Journal of Bioenergetics and Biomembranes, Vol. 30, No. 1, 1998

The Dinuclear Center of Cytochrome bo_3 from *Escherichia coli*

Nicholas J. Watmough,[1] Myles R. Cheesman,[2] Clive S. Butler,[1,2] Richard H. Little,[1] Colin Greenwood,[1] and Andrew J. Thomson[2]

Received September 22, 1997; accepted September 23, 1997

For the study of the dinuclear center of heme-copper oxidases cytochrome bo_3 from *Escherichia coli* offers several advantages over the extensively charactererized bovine cytochrome *c* oxidase. The availability of strains with enhanced levels of expression allows purification of the significant amounts of enzyme required for detailed spectroscopic studies. Cytochrome bo_3 is readily prepared as the *fast* form, with a homogeneous dinuclear center which gives rise to characteristic broad EPR signals not seen in C*c*O. The absence of Cu_A and the incorporation of protohemes allows for a detailed interpretation of the MCD spectra arising from the dinuclear center heme o_3. Careful analysis allows us to distinguish between small molecules that bind to heme o_3, those which are ligands of Cu_B, and those which react to yield higher oxidation states of heme o_3. Here we review results from our studies of the reactions of *fast* cytochrome bo_3 with formate, fluoride, chloride, azide, cyanide, NO, and H_2O_2.

KEY WORDS: *Escherichia coli*; Quinol oxidase; cytochrome bo_3; cytochrome *c* oxidase; nitric oxide reductase; EPR spectroscopy; MCD spectroscopy; oxyferryl heme.

INTRODUCTION

Our understanding of the superfamily of heme-copper oxidases has been considerably advanced by the solution of the three-dimensional structures of the cytochrome aa_3 type oxidases from both *Paracoccus denitrificans* (Iwata *et al.*, 1995) and bovine mitochondria (Tsukihara *et al.*, 1995, 1996). These structures of the oxidized forms resolve many key elements of these protonmotive terminal oxidases. Hence it is now possible to begin to interpret the wealth of mechanistic and spectroscopic studies in terms of plausible structural models. These structures also enable studies of site-directed mutants to be placed on a more rational and systematic basis.

Over the past six years the Norwich group has conducted a systematic study of the dinuclear center of cytochrome bo_3, a quinol oxidase from *Escherichia coli* which complements the earlier studies of bovine C*c*O.[3] The application of a range of biophysical techniques, in particular EPR and MCD spectroscopies, to well-characterized preparations of this enzyme have clarified a number of issues that are of general interest to those working on heme-copper oxidases. In particular, the mechanisms of ligand binding to the dinuclear center (Cheesman *et al.*, 1993; Watmough *et al.*, 1993; Cheesman *et al.*, 1994; Little *et al.*, 1996; Butler *et al.*, 1997), the chemical nature of the P and F forms of cytochrome bo_3 which are proposed as intermediates of O_2 reduction (Cheesman *et al.*, 1994; Watmough *et al.*, 1994), and a series of measurements should permit for the first time a detailed description of the magnetic

[1] Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom.
[2] Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom.
[3] Abbreviations: C*c*O, cytochrome *c* oxidase; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; NO, nitric oxide.

0145-479X/98/0200-0055$15.00/0 © 1998 Plenum Publishing Corporation

coupling between heme o_3 and Cu_B (Watmough *et al.*, 1993; Little *et al.*, 1996).

Cytochrome bo_3 is an attractive subject for investigation by magneto-optical techniques; first, the enzyme contains only three redox active metal centers, lacking the dinuclear Cu_A center of CcO (Farrar *et al.*, 1996) and therefore gives rise to spectra of lower complexity; second, the heme-copper dinuclear center contains heme O, the optical properties of which are similar to the well-characterized protoporphyrin IX (Cheesman *et al.*, 1994). Heme A has somewhat anomalous optical properties because of the strong electron withdrawing nature of the 8-formyl heme substituent. Finally, this enzyme has been overexpressed (Au and Gennis, 1987) and a well-characterized library of site-directed mutants constructed (Hosler *et al.*, 1993). The close sequence homology between subunits I and II of cytochromes bo_3 and aa_3 (Saraste, 1990) leaves little doubt that the two oxidases are structurally very similar.

The enzyme used for most of our work was purified from an over-expressing strain known as RG145 which yields a mixture of two forms of the enzyme; cytochrome bo_3 which contains heme *B* in the low-spin site and cytochrome oo_3 which has heme *O* misincorporated into the low-spin site (Puustinen *et al.*, 1992). The spectroscopic properties of the dinuclear center appear not to be influenced by the species of heme present in the low-spin site. Further, the method used to solubilize cytochrome bo_3 from extensively washed membranes (Cheesman *et al.*, 1993) ensures that the enzyme does not have the molecule of ubiquinone-8 that is associated with enzyme released from membranes using milder regimes (Sato-Watanabe *et al.*, 1994). It is fortunate that our spectroscopic investigations have been unaffected by such preparation-dependent variations in cytochrome bo_3.

In this article we review progress in our laboratories toward a detailed spectroscopic description of the dinuclear center of cytochrome bo_3, understanding the behavior of that site toward exogenous ligands, and characterizing the intermediates of dioxygen reduction.

WHY STUDY LIGAND BINDING TO CYTOCHROME Bo_3?

The coupling of the free energy of O_2 reduction to vectorial proton movement must entail mechanical motion which results from scission of the O–O bond. This may be associated with ligand rearrangements around Cu_B (Mitchell, 1987) or with changes in the Fe–Cu distance; there is therefore considerable interest in clarifying to what extent this center can expand or alter its coordination sphere. The study of this is extremely difficult since no spectroscopic parameters attributable to Cu_B alone have been observed. One approach to this problem is by studying the coordination of small ligands to the dinuclear center, in particular those that induce spectral changes in response to binding to Cu_B.

Fast[4] cytochrome bo_3 binds formate, fluoride, and azide rapidly (Little *et al.*, 1996; Moody *et al.*, 1997) to give rise to compounds with characteristic broad EPR features (Watmough *et al.*, 1993; Little *et al.*, 1996). These forms of the oxidized enzyme are also characterized by shifts in the Soret region of the spectrum and in the position of the 624 nm ligand-to-metal charge-transfer (C-T) band (Table I). This is one of two C-T bands associated with high-spin ferric hemes, which are readily seen in the room-temperature MCD spectrum (Cheesman *et al.*, 1994). The visible region C-T band appears as a trough in the MCD spectrum, while the second feature in the near infrared region (800–1100 nm) is derivative shaped (Cheesman *et al.*, 1994). The energies of both transitions are dependent upon the axial ligands of the heme (Table I). Since the proximal ligand of heme o_3 is invaribly histidine-419 of subunit I, the positions of this pair of bands report the nature of the distal ligand.

LIGANDS WHICH LEAVE Fe (III) HEME o_3 HIGH-SPIN

In the case of the azide derivative, the positions of the C-T bands are little changed relative to *fast* cytochrome bo_3, indicating that water remains bound in the sixth coordination position of heme o_3. This implies that azide binds to Cu_B. In contrast, on binding fluoride, the position of both bands shifts considerably to the blue (Table I), demonstrating that fluoride is a direct ligand to heme o_3. In fact fluoride is atypical of the halide ions which otherwise appear to bind to Cu_B, as judged by the position of the visible region C-T band in the electronic absorption spectrum (Moody *et*

[4] Enzyme prepared in our laboratories which we refer to as *fast* is characterized by a Soret maximum at 406.5 nm, a distinct charge transfer band at 624 nm, and an ability to bind 10 mM cyanide monophasically (k_{obs} = 0.27 s^{-1}).

Table I. Spectroscopic Properties of a Number of Important Dinuclear Center Derivatives of Cytochrome Bo_3

	Ligand-binding properties		UV-visible electronic absorption		MCD high-spin heme O_3 C-T bands		Integer-spin EPR signals		
Derivative	Site	K_D ($\times 10^{-6}$ M)	Soret λ_{max} (nm)	λ_{CT}[a] (nm)	visible[b] λ_{min} (nm)	NIR[c] λ_{cross} (nm)	Low-field feature[d]	High-field features[e]	References
Fast[f]	—	—	406.5	624	635	1100	$g \approx 7.3$	$g \approx 3.7/2.8$	Watmough *et al.*, 1993, Cheesman *et al.*, 1994
Fluoride	Heme o_3	86	405	618	625	940	$g \approx 10.2$	$g \approx 3.6/3.1$	Watmough *et al.*, 1993, Cheesman *et al.*, 1994 Moody *et al.*, 1997
Chloride[f]	Cu_B	210	407	634	647	nd	$g \approx 8.6$	$g \approx 3.2/3.0$	Butler *et al.*, 1997, Moody *et al.*, 1998
Formate	Heme o_3 Cu_B?	<200	406	634	642	1180	$g \approx 13$	$g \approx 3.2, 2.6$	Watmough *et al.*, 1993, Cheesman *et al.*, 1994 Moody *et al.*, 1997
Azide[f]	Cu_B	17	407	634	645	≈1300	$g \approx 9.2$	$g \approx 3.2, 2.8$	Little *et al.*, 1996
Dinitrosyl[f]	Cu_B	2.3, 33	408	634	nd	nd	absent	absent	Butler *et al.*, 1997
Cyanide	Heme o_3 Cu_B	1–2	412	absent	absent	absent	absent	absent	Cheesman *et al.*, 1994 NJW and RHL, unpublished

[a] Determined from the minimum in the reduced-oxidized electronic absorption spectrum.
[b] Measured at the minimum.
[c] Measured at the point of crossover.
[d] The apparent g value given represents the point of crossover of the derivative-shaped feature.
[e] These features are very broad, extending from 150–300 mT. The apparent g values represent the maxima and minima.
[f] These derivatives all appear to retain water as the sixth ligand to Fe(III) heme o_3.

al., 1998) In the case of the Cl^- adduct this assumption is supported by the room-temperature MCD spectrum and the behavior of the Cl^--ligated form of the enzyme toward NO (Butler *et al.*, 1997).

The binding of formate to cytochrome bo_3 is less easy to understand. It has been shown previously that formate binding to *fast* cytochrome aa_3 causes changes in the EPR spectrum and reactivity toward other anionic ligands that are similar to those caused by the the so-called *fast* to *slow* transition (Moody *et al.*, 1991). The dinuclear center of cytochrome bo_3 does not readily revert to a *slow* conformer (Moody *et al.*, 1995). However, it is clear that formate-bound cytochrome bo_3 produces a species that has EPR signatures similar to *slow* cytochrome aa_3 (Watmough *et al.*, 1993; Moody *et al.*, 1995). The magnitude of these *slow* EPR signals is inversely proportional to the amplitude of the optical changes observed in the reaction of H_2O_2 with heme o_3 (Moody *et al.*, 1995). In addition we have shown that formate competes for the binding sites of both cyanide (a bridging ligand) binding and azide (a terminal ligand to Cu_B) binding. This suggests that formate binds to the dinuclear center to make both metal centers inaccessible to other ligands. Spectroscopic evidence would be consistent with direct formate binding to heme o_3 (Cheesman *et al.*, 1994), which taken together with the ligand competition experiments (Little *et al.*, 1996) implies that formate may bind as a bidentate ligand. A similar mode of ligation has been proposed for the binding of formate to the dinuclear center of bovine cytochrome aa_3 (Baker and Gullo, 1994).

CYANIDE CAN BRIDGE BETWEEN HEME o_3 AND Cu_B

In addition to being a potent inhibitor of cytochrome aa_3 in turnover, cyanide is useful as an indicator of the conformation of the dinuclear center (Baker *et al.*, 1987; Mitchell *et al.*, 1992). Further, because cyanide can form a bridge between heme a_3 and Cu_B (Thomson *et al.*, 1982), spectroscopic analysis of the cyano-adduct can yield potentially useful information about the organization of the dinuclear center. Cytochrome bo_3 binds a single equivalent of cyanide (K_D = 1–2 $\times 10^{-6}$M) with monophasic kinetics (NJW and RHL, unpublished). A second-order plot of k_{obs} as a

function of [KCN] is described by a hyperbola (Moody *et al.*, 1997), suggesting that the reaction is a two-step process in which the second process is rate limiting. The room-temperature NIR-MCD spectrum of the product of the reaction of cytochrome bo_3 with cyanide closely resembles that of cyanide-bound cytochrome aa_3 (Thomson *et al.*, 1982; Cheesman *et al.*, 1994). We therefore presume that cyanide also bridges the metals in the dinuclear center of cytochrome bo_3. Since we do not observe a spectroscopically distinct intermediate in the reaction of cytochrome bo_3 with cyanide, this may imply that cyanide binds first to Cu_B as a terminal ligand before rearranging to form a bridge.

NITRIC OXIDE AS A PROBE OF Cu_B

Nitric oxide (NO) is a useful probe of the dinuclear center not only because its binding properties resemble those of O_2, but it carries one unpaired electron which is a useful reporter of interactions with the paramagnetic dinuclear center. Reduced heme o_3 reacts with one equivalent of NO to form a typical EPR-detectable Fe(II) nitrosyl species which shows the nuclear hyperfine coupling arising from the ^{14}N atoms both of NO and the proximal histidine ligand (His^{421}) (Cheesman *et al.*, 1993). However, it is the reaction of NO with the oxidized state of the dinuclear center that provides fascinating insight into the reactivity of this site.

The ability of NO to react with Cu_B to form an even-spin cupric-nitrosyl species with $S = 0$ and release a magnetically isolated high-spin ferric heme visible by EPR spectroscopy is well established in bovine cytochrome aa_3 (Stevens *et al.*, 1979). The intensity of the high-spin heme EPR signal is dependent on the method used to prepare the enzyme (Stevens *et al.*, 1979; Palmer *et al.*, 1988). Moody has pointed out that the preparations that yield the highest occupancy of the magnetically isolated high-spin ferric heme state also contain significant levels of the chloride-ligated form of cytochrome aa_3 (Moody, 1997).

Recently, we have studied the reactions of *fast* cytochrome bo_3 with NO and shown that the dinuclear center can bind sequentially two equivalents of NO (Butler *et al.*, 1997). When the first equivalent of NO binds ($K_D = 2.3 \times 10^{-6}$ M), an EPR signal attributable to high-spin Fe(III) heme o_3 appears. This signal subsequently disappears as a second equivalent of NO binds ($K_D = 3.3 \times 10^{-5}$ M). Since the absorption spectrum clearly shows that heme o_3 remains in the high-spin Fe(III) state throughout, it is unlikely that NO binds to heme o_3. Therefore we have suggested that both NO molecules can bind to Cu_B.

Further evidence for the formation of a Cu_B(II) $(NO)_2$ species was obtained using the Cl^- adduct of cytochrome bo_3. MCD evidence suggests that in this adduct heme o_3 remains coordinated by histidine and water, suggesting that Cl^- binds to Cu_B. The addition of NO to the adduct leads to the appearance of a significant high-spin heme EPR signal. Hence the binding of Cl^- to Cu_B blocks the binding of a second NO molecule. This interpretation is consistent with the observations made earlier by Chan and co-workers (Stevens *et al.*, 1979) and could explain why only certain bovine cytochrome aa_3 preparations appeared reactive toward NO.

The formation of a Cu_B(II) $(NO)_2$ species is of particular interest given the close relationship of nitric oxide reductase (NOR), an enzyme of bacterial denitrification, to the superfamily of heme-copper oxidases (van der Oost *et al.*, 1994). NOR has no copper associated with it, but instead contains one equivalent of non-heme iron (NHI_B) which appears to be magnetically coupled to heme *b* to form a dinuclear center (Girsch and de Vries, 1997). Since cytochrome aa_3 can also reduce NO to N_2O (Brudvig *et al.*, 1980), it may be helpful to make comparisons between the heme-Cu_B and heme-NHI_B, dinuclear centers in order to try to understand the mechanism of the two-electron reduction of 2NO to N_2O. This reaction requires a N–N bond to be formed, and an oxygen atom abstracted and reduced to water. Having two NO molecules bound to Cu_B may place two activated nitrogen atoms in close proximity while the presence of nearby ferric heme may assist this process by the abstraction of the oxygen atom (Fig. 1).

THE DINUCLEAR CENTER OF FULLY OXIDIZED CYTOCHROME Bo_3 HAS UNUSUAL ERP PROPERTIES

Preparations of bovine C*c*O yield an enzyme whose dinuclear center can exist in one or more forms depending on the history of the sample (Moody, 1997). This difficulty appears to be less for cytochrome bo_3. The features of the *fast* conformer which suggest a homogeneous dinuclear center containing high-spin heme o_3 are accompanied by a series of unusual features in the perpendicular mode X-band EPR spectrum. In addition to a rhombic trio, g = 2.98, 2.26, 1.50,

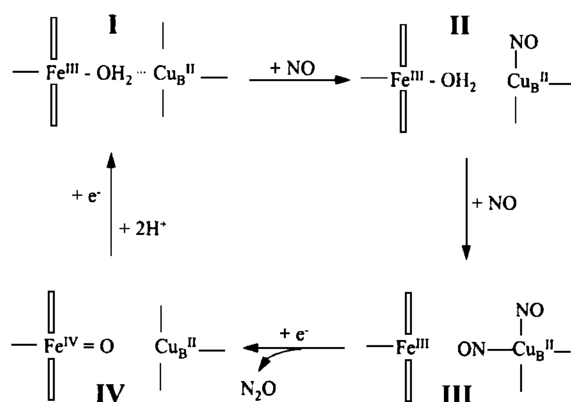

Fig. 1. A plausible mechanism for the reduction of NO to N_2O by cytochrome bo_3. *Fast* cytochrome bo_3 (I) sequentially binds two equivalents of NO at Cu_B (II and III). Input of an electron to the dinuclear center leads to formation N_2O. For this reaction to occur an oxygen atom must be abstracted by the heme, leading to formation of IV. Which is identical to the 555 nm (F) species, an intermediate of O_2 reduction. Finally, a second electron and two protons are required to reduce the oxyferryl species and return the enzyme to the *fast* conformer. This mechanism avoids the reaction of NO with Fe(II) heme o_3 which might be expected to be inhibitory. In principle the same mechanism could be used to describe the same reaction catalyzed by NOR with the two equivalents of NO binding to NHI_B; indeed, iron may be more effective at catalyzing formation of the N–N bond than is copper.

arising from the magnetically isolated low-spin ferric heme *b* (which are unchanged by the misincorpration of heme *O* into this site), a complex set of rather broad features across the field region 50–300 mT is seen. These are most apparent at ultralow temperatures (5 K) and microwave powers > 100 mW (Watmough *et al.*, 1993; Little *et al.*, 1996).

These spectral features are present with minor changes in position and intensity after binding of ligands such as F^-, Cl^- formate and azide at the dinuclear center, but are completely lost on the binding of CN^- which switches heme o_3 low-spin. Clearly these features arise from the interaction of high-spin ferric heme ($S = 5/2$) and Cu_B(II) ($S = 1/2$).

The nature and magnitude of this magnetic interaction between the components of the dinuclear site of C*c*O in several forms has been the subject of a large number of studies employing magnetic susceptibility (Moss *et al.*, 1978; Tweedle and Wilson, 1978; Barnes *et al.*, 1991), Mössbauer (Kent *et al.*, 1983; Rusnak *et al.*, 1987; Fee *et al.*, 1988), and EPR spectroscopies (Hagen, 1982; Dunham *et al.*, 1983; Brudvig *et al.*, 1986), and theoretical treatments ever since the proposal of Beinert thirty years ago that heme a_3 and Cu_B were magnetically coupled (Van Gelder *et al.*, 1967; Van Gelder and Beinert, 1969), but there is still no agreement as to the sign or magnitude of this interaction.

The essential EPR features of the dinuclear center of cytochrome bo_3 are retained in the presence of a range of ligands which either bind to the heme o_3, but leave it high-spin Fe(III), or which bind to Cu_B. The observation of these features depends critically upon the spacings of the electronic ground state-energy levels which are governed by parameters of the system such as the exchange coupling constant (J) and the zero-field splitting constant (D) at heme o_3. Therefore, in spite of the variable chemical nature of the ligands, the values of these parameters vary little between derivatives. Hence it is most unlikely that any one of these ligands is responsible for mediating coupling between the unpaired electrons of Cu_B(II) and Fe(III)o_3. There must exist an alternative route of spin coupling between the two metals which may be intrinsic to the protein structure. We are currently simulating the dinuclear center EPR spectra, measured in both perpendicular and parallel mode, using a theoretical model which will also account for the novel temperature dependence which we have observed for MCD features arising from heme o_3.

THE P AND F FORMS OF CYTOCHROME *Bo*$_3$

For over thirty years the reaction of fully reduced bovine cytochrome aa_3 with O_2 has been studied using the flow-flash approach (Gibson and Greenwood, 1963). The results of these studies, which have recently been reviewed in detail (Babcock and Wikström, 1992; Ferguson-Miller and Babcock, 1996), implicate at least two intermediates in the reaction; peroxide bound to heme a_3 and oxyferryl heme a_3. These are widely believed to correspond to two optically distinct species known as P and F that were first observed in the partial energy-dependent reversal of mitochondria (Wikström, 1981). The participation of P and F in the reduction of O_2 to water has recently been demonstrated by time-resolved electronic absorption spectroscopy at cryogenic temperatures (Morgan *et al.*, 1996). In this experiment P exists in the presence of Cu_B(I) and is referred to P_R (Morgan *et al.*, 1996) in order to distinguish this species from P_M which is the product of mixed valence (2-electron reduced) C*c*O with O_2 (Greenwood *et al.*, 1974) in which Cu_B remains oxidized.

Cytochrome bo_3 is a good system with which to resolve the issue of the chemical nature of the dinuclear center in the P and F states. Several recent studies show the oxygen reaction of cytochrome bo_3 to be broadly similar to that of cytochrome aa_3 (Svensson and Nilsson, 1993; Puustinen *et al.*, 1996) as well as having optically distinct species that correspond to the P and F states (Watmough *et al.*, 1994; Morgan *et al.*, 1995; Brittain *et al.*, 1996; Puustinen *et al.*, 1996). In addition the optical properties of protoporpyhrin IX favor the assignment of the spin, oxidation, and ligation states of heme o_3. However, the major obstacle confronting any spectroscopist wishing to characterize the P and F forms of cytochrome bo_3 is to generate them at sufficiently high occupancy long enough to complete the experiment.

One solution to this problem is to attempt to generate the intermediates by treating the enzyme with H_2O_2. This approach has been used with some success with bovine cytochrome aa_3, although the extent of the peroxide reaction and the relative yields of P and F depend upon a number of factors including the concentration of H_2O_2 used (Wrigglesworth, 1984), the method of preparation of the enzyme (Weng and Baker, 1991), and the pH at which the reaction takes place (Vygodina and Konstantinov, 1989).

Studies of the reaction of *fast* cytochrome bo_3 with micromolar concentrations of H_2O_2 have established that a single species is produced which is characterized by a red-shifted Soret, additional absorbance around 555 nm and the loss of the 624 nm C-T band (Svensson and Nilsson, 1993; Moody and Rich, 1994; Watmough *et al.*, 1994). This species was shown by MCD spectroscopy to contain oxyferryl heme o_3 (Cheesman *et al.*, 1994; Watmough *et al.*, 1994). The electronic absorption spectrum of cytochrome bo_3 after completing this reaction is identical to the species formed in the reaction of the fully (3-electron) reduced enzyme with O_2 (Puustinen *et al.*, 1996) and as such almost certainly corresponds to the F state of C*c*O.

This poses something of a difficulty as oxyferryl heme o_3 is the product of the three-electron reduction of O_2. However, a single equivalent of H_2O_2 which provides only two reducing equivalents reacts with the oxidized enzyme also to yield the same species. Scission of the O–O 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 cation radical (Moody and Rich, 1994; Watmough *et al.*, 1994). Since MCD spectroscopy indicated that the porphyrin macrocycle was not oxidized (Cheesman *et al.*, 1994), we speculated that the extra electron storage site was provided by a modified amino acid side-chain close to the dinuclear center.

The situation was further complicated by the observation of an intermediate in the peroxide reaction with characteristic absorbance at 582 nm which probably corresponds to P_M (Morgan *et al.*, 1995; Brittain *et al.*, 1996). This 582 nm species can react rapidly with a second equivalent of H_2O_2 to yield the oxyferryl product. Both this reaction as well as formation of the 582 nm species show a marked dependence upon pH (Brittain *et al.*, 1996). At first sight these results appear to be incompatible with the observation that only one equivalent of H_2O_2 is required to generate the oxyferryl species (Moody and Rich, 1994). However, these data can be reconciled by a branched reaction mechanism whose contributions differ according to the peroxide concentration used (Fig. 2).

The same 582 nm species appears to be the immediate product of the reaction of O_2 with fully reduced cytochrome bo_3 in which a conserved glutamic acid at position 286 of subunit I which is close to the dinuclear center has been changed to an alanine (Watmough *et al.*, 1997). The rate of electron transfer from heme *b* to the dinuclear center is unaffected by this mutation. These observations are important for two

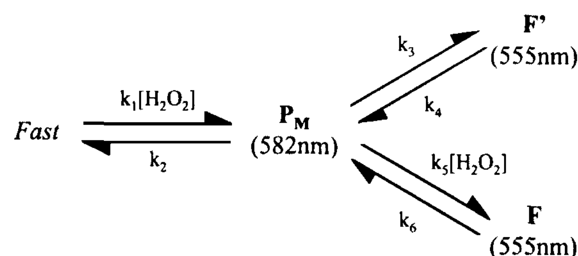

Fig. 2. The reactions of *fast* cytochrome bo_3 with H_2O_2. The branched reaction scheme proposed by Brittain *et al.* (1996) predicts that P_M is formed from *fast* cytochrome bo_3 in a bimolecular reaction with H_2O_2. This can subsequently be reduced to an oxyferryl species either by taking an electron from a site within the protein to form F′ as we have previously suggested (Watmough *et. al.*, 1994), or by reaction with a second equivalent of H_2O_2 to yield F. Simulations based upon this scheme indicate that when $[H_2O_2]$ is low ($k_3 >> k_1[H_2O_2]$, $k_3 >> k_5[H_2O_2]$), the reaction will proceed to F′ with very low transient occupancy of P_M. Such conditions have been used in previously reported titrations (Moody and Rich, 1994; Watmough *et al.*, 1994) and would account for the apparent stoichiometry (H_2O_2: cytochrome bo_3) of 1:1 (Moody and Rich, 1994). However, at high $[H_2O_2]$ ($k_1[H_2O_2]$ and $k_5[H_2O_2] > k_3$) the reaction will proceed via P_M to the F state and the expected stoichiometry will be 2:1. The fundamental rate constants used for these simulations were as follows: $k_1 = 2 \times 10^3\ M^{-1}s^{-1}$; $k_2 = 0.1\ s^{-1}$; $k_3 = 1\ s^{-1}$; $k_4 = <0.001\ s^{-1}$; $k_5 = 2 \times 10^2\ M^{-1}\ s^{-1}$; $k_6 = 0.5\ s;^{-1}$;

reasons. First, they suggest that formation of the 582 nm species from O_2 requires two electrons, and that it is at the same formal redox state as peroxide bound to ferric heme, this intermediate being artificially stabilized by the mutation. Second, it raises the possibility that the transfer of the third electron to heme *o* (presumably from Cu_B) requires a proton to be donated to the dinuclear center via glutamate-286, and that the rate of uptake of this proton is rate limiting for electron transfer.

Transfer of the third electron has been associated with controlled oxygen scission. However, this mechanism assumes the correct assignment of P_R as a bound peroxide species (for a more complete discussion see Ferguson-Miller and Babcock, 1996). For a number of proteins containing protoporphyrin IX, heme-bound peroxide has been proposed as an early intermediate in their reaction mechanisms. These include heme peroxidases (Rodriguez-Lopez *et al.,* 1996) and heme-oxygenases (Wilks and Ortiz de Montellano, 1993). Rodriguez-Lopez and colleagues (Rodriguez-Lopez *et al.,* 1996) described an intermediate in the reaction of a mutant HRP with hydrogen peroxide with a blue-shifted Soret maximum which they assigned to a bound neutral peroxide species. Unfortunately its appearance was too transient to allow examination by EPR spectroscopy.

Recently, we have identified an intermediate in the reaction of H_2O_2 with a number of mutant myoglobins in which the distal pocket histidine-64 has been changed (Brittain *et al.,* 1997). This intermediate has an electronic absorption spectrum typical of low-spin ferric heme but a rhombic EPR spectrum with novel *g* values. If further spectroscopic analysis confirms our assignment of this species as histidine/peroxide coordinated heme, then it may provide a useful model for the P_R form of cytochrome bo_3. A species of the form $Fe(III)–O_2^- :: Cu_B(I)$ should be detectable by EPR spectroscopy since it contains an odd number of unpaired electrons.

CONCLUDING REMARKS

Our decision to work on cytochrome bo_3 was based on the belief that because it contained protoporphyrin IX as a prosthetic group its magneto-optical properties would be easier to understand than the heme A containing *Cc*Os. Fortunately the properties of the heme O, subsequently shown to be present in the dinuclear center, are sufficiently similar to those of protoporphyrin IX not to alter this proposition. Our observation that Cu_B can bind two equivalents of NO is especially interesting, since not only does this provide a useful probe of the spectroscopically inert Cu_B, but also emphasizes the close relationship between NOR and heme-copper oxidases.

Carefully documenting the EPR properties of *fast* cytochrome bo_3 and its derivatives has led us to consider an alternative theoretical treatment of electronic properties of the dinuclear center. This will complement the structural models that have lately become available and provide insight into the mechanism of heme-copper oxidases. Our demonstration by MCD spectroscopy that the 555 nm species, which is equivalent to the F form of *Cc*O, contains oxyferryl heme helped to understand the mechanism of O_2 reduction. Subsequently, both the Helsinki group and ourselves have seen a second species with characteristic absorbance at 582 nm in the facile reaction of the oxidized enzyme with H_2O_2 which corresponds to the P_M state of *Cc*O. We intend to characterize this 582 nm species, which is also an intermediate of O_2 reduction, to determine if it is associated with peroxide-bound Fe(III) heme o_3 or a related compound in the same formal redox state. This discrimination would help us to understand when O–O scission takes place and if an extra electron storage site in the dinuclear center is required for catalysis.

ACKNOWLEDGMENTS

We are most grateful to Professor R. B. Gennis, University of Illinois, USA for the gift of strains of *E. coli* which overexpress cytochrome bo_3. The UK Biotechnology and Biological Sciences Research Council have provided core support for the Centre for Metalloprotein Spectroscopy and Biology (BO 3032-1). BBSRC also supported much of the work reviewed here through the award of project grants (GR/F/83983 and BO 02073) to CG and AJT. NJW is in receipt of a Career Development Award from the Wellcome Trust (042103/Z/94/Z)

REFERENCES

Au, D. C.-T., and Gennis, R. B. (1987). *J. Bacteriol.* **169**, 3237–3242.
Babcock, G. T., and Wikström, M. (1992). *Nature* 301–309.
Baker, G. M., and Gullo, S. M. (1994). *Biochemistry* **33**, 8058–8066.

Baker, G. M., Noguchi, M., and Palmer, G. (1987). *J. Biol. Chem.* **262**, 595–604.
Barnes, Z., Babcock, G. T., and Dye, J. L. (1991). *Biochemistry* **30**, 7597–7603.
Brittain, T., Baker, A. R., Butler, C. S., Little, R. H., Lowe, D. J., Greenwood, C., and Watmough, N. J. (1997). *Biochem. J.* **326**, 109–115.
Brittain, T., Little, R. H., Greenwood, C., and Watmough, N. J. (1996). *FEBS Lett.* **399**, 21–25.
Brudvig, G. W., Stevens, T. H., and Chan, S. I. (1980). *Biochemistry* **19**, 5275–5285.
Brudvig, G. W., Morse, R. H., and Chan, S. I. (1986). *J. Magn. Reson.* **67**, 189–201.
Butler, C. S., Seward, H. E., Greenwood, C., and Thomson, A. J. (1997). *Biochemistry* **36**, 16259–16266.
Cheesman, M. R., Watmough, N. J., Pires, C. A., Turner, R., Brittain, T., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1993). *Biochem. J.* **289**, 709–718.
Cheesman, M. R., Watmough, N. J., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1994). *Eur. J. Biochem.* **219**, 595–602.
Dunham, W. R., Sands, R. H., Shaw, R. W., and Beinert, H. (1983). *Biochim. Biophys. Acta* **748**, 73–85.
Farrar, J. A., Neese, F., Lappalainen, P., Kroneck, P. M. H., Saraste, M., Zumft, W. G., and Thomson, A. J. (1996). *J. Am. Chem. Soc.* **118**, 11501–11514.
Fee, J. A., Zimmerman, B. H., Nitsche, C. I., Rusnack, F., and Münck, E. (1988). *Chem. Scr.* **28A**, 75–78.
Ferguson-Miller, S., and Babcock, G. T. (1996). *Chem. Rev.* **96**, 2889–2907.
Gibson, Q. H., and Greenwood, C. (1963). *Biochem. J.* **86**, 541-.
Girsch, P., and de Vries, S. (1997). *Biochim. Biophys. Acta* **1318**, 202–216.
Greenwood, C., Wilson, M. T., and Brunori, M. (1974). *Biochem. J.* **137**, 205–215.
Hagen, W. R. (1982). *Biochim. Biophys. Acta* **708**, 82–98.
Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M. J., Babcock, G. T., and Gennis, R. B. (1993). *J. Bioenerg. Biomembr.* **25**, 121–136.
Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995). *Nature* **376**, 660–669.
Kent, T. A., Young, L. J., Palmer, G., Fee, J. A., and Münck, E. (1983). *J. Biol. Chem.* **258**, 8543–8546.
Little, R. H., Cheesman, M. R., Thomson, A. J., Greenwood, C., and Watmough, N. J. (1996). *Biochemistry* **35**, 13780–13787.
Mitchell, P. (1987). *FEBS Lett.* **222**, 235–245.
Mitchell, R., Brown, S., Mitchell, P., and Rich, P. R. (1992). *Biochim. Biophys. Acta* **1100**, 40–48.
Moody, A. J. (1997). *Biochim. Biophys. Acta* **1276**, 6–20.
Moody, A. J., and Rich, P. R. (1994). *Eur. J. Biochem.* **226**, 731–737.
Moody, A. J., Cooper, C. E., and Rich, P. R. (1991). *Biochim. Biophys. Acta* **1059**, 189–207.
Moody, A. J., Cooper, C. E., Gennis, R. B., Rumbley, J. N., and Rich, P. N. (1995). *Biochemistry* **34**, 6838–684.
Moody, A. J., Mitchell, R., Jeal, A. E., and Rich, P. R. (1997). *Biochem. J.* **324**, 743–752.
Moody, A. J., Butler, C. S., Watmough, N. J., Thomson, A. J. and Rich, P. R. (1998). The reaction of halides with pulsed cytochrome *bo* from *Escherichia coli*. *Biochem. J.* in press.
Morgan, J. E., Verkhovsky, M. I., Puustinen, A., and Wikstöm, M. (1995). *Biochemistry* **34**, 15633–15637.
Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1996). *Biochemistry* **35**, 12235–12240.
Moss, T. H., Shapiro, E., King, T. E., Beinert, H., and Hartzell, C. (1978). *J. Biol. Chem.* **253**, 8072–8073.
Palmer, G., Baker, G. M., and Noguchi, M. (1988). *Chem. Scr.* **28A**, 41–46.
Puustinen, A., Morgan, J. E., Verkhovsky, M., Thomas, J. W., Gennis, R. B., and Wikström, M. (1992). *Biochemistry* **31**, 10363–10369.
Puustinen, A., Verkhovsky, M. I., Morgan, J. E., Belevich, N. P., and Wikström, M. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 1545–1548.
Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996). *J. Bioinorg. Chem.* **1**, 136–142.
Rusnak, F. M., Münck, E., Nitsche, C. I., Zimmerman, B. H., and Fee, J. A. (1987). *J. Biol. Chem.* **262**, 16328–16332.
Saraste, M. (1990). *Q. Rev. Biophys.* **23**, 331–366.
Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., and Anraku, Y. (1994). *J. Biol. Chem.* **269**, 28908–28912.
Stevens, T. H., Brudvig, G. W., Bocian, D. F., and Chan, S. I. (1979). *Proc. Natl. Acad. Sci. USA* **76**, 3320–3324.
Svensson, M., and Nilsson, T. (1993). *Biochemistry* **32**, 5442–5447.
Thomson, A. J., Eglinton, D. G., Hill, B. C., and Greenwood, C. (1982). *Biochem. J.* **207**, 167–170.
Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawah-Itoh, A., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995). *Science* **269**, 1069–1074.
Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawah-Itoh, A., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996). *Science* **272**, 1136–1144.
Tweedle, M. F., and Wilson, L. J. (1978). *J. Biol. Chem.* **253**, 8065–8071.
van der Oost, J., deBoer, A. P. N., deGier, J.-W. L., Zumft, W. G., Stouthamer, A. H., and van Spanning, R. J. M. (1994). *FEMS Microbiol. Lett.* **121**, 109.
Van Gelder, B. F., and Beinert, H. (1969). *Biochim. Biophys. Acta* **189**, 1–24.
Van Gelder, B. F., Orme-Johnson, W. H., Hansen, R. E., and Beinert, H. (1967). *Proc. Natl. Acad. Sci. USA* **58**, 1073–1079.
Vygodina, T., and Konstantinov, A. (1989). *Biochim. Biophys. Acta* **973**, 390–398.
Watmough, N. J., Cheesman, M. R., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1993). *FEBS Lett.* **319**, 151–154.
Watmough, N. J., Cheesman, M. R., Greenwood, C., and Thomson, A. J. (1994). *Biochem. J.* **300**, 469–475.
Watmough, N. J., Katsonouri, A., Little, R., Osborne, J. P., Furlong-Nickels, E., Gennis, R. B., Brittain, T. and Greenwood, C. (1997). A conserved glutamic acid in helix VI of cytochrome bo_3 influences a key step in oxygen reduction. *Biochemistry* **36**, 13736–13742.
Weng, L., and Baker, G. M. (1991). *Biochemistry* **30**, 5727–5733.
Wikström, M. (1981). *Proc. Natl. Acad. Sci. USA* **78**, 4051–4054.
Wilks, A., and Ortiz de Montellano, P. R. (1993). *J. Biol. Chem.* **268**, 22357–22362.
Wrigglesworth, J. M. (1984). *Biochem. J.* **217**, 715–719.