The spectral and kinetic characteristics of two oxidized states of bovine heart cytochrome c oxidase (CcO) have been compared. The first is the oxidized state of enzyme isolated in the fast form (O) and the second is the form that is obtained immediately after oxidation of fully reduced CcO with O2 (O3). No observable differences were found between O and O3 states in: (i) the rate of anaerobic reduction of heme a3 for both the detergent-solubilized enzyme and for enzyme embedded in its natural membraneous environment, (ii) the one-electron distribution between heme a3 and CuB in the course of the full anaerobic reduction, (iii) the optical and (iv) EPR spectra. Within experimental error of these characteristics both forms are identical. Based on these observations it is concluded that the reduction potentials and the ligation states of heme a3 and CuB are the same for CcO in the O and O3 states.

Mitochondrial cytochrome c oxidase (CcO) transfers electrons from cytochrome c to dioxygen. The reduction of O2 to water is associated with the generation of a transmembrane proton gradient. Two processes contribute to the development of this gradient. The first is the oxidation of ferrocytochrome c from the cytosolic side of the inner mitochondrial membrane coupled to proton consumption for water formation from the matrix compartment of mitochondria. The second process is proton pumping during which protons are translocated from the matrix space to the cytosolic side of the membrane.

In CcO four redox centers participate in electron transfer (ET) and reduction of O2 to H2O. Three of these centers, heme a, heme a3, and copper ion, called CuB, are in subunit I and a dinuclear copper center CuA, is located in subunit II (2). CuA serves as the acceptor of electrons either from ferrocytochrome c or from artificial electron donors (3–7). Electrons received by the oxidase are rapidly distributed between CuA and heme a on the microsecond time scale (4, 5, 7–10). ET continues further to the catalytic center composed of heme a3 and CuB, where O2 is reduced to water.

The part of the catalytic cycle during which electrons are delivered to CcO, prior to reaction with O2, is usually referred to as the reductive phase. In the subsequent oxidative phase the reduced enzyme reacts with O2. It was proposed (11) and demonstrated later that proton pumping takes place in both the reductive and oxidative phases (12–14). Two protons are pumped in the oxidative phase when fully reduced CcO is oxidized by oxygen (12, 14). Subsequently, if this freshly oxidized enzyme is reduced by two electrons two additional protons are translocated across the membrane (12, 14).

However, proton translocation in the reductive phase is only observed with freshly oxidized enzyme. This reoxidized enzyme appears to be in a metastable state, which relaxes relatively slowly in the absence of electron donors to the stable oxidized form on a time scale of seconds. These two forms of oxidized enzyme, CcO as it exists immediately after oxidation and the subsequent relaxed form, are designated as the O and the O3 states, respectively (12, 14).

It has been concluded that the free energy needed for proton pumping in the reductive phase is transiently conserved in the high-energy O3 state of the enzyme. After the enzyme has relaxed from O3 to O, this stored energy dissipated, and the reduction proceeds without associated proton translocation (12, 14). Consequently it was anticipated that there would be differences in the reduction potentials and coordination of one or both metals in the heme a3-CuB site between the O and O3 forms (14).

Reaction 1 summarizes a current view on the steps in which proton pumping occurs. The reduction of oxidized CcO as isolated (O) by four electrons produces the fully reduced enzyme (FR). When fully reduced enzyme reacts with O2, two protons are pumped and the metastable oxidized O3 state is formed. Transfer of two electrons to O3 is associated with the translocation of two additional protons and generation of partially reduced enzyme (PR).

This reaction scheme with its two states for oxidized enzyme does not take into account that there are altogether six forms of oxidized enzyme described in the literature: “resting” (15, 16),

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8 This was supported by the National Institutes of Health Grant GM 55807 and Slovak Grant Agency (VEGA) Grant 6167. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: CcO, cytochrome c oxidase; apNADH, 3-acylpyridine adenine dinucleotide reduced form; ETP, electron transport particles; DM, n-dodecyl-β-D-maltoside; Fepp, iron of heme α; FeN, iron of heme α3; Mes, 2-(N-morpholino)ethanesulfonic acid.
Two States of Oxidized Cytochrome Oxidase

\[
\begin{align*}
O + 4e^{-} & \rightarrow FR + O_2 \\
2H^+ & \rightarrow O_H + 2e^{-} + PR
\end{align*}
\]

REACTION 1

“slow” (17–21), “pulsed” (15, 16), O (12, 14), O_H (12, 14), and “fast” form (18, 20). The term resting is used for oxidase that shows low catalytic activity because of slow internal ET from Cu_a-heme a to the catalytic site (15, 16). This form of enzyme can be activated when fully reduced resting enzyme is reacted with oxygen. The activated state, which exhibits rapid internal ET, was called pulsed enzyme (15, 16). This state is, however, not stable and in the absence of electron flux through oxidase the pulsed CcO relaxes to the resting state (15, 16). It has been suggested that both the resting and pulsed forms constitute an important regulatory mechanism in mitochondrial respiration (15).

Identifying the resting form with the native state of CcO (15, 16) is not supported by the fact that enzyme isolated by different protocols shows variability in the homogeneity of the catalytic site (for review see Refs. 17 and 21). The binding of ligands, such as cyanide and nitric oxide, to the catalytic site of oxidized enzyme exhibits multiple kinetic phases and the form that binds cyanide extremely slowly was named the slow form (18–21).

The recognition of this inhomogeneity led to the development of purification protocols that produce enzyme with a homogenous catalytic site (18, 22, 23). This form was called the fast enzyme (18, 20) to reflect the more rapid binding of cyanide during the anaerobic reduction of the fast CcO in either the “slow” (17–21), “pulsed” (15, 16), O (12, 14), O_H (12, 14), and “fast” form (18, 20). The term resting is used for oxidase that shows low catalytic activity because of slow internal ET from Cu_a-heme a to the catalytic site (15, 16). This form of enzyme can be activated when fully reduced resting enzyme is reacted with oxygen. The activated state, which exhibits rapid internal ET, was called pulsed enzyme (15, 16). This state is, however, not stable and in the absence of electron flux through oxidase the pulsed CcO relaxes to the resting state (15, 16). It has been suggested that both the resting and pulsed forms constitute an important regulatory mechanism in mitochondrial respiration (15).

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The recognition of this inhomogeneity led to the development of purification protocols that produce enzyme with a homogenous catalytic site (18, 22, 23). This form was called the fast enzyme (18, 20) to reflect the more rapid binding of cyanide relative to the slow form. Spectroscopic evidence indicates that fast CcO is equivalent to enzyme in mitochondria (18) and is already in the active state (22). It was suggested later that fast CcO is a native state of oxidase (21).

The term “the O state” belongs to oxidase as isolated (14). Unfortunately this assignment is not unambiguous because the isolated enzyme can be in either the fast or the resting form. It means that the O state can represent either the native enzyme or enzyme with the modified catalytic center.

The O_H state is attributed to the enzyme formed immediately after reoxidation and consequently corresponds to pulsed oxidase. O_H is a metastable form that relaxes to the O state (12, 14). However, the rates of relaxation of O_H and the pulsed enzyme are different. It has been estimated that the lifetime of O_H is about several seconds (14). In contrast the transition of the pulsed to the resting or slow forms occurs on the time scale of minutes (24–26) or even hours (18). Thus it seems that the energy stored in the O_H state dissipates more rapidly than the enzyme relaxes from the pulsed to resting state.

In our recent study on ET to the catalytic site of CcO we have established that the rates of internal ET to heme a_3 during the anaerobic reduction of the fast CcO in either the O or O_H states are practically the same (27). This kinetic equivalence indicated that the redox properties of heme a_3 are identical in these two forms. In the present work the kinetic and spectral characteristics of the fast enzyme in both

EXPERIMENTAL PROCEDURES

Materials—Hepes and Mes buffers, horse heart cytochrome c (prepared without trichloroacetic acid), glass beads size 16, 3-acetylpyridine adenine dinucleotide reduced form (apNADH), phenazine methosulfate, carbonyl cyanide m-chlorophenyl hydrazone, cholic acid, and valinomycin were from Sigma. Hexaamineruthenium (III) chloride and sodium hydrosulfite (dithionite) were from Aldrich, peroxide-free Triton X-100 was from Roche Diagnostics, and n-dodecyl-β-D-maltoside (DM) from Anatrace.

Preparation of Enzyme and Electron Transport Particles—Bovine heart CcO was isolated from mitochondria by the modified method of Soulimane and Buse (23) into DM. Two modifications were introduced into this protocol. First, instead of using KCl in all purification steps we employed K_2SO_4. Replacing KCl by K_2SO_4 was necessary to avoid the binding of Cl^- to the catalytic site of CcO (28). Second, gel filtration chromatography was introduced as the last step of purification. The application of gel filtration is a consequence of our observation that extended exposure of CcO to the anion exchanger Sepharose Q modifies the enzyme surface sites for anion binding (29). Therefore we shortened the time that CcO was exposed to Sepharose Q. As this led to incomplete purification an additional step was required. Briefly, mitochondria are subjected to protein extraction twice with Triton X-100. EDTA (1 mm) and histidine (1 mm) supplemented the original buffer (10 mM Tris, pH 7.6, 250 mM sorcose) to minimize binding of transition metals; the EDTA was omitted in the second extraction. The second extract, containing solubilized CcO, was further purified in two chromatographic steps during which the detergent Triton X-100 is exchanged to DM. The CcO bound to the Sepharose Q fast flow column was washed with 700 ml of 10 mM Tris, pH 7.6, 0.1% DM and then by 500 ml of the same buffer containing 70 mM K_2SO_4. The CcO was eluted from the column with 200 mM K_2SO_4 in 10 mM Tris, pH 7.6, 0.1% DM. The eluted CcO was concentrated and further purified on a Sephacryl S300HR column using 10 mM Hepes, pH 7.8, 50 mM K_2SO_4 and 0.1% DM as buffer. During this chromatography CcO separates into two fractions. The first fraction contains a small amount of CcO contaminated with other proteins. The second, major fraction, containing the purified CcO and used in this study was analyzed for its subunit composition and shown to contain all 13 subunits (data were obtained in collaboration with Dr. A. Musatov, University of Texas Health Science Center, San Antonio, TX). Enzyme concentration was determined from the optical spectra of oxidized CcO using an extinction coefficient at A_424 of 156 mm^-1 cm^-1 (30).
CcO as isolated by this method is in the fast form and is considered to be the native form of the enzyme (21). The key factor that controls the state of purified enzyme is pH. It has been demonstrated that the catalytic site of CcO is highly sensitive to pH and can be easily modified during purification, if the pH is not kept at or slightly above pH 8.0 (18). However, it appears to us that the presence of cholate, a detergent often used for enzyme purification, is also one of the critical factors controlling the conversion of the native enzyme to the resting form. We believe that the combination of low pH plus cholate modifies the catalytic site of oxidase.

For comparison of the kinetic properties of fast and resting CcO we have also prepared the resting enzyme. This form was made by incubation of the isolated fast form (217 μM) for 6 h at pH 7.0 and 23 °C in 10 mM Hepes, 50 mM K₂SO₄, 0.1% DM, and 0.05% sodium cholate. During this process the catalytic site was modified and the most obvious change is observed in the position of the Soret band maximum. The optical spectra collected at pH 8.0 showed that this shifted from 424 to 421 nm.

Mitochondria, the starting material for CcO purification, were also used for isolation of electron transport particles (ETP). The isolated mitochondria were washed twice with 10 mM Tris, pH 7.6, 250 mM sucrose. Then cytochrome c was depleted from the washed mitochondria (31, 32). To extract cytochrome c, the mitochondria were diluted to a protein concentration of about 3.3 mg/ml with cold 10 mM K₂SO₄, stirred at 4 °C for 30 min, and centrifuged at 26,000 × g for 30 min. Cytochrome c was extracted by suspension of the sediment in cold solution of 10 mM Hepes, pH 8.0, 100 mM K₂SO₄, and 10 mM K₃Fe(CN)₆. This suspension was stirred at 4 °C for 10 min and centrifuged at 48,000 × g for 25 min. To ensure that extraction of cytochrome c is complete, this step was repeated twice using the same buffer with ferricyanide omitted and twice with 200 mM Hepes, pH 8.0, containing 30 mM K₂SO₄. These mitochondria were mechanically disrupted by mixing with glass beads and buffer (200 mM Hepes, pH 8.0, 30 mM K₂SO₄) in a vortex mixer for about 1 min at 4 °C. Typically for the mixing we used 1 volume of sedimanted mitochondria, 2 volumes of buffer, and 1 volume of glass beads. The disrupted mitochondria were centrifuged at 27,000 × g for 10 min and the supernatant fraction containing ETP was collected. The CcO concentration in ETP was estimated from the difference optical spectrum of fully reduced minus oxidized CcO using an extinction coefficient at ΔA₄05..615 of 23.2 mm⁻¹ cm⁻¹ (30). The concentration of CcO in ETP was about 1.2 μM.

Stopped-flow Measurements—The kinetics of reduction of oxidized CcO as isolated (O) by hexaamineruthenium were measured under anaerobic conditions in an argon atmosphere using a stopped-flow apparatus. An anaerobic solution of O was mixed in a 1:1 ratio with the mixture of hexaamineruthenium and sodium dithionite. Unless noted otherwise, all kinetics of internal ET to heme a₁ were obtained with CcO in the O state in the presence of 3 mM hexaamineruthenium and 5 mM dithionite (final concentrations). All rate constants for ET are averages of at least three measurements.

For the measurement of ET in the O₄ state anaerobic, fully reduced CcO in the presence of 6 mM hexaamineruthenium and 10 mM dithionite was mixed in the stopped flow with air-saturated buffers. In the ~3-ms dead time of the apparatus, the enzyme is oxidized and any oxygen consumed by dithionite, both Cuₐ and heme a are reduced by the excess reductant, and only the re-reduction of heme a₁ is available for monitoring.

The kinetics of O and O₄ reduction by hexaamineruthenium were determined under anaerobic conditions in an argon atmosphere using the OLIS RSM-1000 stopped-flow apparatus collecting 1000 spectra per second; the light path was 20 mm. The solutions were made anaerobic following a published procedure (27).

To remove oxygen from the stopped-flow instrument the whole flow system was filled with a buffered solution of 5 mM dithionite for about 4 h prior to measurements. The driving syringes were submerged in water that was continually purged with nitrogen. The temperature was 23 °C.

Rapid Freezing and EPR Measurements—For preparation of rapid-freeze samples a System 1000 chemical/freeze quench apparatus was employed (Update Instrument Inc., Madison, WI). In this apparatus O form at CcO (100 μM) was mixed with 5 μM ferrocyanochrome c and 10 mM dithionite at 23 °C, and the samples were rapidly frozen at selected times in a bath of isopentane precooled to 125–130 K. The samples for O₄ were made by mixing reduced CcO in the presence of 5 μM ferrocytochrome c and 10 mM dithionite with air-saturated buffer. EPR spectra of samples prepared by the rapid-freeze method were recorded with a Varian E-6 or Bruker EMX spectrometer. The conditions for EPR measurements were as follows: frequency, 9.26 GHz in the Varian spectrometer, and 9.6 GHz in Bruker instrument; power, 10 or 30 milliwatts; modulation amplitude, 10 G; modulation frequency, 100 kHz; and temperature, 4.2 K. In the Bruker spectrometer the frequency was continually monitored during the EPR scan with the built in frequency counter. In the Varian spectrometer the Hewlett-Packard 5342A Microwave frequency counter was used. The reading of frequency was accurate to 10⁻⁴ GHz. The accuracy of the magnetic field was ± 1.1 G as established by using the pitch standard.

To make samples of O₄ for EPR measurement without excess reductant O was fully reduced by a stoichiometric amount of apNADH in the presence of a catalytic concentration of phenazine methosulfate in the absence of oxygen. This reduced enzyme was then oxidized by air and frozen in a dry ice/methanol bath within 25 s of reoxidation.

The high-spin signal at g = 6 of heme a₁ was quantified by double integration with the lower integration limit taken below the low-field end of the spectrum, and the upper limit at a field corresponding to a g value of 4.67 (33). This integrated intensity was compared with the signal of a standard high-spin complex of met-myoglobin fluoride.

The standard errors in the measurements of g = 6 EPR signals of CcO in the rapid freeze quench samples were estimated from the reaction of oxymyoglobin (Mb-O₂) with NO (data obtained in collaboration with Dr. C. Rogge, University of Texas Medical School, Houston, TX). The reaction of Mb-O₂ with a stoichiometric amount of NO quantitatively produces the high-spin signal in the dead time of the rapid-freeze quench apparatus. From EPR measurement on four samples the variation in both the height of the high-spin signal and the integrated intensity were calculated. The variation in height was
found to be ±19% with ±22% variation in the integrated intensity. The major contribution to this deviation comes from packing the samples. Nevertheless, this uncertainty does not lead to a large scatter of data points as is documented by the time dependence of the $g = 6$ signal (Fig. 2).

**Fitting Model and Estimation of Reduction Potentials for Heme $a_3$ and Cu$_B$**—In the course of reduction of the oxidized catalytic site (Fe$_{a3}^{3+}$ Cu$_B^{2+}$) by two electrons the transient formation of a one-electron reduced state appears (Fe$_{a3}^{3+}$ Cu$_B^{1+}$) that is characterized by the high-spin signal at the $g = 6$ originating from the iron of heme $a_3$. The transient formation of Fe$_{a3}^{3+}$ Cu$_B^{1+}$ and its decay to the fully reduced state, Fe$_{a3}^{2+}$ Cu$_B^{1+}$, was fitted to the equation,

$$g6(t) = \left( k_1 \cdot p / (k_2 - k_1) \right) (e^{-k_1 \cdot t} - e^{-k_2 \cdot t}) \quad \text{(Eq. 1)}$$

where $k_1$ and $k_2$ are the observed rate constants for the entry of the first and second electron into the binuclear site, respectively. The model also assumes that the equilibration of an electron between these two redox centers is more rapid than the rate of electron entry into the catalytic site. The parameter $p$, representing the fraction of the Fe$_{a3}^{3+}$ Cu$_B^{1+}$ state relative to the total concentration of the one-electron reduced binuclear sites, was taken to be 0.5. The value of the parameter $p$, held constant in all fits, is justified by the similarity of the estimated reduction potentials of heme $a_3$ and Cu$_B$.

The reduction potentials of heme $a_3$ and Cu$_B$ were estimated from the optical spectrum of partially reduced oxidase. In this measurement anaerobic oxidized CcO was reduced with 2 eq of ferrocytochrome $c$. After equilibration had been reached the optical spectrum was analyzed for the amounts of reduced heme $a_3$, heme $a_3$, Cu$_A$, and ferrocytochrome $c$. The concentration of Cu$_A$ and ferrocytochrome $c$ was determined by the deconvolution of this difference spectrum for the contribution of heme $a$ and heme $a_3$ and using the extinction coefficients $\Delta A_{444-427} = 112 \text{ mm}^{-1} \text{ cm}^{-1}$ for heme $a$ and $\Delta A_{446-416} = 122 \text{ mm}^{-1} \text{ cm}^{-1}$ for heme $a_3$ (30). The difference between the amount of oxidized cytochrome $c$ and reduced (Fe$_{a3}^{2+}$ + Cu$_A^{1+}$) was assumed to reflect the extent of reduction of Cu$_B$. Taking the standard reduction potential of cytochrome $c$ as $+264 \text{ mV}$ (36) the potentials of all four metal centers were calculated. At pH 8.0 the standard potential for heme $a_3$ was $+290 \text{ mV}$ and for Cu$_B$ about $+280 \text{ mV}$.

**RESULTS**

The reduction kinetics of heme $a_3$ by 3 mM hexaamineruthenium for the fast and resting CcO in both the O and O$_H$ states monitored at 444 nm are presented in Fig. 1. As already mentioned, with this concentration of hexaamineruthenium the redox centers, Cu$_A$ and heme $a$, are reduced in the dead time of the stopped-flow apparatus and only the kinetics of reduction of heme $a_3$ are observed (27). For fast CcO the reduction of heme $a_3$ proceeds in two phases (27, 37, 38), however, the rates of ET are very similar for the O and O$_H$ states (Fig. 1A). The apparent rate constants for the larger and rapid phase of heme $a_3$ reduction in O and O$_H$ are listed in Table 1. Different behavior is observed with resting enzyme (Fig. 1B). In the first second, only about 40% of heme $a_3$ is reduced, with rate of $47 \text{ s}^{-1}$ for oxidase in the O state. The reduction of the remaining 60% proceeds with the rate of 0.01 s$^{-1}$ and it takes $\sim 500 \text{ s}$ to fully reduce heme $a_3$ (not shown). When this resting form is activated by reoxidation heme $a_3$ is reduced completely in the time scale of 1 s in a biphasic process with apparent rates of 56 s$^{-1}$ and 6.7 s$^{-1}$. The contribution of the rapid phase to the overall spectral change at 444 nm is 84%.

When all of CcO molecules are in the slow form the maximum of the Soret band is at 418 nm (18–20). The shift of this band from 424 to 418 nm represents the conversion of the fast to the slow oxidase. The resting enzyme, utilized for the measurements in Fig. 1B, had a Soret band maximum at 421 nm. Thus it appears that the used enzyme is actually not the homog-
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A

B

FIGURE 2. The transient formation of the g = 6 EPR signal during the full reduction of oxidase in the O and O₆₇ state by ferrocytochrome c.

A. EPR spectra in the g = 6 region of O₆₇ and O samples rapidly frozen at 0.2 s in the course of anaerobic reduction of CcO with the ferrocyanochrome c-dithionite mixture. The magnetic fields corresponding to g values of 6.38, 6.00, and 5.35 are indicated below the O₆₇ spectrum. B. The formation and decay kinetics of g = 6 signal, corresponding to the CcO Fe₃⁺⁰ state, during the anaerobic reduction of O and O₆₇. Symbols: data for O (○) and O₆₇ (■). Dashed line: fit to the equation presented under “Experimental Procedures” for oxidase in the O₆₇ state. CcO is reduced by 2.5 μM ferrocyanochrome c in the presence of 5 mM dithionite. The buffer used was 200 mM Hapes, pH 8.0, 22 mM K₂SO₄, 10 mM KCl, 0.1% DM. Final concentration of CcO is 25 μM. EPR spectra were measured at 4.2 K and power, 10 milliwatts.

both the axial high spin signal and the rhombic signal are in agreement with these values (33).

The precise origin of these high spin states of the heme a₃ population is not known. However, it has been shown that these signals are pH-dependent (42). At pH 8.4 the oxidized heme a₃ exhibits nearly axial as well as rhombic high spin signal, whereas at pH 6.4 only axial signals appear (42). Thus the deviation from tetragonality seen in the EPR spectrum at pH 8.0 may arise from: 1) the mechanical constraint on the heme a₃ imposed by the protein (43); 2) an interaction between some acid-base group(s) and heme a₃, or 3) from the mixture of the hexacoordinate and pentacoordinate structures of oxidized heme a₃ with the latter dominating at lower pH.

TABLE 1
Apparent rate constants for the reduction of heme a₃ by 3 mM hexaamineruthenium (III) chloride for both as isolated (O) and activated enzyme (O₆₇) at pH 8.0 and 23 °C

|          | O    | O₆₇  |
|----------|------|------|
| CcO resting | 47 ± 2 (40%) | 56 ± 3 (84%) |
| CcO fast | 87 ± 6 (80%) | 78 ± 5 (90%) |
| ETP      | 121 ± 16 (100%) | 123 ± 16 (100%) |

* CcO fast or CcO resting, purified oxidases in DM buffer.

** Relative contributions of the rapid phase of heme a₃ reduction.

*** ETP, oxidase in electron transport particles.

Tensorous slow form but a mixture of two populations. This explanation is consistent with the two phases in the kinetics of ET to heme a₃ (Fig. 1B and Table 1). Despite this heterogeneity the data demonstrate clearly the phenomenon of activation for the resting enzyme and also the absence of activation for enzyme in the fast or native form.

The kinetics of heme a₃ reduction of CcO in ETP, where it is embedded in its natural membrane, is fitted as a single exponential for both O and O₆₇. The almost identical rate constants obtained from fits for both forms are summarized in Table 1.

The reduction kinetics of CuB, the second metal ion at the catalytic site, cannot be assessed directly from the optical spectra. However, the heme a₃ charge transfer band at 650 nm present in the fully oxidized enzyme can be used as an indicator of ET to CuB (39). The rates of disappearance of this band on reduction by 3 mM hexaamineruthenium were identical for both O and O₆₇ and equivalent to the rates established at 444 nm.

A second indirect approach to monitor the kinetics of CuB reduction is based on EPR spectroscopy (40, 41). In fully oxidized CcO, the binuclear center is EPR silent because of the exchange coupling between the iron of heme a₃ (Fe₃⁺²) and CuB²⁺. When CuB is reduced and heme a₃ is oxidized (40, 41) the coupling is broken and the magnetic resonance of Fe₃⁺² is observable in EPR spectra (40, 41). This state, which is represented by the EPR signal at g = 6 from high-spin heme a₃, appears transiently during the course of anaerobic reduction by ferrocyanochrome c and dithionite (Fig. 2). This signal is composed of at least two species (Fig. 2A), one nearly axial with g values of 6.0 and 5.84 and one a more rhombic signal with g values of 6.38 and 5.35. Despite this heterogeneity, the two high-spin signals of Fe₃⁺² are present in both O and O₆₇ states.

The almost identical EPR amplitudes of these signals indicate that the CuB²⁺ ↔ Fe₃⁺² state is populated with nearly the same intensity in both forms of CcO (Fig. 2A).

Similar signals have already been detected during the reductive titration of oxidase under anaerobic conditions (33, 40). In fact, in the earlier study it has been shown that there must be at least two rhombic types that can be, however, resolved only in the 35 GHz spectrum (33). For the axial signal g values of 5.988 and 5.787 were established. Two rhombic signals were represented by the g values of 6.42, 5.37 and 6.27, 5.486. The g values determined in this work for

|          | O    | O₆₇  |
|----------|------|------|
| CcO resting | 47 ± 2 (40%) | 56 ± 3 (84%) |
| CcO fast | 87 ± 6 (80%) | 78 ± 5 (90%) |
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* CcO fast or CcO resting, purified oxidases in DM buffer.

** Relative contributions of the rapid phase of heme a₃ reduction.

*** ETP, oxidase in electron transport particles.
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The transient formation of the g = 6 signal reflects the production of the one-electron reduced state (Fea$_3^{3+}$/Cu$_B^{1+}$) followed by the decay to the EPR silent and fully reduced state (Fea$_3^{2+}$/Cu$_B^{1+}$). Thus the kinetic behavior of this signal provides information on the initial reduction of Cu$_B$ and the subsequent reduction of heme $a_3$. The apparent rate constants for the entry of the first electron ($k_1$) into the catalytic site, to Cu$_B$, and the second electron transfer ($k_2$), to heme $a_3$, when Cu$_B$ is reduced, obtained from the fits are $k_1 = 5.3$ s$^{-1}$ and $k_2 = 4.9$ s$^{-1}$ for the O state. For the O$_H$ state the rate constants are $k_1 = 4.7$ s$^{-1}$ and $k_2 = 4.8$ s$^{-1}$ (Fig. 2B). There are no marked differences in the reduction of the catalytic site of CcO by the first and second electrons in either the O and O$_H$ states.

An estimation of the concentration of Cu$_B^{1+}$/Fea$_3^{3+}$ from the integration of the g = 6 signal at 200 ms, when the signal amplitude is maximum, suggests that ~12% of heme $a_3$ is detected transiently in the course of full reduction. This amount is about half the maximal concentration of Cu$_B^{1+}$/Fea$_3^{3+}$ observed (23%) during the equilibrium reductive titration of oxidase (33).

In earlier observations it was found that the EPR spectra of pulsed CcO showed a new transient signal, originating from the catalytic site, and characterized by g values of 5, 1.78, and 1.69 (24–26). This EPR signal exists for a short period of time (less than 10 min) following reoxidation (24–26). The EPR spectra of O and O$_H$ are compared in Fig. 3. O$_H$ was frozen 25 s after reoxidation. The EPR signal characterized with g = 5.0, 1.78, and 1.69 was expected to be present in the spectrum of O$_H$ (24–26). As can be seen from Fig. 3 the EPR transitions at g = 1.69 and 1.78 are detectable in both samples. However, reoxidation does not produce any measurable increase or change in the intensity of these two signals as documented by the difference spectrum (Fig. 3). Both the transition at g = 1.69 and 1.78 were diminished when enzyme was reacted with cyanide. This response to cyanide is consistent with the previous assignment of the origin of the EPR signal (g = 5.0, 1.78, and 1.69) to the catalytic center (25). The large truncated signal located at about g = 2 is due to oxidized Cu$_A$.

The novel EPR signal produced by reoxidation is also missing at pH 7.4 (not shown), where this signal is expected to be present at full strength (24). Moreover, the development of these EPR transitions was also absent in O$_H$ trapped by the rapid-freeze quenching method 25 ms after reoxidation of dithionite-reduced CcO (not shown).

Optical spectra, obtained on the stopped-flow instrument between 387 and 613 nm, also do not exhibit any difference between the spectra of O and O$_H$ (not shown). The O$_H$ spectrum was collected 3 ms after the fully reduced enzyme by 1 mm dithionite was oxidized with oxygen. The spectrum for O state was measured similarly in the stopped-flow instrument by mixing oxidized CcO with buffer.

**DISCUSSION**

Previous experiments have demonstrated that freshly oxidized enzyme (O$_H$) behaves differently from enzyme that has not been pulsed (O), and anaerobic reduction of O$_H$ is associated with proton pumping, which is absent when enzyme is in the O state (12, 14). It has been suggested that these differences between O and O$_H$ are the result of distinct reduction potentials and/or ligation states of heme $a_3$ and Cu$_B$ (12, 14). If this is the case, then O and O$_H$ should exhibit different kinetics of internal ET to the catalytic site as well as differences in spectral characteristics.

However, the data presented here, using the fast or native enzyme, do not reveal any observable differences between O and O$_H$ for the detergent-solubilized enzyme nor for the enzyme in its natural membraneous environment. The kinetics of internal ET to heme $a_3$ were identical under conditions in which both Cu$_A$ and heme $a$ are fully reduced (Fig. 1 and Table 1). This result suggests that the reduction potential of heme $a_3$, whether in the O or O$_H$ states, is the same.

A similar conclusion can be made regarding the redox potential of Cu$_B$, based on the identical appearance of the g = 6 EPR signal during the anaerobic reduction of O and O$_H$ (Fig. 2). If the reduction potential of Cu$_B$ in the O$_H$ state were increased relative to the potential of heme $a_3$, the kinetics of the g = 6 signal should be distinctly different for the O and O$_H$ forms. The amplitude of the g = 6 signal observed during reduction of O$_H$ should be larger than that observed with O. However, the identical evolution of the g = 6 signal during full reduction of O and O$_H$ shows that there is no difference in the distribution of one electron between heme $a_3$ and Cu$_B$ in these two forms. It follows that the reduction potentials of Cu$_B$ and Fe$_a^{3+}$ are essentially identical in both states. Taken together, the kinetic data indicate that the redox properties of heme $a_3$ and Cu$_B$ are the same in O and O$_H$.

The possibility of differential ligation of heme $a_3$ and Cu$_B$ was investigated by comparing the optical and EPR spectra of O and O$_H$. The identity of these spectra (Fig. 3) suggests that the cat-
alytic sites in these two forms have the same isoelectronic structure and thus identical coordination. As mentioned in the Introduction, the purification protocols used for isolation of CcO from bovine heart can lead to enzyme with a modified catalytic center (18–21). We believe this is the source of the distinct EPR spectra observed for the $O_H$ or pulsed form in previous experiments (24, 26) and in this study. The purification protocol we utilize (23) yields the $O$ state of the enzyme, which is defined as fast enzyme. This form is fully reduced by dithionite or apNADH plus phenazine methosulfate in less than a minute. However, to fully reduce CcO, in which the new EPR signal has been developed on reoxidation, it takes at least 12 h of anaerobic incubation of enzyme with reductants (24, 26). We believe that the differences in the catalytic site of these two, as isolated, forms is the principal reason for the distinctly different rates of reduction as well as the novel EPR signal.

The kinetic and spectral equivalence of the $O$ and $O_H$ states for the fast form of CcO established in this work does not contradict that there may be proton pumping during the reductive phase. Indeed there is an indication that the transfer of the second electron into the one-electron reduced oxidase isolated from Paracoccus denitrificans leads to proton translocation (13).

Acknowledgment—We thank Dr. A.-L. Tsai for providing generous access to the rapid-freeze instrument.

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