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# The kinetics of electron entry in cytochrome *c* oxidase

**Francesco Malatesta[1], Giovanni Antonini[1], Paolo Sarti[2], Beatrice Vallone[3], and Maurizio Brunori[3]**

[1] Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Universitá di Roma Tor Vergata, Rome, Italy
[2] Dipartimento di Scienze Biomediche, Tecnologia e di Biometria, Universitá dell'Aquila, L'Aquila, Italy
[3] Dipartimento di Scienze Biochimiche, Centro di Biologia Molecolare del CNR, Universitá di Roma La Sapienza, Rome, Italy

**Summary.** The kinetics of electron entry in beef heart cytochrome *c* oxidase have been studied by stopped-flow spectroscopy following chemical modification of the $Cu_A$ site with mercurials. In this derivative $Cu_A$ is no longer reducible by cytochrome *c* while cytochrome *a* may accept electrons from the latter with rates comparable to the native enzyme. The results indicate that $Cu_A$ is not the exclusive electron entry site in cytochrome *c* oxidase.

**Key words:** Electron transfer - Chemical modification - Cytochrome oxidase

## Introduction

Cytochrome *c* oxidase is among the most intriguing proteins known to date. This integral membrane protein catalyses the transfer of electrons between its primary substrate, i.e. cytochrome *c* and molecular oxygen, a process which consumes four protons/molecule dioxygen reduced to water (Brunori et al. 1988; Malmström 1989). These protons appear to be provided from the mitochondrial inner matrix space and are responsible for the build-up of a proton electrochemical potential gradient across the membrane. Apart from this 'scalar' process, cytochrome oxidase is capable of vectorial proton transport towards the cytosol coupled to the electron transfer processes (Wikström et al. 1981). The electron transfer processes are mediated by at least four redox-active metallic sites (Malatesta et al. 1990) called cytochrome *a* and a copper ion, $Cu_A$ (the so-called EPR-detectable copper), which appear to be responsible for redox interaction with cytochrome *c*, and the heterobinuclear cytochrome $a_3$-$Cu_B$ site made up of an otherwise identical heme A porphyrin in close proximity to a copper ion (0.5 nm). This EPR-silent bimetallic site accepts electrons transferred intramolecularly from cytochrome *a* and $Cu_A$ and rapidly transfers them to molecular oxygen (Palmer 1987).

One of many important aspects of cytochrome oxidase function is the modality of interaction of the protein with cytochrome *c*. It is not known with certainty if cytochrome *a* or $Cu_A$ or both are the electron input sites from cytochrome *c*. Yet this is a fundamental problem which may yield relevant information from the mechanistic point of view. Several studies designed to elucidate the mechanism of electron entry into cytochrome oxidase have been carried out over more than 20 years (Gibson et al. 1965; Andreasson et al. 1972; Andreasson 1975; Wilson et al. 1975; Greenwood et al. 1976). The major issue, which is actively debated, is the observation that more than one electron is transferred to cytochrome oxidase at early stages of the reaction with cytochrome *c*. This phase is referred to as the burst phase. What is still not clear is which prosthetic group is directly involved in this process: cytochrome *a* and/or $Cu_A$. In this report we describe stopped-flow experiments to elucidate the complex patterns of electron entry into cytochrome oxidase. By chemical modification of $Cu_A$, it is possible to exclude that this metal represents the exclusive site of electron transfer from cytochrome *c*.

## Materials and methods

Cytochrome *c* oxidase (EC 1.9.3.1) was prepared from fresh beef heart according to the Yonetani method (1960). The final pellet was dissolved in 0.1 M sodium potassium phosphate pH 7.4, containing 0.5% Tween 80, up to approximately 0.3 mM protein. Protein samples were frozen in liquid nitrogen and stored at −70° C. The protein was used either directly or within one month from preparation. Cytochrome oxidase was modified with sodium *p*-(hydroxymercuri)benzoate (pHMB; from Sigma) following Gelles and Chan (1985) and used immediately. Cytochrome *c* concentration was calculated using an absorption coefficient of 17 $mM^{-1}\,cm^{-1}$ at 550 nm (reduced-oxidized). Cytochrome oxidase concentration was determined by the pyridine hemochromogen assay (Morrison and Horie 1965) or by using an absorption coefficient at 605 nm of 11 $mM^{-1}\,cm^{-1}$ (reduced-oxidized). Stopped-

*Offprint requests to:* F. Malatesta, Dipartimento di Scienze Biochimiche, Universitá di Roma "La Sapienza", Piazzale Aldo Moro, 5, I-00185 Rome, Italy

flow experiments were performed by using a thermostatted Durrum-Gibson apparatus equipped with a 2-cm light path cell. All reagents were of analytical grade.

## Results

In this work we have investigated the kinetic properties of pHMB-modified cytochrome oxidase. In this derivative, in which the cysteine ligand(s) to $Cu_A$ have been modified (Gelles and Chan 1985), the symmetry of the metallic site has drastically changed to a type-II copper site (Peisach and Blumberg 1974). At variance with the conclusions of Gelles and Chan (1985), who suggested a rearrangement of the $Cu_A$ center following the treatment, we propose that chemical modification of $Cu_A$ by pHMB induces a displacement of copper from the native site to the protein surface. This is based on the following lines of evidence: (a) Addition of $CuSO_4$ to native cytochrome oxidase (Beinert and Palmer 1965) generates an EPR spectrum virtually identical to the EPR spectrum of the pHMB-modified oxidase. (b) Denaturation of cytochrome oxidase with urea (Beinert and Palmer 1965) and/or sodium dodecyl sulphate (Malatesta et al. 1990) also yields this spectrum, albeit with double intensity (i.e. 2 mol copper/mol enzyme, due to the appearance of both $Cu_A$ and $Cu_B$, the latter being EPR-silent in the native enzyme). (c) The very same EPR spectrum is obtained by treatment of cytochrome oxidase with $\alpha$-chymotrypsin (unpublished experiments from this laboratory). (d) The modified copper is easily removed by treatment with EDTA with loss of the EPR signal (Li et al. 1987). (e) The redox potential of the modified copper is so much lowered as to be non-reducible by ferrocytochrome *c* (Beinert and Palmer 1965; Gelles and Chan 1985). A detailed study on the pitfalls of the preparation of this derivative and its spectroscopic characterization will be presented elsewhere.

These observations prompted us to investigate the initial events of electron transfer from cytochrome *c* to pHMB-modified cytochrome oxidase, since in this protein the only electron acceptor available for interaction with substrate is cytochrome *a*. The dependence of the amount of cytochrome *c* oxidized is shown in Fig. 1, normalized to the oxidase functional unit concentration, on the actual ferrocytochrome *c* concentration for native and pHMB-modified cytochrome oxidase fully saturated with sodium cyanide (to keep the binuclear center in the oxidized state). It is clear from this stopped-flow experiment that, in the case of the pHMB-modified enzyme, the amount of cytochrome *c* oxidized/functional unit is lower than the value of 2 expected and observed for the control enzyme (Wilson et al. 1975). The actual value obtained for the modified enzyme is 1.3, indicating that the removal (physical and/or functional) of $Cu_A$ noticeably decreases the stoichiometry of electron entry. The stoichiometry, however, does not achieve the value of 1 electron/functional unit as expected if modification were complete; the residual amount of protein which has not been modified in this experiment is, on average, 20%–30%.

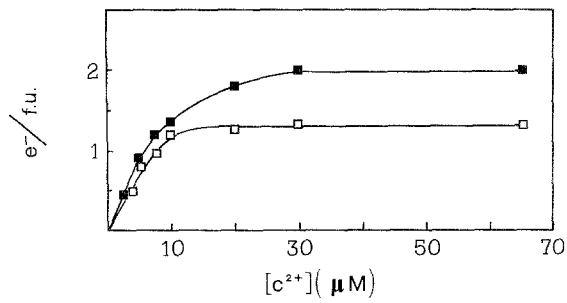


**Fig. 1.** Stoichiometry of the early electron transfer events in cytochrome oxidase. Native (■) or pHMB-modified (□) cytochrome oxidase (5 μM), complexed with 5 mM sodium cyanide, was mixed in the stopped-flow apparatus against the indicated amounts of ferrocytochrome *c* (prepared by reduction with sodium dithionite and passage through a Sephadex G-25 column to remove the excess reductant). The process was followed at 550 nm. The buffer was 0.2 M sodium potassium phosphate pH 7.0 containing 0.5% Tween 80 and the temperature 7° C. Concentrations are before mixing. e-/f.u. is electrons transferred/functional unit

The time course of cytochrome *c* oxidation and cytochrome *a* reduction are comparable for the native and the modified enzyme (not shown), indicating that cytochrome *a*, the only electron acceptor available in the modified enzyme, is fully competent in electron transfer.

## Discussion

At early stages of the reaction between ferrocytochrome *c* and native cytochrome oxidase more than one electron acceptor is being reduced. This is most clearly demonstrated by constructing plots of absorbance changes at 550 nm (representing oxidation of cytochrome *c*) versus those at 605 nm (representing reduction of cytochrome *a*) at identical times. Although these plots are totally dependent on the absorption coefficients used (and especially so for the pHMB-modified enzyme), if only one electron acceptor were present in the oxidase then a plot of the amount of cytochrome *c* oxidized/unit time versus the amount of cytochrome *a* reduced/unit time would yield a straight line with slope equal to 1. Instead, lines with slopes higher than 1 are found (typically 1.4–1.6, at substoichiometric cytochrome *c* concentrations whereas under saturating conditions the plot is not linear) indicating that more than one cytochrome *c* molecule is oxidized in the burst phase (Gibson et al. 1965; Wilson et al. 1975). This finding was the first to indicate that more than one electron acceptor, i.e. cytochrome *a* and $Cu_A$, is available in cytochrome oxidase for direct or indirect interaction with cytochrome *c*. It must be stressed that the 'excess' cytochrome *c* oxidized is not due to electron transfer to the oxygen-binding site, since in these experiments either the dioxygen concentration was very low (Gibson et al. 1965) or the binuclear site was blocked in the oxidized state by cyanide (Wilson et al. 1975 and present paper) or in the reduced state by carbon mon-

oxide (when the mixed valence oxidase was used) (Greenwood et al. 1976). Thus, any model of electron entry into cytochrome oxidase must take into account that, during the burst phase of the reaction, two electrons are involved (however see Andreasson et al. 1972 and Andreasson 1975).

To explain these phenomena one must decide among at least four different kinetic models shown in Fig. 2. Model A represents the situation in which cytochrome *c* directly binds and transfers electrons to cytochrome *a*, which therefore represents the only electron entry site in cytochrome oxidase. This model, where cytochrome *a* is in redox equilibrium (intramolecular) with $Cu_A$, is supported by the results of temperature-jump experiments carried out by Greenwood et al. (1976). Model B describes the symmetrical case with respect to model A, in which however $Cu_A$ is the only electron entry site. Finally, C and D are additional possibilities obtained by combining the former models.

Analysis of these models in terms of the differential equations which describe the transformation rates of each species yields a common result:

$$d[c^{3+}]/dt = d[a^{2+}]/dt + d[Cu_A^+]/dt. \quad (1)$$

This equation states that the rate of oxidation of ferrocytochrome *c* is equal to the sum of rates of formation of reduced cytochrome *a* and $Cu_A$. In so far as two electron acceptors are involved in the burst phase of the reaction, this equation is mechanism-independent. The stoichiometry implies that, at any time, the total amount of ferricytochrome *c* formed (free or bound to cytochrome oxidase) is exactly balanced by the amounts of reduced cytochrome *a* and $Cu_A$. Thus, a plot of cytochrome *c* oxidized versus cytochrome *a* reduced will not, in general, yield a slope equal to unity as stated above. The only assumption is that the off-rates of cytochrome *c* (ferrous and ferric) are never rate-limiting (i.e. these steps are not formally included in the mechanisms). This is a reasonable assumption since, firstly, in the burst phase two electrons are transferred to the protein with bimolecular rate constants in excess of $10^8\ M^{-1}s^{-1}$ at low ionic strength and room temperature. Unless two independent electron acceptors are present in the oxidase (see Fig. 2, models C and D), this result (i.e. two electrons involved) is difficult to reconcile with a rate-limiting cytochrome *c* off-rate. Secondly, the oxidation of ferrocytochrome *c* under steady-state conditions always follows an exponential (first-order) time course at all cytochrome *c* concentrations; as discussed by Robinson et al. (1985), if the off-rate were rate-limiting, a zero-order process would be expected. Last, at very high cytochrome *c* concentrations no rate limit is detected.

Proving one of the four models shown in Fig. 2 is not a trivial matter. Theoretically all that is needed to be known is the dependencies of the rates on the right-hand side of Eq. (1) on cytochrome *c* concentration. Clearly, $d[a^{2+}]/dt$ is a function of cytochrome *c* concentration and the dependence on the latter is always bimolecular (Gibson et al. 1965; see Brunori et al. 1988 for a complete discussion) even at very high cytochrome *c* concentrations. The experiments reported in this paper, carried out using a chemically modified derivative of cytochrome oxidase in which the $Cu_A$ site is redox-inactive exclude that this is the only electron entry site (see Fig. 1). Determining, on the other hand, the properties of $d[Cu_A^+]/dt$ is difficult since the only optical signal attributed to $Cu_A$ (apart from EPR) is a near-infrared low-absorption charge-transfer band centered at 830 nm. The oxidized-minus-reduced absorption coefficient is of the order of 1–2 $mM^{-1}cm^{-1}$ and possible contributions of the hemes have not been ruled out definitively. Temperature-jump experiments (Greenwood et al. 1976) suggested that electron transfer between cytochrome *a* and $Cu_A$ is intramolecular, approaching a rate limit of about $100\ s^{-1}$ at high cytochrome *c* concentrations, which is nevertheless predicted by their proposed model (model A in Fig. 2).

Chemical modification is a very important tool to probe structure and mostly function. Numerous spectroscopic and biochemical investigations have proposed that among the ligands to the copper ion in the $Cu_A$ site of cytochrome oxidase is at least one cysteine residue, which is most likely located in the carboxy-terminal portion of subunit II, in a sequence of residues very similar to that characteristic of known copper proteins (Brunori et al. 1988). Treatment of cytochrome oxidase with pHMB modifies these cysteines and generates a derivative in which $Cu_A$ is no longer capable of accepting electrons from cytochrome *c*. Alternative procedures to modify and remove $Cu_A$ are now available (Hall et al. 1988). These procedures are of crucial importance for comparative purposes and assessment of the quality controls in the pHMB modification procedure. Kinetic experiments with reduced substrate have shown that in pHMB-modified cytochrome oxidase the overall mechanism of electron entry is unchanged. Thus cytochrome *a* may represent the true electron entry site in cytochrome oxidase.

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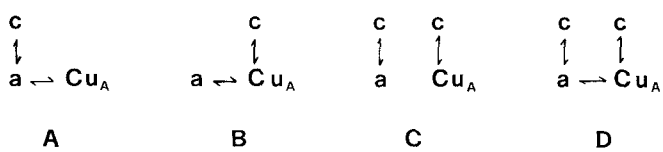


**Fig. 2.** Models for electron entry in cytochrome oxidase. See text for explanation and details

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