Electron Transfer between Cytochrome *a* and Copper A in Cytochrome *c* Oxidase: A Perturbed Equilibrium Study†

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ABSTRACT: Intramolecular electron transfer in partially reduced cytochrome *c* oxidase has been studied by the perturbed equilibrium method. We have prepared a three-electron-reduced, CO-inhibited form of the enzyme in which cytochrome *a* and copper A are partially reduced and in an intramolecular redox equilibrium. When these samples were irradiated with a nitrogen laser (0.6-ns, 1.0-mJ pulses) to photodissociate the bound CO, changes in absorbance at 598 and 830 nm were observed which were consistent with a fast electron transfer from cytochrome *a* to copper A. The absorbance changes at 598 nm gave an apparent rate of 17 000 ± 2000 s^{-1} (1σ), at pH 7.0 and 25.5 °C. These changes were not observed in either the CO mixed-valence or the CO-inhibited fully reduced forms of the enzyme. The rate was fastest at about pH 8.0, falling off toward both lower and higher pHs. There was a small but clear temperature dependence. The process was also observed in the cytochrome *c*–cytochrome *c* oxidase high-affinity complex. The electron equilibration measured between cytochrome *a* and copper A is far faster than any rate measured or inferred previously for this process.

The role of the mitochondrial electron-transport chain is to carry out the controlled sequential oxidation of reducing equivalents derived from various metabolic processes and to conserve part of the free energy associated with each step as an electrochemical potential across the inner mitochondrial membrane. The chain terminates with the oxidation of ferrocytochrome *c* by molecular oxygen. This process is mediated by cytochrome *c* oxidase, which accepts the electrons one at a time from cytochrome *c* and passes them on to molecular oxygen.

In addition to this role in catalyzing the reduction of oxygen, cytochrome *c* oxidase also serves as one of the coupling sites where the respiratory chain generates a membrane potential from redox energy. In fact, two concurrent coupling mechanisms operate in this enzyme. One mechanism is a direct consequence of the redox chemistry of the enzyme: The conversion of one molecule of dioxygen to water requires four electrons and four protons. When this reaction is catalyzed by cytochrome *c* oxidase, the electrons enter the enzyme from the outer (cytosolic) side of the membrane, and the protons are consumed from the inner (matrix) side. In this manner, the membrane sidedness is exploited to produce a charge separation and a proton gradient across the membrane.

Coupled to this transmembrane electron transfer is an electrogenic proton pump. As electrons flow through the enzyme, protons are pumped out of the mitochondrial matrix and into the cytosol. The stoichiometry of this vectorial proton translocation process can be as high as one proton per electron. Thus, for every electron which passes through the enzyme, one proton is consumed, and up to one proton is pumped, resulting in a maximum proton per electron stoichiometry of two [for a review, see Wikström et al. (1981)].

Cytochrome *c* oxidase contains four redox-active metal centers, two iron hemes and two copper ions [see Blair et al. (1983)]. One heme and one copper (Fe_{a_3} and Cu_B)[1] make up the site of dioxygen reduction. The other two metal centers, Fe_a and Cu_A, function as intervening electron carriers between cytochrome *c* and the oxygen binding site, and it is widely believed that one of these two centers is the site of electron gating in the proton pump [see Blair et al. (1986b), Wikström and Casey (1985), Babcock and Callahan (1983), and Chan et al. (1987)].

However, even the role of these two metal centers as electron carriers is not clearly understood. Numerous transient kinetics studies have indicated that Fe_a and Cu_A lie between cytochrome *c* and the oxygen binding site in the sequence in which the metal centers of the enzyme are reduced and oxidized (Wikström et al., 1981; Brunori et al., 1981), but the details of the electron flow remain unclear. At this point, we still do not know which of these two metal centers accepts electrons directly from ferrocytochrome *c*, or which ones donate them to the oxygen binding site. Specifically, we do not know whether, during *turnover*, electrons must pass through both metal centers or whether only one of the two centers functions as the primary carrier, accepting electrons from ferrocytochrome *c* and passing them on to dioxygen. [It is interesting to note that separate models have been proposed in

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; COMV, CO mixed-valence compound of cytochrome *c* oxidase; COMV+1, CO mixed-valence compound reduced by 1 additional reducing equiv; COFR, CO-inhibited, fully reduced cytochrome *c* oxidase; Cu_A, copper A; Cu_B, copper B; Fe_a, cytochrome *a*; Fe_{a_3}, cytochrome a_3; k_{app}, apparent rate constant; k_f, rate constant of forward process; k_r, rate constant of reverse process; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADH, nicotinamide adenine dinucleotide, reduced form; NHE, normal hydrogen electrode; PMT, photomultiplier tube; σ, standard deviation; Tween-20, poly(oxyethylene)sorbitan monolaurate.

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which *both* Fe_a and Cu_A can accept electrons directly from cytochrome *c* (Brunori et al., 1981) and in which *both* metal centers can pass electrons to dioxygen [Blair et al., 1985; see also Hill et al. (1984a,b, 1986)]. These models taken together would provide for at least two discrete pathways of electron flow from cytochrome *c* to the oxygen binding site.]

One quantity that is important to the issue of electron flow within the enzyme is the rate of electron transfer *between* Fe_a and Cu_A. If this rate is slow relative to the rate of electron flow during steady-state turnover, any mechanism which requires electrons to pass sequentially through both metal centers can be excluded. If the rate is fast, such mechanisms become possible, but the possibility of electron flow between these two centers during turnover would place restrictions on the mechanism of energy transduction. Of course, there is also the possibility that the enzyme can modulate the rate of electron transfer between Fe_a and Cu_A as part of its energy transduction mechanism.

In the present study, we report a direct measurement of the Fe_a to Cu_A electron equilibration rate by the perturbed equilibrium method. An experiment was carried out on a three-electron-reduced CO-inhibited (CO mixed-valence plus one electron, or "COMV+1") form of the enzyme in which Fe_{a_3} and Cu_B were both reduced, and Fe_a and Cu_A were partially reduced and in (intramolecular) redox equilibrium (see below). A brief laser flash was used to photodissociate the bound CO from $Fe_{a_3}^{2+}$ at the oxygen binding site, thereby slightly shifting the redox equilibrium between Fe_a and Cu_A. The time course of approach of the enzyme toward the new equilibrium was then monitored by optical spectroscopy. The apparent rate constant observed (k_{app}) for this approach to the new equilibrium is equal to the sum of the forward and reverse rate constants (k_f and k_r) for this reaction. Although Fe_a and Cu_A are not involved in the binding of CO, and, in fact, are some distance from the oxygen binding site (Goodman & Leigh, 1985, 1987; Brudvig et al., 1984), changes in the optical spectrum indicate that flash photolysis of $Fe_{a_3}^{2+}$-CO is followed by a fast electron reequilibration from Fe_a to Cu_A (k_{app} = 17000 ± 2000 s^{-1}). In addition, the lack of transient kinetics in the COMV compound, in which the binuclear center is reduced, leaving Fe_a and Cu_A oxidized, excluded the possibility of electron transfer from the reduced binuclear center to Fe_a and Cu_A in these experiments. The mechanism by which an event at the oxygen binding site could cause a change in the redox potentials of Fe_a and Cu_A is unknown, but presumably a protein conformational change is involved. [The events surrounding the photolysis of $Fe_{a_3}^{2+}$-CO have been studied extensively by Fiamingo et al. (1982), Boelens and Wever (1979, 1980), and Boelens et al. (1982).]

Materials and Methods

Materials. Cholic acid (U.S. Biochemical) was purified by 3-fold recrystallization from 50:50 water–ethanol. Sephadex G-200 (particle size 40–120 μm) was obtained both from Sigma and from Pharmacia. Tween-20 for general use (dialysis buffers used in the preparation etc.) was obtained from Sigma. Purified Tween-20 as a 10% aqueous solution, stored under nitrogen in sealed vials, was obtained from Pierce. Argon for anaerobic work was made oxygen-free by passing it through a column of manganese oxide on vermiculite. Carbon monoxide from Matheson ("Matheson Purity") was used without further purification. NADH (preweighed vials) was obtained from Sigma and sodium dithionite from GFS Chemicals.

Cytochrome *c* oxidase was isolated by the method of Hartzell and Beinert (1974). Enzymatic activity was assayed spectrophotometrically by monitoring ferrocytochrome *c* oxidation (Smith, 1955). Horse heart cytochrome *c* (type VI) was obtained from Sigma and generally used without further purification. However, cytochrome *c* which was used in the preparation of the cytochrome *c*–cytochrome *c* oxidase high-affinity complex ("caa_3 complex") was purified by ion-exchange chromatography according to the procedure of Brautigan et al. (1978).

Preparation of the Cytochrome c–Cytochrome c Oxidase High-Affinity Complex. The caa_3 complex used in these experiments was made by a modification of the method of Kuboyama et al. (1962) [see Morgan (1989) for details].

Sample Preparation. Samples for kinetic studies were prepared in concentrated form at pH 7.4 and then diluted as necessary to achieve the desired concentration and pH. The CO mixed-valence (COMV) compound was made by incubating the resting enzyme under CO at room temperature for about 8 h [see Bickar et al. (1984)]. These samples could then be diluted into buffers of various pH values. This transfer was performed aerobically. The enzyme became reoxidized, but once the solution was made anaerobic again with CO, the COMV compound was regenerated within minutes. The original formation of the COMV compound from resting enzyme requires hours at room temperature and is very slow at lower temperatures, but once the enzyme has been reduced and reoxidized, the regeneration of the COMV compound takes only a few minutes, even at ice temperature (Nicholls & Chanady, 1981; Morgan et al., 1985).

The three-electron-reduced CO-inhibited (COMV+1) samples were prepared as follows: (1) The CO mixed-valence compound was made as described above. (2) One electron equivalent of NADH solution was added aerobically. (3) The sample was made anaerobic again with CO. (4) The sample was incubated in the refrigerator until it was overall three-electrons-reduced (usually about 12 h). The level of reduction was estimated from the sample's visible spectrum. (In practice, the three-electron level of reduction was only a target. What was important was that the sample contain a significant population of the three-quarter-reduced species.) (5) The three-electron-reduced sample was diluted anaerobically into CO-containing buffer of the desired final pH.

When the NADH was added, there was enough air in the solution to reoxidize the enzyme. In spite of this, it was only necessary to add 1 electron equiv of NADH to reach the three-electron reduction level. As described above, when the sample was made anaerobic again with CO, the COMV compound was regenerated within minutes. This regeneration occurred much more quickly than the reduction of the enzyme by NADH. Thus, Fe_{a_3} and Cu_B were reduced quickly (presumably by CO), leaving only the low-potential metal centers to be reduced by NADH.

The anaerobic dilution of the COMV+1 samples was accomplished by transferring the concentrated sample into a cell containing anaerobic diluting buffer. The transfer was carried out by syringe, under argon-flush, in the dark. First, the diluting buffer was made anaerobic and saturated with CO. Next, both the sample cell and the cell containing the dilution buffer were attached to the vacuum line with "argon flush" adapters. These fittings provided an opening into the anaerobic cell, while the atmosphere in the cell was guarded by a counterflow of anaerobic gas. Although both the sample and the diluting buffer were under CO atmosphere, an argon, not CO, flush was used for the transfer. A gas-tight syringe (Hamilton, Reno, NV) with a very long needle was used to transfer the sample. Once the enzyme solution had been

transferred, the dilute solution was deaerated once with argon and 5 times with CO before the lights were turned back on.

We found that the purity of Tween-20 used in the diluting buffer was critical. Unless specially purified Tween-20 (see above) was used, the samples reoxidized upon dilution. In the case of the COMV compound, this was not a problem since under an atmosphere of CO the COMV compound was regenerated after oxidation. However, in the case of the COMV+1 samples, this had the effect of converting the enzyme to the COMV compound.

The fully reduced CO-inhibited compound was prepared in the same way as COMV+1, except that about twice as much NADH was added. Reduction with a large excess of of reductant (NADH or sodium dithionite) was not viable since these reductants absorb strongly at the wavelength of the laser.

Visible spectra of the samples were measured before and after the kinetic experiments. In most cases, some increase in the level of reduction was observed following laser photodissociation, presumably due to photoreduction. After all other measurements were finished, the samples were opened to air, and their pH was measured.

Kinetic Measurements. Transient changes in absorbance after photolysis were measured as follows: Photolysis was accomplished with 337-nm pulses from a N_2 laser (Photochemical Research Associates LN1000) run at a repetition rate of 5 Hz. The photolyzing laser pulses had an energy of about 1.0 mJ with a temporal pulse width of about 0.6 ns. The laser beam was made co-linear with the probe light beam (in the opposite direction) by means of a dichroic mirror and was focused to a spot size of about 2 mm. Probing light was produced by a 75-W tungsten lamp powered by a regulated DC power supply (Kikusui Electronics PAD). The light from the tungsten lamp was passed through filters, the sample, and filters and then focused into a 0.25-m monochromator (Jarrell-Ash 82-410). The purpose of the first set of filters was to minimize sample heating and photolysis by the probe beam. The second set of filters was used to minimize the entry of scattered laser light into the monochromator and to reject other potential stray light. For the 830-nm measurements, RG715 and RG9 filters were placed before the monochromator to cut off all visible light (50% T at 730 nm). The wavelength-selected probe light was detected by a photomultiplier tube (PMT for measurements in the visible range, RCA 1P28A; for measurements in the near-IR, Hammamatsu R406). The PMT output, terminated with a 3900 Ω resistor, was recorded with a transient digitizer (Biomation 805), which was interfaced to a microcomputer (Apple II+). Unless otherwise indicated, signals were averaged for 1024 transients. Temperature control was maintained with a Beckman water-jacketed cell holder (Model INS-TW) attached to a refrigerated water bath.

Kinetic measurements employing a microsecond flash lamp, which are referred to under Discussion, were carried out as described by Morgan (1989).

Calculations and Data Analysis

Equilibrium Redox Calculations. The calculation of subpopulations in the partially reduced enzyme was carried out as described by Copeland et al. (1987).

Base-Line Subtraction. Since the timing and trigger circuitry introduced a significant background signal into the data, background ("dark") signals were acquired with the laser and the probe lamp both shuttered. The kinetic data were base-line-corrected and converted into relative absorbance units by using a computer program [see Morgan (1989)]. What is meant by relative absorbance is that, within a given kinetic trace, the changes are in correct absorbance units but that the entire data set is subject to an unknown offset. In the case of the 830-nm data shown in Figure 4, in order to correct for the background artifacts, the preflash part of the raw data for each of the samples and the background were fit to a single exponential. The amplitudes of these fitted exponential functions were used to scale the base line for each data set. The 10 ms per point 830-nm data shown in Figure 6 were handled in the normal way.

Determination of Rates. The absorbance data were fit by computer to either single- (three-parameter) or double (five-parameter)-exponential functions. When a double-exponential function was used and the rate of CO rebinding had been measured, that rate could be supplied and held constant while the other four parameters were allowed to vary. In other cases, all five parameters were allowed to vary. The type of fit used in a given instance is indicated in the figure caption. Uncertainties were estimated by first fitting the function and finding the average of the residuals. The standard deviation for the residuals was then used as the uncertainty in the original data points. This resulted in a χ^2 value of 1 for the fit. When more than one data set was acquired for a given set of conditions, the uncertainty of the measurement was estimated from a series of rates measured at 25.5 °C between pH 6.5 and 7.5 (see Figure 7). In all cases, except in the temperature dependence (Figure 8) where the uncertainties in the fitted rates were plotted, the larger uncertainties are given. For the temperature dependence, all of the data were obtained from the same sample on the same day.

Results

In order to study the Fe_a to Cu_A electron-transfer rate by the perturbed equilibrium method, it was first neccessary to prepare the enzyme in a state in which these two metal centers were partially reduced and in intramolecular redox equilibrium, but in which the remaining redox centers (Fe_{a_3} and Cu_B) were not able to change oxidation state. To accomplish this, we have taken the CO mixed-valence compound of the enzyme and added 1 additional electron equiv of reductant to partially reduce Fe_a and Cu_A. In the presence of CO, Fe_{a_3} and Cu_B form a cooperative high-potential redox couple (Lindsay et al., 1975). In addition, the redox potentials of Fe_a and Cu_A in the CO-inhibited enzyme are very similar. At 25 °C, the potentials are approximately 288 mV (vs NHE) for Fe_a (when Cu_A is oxidized) and 276 mV for Cu_A (when Fe_a is oxidized) (Wang et al., 1986; Ellis et al., 1986). This translates into an equilibrium constant of 0.68 for electron distribution between the two metal centers ($Fe_a^{2+}Cu_A^{2+} \rightarrow Fe_a^{3+}Cu_A^{1+}$).

When 1 electron equiv of reductant is added to a sample of the CO mixed-valence (COMV) compound, the resultant "CO mixed-valence plus one electron" (COMV+1) sample will contain four subpopulations: (1) a COMV population, in which Fe_a and Cu_A are both in the oxidized form; (ii) a three-electron-reduced population in which Fe_a is reduced and Cu_A is oxidized; (iii) a three-electron-reduced population in which Cu_A is reduced and Fe_a is oxidized; and (iv) a fully reduced (COFR) population in which both Fe_a and Cu_A are in the reduced form (see Figure 1). All of these species will be in equilibrium through intermolecular electron exchange, but the two three-electron-reduced populations, (ii) and (iii), are also in equilibrium through intramolecular electron transfer between Fe_a and Cu_A. This intramolecular electron transfer is the process of interest in this study.

It should be possible to measure the rate of this equilibration by making a small sudden shift in the redox equilibrium and following the relaxation kinetics of the system. In addition

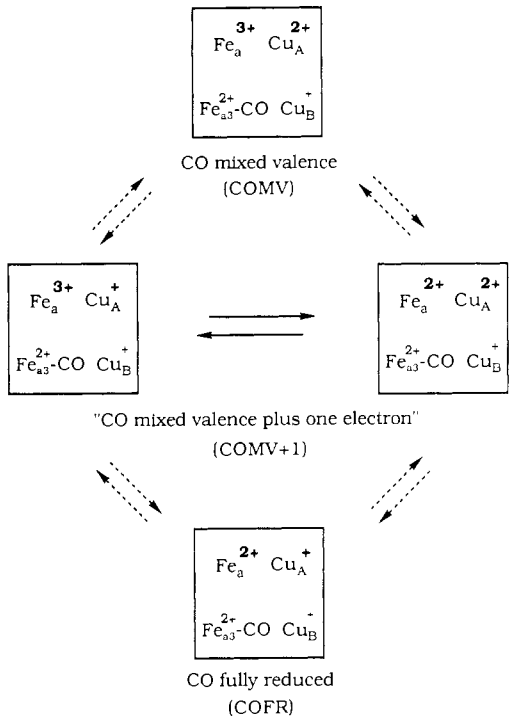

FIGURE 1: Redox equilibria in CO-inhibited cytochrome *c* oxidase. The transitions which must occur through intermolecular processes are indicated by dashed arrows. The solid arrows indicate the intramolecular $Fe_a \leftrightarrow Cu_A$ redox equilibrium [see Wang et al. (1986)].

to the extent of the shift in the redox equilibrium, the amplitude of the observed reequilibration will depend on the size of the three-electron-reduced subpopulation in the sample. One factor that contributes to the size of the three-electron-reduced subpopulation is the redox anticooperativity (about 40 mV) between Fe_a and Cu_A [Ellis et al., 1986; Wang et al., 1986; redox anticooperativity is discussed in detail by Blair et al. (1986a)]. The consequence of this anticooperativity is that even though the upper asymptotic redox potentials of Fe_a and Cu_A in the CO-inhibited enzyme are almost identical, the entry of a second electron into the low-potential metal centers is energetically much less favorable than the entry of the first electron. This has the effect of increasing the subpopulation of the three-electron-reduced enzyme at the expense of the two- and four-electron-reduced subpopulations. Without this anticooperativity, 50% of the molecules in a COMV+1 sample would be at the three-electron level of reduction. Because of the anticooperativity, this number is expected to be 69% [see Copeland et al. (1987)].

Figure 2 shows the visible absorption spectra of CO mixed-valence (COMV) cytochrome *c* oxidase, the CO-bound fully reduced (COFR) enzyme, and a sample poised at an intermediate three-electron level of reduction (COMV+1). As described above, the COMV and COFR are well-defined two- and four-electron-reduced states of the enzyme, while the three-electron-reduced COMV+1 sample is an equilibrium mixture containing small populations of COMV and COFR and a larger population of three-electron-reduced molecules in which two electrons are effectively held at the oxygen binding site and the third electron is shared between Fe_a and Cu_A.

The redox states of both Fe_a and Cu_A can be monitored optically. Reduced Fe_a gives rise to an absorption band at 605 nm, and oxidized Cu_A gives rise to a broad absorption centered at around 830 nm. Figure 2 shows that as Fe_a and Cu_A become more reduced, the 605-nm band increases in intensity, while the absorbance at 830 nm decreases. The absorption

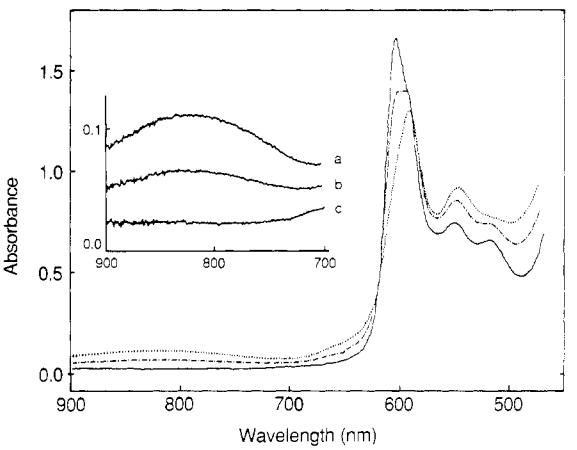

FIGURE 2: Visible absorption spectra of CO-inhibited cytochrome *c* oxidase at the two-, three-, and four-electron reduction levels. COMV (a, dots), COMV+1 (b, dots and dashes), COFR (c, solid line). The inset shows the 830-nm region of the same spectra expanded vertically. All three traces in the inset were drawn in solid lines so that the noise could be reproduced. Enzyme concentration, 48 μM; path length, 10 mm; temperature, 22 °C; pH 7.4.

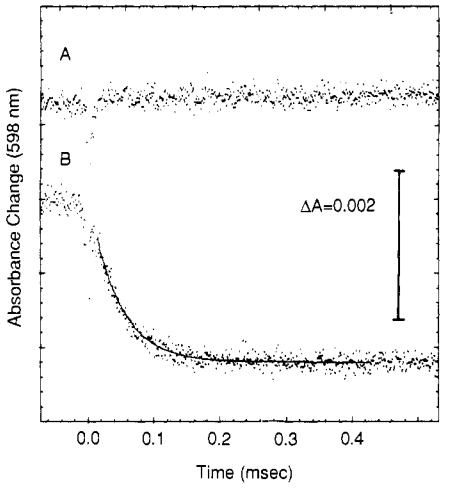

FIGURE 3: Absorption changes at 598 nm following CO photodissociation by a 0.6-ns laser pulse. (A) COMV; (B) COMV+1. Enzyme concentration, approximately 27 μM; path length, 10 mm; temperature, 25.5 °C; buffer, 150 mM NaMOPS–0.5% Tween-20; pH 7.16; 1024 transients averaged. The fit shown is a single-exponential fit with an apparent rate equal to 22 000 s^{-1}.

peak at 590 nm which dominates the α band of the COMV is characteristic of CO-bound $Fe_{a_3}^{2+}$ (Vanneste, 1966). The intensity of this band remains relatively constant as Fe_a and Cu_A go from oxidized to reduced.

$Fe_{a_3}^{2+}$-CO is photolabile. The photolysis of this species is accompanied by a shift in the α band. There is an absorbance decrease at 590 nm and an increase above 600 nm (Wikström, 1981; Greenwood et al., 1974). This spectral change interferes with the observation of Fe_a at 605 nm. However, 598 nm is an isosbestic wavelength for the absorbance changes associated with this photolysis, and the Fe_a reduced minus oxidized band, centered at 605 nm, still has about 75% of its intensity at 598 nm. Thus, by observation at 598 nm, Fe_a can be followed without interference from the absorbance changes associated with the photodissociation. This technique has been used to study electron transfer in the COMV compound after photolysis (Brzezinski & Malmström, 1987).

Figure 3 shows the absorbance changes in COMV+1 and COMV samples at 598 nm following the laser pulse. In COMV+1, there was a decrease in absorbance with an apparent first-order rate constant of 17 000 ± 2000 s^{-1} (1σ, see

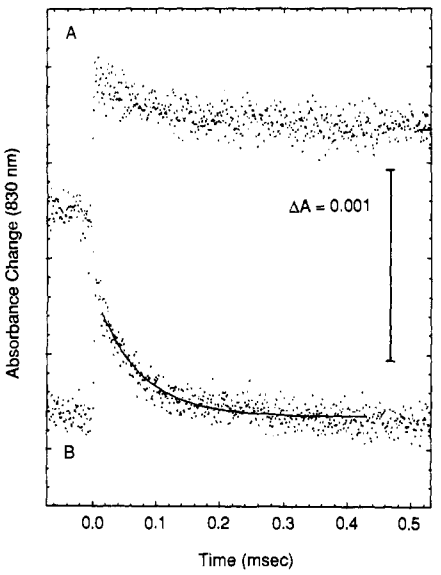

FIGURE 4: Absorbance changes at 830 nm following CO photodissociation by a 0.6-ns laser pulse. (A) COMV; (B) COMV+1. 4096 transients averaged. Because the number of scans was different, a different "dark" base line was collected. However, when the data were processed using this base line, a significant amount of trigger circuit artifact (apparently an exponential decay) was still apparent in the preillumination part of the data. In order to correct for this, the preillumination part of the raw data for both samples and the background were fit to a single exponential. The extents in the fitted exponential functions were used to scale the base line to match each data set (see Data Analysis under Materials and Methods). The base-line offset was not scaled. All other conditions are the same as for Figure 3. The fit shown is a single exponential with an apparent rate equal to 14 000 s^{-1}.

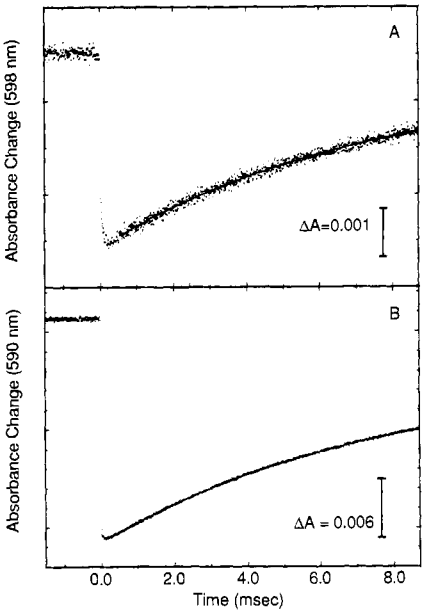

FIGURE 5: Absorbance changes at 598 and 590 nm for a COMV+1 sample following CO photodissociation by a 0.6-ns laser pulse. All conditions are the same as for Figure 3. Fits shown are single exponentials with apparent rates of (A) 132 and (B) 135 s^{-1}.

Materials and Methods). No corresponding change was observed in COMV. The same experiments were repeated, observing at 830 nm (Figure 4). At this wavelength, there was an initial increase in absorbance in both samples, during the dead time of our measurement [see Boelens et al. (1982)]. In the COMV+1 sample, this increase was followed by a decrease with an apparent first-order rate constant of about 14 000 s^{-1} (see below). Again, no corresponding change was seen in the COMV sample, indicating that electron transfer between the reduced binuclear center and Cu_A or Fe_a does not occur.

These results are consistent with a fast electron reequilibration between Fe_a and Cu_A with an apparent rate of 17 000 ± 2000 s^{-1}, following laser-induced dissociation of the CO from COMV+1 enzyme. The observations correspond to a small shift in the Fe_a/Cu_A redox equilibrium by ca. 2% of the oxidase molecules. The absorbance changes which occur on this time scale are seen only in the COMV+1 sample, where there is a subpopulation of enzyme in which electron redistribution between Fe_a and Cu_A is possible; no corresponding changes are observed in the COMV or COFR samples (data for the latter not shown). The direction of the absorbance changes (a decrease at both 598 and 830 nm) is consistent with the simultaneous oxidation of Fe_a and reduction of Cu_A. The data from 598 and 830 nm differ in two ways. The relative magnitude of the absorbance changes (based on the amplitude of the single-exponential fitted curves) indicates that about 1.5 times as much Cu_A is reduced as Fe_a is oxidized, but within experimental error, the Cu_A reduced corresponds to the Fe_a oxidized. The apparent rate observed at 830 nm is also somewhat slower, 14 000 s^{-1}, compared to 17 000 ± 2000 s^{-1}. These differences can be accounted for, in part, by inaccuracies in the base-line subtraction and scaling for the 830-nm data (see Materials and Methods). In addition, we estimate that the experimental error in the 830-nm data is at least ±2000 s^{-1} on the basis of the signal to noise ratio. However, since only one data set was acquired at this wavelength, we can only estimate a lower limit for the error as compared to the 598-nm data (Figure 3). For this reason, more confidence should be placed in the apparent rate constant and the estimated error for the data acquired at 598 nm.

In some COMV samples, we observed absorbance changes which were similar to those seen in the COMV+1 samples, but much smaller. This apparently occurred because the enzyme had become photoreduced, and some of the COMV compound had been converted into COMV+1 (see below). However, no absorbance changes attributable to electron redistribution in the COMV compound were observed in the experiments reported here.

A COMV+1 sample of the cytochrome *c*–cytochrome *c* oxidase high-affinity complex was also studied. The absorbance changes at 598 nm which followed photolysis in this sample were essentially the same as those described above for the COMV+1 samples without cytochrome *c* (data not shown).

Figure 5 shows the absorbance changes in the COMV+1 sample at 590 nm and at 598 nm on a longer time scale. As discussed above, the major contribution to the absorption intensity at 590 nm arises from $Fe_{a_3}^{2+}$-CO, and the decrease and subsequent increase in absorbance at this wavelength are thought to reflect the photodissociation and rebinding of CO. As would be expected, the initial decrease in absorbance corresponding to photodissociation is too fast to be resolved by our apparatus (Findsen et al., 1987). The first part of the recombination which follows can be fitted to a rate of 135 s^{-1}. This rate is generally in agreement with the rates observed at 590 nm in the COMV. This suggests that the recombination of $Fe_{a_3}^{2+}$-CO is not significantly affected by redox states of Fe_a and Cu_A, in agreement with the findings of Boelens et al. (1982). It should be noted, however, that in some of the COMV samples, an early slow phase was observed at 590 nm (data not shown).

At 598 nm, in the COMV+1 sample, the rapid decrease in absorbance was followed by a return to the original level at an apparent rate of 131 ± 20 s^{-1} which is almost identical with the rate of the recovery phase observed at 590 nm. Apparently,

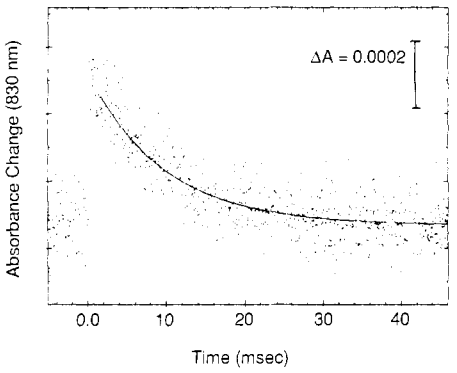

FIGURE 6: Absorbance changes at 830 nm for a COMV sample following CO photodissociation by a 0.6-ns laser pulse. 4096 transients were averaged. All other conditions were the same as for Figure 3. The fit shown is a single exponential with an apparent rate equal to 104 s^{-1}.

the electron population returned to its original distribution at a rate controlled by the re-formation of $Fe_{a_3}^{2+}$-CO. This is to be expected, since the apparent rate of the electron equilibration is much faster than the recombination of CO with $Fe_{a_3}^{2+}$. It should be pointed out that the kinetic trace in Figure 5A was not measured exactly at the isosbestic point. There is a significant initial deflection before the 17 000 s^{-1} phase begins. This initial deflection is a contribution from the photodissociation itself. There should be a corresponding contribution from the CO rebinding in the recovery part of this trace, and this could influence the measured rate. However, the corresponding data for a sample at pH 6.76 were measured almost exactly on the isosbestic point (there is no jump in absorbance in the dead time), and in this case, the recovery phase has a similar rate (143 ± 10 s^{-1}). In this connection, it should be noted that the isosbestic wavelength for the photodissociation of $Fe_{a_3}^{2+}$-CO is pH dependent. At pH 7.0, the isosbestic point is at 598 nm, but at pH 9.0, it is close to 600 nm. One consequence of this is that the isosbestic point does not always correspond to the same point on the Fe_a α band, and so the applicable Fe_a reduced minus oxidized extinction coefficient also changes with pH.

At 830 nm, in the case of the COMV+1 sample, the initial increase in absorbance was exactly offset by the decrease during the 14 000 s^{-1} phase, and the absorbance had already returned to base-line levels after 200 μs (see Figure 4). This appeared to rule out a recovery phase, and no data were taken at longer times. As shown in Figure 6, the initial increase in absorbance at this wavelength was followed by a return to its original level with a rate of about 100 ± 5 s^{-1} in the COMV compound. We attribute this recovery to a small absorbance contribution from the CO photolysis reaction at 830 nm. In the case of the COMV+1 data shown in Figure 4, the magnitude of the approach to equilibrium matches that of the CO photolysis contribution. Therefore, the recovery phase of 100 s^{-1} is not seen.

Figure 7 shows the pH dependence of the apparent rate. What is clear from these data is that the rate is fastest at about pH 8.0 and falls off in either direction. Figure 8 shows the temperature dependence of the apparent rate. The changes in the rate as a function of temperature are much smaller than those observed by changing the pH. On the basis of the uncertainties used in the pH data set (see Data Analysis under Materials and Methods), this temperature dependence could be considered insignificant. However, all these data come from the same sample on the same day, and a similar trend was observed at several pH values. The consistency in these data may be partly fortuitous, but the data do illustrate the fact

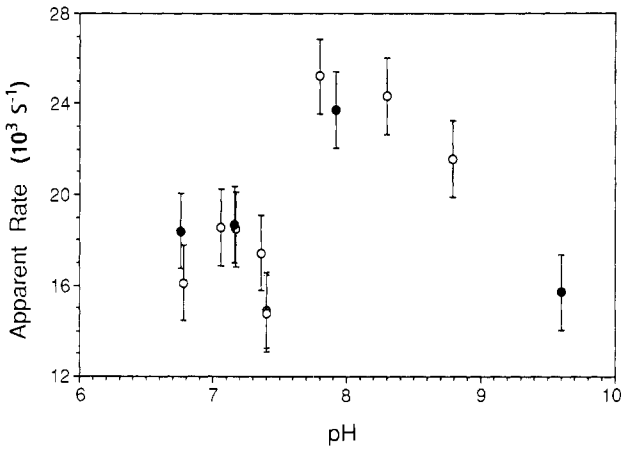

FIGURE 7: pH dependence of the fast absorbance change at 598 nm (25.5 °C). Buffers: pH 8.3 and above, 150 mM NaCHES–0.5% Tween-20; below pH 8.3, 150 mM NaMOPS–0.5% Tween-20. (Closed circles) Four-parameter fit; (open circles) three-parameter fit. Error bars: 1σ (see Data Analysis under Materials and Methods).

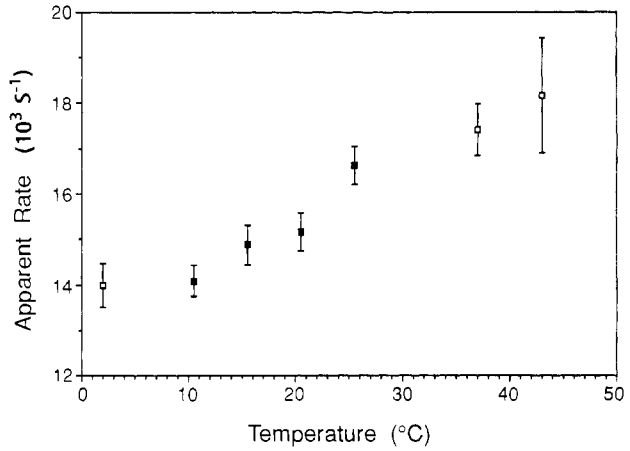

FIGURE 8: Temperature dependence of the fast absorbance change at 598 nm. Buffer: 150 mM NaMOPS–0.5% Tween-20, pH 7.36. Data and error bars (1σ) are from three-parameter fit. Closed squares indicate points where three-, four-, and five-parameter fits are in good agreement (see Data Analysis under Materials and Methods).

that the process is slightly temperature dependent. The error bars for this figure reflect the uncertainties in the fitted rates (see figure caption).

Visible spectra of the samples were acquired before and after the experiments. Almost invariably, the level of reduction was greater at the end than at the beginning. Although we did not perform a controlled study, it appeared as though the extent of reduction was greater in samples which had been illuminated for a longer time in the laser beam. This suggests that the samples were being photoreduced. The extent of photoreduction also appeared to be greatest in samples of high pH.

Discussion

The results reported here show that photodissociation of CO from $Fe_{a_3}^{2+}$-CO in the three-electron-reduced COMV+1 sample brings about a redistribution of electrons from Fe_a to Cu_A, with an apparent rate of 17 000 ± 2000 s^{-1}. In control experiments, no electron redistribution was observed in the COMV or COFR compounds.

Boelens et al. (1982) and Brzezinski and Malmström (1987) have studied the changes in absorption which follow photolysis of the COMV compound. In contrast to the results obtained here, Boelens et al. reported that CO photodissociation was followed by electron transfer from the oxygen binding site to

Cu_A with an apparent rate of about 7000 s^{-1}. About 5% of Cu_A became reduced. Brzezinski and Malmström observed the same process but reported an apparent rate of about 14000 s^{-1}. They also found that the reduction of Cu_A was followed by reduction of Fe_a at an apparent rate of about 500 s^{-1}. Under our experimental conditions, we find no evidence of electron transfer from the reduced binuclear center to Cu_A or Fe_a in the COMV compound.

These results are not necessarily contradictory. In their experiments, Boelens et al. and Brzezinski and Malmström employed flash sources with temporal pulse widths of 200 and 500 ns, respectively, and Brzezinski and Malmström reported that their flash energy was 1.0 J per pulse. The present results were obtained by using 0.6-ns, 1.0-mJ pulses. We obtained some results similar to those of the other investigators, using a flash lamp apparatus which had a pulse width greater that 1 μs. It appears, therefore, that the outcome of the photolysis event may be dependent on the duration or total energy of the excitation pulse.

It is important to note that these differences in the outcome of the photolysis experiment occurred in the COMV and not in the COMV+1 samples. In the case of the COMV+1 samples, we obtained consistent results regardless of which apparatus was used. Thus, the validity of our results does not depend entirely on the resolution of this issue.

In the COMV+1 samples, photolysis of $Fe_{a_3}^{2+}$-CO resulted in a transient redistribution of electrons from Fe_a to Cu_A. The process initiated by the photodissociation of CO apparently brings about a shift in the redox equilibrium between Fe_a and Cu_A, which are about 20 Å away from the oxygen binding site (Goodman & Leigh, 1987; Brudvig et al., 1984). Although the mechanism of this communication is unknown, it is most likely mediated by an enzyme conformational change. The observed rate constant for the Fe_a to Cu_A electron-transfer rate is a little slower than the rate constant for the cytochrome a_3 heme pocket relaxation following photodissociation of CO as measured by resonance Raman spectroscopy (Findsen et al., 1987). This process may be involved in bringing about a shift in the redox equilibrium. One possibility that cannot be ruled out is that the rate of the electron reequilibration which we observe is limited by such a putative conformational change. In this case, the present results would still place a lower limit on the "true" electron equilibration rate, and given that caveat, much of the following discussion would still apply.

On the other hand, the rate-limiting step may be the electron transfer itself. If the process being observed is simply an intramolecular electron equilibration between two metal centers, then the relaxation rate is the sum of the forward and reverse electron-transfer rates ($k_r + k_f$). The individual rates can be deduced if the equilibrium constant ($K = k_f/k_r$) is known. The redox potentials of Fe_a and Cu_A in the CO-inhibited enzyme have been determined electrochemically (Ellis et al., 1986; Wang et al., 1986). At 25 °C, the equilibrium constant is 0.68, in favor of the reduction of Fe_a. From the rate of 17000 ± 2000 s^{-1} observed at 598 nm for the fast kinetic phase at pH 7.0 and 25.5 °C, we obtain k_r = 10200 s^{-1} and k_f = 7000 s^{-1}.

Two significant assumptions are implicit in this analysis: (1) that the equilibrium constant in the photodissociated enzyme is not significantly different from that measured for the CO-inhibited enzyme; (2) that the simple redox equilibrium invoked actually governs the observed relaxation. The validity of these assumptions is discussed below.

The first assumption is that the redox potentials of Fe_a and Cu_A in the CO-photodissociated enzyme where the observed kinetics takes place can be reasonably approximated by the corresponding potentials in the CO-inhibited enzyme. The correct way to solve for k_f and k_r would be to use the redox potentials of Fe_a and Cu_A in the CO-photodissociated enzyme. However, these potentials are not known. They must be different from the corresponding potentials in the unphotolyzed enzyme; the experimental design here relies on a change in these potentials to perturb the redox equilibrium. It appears, however, that this change is small. In the kinetics experiment, the total electron population change upon photolysis of the COMV+1 amounts to no more than 2%.

The second assumption is that the simple redox equilibrium invoked closely describes the situation and governs the observed reequilibration. This would be the case if the relaxation is a monophasic exponential decay. However, if the overall relaxation involves more than one step, other intermediate(s) would enter into the picture (Hiromi, 1979). The measured relaxation appears to be a single-exponential decay, but there is no way to be sure that it is not just the first phase of a more complicated process. There are no equilibrium data with which to compare the extent of the absorbance change, and because of the rapid recombination of CO to $Fe_{a_3}^{2+}$, the relaxation cannot be observed for more than a few milliseconds.

The relaxation process might be multiphasic if either the reduction of Cu_A or the oxidation of Fe_a was followed by a conformational relaxation. This kind of behavior is predicted by many models of proton pumping by the oxidase (Gelles et al., 1986; Blair et al., 1986b; Wikström et al., 1981). As discussed in the introduction, the reduction and oxidation kinetics of Fe_a and Cu_A are biphasic at both sites (Antalis & Palmer, 1982; Hill et al., 1984a,b, 1986). In fact, Wikström and colleagues (Wikström et al., 1981) and Brzezinski and colleagues (Brzezinski et al., 1986) have proposed that biphasicity in the enzyme's kinetics is related to its role as a proton pump. Thus, some multiphasic behavior in the electron redistribution between these two sites would not be unexpected, but cannot be excluded on the basis of this experiment.

Nevertheless, our results do suggest that the electron transfer between Fe_a and Cu_A can be extremely facile. We now review, in some detail, a number of previous results that have been interpreted to mean that the $Fe_a \leftrightarrow Cu_A$ electron equilibration rate is considerably slower than we report here.

(1) Greenwood et al. (1976) reported a temperature-jump experiment in which a sample containing CO-inhibited cytochrome *c* oxidase and cytochrome *c* was poised at a reduction level at which the cytochrome *c*, Fe_a, and Cu_A were all partially reduced. A sudden increase in temperature was followed by an electron redistribution from Fe_a to cytochrome *c*. The relaxation was biphasic. The two phases were interpreted to be (a) a fast reequilibration between Fe_a and cytochrome *c*, followed by (b) a slower reequilibration involving Cu_A as well. The fast phase was analyzed to give electron-transfer rates of 9×10^6 M^{-1} s^{-1} from cytochrome *c* to Fe_a and 8.5×10^6 M^{-1} s^{-1} for the reverse reaction. The slower phase, which was also somewhat concentration dependent, had a relaxation time constant of 40–100 s^{-1}. This was inferred to be the $Fe_a \leftrightarrow Cu_A$ electron equilbration rate. However, since the reduction of Fe_a by cytochrome *c* has itself been shown to be biphasic (Antalis & Palmer, 1982), it is possible that the biphasic behavior observed in this *T*-jump experiment does not arise from slow $Fe_a \leftrightarrow Cu_A$ redox equilibration.

(2) Wilson et al. (1975) reported a stopped-flow experiment in which cytochrome *c* oxidase was reduced by ferrocytochrome *c*. As the concentration of cytochrome *c* was increased, Fe_a became reduced more quickly, and a lag was

observed between the reduction of Fe_a and Cu_A. At higher cytochrome *c* concentrations, the rate of reduction of Cu_A was reported to be independent of cytochrome *c* concentration, with a half-time of about 8 ms. The fact that the lag in the reduction of Cu_A was observed only at higher concentrations, and at faster rates, indicates that it is a kinetic phenomenon and does not arise from a difference between the redox potentials of Fe_a and Cu_A. This result does not appear to be consistent with fast electron equilibration between these two sites. However, it should be noted that the oxygen intermediates absorb at 830 nm (Andréasson et al., 1972), and this contribution may also be convoluted into the reduction kinetics assigned to Cu_A.

However, other workers have reported similar experiments in which no such lag was observed (Antalis & Palmer, 1982; Andréasson et al., 1972). The experiments of Wilson et al. (1975) were all carried out in the presence of some oxygen; in some cases, the cyanide-inhibited form of the enzyme was used. In contrast, the other experiments referred to above were all carried out anaerobically. Andréasson et al. (1972) reported that, when they allowed air into their reactions, the absorbance changes at 550 nm (oxidation of cytochrome *c*) no longer tracked linearly with absorbance changes at 830 nm. This could occur because, in the native enzyme, some electron transfer to the oxygen binding site can take place on this time scale [see also Beinert et al. (1976)]. However, these authors did not report whether the reduction of Fe_a and Cu_A (i.e., absorbance changes at 605 and 830 nm) still tracked together under aerobic conditions.

(3) Halaka et al. (1984) observed a similar lag between the reduction of Fe_a and Cu_A when the enzyme was reduced by using phenazine methosulfate. In this case, a rate of about 20 s^{-1} was resolved for Fe_a to Cu_A electron transfer. Although the reduction of Fe_a was a second-order process, the rate of reduction of Cu_A did not change significantly when the concentrations of the reactants were varied (Barnes, 1986), suggesting that here also the lag is a kinetic and not a thermodynamic phenomenon. The authors noted the fast Fe_a to Cu_A electron transfer invoked by Antalis and Palmer (1982) and suggested that this rate could be different under different experimental conditions.

(4) Brzezinski and Malmström (1987) studied the intramolecular electron transfer in cytochrome *c* oxidase following photolysis of the CO mixed-valence compound. They reported that photodissociation of the CO was followed by partial reduction of Cu_A at an apparent rate of about 14 000 s^{-1}, and partial reduction of Fe_a at an apparent rate of about 500 s^{-1}. They interpreted these results in terms of fast electron transfer from the oxygen binding site to Cu_A, followed by slower electron transfer from Cu_A to Fe_a.

The rate which they have measured for the $Fe_a \leftrightarrow Cu_A$ electron equilibration (k_{app} = 500 s^{-1}) is at least 10 times slower than the one we have observed. The authors have pointed out that their data are also consistent with a model in which Cu_A and Fe_a both accept electrons directly from the oxygen binding site but with different rates. However, this explanation is still inconsistent with the faster $Fe_a \leftrightarrow Cu_A$ electron equilibration rate, because with the fast equilibration measured Fe_a and Cu_A should reduce synchronously.

In the experiment of Brzezinski and Malmström (1987), either Fe_{a_3} or Cu_B must become reoxidized in order for Cu_A or Fe_a to be reduced, whereas in our experiment, it appears that both Fe_{a_3} and Cu_B remain reduced throughout. This could be the key to understanding the difference between the two results. It is possible that the reoxidation of one of the oxygen binding site metal centers causes the $Fe_a \leftrightarrow Cu_A$ electron equilibration rate to become much slower. The reduction of Fe_a which Brzezinski and Malmström observe at 500 s^{-1} could also be driven by a conformational change. The apparent rate of electron redistribution would then reflect the rate of this conformational change and not the electron-transfer rate. If this putative conformational change could occur only when Fe_{a_3} or Cu_B becomes oxidized, it would not be observed in our experiment.

Thus, we cannot be sure that the fast $Fe_a \leftrightarrow Cu_A$ redox equilibration measured applies under all circumstances. Experiments 2–4 discussed above provide some compelling evidence that this rate can be slower, at least in some states of the enzyme. The rate could vary with experimental conditions (Halaka et al., 1984), or even be controlled or "gated" as part of the enzyme's proton pumping mechanism [see Blair et al. (1986b)].

We conclude by exploring two possible ramifications of a fast $Fe_a \leftrightarrow Cu_A$ electron equilibration rate. If the rate of electron equilibration between these two sites were as fast as 17 000 s^{-1} under enzyme turnover conditions, then it would not matter whether electrons entered the enzyme through Fe_a or Cu_A. The maximum turnover rate of the enzyme under typical experimental conditions is 50–100 electrons s^{-1} and is never more than a few hundred (Sinjorgo et al., 1986). Given these rates, electrons from cytochrome *c* could enter the enzyme at Fe_a and be transferred to the oxygen binding site out of Cu_A (or vice versa), without ever disturbing the redox equilibrium between Fe_a and Cu_A. This would not resolve the question of whether electrons could enter the enzyme from more than one cytochrome *c* binding site, but the question of which metal center(s) received electrons directly from cytochrome *c* would be moot.

Second, if the $Fe_a \leftrightarrow Cu_A$ redox equilibration is fast under turnover conditions, it is unlikely that electrons are transferred to the oxygen binding site from more than one of the two centers at a significant rate. It seems unlikely that *both* Fe_a and Cu_A could be coupling sites for the proton pump; in effect, that there would then be *two* pumping centers. Thus, electron transfer out of one of these metal centers might not drive the proton pump; i.e., it would not be "coupled" [see Blair et al. (1985)]. The proton pump site is generally assumed to be a 1.0 H^+/e^- device [see Chan et al. (1987)]. If this is indeed the case, then in order to achieve an average stoichiometry of 0.9 H^+/e^- (Sarti et al., 1985), almost all of the electrons moving through the enzyme would have to pass through a coupled pathway. Thus, the system must be able to run with a minimum uncoupled electron flow. However, if the nonpumping site could transfer electrons to oxygen at an appreciable rate, and if that site was in fast redox equilibrium with the pumping site, a significant fraction of the electron flow would not be coupled.

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Registry No. Cu A, 7440-50-8; cytochrome *a*, 9035-34-1; cytochrome *c* oxidase, 9001-16-5.

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