The redistribution of two electrons in the four redox centers of cytochrome c oxidase following photodissociation of CO from the CO-bound mixed valence species has been examined by resonance Raman spectroscopy. To account for both the kinetic data, obtained from 5 µs to 2 ms, and the equilibrium results, a model is proposed in which the electron redistribution is modulated by a protein conformational transition from a nascent $P_2$ state to a relaxed $P_2$ state in a time window longer than 2 ms. In this model, all six possible two-electron reduced species are considered. The high population of species with a one-electron reduced binuclear center, in which the spectrum of heme $a_5$ is perturbed by the redox state of CuB$_3$, accounts for the significant residuals in the fitting of the kinetic data with four standard spectra derived from redox states with either zero or two electrons in the binuclear center. Under equilibrium conditions, the conformational change to the $P_2$ state destabilizes the redox states with only one electron in the binuclear center with respect to those with either zero or two electrons. As a result, the redox equilibrium is perturbed, and the electrons are redistributed. A simulation based on the new kinetics scheme, in which the electron redistribution is modulated by the protein conformation, gives reasonable agreement with both the equilibrium and the kinetic data, demonstrating the validity of this model.

Cytochrome $c$ oxidase, (CcO$^*$; ferrocytochrome $c$: O$_2$ oxidoreductase, EC 1.9.3.1), the terminal enzyme in the electron transport chain, catalyzes the four-electron reduction of oxygen to water. Associated with the oxygen reduction chemistry, four protons are pumped across the mitochondrial inner membrane, contributing to the proton motive force required for ATP synthesis. The enzyme contains four redox centers: two hemes and two copper centers termed heme $a$, heme $a_3$, Cu$_A$, and Cu$_B$, respectively. Under physiological conditions, the electron donor, cytochrome $c$, donates electrons to Cu$_A$, which is located near the cytochrome $c$ binding site. The electrons in Cu$_A$ are subsequently transferred to heme $a$ and then to the binuclear center consisting of heme $a_3$ and Cu$_B$, where oxygen reduction occurs (1–4). It requires four electrons for one molecule of oxygen to be reduced to two molecules of water. It is very important to understand the electron transfer events to fully determine the molecular mechanism underlying this important enzyme. However, it is a non-trivial task to study the forward electron transfer reactions because they are tightly coupled with the oxygen reduction chemistry. On the other hand, flash photolysis of the so-called mixed valence CO-bound derivative (MVCO) has been extensively used to study the reverse electron transfer reactions in CcO (5–8).

The MVCO species is a two-electron reduced derivative in which heme $a_2$ and Cu$_B$ are reduced, whereas the other two redox centers, heme $a$ and Cu$_A$, remain oxidized. The reduced state of the binuclear center in MVCO is stabilized by a CO molecule bound to the iron atom of heme $a_5$. Photolysis of CO lowers the redox potential of the binuclear center, bringing about electron redistribution with electron flow in the reverse direction from the binuclear center to heme $a$ and Cu$_A$ (9–11). With a known equilibrium constant, the forward electron transfer rate can be extracted from the observed rate constant that is the sum of the forward and reverse rate constants.

Scheme 1 illustrates a generally accepted electron transfer mechanism associated with photolysis of MVCO (species $a$). Immediately following photolysis, a ligand-free species, $b$, is formed. It is followed by an electron transfer reaction from heme $a_5$ to heme $a$ that leads to species $c$. The subsequent electron transfer from heme $a$ to Cu$_A$ produces species $d$. Each step in the electron transfer chain described in Scheme 1 is reversible. In addition, it is believed that one electron always resides on Cu$_B$ because it is proposed to have a midpoint potential that is much higher than those of the two hemes (6, 8).

Two fast kinetic phases have been reported for the electron transfer reactions in bovine CcO. The first phase is assigned to the electron transfer from heme $a_5$ to heme $a$, which occurs at a rate of $-3 \times 10^5$ s$^{-1}$ (8, 12, 13). It is followed by electron transfer from heme $a$ to Cu$_A$ at about $2 \times 10^5$ s$^{-1}$. Similar studies have been carried out on other members of the terminal oxidase super family, including those from the bacterium Rhodobacter sphaeroides and cytochrome $b_5$ from Escherichia coli (12, 14, 15), and the electron transfer events in these enzymes were detected on similar time scales. Recently, the rates and the extent of the electron transfer were questioned by Verkhovsky et al. (16), who reported that an electron transfer event between the two hemes occurs on a time scale much faster than 100 ns, the limit of their instrumental resolution, and accounted for about 50% amplitude of the total amount of electron transfer from heme $a_5$ to heme $a$. However, after reinvestigation of this reaction, Namslauer et al. (17) concluded that no evidence was found for the presence of this early phase. Instead, they reported that the microscopic forward and reverse rate constants for the electron-transfer reactions from heme $a$ to heme $a_5$ are not faster than $-2 \times 10^5$ and $-1 \times 10^5$ s$^{-1}$, respectively.

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Electron Redistribution in Cytochrome c Oxidase

In addition to the fast phases, millisecond time scale changes were also observed that were ascribed to the structural re-arrangement associated with the electron redistribution and/or CO recombination (6, 7). Based on optical absorption spectroscopy, Einarsdottir and co-workers (5, 6) pointed out that in addition to the four species included in Scheme 1, other intermediates have to be considered to account for the changes observed in the optical spectra.

To clarify the electron transfer reaction mechanism, we used resonance Raman spectroscopy to reinvestigate the intramolecular electron transfer events following photolysis of MVCO. Resonance Raman spectroscopy is a sensitive probe for detecting electron density and structural dynamics of heme prosthetic groups in heme proteins. It has been proved to be a powerful technique for characterizing the structural properties of reaction intermediates and associated kinetics in various heme proteins (18). With resonance Raman spectroscopy, we demonstrate that the electron transfer reaction following photolysis of MVCO is associated with a conformational transition that regulates the electron redistribution in the four redox centers of CcO.

**MATERIALS AND METHODS**

CcO was purified from beef hearts by the method described by Yoshikawa et al. (19). The enzyme was dissolved in 0.01 M sodium phosphate buffer at pH 7.4 with 0.1% n-dodecyl-β-maltoside as the detergent. The protein solution was stored in liquid nitrogen until further use. The enzyme concentration was determined by the optical absorption intensity difference of the fully reduced form at 604 nm minus that of fully oxidized form at 530 nm. Prior to the resonance Raman measurements, the enzyme solution was purged with high purity nitrogen (0.5 m), and the deconvolution program was written with a commercial software package, MathCad.

RESULTS

*Generation of the Standard Spectra—Based on Scheme 1, at least four species are expected during the photolysis experiments. The spectrum of species a can be directly obtained from MVCO (Fig. 1, spectrum A). On the other hand, species b and c are metastable species that cannot be measured directly. We sought to calculate their spectra by a linear combination of the spectra of various redox species that can be easily obtained under equilibrium conditions. Fig. 1, spectrum B, is derived by adding the spectrum of the fully reduced form (CuA^1−a^3−a^2−a^1−CuB^4+), to that of the MVCO form (CuA^2+a^2−a^2−2COCuB^5+), and subtracting out that of the CO-bound fully reduced form (CuA^2+a^2−a^2−2COCuB^5+). The result of this calculation is CuA^1−a^3−a^2−a^1−CuB^4+, exactly the same as species b shown in Scheme 1. Likewise, spectrum C is derived by adding the spectrum of the CO-bound oxidized form to that of the fully reduced form to generate the standard spectrum of the fully oxidized form and subtracting out that of MVCO. The result of this calculation is CuA^1−a^3−a^2−a^3−CuB^5−. It is important to note that the redox states of heme a and a3 in this calculated spectrum are the same as that of species c, but the copper centers adopt different redox states. However, with a 413.1-nm excitation, which is nearly in resonance with the Soret absorption of this enzyme, only the vibrational modes of the hemes are enhanced; therefore, the calculated spectrum should represent that of species c if the redox states of the copper centers do not perturb the heme spectra (this assumption came into question as will be discussed later). Likewise, spectrum D from the fully oxidized enzyme with CuA^2+a^2−a^3−3CuB^5− redox centers is used to mimic species d, although the redox states of the copper centers are not identical. It is important to point out that all the initial spectra used to generate the four standard spectra have either zero or two electrons in the binuclear center.

*Equilibrium Measurements—*The output at 413.1 nm from a krypton ion laser was employed to photodissociate CO from MVCO and to probe the extent of the subsequent electron transfer from heme a3 to heme a. To avoid photoreduction, the laser power was kept low, and the MVCO enzyme was placed in a rapidly rotating cell. Under these conditions, a photostationary state was reached at ~15 min following the initiation of the photolysis. Fig. 2 shows the resonance Raman spectra of a series of MVCO samples measured at various laser illumination times. Progressive spectral changes in several of the modes are evident: 1) the decrease in the intensity of the electron density marker line (νS) of the CO bound heme a3^2− at 1368 cm−1; 2) the increase in intensity of the high spin marker line (νH) at 1570 cm−1 of the ligand-free heme a3^2−; and 3) the decrease in the intensity of the reduced heme a3^1− formyl line at 1667 cm−1. These changes reflect the photodissociation of CO from heme a3, and its conversion to a ferric heme due to the subsequent electron transfer to heme a. The electron transfer from heme a3,
to heme a is also evident from the increase in the intensity of the marker lines for the reduced state of heme a at 1519 and 1624 cm$^{-1}$.

To obtain quantitative information, each of the experimental spectra was deconvoluted into the four basis spectra presented in Fig. 1. All the experimental spectra can be fully accounted for by the basis spectra as demonstrated by a typical deconvolution result shown in Fig. 3. The negligible residuals from the fitting displayed at the bottom of the figure demonstrate that there are no additional spectral intermediates present during the reaction other than the four spectra shown in Fig. 1. The population of each spectral component was plotted as a function of the exposure time in Fig. 4. It is evident that the population of the photolyzed species increases as the exposure time increases, and the contribution from spectrum D is negligible during the full course of photolysis. Furthermore, under the photostationary state, 30% of B and 40% of C were observed. The rest of the molecules are in the CO-bound mixed valence state due to limited photolysis resulting from the low laser power and to CO rebinding. Under the conditions applied here, photolyzed CO from MVCO rebinds to heme a$_3$ on the 10–100-ms time scale (22). With the speed of the rotating cell set at $-$6000 rpm, a very low degree of photolysis and a significant amount of CO rebinding is expected under the photostationary conditions. Based on the results shown in Fig. 4, in the photolyzed fraction of the enzyme, $-$60% of the electron density originally stabilized in heme a$_3$ is transferred to heme a. This conclusion is consistent with the results observed by others; for example, see Verkhovsky et al. (16).

**Microsecond Time-resolved Measurements**—A flow-flash-
probe method, as described under “Materials and Methods,” was adapted to resolve the kinetics of the electron transfer reaction. The time-resolved resonance Raman spectra following photolysis are shown in Fig. 5. The spectrum labeled $0 \mu s$ represents the spectrum of the MVCO species that is obtained without photolysis. The spectrum labeled $5 \mu s$, the earliest detectable time point, is obtained by overlapping the pump and the probe beams. Significant changes are observed between the MVCO and the $5-\mu s$ spectrum, confirming that CO is photo-lyzed from MVCO. However, the spectrum at $2 \mu s$ (the longest time point that can be measured with the current setup) is virtually the same as that at $5 \mu s$, indicating that no further electron transfer takes place between $5 \mu s$ and $2 \mu s$. Similar observations were made by using independent preparations of the enzyme confirming the reliability of these results.

The $5-\mu s$ spectrum is deconvoluted with the four standard
spectra shown in Fig. 1. The contributions from A–D are estimated to be 0, 60, 27, and 13%, respectively. However, in contrast to the equilibrium data (Fig. 3), the residuals from the fitting are significant, as shown in Fig. 6, bottom. Due to the unsatisfactory fit, efforts were also made to deconvolute the experimental spectrum with the fully reduced and fully reduced CO-bound spectra in addition to spectra A–D. Although the spectra include all the possible combinations of the heme redox states, \((a^{2+}a_{3}^{2+}), (a^{2+}a_{3}^{2+}), (a^{2+}a_{3}^{2+}),\) and \((a^{2+}a_{3}^{2+})\), we were unable to obtain good fits. Therefore, it is concluded that under the transient conditions applied here in the kinetic experiments, either the four spectra are not a valid set of basis spectra, or the four spectra are a valid set of basis spectra, there is at least one additional intermediate that has not been accounted for.

**DISCUSSION**

The spectral deconvolution method applied here for the quantitative analysis of the intermolecular electron transfer reactions of CcO is carried out based on the assumption that all of the spectral features in the resonance Raman spectra originate from the heme groups, that the calculated basis spectra are representative of the authentic intermediates, and that no additional intermediates are present. The excellent fit of the equilibrium data with the four standard spectra (Fig. 3) confirms the validity of these assumptions with this method. However, the fit does not address the validity of Scheme 1 because the redox states of CuB in species c and d (Scheme 1) are different from those associated with spectra C and D (Fig. 1), as will be discussed later. On the other hand, the calculated spectrum B for the species b is the same as that of the 10-ns photoproduct of MVCO reported by Proshlyakov et al. (23), confirming the authenticity of this spectrum for species b. In addition, the consistency of these two spectra indicates that at 10 ns, the CO has already been cleaved from heme a and that the initial electron transfer from heme a to heme a takes longer than 10 ns. Based on this conclusion, we exclude the possibility for the existence of the ultrafast electron tunneling phase with a rate of \(\approx 10^{10} \text{ s}^{-1}\) reported by Verkhovsky et al. (16).

The failure to obtain a good fit of the kinetic data indicates that there are some new intermediates that cannot be accounted for by the standard spectra shown in Fig. 1. A spectral contribution from non-heme species is excluded because it is well established that in the resonance Raman spectra with Soret excitation, only the heme vibrational modes are detectable due to their strong enhancement. To account for these new intermediates, we propose a new kinetic model in which all six possible oxidation states of the two-electron reduced CcO are considered, as illustrated in Scheme 2. We also postulate that the resonance Raman spectrum of heme a in the redox states c, c', d, and d', which consist of a one-electron reduced binuclear center, is perturbed by the redox state of CuB. As a consequence, the spectra of the intermediates c, c', d, and d' cannot be accounted by the standard spectra derived from the equilibrium species with either zero or two electrons in the binuclear center. An influence on the heme a in the redox states c, c', d, and d', which consists of a one-electron reduced binuclear center, is perturbed by the redox state of CuB.

In the model proposed here, species b is produced within 10 ns following photodissociation of CO from MVCO. The electron in heme a is subsequently transferred to heme a and then to CuA, leading to species c and d, respectively. We propose that the single electron in the binuclear center in species c and d is delocalized on heme a and CuA as reflected by the equilibrium between c and c' and that between d and d'. Electron delocalization in heme a in the binuclear center is not unprecedented. It has been reported in the past that the electrons can reside on heme a, heme a, or the CuA site when CcO is not fully reduced (25–30). It is postulated that the electron in heme a in species d can further transfer to heme a, leading to species e. The significant population of c, c', d, and d' during the reaction accounts for the unsatisfactory fit of the data with the equilibrium spectra because the resonance Raman spectrum of heme a in these redox species is perturbed by the redox state of CuB.

To account for the equilibrium photostationary data, we propose that following photodissociation of the mixed valence spe-
cies, the protein matrix undergoes a transition from the nascent P1 conformation to an equilibrium P2 state on a time scale of several milliseconds. Under equilibrium conditions, the protein matrix converts to the P2 state. With the protein matrix in the P2 state, the redox species consisting of a one-electron reduced binuclear center, including species c, c'/H11032, d, and d'/H11032, are destabilized with respect to configurations in which the binuclear center is occupied by either zero or two electrons. As a result, only a, b, and e accumulate under equilibrium conditions. These species have exactly the same number of electrons in each redox center as those associated with Fig. 1, spectra A–C, respectively. The equilibrium data can thus be well fitted with these standard spectra.

To test the validity of Scheme 2, we simulated the kinetic and photostationary data with the reaction constants available in the literature. The Fe–CO bond breakage following photolysis is reported to be faster than 1 × 10^12 s^-1 (31); however, the amount of photolysis depends on the effective laser power. In the simulations, we thus assumed complete photolysis, and species b was used as the starting point. The rate constant, k1r, for CO rebinding to heme a3 depends on a bimolecular pre-
equilibrium binding to Cu$_B$ followed by a unimolecular transfer to heme a$_3$ (22). Under our concentration conditions, the observed rate of CO rebinding to heme a$_3$ is expected to be $-10^{-10}$ s$^{-1}$. This is consistent with our observation of the absence of any MVCO species in the kinetic measurements in contrast to its presence in the photostationary experiments.

The first set of rate constants, $k_{2r}$ and $k_{3r}$, associated with the electron transfer from heme a$_3$ to heme a are estimated to be $-1 \times 10^8$ and $-2 \times 10^9$ s$^{-1}$, respectively, based on recent studies by Namslauer et al. (17). The second set of rate constants, $k_{4r}$ and $k_{5r}$, associated with the electron transfer from heme a$_3$ to Cu$_A$, are assigned values of $1 \times 10^4$ and $7 \times 10^3$ s$^{-1}$, respectively, based on the photolysis studies carried out in a three-electron-reduced CO-bound form of the enzyme, in which heme a and Cu$_A$ were partially reduced and in a redox equilibrium (32). The rate constants, $k_{4r}$ and $k_{5r}$, associated with the electron transfer from Cu$_A$ to heme a$_3$ have not been reported.

We assume that the redox equilibrium between c and c’ and that between d and d’ are much faster than $k_{4r}$ and $k_{5r}$. For a rough approximation that satisfies the pre-equilibrium assumption, $k_{4r}$ and $k_{5r}$ are assumed to be 50 times faster than $k_{3r}$. It should be noted that the selection of these rate constants does not affect the qualitative results of the simulations, provided they satisfy the pre-equilibrium conditions. The rate constants, $k_{4r}$ and $k_{5r}$, associated with the conversation from d’ to e are approximated to be the same as $k_{2r}$ $(1 \times 10^8$ s$^{-1})$ and $k_{3r}$ $(2 \times 10^9$ s$^{-1})$, respectively, because they reflect electron transfer rates between heme a$_3$ and heme a. Nonetheless, it is important to point out that the electron transfer rates between two redox centers might be affected by the redox states of the other two redox centers.

Fig. 7A shows the simulated result based on the rate constants listed above. The result shows that ~60% of the enzyme is in the c, c’, d, and d’ states (with equal population). In addition, ~30% and 10% of the enzyme are in the b and e states, respectively. The dotted lines show the total amount of the redox species with either a reduced or an oxidized heme a$_3$ by summing up the contribution from each species. The data suggest that about 40% of the electron density in the photolyzed MVCO transfers out of heme a$_3$. The failure to fit the kinetic data with the basis equilibrium spectra is attributed to the high population (~60%) of the c, c’, d, and d’ species that consist only of one electron in the binuclear center.

Under equilibrium conditions, we assume that $k_{2r}$ and $k_{3r}$ are significantly reduced due to the protein structural transition from P$_1$ to P$_2$ that stabilizes the states with either zero (species e) or two (species b) electrons in the binuclear center with respect to those with only a single electron. Fig. 7B shows the simulation results based on this assumption by decreasing $k_{2r}$ and $k_{3r}$ by a factor of 10 and 70, respectively. All of the other rate constants are kept unchanged. With these two new rate constants, ~60% of the electron in the photolyzed MVCO transfers out of heme a$_3$ under the photostationary state conditions, which is consistent with the experimental results shown in Fig. 4. More importantly, under these conditions, the populations of species c, c’, d, and d’ are negligible. As a result, the equilibrium data can be well fitted with the standard spectra.

A kinetic scheme that is similar to Scheme 2 has been proposed by Einarsdottir and co-workers (5, 6) based on optical absorption measurements following photodissociation of MVCO. In that work, spectral perturbations of the intermediate in both the Soret and visible regions of the optical absorption spectrum were observed in the microsecond time domain. The authors proposed that the redox state of Cu$_A$, significantly affects the absorption spectrum of heme a$_3$, similar to what we observed here in the resonance Raman spectra. They postulate that there is a rapid equilibrium between reduced heme a$_3$ and reduced Cu$_A$ when only one electron resides in the binuclear center, and this equilibrium favors the direction of reduced Cu$_A$ because of its relatively higher $E_m$.

Modulation of electron redistribution following photolysis of MVCO by protein conformational changes has never been reported. However, conformational changes on faster time scales were documented by Einarsdottir et al. and Woodruff et al. (32, 31). In these studies, spectral changes in the visible region were observed on the picosecond time scale followed by additional changes on the microsecond time scale. The former is ascribed to be the conformational change triggered by the binding of CO to Cu$_A$, and the latter is suggested to be the relaxation of the conformational changes induced by the departure of CO from Cu$_A$, which occurs at about 1 µs (31). The picosecond time scale conformational change does not appear to disturb the Raman spectrum since the 10-ns spectrum is almost identical to the model spectrum B. It is plausible that the departure of the CO from Cu$_A$ at ~1 µs could leave the binuclear center in an unrelaxed metastable state that could affect the electron transfer rates. Furthermore, kinetic studies following the photodissociation of the CO-bound fully reduced enzyme demonstrate that the heme a$_3$ pocket undergoes significant reorganization upon photolysis, with the frequency of the Fe-His stretching mode evolving from 222 cm$^{-1}$ at 10 ns to 214 cm$^{-1}$ on a ~10-µs time scale (33, 34). The conformational change we reported here in the mixed valence enzyme on the milliseconds time scale could have a similar origin as that observed in these systems.

In summary, we have observed a modulation of the electron redistribution following photodissociation of CO from MVCO due to a conformational transition. Subsequent to photodissociation, the protein matrix is trapped transiently in the P$_1$ metastable state in which the initial electron transfer events occur within 5 µs. It relaxes to the equilibrium P$_2$ conformation on a time scale longer than 2 ms. The conformational change destabilizes the redox states with a one-electron reduced binuclear center with respect to those that have either zero or two electrons. As a result, the redox equilibrium is perturbed, and electrons are redistributed. Under transient conditions when the protein matrix is in the P$_1$ conformation, ~40% of the electron density originally on heme a$_3$ is transferred to heme a$_2$. On the other hand, under equilibrium conditions when the protein matrix is in the P$_2$ conformation, ~60% of the electron density is transferred from heme a$_2$ to heme a. The conformational change-induced electron redistribution is confirmed by the simulation results with a new proposed kinetic model. Future work is planned to determine the molecular basis of the conformational change.

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Electron Redistribution in Cytochrome c Oxidase

9399

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