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Rapid Report

Raman detection of a peroxy intermediate in the hydroquinone-oxidizing cytochrome aa_3 of *Bacillus subtilis*

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Abstract

When the mixed valence, carbon monoxide-bound form of the hydroquinone-oxidizing cytochrome aa_3-600 of *Bacillus subtilis* is illuminated in the presence of O_2, it forms a species that corresponds to 'Compound C', first described for the mitochondrial cytochrome *c* oxidase by Chance, Saronio and Leigh (J. Biol. Chem. 250 (1975) 9226–9237). Resonance Raman spectra of the this species show a mode at 366 cm^{-1} that shifts to 342 cm^{-1} when the experiment is repeated with $^{18}O_2$. The appearance of this mode is insensitive to deuteration exchange within the limits of resolution. High- (1200–1700 cm^{-1}) and low-frequency (200–500 cm^{-1}) data, allow us to assign the 366 cm^{-1} mode to the Fe^{3+}-O stretching vibration of a peroxide adduct where the iron is either low or intermediate spin. This is to our knowledge the first time an $^{18}O_2$-sensitive iron-oxygen stretching mode has been reported for 'Compound C', providing strong support for the notion that this species is a peroxide adduct. The observed 366 cm^{-1} $\nu(Fe^{3+}\text{-}O^{-}\text{-}O^{-})$ frequency is 8 cm^{-1} higher than that previously found for a *transient* peroxy intermediate in the reaction between the fully reduced mitochondrial enzyme and O_2. Our observation indicates that, while similar, the metastable peroxyheme a_3 species reported here differs in the fine details of geometry, protonation state, and/or hydrogen bond status.

Keywords: Cytochrome aa_3-600; Raman spectroscopy; Peroxyheme a_3; Compound C

Cytochrome aa_3-600 in *Bacillus subtilis* transfers electrons from hydroquinone to O_2. In contrast to the mitochondrial cytochrome *c* oxidase, this enzyme contains only three catalytically active metals: two heme irons and one copper [1]. Heme a is the site where the initial electron transfer reaction from quinol takes place, while heme a_3 and its associated Cu_B atom form a binuclear site where electrons from heme a are accepted and subsequent oxygen binding and reduction occur. X-ray absorption spectroscopy of the fully oxidized aa_3-600 revealed a structure for the binuclear center similar to that reported for mitochondrial cytochrome *c* oxidase [2]. Recently, cytochrome aa_3-600 was shown to translocate protons across the cell membrane, in addition to generating charge separation in the oxidation of quinol [3], and to catalyze the reduction of dioxygen to water through intermediates similar to those that occur in the mitochondrial cytochrome *c* oxidase [4]. The hydroquinone oxidase members of the family of heme-copper oxidases, including cytochrome aa_3-600, lack the EPR-visible copper center (Cu_A) which is typical of the cytochrome *c* oxidases [5,6]. As a consequence, only three reducing equivalents are required to fully reduce this enzyme. The absence of the additional electron donor makes the aa_3-600 enzyme particularly attractive for exploring the reaction sequence for the reaction with O_2[7]. Moreover, the absence of Cu_A is likely to simplify the time course of dioxygen reduction, and may therefore allow further insight into the rate constants assigned for the reaction [8].

Using optical spectroscopy, Lauraeus et al. [4] found that two intermediate species were formed during the reaction of dioxygen with the fully reduced aa_3-600 at 0°C. At high pH, a putative peroxy (P) intermediate was relatively stable, and decayed subsequently to a ferryl (F) intermediate, both species having optical spectral proper-

Abbreviations: MCD, magnetic circular dichroism; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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ties similar to those observed previously for the P and F states of the mitochondrial enzyme. On the other hand, when the mixed-valence, two-electron-reduced ($a^{3+}a_3^{2+}Cu_B^{1+}$), enzyme was allowed to react with O_2, a peroxy intermediate was formed with a similar but slightly blue-shifted absorption band as compared with the P state formed from fully reduced enzyme. The former species is analogous to 'Compound C' originally described by Chance et al. [9] for the mitochondrial enzyme. At lower pH this species decomposed to the ferryl intermediate (F) by disproportionation [4]. These findings suggest that the transfer of the third electron to the binuclear center is followed by proton uptake, which occurs before the O–O bond cleavage.

Resonance Raman studies of aa_3-600 [10] revealed strong similarities of ligation and heme environment with those in the mitochondrial enzyme. However, the low-frequency region showed two modes at 194 and 214 cm^{-1} in the fully reduced aa_3-600, while a single vibration at 214 cm^{-1} has been assigned to the iron histidine stretch for the other aa_3-type oxidases. Although plausible interpretations were given for the appearance of the two modes in the low-frequency aa_3-600 spectrum, no definite assignment was made. Nonetheless, comparison of the aa_3-600 data to those reported by Einarsdóttir et al. [11] on the ba_3 oxidase showed that significant differences in the Fe–his interaction at the heme a_3 site may occur in the heme-copper family of enzymes.

Because of the difficulties in studying the structures of the short-lived intermediate species that occur in the cytochrome oxidase/O_2 reaction at room temperature, several attempts were made in the past to trap or generate these intermediates and characterize them by Raman spectroscopy. However, such methods do not always result in faithful reproduction of the structure of the transient catalytic intermediate. For example, a metastable intermediate of mitochondrial cytochrome *c* oxidase was generated by Han et al. [12] by simultaneously reducing the enzyme photoreductively and oxidizing it chemically. With this approach, an oxygen and hydrogen isotope-sensitive ligand vibration was observed at 477 cm^{-1}, which was attributed to an Fe–OH stretching mode. Comparison with the $\nu(Fe^{3+}–OH)$ at 450 cm^{-1} [12,13] or 458 cm^{-1} [8] observed in time-resolved resonance Raman measurements shows that the Fe-OH mode in the metastable species is much stronger than that observed from transient measurements. This difference suggests that the iron spin state and heterogeneities in hydrogen bonding may influence the strength of the Fe–OH bond, and that metastable oxygen species may not exactly reproduce the transient intermediates of the catalytic reaction.

In an attempt to characterize the electronic structure of the dioxygen intermediates Larsen et al. [13] prepared several 'static' derivatives of the mitochondrial enzyme at room temperature with optical spectroscopic properties similar to those of the transient intermediates. Although the optical properties and the high frequency resonance Raman data of one 'static' derivative suggested the formation of 'Compound C' (the two-electron-reduced dioxygen intermediate [9]), no oxygen isotope-sensitive ligand vibrations were reported.

In the work presented here we have extended our resonance Raman approach to study the reaction between the mixed valence (two-electron reduced) aa_3-600 and dioxygen at 4°C [4]. This reaction yields the 'Compound C' previously characterized optically. The present Raman results show an $^{18}O_2$-sensitive Fe–O stretching mode in this compound, indicating that it is a peroxy adduct of heme a_3 with properties similar to those observed for the transient peroxy intermediate in time-resolved measurements of the reaction between fully reduced mitochondrial enzyme and oxygen. This finding provides support for the concept of a peroxy intermediate in the reaction, and an important link between metastable and transient intermediates.

Cytochrome aa_3-600 was isolated as described by Lauraeus et al. [1]. The mixed valence CO complex and its oxygen adduct ('Compound C') were prepared as described previously [4]. The fully reduced form (a^{2+} a_3^{2+} was prepared by adding a few grains of solid sodium dithionite, 15 mM ascorbate, and 1 mM TMPD to the oxidized enzyme. The mixed valence $a^{2+}a_3^{3+}$-CN derivative was prepared by adding 15 mM ascorbate and 1 mM TMPD to $a^{3+}a_3^{3+}$-CN. Resonance Raman spectra were spectra were obtained from 60–80 μM samples, pH 7.5, in 5 mm cuvettes maintained at 2–5°C by a stream of cold nitrogen gas. The Raman spectra were acquired by a SPEX 1877 triplemate with an EG and G Model 1421 diode array detector. A Liconix 4240 helium-cadmium and a Coherent Innova 90 Krypton ion laser were used to provide excitation wavelengths of 441.6 and 413.1 nm, respectively. The power incident on the oxidase samples was typically 5–20 mW. Optical absorption spectra were obtained with a Perkin-Elmer Lamda 5 UV-visible spectrophotometer.

Fig. 1 shows high frequency resonance Raman spectra of reduced, mixed valence cyanide-bound, and the metastable 'Compound C', obtained with 413.1 nm excitation. With this excitation frequency, which lies to the high-energy side of both the a and a_3 Soret bands, resonance of both hemes is enchanced via 0–1 transitions. In particular, modes from ferric hemes, from heme a_3^{2+} and from those of oxygenated cytochrome a_3^{2+} are obtained. The porphyrin π^* electron density-sensitive mode (υ_4) of aa_3-600 in the fully reduced enzyme is at 1358 cm^{-1}, establishing that both hemes are in the ferrous state (Fig. 1, trace A). The core expansion region shows two vibrations, at 1569 and 1549 cm^{-1}. The 1620 cm^{-1} mode arises from the C = C stretching vibrations of hemes a^{2+} and a_3^{2+}. The 1611 and 1666 cm^{-1} modes have been assigned as the C = O stretching vibrations of the formyl group of a^{2+} and a_3^{2+}, respectively. In the mixed-valence cyanide-bound enzyme (Fig. 1; spectrum B) the υ_4 mode of cytochrome

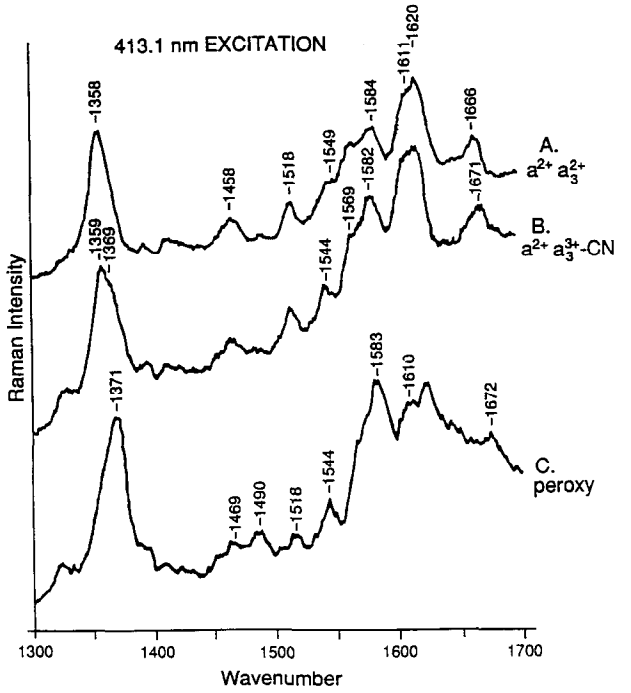

Fig. 1. High-frequency resonance Raman spectra of fully reduced (A), mixed-valence cyanide (B), and peroxy (C) forms of cytochrome aa_3 600. The exciting laser wavelength was 413.1 nm. The accumulation time for each spectrum was 5 min. The measurement was repeated three times independently (not shown).

a^{2+} is at 1359, whereas υ_4 of heme a_3^{3+}-CN is at 1369 cm^{-1}, as previously reported [10]. The formyl vibration of heme a_3, which is located at 1666 cm^{-1} in the fully reduced enzyme (spectrum A), has shifted to 1671 cm^{-1}, indicating the formation of low-spin oxidized heme a_3^{3+}-CN (spectrum B). Trace C (Fig. 1) shows the resonance Raman spectrum of the metastable oxygen adduct of the mixed-valence enzyme. The oxidation state marker is at 1371 cm^{-1}, which indicates essentially complete generation of a form of the enzyme in which heme a_3 is oxidized. The heme a_3 core size-sensitive band (υ_2), which appears at 1569 cm^{-1} in both the a_3^{3+}-CN and a_3^{2+} spectra, has lost intensity and has shifted underneath the υ_2 vibration of heme a^{3+} at 1583 cm^{-1}. The shift of this mode, along with the υ_4 shift, indicate that heme a_3 is either low or intermediate spin [13,14]. The υ_{10} vibration at 1611 cm^{-1} has lost intensity upon O_2 binding, indicating that this mode is sensitive to the ligation state of the cytochrome a_3 site. Rousseau et al. [15,16] observed similar behavior on NO and CN binding to mitochondrial cytochrome aa_3. The heme a_3 formyl stretching vibration has lost intensity and is located at 1672 cm^{-1}. The high-frequency data in Fig. 1 (trace C) are comparable to those from earlier studies of the reaction between mixed valence mitochondrial cytochrome oxidase and O_2 [14,17]. However, chemical characterization of a putative iron-oxygen adduct requires the detection of oxygen isotope-sensitive lines in the Raman spectrum.

Fig. 2 shows the low frequency resonance Raman spectra of the reduced enzyme (trace A), and the mixed-valence cyanide (trace B) and oxygen (trace C) adducts, obtained with 441.6 nm excitation. With this excitation, vibrational modes throughout the 100–1700 cm^{-1} region are enchanced via 0–0 transitions. The reduced and mixed-valence cyanide spectra are similar to those reported earlier [10]. In the spectrum of the reduced enzyme (Fig. 2A) the set of lines at 196 and 206 cm^{-1} is the most prominent difference when compared to the mitochondrial enzyme. This unusual splitting has been attributed to the Fe^{2+}–his vibration(s) of heme a_3 in earlier work. Upon forming the mixed-valence CN species (Fig. 2B), both lines disappear and no new lines appear in the Raman spectrum, consistent with the behavior observed in the mitochondrial enzyme. The Fe–his stretching modes are absent from the spectrum of the oxygen adduct (Fig. 2C), just as in the $a^{2+}a_3^{3+}$-CN spectrum. We attribute the loss of the lines at 196 and 206 cm^{-1} to a change in the redox state of heme a_3 from Fe^{2+} to Fe^{3+}.

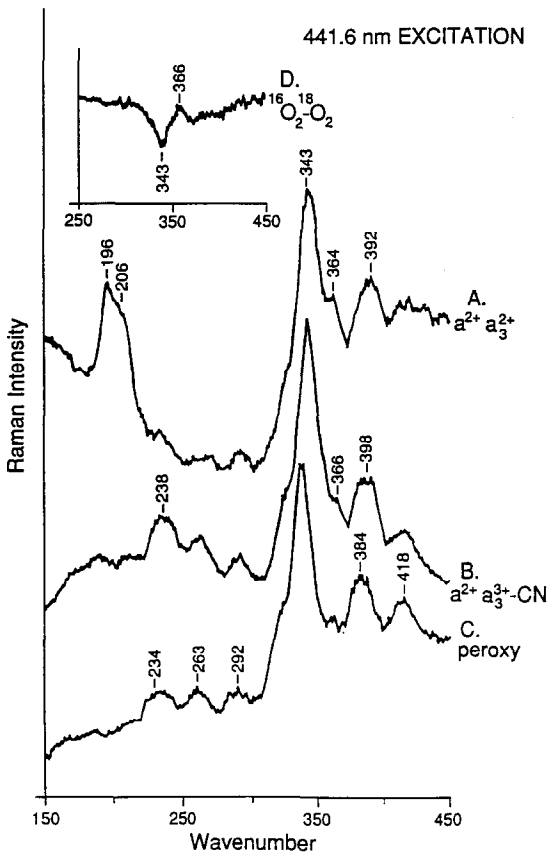

Fig. 2. Low-frequency resonance Raman spectra of fully reduced (A), mixed-valence cyanide (B), and peroxy (C) forms of cytochrome aa_3-600. The inset shows the difference spectrum ($^{16}O_2 - {}^{18}O_2$) of the peroxy enzyme. The exciting laser wavelength was 441.6 nm. The accumulation time was 5 min each for spectra A and B. The difference spectrum is the sum of 6 spectra, each with an accumulation time of 5 min. The measurements were repeated three times (not shown).

The difference spectrum (Fig. 2, inset) was obtained by subtracting the Raman spectrum of the mixed-valence enzyme reacted with $^{18}O_2$ from that of enzyme reacted with $^{16}O_2$. Although the absolute spectra are similar, their difference indicates the presence of one oxygen isotope-sensitive mode. The fact that such difference spectra were obtained for several independent preparations, and that no other porphyrin in-plane bands were observed as positive/negative lines in the difference spectra, makes the presence of the oxygen isotope-sensitive band reliable. In addition, the oxygen-sensitive modes were stable for about 6 min under laser illumination. The difference spectrum shows that the 366 cm^{-1} mode in the $^{16}O_2$ spectrum is downshifted to 343 cm^{-1} when the experiment is repeated with $^{18}O_2$. The frequency of this mode, and the 23 cm^{-1} isotope shift, which is in good agreement with that predicted by a two-body harmonic oscillator approximation, indicate that this vibration arises from a peroxy (Fe^{3+}-O^--O^-) heme a_3 species. The resonance observed at 366 cm^{-1} for 'Compound C' (Fig. 2, inset) is at a frequency approximately 8 cm^{-1} higher than that reported by Ogura et al. [18] and Varotsis et al. [8] for the transient mitochondrial peroxy (Fe^{3+}-O^--O^-(H)) intermediate, observed after reacting fully reduced mitochondrial enzyme with O_2.

The reduction of dioxygen by cytochrome oxidase has been studied extensively by optical, EPR and resonance Raman spectroscopy. Studies with mitochondria energized by ATP suggested the appearance of ferryl and peroxy species of the binuclear center in a reversed electron transfer process [19,20]. Time-resolved resonance Raman studies of the forward reaction with O_2 have strongly supported these assignments. These and other studies have led to the proposal of reaction schemes for the intramolecular transfer of electrons to the dioxygen-reducing site, and the assignment of molecular structures and rate constants of intermediates in the O_2 reaction (for a review, see Ref. [7]). However, the precise structural assignment of certain oxygen intermediates has remained problematic, and this has prevented general agreement on the reaction mechanism.

In earlier time-resolved work on the reaction between mixed-valence mitochondrial cytochrome oxidase and O_2, it was proposed that the primary oxyheme a_3 species ('Compound A') decays to a peroxy-like complex with a rate constant of $(3.5–4.5) \cdot 10^3$ s^{-1} [14,17]. However, no oxygen isotope-sensitive vibrations have been reported for this species up to now, which has made the structural assignment uncertain. The high- and low-frequency spectra reported here clarify the situation and are consistent with the original hypothesis in Refs. [14,17]. In the high frequency region, υ_4 shifts from 1358 cm^{-1} in the $a^{2+}a_3^{2+}$ spectrum (Fig. 1A) to 1371 cm^{-1} in the peroxy complex, supporting the notion that both hemes are oxidized in the latter. It is not possible, however, to assign the precise spin state of the peroxy complex since the frequency of the υ_2 vibration, which is sensitive to the core size of the heme macrocycle, is similar for both low ($S = 1/2$) and intermediate ($S = 1/2$) spin states of the Fe^{3+} species.

Our data indicate that 'Compound C' of cytochrome aa_3-600 consists of a peroxy species which can be detected by its Fe^{3+}-O_2^{2-} stretching vibration. In 'Compound C' of the mitochondrial enzyme this vibration has thus far escaped resonance Raman detection. Our data show that the $\upsilon(Fe^{3+}$-O^--$O^-)$ is located at 366 cm^{-1} in the bacterial oxidase, and shifts down to 342 cm^{-1} when the experiment is repeated with $^{18}O_2$. The frequency of the vibration and the magnitude of the isotope shift are both consistent with the assignment of this compound as a peroxyheme a_3 species. Moreover, this mode is insensitive to D_2O exchange (to within ± 2 cm^{-1}; data not shown), which suggests that the peroxide-bound cytochrome a_3 site is not protonated or hydrogen-bonded, in agreement with the structure proposed from optical studies [4]. The Fe^{3+}-O^--O^- mode observed here is at a 8 cm^{-1} higher frequency than that of the transient hydroperoxy intermediate detected previously in time-resolved Raman measurements, where the fully reduced enzyme was reacted with O_2 [8,18]. We attribute the higher frequency to a stronger Fe–O^- bond that results from differences in geometry, protonation, or hydrogen bonding. Another plausible interpretation of the increased Fe^{3+}-O^--O^- frequency in 'Compound C' from cytochrome aa_3-600 is that electron donation from the split Fe-his (see above) influences the strength of the Fe–O and O–O bonds. Although the $\upsilon(O_2^{2-})$ is Raman-active and should appear in the peroxy region (700–1100 cm^{-1}), it was not possible to resonance-enhance this mode, as is also the case of mitochondrial cytochrome oxidase and other oxy-iron porphyrins [21]. The precise extent of the oxygen isotope shift is important because it is sensitive to the coordination of the peroxide. The isotope shift of 24 cm^{-1} for the $^{16}O_2/^{18}O_2$ exchange that we detect here, when compared to the 18 cm^{-1} shift observed for the transient hydroperoxy intermediate in the mitochondrial enzyme, suggests that the Fe-O^--O^- group maintains a high degree of bent geometry. Although we cannot exclude a bridged but highly bent geometry of the Fe^{3+}-O^--O^- unit with a nearby Cu_B atom, our data are most consistent with a model in which the copper atom (Cu_B) does not have a direct interaction with the bound O^--O^- group.

Recently, Ogura et al. [18] reinvestigated the nature of the bands at 785 and 356 cm^{-1} that they observed transiently in the mitochondrial cytochrome oxidase/O_2 reaction, and reported that they originate from different intermediates, contrary to their previous interpretation [22]. Although the 356 and 785 cm^{-1} modes cannot be separated in time under their experimental conditions, they concluded that the 356 cm^{-1} vibration cannot be ascribed to a hydroperoxy intermediate due to its insensitivity to H/D exchange, and assigned the 785 cm^{-1} mode to the Fe^{3+}-O^--O^-(H) species. In our time-resolved resonance Raman studies [8], we showed that the 358 cm^{-1} mode

appears prior to the 790 cm^{-1} mode, which formed the basis for assigning these modes to different intermediates. We assigned the 790 and 358 cm^{-1} modes to the ferryl and hydroperoxy intermediates, respectively. Moreover, we assigned rate constants to several steps in the linear reaction sequence proposed by Babcock and Wikström [7]. The observation here of a υ ($Fe^{3+} - O^{-}$-O^{-}) vibration at 366 cm^{-1} in the mixed-valence aa_3-600, the only oxygen isotope-sensitive mode in the 200–1100 cm^{-1} region, is consistent with our previous assignment of the transient 358 cm^{-1} mode in the mitochondrial aa_3/O_2 reaction.

These results and other research recently reported provide new insight on the reaction product of mixed-valence enzyme and O_2 ('Compound C'), which has been under debate. Chance et al. [23] originally proposed a ferrous superoxy structure for the Fe_{a3} site in this species, while Clore et al. [24] suggested a ferrous-peroxy structure with an additional oxidizing equivalent as either a porphyrin or an amino acid free radical. Both proposals are inconsistent with the present data, which show that Fe_{a3} is oxidized (and see Refs. [13,14,17]).

Wikström [21] assigned 'Compound C' as a ferric peroxy/cupric species, but a ferryl state was also considered, primarily due to the strong absorption band at 607 nm, which is atypical for a ferric heme [25]. Nicholls [26] dismissed the possibility of a sufficiently stable peroxy state, and suggested an oxyferryl structure with an oxidizing equivalent elsewhere in the molecule, by analogy with 'Compounds I' of other heme proteins. This view was supported by Watmough et al. [27], who, based on MCD and optical studies of cytochrome bo_3 of *E. coli*, concluded that 'Compound C' has an oxyferryl structure and an amino acid radical. Moreover, Kitagawa et al. [28], who recently reinvestigated the reaction between H_2O_2 and oxidized mitochondrial cytochrome *c* oxidase, reported that the resonance Raman spectrum of the produced 607 nm species exhibits an oxygen isotope-sensitive band at 803 cm^{-1} which they attributed to the $Fe_{IV} = O$ motion of a cytochrome a_3 ferryl adduct. They concluded that the ferryl oxygen is hydrogen-bonded to a surrounding residue, and that the signal arises from the same ferryl intermediate as detected in the cytochrome oxidase/O_2 reaction. However, the oxygen isotope-sensitive stretching vibration at 366 cm^{-1} reported here for the product of mixed-valence enzyme and O_2, provides direct support for a non-hydrogen-bonded ferric-peroxide structure, while the strong iron-oxygen bond of an oxyferryl compound should give rise to a stretching mode at much higher frequencies, as observed for other heme proteins, as well as in time-resolved experiments with mitochondrial cytochrome *c* oxidase [29–31].

It seems unlikely that the cytochrome bo_3 of *E. coli* would react profoundly differently with O_2 than the cytochrome aa_3-600 of *B. subtilis* since the primary protein structures of these two hydroquinone oxidases are highly homologous [32]. On the other hand, the structures of the hemes of the binuclear center differ in that the formyl group of heme A is replaced by a methyl in heme O [33,34]. Although we cannot presently resolve this discrepancy, one essential question is whether the species formed with H_2O_2 is, in fact, the same as that formed in the oxygen reaction. Another question relates to the possibility that the precise electronic distribution might differ in otherwise equivalent intermediates, depending on the structure of the heme. Finally, it is also possible that 'Compound C' is a fast equilibrium mixture of species, which differ in electronic distribution, but which may be distinguished by different wavelengths of Raman excitation.

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