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Influence of Reduction of Heme *a* and Cu_A on the Oxidized Catalytic Center of Cytochrome *c* Oxidase: Insight from Organic Solvents†

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ABSTRACT: Purified bovine heart cytochrome *c* oxidase (CcO) has been extracted from aqueous solution into hexane in the presence of phospholipids and calcium ions. In extracts, CcO is in the so-called "slow" form and probably situated in reverse micelles. At low water:phospholipid molar ratios, electron transfer from reduced heme *a* and Cu_A to the catalytic center is inhibited and both heme a_3 and Cu_B remain in the oxidized state. The rate of binding of cyanide to heme a_3 in this oxidized catalytic center is, however, dependent on the redox state of heme *a* and Cu_A. When heme *a* and Cu_A are reduced, the rate is increased 20-fold compared to the rate when these two centers are oxidized. The enhanced rate of binding of cyanide to heme a_3 is explained by the destabilization of an intrinsic ligand, located at the catalytic site, that is triggered by the reduction of heme *a* and Cu_A.

Mitochondrial cytochrome *c* oxidase (CcO)[1] is a membrane protein that catalyzes the oxidation of ferrocytochrome *c* by molecular oxygen. This reduction of oxygen to water requires the availability of four electrons and four protons for the catalytic cycle of the enzyme. Electrons enter the oxidase from the cytosolic domain and protons from the matrix phase of the inner mitochondrial membrane. This redox reaction is one of the two processes that contribute to the generation of a transmembrane potential. The second process, proton pumping, is the translocation of protons from the mitochondrial matrix space to the cytosolic side of the membrane, driven by electron transfer (ET).

Bovine heart CcO is composed of 13 subunits (*1*), though the four redox centers, involved in ET and in the reduction of O_2 to water, are located in subunits I and II (*2*). Three of these centers, heme *a*, heme a_3, and the mononuclear copper site, called Cu_B, are located in subunit I, while the initial acceptor of electrons, Cu_A (the dinuclear copper center), is located in subunit II (*2*). Cu_A is close to the cytosolic surface of the protein and serves as the acceptor of electrons from cytochrome *c* (*3*−*5*). Electrons received by the oxidase are rapidly distributed between Cu_A and heme *a* on the microsecond time scale (*3*−*6*). ET continues then to the catalytic, binuclear, center composed of heme a_3 and Cu_B. At this catalytic center, the interaction of electrons, protons, and oxygen occurs. It is also the site of binding of external ligands, including the classical inhibitors of respiration such as cyanide, azide, and carbon monoxide.

The presence of four redox centers in CcO raises a question about their functional significance in catalysis. Clearly, the binuclear center directly participates in the chemical conversion of dioxygen to water. On the other hand, Cu_A and heme *a* could be considered to function solely as electron transfer sites. That the role of heme *a* and Cu_A may be more than sites for delivery of electrons to the binuclear center has been expressed several times (*7*−*11*). Recent data suggest that heme *a* is probably the driving element of the proton-pumping process (*12*). According to another, earlier, suggestion, the reduction of heme *a* and Cu_A switches the conformation of CcO from a "closed" to an "open" form (*7*−*9*). The difference between the open and closed conformations of CcO is reflected in the different rates of binding of cyanide to the binuclear center (*7*−*9*). In the open form, the rate constant for cyanide binding is several orders of magnitude higher than that of the closed enzyme (*7*−*9*). Because the transition from the slowly to the rapidly cyanide binding form was alternatively demonstrated by one-electron reduction of the binuclear site (*13*, *14*), it led to the suggestion that the closed to open form transition is induced by local redox changes at the heme a_3−Cu_B site (*13*, *14*).

Depending on the preparation protocol, isolated bovine heart CcO can be in two forms called "slow" and "fast" (*15*−*17*), reflecting the relatively slow and rapid kinetics of binding of cyanide to heme a_3 in the fully oxidized enzyme. The fast form is considered to be the native state of the enzyme, while the slow form is a product of modification of the binuclear site of the fast enzyme by acidic pH (*15*, *17*). A single catalytic turnover converts slow CcO to the fast form, and this transiently activated enzyme is called

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[1] Abbreviations: CcO, cytochrome *c* oxidase; CcO-O, fully oxidized CcO; CcO.CN, complex of oxidized CcO with cyanide; HR-CcO, half-reduced oxidase, when two electrons are located at heme *a* and Cu_A and the heme a_3 and Cu_B centers are oxidized; Fe_{a3}, iron of heme a_3; Fe_a, iron of heme *a*; ET, electron transfer; TX, Triton X-100; EPR, electron paramagnetic resonance; DAD, diaminodurene.

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"pulsed" oxidase. In the absence of turnover, the pulsed form relaxes back to slow CcO within hours (*15*). However, it has been concluded that the conversion of the closed to the open conformation is independent of the form of CcO, and it has to be present in both slow and pulsed enzymes (*8*).

Earlier studies demonstrated that the reactivity of CcO toward external ligands is affected by the redox state of the catalytic binuclear center itself (*13*, *14*). However, the possibility that the catalytic center can also be influenced by the redox state of heme *a* and Cu_A, in a manner similar to that of the closed to open transition, was not excluded. In this study, we present spectroscopic and kinetic evidence that the reduction state of heme *a* and Cu_A is sensed by the oxidized catalytic site. When both heme *a* and Cu_A are reduced, the rate of binding of cyanide to the oxidized heme a_3-Cu_B site is increased relative to the rate observed with the fully oxidized enzyme. This increase is not as great as that observed with CcO having one electron localized at the binuclear center (*13*), which indicates that the catalytic site can undergo two qualitatively similar but quantitatively different transitions. One transition is triggered by reduction of heme *a* and Cu_A and the second by the direct one-electron reduction of the binuclear site itself.

EXPERIMENTAL PROCEDURES

L-α-Phosphatidylcholine type II-S (asolectin) was from Sigma. Triton X-100 peroxide-free was from Roche Diagnostics. 2,3,5,6-Tetramethyl-1,4-phenylenediamine (diaminodurene, DAD), perchloric acid, 2,4-diaminophenol dihydrochloride, amonium molybdate(VI) tetrahydrate, sodium metabisulfite, and Karl Fisher reagent were obtained from Aldrich. NaCN was from Mallinckrodt. *n*-Hexane and type 4A molecular sieves were from EM Science and YM 100 centricon filters from Amicon.

Two forms of CcO were purified from bovine heart for extraction into organic solvent. The slow form of CcO was isolated by the method of Yonetani (*18*). For the preparation of fast oxidase, we used the method of Soulimane and Buse (*19*). However, we have found that only CcO isolated by the method of Yonetani, which uses the anionic detergent cholate, can be extracted directly into hexane. Enzyme prepared by the method of Soulimane and Buse utilizes the detergent Triton X-100, and this detergent has to be exchanged for cholate before extraction to obtain a reasonable yield of CcO in the organic solvent. The detergent exchange was accomplished by centrifugation of CcO in a sucrose gradient containing 2% cholate (*20*). This procedure separates CcO into two fractions, both of which can be successfully used for extraction into hexane. However, only the lighter fraction, which presumably corresponds to a monomeric form of CcO, was used in this work. CcO in 2% cholate was then diluted several times with 10 mM Tris-HCl (pH 7.6) and 0.2% cholate and reconcentrated using Amicon YM 100 centricon filters.

All data reported in this study were collected on enzyme isolated by the method of Yonetani in the slow form (*18*). However, we performed the same measurements, as described here, on oxidase purified in the fast form (*19*). It was found that after extraction into hexane there is no difference between these two forms of enzyme in the reducibility of the redox centers or in the kinetics of binding of cyanide to both oxidized and partially reduced CcO.

For extraction of CcO from water solutions into hexane, we have slightly modified the protocol of Bona et al. (*21*). Typically, 200 mg of dry asolectin was added to 1 mL of 100 μM CcO in 10 mM Tris (pH 7.6) and 0.2% cholate. The sample was mixed on a Vortex mixer at room temperature for ~3−5 min to suspend the solid asolectin. The resulting suspension was sonicated under a stream of nitrogen at 4 °C until clear. $CaCl_2$ (4 M) was added to the sonicated sample to a final concentration of 75 mM, and the sample was mixed again. The addition of $CaCl_2$ makes the sample turbid, and the pH decreases to ~5.5. Then 2 mL of hexane was layered on the surface, and the whole sample was mixed thoroughly on the Vortex mixer for ~5 min. Phase separation was facilitated by a short spin on a benchtop centrifuge. The hexane phase containing CcO was removed, and a second portion of hexane (2 mL) was added to the sediment and the extraction repeated.

The hexane fractions were pooled, cooled to −20 °C, and then warmed to room temperature. A precipitated residue at the bottom of the storage test tube, containing water and phospholipid, was discarded and the cycle of cooling and warming repeated until no additional residue was formed. CcO is extracted into hexane with a yield close to 70%. The final enzyme concentration was ~25 μM.

The extraction of cyanide into hexane was performed by adding 4 mL of hexane to 4 mL of 4 M NaCN in water that had been neutralized by HCl, followed by mixing on a Vortex mixer for 5 min. The hexane layer containing HCN was removed and stored at liquid nitrogen temperature until it was used. After extraction, the concentration of HCN was approximately 160 mM as established indirectly by the titration of cyanide in the water phase by silver nitrate (*22*). Both water solutions of cyanide, the initial and the solution after extraction, were quantified for their cyanide content. The decrease in the quantity of cyanide in the aqueous phase after extraction, relative to the initial solution, was taken to represent the amount of cyanide transferred into the organic solvent.

The hexane extract was analyzed for water (*23*) and phosphate content (*24*) using standard procedures. To determine the concentration of CcO in hexane, the complex of CcO with cyanide (CcO.CN) was prepared by addition of HCN in hexane followed by reduction with solid DAD. From the reduced minus oxidized optical difference spectrum, the concentration of CcO was determined using a $\Delta A_m(605-622)$ of 18.6 mM^{-1} cm^{-1} for the reduced minus oxidized heme *a* (*25*). Upon reduction of CcO.CN, there is no contribution from heme a_3 to the difference spectrum.

CcO extracted into hexane is probably in reverse micelles composed of phospholipids (*26*). It is known that the catalytic activity of enzymes in reverse micelles is strongly dependent on the molar ratio of water to surfactant (W_0) (*27*). The molar ratio of water to phospholipids was estimated as the ratio of water to total phosphate ($[H_2O]:[P_i] = W_0$). The W_0 of the extracts was close to 19 and this W_0 can be varied by addition of dry asolectin (1 mg of asolectin contains 30.8 μg of P_i) or by a brief sonication of the extract with an appropriate amount of buffer [10 mM Tris (pH 7.6)]. In this study, only extracts with a W_0 adjusted to 3 were used. Dilution of extracts, without changing W_0, was accomplished using dry hexane prepared by storing hexane over a type 4A molecular sieve. Because hexane is fairly volatile at room temperature,

the volume of the sample, if needed, can be decreased using a stream of nitrogen.

EPR spectra of soybean asolectin in hexane show signals from adventitious iron and manganese. The signal of non-heme iron in asolectin at $g = 4.3$ partially overlapped the $g = 3.55$ feature of the low-spin iron of the oxidized heme a_3−cyanide complex and the contribution of this signal was subtracted from spectra of the enzyme. Conditions for EPR measurements were as follows: modulation amplitude of 20 G, power of 3 mW, temperature of 10 K, frequency of 9.27 GHz, and enzyme concentration of 22.5 μM. Each sample was scanned at least twice using the same conditions, and the data were averaged to improve the signal-to-noise ratio.

Optical spectra and kinetics were recorded in an IBM 9430 spectrophotometer interfaced with a personal computer. EPR spectra were recorded with a Varian E-6 spectrometer equipped with an Air Products low-temperature dewar and transfer line. Data were analyzed and graphs prepared using Igor Pro (Wavemetrics, Lake Oswego, OR).

RESULTS

The optical spectrum of CcO extracted into hexane is composed of the spectra of oxidized enzyme, asolectin with a small contribution from light scattering of the slightly opalescent solution (Figure 1). The extracted enzyme is in the slow form as indicated by two major spectral characteristics: (i) the position of the Soret maximum at 414 nm (Figure 1A) and (ii) the presence of the $g = 12$ signal (*15*) in the EPR spectrum (not shown). The optical spectrum of oxidized CcO also exhibits a peak at 601 nm and a shoulder at 650 nm (Figure 1A). It should be noted that in aqueous solutions the slow form of the enzyme is characterized by a Soret band at 418 nm (*15*−*17*). The observed small spectral difference between the slow form of CcO in hexane and that in aqueous media we ascribe to interaction with asolectin and/or exposure of the protein to the organic solvent. An additional factor that might contribute to the spectral difference is the background slope from the asolectin.

When this slow form of oxidized CcO (CcO-O) in hexane is incubated with cyanide for 3 h at room temperature, the shifts of the absorption maxima to 599 and 428 nm are observed while the intensity of the shoulder at 655 nm band is decreased (Figure 1A). The magnetic circular dichroism spectrum of CcO.CN in the Soret region is almost twice as intense as that of CcO-O, as also observed in aqueous buffers (*28*), confirming that the binding of cyanide converts Fe_{a3}(III) to the low-spin state (data not shown).

It has been shown earlier by us (*21*) and others (*29*) that the reduction of CcO in hexane is dependent on W_0 and at low W_0 heme *a* and Cu_A can be reduced but heme a_3 is not (*21*). The optical spectrum of oxidase reduced by DAD when $W_0 = 3.0$ (Figure 1B) confirms these previous observations. The spectrum of reduced CcO exhibits maxima at 414 and 442 nm in the Soret region plus a single red-shifted maximum at 604 nm. These spectral changes show that heme *a* is reduced, giving rise to the maxima at 442 and 604 nm, and that heme a_3 remains oxidized with a Soret maximum at 414 nm. In addition Cu_A is reduced as judged by the disappearance of the 830 nm band of oxidized Cu_A (cf. ref *21*). Both heme *a* and Cu_A are almost completely reduced within 20 min following addition of solid DAD.

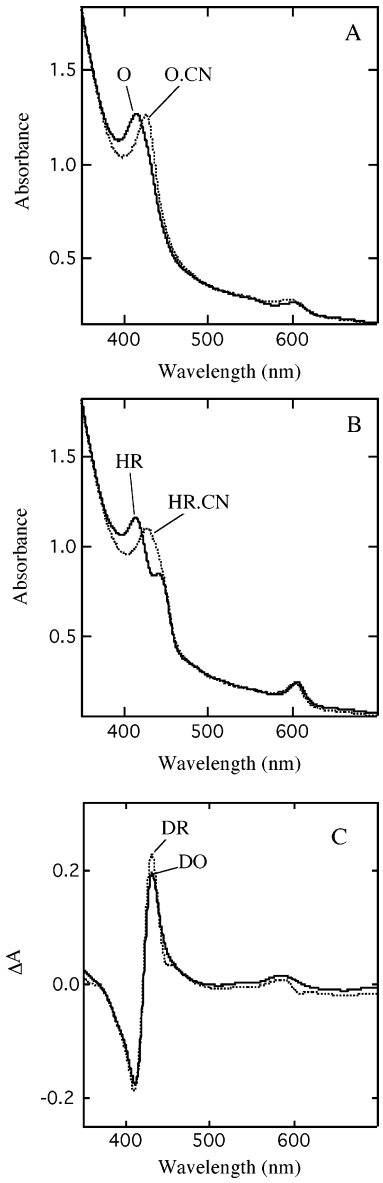

FIGURE 1: Optical spectra of cytochrome *c* oxidase in the fully oxidized or half-reduced state and in complex with cyanide in hexane when $W_0 = 3$. (A) Oxidized CcO (—, O) and the complex of oxidized CcO with cyanide (•••, O.CN). The spectrum of the complex was recorded 3 h after cyanide addition. (B) Half-reduced CcO (—, HR) and the complex of half-reduced CcO with cyanide (•••, HR.CN). The spectrum was taken 20 min after cyanide addition. (C) Difference spectra of HR.CN minus HR (•••, DR) and O.CN minus O (—, DO) for the samples presented in panels A and B. Spectra were corrected for dilution. Concentrations of CcO and cyanide were 7.2 μM and 7.8 mM, respectively.

The changes produced by binding of cyanide to the half-reduced oxidase (HR-CcO) are similar to those observed for the fully oxidized enzyme. After incubation of CcO with cyanide for 20 min, the absorption maxima are at 603 and 428 nm with a shoulder at 442 nm, and a decrease in intensity of the 650 nm absorption band is observed (Figure 1B).

The difference spectra of the half-reduced cyanide complex minus HR-CcO and of CcO.CN minus CcO-O are almost identical (Figure 1C). In both spectra, the absorption decrease of the 650 nm band, the maxima at 586 and 432 nm, and the minimum at 412 nm are apparent. These spectral characteristics are equivalent to those monitored for cyanide

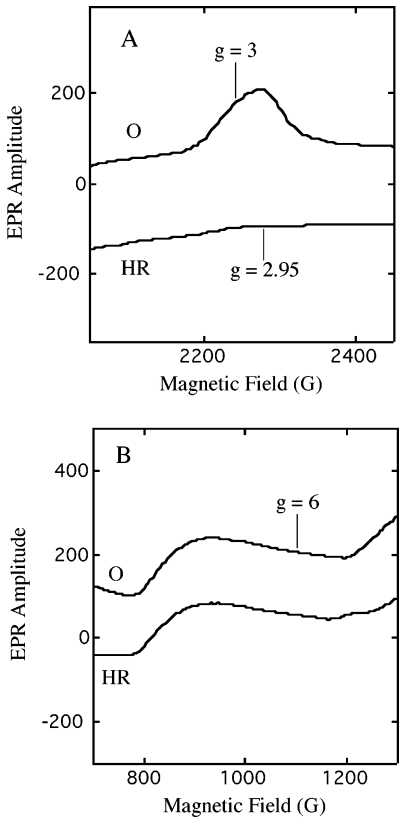

FIGURE 2: EPR spectra of oxidized and half-reduced CcO in hexane: (A) the $g = 3$ region and (B) the $g = 6$ region. Oxidized CcO (O) and half-reduced CcO (HR) in hexane at $W_0 = 3$. The HR sample was prepared by the reduction of oxidized CcO with DAD for 20 min at room temperature and then frozen in liquid nitrogen. The CcO concentration in both solutions is 22.5 μM. For conditions of EPR measurements, see Experimental Procedures.

binding to oxidized oxidase in aqueous solutions (*30*) and show that (i) heme a_3 remains oxidized in HR-CcO and (ii) the spectral state of oxidized heme a_3, before and after ligation with cyanide, is the same for both forms of the enzyme. From the difference spectra (Figure 1C), using a $\Delta A_m(432-412)$ of 58 mM^{-1} cm^{-1} (*29*), it can be calculated that 99% of oxidized heme a_3 in HR-CcO and 89% in oxidized CcO reacted with cyanide during the experiment. The small decrease in absorption at 606 and 450 nm in the difference spectrum of HR-CcO, which is absent in oxidized oxidase, is caused by a minimal oxidation of reduced heme *a* (~3%) during the reaction with cyanide.

Using optical spectroscopy, it was confirmed that at low W_0 the electron transfer to heme a_3 is inhibited and heme a_3 remains in the oxidized state. The redox state of Cu_B, the second metal ion at the catalytic site, cannot be assessed directly from the optical spectra. However, the presence of the band at 650 nm in HR-CcO (Figure 1B) indicates that this center is also oxidized (*31*). To verify the redox state of Cu_B, we have employed EPR spectroscopy. In the EPR spectra of the slow form of CcO in aqueous solutions, the one-electron reduction of the catalytic site, with an electron localized in Cu_B, produces two new signals (*14*, *32*): one is an intense high-spin signal at $g = 6$ (*14*) and the second a low-spin signal at $g = 2.95$ (*32*). These two possible signals are, however, missing in the EPR spectra of HR-CcO in hexane (Figure 2). In Figure 2, the initial EPR spectrum of CcO-O is compared with the final spectrum of HR-CcO in

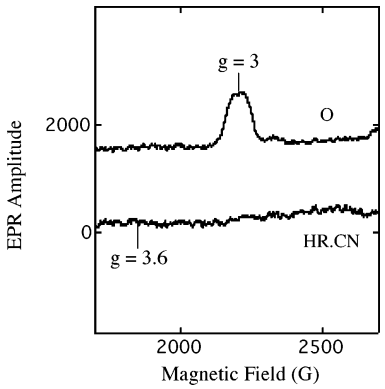

FIGURE 3: EPR spectra of oxidized CcO and the reduced complex of CcO with cyanide in hexane. Oxidized CcO (O) and half-reduced cyanide complex (HR.CN). The complex of O with cyanide was prepared first by incubation of oxidized CcO with cyanide for 3 h and then reduced by DAD for an additional 20 min at room temperature. The CcO concentration in both solutions is 22.5 μM. For conditions of EPR measurements, see Experimental Procedures.

the $g = 3$ and 6 regions. The major change produced by the reduction is that the $g = 3$ signal of the oxidized low-spin iron of heme *a* in the spectrum of CcO-O disappears in HR-CcO, which confirms again that heme *a* is fully reduced in HR-CcO.

It has to be also noted that the $g = 3$ signal of CcO-O is broader and shifted to a higher field relative to the signal in aqueous buffer (*33*). Because 150 mM chloride is present during the extraction, we explain the shape and position of the $g = 3$ signal by the superposition of two signals, namely, those from chloride-free and chloride-containing enzyme (*33*).

The conclusion that Cu_B is in the oxidized state in HR-CcO is also supported by the EPR spectra measured after the complex of oxidized CcO with cyanide was reduced (Figure 3). In this case, the iron of heme a_3 was first stabilized in the oxidized low-spin state by the bound cyanide and subsequently reduced by addition of solid DAD. If Cu_B were reduced in CcO.CN, then the new low-spin EPR signal would have appeared at $g = 3.6$ due to the low-spin ferric heme a_3−cyanide complex (*34*). However, this is not a case; only the $g = 3$ signal of heme *a* disappears on reduction (Figure 3), and no $g = 3.6$ signal is developed even after incubation of CcO.CN with DAD for 3 h at room temperature.

Based on EPR and optical spectra we conclude that at $W_0 = 3$ neither component of the binuclear center is reduced by an external electron donor. Under these conditions only heme *a* and Cu_A are reduced and we have referred to this species as half-reduced CcO.

Even though both CcO-O and HR-CcO have the binuclear center in the same oxidized state, the rates of cyanide binding are distinctly different (Figure 4). The kinetics of the rate of cyanide binding, monitored as an absorption change at 415 nm, can be fitted in both cases by a single exponential. The rate constants obtained from fits for CcO-O and HR-CcO are 4.2×10^{-4} and 8.8×10^{-3} s^{-1}, respectively. The corresponding bimolecular rate constant for CcO-O is ~0.05 M^{-1} s^{-1}, while for HR-CcO it is 1 M^{-1} s^{-1}. Thus, the rate of binding to HR-CcO shows an ~20-fold increase compared to that for CcO-O. From the absorption decrease for HR-CcO at 415 nm and using a ΔA_m of 30 mM^{-1} cm^{-1} (*30*),

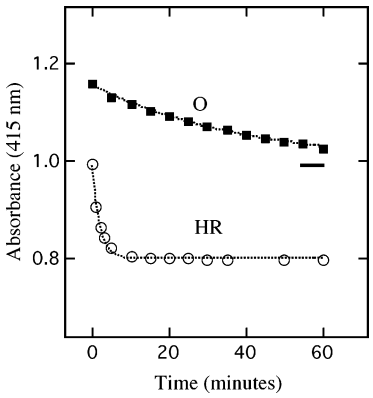

FIGURE 4: Kinetics of binding of cyanide to oxidized CcO and to half-reduced CcO in hexane at $W_0 = 3$. (O) Oxidized CcO and (HR) half-reduced CcO. The short bar under the data for oxidized CcO indicates the value of absorption reached in 3 h after the addition of cyanide. Points are experimental data. Lines are single-exponential fits. At time zero, extracted 7.8 mM HCN was added to 7.2 μM oxidase. The temperature was 21 °C.

it was calculated that ~99% of the enzyme had reacted with cyanide during the measurements.

DISCUSSION

State of CcO in Organic Solvent. Several previous studies have shown that bovine heart CcO can be transferred into apolar solvents either from submitochondrial particles (*29*, *35*, *36*) or from the purified state (*21*, *29*). In this variety of apolar solvents, oxidase retains catalytic activity, displaying oxygen consumption and inhibition of this activity by cyanide (*21*, *36*). In addition, the optical spectra and the spectral changes of CcO in organic solvents, brought about by reduction or cyanide binding, were very similar if not identical to those observed for CcO in aqueous solutions (*21*, *29*). These observations, together with the spectroscopic data presented here, indicate that CcO in the organic medium is in a functional state and retains the catalytic and spectral integrity of the protein in water buffers.

The structure of the oxidase−lipid complex in organic solvent is, however, less certain. Light scattering measurements on hexane extracts of CcO showed the presence of particles that are larger than CcO itself (*21*), and we have assumed that these particles are reverse micelles formed from the phospholipids and oxidase (*21*). The micellar structure of CcO in hexane extracts may be similar to that suggested for another integral membrane protein, rhodopsin, transferred into hexane by a very similar procedure (*38*). According to this suggestion, the hydrophobic core of CcO is probably in direct contact with hexane and the hydrophilic domains at both the matrix and cytosolic surfaces are covered by the polar headgroups of the phospholipids in a reverse micelle configuration. In these reverse micelles, the polar groups of the phospholipids are directed toward the hydrophilic domains of the enzyme and form a polar core that contains water, whereas the hydrophobic chains are exposed to the organic solvent.

Inhibition of the Reduction of the Heme a_3−Cu_B Center. It is known that the properties of enzymes in reverse micelles are sensitive to the molar ratio of water to surfactant (W_0) that is present in solution (*27*). This ratio, rather than the absolute amount of water or surfactant, determines most of the properties of the entrapped proteins (*27*). This is also true for CcO where it has been shown that with the decrease in W_0 there is a decrease in the catalytic activity in the organic solvent. We have observed the complete inhibition of oxygen consumption by oxidase at a W_0 of ~2 (*21*). According to both published data (*21*, *29*) and the data presented here, the loss of catalytic activity of CcO at this low W_0 is caused by a blocking of electron transfer (ET) from heme *a* and Cu_A to the catalytic site and both heme a_3 and Cu_B remain oxidized.

The reason for this inhibition of ET to the catalytic site may be sought in the reactions coupled to this transfer. The transfer of two electrons to the oxidized catalytic center is accompanied by the uptake of two protons from solution (*39*, *40*), and according to the existing view (*41*), this proton transfer controls ET to the catalytic site. If for some reason proton access is blocked, this in turn inhibits the reduction of the catalytic site (*42*). Moreover, the transition of CcO from the oxidized to the reduced state is associated with structural changes directly at the catalytic site (*43*, *44*). In the fully oxidized enzyme, there is a bridge between the iron of heme a_3 (Fe_{a3}) and Cu_B, suggested to be water with hydroxide (*45*) or possibly peroxide (*43*). Since no ligand can be detected in the reduced state (*43*, *44*), the ET to the catalytic site is also coupled with inner ligand release. Both these processes, proton uptake and possible water release, associated with ET to the catalytic site, might be severely restricted in the organic solvent at low W_0 and be responsible for the observed inhibition of the reduction of the catalytic site.

The putative state of CcO in hexane assumes the hydrophobic surface of CcO is in contact with hexane. It is then conceivable that lipids, tightly bound to CcO in aqueous buffers, can be removed from this surface in hexane. However, the removal of lipids, specifically cardiolipin, causes the decrease in electron transport activity of CcO (*46*, *47*), which can also contribute to the observed inhibition of internal ET.

Cyanide Binding. Cyanide is a well-known inhibitor of the catalytic activity of oxidase being bound to the iron of heme a_3. Although the affinity of cyanide for heme a_3 in fully oxidized CcO is high ($K_D \sim 10^{-6}$ M), the rate constant is extremely small ($k = 2$ M^{-1} s^{-1} for the fast form) (*30*). However, partially or fully reduced CcO in aqueous solutions can bind cyanide more rapidly than fully oxidized CcO (*48*−*50*). In aqueous buffers, the rate constant for binding of cyanide to one-electron reduced fast CcO was estimated to be greater than 10^4 M^{-1} s^{-1} (*13*). This enhanced rate is believed to be due to reaction of cyanide with enzyme species having the single electron located at the binuclear center (*13*). It was suggested, for both fast and slow forms of CcO, that it is the reduction of Cu_B that triggers the enabling conformational transition at the binuclear site (*13*, *14*), leading to the faster binding of cyanide.

The enhanced reactivity of partially reduced CcO with cyanide in the hexane extracts has already been observed (*29*). However, the oxidation state of Cu_B was not established. The new contribution of this study is the spectroscopic evidence that, at low W_0, the catalytic site is stabilized in the oxidized state and not accessible to ET from reduced Cu_A and heme *a*. Nevertheless, in this half-reduced state, the rate of binding of cyanide to oxidized heme a_3 is enhanced compared to that in fully oxidized CcO (Figure

4). The relative enhancement of the rate of binding of cyanide to HR-CcO in hexane is, however, not as large as that observed in aqueous solutions for CcO having one electron at the binuclear center (*13*). In HR-CcO, the rate constant is increased only 20-fold compared to the rate when these centers are oxidized (Figure 4), which is very similar to the transition from the slow form to the fast form of oxidase in water solutions. The considerably different magnitude of this effect, between HR-CcO and CcO with one electron localized at the binuclear center, suggests that the catalytic site can undergo two qualitatively similar but quantitatively different transitions. One transition is triggered by reduction of heme *a* and Cu_A and the second by the direct one-electron reduction of the binuclear site itself.

There are several indications that binding of cyanide to fully oxidized (*51*−*53*) and partially reduced CcO (*50*) involves the transient coordination of the incoming ligand to Cu_B. Our previous study with the fast form of CcO in aqueous buffers (*52*) indicated that the rate-limiting step is the intramolecular ligand rearrangement at the catalytic site. We assume that the same step limits the rate in the reaction of cyanide with slow CcO in hexane, and the faster kinetics of binding of cyanide to HR-CcO is achieved by increasing the rate of cyanide rearrangement from Cu_B to heme a_3. This could be accomplished by destabilizing the endogenous ligand in the binuclear center of HR-CcO compared to that in CcO-O. This explanation is consistent with earlier observations that demonstrated that the redox transitions of heme *a* and/or Cu_A affect the redox (*54*, *55*) and spectroscopic characteristics of the catalytic site (*56*, *57*).

Our data do not allow us to decide if one or two electrons are necessary to trigger the observed transition of the catalytic site. From the proximity of heme *a* and the heme a_3−Cu_B center (*56*), we would expect that this transition is induced by an electron localized at heme *a*. Hemes *a* and a_3 are bridged by three successive amino acid residues, His376, Phe377, and His378, located in transmembrane helix X of subunit I (*56*). His378 is an axial ligand to the iron of heme *a*, and His376 is coordinated to Fe_{a3}. Thus, it is conceivable that these three residues can communicate the reduction of heme *a* to heme a_3.

An alternative explanation for the closed to open CcO transition could assume some distal alteration in the protein structure. It might be that the diffusion of ligand via the access channel to the catalytic site, buried in the middle of the protein, in the fully oxidized slow CcO in hexane is dramatically restricted. In this case, the increase in the rate of ligand binding by the reduction of heme *a* and Cu_A might be accomplished by slowing the inhibition for ligand diffusion through the channel. However, a redox-coupled conformational transition was identified at the remote surface segment from Gly49 to Asn55 on the cytosolic side of subunit I of bovine CcO (*43*), and this does not involve the putative ligand access channels (*2*).

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