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Microsecond Laser Flash Photolysis of the CO-Complex of Bovine Heart Cytochrome c Oxidase

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ABSTRACT: Bovine heart cytochrome c oxidase was prepared according to the method of Yonetani (1961), the CO-complex of the fully reduced and mixed valence enzyme was prepared as described by Bicker *et al.* (1984). Kinetics of CO rebinding subsequent to Laser photolysis was studied at 430nm by using a 20ns pulsed at 532nm vertically polarized Laser beam of 2mJ energy. The results suggest differences in photodynamic behaviour of the fully reduced and mixed valence forms of the enzyme. The most important implication of this, is at the conformational changes associated with the reduction of the low spin cytochrome a affects the ligation dynamic of the oxygen reduction site via heterotropic interaction.

INTRODUCTION

Cytochrome c oxidase (E.C. 1.9.3.1) is a multisubunit enzyme which couples the oxidation of ferrocytochrome c and the reduction of dioxygen to electrogenic separation of protons in the inner mitochondrial membrane (Wikstrom, 1977). The enzyme is made up of two redox centres, the cytochrome a/CuA and cytochrome a3/CuB centres. The cytochrome a3/CuB binuclear centre constitutes the site of linkage of dioxygen reduction to the proton translocation function of the enzyme (Rich, 1995). CuA is situated on the cytoplasmic side of the inner mitochondrial membrane in close proximity to the docking site of ferrocytochrome c and, therefore, provides the electron entry port into the enzyme. Cytochromes c and a are, however, known to be in rapid redox equilibrium. Electron transfer from cytochrome a to the cytochrome a_3/Cu_B binuclear cluster is generally believed to be the repository of the rate determining step in the cascaded catalytic cycle of the enzyme (Brunori *et al.*, 1994).

Cytochrome a is low spin, hexacoordinate and bisligated to histidines 102 and 421 of subunit 1 in the two axial positions, while cytochrome a_3 is high spin and pentacoordinate with histidine 419 of the same subunit as the ligand in the proximal position (Hosler *et al.*, 1993). The sixth axial position is free and constitutes the site of binding and subsequent reduction of dioxygen to water. Other ligands, such as CN and CO, are also capable of binding to the sixth axial coordination position. The binding of these ligands is inhibitory and, therefore, useful in the study of the kinetics and thermodynamics of ligand to the binuclear centre due to the absence of concomitant reactions. Coupled with this is that the binding of these ligands is associated with measurable spectroscopic signatures which provide easy and convenient monitoring of binding events (Yonetani, 1960).

The two redox centres in the enzyme are, however, known to be engaged in long-range heterotropic interaction, such that the value of an observable for one depends on the chemistry of the other. It has been known, for example, that despite a separation of about 19A, the reduction potential of the two cytochromes are interdependent in an anticooperative manner. Similarly, Greenwood *et al.* (1974) have demonstrated that the rate of inhibitory binding of CO to the binuclear centre depends on the redox

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state of the cytochrome a_3/Cu_A centre. On the other hand, it has been shown that the ligation dynamics of the distal pocket of cytochrome a3, subsequent to CO photodissociation also exhibited some dependence on the redox state of cytochrome a (Lou *et al.*, 1993).

However, although considerable work has been done on the kinetics of CO rebinding subsequent to photolysis (Lou *et al.*, 1993) in fully reduced (a^{2+} a_3^{2+}- CO) and mixed valence cytochrome c (a^{2+} a_3^{3+}-CO) oxidase, there is a continuing interest in this process because of the intriguing complexity of the process and the importance of the binuclear centre. The aim of this work is to probe further into the kinetic determinants of the rate of CO rebinding in fully reduced and mixed valence cytochrome c oxidase with a view to shedding more light into the subtle differences in these two versions of this enzyme of immense functional significance.

MATERIALS AND METHODS

Bovine heart cytochrome c oxidase was prepared according to the method of Yonetani (1961), the final pellet was dissolved in 20 µM phosphate buffer, pH 7.4, containing 2% sodium cholate. The concentration of haem a is determined from the difference in absorbance at 605nm using an extinction coefficient of 11 um-1cm-1 (Brunori *et al.*, 1983). The enzyme (4 µM total haem) was taken into two separate cuvettes and subjected to alternating cycles of vacuum and CO gas and sealed under oxygen-free CO. To one of the cuvettes was added 50 µl of 1 M solution of the reductant, sodium dithionite. The two cuvettes were kept on the bench for 12 hours at 20°C. The formation of the fully reduced and the mixed valence forms of the enzyme was confirmed by optical spectroscopy between 400 and 600nm prior to photolysis measurement.

Laser flash photolysis studies were carried out by photolysing the cytochrome a_3-CO complex of the two enzyme forms using a 20ns pulse of 532nm vertically polarized laser beam of 2mJ energy. The time-resolved absorbance change due to the reformation of the cytochrome a_3-CO complex were measured in the dark at 430nm. The flash repetition rate was 11 s-1. The photomultiplier output were digitized to 20 µs per point and 100 points were recorded in the two channels of Datalab 102 signal averager. Each experiment averaged 512 flashes and were repeated three times. If these gave coincident time-resolved absorbance change, they were averaged together. The resulting time-resolved absorbance changes were fitted to a single or double exponential of the forms:

$$A = Ao \exp(-t/\tau)$$

or

$$A = A1\exp(-t/\tau_1) + A2\exp(-t/\tau_2)$$

using non-linear least squares interative procedure, where τ's represent the lifetime, i.e. reciprocal of the pseudo first rate constant.

RESULTS AND DISCUSSION

Figure 1 illustrates typical absorption spectra for fully reduced and mixed valence CO complex of cytochrome c oxidase. The spectrum of the fully CO complex exhibited a peak at 430nm, attributable to the CO-complex of cytochrome a3, and a shoulder of 445nm representing the absorbance contribution of reduced cytochrome a which is not receptive to exogenous ligans (Yonetani, 1960). The spectrum of the mixed valence CO derivative of the enzyme exhibited a single peak centred around 430nm which is composite, due to both the CO-bound reduced cytochrome a3 and the oxidized histidine bis-ligated cytochrome a (Abubakar, 1994). These spectral signatures are, in general, in agreement with those reported in the literature (Bickar *et al.*, 1984). In addition, the enzyme preparation was found to be catalytically competent in respect to the oxidation of ferrocytochrome c and the reduction of dioxygen (Abubakar, 1994).

A typical semilogarithmic plot of the time resolved absorbance change subsequent to laser flash photolysis is shown in Figure 2. The time courses were characterized by a fast phase which is resolved from a slower phase. These phases are, however, less resolved in fully reduced CO-bound enzyme at temperatures above 286K. The second order rate constants, at 286K, for the slow and fast phases of the fully reduced and mixed valence versions were determined to be 1.09×10^5 and 1.05×10^5 $M^{-1}S^{-1}$, and 9.56×10^6 and 7.56×10^6 $M^{-1}S^{-1}$, respectively. These values are, however, considerably higher than the values of the order of 5×10^4 $M^{-1}S^{-1}$ for the monoexponential decay observed by Greenwood *et al.* (1974) in the study of the mixed valence and fully reduced forms of the enzyme using ordinary, isotropic, flash photolysis.

The second order rate constants for the slow phases of the fully reduced, in which both the cytochromes are reduced and cytochrome a_3 bound to CO, and mixed valence version of the enzyme, in which only cytochrome a_3 is reduced and CO-bound, exhibited some slight but reproducible difference in agreement with the result of Greenwood *et al.* (1974). In addition, however, the present work demonstrates that this difference is temperature dependent (Table 1), the difference being higher as the temperature was increased. However, both the rate constants exhibited Arrhenius temperature dependence (Figure 3).

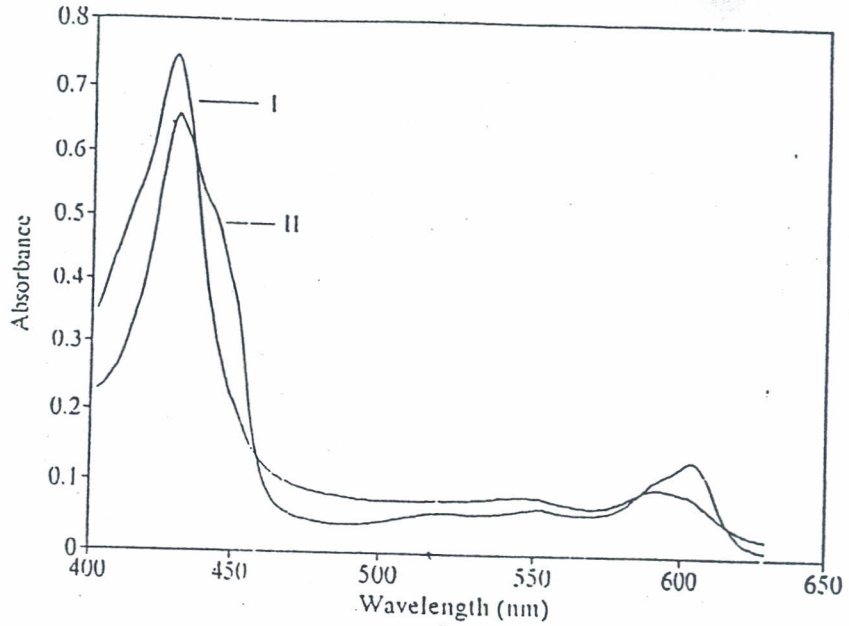

Figure 1: Absorption spectra of mixed valence-CO (trace) and fully reduced-CO (trace II) cytochrome c oxidase (4 μM). The spectra were recorded at 20°C in 10 mM HEPES-KOH buffer, pH 7.4 containing 2% sodium cholate.

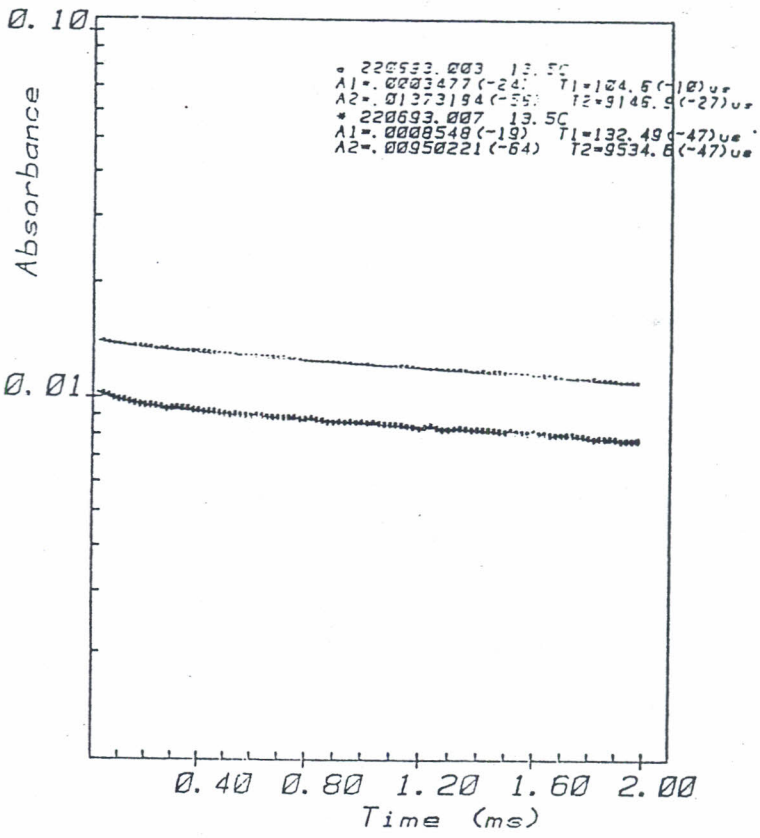

Figure 2: A representative figure of the semilogarithmic plots of time-resolved absorbance change in the laser flash photolysis of CO mixed valence (red coloured trace) and fully reduced (green coloured trace) cytochrome c oxidase. Inset are the changes in absorbance and the life-times for the fast (A1 and T1) and slow (A2 and T2) processes. See materials and methods for details.

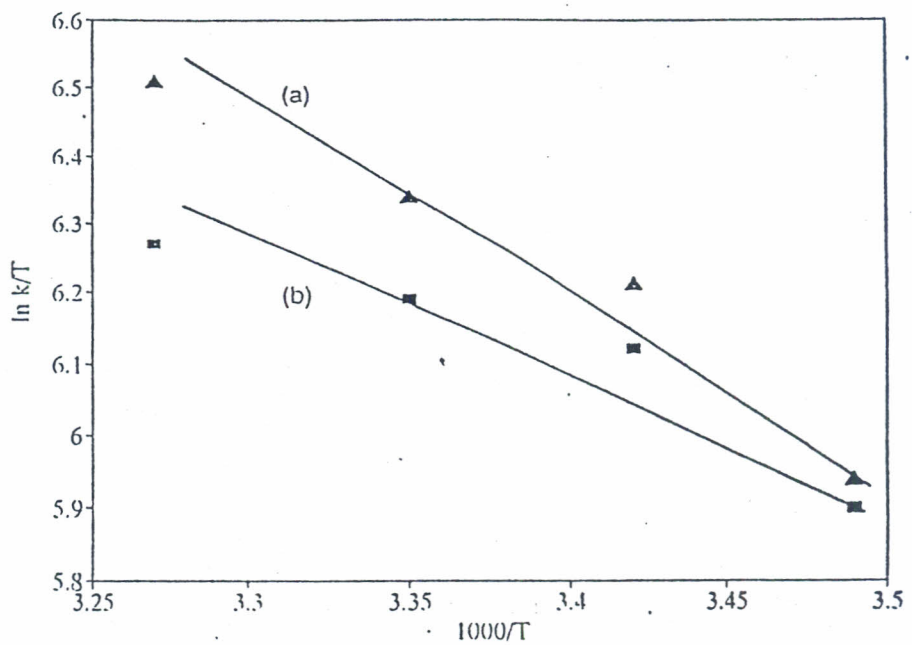

Figure 3: Arrhenius temperature dependence of the second order rate constant of the slow phase of CO recombination of fully reduced (a) and mixed valence (b) cytochrome c oxidase.

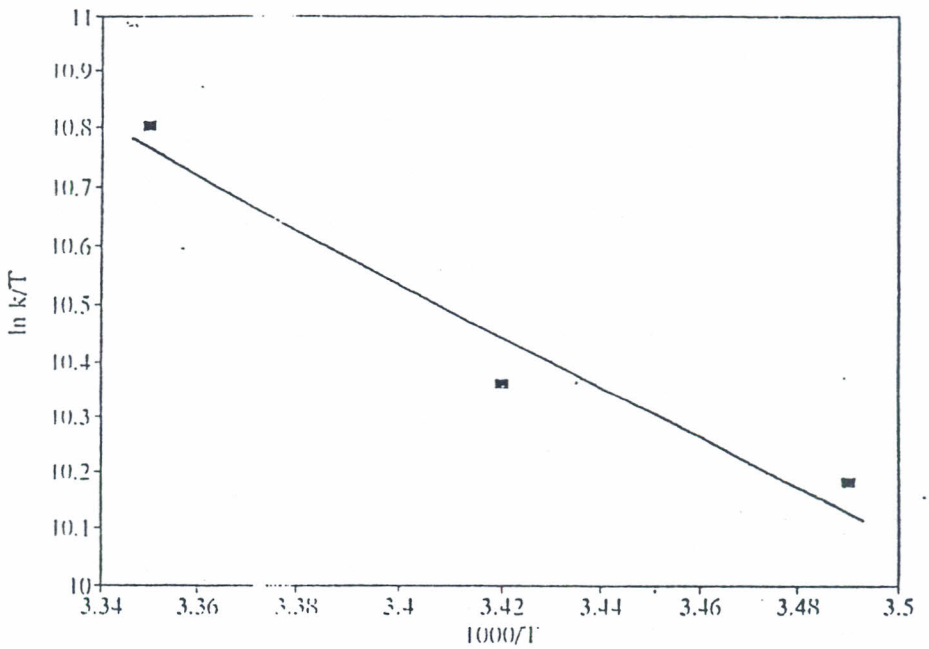

Figure 4: Arrhenius temperature dependence of the second order rate constant of the fast phases of CO recombination to the mixed valence cytochrome oxidase.

Table 1: Summary of life-time (slow phase) for CO rebinding to mixed-valence and fully reduced cytochrome c oxidase at the different temperatures studied.

Temperature °C	Life-Time τmv	(τ,us) FR	τ(FR)/ τ (mv)
13.50	9533	9145	0.96
19.60	7544	6843	0.91
25.50	6894	6004	0.87
32.50	6222	4868	0.78

The activation parameters ΔS^* and ΔH^*, were estimated using the Arrhenius equation of the form:

$$\ln k/T = \ln BT/h + \Delta S^*/R - \Delta H^*/RT \ldots\ldots (1)$$

where k is the rate constant, T, the absolute temperature, B, the Boltzman constant, h, the Plack's constant, R, the Universal gas constant and, ΔS^* and ΔH^* are the entropy and enthalpy changes of activation respectively.

The reaction enthalpies for the slow phases were determined to be 23.3KJ/mole and 19.4KJ/mole with entropies of activation of -67J/mole.K and -88J/mole.K respectively. These values correspond to activation free energies of 42.2KJ/mole and 43.6KJ/mole at 298K for the fully reduced and the mixed valence forms respectively. The activation enthalpy and entropy for the fast phase of the mixed valence form (Figure 4) were found to be 33.3KJ/mole and 3J/mole.K respectively, corresponding to activation free energy of 32KJ/mole at 298K. The free energy of activation for the fast phase of the fully reduced enzyme was estimated from its second order rate constant at 286K to be 31.8KJ/mole using equation (1).

Figure 5 illustrates the semilogarithmic plot of the ratio of the lifetime (reciprocal pseudo-first order rate constant at 1 mM CO), for the slow phases of the fully reduced and mixed valence enzymes, against reciprocal of absolute temperature. The linear plot is described by the equation.

$$\text{Log}\ \frac{\tau(FF)}{\tau(mv)} = \text{Log}\ \frac{X(mv)}{X(fr)} + \frac{\Delta G^+}{2.303RT} \ldots\ldots (2)$$

where τ's are the life-times and X's the transmission coefficients and AG+ the difference is free energy of activation for CO rebinding between the fully reduced and mixed valence forms. The difference in free energy and the ratio of the transmission coefficients were determined to be 1.01KJ/mole and 0.77 from the slope and the intercept of the plot respectively.

This work demonstrates that the kinetics of CO-rebinding, subsequent to laser photolysis, in fully reduced and mixed valence CO derivative of bovine heart cytochrome c oxidase, proceed through two disntict phases characterized by different rate constants and activation parameters. THis indicates that the bimuclear cytochrome a3/CuB centre, in both the enzyme forms, exhibit kinetic heterogeneity. The underlying basis of this heterogeneity, though largely unknown, is fully documented and is generally believed to be due to the different coordination and configurational states which can be assumed by the binuclear centre (Wilson *et al.*, 1994 and Woodruff, 1993). Most importantly, however, the results in this work, in conjunction with the report of other workers (Lou *et al.*, 1993), clearly demonstrate long range co-factor interaction in cytochrome c oxidase in the sense that the ligand binding kinetics of the oxygen reduction site, the binuclear centre, depends on the redox state of cytochrome a. Differences were observed in both rates and activation parameters of CO recombination, reflecting a difference in the structural evolution of the distal pocket of cytochrome a subsequent to photlysis, which is shown to be determined by the redox state of cytochrome a. The most important implication of this is that the conformational change associated with the reduction of cytochrome a affects the ligation dynamics of the oxygen reduction centre via heterotropic interaction.

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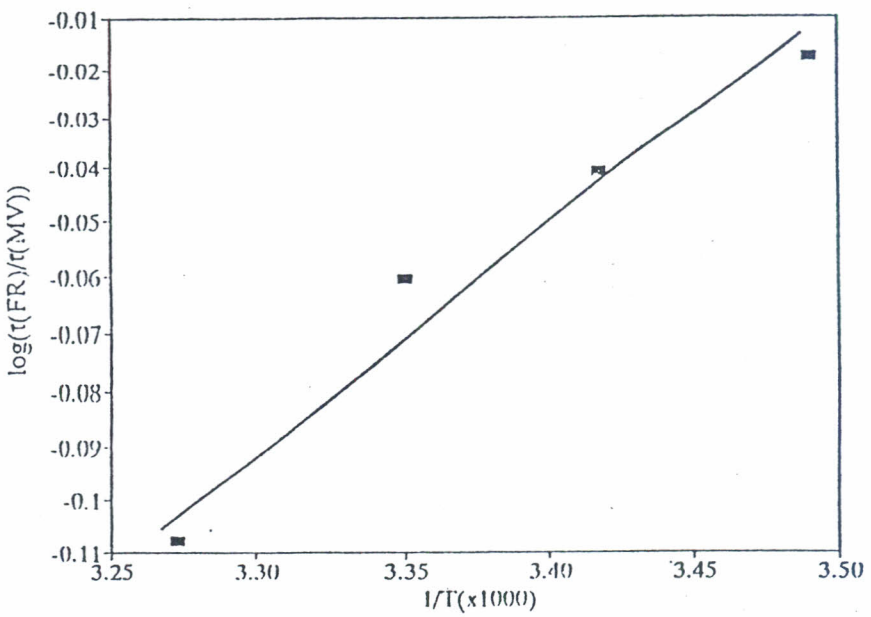

Figure 5: Semilogarithmic plot of the ratio of the lifetimes of CO recombination of the slow phase for ful reduced to mixed valence enzyme against reciprocal absolute temperature. See materials and methods fo experimental details.