Filling the Catalytic Site of Cytochrome c Oxidase with Electrons

REDUCED CuB FACILITATES INTERNAL ELECTRON TRANSFER TOHEME a3

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In the reductive phase of its catalytic cycle, cytochrome c oxidase receives electrons from external electron donors. Two electrons have to be transferred into the catalytic center, composed of heme a3 and CuB, before reaction with oxygen takes place. In addition, this phase of catalysis appears to be involved in proton translocation. Here, we report for the first time the kinetics of electron transfer to both heme a3 and CuB during the transition from the oxidized to the fully reduced state. The state of reduction of both heme a3 and CuB was monitored by a combination of EPR spectroscopy, the rapid freeze procedure, and the stopped-flow method. The kinetics of cytochrome c oxidase reduction by hexaamineruthenium under anaerobic conditions revealed that the rate-limiting step is the initial electron transfer to the catalytic site that proceeds with apparently identical rates to both heme a3 and CuB. After CuB is reduced, electron transfer to oxidized heme a3 is enhanced relative to the rate of entry of the first electron.

Mitochondrial cytochrome c oxidase (CcO) is a membrane protein that catalyzes the oxidation of ferrocytochrome c by molecular oxygen. The reduction of oxygen to water requires the delivery of four electrons and four protons into the catalytic center of the enzyme. Electrons enter oxidase from the cytosolic domain and protons from the matrix side of the inner mitochondrial membrane. This redox reaction is coupled to the pumping of four additional protons across the membrane. Both of these processes contribute to the generation of a transmembrane proton gradient.

Bovine heart CcO contains 13 polypeptides; however, four redox centers involved in electron transport (ET) and in the reduction of O2 to water are located in two subunits (1). Three of these centers, heme a, heme a3, and copper ion called CuB, are in subunit I, and the dinuclear copper center CuA is located in subunit II (1).

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4 The abbreviations used are: CcO, cytochrome c oxidase; TX, Triton X-100; DM, n-dodecyl-β-D-maltoside; Fe3+, iron of heme a3; Ru, hexaamineruthenium; DT, sodium dithionite; ET, electron transport.

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The proposal for the effect of the reduction state of CuB on ET to heme a3, however, lacks experimental support. One approach for assessing the role of the redox state of CuB on ET to heme a3 during the reductive phase is EPR spectroscopy (14, 15). In fully oxidized enzyme, the binuclear center is EPR-silent because of the exchange coupling between the iron of heme a3 (Fe3+) and CuB2+. When CuB is reduced and heme a3 is oxidized (Fe3+CuB2+), this coupling is broken and the magnetic resonance of Fe3+ is observable in EPR spectra (14, 15). This state, which is represented by the EPR signal at g = 6 from high spin heme a3, appears transiently during the course of reduction. The transient formation of the g = 6 signal reflects the initial formation of the one-electron-reduced state (Fe3+CuB2+) followed by the decay to the EPR silent and fully reduced state (Fe3+CuB2+). Thus the kinetic behavior of the g = 6 signal provides information on the initial reduction of CuB and the subsequent reduction of heme a3.

This study demonstrates that transfer of the first electron into the catalytic site is the rate-limiting step in the overall
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two-electron process. The rate for the transfer of the second electron to oxidized heme $a_g$, when Cu$_b$ is reduced, is enhanced relative to the rate of the first electron transfer. The data also suggest that the biphasic reduction of heme $a_g$, interpreted previously as reflecting the effect of the redox state of Cu$_b$ (11, 12), is because of the presence of different populations of purified CcO.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hepes buffer, EDTA, and riboflavin were from Sigma, hexaaminerthenium (III) chloride (Ru) and sodium hydrosulfite (dithionite, DT) were from Aldrich, peroxide-free Triton X-100 (TX) was from Roche Diagnostics, and $n$-dodecyl-$eta$-D-maltoside (DM) was from Anatrace.

**Preparation of Enzyme and Electron Transport Particles**—Bovine heart CcO was isolated from mitochondria by the modified method of Souloum and Buse (16) into DM-containing buffer. The enzyme concentration was determined from the optical spectrum of oxidized enzyme using an extinction coefficient $E_{424}$ of 156 mm$^{-1}$ cm$^{-1}$ (17). CcO isolated by this method is in the “fast” form considered to be the native form of the enzyme (18).

Mitochondria, the starting material for CcO purification, were also used for isolation of electron transport particles. The isolated mitochondria were washed twice with 10 mM Tris, pH 7.6, and 250 mM sucrose. Thereafter, cytochrome $c$ was depleted from the washed mitochondria (19, 20). To extract cytochrome $c$, the mitochondria were diluted to a protein concentration of $\sim$3.3 mg/ml with cold 10 mM K$_2$SO$_4$, 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 K$_2$SO$_4$, and 10 mM K$_3$Fe(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 $c$ was 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 K$_2$SO$_4$. These mitochondria were mechanically disrupted by mixing with glass beads and buffer (200 mM Hepes, pH 8.0, 30 mM K$_2$SO$_4$) in a Vortex mixer for $\sim$1 min at 4°C. Typically, for the mixing, we used 1 volume of sedimented 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 electron transport particles was collected. The CcO concentration in the electron transport particles was established from the difference of the optical spectrum of partially reduced $minus$ oxidized CcO using the extinction coefficients $E_{446-427} = 112$ mm$^{-1}$ cm$^{-1}$ for heme $a$ and $E_{446-416} = 122$ mm$^{-1}$ cm$^{-1}$ for heme $a_3$ (17).

The amount of reduced Cu$_b$ is based on the estimated reduction potential in independent measurement. In this measurement, anaerobic oxidized CcO was reduced with two equivalents of ferrocyanochrome $c$. After equilibrium was reached, the optical spectrum was analyzed for the amounts of reduced heme $a$, heme $a_3$, Cu$_b$, and ferrocyanochrome $c$. The difference between the amount of oxidized cytochrome $c$ and reduced (a$^{2+} + a_3^{2+} + Cu_b^{+}$) was assumed to reflect the extent of reduction of Cu$_b$. Taking the standard reduction potential of ferrocyanochrome $c$ as 264 mV (23), the potentials of all four metal centers were calculated. At pH 8.0, the standard potential for heme $a_3$ was +290 mV and for Cu$_b$ $\sim$ +280 mV.

**Rapid Freezing and EPR Measurements**—For preparation of rapid freeze samples, a System 1000 chemical/Ar-lincequench apparatus was employed (Update Instruments, Madison, WI). In this apparatus, anaerobic oxidized CcO (160 μM) was mixed with Ru-DT at 23°C and the samples rapidly frozen at selected times in a bath of isopentane precooled to 125–130°C. EPR spectra of samples prepared by the rapid freeze method were recorded with a Varian E-6 or Bruker EMX spectrometer. All spectra were collected at a nominal temperature of 4.2 K as read by the temperature sensor located 3 mm below the bottom of the sample tube, which is $\sim$17 mm below the center of the EPR cavity used. The stability of the temperature was controlled to the second decimal point by the temperature controller unit ITC 503. The Oxford continuous flow cryostat ES 900 was used for the transfer of helium to the sample. The conditions for EPR measurements were: frequency, 9.26 or 9.6 GHz; power, 3 milliwatts; modulation amplitude, 10 G; and modulation frequency, 100 kHz. The high spin signal at $g = 6$ of heme $a_3$ was quantified.

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

**Preparation of Samples**—The solutions of oxidase and the mixture of Ru-DT were made anaerobic following the previously published procedure (13).

The anaerobic partial reduction of CcO was achieved by photoreduction using riboflavin as the photosensitizer and EDTA as the sacrificial electron donor (21, 22). To partially reduce CcO, a sample of 10 μM oxidized CcO in 200 mM Hepes, pH 8.0, 0.1% DM or TX, 22 mM K$_2$SO$_4$, 10 mM KCl containing 2 μM riboflavin, and 5 mM EDTA was made anaerobic in the stopped-flow tonometer in the dark. An optical cell was attached as the side arm to the tonometer, which permitted monitoring the progress of photoreduction by recording the absorption spectrum. The spectrum of the anaerobic oxidized CcO was collected as a reference. The sample was then exposed to several light flashes from a Sunpack 544 photoflash until the desired level of reduction was obtained. The absorption spectrum of this partially reduced form was analyzed for the level of reduction of the individual hemes.

The concentrations of reduced heme $a$ and heme $a_3$ were determined by the deconvolution of the optical absorption difference spectra of partially reduced $minus$ oxidized CcO using the extinction coefficients $E_{446-427} = 112$ mm$^{-1}$ cm$^{-1}$ for heme $a$ and $E_{446-416} = 122$ mm$^{-1}$ cm$^{-1}$ for heme $a_3$ (17).

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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 (24). This integrated intensity was compared with the signal of a standard high spin complex of metmyoglobin fluoride.

The S.D. in the measurements of g = 6 EPR signals of CcO in the rapid freeze-quench samples were estimated from the reaction of oxyhemoglobin (Mb-O2) with NO.5 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 was calculated. The variation in height was found to be ±19% with a ±22% variation in the integrated intensity. The major contribution to this deviation comes from the packing of the samples. Nevertheless, this uncertainty does not lead to a large scatter of data points as is documented by the time dependences of the g = 6 signal (see Fig. 3).

Model—In the course of reduction of the oxidized catalytic site (Fe3+CuB2+) by two electrons, the transient formation of a one-electron-reduced state appears (Fe3+CuB1+) that is characterized by the high spin signal at the g = 6 originating from the iron of heme a3. The transient formation of Fe3+CuB1+ and its decay to the fully reduced state, Fe2+CuB1+, was fitted to the model represented by the following scheme,

\[ \text{Fe}_{3+}^{\text{CuB}_{2+}} \rightarrow (k_1) \rightarrow \text{Fe}_{3+}^{\text{CuB}_{1+}} \rightarrow (k_2) \rightarrow \text{Fe}_{2+}^{\text{CuB}_{1+}} \]

where \( k_1 \) and \( k_2 \) are the observable 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. Based on this model, the time development of the normalized g = 6 signal was fitted to the equation,

\[ g_6(t) = (k_1 \cdot p / (k_2 - k_1)) \cdot (e^{-k_1t} - e^{-k_2t}) \]  

(Eq. 1)

with parameter \( p = 0.5 \) representing the fraction of the \( \text{Fe}_{3+}^{\text{CuB}_{1+}} \) state relative to the total concentration of the one-electron-reduced binuclear sites. The value of the parameter \( p \), held constant in all fits, is justified by the similarity of the estimated reduction potentials of heme a3 and CuB.

RESULTS

For the investigation of ET in CcO during the anaerobic reduction, the mixture of two artificial electron donors, dithionite and hexamine ruthenium, was employed. There are three reasons that makes this system advantageous: (i) excess DT ensures the anaerobic conditions during the measurements and also maintains the concentration of reduced Ru constant; (ii) ET from Ru to CcO is substantially fast (12), faster than that from DT to CcO (25), which allows us to identify and study the factors regulating the internal ET; and (iii) the spectral changes of both hemes and CuA can be monitored over the relevant wavelength range without optical interference from reduc tant.

Reduction of Heme a and Heme a3, Stopped-Flow Results—The transition of heme a and a3 from the oxidized to the reduced state can be assessed from the changes in the optical spectrum of CcO. In the Soret band, the reduction of both hemes can be separated by recording data at 428 and 436 nm (Fig. 1). 428 nm is an isosbestic point in the spectral transition of heme a3 from oxidized to reduced state, and the kinetics at this wavelength correspond to the reduction of heme a3 (Fig. 1A). Similarly, the kinetics at 436 nm represents reduction of heme a, as this wavelength is an isosbestic point of heme a (Fig. 1B). The reduction of heme a, at the presence of either 25 or 100 µM Ru, is monophasic and fitted by a single exponential. With 3 mM Ru, the reduction of heme a is complete in the dead time of the apparatus, and only the reduction of heme a3 is observed. With Ru concentrations of 25 µM, 100 µM, and 3 mM, the reduction of heme a3 is biphasic and the relative contribution of the rapid phase, obtained from two exponentials fits, is ~80%. The rate constants for the reduction of heme a and a3 in the rapid phase are summarized in Table 1.

Reduction of CuB and Heme a, Rapid Freeze EPR Results—In Fig. 2, we show the EPR spectra of samples rapidly frozen at 6, 125, and 600 ms after reaction of oxidized CcO with 25 µM Ru. There is a monotonic decrease of the signal at g = 3, together with the copper signal at g = 2, reflecting the reduction of the low spin iron of heme a and CuA, respectively. The g = 6 signal, corresponding to the Fe3+CuB1+ state exhibits different behavior. Initially, there is an increase in the intensity of the g = 6 signal, which is subsequently followed by its decay. This transient formation of the high spin signal results from the initial accumulation of Fe3+CuB1+, the one-electron-reduced catalytic site with an electron located on CuB. The decay of this signal represents the transfer of the second electron to heme a3 and the formation of a fully reduced catalytic site (Fe2+CuB1+).

We have determined the evolution of the g = 6 signal during the reduction of oxidized CcO at three Ru concentrations (Fig. 3). The experimental data for 25 and 100 µM Ru (Fig. 3A) are fitted by the equation that represents the model described under “Experimental Procedures.” The rates for both the formation of Fe3+CuB1+ and for its decay to the fully reduced state, Fe2+CuB1+, obtained from the fitting are summarized in Table 2.

The maximum observed yield of Fe3+CuB1+ decreases with increasing Ru concentration. When reduction is initiated with 3 mM Ru, the increase of the g = 6 signal, corresponding to the formation of the Fe3+CuB1+ state, is missing (Fig. 3B). In the first available data point, collected 6 ms after mixing, the population of Fe3+CuB1+ is at maximum with the appearance of a small amount of Fe2+CuB1+, which quantitation suggests is of the order of 2–4%. The residual g = 6 signal present at 150 ms belongs to the population of CcO, with the catalytic site modified during purification, because it is also present in the EPR spectrum of oxidized enzyme. Even

5 Data obtained in collaboration with Dr. C. Rogge, University of Texas Medical School, Houston, TX.
though there is the uncertainty in the absolute concentration of CuB$^{3+}$/H$^{+}$, we consistently observed the very small increase (1.5%) of the high spin signal in the reaction of CcO with 3 mM Ru compared with the signal present in the fully oxidized oxidase. This signal is significantly smaller and disappears much more rapidly than the signals in experiments with 25 or 100 mM Ru (Fig. 3).

The experimental data in Fig. 3 are overlaid with the kinetic trace obtained from the model using the rate constants presented in Table 2. In the calculation of this trace, the residual g = 6 signal was taken as an offset. It was also assumed that the first electron transfer to the catalytic site to CuB occurs with the rate similar to the rate observed for heme a$_3$ reduction in the stopped-flow measurements. With these two values inserted into the model equation, the best agreement was found with the rate constant for the second electron transfer, to be $2.2 \times 10^3$ s$^{-1}$.

ET to Heme a$_3$ in Oxidized and Partially Reduced Oxidase— The apparent rate constants for reduction of heme a$_3$, obtained from the EPR rapid freeze experiments (Table 2), show that reduced CuB could facilitate ET to the heme a$_3$. This observation is strengthened by the kinetics of reduction of heme a$_3$ in the stopped-flow instrument for CcO in two states, fully oxidized CcO and partially reduced CcO. Enzymes in both DM and TX detergents were used for this measurement. By deconvoluting the initial optical spectrum of partially reduced CcO (10 mM) in DM containing buffer (200 mM Hepes, pH 8.0, 0.1% DM, 22 mM K$_2$SO$_4$, 10 mM KCl), we have determined that 3.9 mM heme a and 3.8 mM heme a$_3$ were reduced. Because of the dilution of CcO after mixing with the reductant in the stopped-flow apparatus, we should have detected the reduction of 3.1 mM heme a$_3$ (6.2 mM before dilution). However, from the observed kinetics of heme a$_3$ reduction with 3 mM Ru, we calculated that 1.2 mM heme a$_3$ was reduced rapidly in the dead time of the apparatus. The residual 1.9 mM heme a$_3$ was reduced with the rate constant equivalent to that for the reduction of heme a$_3$ in fully oxidized CcO (Table 1, Fig. 1B). The rapid reduction of the population of heme a$_3$ (1.2 mM) we ascribe to the state of the catalytic site, with the CuB reduced and heme a$_3$ oxidized.

The measurement on partially reduced enzyme in DM detergent does not, however, bring information on the extent of acceleration of ET to heme a$_3$ when CuB is reduced. To estimate the extent of acceleration, we have used enzyme in TX deterg-
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Table 2

| Ru     | $k_1$  | $k_2$  |
|--------|--------|--------|
| 0.025  | $5 \pm 1$ | $5 \pm 1$ |
| 0.1    | $7 \pm 4$ | $30 \pm 18$ |
| 3      | $85^*$ | $2200^*$ |

* Rate constants used to generate the model kinetic trace in Fig. 3B.

DISCUSSION

In cytochrome c oxidase, electron transfer reactions are intertwined with proton translocation and reduction of oxygen to water. Understanding of this complex process relies on the dissection and characterization of individual ET steps and the possible interactions among the redox-active centers. The present study is focused on the first part of the catalytic cycle, the reductive phase, when electrons are delivered to the metal centers of oxidized CcO from external electron donor.

**ET to the Catalytic Site**—When utilizing the artificial electron donor, it is essential that the reduction of enzyme occurs by the natural ET pathway(s). There are several observations that show the participation of the physiological pathway(s) of ET into CcO and to the catalytic center in the presence of Ru. It has been demonstrated previously that the reduction of CcO by Ru is biphasic (26–28). In the first, rapid phase, heme $a_3$ is reduced, and this is followed by the reduction of heme $a_3$ (26–28). Our data are consistent with these earlier observations.

The present results, however, differ from the published data on the reduction of heme $a_3$. It was found earlier that the kinetics of the reduction of heme $a_3$ are independent of the concentration of Ru (26, 28). This fact itself indicates that heme $a_3$ cannot be reduced directly by electron transfer from Ru. In our case, the rate of heme $a_3$ reduction is, however, dependent on the concentration of Ru (Table 1). Our preliminary study shows that the dependence of the apparent rate constant of ET to heme $a_3$ on the concentration of Ru (from 2 $\mu$M to 10 mM) or of cytochrome c approaches a limiting value. The limiting rate constants with the rate constants of $10 \pm 0.5$ and $3 \pm 1$ s$^{-1}$, respectively, with the rapid phase accounting for the reduction of 4.1 $\mu$M heme $a_2$. In the sample of partially reduced enzyme, with 4.3 $\mu$M heme $a$ and 5.9 $\mu$M heme $a_2$ reduced, the observed kinetics was two exponentials as well. However, the apparent rate constants increased in the partially reduced oxidase, and from the best fit to the kinetics in Fig. 4B, the rates of $108 \pm 14$ and $13 \pm 2$ s$^{-1}$ were obtained; the fast phase accounted for 1.1 $\mu$M and the slow phase for 0.7 $\mu$M heme $a_3$ reduction.

The rate constant for the slow phase of heme $a_3$ reduction in the partially reduced CcO in TX buffer corresponds to the rate for the rapid phase in oxidized CcO. The equivalence of these rates implies that this population of the partially reduced CcO has the catalytic site in the fully oxidized state. Yet, in the partially reduced CcO, there is a fraction of heme $a_3$ reduced with $\sim$10-fold the enhanced rate. This fraction is attributed to the one-electron-reduced state of the catalytic site with the electron localized on Cu$_{ap}$.

![Figure 3](image-url)
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FIGURE 4. The Soret band spectra and kinetics of the reduction of heme $a_3$ by 3 mM Ru for both fully oxidized and partially reduced oxidase. A, the Soret band spectra of oxidized (O) and −50% reduced CcO (PR) collected at 1 ms in the reduction reaction with 3 mM Ru. The final spectrum of fully reduced CcO collected at 1 s is shown (FR). B, the kinetics of the reduction of heme $a_3$ in fully oxidized (O) and −50% reduced CcO (PR) monitored at 444 nm. The final concentrations were 5 μM CcO, 3 mM Ru, and 5 mM DT. Temperature, 23 °C. Buffer used was the same as described in the legend to Fig. 1, except the detergent was 0.1% TX.

constant, using conditions identical to those employed in this study, was 84 ± 4 and 72 ± 7 s$^{-1}$ with Ru and cytochrome $c$, respectively. Because this rate is almost independent of the nature of the electron donor, it implies that there is a common process that controls ET to heme $a_3$. We conclude that, when using either of these two electron donors, physiological or artificial, electrons are delivered to the catalytic site by the same intramolecular pathway from Cu$_{A}$ and heme $a$.

This conclusion is supported further by several studies on the reduction of CcO initiated by flash photolysis of different Ru complexes (29–31). It has been demonstrated that none of these Ru photosensitive compounds reduce the catalytic site directly and it is Cu$_{A}$ that is the first electron acceptor from the excited state of Ru. This initial ET step is followed by rapid electron distribution between Cu$_{A}$ and heme $a$ (29–31). The described sequence of ET steps is true not only for fully oxidized CcO but also for the “peroxy” (P) and ferryl (F) forms of oxidase (30, 31). In P and F, the reduction potential of the catalytic site has been estimated to be close to +1.0 V (32), and therefore this site is the thermodynamically favored acceptor. However, despite the favorable reduction potential, ET to the catalytic site occurs by the intramolecular transfer from heme $a$ to heme $a_3$ (30, 31).

The contrasting observation on the dependence of the rate of heme $a_3$ reduction on Ru concentration between this and previous reports is most likely a consequence of two circumstances. First, the previous measurements were conducted with a relatively high concentration of Ru. The starting Ru concentration was at 0.04 (26) or 0.1 mM (28). The second factor is that the enzyme used in these studies exhibits a slow maximal rate of internal ET to heme $a_3$. We believe that with the low rate of internal ET from heme $a$ to $a_3$ and the Ru concentration employed, the rate of reduction of heme $a_3$ had already reached its limiting value. Consequently, the increase of the Ru concentration from 0.04 or 0.1 mM to 2 mM did not produce any effect on the rate of ET to heme $a_3$ (26, 28).

Influence of Reduced Cu$_B$ on ET to Heme $a_3$—The reduction of Cu$_B$ has been suggested to have an inhibitory impact on the internal ET to heme $a_3$ (11, 12), and this has led to an explanation for the observed biphasic reduction of heme $a_3$ during the anaerobic reduction of CcO. The rapid and more extensive phase was ascribed to ET to heme $a_3$ when Cu$_B$ is oxidized, and the slow phase represented the reduction of heme $a_3$ in the population of CcO with Cu$_B$ reduced. However, our present rapid freeze EPR data for the reduction of the catalytic site in the presence of 25 and 100 μM Ru (Table 2) indicate that the rate of ET to heme $a_3$, when Cu$_B$ is reduced, is not decreased at all.

The transfer of the first electron to Cu$_B$ (Table 2) proceeds with nearly the same rate as the rate of reduction of heme $a_3$ observed in stopped-flow measurements (Table 1). Yet the rate of entry of the second electron to the binuclear site shows a tendency to increase relative to that of the first electron (Table 2). The enhanced rate of the second electron is also suggested by the almost complete absence of the g = 6 signal during the reduction of CcO with 3 mM Ru (Fig. 3B). Even though the data from the EPR rapid freeze experiments have inherently larger standard deviations relative to stopped-flow data, we can quite safely conclude that the presence of an electron on Cu$_B$ stimulates, rather than inhibits, the transfer of electron to heme $a_3$. The rate constants used for the model kinetic trace with 3 mM Ru (Fig. 3B, Table 2) show that the rate of the second electron is at least one order of magnitude faster than the rate of the first electron entry.

Our inference about the kinetics of the reduction of the catalytic site by the second electron is further strengthened by the comparison of ET to heme $a_3$ in the fully oxidized enzyme and enzyme that was partially reduced (Fig. 4). In partially reduced CcO, both in DM and TX buffers, we have found a population of oxidized heme $a_3$ that is reduced with a rate that is greater than any rate for the reduction of heme $a_3$ in fully oxidized oxidase. Taking into account the similarity of the estimated reduction potentials of heme $a_3$ and Cu$_B$, it has to be a similar fraction of both reduced heme $a_3$ and Cu$_B$ in the partially reduced CcO.
Based on this, we surmise, just as from rapid freeze experiments, that the enhanced rate of ET to oxidized heme $a_3$ is a consequence of the reduced Cu$_b$.

According to stopped-flow data, during the reduction of fully oxidized CcO with 3 mM Ru (Table 1), 18% of heme $a_3$ is reduced in the slow phase. If this 18% were to correspond to the population with Cu$_b$ reduced, as was thought earlier, it should be easily detectable in the EPR rapid freeze measurements. However, the maximal observable population of Fe$_{a_3}^{3+}$Cu$_b^{1+}$ in this reaction, represented by the g = 6 signal (Fig. 3B) is ~1.5%. Therefore, we assume that the biphasic characteristic of the reduction of heme $a_3$ reflects two populations of isolated CcO.

The discussion above shows that there is a principal difference between the rapid phase of ET to heme $a_3$ in fully oxidized CcO and the rapid phase in the partially reduced enzyme. Origin of the rapid and slow phases in the fully oxidized CcO is a result of the heterogeneity in the catalytic site of purified enzyme. On the other hand, the rapid phase of ET to heme $a_3$ in partially reduced CcO comes from the population where Cu$_b$ is reduced.

Our observations can be summarized with an overall view of how the catalytic site of CcO is filled with electrons. It is known that the reduction of this site is associated with the uptake of two protons from solution (33, 34). These two protons are assumed to compensate for the charge of the electrons delivered to heme $a_3$ and Cu$_b$ keeping the catalytic center in the electrically neutral state (33). Because the catalytic site is buried within the protein (1), protons are delivered by two channels called K and D that lead from the matrix surface of the enzyme to the binuclear center (1, 35–37). Because the rate of heme $a_3$ reduction appears to be synchronous with proton uptake (12), it has been concluded that the rate-limiting process for this internal ET is the stabilization of the transferred electrons by these protons (12).

In addition to proton transfer, the conversion of CcO from the oxidized to the reduced state is also associated with structural changes directly at the catalytic site (38, 39). In the x-ray structure of the fully oxidized enzyme, only a diffuse electron density is observed between heme $a_3$ and Cu$_b$ and this has yet to be convincingly interpreted. In the bovine enzyme, a peroxo-anion has been proposed to bridge the two metal centers (1, 35–37). Because the catalytic site is buried within the protein (1), protons are delivered by two channels called K and D that lead from the matrix surface of the enzyme to the binuclear center (1, 35–37). Because the rate of heme $a_3$ reduction appears to be synchronous with proton uptake (12), it has been concluded that the rate-limiting process for this internal ET is the stabilization of the transferred electrons by these protons (12).

Thus there are at least two processes associated with the reduction of the catalytic site that can control the apparent rate of ET: the first is proton transfer and the second is, possibly, a release of water molecule. The present study showed, for the first time, that the rate-limiting step in the reduction of the catalytic site under anaerobic conditions is transfer of the first electron. This transfer occurs with the same observable rates to either heme $a_3$ or Cu$_b$. It is conceivable that the first electron transfer is restricted by the coupled proton uptake via the K channel (44) and/or the subsequent release of the inner ligand located between redox centers. As a consequence of changes at the catalytic site, induced by the first electron, the second electron can be transferred more rapidly. These changes might involve the switching of the channel for delivery of the second proton to the catalytic site, or the absence of the inner ligand at the site results in an increase of the reduction potential of heme $a_3$ and/or a decrease of the reorganizational energy for the next electron transfer.

Present observation of the enhanced rate of heme $a_3$ reduction, when Cu$_b$ is reduced, also rationalizes seemingly contrasting results on the internal ET measured by two experimental approaches. The first is the measurement of the forward kinetics as described in this work when enzyme, initially in the oxidized state, is reduced anaerobically. The second is based on the reverse ET that occurs following photodissociation of the adduct of carbon monoxide from the two-electron-reduced enzyme, the so-called mixed valence CcO (MV.CO) (45–47). In MV.CO, heme $a_3$ and Cu$_b$ are reduced (heme $a$ and Cu$_A$ are oxidized) and stabilized by bound CO. Photolysis of CO lowers the apparent midpoint potential of heme $a_3$ causing the reverse flow of electrons from heme $a_3$ to oxidized heme $a$ and Cu$_A$. Under these conditions, ET between the two hemes takes place in several phases distinguished by significantly different reaction time scales (45–47). The last phase of ET, associated with proton transfer to and from the catalytic site, proceeds on the millisecond time scale (47). However, even in this slowest phase, the rate of ET from heme $a$ to $a_3$ was found to be at least one order of magnitude larger than the rate observed in experiments when oxidized CcO is reduced anaerobically (47). This movement of electrons between hemes initiated by photolysis of the CO from MV.CO occurs, however, with Cu$_b$ reduced (48), which means that this transfer should be correlated with the transfer of the second electron to Fe$_{a_3}^{3+}$Cu$_b^{1+}$. This is more rapid than the rate of entry of the first electron observed in the forward reaction.

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