Electron Redistribution in Mixed Valence Cytochrome Oxidase Following Photolysis of Carboxy-Oxidase

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Abstract

Absorbance changes at 446 nm in purified cytochrome oxidase following flash photolysis of carboxy-oxidase poised in the mixed valence state at $+ 220$ mV show biphasic kinetics. One phase corresponds to CO recombination to ferrous cytochrome a_3 with an energy of activation of 9 kcal/mol; the second phase is 3–5 times faster with an energy of activation of 9.15 kcal/mol. Following flash photolysis at approximately $- 60°C$, cytochromes a and c and the 840-nm CuA species are observed to undergo reduction as electrons from ferrous unliganded cytochrome a_3 equilibrate with the equipotential redox centers of the oxidase; as CO recombines with ferrous cyochrome a_3, these centers are oxidized and the mixed valence carboxy-oxidase is regenerated. Electron redistribution between centers of the oxidase in the forward and reverse directions occurs faster than does the binding of CO.

Key Words: Cytochrome oxidase; carboxy cytochrome oxidase; CO recombination; mixed valence; cytochrome c; electron transport.

Introduction

In the reduction of molecular oxygen, the redox centers of cytochrome c oxidase (EC 1.9.3.1) are oxidized by oxygen and re-reduced by the mitochondrial respiratory chain. Chance and coworkers have described not only the sequential low-temperature formation of oxygen-intermediate compounds and their correlation to the oxidation of cytochrome a, cytochrome c, and the infrared detectable copper associated with cytochrome a (Chance et al., 1975a,–d; Chance and Leigh, 1977; Wikstrom et al., 1976a; Chance, 1978), but also energy-dependent reverse electron transport of cytochrome oxidase (Chance, 1961a, b; Chance and Hollunger, 1961a–c).

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Electrons are transferred from cytochrome c via oxidase centers along a thermodynamic potential gradient from $+220\,mV$ at cytochrome c to $+800\,mV$ at oxygen; the energy given off in this process is utilized in the phosphorylation of ADP to ATP. Only by the input of energy (such as by ATP hydrolysis) are electrons capable of going up the "thermodynamic hill," eventually capable of reaching NADH dehydrogenase (Chance, 1961a, b; Chance and Hollunger, 1961a–c).

Wikstrom et al. (1976a) described the effects of an interaction between the hemes of the oxidase where reduction of one equipotential heme causes a decrease in the E_m of the companion heme, a form of heme–heme interaction. The result of this neoclassical concept is the equilibrium between cytochromes a and a_3 in unliganded, partially reduced "mixed valence" oxidase involving electron transfer between cytochrome oxidase centers. The midpotential of one cytochrome is $+240\,mV$ while that of the other cytochrome in mixed valence oxidase is $+380\,mV$; electrons can move from ferrous cytochrome to ferric cytochrome down an electropotential gradient. In unliganded oxidase, cytochrome a, like cytochrome a_3, has an E_m of ca. $+240\,mV$, similar to that of cytochrome c $(+225\,mV)$ and the copper associated with cytochrome a (CuA; $+240\,m$); thermodynamically, electron equilibration should occur between all oxidase centers.

The transport of electrons in the oxidase is frequently viewed as unidirectional except when energy-dependent reverse electron transport occurs. In this communication, electron redistribution between redox centers in nonliganded mixed valence oxidase will be demonstrated.

Materials and Methods

Beef heart mitochondria were isolated from fresh heart by the procedure of Crane et al. (1956).

Lipid-depleted cytochrome oxidase was purified from been heart mitochondria by a slight modification of the cholate procedure of Yu et al. (1975); beef heart mitochondria were used in place of Keilin–Hartree particles as the starting material. Cholic acid (Aldrich) was used in place of potassium cholate and the additions of 20% cholate (w/v; neutralized with KOH) in the first two detergent fractionations were $3.75\,ml/100\,ml$ of $20\,mg/ml$ mitochondrial protein and $5.8\,ml/100\,ml$ of $20\,mg/ml$ mitochondrial protein suspension, respectively, as described previously (Harmon, 1988).

Visible absorbance spectra were recorded with a model DBS-3 scanning dual wavelength spectrophotometer (Johnson Research Foundation, University of Pennsylvania).

CO recombination kinetics were measured using a Gilford single-beam spectrophotometer (model 252). A clear glass dewar with a 2-mm light-path 0.5-ml sample holder (Plexiglas faces) was used in place of the cuvette holder as described previously (Harmon and Sharrock, 1978; Sharrock and Yonetani, 1977; Harmon, 1988); the sample was placed above the liquid nitrogen level in the dewar. Temperature was measured by a copper–constantan thermo-couple in the frozen sample and regulated with a small electric heater immersed in the liquid nitrogen in the dewar; increasing the heater tempera-ture boils off liquid nitrogen, decreasing the temperature of the gas above the liquid. The output of the Gilford was connected to a 16-bit A/D converter in an IBM-PC computer and/or to a strip-chart recorder.

Low-temperature kinetics were initiated by a single flash of 1500 BCPS xenon flash tubes. For measurements in the Soret region, the xenon tubes were fitted with Wratten no. 9 and no. 15 filters (do not transmit 500 nm or longer wavelengths), and a Corning 5113 filter (does not transmit 500 nm or shorter wavelengths) was placed in front of the photomultiplier. For measurements at wavelengths longer than 500 nm, the flash tubes were fitted with Corning 5113 filters, and Wratten no. 9 and no. 15 filters were used at the photomultiplier.

Fully reduced carboxy cytochrome oxidase was formed by the addi-tion of 5 μg N,N,N′,N′, tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and 90 mM sodium ascorbate (pH 6.8) to a 100% CO-flushed mixture of 100 μg cytochrome c (8 μM final concentration, type VI, Sigma) and resting-state ferric cytochrome oxidase ($\sim$ 6 mg/ml final concentration) in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4). The mixture was bubbled with 100% CO in the dark for 20 min and then loaded into the sample holder in the dark prior to freezing in liquid nitrogen. Partially reduced (mixed valence) CO-bound oxidase was generated in the same mix-ture above except that only enough ascorbate (2–5 mM final concentration) was added to 3 ml of the 6 mg/ml oxidase suspension. The reduction state was determined from the absorbance of cytochrome c at 550 nm and cytochrome a at 604 nm taken from ascorbate-reduced *minus* oxidized difference spectra. The partially reduced oxidase was then bubbled with 100% CO for an additional 10 min in the dark to insure CO saturation of the assay medium. The level of reduction was again calculated from the reduced *minus* oxidized difference spectrum. The partially reduced sample was then loaded into the low-temperature sample holder and frozen in liquid nitrogen until use.

Results

As shown in Fig. 1, addition of 2.5 mM ascorbate results in 28% reduction of cytochrome a and 47% reduction of cytochrome c. Insertion
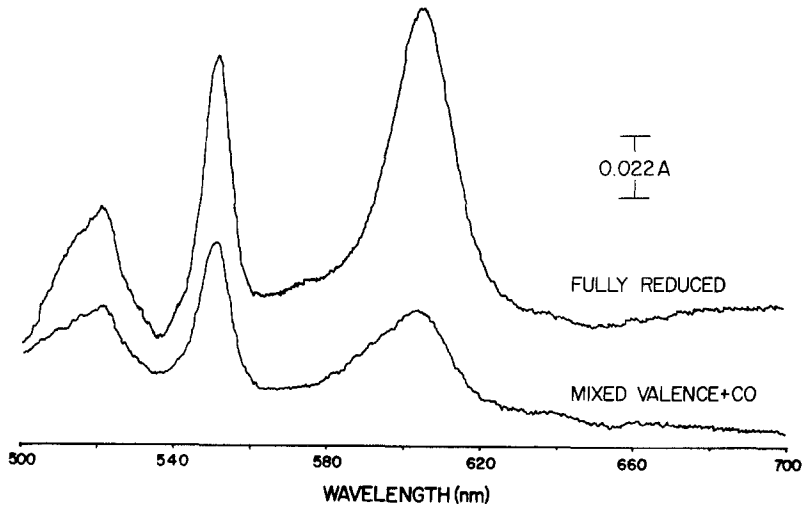
Fig. 1. Absorbance difference spectra (reduced *minus* oxidized) of a 1 : 1 cytochrome *c*-purified cytochrome-oxidase complex at room temperature. The mixed valence + CO spectrum was obtained by the addition of ascorbate + TMPD as described in *Materials and Methods*. The fully reduced spectrum was obtained by addition of dithionite to an identical sample in the absence of CO.

of these values into the Nernst equation using the midpotentials stated above indicates that the system was poised at ca. $+220$ mV.

Following flash photolysis of fully reduced CO-bound oxidase, the recombination of CO follows monophasic kinetics that can be fitted well by a simple exponential expression as reported previously (Harmon and Sharrock, 1978; Sharrock and Yonetani, 1977) (cf. Fig. 2). Data in Fig. 3 show that the value of the rate constant, k, is a logarithmic function of temperature. The activation energy is ~ 9 kcal/mol in agreement with previously reported values (Erecinska and Chance, 1972; Sharrock and Yonetani, 1977; Harmon and Sharrock, 1978).

In contrast, the kinetics of CO recombination at 446 nm with partially reduced mixed valence oxidase poised at $+220$ mV do not follow a single simple exponential expression. The biphasic curve of Fig. 4 can be fitted by at least two exponential curves as shown, one fitting the early stage and one fitting the later stage of absorbance change. Plotting log k vs. $1/T$ reveals two linear relationships (Fig. 5). One corresponding to the slower of the two phases has an energy of activation of ~ 9 kcal/mol, similar to that of CO recombination to fully reduced oxidase; this curve is indistinguishable from the data obtained for fully reduced oxidase. The second phase also has an energy of activation of ~ 9 kcal/mol but has larger rate constants, indicating faster kinetics.
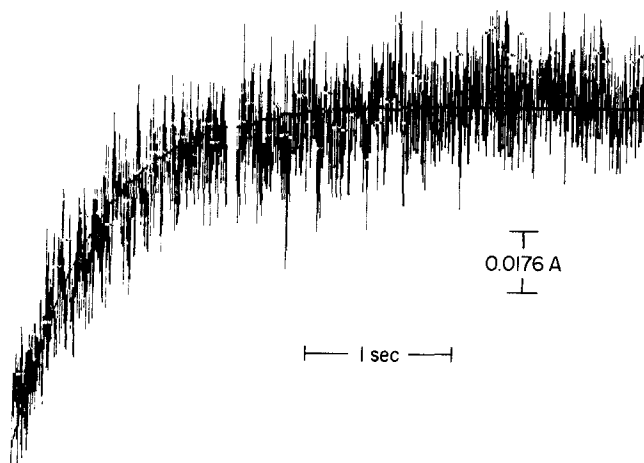
Fig. 2. Recording of the 446-nm absorbance changes vs. time following flash photolysis of fully reduced purified carboxy-cytochrome oxidase at $-85°C$. Absorbance decrease is in the upward direction. The solid line is a computer-generated best fit of the experimental data to a single exponential equation as described previously (Harmon and Sharrock, 1978).

At $+220$ mV, ~ 20–25% of the cytochrome oxidase molecules will be fully reduced in the a^{2+} a_3^{2+} CO form. The remaining 75–80% are in the a^{3+} a_3^{2+} CO mixed valence state. It is therefore plausible to suspect that the two clearly different phases of CO recombination represent CO recombinations to each type of oxidase. A mixture of oxidase reduction states could result in biphasic kinetics. Harmon and Sharrock (1978), however, showed no difference in CO recombination kinetics in fully reduced and mixed valence oxidase in intact mitochondria when measured at 594 nm, suggesting that the biphasic kinetics have their basis in a reaction other than CO recombination to oxidase molecules of different reduction states.

In fully reduced oxidase, both cytochromes a and a_3 are reduced and absorb equally at 446 nm (Wikstrom $et\ al.$, 1976a); in the carboxy form, the 446-nm absorbance is due solely to a^{2+} with a_3^{2+} CO absorbing at 430 nm. Upon photolysis, the absorbance at 446 nm increases twofold and then exponentially decays to the preflash level as CO binds to cytochrome a_3^{2+}; all 446-nm absorbance changes are due to binding of CO to ferrous cytochrome a_3.

In the partially reduced oxidase, however, decreases in 446-nm absorbance can be due to ligand binding to ferrous cytochrome a_3 or the oxidation of cytochrome a_3 (assuming cytochrome a is not reduced in the process). Previous reports indicate that the kinetics of CO binding in intact mitochondria are the same in both mixed valence and fully reduced oxidase
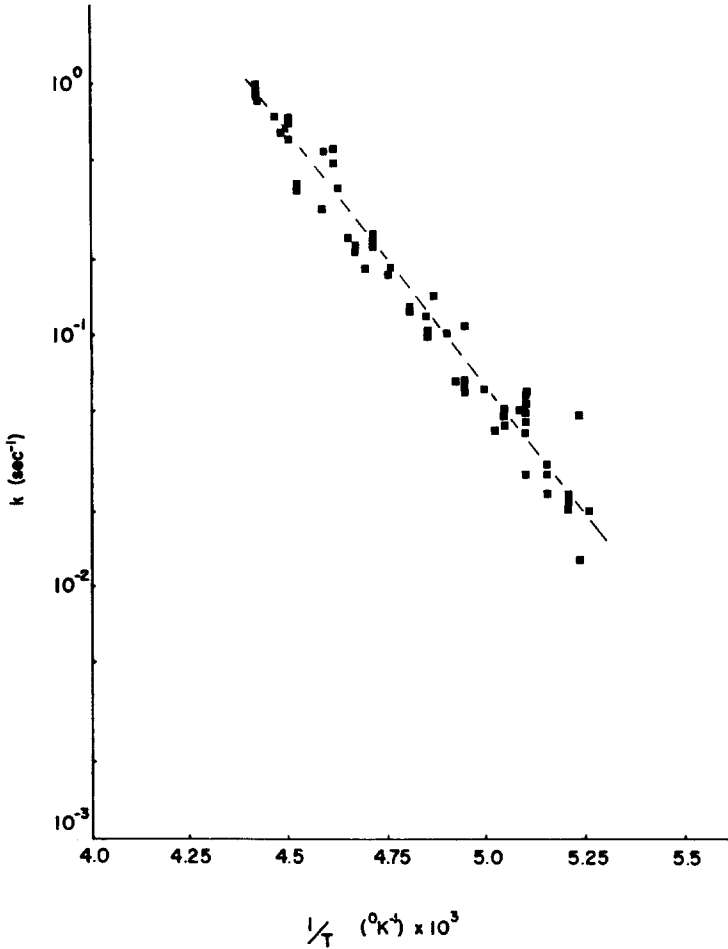
Fig. 3. Plot of kinetic constant k of 446-nm absorbance change vs. inverse temperature in fully reduced carboxy-cytochrome oxidase. The slope of the line (computer linear regression fit) represents the energy of activation; the value is 9.3 kcal/mol.

(Harmon and Sharrock, 1978), suggesting that the change in 446-nm kinetics is due to cytochrome oxidation occurring at the same time as CO recombination.

If the postphotolysis kinetics are measured at 604 nm, a clearly multi-phasic reaction is observed (cf. Fig. 6). Photolysis of a_3^{2+} CO is expected to result in an increase in 604-nm absorbance (assuming cytochrome a_3 is not immediately oxidized following photolysis). Cytochrome a_3^{2+} contributes 20% of the 604-nm absorbance in fully reduced oxidase according to the neoclassical model (Wikstrom et al., 1976a); in 30% reduced oxidase where
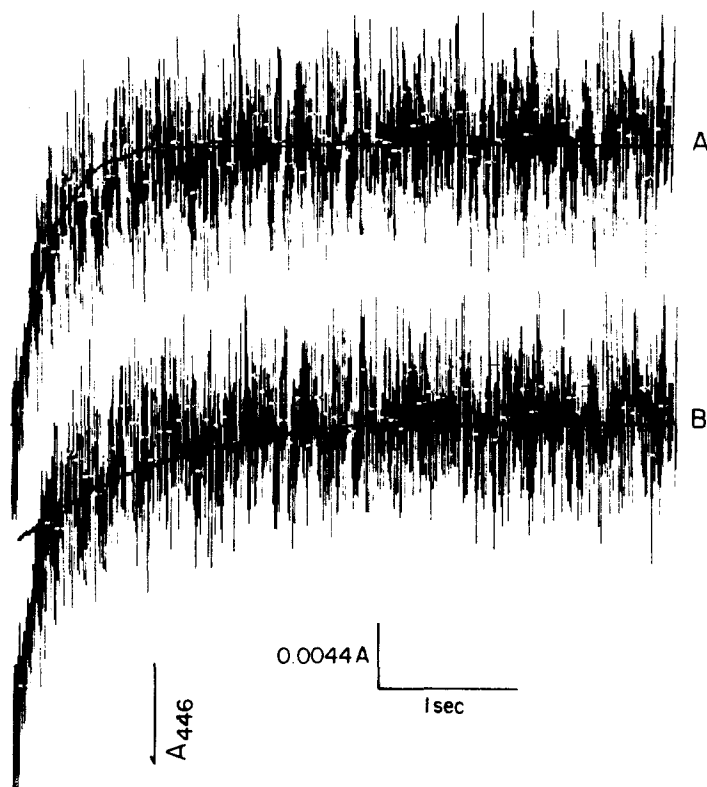
Fig. 4. Recording of 446-nm absorbance changes vs. time following flash photolysis of mixed valence carboxy-cytochrome oxidase at $-65°C$. The computerized fit to a single exponential for the data in trace A in the first 1.5 sec is shown and yields values of $k = 0.65/\text{sec}$ and a half-time of 1.07 sec. For trace B (same raw data as trace A), the half-time is 3.9 sec and the value of k is 0.178/sec.

30% of cytochrome a and all cytochrome a_3 is reduced, a_3^{2+} will contribute $\sim 40\%$ of the 604-nm absorbance observed in the photolyzed mixed valence sample. As CO rebinds to ferrous cytochrome a_3, the absorbance at 604 should decrease at the same time as the 590-nm absorbance increases and the 446-nm absorbance decreases. As shown in Fig. 6, 604-nm absorbance increases at photolysis; a rapid absorbance decrease is observed immediately after this increase, indicating either recombination of CO to ferrous cytochrome a_3, oxidation of either cytochrome a and/or a_3, or both. The absorbance decreases to a value less than the preflash level, however. At ~ 1 min after the flash, the absorbance at 604 nm begins to increase and eventually stabilizes at a constant absorbance value. When absorbance at 446 nm is measured on a longer time scale than Fig. 2, a similar sequence of changes
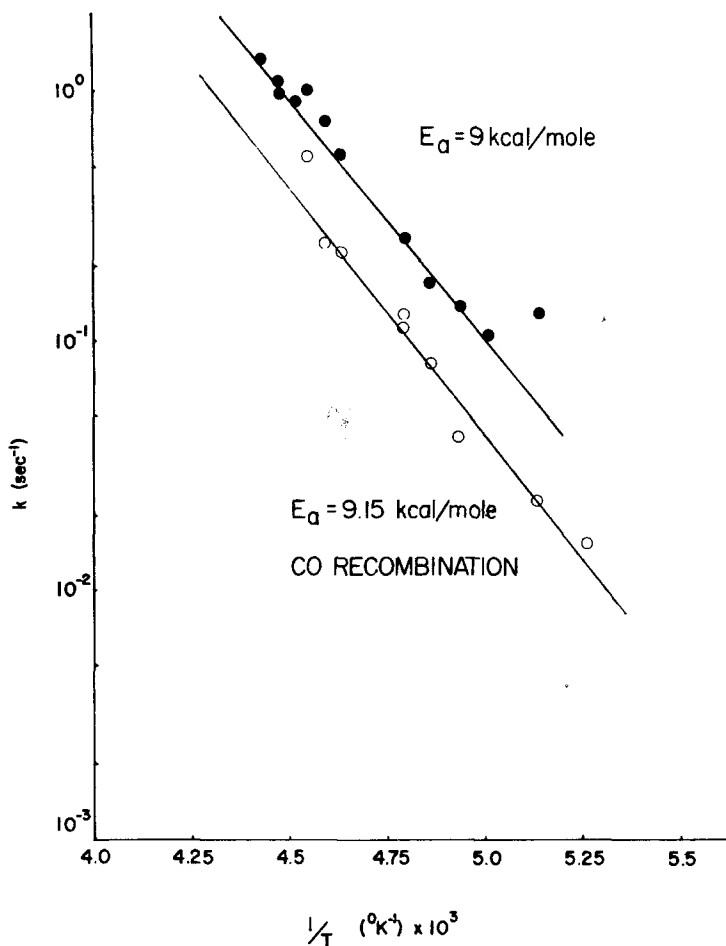
Fig. 5. Plot of log k vs. inverse temperature for the fast (●) and slow (○) phases of 446-nm absorbance changes following flash photolysis of mixed valence carboxy-cytochrome oxidase. The energy of activation of the fast phase is 9 kcal/mol while that of the slow phase is 9.15 kcal/mol. The slow phase data are very similar to those obtained from the monophasic 446-nm changes in fully reduced carboxy-oxidase.

is observed (cf. Fig. 7). At photolysis, absorbance increases; an absorbance decrease followed by a slight increase in absorbance is observed before the absorbance reaches a constant value.

When the postflash kinetics are measured at 550 nm, an increase in absorbance followed by a decrease in absorbance is observed (Fig. 8), indicative of a reduction and then oxidation of cytochrome c in the sample. The
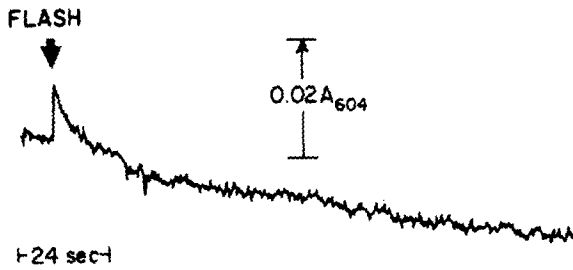
Fig. 6. Plot of 604-nm absorbance vs. time following flash photolysis at −60°C in mixed valence carboxy-oxidase. The arrow denotes the direction of absorbance increase.
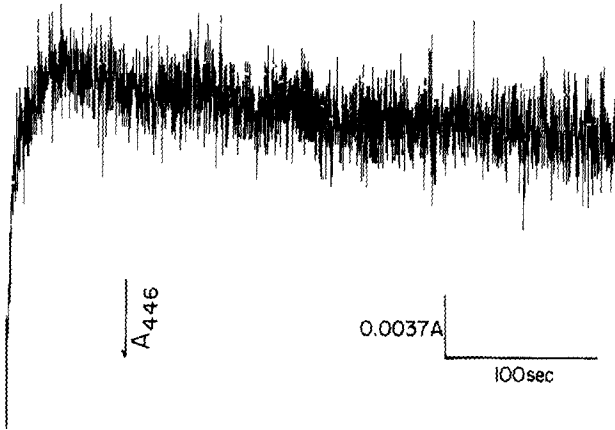

Fig. 7. Recording of 446-nm absorbance decrease (upward) vs. time following flash photolysis of mixed valence carboxy-oxidase at −68°C.
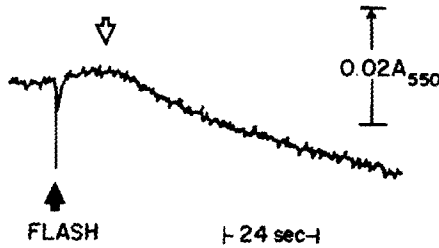

Fig. 8. Recording of 550-nm absorbance changes (increase upward) vs. time following flash photolysis of 1 : 1 cytochrome c–mixed valence carboxy-oxidase complex at −60°C. The reduction–oxidation cycle is indicated in the region of the open arrow.

absorbance change in Fig. 8 involves 19% of the oxidized cytochrome c in the sample (calculated using a millimolar extinction coefficient of 19.5), representing $\sim 1/3$ of the total electrons in the oxidase complex. This fraction of electrons in one individual center is expected if electron equilibration among four equipotential centers (cytochrome c, cytochrome a, and the two coppers) were to occur.

Following flash photolysis, a decrease followed by an increase in 840-nm absorbance (the IR-detectable copper) is also observed, indicative of a reduction–oxidation cycle similar to that observed for cytochrome c (data not shown).

Discussion

Our interpretation of the observed changes at 446 nm is that the rapid phase represents the oxidation of cytochrome a_3 and that the slower phase corresponds to the recombination of CO to cytochrome a_3^{2+}. The absorbance chages at 604 nm (cytochrome a reduction–oxidation) and the changes in 550 nm (cytochrome c reduction–oxidation) are consistent with this model. Upon photolysis, 604-nm absorbance increases due to the formation of unliganded a_3^{2+}. Absorbance at 604 nm decreases as a result of the recombination of CO with cytochrome a_3 as well as oxidation of reduced cytochrome a either by oxidized cytochrome c or by electron transfer back to cytochrome a_3 (and subsequent binding with CO). Following photolysis, 550-nm absorbance increases and then decreases as electrons equilibrate between cytochromes c, a, and a_3.

Wikstrom et $al.$ (1976a) state that, in the absence of ligand, a and a_3 have similar midpotentials and $K_{eq} = (a^{3+} a_3^{2+})/(a^{2+} a_3^{3+}) = 1$. After photolysis, the value of K is extremely large. Electron redistribution to equipotential centers occurs to achieve equilibrium where $K_{eq} = 1$, explaining the partial reduction of cytochrome a and equipotential copper and cytochrome c as well as the partial oxidation of cytochrome a_3. The $a^{3+} a_3^{2+}$ species is capable of binding CO with an $E_m > 500$ mV; the K_{eq} of the unliganded mixed valence oxidase after equilibration is again unity. As $a^{3+} a_3^{2+}$ is depleted as CO binds, $a \rightarrow a_3$ electron transfer occurs.

Forward Electron Transfer at Low Temperatures

Cytochrome c oxidation during oxygen reduction has been observed at low temperatures. Ferguson–Miller et $al.$ (1978) and Waring et $al.$ (1980) studied the oxidation of cytochrome c at temperatures down to $-50°C$. Chance et $al.$ (1978) demonstrated little cytochrome c oxidation below

$-70°C$. These findings are in agreement with earlier findings of Harmon et al. (1976), who observed cytochrome c oxidation down to $-70°C$; they also noted that protamine sulfate, which displaces cytochrome c from its mitochondrial binding site and inhibits cytochrome c oxidation and oxygen reduction at room temperature (Harmon et al., 1974), accelerates the formation of cytochrome oxidase oxygen intermediates by apparently blocking the reduction of cytochrome oxidase centers via electron transfer from cytochrome c.

Wikstrom et al. (1976b) observed cytochrome a oxidation down to $-80°C$. Clore et al. (1980) observed the oxidation of cytochrome a and its copper during the reduction of oxygen by fully reduced oxidase. Thus, at these low temperatures, electron transfer between the centers readily occurs and results in the oxidation of cytochromes a and c and the copper associated with cytochrome a.

Chance et al. (1978) state that, at room temperature, electron transfer from cytochrome $a \rightarrow a_3$ occurs faster than the $c \rightarrow a$ transfer (which would result in a decrease in 446-nm absorbance due to cytochrome a oxidation; as cytochrome a_3 is reduced, it will bind CO and absorb at 430 nm). If this were the case in our experiments, as the electron passes from cytochrome a to cytochrome a_3, a loss of 446-nm absorbance would occur as a_3^{2+} CO is formed, but it would be limited by the CO recombination rate. We would expect a slower phase of 446-nm absorbance decrease as cytochrome c reduces cytochrome a while cytochrome a is oxidized by cytochrome a_3, instead of a faster phase as we observed. There is no reason to expect the relationship of room temperature rates to hold at low temperatures, however.

Reverse Electron Transport at Low Temperatures

Leigh and Wilson (1972) and Wever et al. (1974) suggested the transfer of electrons from cytochrome a_3^{2+} to cytochrome a on the basis of a decrease in $g = 3$ EPR signal at 10 K; Wever et al. (1974) and Boelens et al. (1982a) observed decreases in $g = 3$ cytochrome a intensity at 77 K and 20 K, respectively. Boelens and Wever (1979) demonstrated the reduction of cytochromes a and c and the oxidation of a_3 at room temperature when ferricyanide-induced mixed valence NO-bound oxidase was constantly illuminated. Boelens et al. (1982b) indicated fast and slow phases of 445-nm absorbance changes in mixed valence oxidase as well as CuA reduction and a_3 oxidation evidenced by a trough at 445 nm. The formation of this trough in mixed valence oxidase must indicate the oxidation of both cytochromes a and a_3 (if electron transfer from a_3 to a occurs, then the absorbance at 445 would remain constant unless cytochrome a were subsequently oxidized) or alternatively, the electron from a_3 was transferred not to cytochrome a, but to

another undetected center (this seems unlikely since those experiments were conducted at room temperature where electron equilibration would be rapid). Boelens and Wever (1979: 307) state that biphasic kinetics "might be expected when the electron transfer between cytochrome a and cytochrome a_3 is slow compared to the recombination of CO with cytochrome a_3^{2+}. However, biphasic kinetics can also be expected if the $a \leftrightarrow a_3$ transfer rates are faster than the CO recombination as demonstrated here.

The oxidation of cytochrome a_3 even at 5 K occurs in a few milliseconds (Leigh et al., 1974), after which time the electron should reside on cytochrome a with the same 446-nm absorbance as if the a_3–a transfer never occurred. Any subsequent 446-nm absorbance decrease must be due to two processes (coppers omitted):

1) $a^{2+} a_3^{3+} \to a_3^{+} a_3^{2+} + CO \to a^{3+} a_3^{2+} CO$ with 446-nm absorbance decreasing as CO recombination occurs, or

2) $c^{3+} a^{2+} a^{3+} \to c^{2+} a^{3+} a_3^{3+}$ electron equilibration.

If reaction 2 were to occur reversibly, then reaction 2 would cause a second phase of 446-nm change upon CO recombination to ferrous a_3. If, however, $a \to c$ occurs faster than $c \to a$ and $a \to c$ occurs faster than reaction 1, then a faster phase of 446-nm absorbance decrease corresponding to cytochrome a_3 oxidation would be seen in the biphasic kinetics as in Fig. 4.

De Fonseka and Chance (1979) observed single exponential kinetics in FeCN-induced mixed valence oxidase, but observed that, at $-95°C$, the rate of what was believed to be CO recombination was slower than in fully ferrous oxidase. Clore and Chance (1980) found that the rate of 444–463-nm absorbance change was slower than either the 590–630-nm or 609–630-nm absorbance changes, and that the kinetics of all three wavelength pairs were significantly different from each other. This is understandable since each pair reflects changes in either CO complex (590 nm) or the ratio of a^{2+} to a_3^{2+} (4 : 1 or 1 : 1 at 608 and 444 nm, respectively). The differences in the kinetic progress of the three wavelength pairs decreased with decreasing temperature, suggesting that a process other than simple CO recombination was involved.

Two factors are involved in the previous work of others: (1) the temperature dependence of the intraoxidase electron transfer and (2) the actual potential of the enzyme system.

1) If CO recombination were slightly faster than the $a \leftrightarrow a_3$ transfer rate at temperatures below $-60°C$, the $a \leftrightarrow a_3$ transfer rate (no change in 446-nm absorbance when this occurs) would cause a slower rate of 446-nm decrease, as observed by Clore and Chance (1980) and de Fonseka and Chance (1979). At lower temperatures (ca. $-80°C$) where the $a \leftrightarrow a_3$ and $a \leftrightarrow c$ rates are significantly slowed or do not occur at all (de Fonseka and Chance, 1979; Chance et al., 1978) during the period in which CO recombination occurs,

single exponential kinetics will be observed. At temperatures above $-60°C$, if $a \leftrightarrow a_3$ and $a \leftrightarrow c$ reactions are, as suggested here, faster than CO recombination, biphasic kinetics will be observed if excess oxidant is not present to remove electrons from the oxidase.

2) Denis and Richaud (1985) observed the failure of the 445-nm absorbance to return to its preflash level following photolysis of ferricyanide-induced mixed valence carboxy-oxidase; they attributed this to the formation of Compound C due to contaminant oxygen in the sample (Chance *et al.*, 1975a, b) or to electron distribution. Boelens and Wever (1979) report similar findings. If an electron from a_3^{2+} were to reach cytochrome c, it would reduce ferricyanide. Because of the large excess of ferricyanide over ferrocyanide (the concentration of the latter limited to no more than $1/2$ the concentration of oxidase), the potential of the system would not be less than $+400\,mV$ or so, inadequate to reduce cytochromes a/a_3. Thus, just as observed by Denis and Richaud (1985), the 445-nm absorbance change decreases in magnitude with successive flashes as electrons redistribute and reduce ferricyanide. We do not observe these decreases in magnitude since the entire system is poised at $+220\,mV$.

That earlier reports of flash photolysis of mixed valence oxidase failed to observe the transient reduction of cytochrome c (or cytochrome a or its copper) is also likely due to the use of ferricyanide to generate the mixed valence state in those studies (Chance *et al.*, 1975b–d; Wikstrom *et al.*, 1976; Harmon and Sharrock, 1978; Harmon and Wikstrom, 1978). Mixed valence oxidase is generated by the addition of excess ferricyanide ($E_m = 400\,mV$) to fully reduced carboxy-oxidase; the high potential ($+580\,mV$) a_3^{2+} CO remains reduced. Since ferricyanide binds to the exposed heme edge as well as the back side of the cytochrome c (Stellwagen and Cass, 1975), any electron that makes its way to cytochrome c from the oxidase will be taken up by ferricyanide; electron equilibration between equipotential cytochromes c, a, and CuA will not be observed.

Both CO–ligand recombination and intercenter electron transfer have similar energies of activation, but in these experiments electron transfer is fivefold faster than ligand binding. This suggests that the rate-limiting step in oxygen reduction involves ligand binding and not the transfer of electrons between redox centers. From these data, it is clear that, at low temperatures, reverse electron transfer is faster than ligand binding.

Acknowledgments

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