Although the use of a solubilizing detergent results in an underestimation of ΔG_{diss}, its presence is essential to minimize irreversible protein aggregation and to permit thermodynamical analysis according to the Lumry−Eyring model, e.g., $N \overset{K_{eq}}{\longleftrightarrow} D \xrightarrow{k_{irrev}} F$, where N is native, D is the native dissociated state, F is the final state, and $D \xrightarrow{k_{irrev}} F$ is irreversible unfolding of the dissociated subunit. After exposure to urea or GdmCl for 10 min, the second irreversible, unfolding step is minimized and no changes are detected in either the UV CD spectrum or the second-derivative visible heme spectrum. To properly analyze the data according to eq 1, it is important to minimize the second, irreversible step. Irreversible unfolding is unlikely to be identical for CL-containing and CL-free CcO because the same subunit dissociates at a different denaturant concentration with the two forms of oxidase. Only if the rate for the irreversible step is equal for the two forms of oxidase does $\Delta\Delta G_{diss}$ reflect the difference in the free energy of dissociation. The more probable case is that the rates for the irreversible steps are slightly different, which would slightly affect each $\Delta\Delta G_{diss}$ value. However, it needs to be emphasized that even in the presence of a solubilizing detergent, the subunit dissociations do not behave as simple reversible reactions. Once the dissociated subunit is embedded in a micelle, and the uncovered site in CcO is coated with detergent, future protein−protein contacts between the dissociated subunit and CcO are precluded. In fact, we have never achieved reassociation of any of the dissociated subunits with subunit-depleted CcO, even in the presence of stabilizing additives such as glycerol or poly(ethylene glycol).

Furthermore, the ability of cardiolipin to function as a type of "glue" relies not only on the strength of its stabilizing interaction but also upon its flexibility and unusual bidentate structure, both of which allow it to link relatively distant sites. In fact, cardiolipin, in its extended conformation, can connect sites ~20 Å apart. The flexibility of the cardiolipin acyl chains also may allow cardiolipin (i) to maintain an interaction between two (distant) sites even after a conformational change, i.e., as might occur during the catalytic cycle, or (ii) to transfer information about a conformational change at one site to a distant site within the protein complex. Support for its universal importance is indirectly evident from the fact that it is indispensable for the activity and/or stability of numerous multisubunit and/or oligomeric proteins, including complex II,[6] complex III,[7] glycerol-3-phosphate dehydrogenase,[30] NADH dehydrogenase,[31] the ADP/ATP carrier,[32] and supercomplexes within the inner mitochondrial membrane.[8]

Although subunit associations within the CL-free complex are clearly weakened, it is difficult to determine whether this is a

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direct or indirect consequence of CL removal. Because CcO^{CL-} does not contain CL or subunits VIa and VIb, the decreased level of association of subunits III, Vb, and VIIa within CcO^{CL-} could be caused by one of several possibilities: (1) destabilization of the entire complex because of removal of subunits VIa and VIb, (2) a structural perturbation of the entire complex upon CL removal that weakens subunit associations, or (3) loss of energetically favorable contacts with CL, which destabilize specific subunit associations.

We believe the first possibility is unlikely because removal of subunits VIa and VIb without removal of bound CL only mildly affects the stability of CcO and does not alter the electron-transfer activity of the enzyme.[5] It is known that subunits VIa and VIb stabilize the CcO dimer because they participate in many of the contacts between CcO monomers within dimeric CcO.[2] Dimerization also increases the quaternary stability of CcO as probed by elevated hydrostatic pressure.[11] However, in this study, dodecyl maltoside-solubilized CcO^{CL+} and CcO^{CL-} are both monomeric, which negates the stabilizing effect of dimerization. Furthermore, the value of $[urea]_{1/2}$ for activity loss using an 11-subunit enzyme that still contains CL is only slightly lower than the value obtained with the 13-subunit enzyme containing CL [$[urea]_{1/2}$ = 3.9 M vs 4.4 M (data not shown)]. The value for the CL-containing 11-subunit enzyme is significantly higher than that for the CL-free 11-subunit enzyme ($[urea]_{1/2}$ = 2.6 M). All of this suggests that the lower affinity of subunits III, Vb, and VIIa within CcO^{CL-} is not a direct consequence of removal of subunit VIa and VIb.

Differentiating between the second and third possibilities, i.e., whether CL removal induces global or local structural perturbations, is more difficult. Previous thermal denaturation studies of CcO suggest the effect may be global, while arylazido-CL photolabeling experiments[30] and coarse grain molecular dynamics simulation studies[31] indicate specific molecular contacts exist between CL and the perturbed subunits with CL, suggesting important local effects.

Evidence That CL Removal Does Not Induce Global Destabilization of CcO. Comparison of the thermal and kinetic stability of CcO^{CL+} and CcO^{CL-} indicates that bound CL enhances the kinetic stability of CcO.[10] Without bound CL, the first thermal transition, which involves dissociation of subunits III and VIIa, occurs at a lower temperature. These are the same subunits that dissociate with a lower urea or GdmCl concentration when CL is removed from CcO. Although removal of CL decreases the kinetic stability of CcO, its removal does not significantly perturb either heme *a* or a_3,[5] nor are these heme environments perturbed during the 10 min exposure to urea, which is sufficient for dissociation of these subunits.[11] Because the structural perturbations in CcO upon CL removal are limited to those subunits that directly contact CL, and regions distal from CL, e.g., heme *a* and a_3 remain unperturbed, we believe that perturbation of the entire CcO structure during CL removal is probably not the primary cause of the decreased affinity for subunits III, VIIa, and Vb in CcO^{CL-}.

Evidence That Specific Molecular Contacts with CL Stabilize the Association of Subunits III, Vb, and VIIa. Four CL molecules are resolved within the dimeric CcO crystal structure, two within each monomer.[4] Two CL are symmetrically located at the dimer interface, bridging between subunits VIa and III on one side of the interface with subunits I and II on the other. The other CL resolved in each monomer is located in a crevice on subunit III with the headgroup touching subunit VIIa. Each of these locations is consistent with the decreased affinity we have observed for subunits III, VIa, VIb, and VIIa upon removal of CL from CcO. Although the crystal structure has been invaluable to the interpretation of our results, it cannot predict which subunits, if any, would be destabilized by CL removal. In fact, there is no evidence that the binding affinity of all subunits in contact with CL is affected upon CL removal; e.g., subunits I and II are clearly in contact with CL but are not sensitive to denaturant-induced dissociation. Furthermore, our previous CL removal studies[5] and arylazido-CL photolabeling results[33] suggest that at least one more CL is tightly bound to CcO, most likely on subunit I between subunits VIIa and VIIc,[33] which would place it near the entrance to the proton D-channel. Such a location could explain the sensitivity of CcO activity upon subunit VIIa removal. A recent in-depth coarse grain molecular dynamics simulation study supports the possibility that three CLs are bound to each CcO monomer: a low-affinity site localized at the interdimeric space close to subunits VIa and VIb, a high-affinity site localized adjacent to subunits III and VIIa, and a third high-affinity site between subunits I and VIIc.[34] With the exception of the third CL adjacent to subunits I and VIIa, the bound CLs are in direct contact with the subunits whose binding affinity is directly perturbed by CL removal. Removal of CL from these high-affinity sites would remove important stabilizing molecular contacts. For example, the CL bound in the cleft between subunits III and VIIa participates in numerous hydrogen bonds as well as electrostatic and hydrophobic interactions with both subunits III and VIIa (contacts with subunit III include Gln56, Arg59, Phe68, Arg221, and His226; contacts with subunit VIIa include Lys8, Phe12, Thr27, and Asp28). All of these contacts and interactions have been discussed in detail by Arnarez et al.[34] Removal of CL would disrupt these interactions and explain the decreased affinity of subunits III and VIIa for the core of CcO. Because subunit Vb is located adjacent to subunit III, it is not surprising that its association is also destabilized.

Removal of Subunit III and/or VIIa Inactivates CcO. Coincident with the removal of subunit III and/or VIIa from either CcO^{CL+} or CcO^{CL-} is a complete loss of enzymatic activity. The remaining electron transport activity for CcO^{CL+} and CcO^{CL-}, as well as the ability of exogenous CL to reactivate CcO^{CL-}, directly correlates with the content of subunit III and/or VIIa. It is difficult to distinguish which subunit is responsible for activity loss because both dissociate from either CcO^{CL+} or CcO^{CL-} at nearly the same denaturant concentration. However, inclusion of the structure-stabilizing anion sulfate stabilizes the association of subunit VIIa slightly more than subunit III. Because the subunits no longer dissociate at the same perturbant concentration, only the dissociation of subunit VIIa remains correlated with activity loss, although the correlation is less than perfect. Because subunit VIIa does not contain a redox center (nor does subunit III), the loss of activity must reflect a structural perturbation in a catalytically important pathway when it dissociates from CcO. What the perturbation might be is not obvious, but in CcO^{CL+}, removal of subunit VIIa would almost certainly disrupt binding of the third CL (the one between VIIa and VIIc), which is the CL located near the entrance to the catalytically important D-channel.

In conclusion, denaturant-induced dissociation of subunits from bovine heart CcO confirms the structural importance of bound CL in maintaining a stable quaternary structure.

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Removal of CL not only results in an irreversible loss of proton pumping activity,[3,5] and a reversible ~50% loss of electron-transfer activity,[3,5] but also significantly decreases the level of association of subunits III, Vb, VIa, VIb, and VIIa. The binding affinity of each of these subunits is decreased by 9−10 kJ when CL is removed from CcO, which is the first time that the stabilizing effect of CL has been quantified. Because each of these subunits either is in direct contact with two of the bound CLs or interacts with a subunit that binds CL, we conclude that specific molecular interactions between CL and protein stabilize the CcO quaternary structure. In this sense, CL is acting like a type of "subunit glue" to maintain a stable quaternary structure and fully active complex. The third CL, which is bound near subunits I and VIIc, does not alter the affinity of these subunits for the remainder of the complex, but it may play a critical role in the activity of the enzyme if it is bound near the entrance to the proton D-channel.

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Notes

The authors declare no competing financial interest.

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