A Conserved Glutamic Acid in Helix VI of Cytochrome bo_3 Influences a Key Step in Oxygen Reduction†

Nicholas J. Watmough,*,‡ Andromachi Katsonouri,§ Richard H. Little,‡ Jeffrey P. Osborne,§ Elizabeth Furlong-Nickels,§ Robert B. Gennis,§ Thomas Brittain,|| and Colin Greenwood‡

Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norfolk, NR4 7TJ, United Kingdom, Department of Chemical & Life Sciences, University of Illinois, 600 S. Mathews, Urbana, Illinois 61801, and Biochemistry & Molecular Biology, School of Biological Sciences, University of Auckland, Auckland, New Zealand

Received June 16, 1997; Revised Manuscript Received August 29, 1997⊗

ABSTRACT: We have compared the reactions with dioxygen of wild-type cytochrome bo_3 and a mutant in which a conserved glutamic acid at position-286 of subunit I has been changed to an alanine. Flow-flash experiments reveal that oxygen binding and the rate of heme-heme electron transfer are unaffected by the mutation. Reaction of the fully (3-electron) reduced mutant cytochrome bo_3 with dioxygen yields a binuclear center which is substantially in the P (peroxy) state, not the well-characterized F (oxyferryl) state which is the product of the reaction of the fully reduced wild-type enzyme with dioxygen [Puustinen, A., et al. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1545−1548]. These results confirm that proton uptake is important in controlling the later stages of dioxygen reduction in heme-copper oxidases and show that E286 is an important component of the channel that delivers these protons to the active site.

Cytochrome bo_3, a quinol oxidase from *Escherichia coli*, is a member of a conserved family of protonmotive heme-copper terminal oxidases which includes mitochondrial C*c*O[1] (*1*). Although cytochrome bo_3 lacks the dinuclear copper center Cu_A, the close sequence homology between subunits I and II of cytochromes bo_3 and C*c*O (*2*) leave little doubt that the two oxidases are structurally very similar. Subunit I of cytochrome bo_3 contains three metal centers, heme *b* which is a magnetically isolated low-spin species, heme o_3, and a copper ion known as Cu_B. Heme o_3 is a high-spin species that is magnetically coupled to Cu_B to form a binuclear center which is the site of oxygen reduction and the presumed site of proton translocation.

Like other members of the superfamily, cytochrome bo_3 catalyzes the four electron reduction of dioxygen to water, a reaction which has been studied for many years using the flow-flash approach (*3*). The results of these studies on C*c*O, which have recently been reviewed in detail (*4, 5*), 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[2] that were first observed in the partial energy dependent reversal of mitochondria (*6*). 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 (*7*). In this experiment, P exists in the presence of Cu_B(I) and is referred to as P_R (*7*) in order to distinguish this species from P_M, which is the product of the reaction of mixed valence (2-electron reduced) C*c*O with O_2 (*8*) in which Cu_B remains oxidized.

Several recent studies show the oxygen reaction of cytochrome bo_3 to be broadly similar to that of C*c*O (*9, 10*), as well as having optically distinct species that correspond to the P and F states (*9, 11*−*13*). Studying oxygen reduction in cytochrome bo_3 may offer a number of advantages compared to C*c*O. First, the presence of three rather than four redox centers should in principle give rise to simpler reaction kinetics. Secondly, the optical properties of the extensively characterized protoporpyhrin IX favor the assignment of the spin, oxidation, and ligation-states of heme o_3 (*14*). 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 (*15*) and a well-characterized library of site-directed mutants constructed (*16*).

Used in conjunction with the recently solved three-dimensional structures of the C*c*Os from bovine mitochondria (*17, 18*) and *Paracoccus denitrificans* (*19*), these site-directed mutants of cytochrome bo_3 provide the basis for elucidating

† Supported by grants from the Wellcome Trust (042103/Z/94/Z) and the Royal Society, to N.J.W., from the U.K. BBSRC (BO 3032-1) and the E.U. (Biotechnology of Extremophiles Contract Bio2-CT93-0274) to C.G., and the U.S. Department of Energy (DE-FG02-87ER13716) to R.B.G.

* To whom correspondence should be addressed. Tel: +44 (1603) 592179. Fax: +44 (1603) 592250. E-mail: n.watmough@uea.ac.uk.

‡ University of East Anglia.

§ University of Illinois.

|| University of Auckland.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1997.

[1] Abbreviations: C*c*O, cytochrome *c* oxidase; DBQ, decylbenzoquinone; $DBQH_2$, decylbenzoquinol; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FRCO, fully reduced carbon monoxy adduct; HPLC, high-performance liquid chromatography; k_{obs}, observed rate constant; LM, dodecyl-β-D-maltoside; MCD magnetic circular dichroism spectroscopy; PMS, phenazine methosulfate, TAPS, (*N*-tris[hydroxy-methyl]-3-aminopropanesulfonic acid.

[2] P and F: the terms P and F are usually used to denote two spectroscopically distinct forms of C*c*O that are one reducing equivalent apart. The P form is characterized in C*c*O by characteristic absorbance at 607 nm and the F form by absorbance at 580 nm. The P form contains oxygen bound to the high-spin heme reduced to the level of peroxide, while in the F form oxygen has been reduced to the level of an oxyferryl [Fe(IV)-oxo] species. Unless stated otherwise in the text, we use these terms to indicate the degree of reduction of oxygen, rather than describe the chemical structure of the intermediate.

S0006-2960(97)01434-7 CCC: $14.00

the role(s) of specific residues in heme-copper oxidases. We have been particularly interested in the role of the glutamic acid at position-286[3] in transmembrane helix VI of subunit I. This highly conserved residue lies close to the binuclear center, at the head of a channel which may conduct protons from the cytoplasmic face of the membrane to the heme-Cu_B binuclear center (*18*, *19*). Mutation of this residue in both cytochrome bo_3 and the C*c*O from *Rhodobacter sphaeroides* appears to abolish uptake of a proton associated with formation of compound F (*20*, *21*).

Here, we report the reaction of fully reduced cytochrome bo_3 containing the E286A mutation with oxygen. The mutation has no effect upon oxygen binding, or heme-heme electron transfer. However, the product of the reaction contains a form of heme o_3 characterized by an absorption band at 582 nm, which corresponds to the P form of cytochrome bo_3 (*11*, *12*). These results confirm this conserved glutamic acid as a key component of one of the proton channels in heme-copper oxidases. In addition, they show this residue to have a pivotal role in controlling the P to F transition by modulating the delivery of protons to the binuclear center.

MATERIALS AND METHODS

Cell Growth and Enzyme Preparation. The purification of the cytochrome bo_3 from the *E. coli* strain RG145, which overexpresses cytochrome bo_3 and lacks cytochrome *bd* (*15*), was as previously described (*22*). Enzyme in which the low-spin site is fully occupied by heme *B* was prepared from the strain GO105/pJRhisA. This strain contains a plasmid with a histidine tag engineered to the carboxyl-terminus of subunit II, which allows rapid isolation of homogenous cytochrome bo_3 using metal chelate affinity chromatography (*23*). The binuclear centers of the *fast*[4] oxidized enzyme isolated from each of these sources are spectroscopically identical and behave identically toward exogenous ligands.

Biochemical Characterization of Cytochrome bo_3 Preparations. The steady-state turnover rates (catalytic activities) were determined spectrophotometrically by measuring the rate of oxidation of the water soluble substrate analog $DBQH_2$ at 280 nm ($\epsilon_{red-ox} = 10.5 \times 10^3\ M^{-1}cm^{-1}$) using a Hewlett-Packard 8453 photodiode array spectrophotometer. Assays were made in 1.0 mL in buffer containing 50 mM Hepes, 1 mM EDTA, and 0.01% (w/v) LM, pH 7.5 at 25 °C. DBQ was reduced with sodium borohydride as described by Rieske (1967) (*24*), and stored at −20 °C in DMSO containing 10 mM HCl prior to use. Reactions were initiated by the addition of 1 nM enzyme and the $DBQH_2$ concentration was varied in the range 1.5−40 μM.

Extraction and analysis of hemes and quinone and their subsequent analysis by HPLC was as previously described (*23*).

[3] Numbering of residues: the numbers given in the text refer to residues in subunit I of *Escherichia coli* cytochrome bo_3. The conserved glutamic acid which is E286 in the cytochrome bo_3 sequence corresponds to E242 in subunit I of the bovine mitochondrial cytochrome aa_3 and E278 in subunit I of the enzyme from *Paracoccus denitrificans*.

[4] Fast cytochrome bo_3: As prepared in our laboratories, this is defined as having a Soret maximum of 406.5 nm, a charge transfer band centered upon 624 nm, broad EPR signals with distinctive features at $g = 9.1$, $g = 3.7$, and $g = 2.68$ and binding 10 mM cyanide monophasically with a k_{obs} of 0.27 s^{-1}.

Spectroscopy. The concentration of samples of cytochrome bo_3 used for kinetic experiments was calculated from electronic absorption spectra of the oxidized enzyme [$\epsilon_{407} = 1.83 \times 10^5\ M^{-1}\ cm^{-1}$ (*22*)] recorded using either a Hewlett-Packard 8453 photodiode array spectrophotometer or a Hitachi U3000 spectrophotometer.

Sample Preparation. All samples were prepared in 50 mM TAPS, 0.1 mM EDTA, and 0.01% (w/v) LM, pH 8.0, unless stated otherwise. In order to determine the product of the reaction of cytochrome bo_3 with oxygen, approximately 2.0 mL of enzyme (20−40 μM) containing 60 units of catalase was placed in the barrel of a 2.5 mL glass syringe that was attached with a luer-lock fitting to the sample handling unit of an Applied Photophysics DX18.MV kinetic spectrophotometer. The top of the syringe was sealed with a rubber bung fitted with two needles, one of which was attached to a supply of oxygen-free wet Argon which was flowed over the sample for about 20 min. After this time, sufficient dithionite (10 mM dithionite in 50 mM sodium pyrophosphate pH 9.0) was transferred anaerobically to the enzyme solution to give a final concentration of dithionite of 2−300 μM. The extent of reduction was checked after 5 min by mixing the sample with oxygen free buffer in the stopped-flow spectrophotometer.

For flow-flash experiments, samples of FRCO cytochrome bo_3 were prepared by diluting stock enzyme to approximately 15 μM in buffer and bubbling the sample with oxygen free CO for 10 min. Ascorbate and PMS were added to final concentrations of 10 mM and 0.5 μM, respectively, and the vessel sealed and the sample allowed to become anoxic. After 45 min the sample was transferred anaerobically to a 10 mL gas-tight syringe which was then attached to the sample handling unit of an Applied Photophysics DX18.MV kinetic spectrophotometer. Saturated solutions of oxygen were prepared by equilibrating buffer with oxygen for a minimum of 30 min prior to use. Lower oxygen concentrations were obtained by diluting this stock solution in gas-tight syringes with oxygen-free buffer.

Stopped-Flow Kinetic Measurements. The immediate product of the reaction of fully reduced cytochrome bo_3 with oxygen was determined in an Applied Photophysics Bio-Sequential DX.17MV stopped-flow spectrophotometer using a 1 cm pathlength cell. Detection was with an Applied Photophysics photodiode array accessory. A minimum of 100 spectra were collected per experiment with a time resolution of 3.8 ms/spectrum.

Flow-Flash Kinetic Measurements. The reaction of oxygen under pseudo first order conditions with FRCO cytochrome bo_3 was measured using a LKS.50 laser kinetic spectrophotometer (Applied Photophysics, Leatherhead, U.K.) modified to permit flow-flash measurements. The reaction was initiated by a pulse of laser light (6 ns, 100 mJ, 532 nm) provided by a SL282G Nd:YAG laser equipped with frequency doubling optics (Spectron Laser Systems, Rugby, U.K.). Absorption changes at a single wavelength were recorded using a Hewlett-Packard 54520A digitising oscilloscope.

Mixing the enzyme with oxygen prior to photolysis was done using the sample handling unit of the Bio-Sequential DX.18MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, U.K.), under the control of the LKS 50 software. To achieve the widest possible range of oxygen

Table 1: Relationship of the Amplitude of the Fast Phase of the Oxygen Reaction to Enzyme Type

	ubiquinone-8[a] (mol/mol enzyme)	ratio[b] heme B:heme O	cytochrome bo_3 (%)	fast phase[c] (%)
wild-type (RG145)	0.05	14:86	28	45
wild-type (pJRhisA)	0.27	46:54	92	85
E286A (pJRhisA)	0.1	54:46	100	89

[a] Determined by reverse-phase HPLC. [b] Determined by reverse-phase HPLC. [c] Percentage of total DA430 accounted for by fast phase in flow-flash experiment (data from Figure 2A).

concentrations, enzyme and oxygen were mixed in a ratio of 1:5.

Analysis of Kinetic Data. Experimental traces recorded at a single wavelength were exported as ASC II files and analyzed as the sum of one or two exponentials using TableCurve 2D for Windows (Jandel Scientific, San Rafael, CA). A variety of kinetic mechanisms were investigated to determine if they could account for the observed kinetic characteristics exhibited by the fully reduced cytochrome bo_3 with oxygen. Each mechanism was explored in the time domain using the GEAR variable step numerical integration method to generate theoretical time courses (*25*). The time courses were then fitted to the experimental data by varying the rate constants in the particular scheme being tested. When an adequate mechanism was found, the theoretical time courses were synthesized for oxygen concentrations from 50 μM to 1 mM to allow comparison with the experimentally determined concentration dependence.

Time-resolved kinetic difference spectra in the Soret region were constructed from the amplitudes of kinetic traces recorded between 380 and 460 nm using the LKS.50 software. These spectra were analyzed globally at all times and all wavelengths simultaneously using an Acorn A5000 personal computer running the SVD and global exponential fitting routines found in the software package Glint (Applied Photophysics, Leatherhead, U.K.). Diode array spectra and kinetic traces at a single wavelength from the stopped-flow experiments were exported as ASCII files and replotted in Axum v4.0 (TriMetrix Inc, Seattle, WA).

RESULTS

In the absence of bound quinone (Table 1) (*9, 26*), purified cytochrome bo_3 can only store three electrons. Therefore, it is expected that the immediate product of the reaction with oxygen has a binuclear center in the F state, e.g., containing oxyferryl heme o_3 (*9*). To investigate the effects the E286A mutation on dioxygen reduction, we determined the electronic absorption spectra of the immediate products of the reactions with dioxygen of both fully reduced wild-type cytochrome bo_3 and the mutant.

Mixing fully reduced wild-type cytochrome bo_3 with oxygen in the stopped-flow spectrophotometer yields a species whose difference spectrum calculated with respect to *fast* cytochrome bo_3 shows maxima at 532 and 558 nm and a minimum at 624 nm (Figure 1). This is identical to the difference spectrum reported by Puustinen and coworkers (*9*) for the product of the same reaction. It is also similar in form and intensity to the difference spectrum generated by treating fast oxidized cytochrome bo_3 with hydrogen peroxide (*10*), which was subsequently characterized by MCD spectroscopy as an oxyferryl species (*13, 14*) and likely corresponds to the F state of C*c*O.

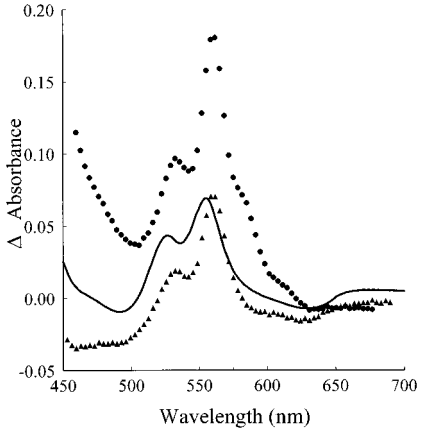


FIGURE 1: Reaction of fully reduced wild-type (▲) and mutant (E286A) (●) cytochrome bo_3 with dioxygen. The experiments were done as described in Materials and Methods. The spectra recorded after 3.8 ms are shown as difference spectra with respect to oxidized *fast* wild-type cytochrome bo_3 (see Results). The concentrations of the reactants after mixing were; wild-type cytochrome bo_3 (10 μM), E286A (15.25 μM), and O_2 (500 μM). To aid comparison the wild-type spectrum has been scaled by a factor of 1.525. The solid line is the difference spectrum (oxyferryl minus oxidized) of 15.25 μM fast cytochrome bo_3 after reaction with hydrogen peroxide.

The visible region difference spectrum of the F state generated in the peroxide reaction has maxima variously reported as being at 526 nm/555 nm (*10, 13*), 526 nm/556 nm (*27*), and 526 nm/558 nm (*7*). It is possible that the red-shift of the visible region maxima in the difference spectrum of the F state formed in the oxygen reaction is the result of the limited spectral resolution of the diode array detector. However, close inspection of the difference spectra associated with the F states formed in the two different reactions indicates other minor differences (Figure 1). The difference spectrum associated with the F state of cytochrome bo_3 formed in the peroxide reaction shows a distinct minimum at 494 nm and a shoulder at approximately 468 nm (*13, 27*). These features are absent when the F state is generated in the oxygen reaction, the difference spectrum showing a broad minimum between 450 and 495 nm (Figure 1) (*9*). Upon the basis of the difference extinction coefficent of the peroxide generated F state ($\Delta\epsilon_{555} = 4.5 \times 10^3$ M^{-1} cm^{-1}), we estimate that this state is >90% populated after the oxygen reaction is complete.

The reaction of the fully reduced E286A mutant with dioxygen gave a strikingly different result. The difference spectrum calculated with respect to the fast conformer of wild-type cytochrome bo_3 shows a peak at 561 nm and shoulders at 532 and 582 nm (Figure 1).[5] Unfortunately, this difference spectrum is not readily identifiable as a single form of cytochrome bo_3. However, the obvious transition centered upon 582 nm is diagnostic of the P form of the enzyme (*11*). Compared with authentic P, the difference spectrum shows extra intensity centered upon 561 nm, which

[5] As isolated the E286A mutant contained varying proportions of the P and F states which we were unable to remove by "pulsing" the enzyme according to the method of Moody and Rich (1994) (*27*). To obtain the difference spectrum presented in Figure 1 we used oxidized wild-type enzyme containing a similar amount of cytochrome bo_3 as a reference spectrum.

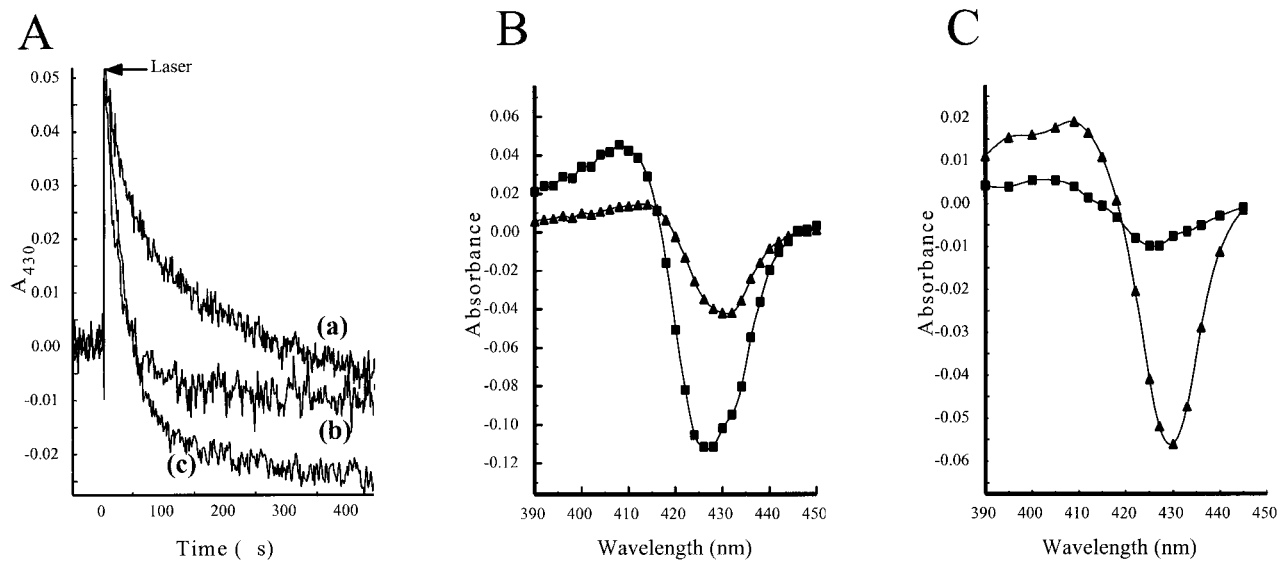


FIGURE 2: (A) Absorbance changes associated with the reaction of FRCO wild-type, RG145 (a), E286A (b), and wild-type, GO105/pJRhisA (c) cytochrome bo_3 after initiation of the reaction with a laser pulse. The traces have been normalized to the amplitude of the laser induced jump in absorbance. (B) Kinetic difference spectra calculated with respect to the post-photolysis state (fully reduced enzyme) in the Soret region of the reactions of cytochrome bo_3 (▲) and cytochrome oo_3 (■) with O_2. The enzyme used in this experiment was purified from the strain RG145. (C) Kinetic difference spectra calculated with respect to the post-photolysis state (fully reduced enzyme) in the Soret region of the reactions of cytochrome bo_3 (▲) and cytochrome oo_3 (■) with O_2. The enzyme used in this experiment was purified from the strain GO105/pJRhisA.

we attribute to reduced low-spin heme *b*. The values of $\Delta\epsilon_{560}$ of ferrous heme *b* and P are 2×10^4 M^{-1} cm^{-1} and 4×10^3 M^{-1} cm^{-1}, respectively (*11*, *28*). From these values we estimate that 20−25% of the heme *b* remains reduced after reaction with oxygen which is in good agreement with our flow-flash experiments (see below).

We wished to determine if the mutation only blocks the P to F transition, or if other processes involved in a single turnover of the enzyme are affected. To address this issue, we used the flow-flash technique (*3*) to compare the reactions of the FRCO forms of mutant and wild-type enzyme with dioxygen. The time course of this reaction monitored at 430 nm depends upon the enzyme preparation (Figure 2A). Wild-type preparations containing only cytochrome bo_3 exhibit an initial increase in absorbance due to CO photolysis followed by a monophasic decay ($k_{obs} = 3.3 \times 10^4$ s^{-1}). In contrast, the decrease in absorbance shown by wild-type preparations containing a large amount of cytochrome oo_3 show two kinetic phases. The amplitude of each phase correlated with the relative proportions of the two forms determined analytically (Table 1) (*20*). The E286A mutant exhibited a time course described by a single exponential of the same k_{obs} as the fast phase of wild-type cytochrome bo_3, but with a total decrease in absorbance that was only 82% of the wild-type preparation (Figure 2A).

To explain this last observation, it is necessary to understand which process(es) are monitored at 430 nm. It is known that the principle effect of heme O misincorporation into the low-spin site is to decrease the rate of heme-heme electron transfer (*29*). Since k_{obs} associated with cytochrome bo_3 is about an order of magnitude greater than that associated with cytochrome oo_3, it is likely that the main contribution to the decrease in A_{430} is the oxidation of the low-spin heme. This is confirmed by the kinetic difference spectra presented in Figure 2 (panels B and C). The kinetic difference spectrum associated with the fast (cytochrome bo_3) phase is red-shifted by about 6 nm relative to that of of the slow (cytochrome oo_3) phase. This difference corresponds to that reported between the reduced minus oxidized spectra of low-spin heme *b* and low-spin heme *o* (*28*).

Having established that the decrease in absorbance at 430 nm is mainly due to the oxidation of low-spin heme, we now consider the decreased amplitude associated with the E286A mutant (Figure 2A). The preparation of E286A consisted entirely of cytochrome bo_3 (Table 1), and therefore, the decreased amplitude cannot be due to the mutation selectively inhibiting heme-heme electron transfer of cytochrome oo_3. Instead, it clearly indicates that a fraction (*c* 18%) of the heme *b* remains reduced.

Since the mutation did not affect the rate of heme-heme electron transfer, we wished to ascertain if it affected the binding of oxygen to the reduced binuclear center. In keeping with previous studies (*10*), we found that k_{obs} exhibited a hyperbolic dependence on oxygen concentration (Figure 3). Moreover, the E286A mutation appeared to have little effect on substrate binding exhibiting the same dependence of k_{obs} on oxygen concentration as wild-type cytochrome bo_3 (Figure 3).

On the basis of these data, we have developed a kinetic model to account for both the dependence of k_{obs} on oxygen concentration and the impairment of the P to F transition by the mutation. This model, which can be applied to both cytochrome bo_3 and cytochrome oo_3 (Scheme 1), requires only the second step to be irreversible. This step depends upon the type of heme occupying the low-spin site (cytochrome bo_3, $k_2 = 4.5 \times 10^4$ s^{-1}; cytochrome oo_3, $k_2 = 4.0 \times 10^3$ s^{-1}) and is assigned to heme-heme electron transfer.

Prior to oxidation of the low-spin heme, oxygen binds to the reduced binuclear center to yield a ferrous-oxy species (*30*). The forward (k_1) and reverse rate (k_{-1}) constants assigned to this step are calculated to be $(1-2) \times 10^8$ M^{-1} s^{-1} and 3×10^3 s^{-1} and are consistent with the values assigned to the equivalent step in the reaction of oxygen with C*c*O (*3*, *31*, *32*). A reasonable fit to the wild-type and mutant data may be obtained by treating the reaction as a two-step

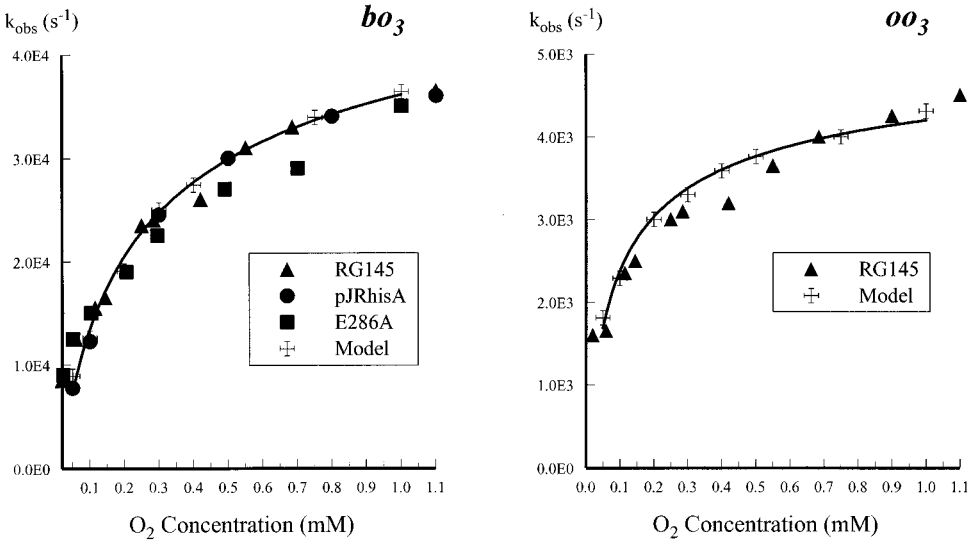
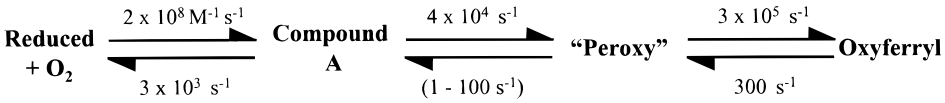


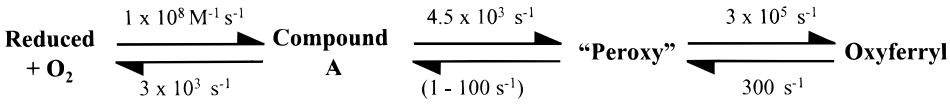
FIGURE 3: The dependence of k_{obs} for the reactions of cytochrome bo_3 and cytochrome oo_3 with O_2. Observed data points are shown as filled symbols according to the inset. Data points simulated using the fundamental rate constants given in Scheme 1, using the GEAR process (see Materials and Methods), are shown as cruciforms (see inset). The solid lines are approximate fits of polynomials to the simulated data in order to aid comparison with the observed data points.

Scheme 1

Cytochrome bo_3



Cytochrome oo_3



process and fitting a simple binding curve (*10*, *33*). However, our experience is that simulated values of k_{obs} best fitted the data when a third step is included. This view is reinforced by our experiments with the E286A mutant, which clearly demonstrate that after oxygen binding and heme-heme electron transfer has taken place the binuclear center is in the P form. This implies that in wild-type enzyme there must be a third process which requires transfer of both a proton and an electron to the binuclear center in order to complete the P to F transition.

DISCUSSION

With the exception of the caa_3 type oxidase from *Thermus thermophilus* (*34*), the glutamic acid which is at position-286 in the subunit I of cytochrome bo_3 is absolutely conserved in eukaryotic and eubacterial cytochrome *c* and quinol oxidases (*2*). However, it is not conserved in the ancient (in terms of evolution) cytochrome cbb_3 oxidases (*1*) nor in the archeal heme-copper oxidase (SoxABC) from *Sulfolobus acidocauldarius* (*35*), which may reflect different strategies used by these enzymes to move protons to the binuclear center (*36*). Structural studies place E286 close to the binuclear center. Together with a conserved aspartic acid (D132) and a chain of solvent molecules it forms a channel able to conduct protons to the active site. To gain further evidence in support of this proposed function for E286, we studied the reaction of a mutant form of cytochrome bo_3 in which it had been changed to an alanine residue.

A flow-flash study of the *R. sphaeroides* C*c*O in which the equivalent glutamate had been changed to glutamine showed two differences compared to wild-type enzyme. Firstly, only one reaction phase ($k_{obs} = 3 \times 10^4$ s^{-1}) which is attributed to oxidation of the hemes was seen in the mutant enzyme rather than the three reaction phases seen in wild-type C*c*O. Secondly, the uptake of a proton which is temporally associated with the second phase in wild-type C*c*O is blocked in the mutant (*21*). Similar results were obtained with the E286A variant of *E. coli* cytochrome bo_3. Again the uptake of a proton was blocked by the mutation, although heme-heme electron transfer appeared normal (*20*). This observation was complicated because the preparation of mutant enzyme used contained a high proportion of cytochrome oo_3, but showed a rate of electron transfer normally associated with homogeneous cytochrome bo_3.

We have re-examined the reaction with dioxygen using cytochrome bo_3 in which the glutamic acid at position-286 of subunit I has been changed to an alanine residue. We chose to work with the E286A mutant rather than the more conservative E286Q in order to facilitate comparison between our work and that of Svensson-Ek et al. (*20*). We expressed mutant cytochrome bo_3 from the plasmid pJRhisA(E286A) in a protease deficient background (*37*) and purified it with the aid of a histidine-tag on the carboxyl-terminus of subunit

II (*23*). Heme analysis showed that, unlike the preparation used by Svensson-Ek and co-workers, the preparation of E286A used in the present study contained little or no cytochrome oo_3. It also differed in that it had somewhat greater catalytic activity (*c* 20% of wild-type enzyme) compared to the 5% reported by Svensson-Ek et al. (*20*). However, in keeping with the previous study, we demonstrated the rate of heme-heme electron transfer to be normal and, in addition, could demonstrate that the mutation does not affect the binding of oxygen to the reduced binuclear center.

In order to determine the effect of the blocked proton uptake reported in the E286A mutant, we followed the reactions of fully (3-electron) reduced wild-type cytochrome bo_3 and the mutant with dioxygen in a stopped-flow spectrophotometer. After 3.6 ms, wild-type enzyme yields a species with spectral properties that are broadly similar to the oxyferryl species that we have previously characterized by MCD spectroscopy (*13, 14*). The slight spectral differences between the F state reported here and that formed in the reaction of oxidized cytochrome bo_3 with hydrogen peroxide are attributed to differences in the organisation of the binuclear center during turnover.

In contrast, the E286A mutant yields a mixture of species, one of which is characterized by a weak absorbance band at 582 nm. This transition at 582 nm is uniquely associated with the P state of cytochrome bo_3 (*11*). Unfortunately the details of the P state spectrum are obscured by extra absorbance at 560 nm, which is due to reduced heme *b*. That as much as 20% of the heme *b* stays reduced is not surprising. There is some evidence in C*c*O that the second electron required by the ferrous-oxy species to yield the P state can be supplied by either the low-spin heme or Cu_B. However, the relative rates of electron transfer from these two centers to the binuclear center differ by about an order of magnitude (*38*). Thus, the majority of the enzyme is in the form b^{3+}, $\{o_3^{3+}\text{-}O_2^{2-}\}$, Cu_B^{1+}, while the remainder is of the form, b^{2+}, $\{o_3^{3+}\text{-}O_2^{2-}\}$, Cu_B^{2+}; the ratio being largely dictated by the ratio of the two appropriate rate constants (*5, 38*).

Our results suggest that the majority of the 582 nm absorbing species is formed after oxidation of the low-spin heme, but before formation of the oxyferryl species. Formation of the 582 nm species requires just two electrons and, as such, is formally equivalent to the P (607 nm) form of C*c*O. However, our experiments using the E286A mutant show that this species can exist when all three electrons are in the binuclear center. Since P has long been thought to be a ferric peroxide species (*4*), we presume that the third electron must remain on Cu_B. This intermediate of oxygen reduction, in which Cu_B remains reduced, was designated P_R by Morgan and co-workers (1996) in order to differentiate it from P_M, the 607 nm species of C*c*O formed in the reaction of the mixed-valence (two-electron reduced) form of the enzyme with oxygen (*8*), or the reaction of the oxidized enzyme with hydrogen peroxide where Cu_B remains oxidized (*39*). It is now established that P_M is formed under similar circumstances in cytochrome bo_3 (*12*), although it is apparently only stabilized at high pH (*11*).

If the 582 and 558 nm (oxyferryl) species are equivalent to the P and F forms of CcO then these forms of cytochrome bo_3 will be one reducing equivalent apart. However, the number of electrons present in the binuclear center during their existence is the same, three, which is sufficient to reduce oxygen to an oxyferryl species. Formation of P_R from ferrous-oxy heme o_3 requires two electrons, and this appears to be the endpoint of the reaction of the binuclear center with dioxygen in the E286A mutant. Mutation of this residue also blocks the uptake of a proton (*20, 21*). Therefore, it would appear that transfer of the third electron to heme o_3 is normally governed by the rate of uptake of that proton by the binuclear center and that E286 is the immediate proton donor. This adds considerable weight to the view that heme-copper oxidases function by rapidly transferring an electron from the low-spin center to kinetically trap weakly bound oxygen as peroxide (*29*) and that subsequent steps, involved in the reduction of peroxide to water, which are coupled to energy transduction, are controlled by proton movements.

The transfer of the third electron has long been thought to be associated with controlled oxygen scission; however, this scenario assumes the correct assignment of P_R as a bound peroxide species [the reader is once again referred to Ferguson-Miller and Babcock (1996) (*5*) for a more complete discussion]. This has always been a difficult issue to resolve in the cytochrome aa_3 type oxidases as there are no other heme *a* containg proteins that can function as models and, thus far, analysis. In contrast, there are a number of protoporphyrin IX containing proteins in which heme bound peroxide has been proposed as an intermediate in their reaction mechanisms. These include peroxidases (*40*) and and heme-oxygenases (*41*), although the spectral properties of such species have proved somewhat elusive. Rodriguez-Lopez and colleagues (*40*) reported an intermediate in the reaction of a mutant HRP with hydrogen peroxide with a blue-shifted Soret maximum which they assign to a bound neutral peroxide species. Unfortunately, its appearance was so transient as to preclude examination by EPR spectroscopy.

More recently, one of our laboratories has reported an intermediate in the reaction of hydrogen peroxide with some mutant myoglobins in which the distal pocket histidine-64 has been changed (*42*). This intermediate has an electronic absorption spectrum typical of low-spin ferric heme and a rhombic EPR spectrum with novel *g* values. If further spectroscopic analysis confirms our provisional assignment of this species as histidine/peroxide coordinated heme, then it will provide a useful model for the P_R form of cytochrome bo_3. If this intermediate does contain a binuclear center of the form $Fe(III)\text{-}O_2^{2-}::Cu_B(I)$, then the histidine/peroxide coordinated heme *o* should be detectable by EPR spectroscopy. However, if such an EPR active heme moiety is not associated with the 582 nm species, it will raise the possibility that the third electron is stored at a site other than Cu_B or that oxygen scission takes place prior to transfer of the third electron to heme *o*. We are currently engaged in experiments designed to resolve this issue.

REFERENCES

1. 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.
2. Saraste, M. (1990) *Q. Rev. Biophys. 23*, 331−366.
3. Gibson, Q. H., and Greenwood, C. (1963) *Biochem. J. 86*, 541.
4. Babcock, G. T., and Wikström, M. (1992) *Nature*, 301−309.
5. Ferguson-Miller, S., and Babcock, G. T. (1996) *Chem. Rev. 96*, 2889−2907.
6. Wikström, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4051−4054.

7. Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1996) *Biochemistry 35*, 12235−12240.
8. Greenwood, C., Wilson, M. T., and Brunori, M. (1974) *Biochem. J. 137*, 205−215.
9. Puustinen, A., Verkhovsky, M. I., Morgan, J. E., Belevich, N. P., and Wikström, M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1545−1548.
10. Svensson, M., and Nilsson, T. (1993) *Biochemistry 32*, 5442−5447.
11. Brittain, T., Little, R. H., Greenwood, C., and Watmough, N. J. (1996) *FEBS Lett. 399*, 21−25.
12. Morgan, J. E., Verkhovsky, M. I., Puustinen, A., and Wikstöm, M. (1995) *Biochemistry 34*, 15633 - 15637.
13. Watmough, N. J., Cheesman, M. R., Greenwood, C., and Thomson, A. J. (1994) *Biochem. J. 300*, 469−475.
14. Cheesman, M. R., Watmough, N. J., Gennis, R. B., Greenwood, C., and Thomson, A. J. (1994) *Eur. J. Biochem. 219*, 595−602.
15. Au, D. C.-T., and Gennis, R. B. (1987) *J. Bacteriol. 169*, 3237−3242.
16. 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.
17. 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.
18. 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.
19. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature 376*, 660−669.
20. Svensson-Ek, M., Thomas, J. W., Gennis, R. B., Nilsson, T., and Brzezinski, P. (1996) *Biochemistry 35*, 13673−13680.
21. Svensson-Ek, M. (1996) *Oxygen Reduction by Terminal Oxidases; The Coupling Between Electrons and Proton Transfer*, Ph.D. Thesis, Göteborg University and Chalmers Institute of Technology Göteborg
22. 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.
23. Rumbley, J. N., Furlong Nickels, E., and Gennis, R. B. (1997) *Biochim. Biophys. Acta 1340*, 131−142.
24. Rieske, J. S. (1967) *Methods Enzymol. 10*, 239−245.
25. Blackmore, R. S., Brittain, T., and Greenwood, C. (1990) *Biochem. J. 271*, 457−461.
26. Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., and Anraku, Y. (1994) *J. Biol. Chem. 269*, 28908−28912.
27. Moody, A. J., and Rich, P. R. (1994) *Eur. J. Biochem. 226*, 731−737.
28. Morgan, J. E., Verkhovsky, M. I., Puustinen, A., and Wikström, M. (1993) *Biochemistry 32*, 11413−11418.
29. Verkhovsky, M. I., Morgan, J. E., Puustinen, A., and Wikström, M. (1996) *Nature 380*, 268−270.
30. Poole, R. K., Salmon, I., and Chance, B. (1994) *Microbiology 140*, 1027−1034.
31. Hill, B. C., and Greenwood, C. (1984) *Biochem. J. 218*, 913−921.
32. Sucheta, A., Georgiadis, K. E., and Einarsdóttir, O. (1997) *Biochemistry 36*, 554−565.
33. Orii, Y., Mogi, T., Kawasaki, M., and Anraku, Y. (1994) *FEBS Lett. 352*, 151−154.
34. Mather, M. W., Springer, P., Hensel, S., Buse, G., and Fee, J. A. (1993) *J. Biol. Chem. 268*, 5395−5408.
35. Lubben, M., Kolmerer, B., and Saraste, M. (1992) *EMBO J. 11*, 805−812.
36. de Gier, J.-W. L., Schepper, M., Reijnders, W. N. M., van Dyck, S. J., Slotboom, D. J., Warne, A., Saraste, M., Krab, K., Finel, M., Stouthamer, A. H., van Spanning, R. J. M., and van der Oost, J. (1996) *Mol. Microbiol. 20*, 1247−1260.
37. Gottesman, S. (1990) *Methods Enzymol. 185*, 119−129.
38. Han, S., Ching, Y., and Rousseau, D. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8408−8412.
39. Wrigglesworth, J. M. (1984) *Biochem. J. 217*, 715−719.
40. Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) *J. Bioinorg. Chem. 1*, 136−142.
41. Wilks, A., and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem. 268*, 22357−22362.
42. 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.

BI971434I