The reaction of *Escherichia coli* cytochrome *bo* with H_2O_2: Evidence for the formation of an oxyferryl species by two distinct routes

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Abstract We have re-examined the reaction of *fast* oxidised cytochrome *bo* with H_2O_2 in a stopped-flow spectrophotometer. Monitoring the reaction at 582 nm allows us to observe the formation and decay of a spectroscopically distinct intermediate which accumulates transiently prior to the formation of an oxyferryl species previously characterised in this laboratory (Watmough, N.J., Cheesman, M.R., Greenwood, C. and Thomson, A.J. (1994) Biochem. J. 300, 469–475 [1]). The reaction shows three distinct phases of which the fast and intermediate phases are bimolecular and show a marked pH dependence. Initially these results appeared incompatible with the report that only one equivalent of H_2O_2 is required to generate the oxyferryl species (Moody, A.J. and Rich, P.R. (1994) Eur. J. Biochem. 226, 731–737 [2]). However, these data can be reconciled by a branched reaction mechanism whose contributions differ according to the peroxide concentration used.

Key words: *Escherichia coli*; Cytochrome *bo*; Quinol oxidase; Hydrogen peroxide; Oxyferryl heme

1. Introduction

Cytochrome *bo*, a quinol oxidase from *Escherichia coli*, is a member of the conserved superfamily of protonmotive haem-copper terminal oxidases which includes mitochondrial cytochrome aa_3 [3] which catalyse the four-electron reduction of dioxygen to water. Unlike the aa_3 type cytochrome *c* oxidases, quinol oxidases lack the dinuclear Cu_A site in subunit II and contain only three redox active metal centres; a magnetically isolated low-spin heme and a high-spin heme coupled to a copper ion (Cu_B) which forms the site of dioxygen reduction.

Given their close structural relationship, cytochrome *bo* might be expected to follow a reaction mechanism similar to that proposed for cytochrome aa_3 [4]. This mechanism proposes the formation of at least two stable intermediates in the reduction of dioxygen which are spectroscopically distinct and are one oxidation level apart. One intermediate, generated from the reaction of dioxygen with the reduced binuclear centre, has been assigned to a 'peroxy' or P species; formally Fe(III)-O^{2-}::Cu(II) which may have protonated states. The other is assigned to an oxyferryl or F species, Fe(IV)=O::H_2O-Cu(II). These intermediates have also been observed in cytochrome *aa_3-600* a quinol oxidase from *Bacillus subtilis* [5]. Another way to generate these intermediates in cytochrome aa_3 is by the reaction of the oxidised enzyme with H_2O_2 [6]. The extent of this reaction and the ratio of the two forms produced are dependent upon several factors, including the concentration of peroxide used [6], the method of preparation of the enzyme [7] and the pH at which the reaction takes place [8].

Previous studies on the reaction of *fast* cytochrome *bo* with micromolar concentrations of H_2O_2 have established that a single species is produced which is characterised by a red-shifted Soret, additional absorbance around 555 nm and the loss of the 624 nm charge transfer band [1,2]. This species was shown by magnetic circular dichroism spectroscopy to contain oxyferryl heme *o* [1,9]. If the reaction is similar to that of cytochrome aa_3 the products of the reaction of fully reduced quinol oxidase with dioxygen are predicted to be water and a binuclear centre containing oxyferryl heme *o* [5,10]. Therefore, it is not surprising that the electronic absorption spectrum of cytochrome *bo* after undergoing this reaction is identical to that of the oxyferryl species formed in the reaction of the oxidised enzyme with H_2O_2 [11].

However, one question remains. Although oxyferryl heme *o* is the product of the three-electron reduction of dioxygen, the reaction of a single equivalent of H_2O_2 with oxidised enzyme should yield a species that is formally equivalent to the product of the reduction of dioxygen by two electrons. Scission of the dioxygen bond and formation of the oxyferryl species require a third electron which it has been proposed is donated by the protein by the formation of a π-cation radical [1,2].

Recently, Morgan and co-workers [12] studied the reaction of *fast* cytochrome *bo* in a stopped-flow spectrophotometer equipped with a multiwavelength detector. Under their reaction conditions (5 mM H_2O_2 at pH 9.0), these authors demonstrated the formation and decay of a spectroscopically distinct intermediate prior to formation of the oxyferryl product. Since these authors used a single concentration of H_2O_2 at alkaline pH the nature of the reaction remained unclear. Either the peroxy intermediate is formed in a bimolecular reaction with H_2O_2 and the decay phase is a unimolecular process representing radical formation or the peroxy intermediate reacts with a second equivalent of H_2O_2 to yield the oxyferryl product. Whilst the first mechanism would be consistent with earlier titrations which demonstrated that a single equivalent of H_2O_2 is required to form the oxyferryl species [2], the second is not.

In order to resolve this issue, we have undertaken a detailed kinetic examination of this reaction under a variety of conditions with the aim of determining the following: the reaction order of the two phases, the pH dependence of the two phases and the electronic absorption spectrum of the intermediate and product(s). The results presented here demonstrate that the oxyferryl form of cytochrome *bo* can be produced in the reaction with H_2O_2 by more than one route.

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2. Materials and methods

2.1. Reagents

Biological buffers, EDTA, lauryl-β-D-maltoside and octyl-β-D-glucopyranoside were all purchased from Sigma. H_2O_2 was obtained from Fisons.

2.2. Cell growth and enzyme preparation

The *E. coli* strain RG145 which overexpresses cytochrome *bo* and lacks cytochrome *bd* [13] was used as the source of cytochrome *bo*. Conditions for batch cell culture and subsequent purification of the cytochrome *bo* were as previously described [14].

2.3. Sample preparation

The amount of cytochrome *bo* in the *fast* conformer was determined from the extent ($\Delta\varepsilon_{430-415} = 7.2\times10^4$ M^{-1} cm^{-1} of the rapid ($k_{obs} = 0.15$ s^{-1}) reaction with 10 mM KCN and the presence of diagnostic EPR signals [15]. When less than 90% of a preparation was in this conformer the enzyme was 'pulsed' by a modification of the method described in [2].

2.4. Kinetic measurements

The reaction of *fast* cytochrome *bo* with H_2O_2 under pseudo first order conditions was measured in an Applied Photophysics Bio-Sequential DX.17MV stopped-flow spectrophotometer using a 1 cm pathlength cuvette. Detection at a single wavelength was with a side window photomultiplier. In this configuration a minimum of 500 data points were collected per experiment. Detection at multiple wavelengths was achieved using an Applied Photophysics photodiode array accessory A minimum of 200 spectra were collected per experiment with a maximum time resolution of 2.38 ms/spectrum.

2.5. Treatment of kinetic data

The experimental traces recorded at 582 nm were exported as ASCII files and analysed as the sum of two or three exponentials using TableCurve 2D for Windows (Jandel Scientific, San Rafael, CA, USA).

A variety of kinetic mechanisms were investigated to determine if they could account for the observed kinetic characteristics exhibited by the reaction of cytochrome *bo* with H_2O_2. Each mechanisms was explored in the time domain using the GEAR variable-step numerical integration method to generate theoretical time courses [16]. The time courses were then fitted to the experimental data by varying the rate constants in the mechanism. When an adequate mechanism was found the theoretical time courses were synthesised for H_2O_2 concentrations from 0.1 to 90 mM to allow comparison with the experimental data. From this iterative procedure Scheme 1 was identified as possessing all the characteristic necessary to describe the kinetic properties determined in this study.

The time-resolved spectra were analysed globally at all times and wavelengths simultaneously. The analysis was performed on an Acorn A5000 personal computer using the singular value decomposition (SVD) and global exponential fitting routines found in the software package Glint (Applied Photophysics, Leatherhead, UK).

3. Results and discussion

3.1. Effects of H_2O_2 concentration and pH

In order to investigate the kinetics of formation and decay of the peroxy intermediate in the reaction of *fast* cytochrome *bo* with H_2O_2 we monitored the reaction at 582 nm. At this wavelength there is an increase in absorption which corresponds to the formation of the peroxy intermediate followed by a decrease in absorbance which corresponds to the transition to the oxyferryl intermediate [12]. At pH 10 the reaction with 90 mM H_2O_2 appears essentially biphasic (Fig. 1) with the rapid formation of the peroxy species followed by a decay. However, at pH 6 (Fig. 1) three phases are clearly resolved with the rise phase (peroxy formation) being followed by a

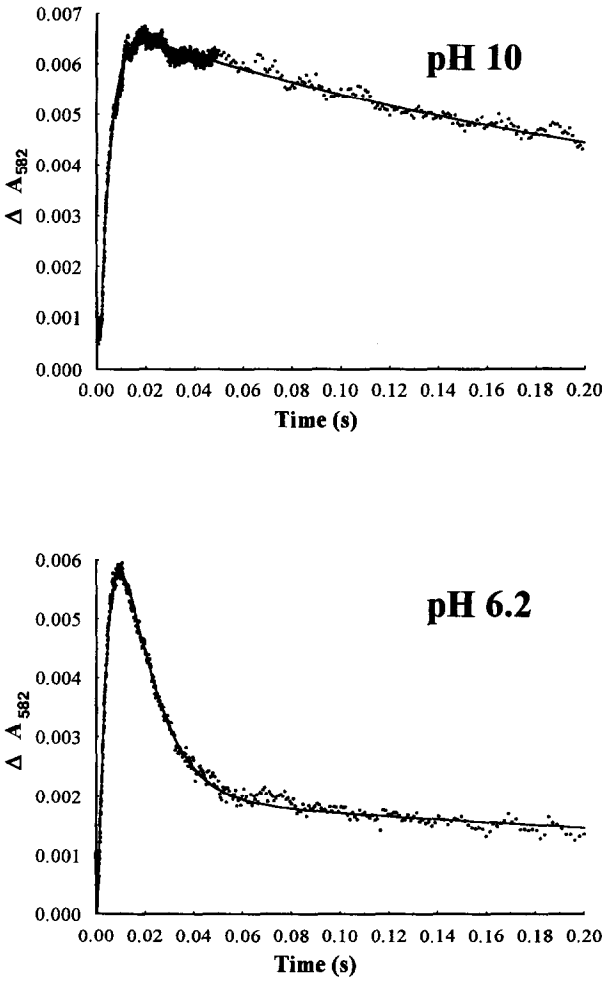


Fig. 1. Reaction of 8 μM *fast* cytochrome *bo* with 90 mM H_2O_2 monitored at 582 nm. The solid circles represent experimental data points, the solid lines the calculated fit to the sum of three exponentials.

Table 1
Dependence of the reaction of *fast* cytochrome *bo* on H_2O_2 concentration and pH

	Fast phase	Intermediate phase	Slow phase
Cytochrome *bo* pH 10.0	1.6×10^3 M^{-1} s^{-1}	2.1×10^2 M^{-1} s^{-1}	1–5 s^{-1}
Cytochrome *bo* pH 8.0	2.1×10^3 M^{-1} s^{-1}	6.7×10^2 M^{-1} s^{-1}	1–5 s^{-1}
Cytochrome *bo* pH 6.3	2.5×10^3 M^{-1} s^{-1}	9.33×10^2 M^{-1} s^{-1}	1–5 s^{-1}
Cytochrome *c* oxidase pH 9.0[a]	0.39×10^3 M^{-1} s^{-1}	0.38×10^2 M^{-1} s^{-1}	–

[a]Data taken from [7].

biphasic decay. Thus our experimental data were all analysed in terms of a triple-exponential time course.

In order to determine the molecularity of the three kinetic phases, the observed rate constant (k_{obs}) for each phase was plotted as a function of H_2O_2 concentration over the range 0.1–90 mM. The values of k_{obs} for the fast and intermediate phases show a linear dependence on H_2O_2 concentration at pH 6, 8 and 10 (data not shown), demonstrating that they are bimolecular processes. This is qualitatively similar to the reaction of *fast* cytochrome *c* oxidase with H_2O_2 where the formation and decay of the peroxy (607 nm) intermediate can be described by two exponential processes, both of which are dependent on H_2O_2 concentration (Table 1) [7]. Furthermore, the bimolecular rate constants for the fast and intermediate phases are sensitive to the pH of the medium (Table 1). In contrast, the third (slow) phase was unaffected by H_2O_2 concentration and pH independent.

In order to examine in greater detail the nature of the pH dependence of the first two phases of the reaction, we measured the formation and decay of the peroxy intermediate at 20 mM H_2O_2 in the range pH 6–10. The resultant traces were again fitted to the sum of three exponentials and k_{obs} for the fast and intermediate phases plotted as a function of pH (Fig. 2). These data could be fitted to the ionisation of single titratable groups with apparent pK_a values of 8.3 and 7.7, respectively. Since H_2O_2 is essentially fully protonated in the pH range studied it would appear that an ionisable group within the protein in equilibrium with the solvent affects these processes.

Such dependence has not been reported for the reaction of *fast* cytochrome *c* oxidase with H_2O_2, however, such experiments are complicated by the rapid conversion of that enzyme to a slow conformer (which is unreactive towards H_2O_2) below pH 8 [17]. It is of interest, however, that the reaction of fully reduced cytochrome *c* oxidase with dioxygen exhibits four kinetic phases which can be resolved by the flow-flash technique [18,19]. The observed rate constants for the third phase show dependence upon a single ionisable residue with a pK_a between 7.9 and 8.3 [19,20]. This phase of the reaction

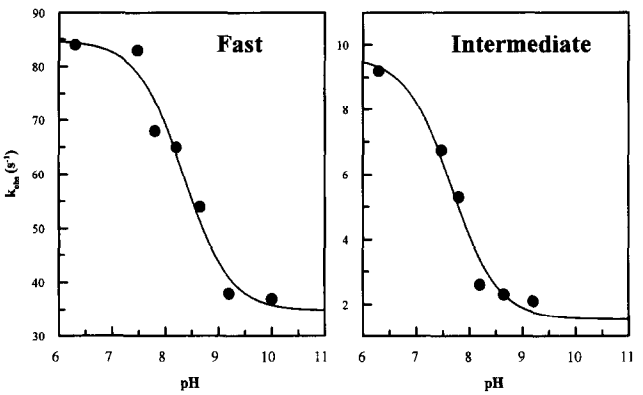


Fig. 2. pH dependence of the fast and intermediate phases of the reaction of 8 μM *fast* cytochrome *bo* with 5 mM H_2O_2 monitored at 582 nm. The values of k_{obs} for each phase (●) were determined by fitting the sum of three exponentials to the experimental traces. The solid line in the left-hand panel is a fitted titration curve with pK_a = 8.3 and limiting k_{obs} at low and high pH of 84.8 and 34.8 s^{-1}, respectively. The solid line in the right-hand panel is a fitted titration curve with pK_a = 7.7 and limiting k_{obs} at low and high pH of 9.7 and 1.5 s^{-1}, respectively.

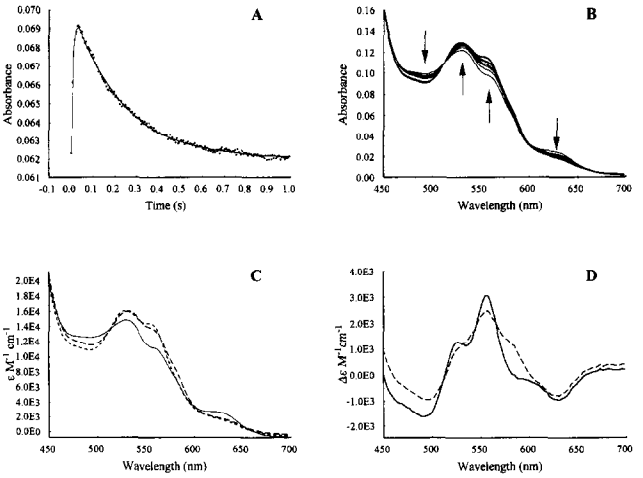


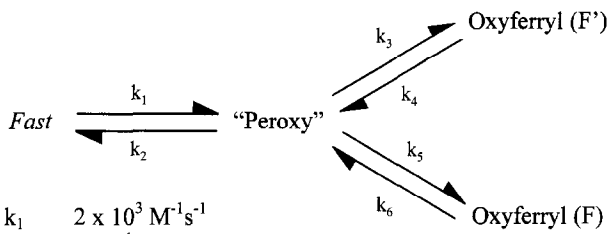
Fig. 3. Spectral changes associated with the reaction of 8 mM *fast* cytochrome *bo* with 90 mM H_2O_2. (A) Time course of the reaction monitored at 582 nm. The data points were determined experimentally; the solid line is a fit to a model as described in the text. (B) Changes in the absolute spectra in the visible region during the course of the reaction. The spectra shown were recorded after 2.56, 7.68, 12.8, 17.9, 28.2, 38.4, 253 and 500 ms. Arrows indicate maximal changes in absorbance. (C) Absolute spectra calculated using the global fitting procedure: *fast* (———), 'peroxy' (– – –), oxyferryl (F) (- - -). (D) Calculated difference spectra: 'peroxy' minus *fast* (– – –) and oxyferryl minus *fast* (———).

corresponds the reduction of peroxy heme a_3 to oxyferryl heme a_3, a process which requires the uptake of a proton from solution [4]. Given the proton channels identified in heme-copper oxidases by a combination of structural studies and site-directed mutagenesis [21–23] are highly conserved, it is conceivable that the conserved residue which governs the rate of the 'P' to 'F' transition in the catalytic cycle of cytochrome *c* oxidase is the same as that which affects the peroxide reaction in cytochrome *bo*.

3.2. *A quantitative model of the reaction of fast cytochrome bo with* H_2O_2

Using the iterative process described in Section 2, it was determined that the quantitative characteristics of the reaction of *fast* cytochrome *bo* with H_2O_2 were best described by Scheme 1. This branched reaction scheme predicts that the peroxy intermediate is formed from *fast* cytochrome *bo* in a bimolecular reaction with H_2O_2. This can subsequently be reduced to an oxyferryl species either by taking an electron from a site within the protein to form F′ as we have previously suggested [1] or by reaction with a second equivalent of to yield F as has been demonstrated for cytochrome *c* oxidase [7].

Numerical integration studies based on this scheme predict the existence of three kinetic phases and which are able to describe fully both the time courses and concentration dependences of each of the phases that we had determined experimentally. Furthermore, simulation of population profiles (data not shown) indicate that under conditions of low H_2O_2 concentration ($k_3 \gg k_1$, $k_3 \gg k_5$) the reaction will proceed to F′ with very low transient occupancy of the peroxy intermediate. Such conditions have been used in previously reported titrations [1,2] and would account for the apparent stoichiometry (H_2O_2:cytochrome *bo*) of 1:1 [2]. However, at high H_2O_2 concentrations (both k_1 and $k_5 > k_3$) the reaction



k_1	2×10^3 $M^{-1}s^{-1}$
k_2	0.1 s^{-1}
k_3	1 s^{-1}
k_4	negligible
k_5	2×10^2 $M^{-1}s^{-1}$
k_6	0.5 s^{-1}

Scheme 1.

will proceed via the peroxy intermediate to the F state and the expected stoichiometry will be 2:1.

3.3. The electronic absorption spectrum of the peroxy intermediate

The predicted populations generated from Scheme 1, synthesised under different H_2O_2 concentrations allowed us to identify experimental conditions optimal for the resolution of the spectrum of the peroxy intermediate and the oxyferryl product F. These conditions (8 μM cytochrome *bo*, 90 mM H_2O_2, pH 10) were used in an experiment to measure the spectral changes in the visible region (500–700 nm) associated with the sequential formation of the peroxy intermediate and the oxyferryl product.

After reducing the data set by singular value decomposition [24] to the eight most significant components the data were reconstructed and subjected to a global fitting routine [25]. Since the contribution of F′ could essentially be ignored under the conditions used the reaction was fitted to the following model:

Fast + $H_2O_2 \leftrightharpoons$ 'Peroxy' $\leftrightharpoons$ Oxyferryl (F)

Fig. 3A shows the formation and decay of the peroxy intermediate monitored at 582 nm. This demonstrates that, as predicted, the occupancy of the intermediate peroxy state is maximal after 20 ms. The spectral changes in the visible region associated with the formation of the peroxy species are shown in Fig. 3B. These demonstrate two features: the loss of intensity at 624 nm associated with a ligand to metal charge-transfer band that is a marker of high-spin ferric heme *o* and the appearance of additional intensity at 558 and 582 nm. The final two spectra in this figure represent the formation of the oxyferryl (F) product which exhibits a small further increase in absorbance at 555 nm and a loss of intensity at 582 nm.

From this data set the global fitting routine was used to calculate the start spectrum (which should correspond to that of *fast* cytochrome *bo*), the spectrum of the intermediate peroxy species and that of the oxyferryl (F) product (Fig. 3C). The calculated spectra of the peroxy and oxyferryl species were unaffected by constraining the starting spectrum to that of authentic *fast* cytochrome *bo* (data not shown).

The spectral changes relative to the starting spectrum alluded to above become more apparent when the data are plotted in the form of difference spectra peroxy minus *fast* and oxyferryl (F) minus *fast* (Fig. 3D). The form and intensity of the difference spectrum of the oxyferryl species are essentially identical to those previously reported [1,2,26]. However, the difference spectrum of the peroxy intermediate differs from that reported by Morgan and colleagues [12] which shows the expected minimum at 624 nm (see above) and a broad absorption maximum at 580 nm with a shoulder at 540 nm. In fact the difference spectrum we report has more in common with that induced by turning over cytochrome *bo* aerobically using ascorbate/phenazine methosulfate as an electron donor [27].

3.4. The nature of the peroxy intermediate

Our model demonstrates that the product of the reaction of *fast* cytochrome *bo* with H_2O_2 may be formed from the peroxy intermediate by one of two routes. Both involve the donation of an electron to reduce the peroxy species to an oxyferryl species in which the porphyrin ring is not oxidised [1,9]. Since the peroxy species is formed in a bimolecular process with H_2O_2 it is reasonable to assume that if it does not represent peroxide ion bound to ferric heme *o*, then it is isoelectronic with that species. Two realistic alternatives exist, that it is an analogue of HRP compound I, i.e. an oxyferryl species in which either the porphyrin macrocycle is oxidised to a π-cation radical [28] or an oxyferryl species in which the extra reducing equivalent is provided by Cu_B, which then must exist as Cu(III). Our current data do not allow us to discriminate between these possibilities, however, we would note the following. First of all, the broad rather featureless difference spectrum of the peroxy intermediate is similar in form and intensity to that of the product of the reaction which we have previously characterised as an oxyferryl species. Secondly, the spectrum of the peroxy intermediate shares some features with that of Fe(IV) myoglobin, in particular increased absorbance at 555 nm and a broad transition centred around 580 nm [29]. Whilst we accept that electronic absorption spectroscopy will not diagnose the chemical nature of the peroxy intermediate we feel that our model of the reaction of cytochrome *bo* with H_2O_2 defines the experimental conditions needed to generate this intermediate at high yield necessary for probing by other more conclusive spectroscopies.

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