Identification of a "Peroxy" Intermediate in Cytochrome bo_3 of *Escherichia coli*†

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ABSTRACT: The respiratory heme−copper oxidases catalyze the reduction of dioxygen to water and link this chemistry to proton translocation. The main subgroups of the enzyme family are the cytochrome *c* oxidases and the quinol oxidases. For the cytochrome *c* oxidases, several key intermediates have been described in the oxygen reaction. Two of these (suggested to be "peroxy" and "ferryl" species) are also produced in the reaction of the oxidized enzyme with hydrogen peroxide. However, only a single product (a "ferryl" species) has been reported for the reaction of hydrogen peroxide with the quinol oxidase cytochrome bo_3 from *Escherichia coli.* The same "ferryl" species has also been reported to be produced when two-electron reduced cytochrome bo_3 reacts with oxygen, whereas this reaction leads to the "peroxy" intermediate in the cytochrome *c* oxidases. Consequently, the oxygen reaction has been considered to be different in the two enzyme subgroups. Here we show that both the peroxide reaction and the reaction of the two-electron reduced enzyme with oxygen actually result in primary formation of a hitherto unreported "peroxy" species in cytochrome bo_3. This intermediate subsequently relaxes into the "ferryl" species which has been described previously. We conclude that the oxygen reaction is similar in the cytochrome *c* and quinol oxidases.

Enzymes of the heme−copper oxidase family play a central role in aerobic respiration. They reduce oxygen to water and direct the energy from this redox process into maintaining a proton electrochemical membrane gradient, which in turn supplies the energy for ATP synthesis and thus for most of the work done by the cell.

Until recently, the study of these enzymes was focused almost exclusively on mammalian cytochrome *c* oxidase (cytochrome aa_3),[1] but today, cytochrome bo_3 of *Escherichia coli* has become one of the most intensively studied members of this family. This has happened because cytochrome bo_3 has been found to be very similar to cytochrome *c* oxidases in structure and overall function and because *E. coli* has proved more amenable to genetic manipulation than previous enzyme sources.

Although cytochrome bo_3 takes its electrons from quinol, rather than from cytochrome *c*, it is a close evolutionary relative of cytochrome aa_3. The protein primary sequences of these enzymes are strongly homologous, and both enzymes reduce oxygen to water employing similar redox cofactors. In both types of enzyme, the chemistry of oxygen reduction takes place at a heme−copper site, and in both there is an additional (low-spin) heme which serves as the immediate electron donor to the oxygen-reduction site.[2]

All heme−copper oxidases conserve energy by "vectorial chemistry" (Mitchell, 1976), that is, the chemical reactions carried out by the enzyme are arranged in such a way that charged reactants and products are taken up or released specifically on one side of the membrane or the other. For example, the protons that are consumed when oxygen is turned to water come exclusively from the "inner" side of the membrane. In addition, both cytochrome *c* oxidases and quinol oxidases pump protons across the membrane and thus achieve a higher stoichiometry of charge translocation than would be possible by vectorial chemistry alone (Wikström, 1977; Puustinen et al., 1989).

These structural and functional parallels would seem to be strong evidence of an essentially identical mechanism at work in cytochrome aa_3 and cytochrome bo_3. Recently, however, functional and kinetic studies on cytochrome bo_3 have raised questions as to whether this is the case and, in particular, whether the chemistry of oxygen reduction is the same in both types of enzyme (see below).

The reaction of fully reduced cytochrome aa_3 with oxygen has been studied intensively by fast kinetic methods. A number of intermediates in the oxygen-reduction reaction have been identified by resonance Raman monitoring of the flow−flash reaction of cytochrome aa_3 with oxygen. These include a ferrous−oxygen compound, a ferric−peroxy compound, an oxy−ferryl compound, and a ferric-hydroxy compound [see Babcock and Wikström (1992)].

Another approach to studying the oxygen reaction has been to try to trap the enzyme at intermediate points in the reaction by starting with fewer than the full four electrons needed to complete the reduction of oxygen to water. Chance and co-workers (1975) showed that the two-electron reduced (CO−

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[1] A good introduction can be found in a collection of "mini reviews" edited by S. Ferguson-Miller (1993).

[2] Nomenclature: In cytochrome bo_3, the low-spin heme is known as heme *b* or Fe_b (the chemical entity is heme **B**). The oxygen-binding heme is known as heme o_3 or Fe_{o3} (chemically heme **O**). The corresponding hemes of cytochrome aa_3 are heme *a* (Fe_a) and heme a_3 (Fe_{a3}), both chemically hemes **A**. In both enzymes, the copper ion of the oxygen-reduction site is known as Cu_B. Cytochrome *c* oxidases contain an additional copper site known as Cu_A, which serves as the initial electron acceptor from cytochrome *c*. Cu_A is not found in the quinol oxidases (Puustinen et al., 1991). Cytochrome bo_3 is categorized under cytochrome *c* oxidase (aa_3) is EC 1.9.3.1.

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mixed valence) enzyme reacts with oxygen to produce a species with a 607 nm absorbance maximum,[3] while Witt and co-workers (1986) found that reaction of the three-electron reduced enzyme with oxygen leads to a species with a 580 nm peak.

The 580 nm species was originally identified by Wikström (1981) in experiments on intact mitochondria. He found that the oxygen reaction could be partially reversed by applying a backward driving force in the form of a membrane potential together with an oxidizing redox potential. In this reverse reaction, the first one-electron step led to the 580 nm species, which was assigned as oxy−ferryl (intermediate "F"), while the second one-electron step produced the 607 nm species, which was assigned as a ferric−peroxy [intermediate :P"; see also Wikström and Morgan (1992)]. However, Proshlyakov and co-workers (1994) have recently presented resonance Raman data which suggests that the electronic structure of Fe_{a3} in the 607 nm species may be oxy−ferryl. If so, the two cytochrome aa_3 intermediates, "P" and "F", would be very much like compounds "**I**" and "**II**" of peroxidases, two oxy−ferryl species that lie one oxidizing equivalent apart (Erman et al., 1989).

The 607 and 580 nm species can also be formed by the reaction of hydrogen peroxide with cytochrome aa_3. The 607 nm species is produced first, probably by the binding of a molecule of peroxide at the oxygen-reduction site. In a subsequent step, a second molecule of peroxide can convert this 607 nm species into the 580 nm species (Wrigglesworth, 1984; Witt & Chan, 1987; Weng & Baker, 1991; Vygodina et al., 1993). In the Soret region, the spectra of the 607 and 580 nm species are very similar (Vygodina & Konstantinov, 1988).

When researchers began to study the chemistry of oxygen reduction in cytochrome bo_3, they found that they could produce a compound analogous to the 580 nm species, but no analog of the 607 nm species could be found. In the reaction with hydrogen peroxide only one product was observed: a species with an absorbance maximum at 556 nm. This compound was originally reported by Svensson and Nilsson (1993) and later assigned as an oxy−ferryl on the basis of its magnetic circular dirchroism spectrum (Cheesman et al., 1994; Watmough et al., 1994). The reaction of oxygen with the CO−mixed valence compound of cytochrome bo_3 gave a product with the same visible spectrum (Watmough et al., 1994; Moody & Rich, 1994).

The failure to find an intermediate corresponding to the 607 nm species in either of these two reactions, both of which produce this compound in cytochrome aa_3, has raised doubts as to the similarity of the oxygen chemistry in the two types of enzyme. These enzymes are so closely related in structure and function that we might expect all important aspects of mechanism to be the same. This is especially true of the oxygen chemistry, since both enzymes not only reduce oxygen to water but use this reaction to drive the pumping of protons. Differences in the chemistry of oxygen reduction could thus have much wider implications for the mechanism of these enzymes.

We now report, however, that a counterpart of the 607 nm species of cytochrome aa_3 has been found in cytochrome bo_3. This intermediate can be observed in the reaction of oxygen with the CO−mixed valence form of the enzyme as well as the reaction of the oxidized enzyme with hydrogen peroxide. We conclude that there is no strong evidence that the mechanism of oxygen reduction in cytochrome bo_3 is fundamentally different from that in cytochrome aa_3.

MATERIALS AND METHODS

Reagents. Hydrogen peroxide, Merck; ethyl hydrogen peroxide, Accurate Chemical and Scientific (Westbury, NY); catalase, Sigma type C-30 (19 000 units mL^{-1}).

Cytochrome bo_3. In order to facilitate preparation of cytochrome bo_3 a C-terminal "histidine tag" has been genetically added to subunit II of the enzyme (bacterial strain GO 105 with the plasmid pJRHisA; J. N. Rumbley and R. B. Gennis, personal communication). This "his tag" makes it possible to isolate homogeneous cytochrome bo_3 enzyme in one step, using a metal chelate affinity column and elution with an imidazole gradient.

Cells were grown aerobically in a fermenter (Medical Braun) containing 15 L of 1% (v/v) lactate minimal medium and harvested in the midexponential growth phase. Cells (40 g) were washed with 500 mM NaCl, 10 mM Tris-HCl, pH 7.5, 20 mL/g of cells. Lysozyme treatment was done in 200 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 20 mL/g of cells. After an osmotic lysis step in 10 mM potassium phosphate, 2 mM EDTA, 20 mL/g of cells, membranes were washed with 5 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 7.8. The washed membranes were solubilized with 1% (w/v) *n*-dodecyl β-D-maltoside (DM)[4] (Anatrace) in 5 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 7.8, and 5−10 mg of protein/mL. After ultracentrifugation the supernatant was applied to a Ni-NTA-agarose (Qiagen) column, which had been equilibrated with 0.05% DM, in 5 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 7.8. The column was washed with 3 bed volumes of the equilibration buffer. The enzyme was eluted from the column with a 5−110 mM imidazole gradient (10 bed volumes). Fractions containing pure four-subunit cytochrome bo_3 were pooled and concentrated using a stirred Amicon cell with a YM 100 membrane. In order to lower the concentrations of imidazole and NaCl in the preparations, samples were diluted 10-fold in 20 mM Tris-HCl, pH 7.8, with detergent and concentrated again.

Sample Preparation. The data in Figures 1 and 2 were acquired with enzyme which had been activated by the "pulsing" procedure of Moody and Rich (1994), in which the fully reduced enzyme is reoxidized and then dialyzed to remove the remaining reductant. Using a global exponential fitting program, we were able to find the transition between intermediates **I** and **II** (see results) in enzyme which had not been through any activation procedure, but the amplitude was small, apparently because the initial reaction was rate limiting in most of the enzyme sample.

Stopped-Flow Kinetic Measurements. Rapid mixing experiments were carried out using a stopped-flow spectrophotometer equipped with a diode array detector which can record spectra as rapidly as one per millisecond (Unisoku Instruments).

[3] In all cases peak position wavelengths refer to difference spectra between the species in question and the fully oxidized enzyme.

[4] Abbreviations: COMV, CO−mixed valence (two-electron reduced, CO-bound form of heme−copper oxidase enzyme); DM, *n*-dodecyl β-D-maltoside; PMS, phenazine methosulfate.

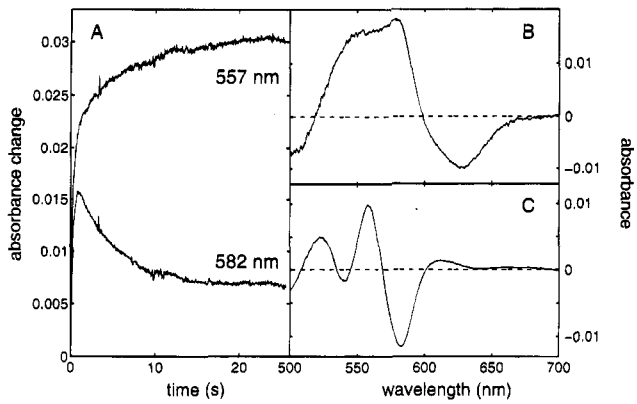

FIGURE 1: Reaction of cytochrome bo_3 with hydrogen peroxide. A, upper trace, 557 nm; lower trace, 582 nm. B, spectrum of first component from global exponential fit ($k_1 = 3.55\ s^{-1}$); C, spectrum of second component from global exponential fit ($k_2 = 0.21\ s^{-1}$). Cytochrome bo_3, 7 μM, hydrogen peroxide, 5 mM; pH 9.0, 100 mM CHES, 0.1% DM (all concentrations *after* mixing). The baseline for the spectra is the fully oxidized enzyme. (Since the spectra are phase components from the kinetic fit, their amplitudes may not give correct extinction coefficients; see Materials and Methods.)

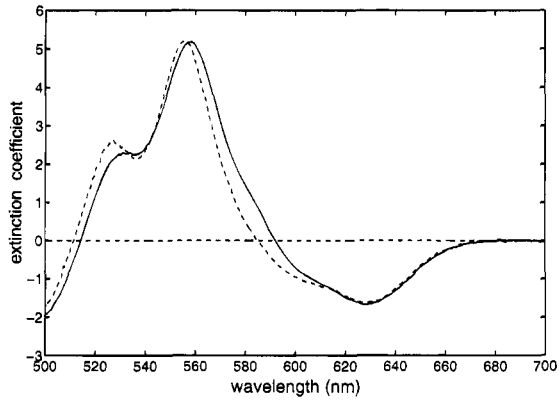

FIGURE 2: Final products of the reaction of cytochrome bo_3 with hydrogen peroxide (solid line) and ethyl hydrogen peroxide (dashed line). Hydrogen peroxide, 5 mM; ethyl hydrogen peroxide, 10 mM, other conditions as in Figure 1. The spectra are the "constant" terms from global exponential fits [$c_0(\lambda)$] and thus represent the steady state toward which the initial reactions converge; see Materials and Methods.

CO−Mixed Valence Reoxidation. The CO−mixed valence compound (COMV) was made by incubation of anaerobic enzyme under an atmosphere of CO. For details of anaerobic sample handling procedures see Morgan et al. (1993) and references therein. After the COMV enzyme had formed, almost all of the CO in the sample was removed by exchanging several times with argon. This was done under low light in order to retain CO bound to the enzyme and thus the uniform electron distribution in the sample. The spectral changes following reoxidation were followed using the same diode array measuring system used for the stopped-flow experiments. COMV samples were prepared in four-window cuvettes. A 100 μL volume of oxygen-saturated water was mixed into the enzyme solution under low light conditions, after which the cuvette was returned to the spectrometer sample holder and data acquisition started. A few seconds later, the sample was illuminated with a camera flash to photolyze the Fe_{o3}−CO bond and ensure complete reaction. A BG3 filter was used between the flash and the sample, and an OG515 filter was used between the sample and the detectors. Just prior to this measurement, the results of photolyzing the same sample with no added oxygen were recorded in order to have an accurate measure of the amount of CO-bound enzyme and the amount of CO, for calibration purposes.

Data Analysis. Basic data matrix manipulations and presentation were done with Matlab (The Mathworks, South Natick, MA). The data from the stopped-flow instrument is a surface of absorbance values covering a 512 × 512 point time/wavelength plane. Decomposition of these surfaces was done using SPLMOD (Provencher & Vogel, 1983), a global exponential fitting program. The SPLMOD algorithm works from the assumption that the kinetics at any wavelength in the surface can be described by a limited number (1−9) of exponential processes

$$A(\lambda,t) = c_0(\lambda) + c_1(\lambda)\exp(-k_1/t) + c_2(\lambda)\exp(-k_2/t) + c_3(\lambda)\exp(-k_3/t) + \ldots$$

where $A(\lambda,t)$ is the absorbance at a given time (t) and wavelength (λ). The rate constants, k_1, k_2, k_3, etc., will be the same for the kinetic data for any wavelength. The amplitudes of the exponential processes will be different at different wavelengths, and the vectors $c_1(\lambda)$, $c_2(\lambda)$, $c_3(\lambda)$, etc. are the spectra of processes which take place in the corresponding exponential process. $c_0(\lambda)$ is a "constant term" spectrum which corresponds to the final state toward which the entire system is decaying.

In practice, SPLMOD was used to determine the rate constants (k_1, k_2, k_3, etc.) from a small number of wavelengths (usually 32−64) distributed uniformly across the spectrum, and then the amplitudes (c_0, c_1, c_2, etc.) were determined for all 512 wavelengths using a simple fitting routine.

RESULTS

When oxidized cytochrome c oxidase (cytochrome aa_3) reacts with excess hydrogen peroxide, a two-phase reaction is observed (Orii, 1988). As described in the introduction, in the first phase of this reaction, the oxidized enzyme is converted to a species with an absorbance maximum[3] at 607 nm. In the second phase, this 607 nm species is converted to a species with an absorbance maximum at 580 nm.

$$\underset{\mathbf{0}}{\text{oxidized enzyme}} \rightarrow \underset{\mathbf{I}}{\text{607 nm species ("P")}} \rightarrow \underset{\mathbf{II}}{\text{580 nm species ("F")}}$$

A qualitatively similar two-phase reaction is seen when cytochrome bo_3 is mixed with hydrogen peroxide (Figure 1A): at 582 nm (lower trace), there is a fast increase in absorbance ($k_1 = 3.55\ s^{-1}$), followed by a slower decrease ($k_2 = 0.21\ s^{-1}$). These two kinetic processes can also be discerned at 557 nm (upper trace), although here the absorbance increases in both phases. The spectra of the fast and slow phases are shown in panels B and C of Figure 1, respectively. The kinetic spectrum of the first phase has an absorbance maximum at 582 nm and a shoulder near 550 nm (Figure 1B). The kinetic spectrum of the second phase has maxima at 523 and 557 and a minimum at 582 (Figure 1C).

Thus, the reaction of peroxide with cytochrome bo_3 appears to produce two sequential products, as it does in the case of cytochrome aa_3. We will refer to these products

as **I** and **II**. We can find the spectra of these intermediates from the results of the two-exponential global fit (see Materials and Methods): Since the baseline for these measurements is the oxidized enzyme (**O**), the spectrum of intermediate **I** should have the same shape, to a first approximation, as the kinetic spectrum for the first (**O** → **I**) phase, shown in Figure 1B. The spectrum of intermediate **II** should be approximately the same as the spectrum of the final product of the two-phase reaction, shown as the solid line in Figure 2 [this is $c_0(\lambda)$ for a two-exponential global fit; see Materials and Methods]. This spectrum has an absorbance maximum near 558 nm and is similar to spectra of the compound reported to be the sole product of this reaction, and assigned as an oxy−ferryl species (Watmough et al., 1994; Moody & Rich, 1994). The spectrum has a small shoulder in the region of 580 nm, which suggests that there is some contribution of intermediate **I** here (see below).

In terms both of its place in the reaction sequence and its spectrum, intermediate **I** bears very much the same relationship to **II** as the 607 nm species in cytochrome aa_3 does to the 580 nm species in that enzyme. In fact, the spectra of intermediates **I** and **II** look very much like those of the 607 and 580 nm species, respectively, shifted about 25 nm to higher energy, which is reasonable given that this reaction is taking place at heme **O** rather than at heme **A**. Two other parallels to cytochrome aa_3 should be noted. First, intermediates **I** and **II** both show spectral minima near 630 nm, indicating that the Fe_{o3}^{3+} charge transfer band is absent. The 607 and 580 nm species of cytochrome aa_3 show a corresponding minimum near 655 nm (Weng & Baker, 1991). Secondly, the Soret spectra of **I** and **II** are very similar (not shown).

In the case of cytochrome aa_3, addition of excess hydrogen peroxide is believed to lead to a steady state reaction in which the 580 nm species reacts slowly with peroxide to form the fully oxidized enzyme, which can then bind another molecule of peroxide and be returned to the 607 nm species (Vygodina et al., 1993). Results of an experiment in which ethyl hydrogen peroxide was mixed with cytochrome bo_3 suggest that a similar steady state reaction may take place in this enzyme: When ethyl hydrogen peroxide was added to cytochrome bo_3, we were able to observe two spectral transitions much like those seen with hydrogen peroxide. However, the spectrum of the final product [dashed line, Figure 2; $c_0(\lambda)$; see Materials and Methods] lacks the shoulder near 580 nm seen in the hydrogen peroxide product. This suggests that the steady state concentrations of intermediates in the reaction with ethyl hydrogen peroxide may be slightly different than with hydrogen peroxide. This is borne out by the finding that the ratio of the first two rate constants with ethyl hydrogen peroxide is 2.5 [$k_1 = 0.93$ s^{-1}; $k_2 = 0.36$ s^{-1}, [EtOOH] = 10 mM], whereas the ratio with hydrogen peroxide is 17 [$k_1 = 3.55$ s^{-1}, $k_2 = 0.21$ s^{-1}, [H2O2] = 5 mM]. Thus, it seems that intermediate **I** is converted to intermediate **II** relatively more quickly in the ethyl hydrogen peroxide reaction, consistent with the smaller contribution from intermediate **I** in the final spectrum.

A similar progression of intermediates is also seen when the CO−mixed valence form of cytochrome bo_3 is photolyzed in the presence of oxygen (Figure 3). The initial product, resolved on a millisecond time scale (Figure 3A), has an absorbance maximum at 582 nm and is very similar to the product of the first phase of the peroxide reaction (**I**).

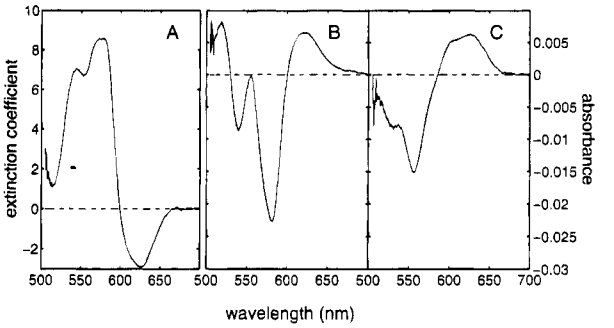

FIGURE 3: Progression of intermediates after reaction of CO−mixed valence cytochrome bo_3 with oxygen. A, spectrum immediately after reoxidation. B, first component from global exponential fit ($k_1 = 7.0 \times 10^{-3}$ s^{-1}); C, second component from global exponential fit ($k_2 = 5.8 \times 10^{-4}$ s^{-1}). Cytochrome bo_3, 3.9 μM (this concentration is based on the amplitude of photolysis measured a few minutes before reoxidation), CO, 30 μM (based on recombination rate measured a few minutes before reoxidation); pH 9.5, 75 mM CHES, 75 mM CAPS, 0.1% DM, catalase 40 μg mL^{-1}. (Since B and C are phase components from the kinetic fit, their amplitudes may not give correct extinction coefficients; see Materials and Methods.)

This accords well with the fact that in cytochrome aa_3, the same reaction leads to the 607 nm species. The appearance of this initial product is followed by a two-phase decay process. The first phase (Figure 3B) is similar to the second phase of the peroxide reaction (Figure 1C). Apparently the initial product (**I**) undergoes a similar conversion to the 556 nm species (**II**), though perhaps with a lower yield. The second phase of this decay process (Figure 3C) appears to be the relaxation of this 556 nm species (**II**) to the oxidized form of the enzyme.

It is important to note that the transition from intermediate **I** to intermediate **II** takes place even though the sample contains catalase and thus cannot be due to reaction of intermediate **I** in one subpopulation with hydrogen peroxide which has diffused out from the oxygen-binding site of enzyme from another subpopulation (see Discussion).

The CO−mixed valence enzyme was formed by incubating the oxidized enzyme in the presence of about 1 mM CO. In this experiment, the amount of CO in the sample was reduced to about 30 μM, by exchange with argon, just before the measurement. If the concentration of CO was not lowered, intermediate **I** was seen in much the same way, but the decay to intermediate **II** was not observed. Presumably, under these conditions, CO is able to re-reduce the enzyme as rapidly as it decays, creating a steady state, as is believed to be the case in cytochrome aa_3 (Morgan et al., 1985; Witt et al., 1986).

DISCUSSION

The fact that the first intermediate has been observed in this study while it was not found earlier could be due to a number of factors, including the higher pH, the high concentrations of hydrogen peroxide used with the rapid mixing experiments, and the ability to follow the development of the reaction at a number of wavelengths simultaneously.

As described in the introduction, in cytochrome aa_3, the conversion of the 607 nm species to the 580 nm species in the peroxide reaction has been described as the reduction of a ferrous−peroxy intermediate to an oxy−ferryl by an electron from hydrogen peroxide, resulting in the production

of a molecule of superoxide. It would be natural to expect the same chemistry to take place in cytochrome bo_3, seen as conversion of intermediate **I** to intermediate **II** in the peroxide reaction.

It is less clear why this transition should also be seen when **I** is formed by oxidation of the CO−mixed valence enzyme. In the cytochrome aa_3 of *Bacillus subtilis*, this formation of the 580 nm species is observed, but only if no catalase is included in the sample, and is apparently due to reaction of the first product with peroxide which has dissociated from other enzyme molecules (Lauraeus et al., 1993). In our experiments on cytochrome bo_3, intermediate **II** is formed even in the presence of catalase, indicating that the electron which effects this transition did not come from peroxide. It is possible that this electron which creates the oxy−ferryl is taken from a site in the protein (Watmough et al., 1994; Moody & Rich, 1994). However, it is unlikely that this is part of the catalytic cycle of the enzyme, since the transition from intermediate **I** to intermediate **II** is much slower than enzyme turnover.

CONCLUSION

A compound corresponding to the 607 nm species of cytochrome aa_3 exists in cytochrome bo_3. It is produced both in the reaction of the oxidized enzyme with peroxide and in the reaction of the two-electron reduced CO−mixed valence enzyme with oxygen (under light). This species has an absorbance maximum at about 582 nm and a shoulder near 545 nm. In both the peroxide reaction and the two-electron reaction, the 582 nm species is subsequently converted to the 556 nm species, which has previously been reported as the sole product of these reactions. This leads us to believe that the oxygen-reduction chemistry of cytochrome bo_3 and of cytochrome aa_3 do not differ in any mechanistically important way.

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