*Journal of Bioenergetics and Biomembranes, Vol. 25, No. 2, 1993*

# The Sequence of Electron Carriers in the Reaction of Cytochrome *c* Oxidase with Oxygen

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*Received November 4, 1992; accepted November 12, 1992*

Kinetic studies of the electron transfer processes performed by cytochrome oxidase have assigned rates of electron transfer between the metal centers involved in the oxidation of ferrocytochrome *c* by molecular oxygen. Transient-state studies of the reaction with oxygen have led to the proposal of a sequence of carriers from cytochrome *c*, to $Cu_A$, to cytochrome *a*, and then to the binuclear (i.e., cytochrome $a_3$–$Cu_B$) center. Electron exchange rates between these centers agree with relative center-to-center distances as follows; cytochrome *c* to $Cu_A$ 5–7 Å, cytochrome *c* to cytochrome *a* 20–25 Å, $Cu_A$ to cytochrome *a* 14–16 Å and cytochrome *a* to cytochrome $a_3$–$Cu_B$ 8–10 Å. It is proposed that the step from cytochrome *a* to the binuclear center is the key control point in the reaction and that this step is one of the major points of energy transduction in the reaction cycle.

**KEY WORDS:** Cytochrome oxidase; electron transfer; energy transduction.

## INTRODUCTION

Cytochrome oxidase has four redox active metal centers: two hemes (cytochrome *a* and $a_3$) and two copper atoms ($Cu_A$ and $Cu_B$), involved in electron transfer from cytochrome *c* to oxygen. The free energy of this redox process is conserved in the form of an electrochemical gradient. Energy conservation during the cytochrome oxidase reaction may be achieved by transmembrane, vectorial electron transfer and coupled, transmembrane proton transfer. The cytochrome oxidase reaction *in vivo* is under control such that the respiratory rate and therefore production of transmembrane electrochemical gradient does not exceed the metabolic demand for energy (Babcock and Wikström, 1992).

Mitochondrial cytochrome oxidase is composed of 13 different subunits (Kadenbach *et al.*, 1987). Studies of a variety of bacterial cytochrome oxidases has demonstrated a much simpler protein structure relative to the mammalian enzyme. A number of bacterial cytochrome oxidases have been isolated that are composed of two subunits and yet retain the electron transfer and energy conservation properties of the more complicated mammalian enzyme. Subunits I and II of mammalian cytochrome oxidase are homologous to the two subunits found in these simpler bacterial oxidases (Saraste, 1990). Therefore, the four redox active metal centers must be found in subunits I and II, and this is the core structure for all the enzymes in this family. Cytochrome $a_3$ and $Cu_B$ are closely situated within subunit I so as to form a binuclear center that is the site for oxygen binding, cytochrome *a* is also located in subunit I and the $Cu_A$ center is in subunit II.

Kinetic studies of electron transfer reactions of cytochrome oxidase have characterized the reactivity of the metal centers in different forms of the enzyme. A number of models outlining electron transfer properties have been advanced derived from transient-state optical spectroscopic studies. This paper will concentrate on models derived from studies of the single turnover of reduced oxidase with oxygen and will be considered in the light of new information

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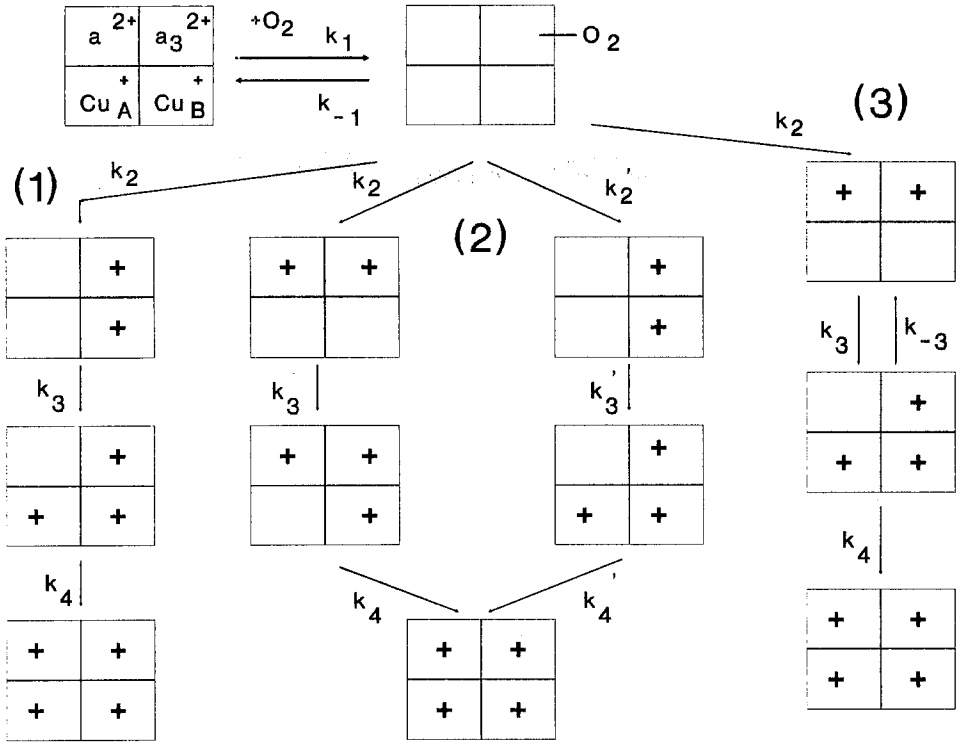


**Fig. 1.** Models for the reaction of reduced cytochrome oxidase with oxygen. The oxidase is represented as a box and each of the four redox active metals is allocated a compartment.

about the arrangement of the metal centers in the protein structure.

## KINETIC MODELS OF ELECTRON TRANSFER IN THE CYTOCHROME OXIDASE REACTION

Figure 1 outlines three schemes which have been used to account for the single-turnover reaction of fully reduced cytochrome oxidase with oxygen. The oxidase is depicted in these schemes as a box with a compartment for each of the four metal centers. All of these reaction sequences begin with the binding of oxygen to reduced oxidase via the binuclear center at cytochrome $a_3$ (Hill and Greenwood, 1983; Orii, 1984; Babcock *et al.*, 1985). There is also evidence that $Cu_B$ may be involved in this initial oxygen ligation reaction (Woodruff *et al.*, 1991; Blackmore *et al.*, 1991; Hill and Marmor, 1991; Oliveberg and Malmström, 1992). In the model outlined along path 1 the electron transfer reaction begins with two electrons transferred from the binuclear center to form a peroxy intermediate. This is followed by electron transfer from $Cu_A$ and then from cytochrome *a* (Greenwood and Gibson, 1967). The major shortcoming of this model is that the kinetic phases assigned to the individual centers give rise to kinetic spectra that do not correspond to the spectra of cytochrome *a* and cytochrome $a_3$ (Wrigglesworth *et al.*, 1988). In this model $Cu_A$ is capable of direct electron transfer to the binuclear center and cytochrome *a* is the site of electron entry from the reducing substrate ferrocytochrome *c*. Path 2 in Fig. 2 specifies a branched reaction that allows for two-electron transfer from either cytochrome $a_3$ and $Cu_B$ or cytochrome $a_3$ and cytochrome *a*. This model can account better for the spectral properties of the heme centers and also for the complex reactivity observed for the $Cu_A$ center, which is oxidized with a heterogeneous time course. This model specifies direct electron transfer from cytochrome *a* to the binuclear center (Hill and Greenwood, 1984), and this feature has now been confirmed in time-resolved resonance Raman experiments (Han *et al.*, 1990).

Path 3 is a linear reaction pathway which also specifies direct electron transfer from cytochrome *a* to the binuclear center, but allows electron transfer from $Cu_A$ to the binuclear center only via cytochrome *a*. In addition, electron input from ferrocytochrome *c* is specified to occur via $Cu_A$. The mediation of cyto-

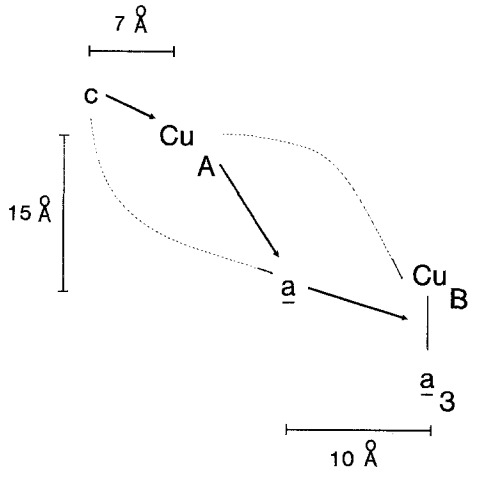


**Fig. 2.** Pathways of electron transfer from cytochrome *c* to the cytochrome $a_3$–$Cu_B$ binuclear center. The solid lines define the forward reactions outlined in path 3 of Fig. 1. The dotted lines show other possibilities discussed in the text.

chrome *a* in electron transfer to the binuclear center is an important feature of this model. The rate of electron transfer from cytochrome *a* to the binuclear center occurs at two different rates in the model outlined along path 3. It is this feature that leads to the heterogeneous oxidation kinetics seen in the single-turnover experiments for $Cu_A$ and cytochrome *c* in spite of the single electron transfer pathway available for their oxidation.

Table I shows the rates of electron transfer that have been measured for electron exchange between the metal centers of the cytochrome oxidase. The cytochrome *c* to $Cu_A$ rate is estimted from single-turnover studies of the noncovalent cytochrome *c*–cytochrome oxidase complex (Hill, 1991). The other slower rates for electron input from cytochrome *c* to the oxidase are for reaction with the resting, oxidized enzyme. The slower rate for electron exchange between cytochrome *c* and resting cytochrome oxidase may reflect an interaction with the binuclear center, and that this interaction limits the rate of electron input to the resting enzyme.

**Table I.** Reaction Rates for Electron Transfer between the Metal Centers of Cytochrome Oxidase

| Center to center | Rate ($s^{-1}$) | Reference |
|---|---|---|
| Cytochrome *c* to $Cu_A$ | $1 \times 10^5$ | Hill (1991) |
| | $1.5 \times 10^3$ | Pan *et al.* (1991) |
| | 800 | Antalis *et al.* (1982) |
| $Cu_A$ to cytochrome *a* | $7 \times 10^3$ | Hill (1991) |
| | $2.0 \times 10^4$ | Morgan *et al.* (1989) |
| Cytochrome *a* to cytochrome $a_3$-$Cu_B$ | $6 \times 10^4$ | Hill (1991) |
| | $2.5 \times 10^5$ | Oliveberg and Malmström (1991) |
| | $8 \times 10^2$ | Greenwood and Gibson (1967) |
| | 10 | Antonini *et al.* (1977) |
| | 1 | Greenwood and Gibson (1967) |

There is a good agreement for the rate of electron transfer between $Cu_A$ and cytochrome *a* from single-turnover oxidation experiments (Hill, 1991) and perturbed equilibrium experiments (Morgan *et al.*, 1989). Electron input by artificial donors first reduces $Cu_A$ and then internal electron transfer to cytochrome *a* is observed (Kobayashi *et al.*, 1989; Nilsson, 1992). The rate reported here is $2 \times 10^4$ $s^{-1}$ and slightly faster than the rates reported in the experiments cited above and in Table I. It is not known if this difference in rate is due to experimental variation or reflects kinetic control of the reducibility of cytochrome *a* which is dependent on the state of the binuclear center.

The widest variation seen for reported electron exchange rates is for the reaction between cytochrome *a* and the binuclear center. The fastest rate is from a relaxation experiment and thus involves no net electron transfer (Oliveberg and Malmström, 1991) and is about five times faster than the fastest rate reported from the single-turnover oxidation experiment which involves electron transfer to an oxygen adduct of the binuclear center (Hill, 1991). The rate of $800\,s^{-1}$ was originally assigned by Greenwood and Gibson (1967) as the oxidation rate of cytochrome *a* in the oxygen reaction of fully reduced oxidase. This rate is retained in the current model (i.e., path 3 of Fig.1) of the single-turnover reaction for the reoxidation of cytochrome *a* following the formation of the peroxy adduct at the binuclear center. The very slow rates of electron transfer from cytochrome *a* to the binuclear center are for reduction of the pulsed, oxidized enzyme (Antonini *et al.*, 1977) or resting, oxidized enzyme (Greenwood and Gibson, 1967).

## ARRANGEMENT OF ELECTRON CARRIERS IN THE PROTEIN MATRIX OF CYTOCHROME OXIDASE

Figure 2 outlines a scheme of the possible electron transfer pathways between the metal centers of

cytochrome oxidase. The solid lines in the scheme connect the centers in a linear electron transfer sequence that specify electron transfer to the binuclear center via cytochrome *a* and electron entry from cytochrome *c* via $Cu_A$. The broken lines in this scheme allow for electron transfer from $Cu_A$ directly to the binuclear center and from cytochrome *c* directly to cytochrome *a*. How does this kinetic picture fit what is known about the structural arrangement of the metal centers in cytochrome oxidase?

Holm *et al.* (1987) described a structural model of cytochrome oxidase which has served as a framework for interpretation of many subsequent experiments. One of the main features of their model is the subunit location of the four redox active metal centers; $Cu_A$ is located in subunit II, whereas cytochrome *a* and the binuclear center are all located in subunit I. The four ligands to $Cu_A$ are all contained in a highly conserved region of subunit II that includes a set of conserved acidic residues that act as the binding site for cytochrome *c*. In the noncovalent, cytochrome *c*–cytochrome oxidase complex the heme of cytochrome *c* would be located very close to $Cu_A$, consistent with the rapid electron exchange observed between these two centers. These features of subunit II are conserved in the sequences of cytochrome *c* oxidases from a variety of species (Saraste, 1990). In contrast, the recent sequence for the cytochrome $aa_3$-600 nm oxidase from *Bacillus subtilis* does not retain the $Cu_A$ ligands and it is not a cytochrome *c* oxidase (Santana *et al.*, 1992).

The predictions from the model of Holm *et al.* (1987) in regard to the conserved histidines of subunit I have recently been tested by the approach of site-directed mutagenesis (Shapleigh *et al.*, 1992). There are seven highly conserved histidine residues in subunit I that could act as inner-sphere ligands to cytochrome *a* (2 his), cytochrome $a_3$ (1 his) and $Cu_B$ (3 his). Shapleigh *et al.* (1992) produced mutants at each of these positions and examined the UV-visible and resonance Rama spectra of the mutated proteins to provide a modified model of the ligation state of these centers in subunit I. A picture of subunits I and II of the oxidase and cytochrome *c* is shown in Fig. 3 to highlight the relative location of the metal centers with respect to one another and the membrane bilayer. This model predicts heme-to-heme separation between cytochrome *a* and cytochrome $a_3$ of 8–10 Å, which is rather smaller than previously assumed (e.g., Gray and Malmström, 1989). Such a distance is consistent with rapid electron transfer observed between these two

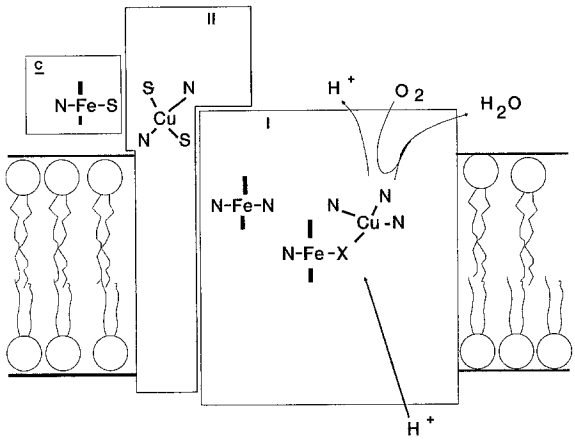


**Fig. 3.** The arrangement of the metal centers in the cytochrome *c*–cytochrome oxidase complex. Only subunits I and II, the core subunits, of the oxidase are shown.

centers in single-turnover and perturbed-equilibrium experiments.

The distance from cytochrome *c* to cytochrome *a* is difficult to predict from this model as the disposition of subunits I and II relative to one another is not known. However, energy transfer experiments using porphyrin cytochrome *c* estimate the distance from cytochrome *c* to cytochrome *a* at about 20 Å (Dockter *et al.*, 1978). This distance makes direct electron transfer from cytochrome *c* to cytochrome *a* unlikely and defines the role of $Cu_A$ as intermediary between cytochrome *c* and cytochrome *a*. Experiments with $Cu_A$-modified oxidase demonstrate a key role for $Cu_A$ in mediating electron transfer from cytochrome *c* to cytochrome *a* (Pan *et al.*, 1991). The distance from $Cu_A$ to cytochrome *a* is not known with certainty but spectroscopic measurements place the two centers about 15 Å apart (Brudvig *et al.*, 1984; Goodman and Leigh, 1985). This picture of the relative distance between the metal centers of the oxidase is consistent with the fastest reactivities observed for electron exchange between these centers (see Table I).

The distance from $Cu_A$ to the binuclear center probably approaches 20 Å and suggests that direct electron transfer between these centers would be much slower than that mediated by cytochrome *a*. Earlier views that $Cu_A$ could donate electrons to the binuclear center in a rapid reaction, independent from cytochrome *a* (Greenwood and Gibson, 1967), or that $Cu_A$ alone donates electrons directly to the binuclear center (Brzezinski and Malmström, 1987) are not supported in this current model.

## CONTROL OF ELECTRON TRANSFER RATES WITHIN CYTOCHROME OXIDASE

In this paper a model is advanced which details a linear path of electron transfer from cytochrome *c* to oxygen. $Cu_A$ is the primary site of electron acceptance from cytochrome *c*. The oxygen binding site is composed of the binuclear center: cytochrome $a_3$–$Cu_B$. Cytochrome *a* acts to bridge the electron receiving site and the oxygen binding site. This simple model can be shown to account for the complex kinetic behavior seen during the oxidation of the fully reduced enzyme with oxygen. In this model there are two different rates specified for the oxidation of cytochrome *a*, and neither of these measured rates are as fast as found in simple relaxation experiments. In addition, the rate of electron transfer in the oxidized enzyme is very slow. The rate of electron transfer from cytochrome *a* to the binuclear center is one which is highly sensitive to the enzyme's overall redox and structural status.

The Marcus theory of electron transfer (Marcus and Sutin, 1985) tells us the physical factors that determine electron transfer between a donor and acceptor pair. If we consider the intramolecular electron transfer from cytochrome *a* to the binuclear center in different states of cytochrome oxidase, we may consider if any of these factors account for the variation in rate seen for this process. Is there a change in distance between these two centers in different states of the oxidase? Tryptophan fluorescence measurements both in the steady-state (Ferreira-Rejabi and Hill, 1989) and time-resolved modes (Hill, Ferreira-Rejabi, and Sharma, unpublished results) indicate that there are no redox- or ligation-state-dependent, large-scale, conformational changes in the protein that would be expected to accompany such inter-site distance changes.

What about the free energy of the redox process? In the case of the relaxation experiment, electron transfer is between cytochrome *a* and cytochrome $a_3$ in a form of the protein in which there is no more than 100 mV difference in their redox potentials. One would expect this difference to increase when cytochrome $a_3$ is liganded with oxygen, in favor of cytochrome $a_3$ reduction, and it might reach a redox difference of as much as 0.8 V (Wikström and Morgan, 1992). Therefore, the forward reaction rate is expected to increase, but the rate of this step is observed to be slightly lower in the single-turnover reaction as compared to the relaxation experiment (see Table I).

The other rate-determining factor considered by the Marcus theory is from reorganizational terms: either of the inner-sphere ligands to the metal center or of the surrounding medium (i.e., protein or solvent). The fluorescence data cited above preclude any large-scale, redox-linked rearrangement in the protein's conformation. The slow rate of electron transfer to the binuclear center in resting cytochrome oxidase may arise from inner-sphere rearrangements about cytochrome $a_3$ (e.g., spin-state change of cytochrome $a_3$; Nicholls and Hildebrandt, 1978). The change in the rate of cytochrome *a* oxidation in the single-turnover experiment may arise from outer-sphere contributions to the reorganization energy. Gray and Malmström (1989) suggest that this reorganization is linked to the protein conformation and leads to limiting the electron transfer rate. There is evidence from both hydrostatic (Kornblatt *et al.*, 1988) and osmotic pressure (Kornblatt and Hui Bon Hoa, 1990) effects on the oxidase that rearrangement of water molecules is connected to electron transfer reactions and this might be the origin of the reorganization energy associated with electron transfer from cytochrome *a* to cytochrome $a_3$ in the step limited at a rate of $800\,s^{-1}$. I would like to suggest that this reorganization energy is found in highly localized protein and solvent rearrangements that are linked to the protonation of reduced oxygen and the proton binding and or dissociation required for the catalysis of transmembrane proton transfer. The electron transfer rates are gated by the chemical events of protonation and deprotonation reactions performed by cytochrome oxidase. Thus, the binuclear center is intimately involved in both the electronation and protonation reactions carried out by cytochrome oxidase. This site is demonstrated to exhibit great flexibility in its ligand-binding reactivity and these properties may suit it for a complex role in the energy conservation reaction catalyzed by cytochrome oxidase. The current structural view of cytochrome oxidase (see Fig. 3) which places cytochrome *a* in close proximity to the binuclear center could link solvent rearrangements to confined conformational changes to bring about changes in the intromolecular electron transfer rate from cytochrome *a* to cytochrome $a_3$. The trigger for these changes could be the redox state of $Cu_B$; when $Cu_B$ is oxidized, the electron transfer rate from cytochrome *a* is slow, whereas when $Cu_B$ is reduced, electron transfer from cytochrome *a* is ultrafast (i.e., faster than the known turnover of the enzyme).

This paper proposes a sequence for the involvement of the metal centers of cytochrome oxidase in the

transfer of electrons from cytochrome *c* to $O_2$. Information on the role of the protein in defining more precisely the electron transfer pathways in the oxidase should become available from electron transfer studies of site-directed mutants of bacterial cytochrome oxidases.

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