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Review

Intermediate forms of cytochrome oxidase observed in transient kinetic experiments and those visited in the catalytic cycle

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Abstract

The cytochrome oxidase family of heme-copper oxidases has been the subject of intense kinetic and mechanistic enquiry. Much of this work has focussed on transient kinetic studies of the partial reactions of the enzyme with the goal being to build a kinetic model describing the catalytic cycle that the enzyme undergoes to direct the oxidation of substrate, reduction of oxygen and vectorial proton transfer. A key aspect of such a model is to define the structures of each of the intermediate forms the enzyme takes up as it traverses the catalytic cycle. One complication that has been prevalent with mitochondrial cytochrome *c* oxidase is the existence of structural variants of the enzyme, as isolated, that may not be participants in catalysis. Studies of structurally simpler procaryotic members of the family may offer new insight on the intermediates of catalysis. In this paper transient-state and steady-state kinetic studies of cytochrome aa_3-600 from *Bacillus subtilis* are integrated into a model of the catalytic cycle. This model specifies that the P intermediate accumulates in the steady-state and it is proposed that the step following its formation is limited by proton uptake.

Keywords: Cytochrome oxidase; Transient kinetics; Steady state kinetics; Oxygen reaction

1. Introduction

Studies on the family of cytochrome oxidase enzymes include work on the protein from a diverse array of biological sources ranging from mammalian mitochondria to bacterial plasma membranes. It is very useful that high-resolution structures of the enzyme from bovine heart mitochondria [1] and the plasma membrane of *Paracoccus denitrificans* [2] became available at virtually the same time. The most highly conserved structural elements across the members of the cytochrome oxidase enzyme family reside in subunit I, the largest subunit. Cytochrome *a* is composed of a heme A moiety ligated to the protein via two coordination linkages to two axially positioned histidine residues. The binuclear center is composed of cytochrome a_3 and Cu_B groups that are positioned at a distance of about 5 Å from one another. In the cytochrome a_3 center the heme A group is ligated by a single histidine and the sixth position is available for interaction with the substrate oxygen or inhibitory ligands such as cyanide. The Cu_B center is ligated by three histidine residues. One of these histidines is structurally distinct in that it has been physically fused with the side chain of a tyrosine residue [3] and this group is proposed to be redox active [4]. In the subgroup of the family that uses cytochrome *c* as the reductive substrate, the cytochrome *c* oxidases, there is an additional redox active metal center located in subunit II. This center is composed of a tightly coupled pair of copper ions that function together as a one-electron center. This center is known as Cu_A and it functions to receive electrons from cytochrome *c* and pass them onto cytochrome *a* [5]. In quinol oxidases, such as the cytochrome aa_3-600 from *Bacillus subtilis*, this center is missing and the initial electron acceptor from the quinol substrate is proposed to be cytochrome *a* (see Fig. 1).

The kinetic characteristics of cytochrome oxidases have been studied with a view to describing the interrelationships between proton and electron transfer processes, and in terms of a molecular mechanism of enzyme activity. Kinetic studies of electron transfer have been performed to look at steady-state properties of the oxidase (e.g., Ref. [6]), whereas transient-state experiments have looked at the kinetics of electron input to the oxidized enzyme [7,8]

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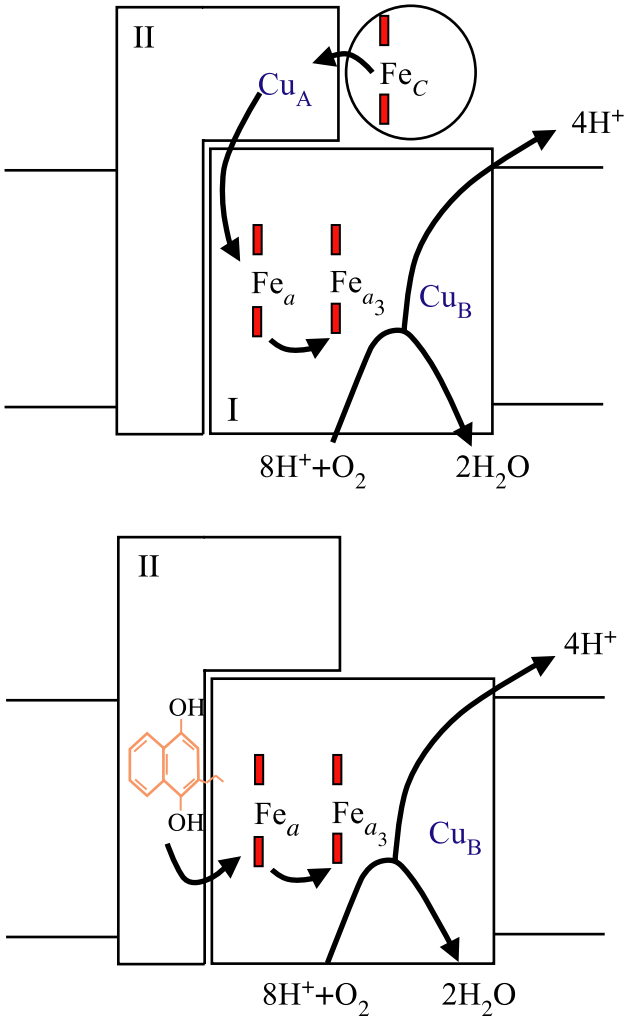

Fig. 1. Schematic diagram of the two-subunit forms of (A) cytochrome *c* oxidase and (B) menaquinol oxidase or cytochrome aa_3-600. The electron transfer sequence within cytochrome *c* oxidase from cytochrome *c* to Cu_A, to cytochrome *a* and then to cytochrome a_3-Cu_B is indicated. In (B) the electron transfer sequence is from menaquinol to cytochrome *a* and then onto the cytochrome a_3-Cu_B group.

and electron output from the reduced enzyme [9]. In the context of transient kinetic studies of cytochrome *c* oxidase, emphasis has been placed on the reactions of the reduced enzyme with molecular oxygen. One of the rationales for this emphasis is that the observed rates of reactivity of the enzyme with oxygen equal or exceed the maximal turnover number ($\sim$ 1000 s^{-1} for bovine mitochondrial cytochrome *c* oxidase).

My own participation in this work began as a graduate student in Colin Greenwood's lab at the University of East Anglia and continues in my own lab today. Colin Greenwood and I were looking for kinetic and spectroscopic evidence for the existence of compound A, or the oxygen complex with ferrocytochrome a_3, that was proposed from low temperature studies by Chance et al. [10]. We concluded that we had found evidence of compound A in the transient reaction of partially reduced, or mixed-valence, cytochrome *c* oxidase with oxygen [11]. Our interpretation of the data met with some scepticism from the reviewers of our initial manuscript on the basis that the lifetime of this species would be so short as to make it impossible to observe in the reaction and, in any case, simple chemical logic argued against the idea of a discrete complex in which a dioxygen molecule would sit on a reduced heme center without electron transfer ensuing immediately. This was some of the context of my first encounter with Jerry Babcock. I was bringing our studies on the oxygen reaction of the oxidase on a trans-Atlantic trip to a meeting of the American Society of Biological Chemistry in San Francisco. We had an occasion at that meeting to discuss our results on the transient intermediates of the oxidase observed during reaction with oxygen, and Jerry was very quick to lend support for our work from his own transient Raman studies [12]. This was the beginning of a synergistic interplay between transient Raman and transient absorption spectroscopies in mapping spectroscopic, structural and kinetic features of the intermediates in the reaction of cytochrome *c* oxidase with oxygen. Jerry played a very important role in facilitating discussions between the different groups working in this area, providing key insights to interpret data and devising incisive new experiments. As a young scientist, I was very deeply impressed with Jerry's clarity of thought and generosity of spirit. And I am certain Jerry left this mark on many impressionable young scientists, and perhaps some older impressionable ones as well.

In recent years, mechanistic attention has shifted from the inherently complex mitochondrial cytochrome *c* oxidase to its simpler cousins that are found in a range of procaryotic organisms. High-resolution structures have been reported for both four-subunit and two-subunit forms of cytochrome *c* oxidase from *P. denitrificans* [2,13], wild-type and mutant forms of cytochrome *c* oxidase from *Rhodobacter sphaeroides* [14] and ubiquinol oxidase from *Escherichia coli* [15]. Recombinant DNA methodologies have allowed for the generation of many different molecular forms for detailed structural–functional analysis (e.g., Ref. 16). We have been looking at the highly aerobic bacterium *B. subtilis* and studying two native versions of the cytochrome oxidase molecule found in wild-type *Bacillus*. The main subject of this mini-review is the menaquinol oxidase, or cytochrome aa_3-600, and the particular problem that will be addressed is the relationship between the intermediates observed in transient state experiments and those that are populated in the steady-state catalytic cycle. As with other quinol oxidases of the heme-copper family, cytochrome aa_3-600 oxidizes a lipid-soluble quinol substrate and not cytochrome *c* (see Fig. 1). Cytochrome aa_3-600 does not have the dinuclear copper center, CuA, that is found in subunit II of the cytochrome *c* oxidases.

2. Reaction of fully reduced menaquinol oxidase (cytochrome aa_3-600) with O_2

Fully reduced cytochrome aa_3-600 contains three reducing equivalents, one each in cytochrome *a*, cytochrome a_3 and Cu_B. There does not appear to be a tightly bound quinone or semiquinone in our purified preparations as is observed with ubiquinol oxidase (cytochrome bo_3) from *E. coli* [17]. The reaction of fully reduced cytochrome aa_3-600 with O_2 has been studied by the method of flow-flash photolysis in which fully reduced, ligand-free enzyme is generated from the corresponding CO derivative by flash-photolysis. Fig. 2 shows the time course at two different wavelengths and three O_2 concentrations. At 590 nm (panel A) the trace begins with an immediate decline in absorption due to a small interference from the laser flash and photolysis of the CO adduct. There follows a phase of increasing absorption that becomes more prominent as the O_2 concentration is increased. The absorption then declines at a rate that increases as the O_2 concentration is raised. At 600 nm, photolysis of the CO adduct leads to an immediate increase in absorption and then a decline. The reaction traces can be modelled as a two-step process, following the photolytic removal of CO, that is assigned to formation and decay of the O_2 adduct of ferrocytochrome a_3. The decay of the oxygen adduct results from rapid electron transfer from cytochrome *a* and cytochrome a_3 to form the ferryl-oxo species (see below). The rate of this electron transfer step exhibits an apparent dependence on oxygen concentration due to the rate-limiting formation of oxy-ferrocytochrome a_3. This also accounts for the observed, limited accumulation of the oxygen adduct. There is no intermediate that is apparent between the oxy-adduct and the final state reached after a few milliseconds, and this is consistent with our earlier transient studies of the Soret region [18]. Our earlier data were obtained at pH 7.4, the present results are at pH 8.5 and similar results have been obtained at pH 6.5. This is distinct form the mitochondrial oxidase in which oxy-cytochrome oxidase decays through a series of intermediates (e.g., Ref. [19]) and the ubiquinol oxidase in which tightly bound quinol complicates the reaction [20].

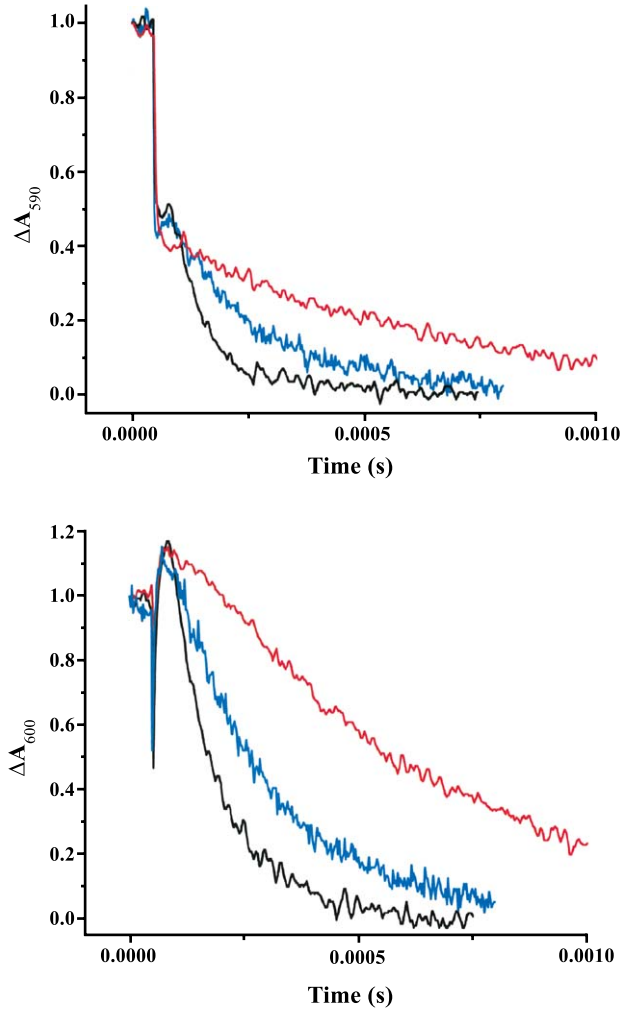

Fig. 2. Transient reaction of menaquinol oxidase with oxygen by stopped-flow, flash photolysis. The enzyme concentration after mixing was 3.86 μM in 50 mM Tris buffer (pH 8.5) with 0.5 mg/ml lauryl maltoside at 20 °C. The enzyme was reduced after equilibration with N_2 by incubation with 2 mM sodium ascorbate and 25 μM TMPD. The reduced enzyme was mixed in a 1:1 ratio with a mixture of O_2-equilibrated and N_2-equilibrated buffers in a Bio-Logic, three-syringe stopped-flow. Panel A is recorded at 590 nm and panel B at 600 nm. The oxygen concentration for each trace in both panels is indicated by the color 100 μM (red), 240 μM (blue) and 600 μM (black). The absorbance of the CO complex prior to the flash is taken as the reference point and the absorption change between this point and the final level is normalized to one. The yellow dashed line through the data following CO removal is fitted from a two-step model in which the reduced enzyme reacts with O_2 in a bimolecular reaction with a rate of 1.5×10^8 M^{-1} s^{-1}. The resulting oxy complex decays to the ferryl complex in a first-order reaction at a rate of 1.2×10^4 s^{-1}. Details of the flow flash set up and data reduction are described in Ref. [6].

Diode array spectra of the complex formed following the initial rapid reaction with oxygen reveal the presence of a species with absorption peaks at 580 nm and 432 nm (Fig. 3). These features indicate the formation of compound F with a ferryl-oxo state of cytochrome a_3. This species decays back to the oxidized enzyme with a half-time of about 2 s at 20 °C. This pattern is the same over the pH range from 6.5 to 8.5. There does not appear to be, therefore, any evidence for participation of a P-type species in the reaction of fully reduced cytochrome aa_3-600 with O_2, or its conversion may be so efficient as to make its detection difficult.

3. Reaction of oxidized cytochrome aa_3-600 with H_2O_2 generates a transient P intermediate

A species with the spectral features of a P intermediate is observed transiently in the reaction of oxidized cytochrome aa_3-600 with hydrogen peroxide at pH 8.5. Fig.

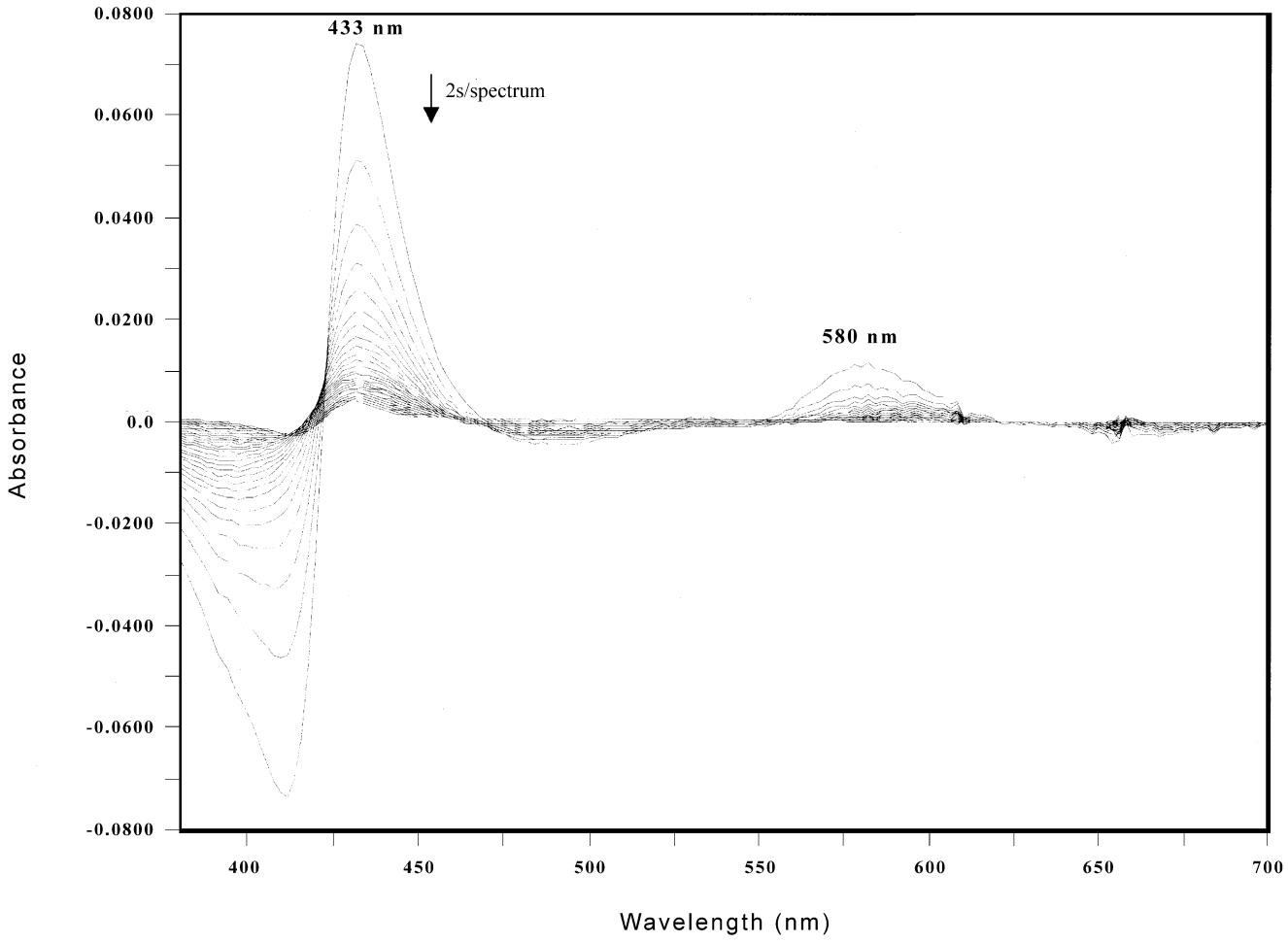

Fig. 3. Diode array spectra of the product of the reaction of fully reduced cytochrome aa_3-600 with oxygen. The enzyme concentration was 3.9 μM in 50 mM HEPES pH 7.8 with 0.5 mg/ml lauryl maltoside at 20 °C. The enzyme was reduced anaerobically with 1 mM sodium ascorbate plus 5 μM TMPD. Oxygen was added (~ 40 μM) by vigorous stirring and spectra were recorded on a Hewlett-Packard 8452A spectrometer at 2-s intervals for 100 s. The spectra were processed using the multi-wavelength analysis program Specfit 32 (Spectrum Software Associates).

4A compares the reaction profile at 604–630 nm of the oxidized enzyme with H_2O_2 at pH 7.5 and pH 8.5. The time courses illustrated in Fig. 4A are fit by a two-step process. At pH 8.5 there is evidence for the appearance of an initial, spectrally distinguishable intermediate which then disappears. However, at pH 7.5 evidence of this initial species is much less apparent in the trace at 604–630 nm. When this reaction is subjected to multi-wavelength analysis, only a single spectral species with maxima at 432 and 580 nm makes up the transition at pH 7.5 (Fig. 4B). In contrast, two spectrally distinguishable species are resolved at pH 8.5 (Fig. 4C). The initial species at pH 8.5 has the characteristics of a P intermediate with absorption maxima at 604 and 434 nm. This pattern is similar to what has been observed with bovine heart mitochondrial oxidase [21]. Much of the early debate on the nature of compounds P and F centered on the redox state of the oxidase in the two species [21,22] whereas the difference may lie in the protonation state of the oxidase [23]. The proposal that these two forms may house a radical centered either on a protein side chain, such as tyrosine or tryptophan, or on the porphyrin ring [21,24,25] is still open to question [26].

4. A P-type intermediate accumulates in the steady-state of cytochrome aa_3-600

Steady-state studies of cytochrome *c* oxidase and heme-containing enzymes in general have a long tradition in the field of enzymology because their readily observable spectral properties hold the promise of direct detection of elusive turnover intermediates (e.g., Ref. [27]). Such studies of mitochondrial cytochrome *c* oxidase have been hampered by the seemingly inherent complexity of the system. Purified mitochondrial oxidase does not retain the high molecular turnover observed in situ (i.e., $\sim 1000\ s^{-1}$) at concentrations that allow for spectral properties of the intermediates to be observed. In addition, the enzyme as isolated tends to relax to a form with relatively low reactivity which, during turnover, shifts to a form with

higher inherent activity [28]. The enzyme also exhibits a separate conformational heterogeneity reflected in the reactivity of cytochrome a_3-Cu_B [29]. Fig. 5 shows a steady-state, spectrophotometric assay of cytochrome aa_3-600. The time course of absorption at 444–460 nm begins with the enzyme in the oxidized state at time zero. NADH is added as reductant, followed by addition of the enzyme D,T-diaphorase which is used to generate the reduced form of the substrate analog 2,3-dimethyl-1,4-naphthoquinone (DMN). The enzyme enters the steady state upon addition of DMN and the steady state persists until the dissolved O_2 is exhausted at which time the absorption increases rapidly. The time taken for the enzyme to consume all the dissolved oxygen allows for the calculation of activity. The enzyme retains the molecular activity observed for the enzyme in O_2 electrode assays where the enzyme concentration is 100- to 200-fold more dilute. The spectrum of the steady-state species (Fig. 5B) has peaks at 604 and 438 nm that are similar to a P-type intermediate. There is no evidence for the presence of reduced cytochrome *a*, which is consistent with the relative electron input and output rates measured in transient state experiments. EPR spectra of the enzyme rapidly frozen in the steady state confirm that cytochrome *a* remains completely oxidized [30].

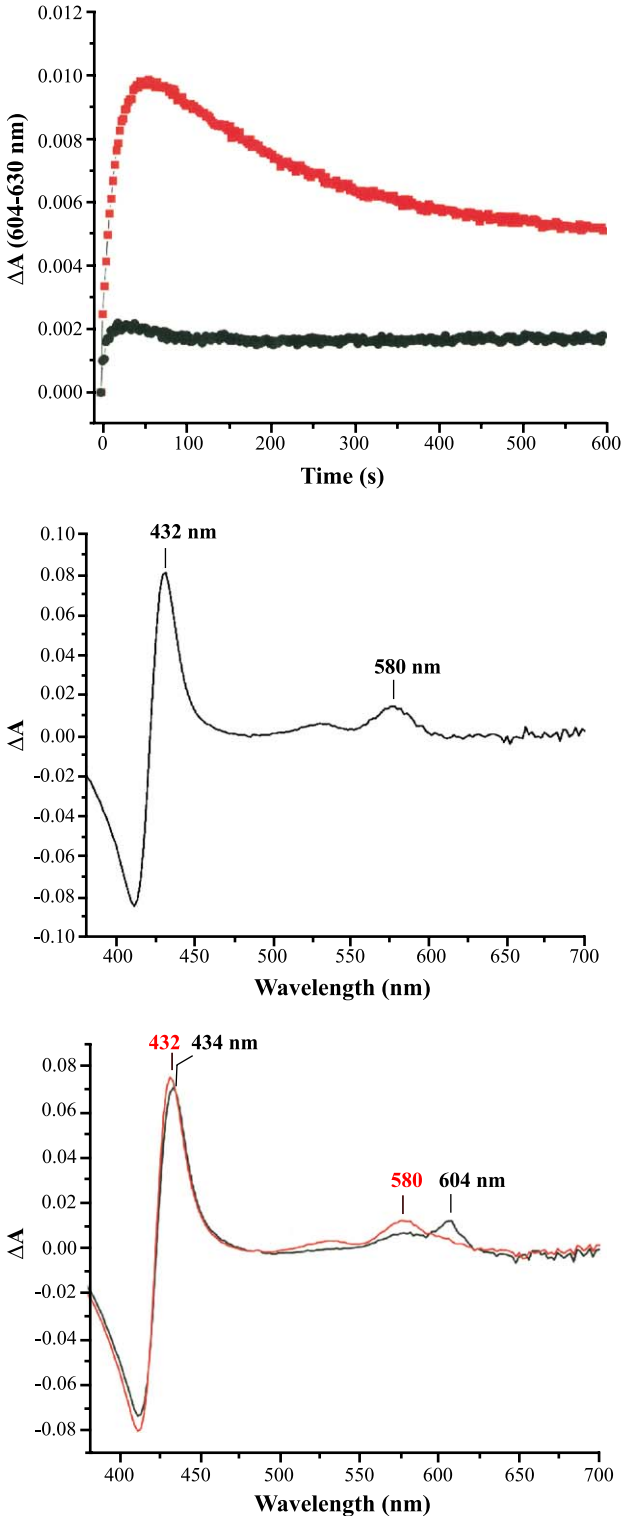

Fig. 4. The reaction of oxidized cytochrome aa_3-600 with hydrogen peroxide. The enzyme concentration was 4.4 μM in either 100 mM sodium phosphate pH 7.5 or pH 8.5. Both buffers contained 0.5 mg/ml lauryl maltoside. In panel (A) the time course of the reaction with 80 μM H_2O_2 is shown at pH 7.5 (black) and pH 8.5 (red). Multi-wavelength analysis on these reactions was performed using Specfit 32 software and a single spectral form fit the data set at pH 7.5 (panel B). Two spectral species were distinguished in the reaction at pH 8.5 and these are shown in panel C. The initial species at pH 8.5 has spectral peaks at 604 and 434 nm whereas the final spectral form has peaks at 580 and 432 nm. The kinetic model used to fit the reaction at both pHs shown here was a two-step process consisting of an initial bimolecular reaction of the oxidized enzyme and H_2O_2 (i.e., $k_1 \sim 8.6 \times 10^2$ M^{-1} s^{-1}) followed by a first-order evolution to the final species ($k_2 \sim 0.028$ s^{-1}). Neither of the two rate constants changed with pH, but the spectral form of the initial intermediate differed as shown in panels B and C.

5. A catalytic cycle for cytochrome aa_3-600

The scheme shown in Fig. 6 depicts two catalytic cycles for cytochrome aa_3-600. Along the outer cycle the conversion of oxidized cytochrome aa_3-600 to its fully reduced state (i.e., FR) requires the uptake of three electrons and at least three protons. When the fully reduced and fully protonated enzyme reacts with oxygen, it goes directly to the F-state with absorption at 580 and 432 nm. In contrast, when cytochrome aa_3-600 is reduced in the presence of oxygen, depicted on the inner cycle here, it reaches only a partially reduced (i.e., PR), but oxygen reactive state. This partially reduced form is also only partially protonated and when it reacts with oxygen it forms the P intermediate. This species must take up additional electrons and protons to complete the reduction of dioxygen to water. It is suggested that the progression of the P intermediate is limited by the arrival of a proton from the medium. Once this proton arrives, then further electron transfer will allow the enzyme to convert back to the oxidized enzyme and enter another round of catalysis. The control of electron transfer by the rate of a coupled proton uptake step has been proposed previously for cytochrome *c* oxidase based

on transient kinetic studies of mutants of the oxidase from *R. sphaeroides* [30].

The steady-state behaviour of cytochrome aa_3-600 is somewhat different than what has been observed for mitochondrial cytochrome *c* oxidase (e.g., Ref. [6]). It does not appear to undergo a resting to pulsed conversion when the oxidized enzyme is taken into the steady-state by the addition of reductants and the cytochrome aa_3-600 retains its maximal molecular turnover over a wide range of protein concentration. The spectral form of cytochrome aa_3-600 in the steady state does not appear to contain reduced cytochrome *a*, even though cytochrome *a* is the initial electron acceptor from menaquinol [31]. This implies very rapid internal electron transfer from cytochrome *a* to cytochrome a_3 and rapid progress of the oxygen reactive form of the enzyme in the catalytic cycle. The accumulation of reduced cytochrome *a* in steady-state spectra of the mitochondrial cytochrome *c* oxidase and the nature of the steady-state form have been the subject of some debate [32–34]. We have also reported the presence of a radical in the steady state species of cytochrome aa_3-600 [31]. At present, the radical could either arise from a protein based group such as a tyrosine or a tightly bound semi-menaquinone.

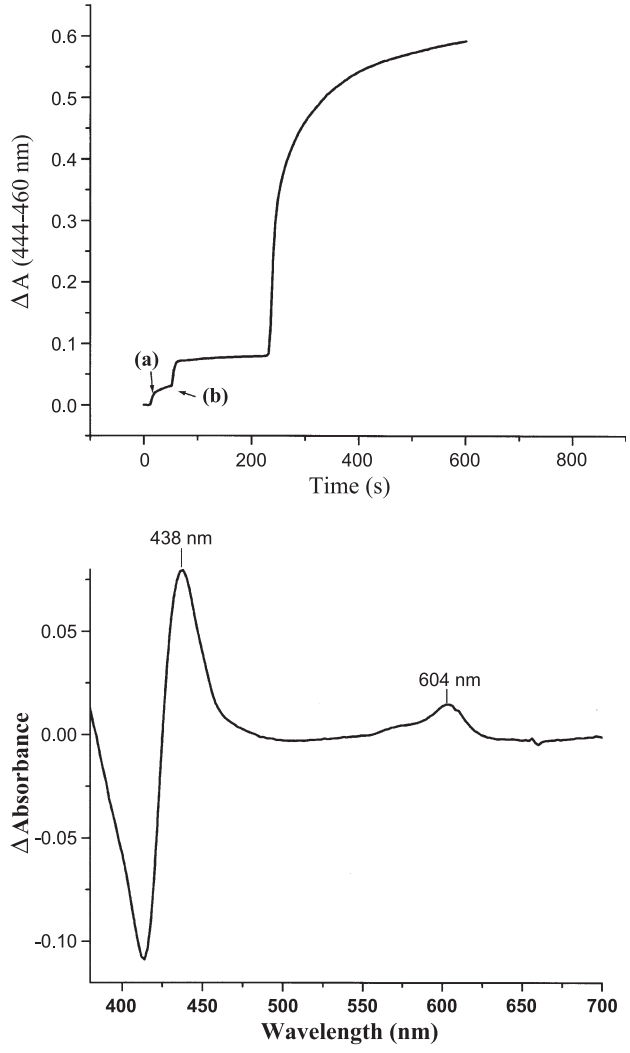

Fig. 5. Steady-state spectrophotometric assay of cytochrome aa_3-600. The concentration of cytochrome aa_3-600 was 3.5 μM in 50 mM sodium phosphate pH 6.4 with 0.5 mg/ml lauryl maltoside. At position (a) in the trace in panel (A) 2 mM NADH and 2 mg/ml D,T-diaphorase (Sigma) were added. At position (b) 6 μM DMN was added to initiate steady-state activity. Panel (B) is a difference spectrum generated by subtracting the spectrum of the oxidized enzyme from the species generated in the steady-state.

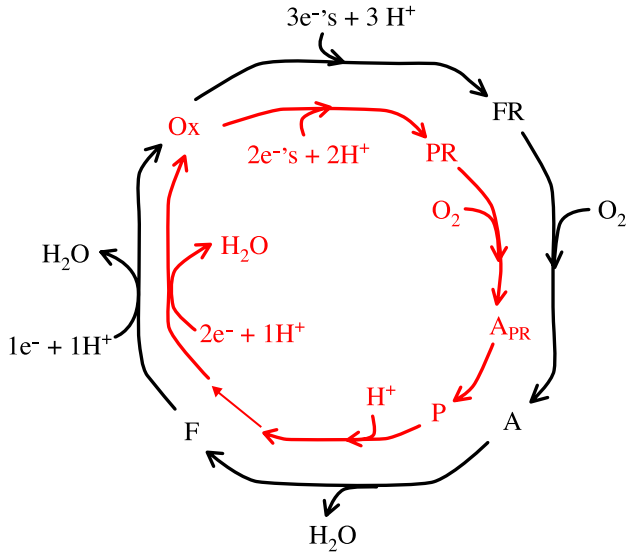

Fig. 6. Catalytic cycles of cytochrome aa_3-600. Around the outside the oxidized enzyme (Ox) is reduced (FR) by the addition of three electrons and three protons. FR reacts with oxygen to form a ferro-oxy complex (A). Species A reacts in a single transition to F and then slowly proceeds to Ox in the presence of limiting reductant. The steady-state cycle is depicted on the inner path in red. The oxidized enzyme is partially reduced by addition of two protons and two electrons to form PR. PR can react with dioxygen to form A_{PR} with oxy-ferrocytochrome a_3. A_{PR} converts to the P intermediate by electron transfer to bound O_2 and cleavage of the O–O bond to form a ferryl-oxo complex. The full reduction of dioxygen and recovery of the oxidized enzyme then await further proton and electron delivery.

6. Conclusions

(1) Steady-state studies of bacterial cytochrome oxidases may offer new opportunities to observe catalytically competent intermediate forms that are populated during steady state catalysis.
(2) Accumulation of reduced cytochrome *a* is not seen in the steady-state reaction of cytochrome aa_3-600 and is not expected if intrinsic electron transfer rates measured in transient-state reactions are functional during turnover.
(3) The fully reduced state is not required to participate in the turnover cycle of cytochrome aa_3-600.
(4) In the turnover cycle of cytochrome aa_3-600 the step that limits turnover is the transition from compound P and this may be controlled by the rate of proton delivery. Once F is formed it decays immediately.

Acknowledgements

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