Cholate-Induced Dimerization of Detergent- or Phospholipid-Solubilized Bovine Cytochrome *c* Oxidase†

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ABSTRACT: Bovine heart cytochrome *c* oxidase (CcO), solubilized by either nonionic detergents or phospholipids, completely dimerizes upon the addition of bile salts, e.g., sodium cholate, sodium deoxycholate, or CHAPS. Bile salt induced dimerization occurs whether dodecyl maltoside, decyl maltoside, or Triton X-100 is the primary solubilizing detergent or the enzyme is dispersed in phosphatidylcholine, phosphatidylethanolamine, or mixtures thereof. In each case, complete CcO dimerization can be verified by sedimentation velocity and sedimentation equilibrium after correction for bound detergent and/or phospholipid. The relative concentration of the bile salt is critical for production of homogeneous, dimeric CcO. For example, enzyme solubilized by 2 mM detergent requires an equal molar concentration of sodium cholate. Similarly, enzyme dispersed in 20 mM phospholipid requires 50 mM sodium cholate, concentrations that are commonly used to reconstitute CcO into small unilamellar vesicles. Bile salts do more than just stabilize dimeric CcO and prevent detergent-induced dissociation into monomers. They are able to completely reverse detergent-induced monomerization and cause completely monomeric CcO to reassociate. Dimeric CcO so generated is no more stable than the original complex and easily dissociates into monomers if the bile salt is removed. The dimerization process is dependent upon a full complement of subunits; e.g., if subunits VIa and VIb are removed, the resulting monomeric CcO will not reassociate upon the addition of sodium cholate. These results support four important consequences: (1) dissociation of dimeric CcO into monomers is reversible; (2) stable dimers can be produced under solution conditions; (3) dimers can be stabilized even at relatively high pH and low enzyme concentration; and (4) subunits VIa and VIb are required for dimerization.

Bovine heart cytochrome *c* oxidase (CcO;[1] EC 1.9.3.1) is an integral membrane complex of the mitochondrial respiratory chain and is comprised of 13 nonidentical subunits (*1*). The monomeric unit of CcO contains a single copy of each subunit with a total molecular weight of 204005. Crystallographic data indicate that the enzyme is almost always dimeric in two- or three-dimensional crystal lattices (*2*−*4*); the only exception is that CcO is monomeric in a two-dimensional crystal prepared from deoxycholate-solubilized enzyme (*5*). However, self-association of the detergent-solubilized, purified complex is more complicated. CcO is never homogeneous and dimeric when it is solubilized in a single nonionic detergent. It is either homogeneous and monomeric, a mixture of the monomeric and dimeric forms, or contains higher aggregates (*6*). Moreover, the relative proportion of monomer and dimer varies significantly between individual enzyme preparations when examined under identical experimental conditions; e.g., with 1 mg of dodecyl maltoside/mg of protein the percentage of dimer can vary from 20% to 80%.

The functional role of dimeric CcO is, as yet, poorly understood, but it is generally believed that the dimeric form occurs in vivo (*7*, *8*). There is also evidence that the dimeric form of several electron transport complexes plays an important role in electron transfer and possibly proton translocation (*7*, *9*−*11*). If the dimeric form does have a functional role in vivo, the experimental difficulty in obtaining homogeneous dimeric CcO with the purified, detergent-solubilized enzyme obviously compromises the interpretation of many solution studies on CcO. Therefore, it is important to determine solution conditions in which detergent-solubilized CcO is homogeneous and dimeric.

Here we describe a completely dimeric CcO produced by the addition of bile salts (cholate, deoxycholate, or CHAPS) to either detergent- or phospholipid-solubilized enzyme. Not only does addition of a bile salt stabilize the dimeric form but it also induces monomeric CcO to dimerize.

EXPERIMENTAL PROCEDURES

Materials. Dodecyl maltoside, decyl maltoside, and CHAPS were purchased from Anatrace Inc. Ultrapure Triton X-100 was from Boehringer-Mannheim and Tween 20 from Cal-

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[1] Abbreviations: CcO, bovine heart cytochrome *c* oxidase; Tween 20, polyoxyethylene(20)sorbitan monolaurate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, phosphatidylcholine; DOPC, dioleoylphosphocholine; DOPE, dioleoylphosphoethanolamine.

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biochem. Sodium cholate and deoxycholate were purchased from Sigma Chemical Co. and recrystallized from ethanol. DOPC, DOPE, and egg PC were purchased from Avanti Polar Lipids. Asolectin (type II-S) was from Sigma and was used without purification. Other chemicals were of analytical grade.

Cytochrome c Oxidase. Cytochrome *c* oxidase was purified from frozen bovine heart using a modification of the Fowler procedure (*12*). After the final ammonium sulfate precipitation, the oxidase pellet was dissolved in 0.1 M NaH_2PO_4, pH 7.4, buffer, containing 10 mg/mL sodium cholate and 1.0 mM EDTA. Droplets of the purified enzyme (20−30 mg/mL) were quickly frozen in liquid nitrogen, and the resulting pellets containing ~1 mg each of CcO were stored at −80 °C. Four different enzyme preparations were used in the present investigation. They contained 15−20 mol of PL/mol of monomeric CcO and had electron transport activity of 350−400 s^{-1} at pH 7.0 in 25 mM phosphate buffer containing 2 mM dodecyl maltoside determined spectrophotometrically as previously described (*6*). Enzyme isolated in Triton X-100 according to Soulimane and Buse (*13*) was a gift from Dr. M. Fabian in Dr. G. Palmer's laboratory at Rice University.

Cytochrome c Oxidase in Single Detergents. To prepare CcO samples in a single detergent, e.g., dodecyl maltoside or Triton X-100, the stock enzyme solution was diluted to 1 mg/mL in 20 or 50 mM HEPES buffer, pH 7.4, containing 1 mg/mL new detergent, dialyzed versus two changes of buffer containing at least 0.1 mg/mL detergent for 24 h at 4 °C. By this procedure the concentration of sodium cholate was lowered to less than 0.03 mg/mL as quantified with [^{14}C]-cholate (*6*).

Cytochrome c Oxidase in Nonionic Detergent/Sodium Cholate Mixtures. CcO samples in detergent/cholate mixtures were prepared from stock enzyme solution by dilution to 1 mg/mL with the 20 or 50 mM HEPES buffer, pH 7.4, containing 2 mM nonionic detergent and 2 mM sodium cholate. The resulting solution was then dialyzed for 24 h against buffer containing 0.2 mM nonionic detergent and 2 mM sodium cholate (both concentrations are sufficiently high to prevent removal of either detergent during dialysis of the CcO sample).

Cytochrome c Oxidase in Phospholipid/Cholate Mixed Micelles. Phospholipids, either egg PC, DOPC, DOPE, or a 1:1 mixture of DOPC/DOPE, were dried under nitrogen, and the resulting phospholipid film was suspended by vortexing in 0.1 M HEPES, pH 7.4, buffer, containing 50 mM sodium cholate. The resulting mixture was clarified by sonication for a few minutes on ice under nitrogen using a Branson model 250 sonifier in pulsed mode at 30 W power followed by centrifugation for 15 min at 12000*g*. Cytochrome *c* oxidase that had been dissolved in sodium cholate was then added to this clear solution and the self-association of the resulting enzyme/phospholipid/cholate mixture analyzed by analytical ultracentrifugation.

Analytical Ultracentrifugation. The sedimentation coefficients and molecular weight of cytochrome *c* oxidase were assessed by sedimentation velocity and sedimentation equilibrium experiments in a Beckman Optima XL-A analytical ultracentrifuge. All analytical ultracentrifugation experiments and data analysis were as previously described (*6*, *14*). The initial protein concentration was 5 μM (1 mg/mL) for sedimentation velocity experiments and 1.5 μM (0.3 mg/mL) for sedimentation equilibrium experiments. All enzyme solutions were dialyzed for 24 h against the appropriate buffer (see above) prior to analysis.

Preparation of 11-Subunit Cytochrome c Oxidase by HiTrapQ FPLC Ion-Exchange Chromatography. Eleven-subunit CcO was prepared by anion-exchange column chromatography using the HiTrapQ column procedure in dodecyl maltoside described by Sedlák and Robinson (*15*). Complete removal of subunits VIa and VIb was confirmed by C_{18} reversed-phase HPLC (*16*). The 11-subunit CcO preparation routinely had 85−90% of the normal enzymatic activity.

RESULTS

Cytochrome c Oxidase Is Dimeric in Phospholipid/Cholate Mixtures. Mixed micelles of phospholipid and cholate were used as a model system to determine the self-association state of CcO in phospholipid environments. In such mixed micelles, CcO is surrounded by an annulus of phospholipid that is coated and solubilized by a secondary layer of cholate. Therefore, CcO (5 μM) was solubilized in a mixture of 20 mM egg phosphatidylcholine and 50 mM sodium cholate, a composition similar to that commonly used to reconstitute CcO into small unilamellar vesicles. The result was unambiguous. In these mixed micelles, CcO was nearly homogeneous and dimeric with 85−95% of the enzyme sedimenting with $s_{20,w}$ between 9.5 and 9.8 S (Figure 1). This result is in direct contrast to its behavior in pure nonionic detergent environments where CcO either is a mixture of monomers and dimers or is completely monomeric (*6*). Confirmation that the 9.5−9.8 S species corresponded to dimeric oxidase was proved by the more rigorous method of sedimentation equilibrium. Sedimentation equilibrium data were readily fitted by nonlinear least squares regression analysis to a single noninteracting model system as evident by the nearly random residual errors (Figure 1, main panel). The resulting protein molecular weight of 405000 ± 5000, after correction for bound cholate and phospholipid, confirms the dimeric state for PC/cholate-solubilized CcO. Nearly identical results were obtained in DOPC/cholate, DOPE/cholate, or DOPC/DOPE/cholate mixtures. However, CcO was found to be a very heterogeneous mixture of high aggregates in a mixture of cholate and asolectin, i.e., soybean phospholipids, a mixture that is often used to reconstitute CcO into phospholipid vesicles.

Cholate-Induced Dimerization of Detergent-Solubilized Cytochrome Oxidase. Solubilization of purified CcO by low concentrations of alkyl maltosides, e.g., 2 mM dodecyl maltoside or decyl maltoside, usually results in a mixture of monomers and dimers with $s_{20,w}$ values of 11.5−12.0 S and $s_{20,w}$ of 15.5−16.0 S for monomers and dimers, respectively [Figure 2 (open circles in panels A and C; thin line in panels B and D); see ref *6*]. To our surprise, addition of 2 mM sodium cholate to these detergent-solubilized CcO mixtures produced nearly homogeneous dimeric enzyme with $s_{20,w}$ of 15.5 S [Figure 2 (filled circles in panels A and C; thick line in panels B and D)]. This was the first time that we have been able to produce homogeneous, dimeric enzyme in an entirely detergent environment. It suggests that sodium cholate, not the phosphatidylcholine, was responsible for CcO

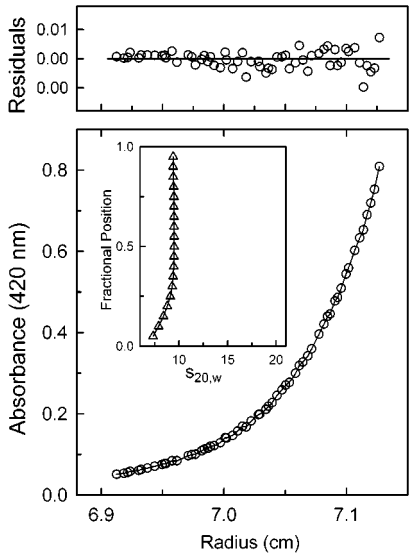

FIGURE 1: Dimerization of cytochrome *c* oxidase in phospholipid/cholate mixed micelles. Self-association was determined by analytical ultracentrifugation with correction for bound phospholipid and cholate. Cytochrome *c* oxidase, ~100 μM in 25 mM sodium cholate, was diluted to 5 μM with 0.1 M HEPES buffer, pH 7.4, containing 50 mM sodium cholate and 20 mM egg phosphatidylcholine. Analysis by sedimentation velocity (inset): The resulting solution of CcO was used directly, and 420 nm data were collected during centrifugation at 30000 rpm and 20 °C. Velocity data are presented as an integral distribution plot (inset) after data analysis by the van Holde−Weischet method using the UltraScan software as described in Experimental Procedures. Analysis by sedimentation equilibrium (main panel): The solution was diluted 8-fold to 0.6 μM CcO using the cholate/PC/HEPES buffer; equilibrium data were collected at 420 nm after centrifugation for 24 h at 6000 rpm. The experimental data (open circles) were fitted by nonlinear least squares to a single component with a molecular weight of 405000 (solid line). Residual differences between the best-fit line and experimental data are shown in the top panel.

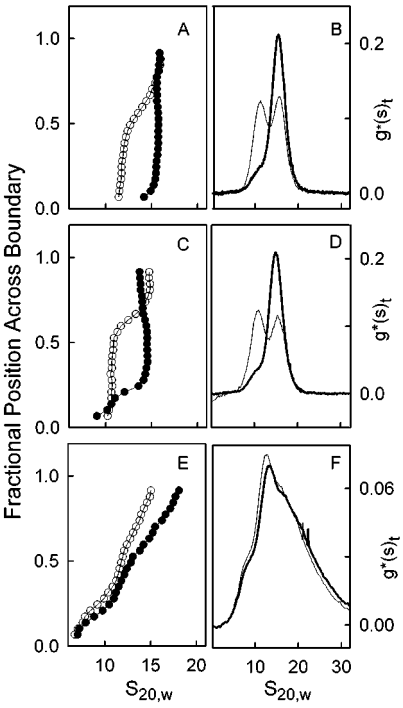

FIGURE 2: Stabilization of dimeric cytochrome *c* oxidase by sodium cholate. Cytochrome *c* oxidase self-association was quantified by sedimentation velocity at 40000 rpm and 20 °C. The resulting data were analyzed by both the van Holde−Weischet (panels A, C, E) and the Stafford $g^*(s)_t$ methods (panels B, D, F). In each sample, CcO (5 μM) was solubilized in 50 mM HEPES buffer, pH 7.4, in the presence of 2 mM sodium cholate (filled circles, panels A, C, E; thick line, panels B, D, F) or its absence (open circles, panels A, C, E; thin line, panels B, D, F). The solutions also contained either 2 mM dodecyl maltoside (A, B), 2 mM decyl maltoside (C, D), or 0.8 mM Tween 20 (E, F). Samples were prepared as described in Experimental Procedures.

dimerization in the PC/cholate mixtures of Figure 1. The sedimentation coefficient for CcO in alkyl maltoside/cholate mixtures was independent of both protein concentration (0.025−1 mg/mL) and solution pH (7.2−8.8), a result that is quite different from that previously observed in pure alkyl maltosides. However, with cholate concentrations above 2 mM, or lower than 1 mM, CcO was not homogeneous and dimeric but was a heterogeneous mixture of monomer, dimer, and higher aggregates, with behavior similar to that of CcO solubilized by sodium cholate or nonionic detergents alone (*6*, *17*).

The continued presence of sodium cholate is absolutely essential to prevent partial monomerization of CcO by nondenaturing detergents. Once the sodium cholate is removed, CcO self-association is similar to that in pure alkyl maltoside; i.e., sodium cholate induces dimers that are no more stable than those present in the original preparation (results not shown). Moreover, the effect of cholate depends on the nature of the primary nonionic detergent. For example, dimers predominate when sodium cholate is added to dodecyl maltoside-solubilized CcO that is 50% monomeric (Figure 2A,B). Dimers also predominate when sodium cholate is added to decyl maltoside-solubilized cytochrome oxidase that is ~70% monomeric (Figure 2C,D) or to the Triton X-100-solubilized CcO that is 60−90% monomeric (data not shown). However, sodium cholate is ineffective in producing dimeric enzyme from a highly aggregated, heterogeneous CcO. For example, Tween 20-solubilized CcO is a heterogeneous mixture of monomers, dimers, and higher aggregates, and this heterogeneity remains after the addition of sodium cholate (Figure 2E,F). Sodium cholate induces or stabilizes CcO dimers only if CcO is solubilized with an effective nonionic detergent. (Refer to ref *6* for a description of which detergents effectively solubilize CcO.)

Reassociation of Monomeric Cytochrome Oxidase. Incubation of CcO in high concentrations of nonionic detergents produces fully active, monomeric enzyme that contains all 13 subunits (*6*, *18*). For example, with 2−3 mg of Triton X-100/mg of protein, i.e., 500−1000-fold molar excess, CcO is 100% monomeric [Figure 3, panel A (open circles) and panel B (solid lines)]. Addition of 2 mM sodium cholate to these samples induces significant dimerization: i.e., 80−90% dimers with 2 mg of Triton X-100/mg of CcO [Figure 3, panel A (filled circles) and panel B (dashed line)]; 55−60% dimers with 3 mg of Triton X-100/mg of CcO [Figure 3, panel A (filled squares) and panel B (dotted line)]. Sodium cholate-induced dimerization of completely monomeric enzyme is even more pronounced when CcO is solubilized with dodecyl maltoside (Figure 3, panels C and D). Clearly, detergent-induced monomerization is a reversible process. However, addition of sodium cholate to monomeric CcO isolated according to Soulimane and Buse (*13*) did not result in dimeric CcO. Presumably this is due to the very high concentration of Triton X-100 in such preparations.

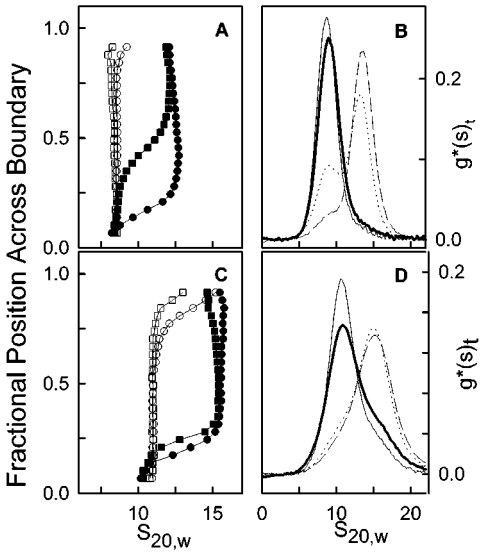

FIGURE 3: Dimerization of monomeric cytochrome *c* oxidase induced by sodium cholate. Monomeric CcO was prepared by exposing the enzyme (5 μM) in 50 mM HEPES buffer, pH 7.4, to either 3−5 mM Triton X-100 or 6−10 mM dodecyl maltoside. After verification that the enzyme was monomeric by sedimentation velocity, dimerization was induced by the addition of 2 mM sodium cholate. Sedimentation velocity conditions were identical to those of Figure 2. The resulting 420 nm absorbance data were analyzed by both the van Holde−Weischet (panels A and C) and Stafford $g^*(s)_t$ methods (panels B and D). Top panels (A and B): CcO (5 μM) containing 3 mM Triton X-100 with (● and dotted line) or without (○ and thin solid line) 2 mM sodium cholate; 5 mM Triton X-100 with (■ and dashed line) or without (□ and thick solid line) 2 mM sodium cholate. Bottom panels (C and D): CcO (5 μM) containing 6 mM dodecyl maltoside with (● and dotted line) or without (○ and thin solid line) 2 mM sodium cholate; 10 mM dodecyl maltoside with (■ and dashed line) or without (□ and thick solid line) 2 mM sodium cholate.

Role of Subunits VIa and VIb in Dimerization of Cytochrome Oxidase. The major contacts between monomers in dimeric CcO involve subunits VIa and VIb. It has been suggested that contacts involving these subunits stabilize the dimer and are necessary for CcO dimerization (*8*, *19*). Selectively removing both subunits produces an 11-subunit complex that can be purified by either Mono-Q or HiTrapQ anion-exchange chromatography (*15*). Sedimentation velocity analysis of this purified form of CcO confirms the prediction that removal of subunits VIa and VIb would produce a monomeric complex (Figure 4, open circles and thin line). Furthermore, in contrast to monomeric enzyme that contains all 13 subunits, sodium cholate does not induce dimerization of the monomeric complex that is missing subunits VIa and VIb; i.e., the sedimentation behavior before and after exposure to cholate is identical (compare filled and open circles, or thick and thin lines, in Figure 4).

DISCUSSION

Bovine heart cytochrome *c* oxidase is almost always dimeric in two-dimensional and three-dimensional crystals. The only exception is that it is monomeric in two-dimensional crystals prepared from deoxycholate-solubilized enzyme (*5*). The contacts between monomers within the crystalline dimer are strong enough that the dimer is thought to be the active form of the enzyme in vivo (*7*, *8*). However, the dimeric form is unstable in pure nonionic detergent

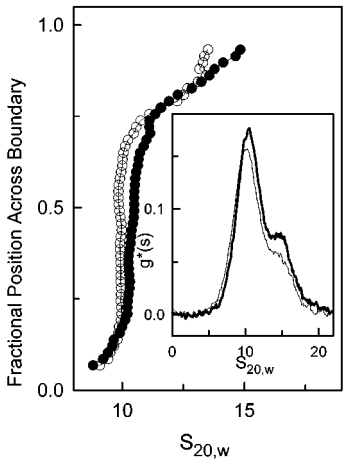

FIGURE 4: Lack of cytochrome *c* oxidase dimerization if it is missing in both subunit VIa and subunit VIb. Sedimentation velocity conditions were identical to those in Figure 2. The resulting 420 nm absorbance data were analyzed by both the van Holde−Weischet (main panel) and Stafford $g^*(s)_t$ methods (inset). Samples of purified 11-subunit CcO were prepared in 20 mM Tris−SO_4 buffer, pH 7.5, containing 2 mM dodecyl maltoside with (filled circles and thick line) or without (open circles, thin line) 2 mM sodium cholate.

environments, and CcO is only partially dimeric after detergent solubilization (*6*). To date, it has not been possible to prepare homogeneous dimeric detergent-solubilized enzyme. If dimeric CcO is really the most favored state in vivo, it should also be favored in phospholipid bilayer environments that mimic the native mitochondrial inner membrane. Therefore, we investigated the self-association behavior of CcO in a model mixed micelle system comprised of phospholipid and sodium cholate.

Bile salts solubilize phospholipids by coating the apolar faces of bilayer patches, not by forming random uniform mixtures. We reasoned that solubilization of CcO by mixtures of sodium cholate and phospholipid should produce a similar bilayer patch with CcO inserted in its center. If this hypothesis is correct, a mixture of sodium cholate and phospholipid would place CcO into a soluble environment that mimics its native state. The self-association behavior of CcO in such a mixed micelle system should reflect its behavior within the mitochondrial inner membrane better than solubilization by only nonionic detergents.

The above hypothesis was confirmed by analyzing the sedimentation behavior of CcO in phospholipid/bile salt mixtures. Dimeric CcO is the favored self-association state in a number of such mixtures, a result that is quite different than we previously found when the enzyme was placed in pure nonionic detergent environments. Sodium cholate, sodium deoxycholate, and CHAPS are equally effective in producing dimeric enzyme that is placed in DOPC, DOPE, a DOPC/DOPE mixture, or egg PC. However, dimers are not produced if asolectin is used as a source of phospholipids, and CcO is highly aggregated in any of the asolectin/bile salt mixtures. Similar bile salt/phospholipid mixtures are routinely used to reconstitute CcO into small unilamellar phospholipid vesicles (SUV's) for determination of its proton pumping activity. If we are correct and sodium cholate only coats and solubilizes a bilayer patch with CcO at its center, it is likely that CcO would remain dimeric as the sodium cholate is slowly removed and SUV's are formed. Therefore,

we conclude that CcO is probably dimeric within most proton pumping assays that involve fairly pure sources of phospholipids. In fact, cross-linking studies have shown that CcO is at least partially dimeric after reconstitution into phospholipid vesicles (*20*). However, the self-association state after reconstitution into asolectin vesicles is poorly defined and is unlikely to be entirely dimeric.

The ability of bile salts to alter the self-association behavior of CcO is not limited to only phospholipid environments. Dimeric CcO is also favored in mixtures of bile salts and nonionic detergents. The concentration of bile salt that is necessary to produce dimeric CcO (~2 mM) is significantly less than the CMC (5−10 mM) but is approximately equal to the concentration of nonionic detergent that is present (2 mM) (refer to Figure 2). In these experiments, sodium cholate is probably reorganizing the bound detergent and altering CcO self-association in one of two ways: (1) stabilizing dimers present in the initial preparation, thereby preventing their dissociation by addition of nonionic detergent, or (2) making the dimer form of CcO thermodynamically favored, i.e., inducing monomeric CcO to dimerize. Our results support the second hypothesis. Fully monomeric CcO dimerizes upon the addition of sodium cholate (refer to Figure 3); i.e., detergent-induced monomerization is fully reversible. Full reversibility is somewhat unexpected. In fact, the unusual "hooking" of subunit VIa of one CcO monomer into the cleft between helix V and helix VII of subunit I of the other CcO monomer led Yoshikawa and co-workers to predict that monomerization of bovine cytochrome oxidase would likely produce such a large conformational change in the N-terminal region of subunit VIa that dissociation of CcO dimers was probably not reversible (*8*, *19*). Whether the contacts between monomers in the cholate-induced dimers are exactly the same as those in the crystal structure is, of course, not known. However, we have not been able to detect any difference in terms of stability or subunit loss between those induced to form by sodium cholate and the original dimers.

These results are entirely consistent with the two previous studies of bovine CcO in which the enzyme had been solubilized by cholate/detergent or cholate/phospholipid mixed micelles. Nearly 30 years ago, Kuboyama et al. (*21*) reported that bovine CcO was dimeric in mixed micelles of Emasol-1130 and sodium cholate with a molecular weight of 430000. Finel and Wikström (*22*) also produced dimeric CcO in a cholate/phospholipid mixture. In fact, both the two-dimensional and three-dimensional crystals of bovine CcO, which produced crystalline dimeric CcO, were produced from cholate-solubilized CcO. These studies together with our results clearly support the predominance of dimeric CcO in detergent and phospholipid environments that also include sodium cholate or some other bile salt.

The dimerization step itself cannot involve a general requirement for a phospholipid as is required for the dimerization of photosystem II (*23*). CcO from which all phospholipid has been removed, except for the four tightly bound cardiolipins (*15*), still completely dimerizes in the absence of exogenous phospholipid (refer to Figures 2 and 3). The remaining few molecules of cardiolipin may possibly stabilize dimeric CcO, but we presently believe their role in dimerization is largely indirect. For example, two of these cardiolipins prevent the dissociation of subunits VIa and VIb, both of which directly participate in monomer−monomer contacts. However, the possibility that these two cardiolipins directly participate in dimer formation cannot be ruled out. Coincident removal of these CL's together with subunits VIa and VIb precludes directly testing this hypothesis.

The most likely reason that bile salts are able to induce CcO dimerization is that they structurally rearrange detergent or phospholipid located at the protein surface, thereby permitting the necessary protein−protein contacts. The planar bile salts probably cause the hydrophobic tails of the detergent or phospholipid to line up parallel to the bile salt and, therefore, roughly parallel to the CcO transmembrane helices. This would cause the protein−detergent complex to more closely resemble a native membrane bilayer rather than an artificial micellar environment with its radially oriented detergent tails. At least transient fusion between the cholate-solubilized protein/mixed micelles must then occur to permit the reassociation of CcO monomers into dimers.

An alternative possibility is that sodium cholate binds directly at specific site(s) on CcO and induces dimerization through a cholate-induced conformational change. Such sites may exist since 2−10 molecules of sodium cholate are associated with CcO in the three-dimensional crystal structure (*4*, *24*). According to Napiwotzki et al. (*24*), these cholate molecules may occupy important ADP or ATP binding sites on CcO. Two of these sites are localized on subunit VIa, which is known to stabilize the CcO dimer (*19*); therefore, ADP or ATP binding may alter CcO self-association. ATP does induce dissociation of dimeric *Thiobacillus novellus* CcO (*25*), but this must occur by way of a very different mechanism since bacterial CcO's do not contain a subunit analogous to subunit VIa. Furthermore, the ATP effect on bacterial CcO's is exactly opposite to what we observe for the effect of cholate on a mitochondrial CcO, i.e., dimer stabilization. To date, we have been unable to detect any perturbations of the oligomeric structure of bovine heart CcO by ATP, ADP, or ADP/ATP mixtures.

A question that remains to be answered is whether self-association of CcO occurs in vivo. Because dimers are favored in the cholate/phospholipid mixture, it suggests that dimers would also be favored in membrane environments. However, it should be remembered that, to date, dimers have been detected only with enzyme that has been exposed to sodium cholate or some other bile salt. Purification of bovine CcO from mitochondrial membranes almost always involves detergent solubilization by either sodium cholate or Triton X-100. We know that extraction of CcO by high concentrations of Triton X-100 produces homogeneous, monomeric CcO (*6*); therefore, it is impossible to obtain any information regarding the intrinsic dimerization of CcO from enzyme isolated in Triton X-100. In fact, we were not able to induce dimerization of CcO by addition of sodium cholate to enzyme prepared in Triton X-100 according to Soulimane and Buse (*13*). Purification of CcO after its exposure to deoxycholate and cholate introduces the opposite problem. Because the bile salts used in purifying the enzyme would induce dimerization, it becomes difficult to interpret such studies as supporting an intrinsic propensity of CcO to dimerize in vivo. Unfortunately, almost all structural work, including our own, has been done on enzyme that has been exposed to either Triton X-100 or bile salts. However, if the above cholate-induced fusion model is correct, then sodium cholate

is only making the model micellar system more closely resemble a naturally occurring phospholipid bilayer. If lateral diffusion of phospholipid and protein within cholate/phospholipid mixed micelles is favored and results in CcO dimerization, then similar lateral diffusion and dimerization should also occur within the native bilayer environment. Therefore, the cholate/phospholipid mixed micelle system that both we and Wikström and Finel (*22*) have utilized to produce CcO dimers seems to be the best evidence yet that CcO has an intrinsic propensity to dimerize within a bilayer environment.

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REFERENCES

1. Kadenbach, B., Jaraush, J., Hartmann, R., and Merle, P. (1983) *Anal. Biochem. 129*, 517−521.
2. Henderson, R., Capaldi, R. A., and Leigh, J. S. (1977) *J. Mol. Biol. 112*, 631−648.
3. Yoshikawa, S., Tera, T., Takahashi, Y., Tsukihara, T., and Caughey, W. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1354−1358.
4. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science 272*, 1136−1144.
5. Fuller, S. D., Capaldi, R. A., and Henderson, R. (1979) *J. Mol. Biol. 134*, 305−327.
6. Musatov, A., Ortega-Lopez, J., and Robinson, N. C. (2000) *Biochemistry 39*, 12996−13004.
7. Klingenberg, M. (1981) *Nature 290*, 449−454.
8. Lee, S. J., Yamashita, E., Abe, T., Fukumoto, Y., Tsukihara, T., Shinzawa-Itoh, K., Ueda, H., and Yoshikawa, S. (2001) *Acta Crystallogr. D57*, 941−947.
9. Wikström, M., Krab, K., and Saraste, M. (1981) *Cytochrome Oxidase: A synthesis*, Academic Press, London.
10. Yu, C. A., Xia, D., Kim, H., Deisenhofer, J., Zhang, L., Kachurin, A. M., and Yu, L. (1998) *Biochim. Biophys. Acta 1365*, 151−158.
11. Sadoski, R., C., Zaslavsky, D., Gennis, R. B., Durham, B., and Millett, F. (2001) *J. Biol. Chem. 276*, 33616−33620.
12. Robinson, N. C., Neumann, J., and Wiginton, D. (1985) *Biochemistry 24*, 6298−6304.
13. Soulimane, T., and Buse, G. (1995) *Eur. J. Biochem. 227*, 588−595.
14. Robinson, N. C., Gomez, B., Musatov, A., and Ortega-Lopez, J. (1998) *Chemtracts: Biochem. Mol. Biol. 11*, 960−968.
15. Sedlák, E., and Robinson, N. C. (1999) *Biochemistry 38*, 14966−14972.
16. Liu, Y.-C., Sowdal, L. H., and Robinson, N. C. (1995) *Arch. Biochem. Biophys. 324*, 135−142.
17. Robinson, N. C., and Capaldi, R. A. (1977) *Biochemistry 16*, 375−381.
18. Georgevich, G., Darley-Usmar, V. M., Malatesta, F., and Capaldi, R. A. (1983) *Biochemistry 22*, 1317−1322.
19. Yoshikawa, S., Shinzawa-Itoh, K., and Tsukihara, T. (1998) *J. Bioenerg. Biomembr. 30*, 7−14.
20. Estey, L. A., and Prochaska, L. J. (1993) *Biochemistry 32*, 13270−13276.
21. Kuboyama, M., Yong, F. C., and King, T. E. (1972) *J. Biol. Chem. 247*, 6375−6383.
22. Finel, M., and Wikström., M. (1986) *Biochim. Biophys. Acta 851*, 99−108.
23. Kruse, O., Hankamer, B., Konczak, C., Gerle, C., Morris, E., Radunz, A., Schmid, G. H., and Barber, J. (2000) *J. Biol. Chem. 275*, 6509−6514.
24. Napiwotzki, J., Shinzawa-Itoh, K., Yoshikawa, S., and Kadenbach, B. (1997) *Biol. Chem. 378*, 1013−1021.
25. Shoji, K., Tanigawa, M., Hori, K., Tomazawa, Y., and Yamanaka, T. (1999) *Eur. J. Biochem. 264*, 960−964.

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