Peroxy and Ferryl Intermediates of the Quinol-Oxidizing Cytochrome aa_3 from *Bacillus subtilis*†

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ABSTRACT: The quinol-oxidizing cytochrome aa_3-600 from *Bacillus subtilis* has a binuclear heme a_3–Cu_B center of O_2 reduction and a low-spin heme *a*, but lacks a fourth redox center, Cu_A, which is a typical component of cytochrome *c* oxidases. Fully reduced ($3e^-$) cytochrome aa_3-600 and the two-electron-reduced CO complex were allowed to react with O_2 at 0 °C, and the reaction products were studied by optical spectroscopy. When the two-electron-reduced CO complex (heme a_3 and Cu_B are reduced, but the low-spin heme is oxidized) reacts with O_2 at neutral pH, a compound is produced that may be assigned a ferric–cupric peroxy structure (P). At low pH, this species spontaneously decomposes into another compound, which may be assigned a ferryl structure (F). When fully reduced enzyme ($3e^-$) reacts with O_2 at high pH, a peroxy species is the primary product. This subsequently decays into F, followed by very slow decay of the latter. Our data show that at high pH the third electron, which is required to convert P into F, resides for a relatively long time in either Cu_B or heme a_3. This suggests that transfer of the third electron to the binuclear center is followed by proton uptake, which must occur before scission of the O–O bond. The present data strongly support the involvement of discrete peroxy and ferryl intermediates in the catalytic cycle of cytochrome aa_3–600. The dioxygen reduction mechanism in the binuclear site is thus very similar in the quinol and the cytochrome *c* oxidases.

Bacillus subtilis expresses a cytochrome aa_3 type quinol oxidase during vegetative growth, called aa_3-600 (Lauraeus et al., 1991). This enzyme has only three redox-active metal centers: two hemes (hemes *a* and a_3) and one copper (Cu_B). Heme a_3 and Cu_B are closely apposed; an Fe–Cu distance of 3.65 Å has recently been deduced from an EXAFS study of the fully oxidized enzyme (B. Chance, personal communication). They are thought to form a binuclear O_2 reduction center, as in the other members of the family of terminal oxidases [see Saraste (1990)]. The primary structures of the subunits of cytochrome aa_3-600 were recently deduced (Santana et al., 1992), showing particular resemblance to cytochrome *o* of *Escherichia coli*. Resonance Raman spectroscopy has also shown that the redox centers of cytochrome aa_3-600 are in an environment very similar to that of mitochondrial cytochrome oxidase (Lauraeus et al., 1992).

The quinol-oxidizing members of the terminal oxidase family lack the EPR-visible Cu_A center (Anemüller & Schäfer, 1990; Puustinen et al., 1991; Lauraeus et al., 1991), which is typical of cytochrome *c* oxidases and is thought to be the entry port of electrons from cytochrome *c*. In the quinol oxidases, such as cytochrome *o* (Anraku & Gennis, 1987) and the enzyme studied here, the low-spin heme (heme *a*) is thought to accept electrons directly from the membranous quinol.

The mechanism by which enzymes of this family catalyze the four-electron reduction of O_2 to water has been the subject of a large number of studies [see Figure 1 and review by Babcock and Wikström (1992)]. Following the binding of O_2 at the binuclear site, the first redox step in this process is thought to be a concerted two-electron reduction of O_2 to peroxide by electrons from ferrous heme a_3 and cuprous Cu_B, resulting in a peroxy intermediate (P).[1] Transfer of a third electron to the binuclear site is thought to convert P to a ferryl intermediate (F), and a fourth electron to reduce F to a ferric–cupric intermediate (O = fully oxidized).

An intermediate proposed to be the ferric–cupric peroxy compound (P) was revealed in reversed electron-transfer studies using intact mitochondria (Wikström, 1981; Wikström & Morgan, 1992). A compound with similar spectroscopic properties had been found earlier in the reaction of two-electron-reduced (mixed-valence CO) enzyme with O_2, but at that time, it was assigned a different electronic structure or was not deemed to be a true catalytic intermediate, since it has not been possible to observe P as an intermediate in the reoxidation of the fully reduced enzyme (Greenwood et al., 1974; Chance et al., 1975; Clore et al., 1980).

The ferryl compound (F) was first discovered in reversed electron-transfer studies (Wikström, 1981), and it has subsequently been demonstrated as an intermediate in the forward reaction by optical (Orii, 1988) and time-resolved resonance Raman work (Varotsis & Babcock, 1990; Ogura et al., 1990; Han et al., 1990). Time-resolved low-temperature EPR spectroscopy revealed an intermediate which was suggested to have the ferryl structure (Karlsson et al., 1981; Hansson et al., 1982), but which was subsequently ascribed to an earlier ferrous peroxy intermediate (Blair et al., 1985).

The relationship between intermediates P and F (Figure 1) has been demonstrated both in reverse electron-transfer studies and in studies on the interaction of peroxide with the enzyme: In the reverse electron-transfer studies, the final two steps of the dioxygen cycle are reversed by means of a high redox potential of cytochrome *c* and a high $\Delta\mu_{H^+}$. When mito-

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DS, dodecylsucrose or dodecyl β-D-fructofuranosyl-α-D-glucopyranoside; F, O, P, and R, see Figure 1.

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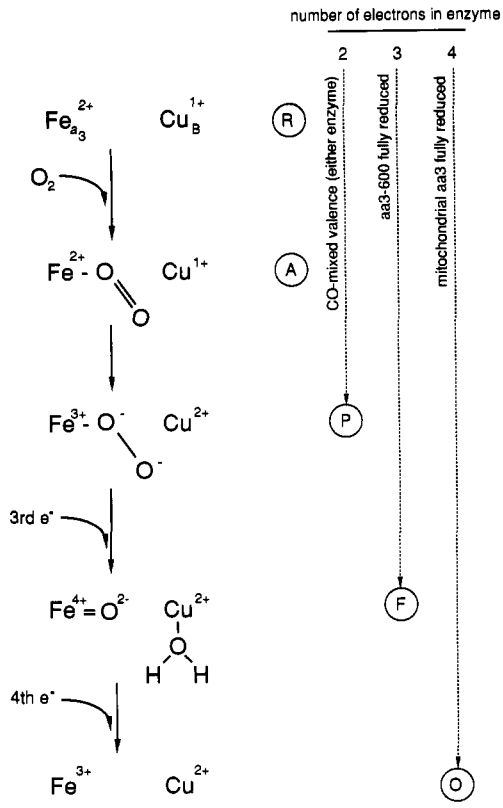

FIGURE 1: Simplified mechanistic scheme for reduction of O_2 to water by related oxidases based on Babcock and Wikström (1992). The circled letters indicate the various intermediates: R, reduced; A, dioxygen-bound; P, peroxy; F, ferryl; O, oxidized. The arrows show how far the reaction can proceed starting at various levels of reduction of the enzyme. Uptake and release of protons and water are now shown explicitly.

chondria are thus "energized", the enzyme, initially in the O state (Figure 1), is converted sequentially to F and then to P, while as $\Delta\mu_{H^+}$ dissipates, the enzyme returns to F and then to state O (Wikström, 1981; Wikström & Morgan, 1992). Compound P can also be formed by the binding of hydrogen peroxide to the fully oxidized enzyme (state O). At higher concentrations, hydrogen peroxide can reduce the compound P thus produced to F (Kumar et al., 1984b; Wrigglesworth, 1984; Witt & Chan, 1987; Vygodina & Konstantinov, 1989).

In this study, we have analyzed the intermediates produced in the reaction of O_2 with partially and fully reduced cytochrome aa_3-600. The results suggest that this enzyme has a catalytic cycle of dioxygen reduction similar to that deduced for cytochrome *c* oxidase from mitochondria. The fact that cytochrome aa_3-600 has only three redox-active metal centers provides an important addition to this analysis. The lack of the fourth electron equivalent makes the three-electron-reduced state stable at full occupancy, allowing the fate and route of the third electron to be studied much more amenably (see Figure 1).

MATERIALS AND METHODS

Cytochrome aa_3-600 was isolated as described by Lauraeus et al. (1991). Its concentration was determined from the dithionite-reduced minus air-oxidized optical difference spectrum at the wavelength couple 600–626 nm using a millimolar absorptivity of 26.4 cm^{-1} for the two-heme enzyme unit (Lauraeus et al., 1992).

The two-electron-reduced "mixed-valence CO" enzyme was prepared as follows: The purified enzyme was dissolved in 75 mM K-Hepes, 50 mM NaCl, and 0.2% DS, pH 7.5. Samples in Thunberg cuvettes were first deaerated with oxygen-free argon. Then 1 atm of O_2-free CO gas was added, and the samples were incubated at room temperature until the mixed-valence CO derivative had formed, as ascertained by optical spectroscopy.

To form the fully reduced state of cytochrome aa_3-600, the sample was deaerated as described above, and a 5-7-fold excess of dithionite over enzyme was added from the side arm of the cuvette. Complete reduction was ascertained by following the reaction spectroscopically.

The mixed-valence CO enzyme was reacted with O_2 by addition of 25 μL of O_2-saturated water (0 °C) to 1 mL of the sample under an argon atmosphere, followed by mixing and illumination by bright light. The fully reduced enzyme was oxygenated by flushing it with pure oxygen for 1 min to ensure oxidation of all unreacted dithionite. About 60 nM catalase (Sigma) was present to destroy any hydrogen peroxide formed during oxidation of excess dithionite [cf. Kumar et al. (1984a)].

A Shimadzu UV-3000 dual-beam spectrophotometer with a thermostated cuvette holder was used to perform optical difference spectroscopy at 0 °C. The scan rate was 100 nm/min, and scanning occurred from longer to shorter wavelengths. Times in the figure legends indicate starting times of scans. Cuvettes with 1-cm path length were used throughout.

RESULTS

Mixed-Valence CO Compound. Bickar et al. (1984) showed that CO reduces bovine heart cytochrome aa_3, yielding CO_2 and the "mixed-valence" CO derivative of the enzyme. In this derivative, only heme a_3 and Cu_B are reduced [see Wikström et al. (1981)]. Figure 2 shows a comparison of optical difference spectra of the fully reduced and the mixed-valence CO forms, both with respect to fully oxidized enzyme. The fully reduced minus oxidized difference spectrum peaks at 600 nm (Figure 2B, trace a) in contrast to the characteristic peak at 605 nm of the enzyme from bovine heart. The reduced minus oxidized spectrum of the bacterial oxidase has a clear trough at approximately 655 nm, as has the bovine counterpart (not shown). The characteristic features of the mixed-valence CO difference spectrum include an α-band at 591 nm, a β-band at 550 nm, and a Soret band at 431 nm with a trough near 410 nm. This spectrum is very similar to the mixed-valence CO compound of bovine cytochrome aa_3 [see, e.g., Bickar et al. (1984)] and clearly shows that the low-spin heme *a* remains oxidized. However, reduction of the enzyme by CO is not limited to two electrons; reduction will continue beyond this point with a slower rate. It was therefore important to carefully follow this reaction spectroscopically to avoid reduction of heme *a*.

Reaction of the Mixed-Valence CO Compound with O_2. After replacement of the CO atmosphere of the mixed-valence CO enzyme sample with argon, oxygen-saturated water was added, and after being mixed, the sample was illuminated with bright light to ensure photolysis of Fe–CO and a complete concerted reaction with O_2 (see Materials and Methods). Figure 3 shows a difference spectra relative to the oxidized enzyme at various times after initiation of the reaction. The Soret spectrum (Figure 3A) is characteristic of a high- to low (or intermediate)-spin conversion of a ferric heme, and is similar to the difference spectrum of the peroxy species P of the mitochondrial enzyme (Wikström, 1981), or "Compound C" (Chance et al., 1975). The corresponding α-band (Figure

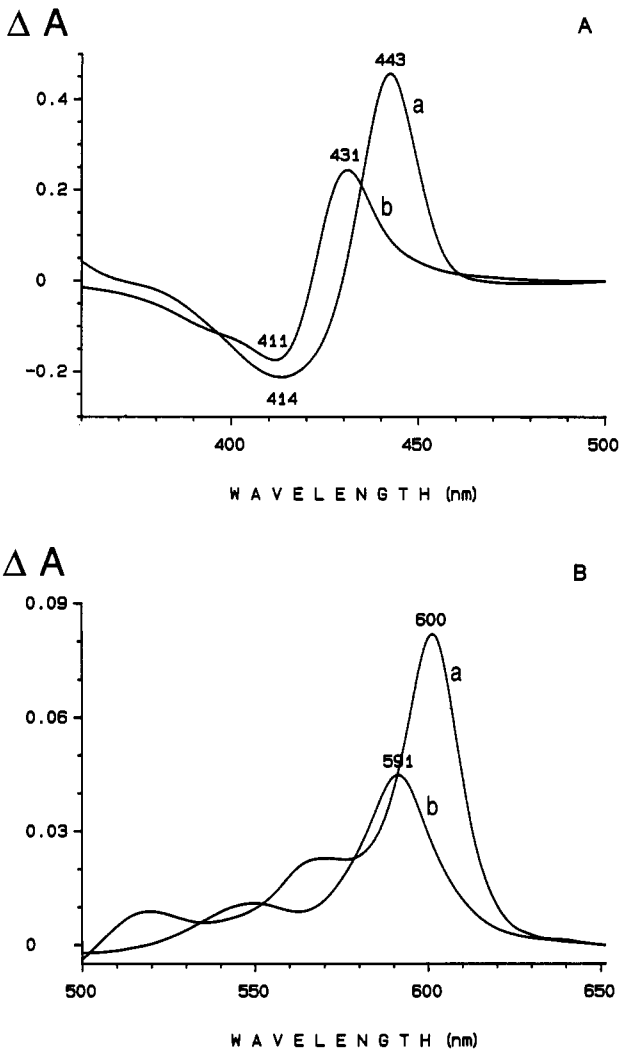

FIGURE 2: (a) Reduced minus oxidized difference spectra of cytochrome aa_3-600, reduced anaerobically with a small excess of dithionite. The sample contains 60 nM catalase (see Materials and Methods). (b) Mixed-valence CO minus air-oxidized difference spectra of cytochrome aa_3-600. [aa_3-600] = 3.1 μM; 75 mM Tris-HCl buffer, pH 8.5, containing 50 mM NaCl and 0.2% DS. All spectra were recorded at O °C. Panels A and B show the Soret and α-regions, respectively.

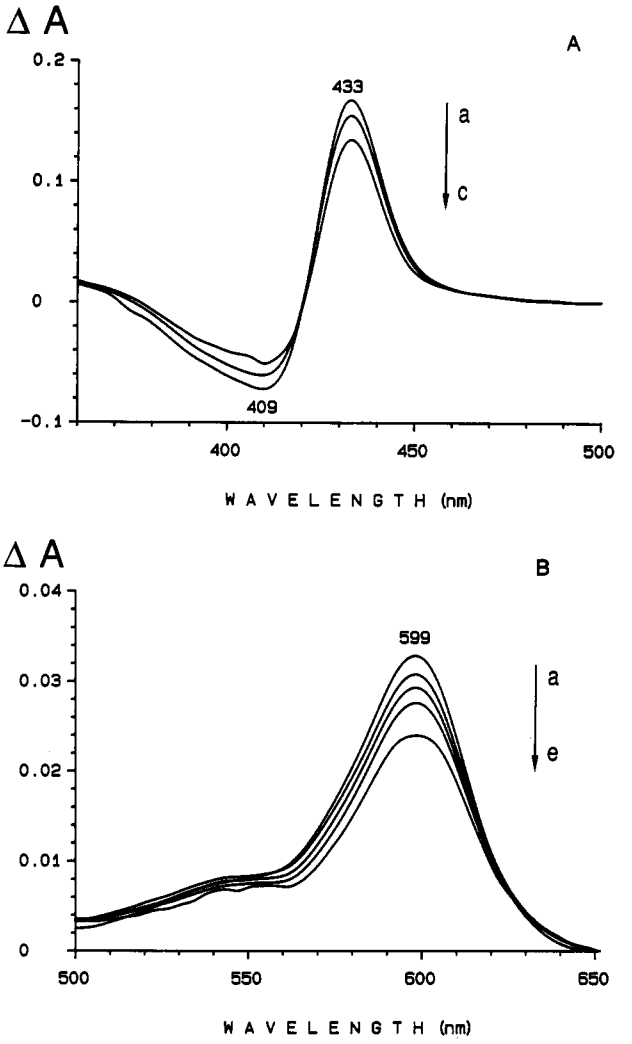

FIGURE 3: Reoxidation by O_2 of the mixed-valence CO enzyme at pH 7.5: difference spectra relative to fully oxidized enzyme. Panel A: (a) 3.10 min after reoxidation; (b) 7.10 min; (c) 19.00 min. Panel B: (a) 1.10 min; (b) 5.10 min; (c) 17.00 min; (d) 70 min. The spectrum after decay to the fully oxidized form is used as a baseline. See legend to Figure 1 and Materials and Methods for other conditions.

3B) is broad with a maximum at 599 nm. This is somewhat reminiscent of a reduced low-spin heme A chromophore. However, the Soret spectrum shows no evidence of reduction of heme *a*. The α-band is, in fact, similar to "Compound C", as formed by reacting the mixed-valence CO bovine cytochrome aa_3 with O_2 (Greenwood et al., 1974; Chance et al., 1975; Clore et al., 1980), or with enzyme incubated aerobically in the presence of CO (Nicholls, 1978). The α-band is also reminiscent of the ferric–cupric peroxy state P formed by reversed electron transfer (Wikström, 1981; Wikström & Morgan, 1992), or after hydrogen peroxide addition to fully oxidized mitochondrial enzyme (Wrigglesworth, 1984; Vygodina & Konstantinov, 1989). However, in contrast to all the aforementioned data with the mitochondrial enzyme, the absorption maximum of this peroxy state of cytochrome aa_3-600 is at 599 nm, whereas it is at approximately 607 nm in the mitochondrial counterpart.

The millimolar absorptivity of this peroxy species is 10.1 cm^{-1} at the wavelength couple 599 minus 626 nm, relative to the oxidized enzyme base line, assuming full occupancy of this state in the conditions of the first spectrum of Figure 3B. This is similar to the value of 11 mM^{-1} cm^{-1} reported for the P intermediate of rat liver cytochrome aa_3 (Wikström & Morgan, 1992).

Stability and Decomposition of the Peroxy Species. In the absence of further reductant, the peroxy species is relatively stable at pH 7.5 and 0 °C, as shown in Figure 3, but when the same experiment is repeated at pH 5.5, the result is different (Figure 4). The Soret spectrum (Figure 4A) is similar to that at pH 7.5, but the peak position is shifted to 431 nm and the product is less stable. However, the α-band is quite different (Figure 4B). Absorption bands are now seen at 580 and 535 nm, with a small shoulder near 595 nm. Such a difference spectrum has been previously assigned to a ferryl (F) intermediate of the mitochondrial enzyme (Wikström, 1981; Wrigglesworth, 1984; Kumar et al., 1984b; Blair et al., 1985; Orii, 1988; Vygodina & Konstantinov, 1989; Morgan & Wikström, 1992).

The appearance of compound F following oxidation of the two-electron-reduced enzyme was surprising because the product of this reaction is expected to be the peroxy (P) intermediate, as it is at higher pH, and the transition from P to F is known to be a redox process requiring one more electron. The answer to this may lie in the fact that although the observed product is F, it is produced in only 50% (approximately) of the enzyme in the sample; the ratio of absorptivities between

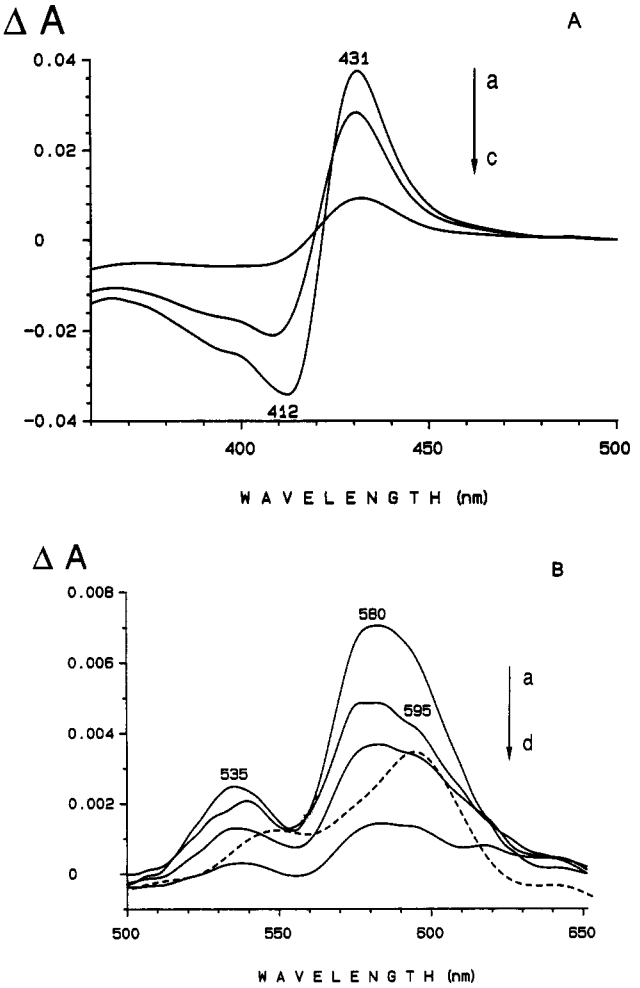

FIGURE 4: Reoxidation of the mixed-valence CO compound by O_2 at pH 5.5: difference spectra relative to fully oxidized enzyme. Panel A: (a) 3.20 min after reoxidation; (b) 7.20 min; (c) 21.00 min. Panel B: (a) 1.10 min; (b) 5.15 min; (c) 9.20 min; (d) 19.20 min. Dashed line: 3.10 min with 0.71 μM catalase added before reoxidation. The spectrum after decay to the fully oxidized form is used as a base line. See legend to Figure 1 and Materials and Methods for other conditions.

the P and F species of cytochrome *c* oxidase is about 2.1 (Wikström & Morgan, 1992; see below), whereas the absorbance ratio between the peroxy species at 599 nm (Figure 3B) and the present ferryl species at 580 nm (Figure 4B) is about 4.5. It seems likely that at low pH the reaction first produces the peroxy compound quantitatively, just as at high pH, but that immediately upon formation 2 equiv of the peroxy form reacts to produce 1 equiv of the ferryl form. We suggest (see Figure 5) that acidic pH allows the bound peroxide to dissociate out of the binuclear center, leaving it in the "pulsed" (activated, fully oxidized) state. The dissociated, free H_2O_2 is, in turn, able to reduce undissociated peroxy intermediate to the ferryl state, as has been shown previously (Witt & Chan, 1987; Vygodina & Konstantinov, 1989; Wrigglesworth, 1984; Mitchell et al., 1992). This mechanism is strongly supported by the finding that inclusion of catalase in the reaction mixture prevented generation of the F intermediate. Instead, the end product now has an absorption maximum at 595 nm (see Figure 4B, dashed line).

This 595-nm feature, which is a minor one on the other spectra of Figure 4B, may be assigned to the "pulsed" state of the enzyme due to its spectral appearance and decay rate. These are similar to those of the corresponding state in the bovine heart enzyme (Armstrong et al., 1983). The "pulsed"

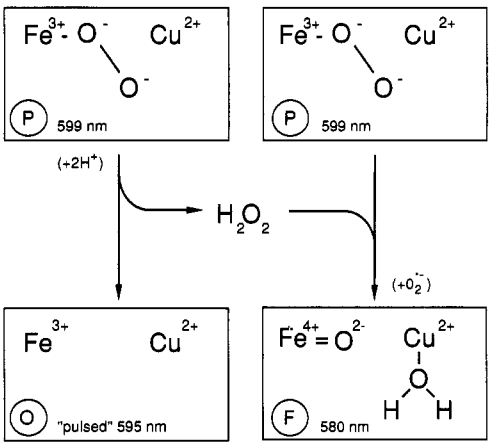

FIGURE 5: Scheme for the disproportionation of the peroxidic compound (P) at low pH. Beginning with 2 equiv of P, peroxide dissociates out of the binuclear site of 1 equiv, leaving that enzyme in the pulsed state (O). The free peroxide can now reduce the remaining equivalent of P, resulting in 1 equiv of F (with production of superoxide; Ksenzenko et al., 1992). The product is thus a 1:1 mixture of F and O.

form of the enzyme has been proposed to have an oxidized, ferric–cupric binuclear center. It is formed upon oxygenation of the four-electron-reduced enzyme and has a ligation state of the binuclear center that is different from the less active "resting" enzyme (Chance et al., 1983).

Fully Reduced Enzyme and Its Reaction with O_2. Cytochrome aa_3-600 reacts slowly with a small molar excess of dithionite, complete reduction being achieved in about 3 h (not shown). Studies with the bovine heart enzyme have shown complete reduction within 30 min [e.g., see Kumar et al. (1984a)]. We attribute this difference to the lack, in cytochrome aa_3-600, of the Cu_A center (Lauraeus et al., 1991), which is the likely entry port for electrons from water-soluble electron donors [see Holm et al. (1987), Rich et al. (1988), and Babcock and Wikström (1992)].

When the fully reduced enzyme is allowed to react with O_2, it is imperative to ensure than any H_2O_2 produced by reaction of O_2 with excess dithionite is rapidly destroyed. Catalase was present for this purpose (see Kumar et al. (1984a) and Materials and Methods]. If this precaution is not taken, the H_2O_2 generated will react with oxidized enzyme to produce both peroxy and ferryl intermediates secondarily (see the introduction), and not as true intermediates in the dioxygen reaction (cf. Discussion).

Figure 6 shows the spectra, and their time courses, of products of the reaction of fully reduced enzyme with O_2 at 0 °C and pH 8.4. The first intermediate trapped (spectrum 1; Figure 6B; see legend) appears to be a mixture of a peroxy (603-nm peak) and a ferryl form (534- and 578-nm peaks). The possibility that the 603-nm absorption peak is due to ferrous low-spin heme *a* can be excluded because of the lack of any significant corresponding feature at 445 nm (Figure 6A). Figure 6B shows that the peroxy species decays to form the ferryl compound (isosbestic point near 590 nm in spectra 1–4), after which the latter decays more slowly to fully oxidized enzyme (O; spectra 4–7). A very similar decay of P to O via F was recently demonstrated in rat liver mitochondria using the reversed electron-transfer approach (Wikström & Morgan, 1992). At maximal occupancy of the ferryl state (Figure 6B, trace 4), the millimolar absorptivity at 578–630 nm (relative to oxidized enzyme) is approximately 5.3 cm^{-1}, which is similar to that found for F in the mitochondrial enzyme (Wikström & Morgan, 1992). Figure 6C shows the early stages of the

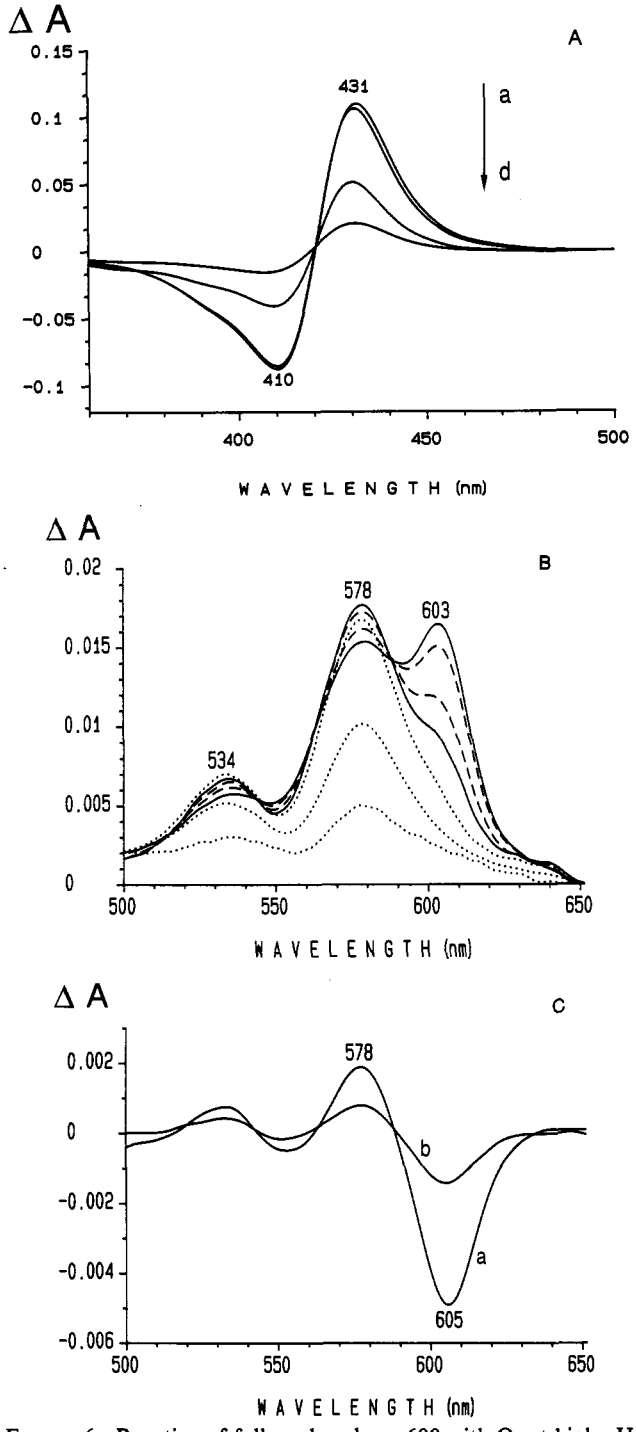

FIGURE 6: Reaction of fully reduced aa_3-600 with O_2 at high pH: difference spectra relative to fully oxidized enzyme. Panel A: Soret difference spectra at (a) 5.20 min after reoxidation, (b) 11.20 min, (c) 120 min, and (d) 240 min. Panel B: spectrum 1 (1.30 min after oxygen addition), solid line with a maximum at 603 nm; spectra 2 and 3 (3.30 and 8.00 min, respectively), dashed lines; spectrum 4 (14.00 min), solid line with a maximum at 578 nm (maximum occupancy of F); spectra 5–7 (32.00, 120, and 240 min), dotted lines. The spectrum of the final air-oxidized enzyme is used as a base line. Panel C: difference spectra from data in panel B at (a) 8.00 min *minus* 1.30 min and (b) 3.30 min *minus* 1.30 min. See legend of Figure 1 and Materials and Methods for other conditions.

decay by difference spectra, clearly demonstrating the conversion of the 603-nm species into the 578-nm species. Their absorbance ratio is approximately 2, which is again similar to previous data with the mitochondrial enzyme (Wikström & Morgan, 1992), and consistent with the data presented above.

In contrast to P formed from the mixed-valence, two-electron-reduced enzyme (Figure 3), the P state obtained from the fully reduced enzyme is unstable, and much of it has already been converted to F at the first time point of Figure 6B. Clearly, this is due to the presence of 1 additional electron equiv in the enzyme, as compared to the mixed-valence state (see Figure 1). This conversion is faster at lower pH (at pH 7.4, the rate is approximately 3–4 times faster than at pH 8.4; not shown); at neutral pH, very little of the P form can be trapped in this reaction. In contrast, the F state is quite stable, apparently because of the lack of a fourth electron in cytochrome aa_3-600. This is very different from cytochrome *c* oxidase, which is rapidly converted to the (pulsed) O state upon reaction with O_2 (Figure 1).

DISCUSSION

The results presented here show that a quinol oxidase member of the terminal oxidase family catalyzes the reduction of dioxygen to water through the same, or very similar, intermediates as the mitochondrial cytochrome *c* oxidase (Babcock & Wikström, 1992). This is not unexpected in view of the similar active-center structures of the two kinds of enzyme, as revealed by optical spectroscopic and resonance Raman studies (Lauraeus et al., 1992), and by the similar primary structures of the major subunits (Santana et al., 1992). However, the study of a terminal oxidase, which only contains 3 reducing equiv in its fully reduced state, instead of 4, can provide additional useful information on the catalytic mechanism, as shown here.

The two-electron cytochrome aa_3-600 produces a relatively stable peroxy intermediate upon reaction with O_2, whereas the fully reduced (three-electron) enzyme yields a stable ferryl form albeit with a clear peroxy intermediate. The specific absorptivities of the ferry and peroxy intermediates reported here are similar to those reported earlier for the corresponding intermediates of the mitochondrial enzyme, which were produced by a very different technique (Wikström & Morgan, 1992). These intermediates are stable in cytochrome aa_3-600, especially in the alkaline pH range. Notably, there is no indication of a continuous cyclic process, which is often detected when these intermediates are produced by addition of hydrogen peroxide to the oxidized enzyme (Wrigglesworth, 1984; Vygodina & Konstantinov, 1989).

In the past, it has not been possible to detect the peroxy intermediate, at any temperature, in so-called flow-flash experiments with the fully reduced mitochondrial enzyme. This has sometimes been taken to mean that the peroxy state is not a true intermediate in the catalytic mechanism [see Babcock and Wikström (1992)]. Babcock and Wikström (1992) suggested that the failure to detect the P state in such experiments might reflect fast transfer of the (third) electron in heme *a* to the binuclear site. In agreement with this, Verkhovsky et al. (1992) recently showed that the rate of oxidation of heme *a* by the binuclear site is very fast ($k = 2.4 \times 10^5\ s^{-1}$) and cannot be rate-limiting in cytochrome *c* oxidase. In contrast to the mitochondrial enzyme, fully reduced cytochrome aa_3-600, in which a third electron is in the low-spin heme *a*, exhibits a clear transient intermediate with optical characteristics of a peroxy state in which heme *a* is oxidized (Figure 6). The lack of cuprous Cu_A in cytochrome aa_3-600 might explain the relative stability of this peroxy state. In the absence of the fourth electron, the enzyme probably assumes a ferric heme a_3–peroxy–Cu_B^{1+} state with ferric heme *a*. It is possible that if heme *a* can be re-reduced in this situation, as in the mitochondrial enzyme, it forces fast electron transfer

from Cu_B to the heme a_3–peroxy structure due to the anticooperative redox interaction between heme *a* and Cu_B [see Babcock and Wikström, (1992)].

There is another difference of technical nature, however, which may be significant. In the "flow-flash" technique, the reactant is the CO derivative of the fully reduced enzyme, whereas no CO was used in the presently described experiment (Figure 6). As shown originally by Fiamingo et al. (1982), the photolysis of CO from heme a_3 iron initially yields CO bound to Cu_B^+ in the binuclear site. If the rate of dissociation of CO from Cu_B limits the velocity of the subsequent reaction steps [see Einarsdottir et al. (1992)], as was already proposed in the past (Wikström et al., 1981; Blair et al., 1985), this may well cause artificial reaction dynamics where the occupancy of the peroxy state is minimized.

In this connection, we should also recall the pioneering experiments of Orii and King (1972, 1976). These workers described intermediates, which may today be assigned as P and F, after reacting the fully reduced bovine heart enzyme with O_2 (without CO). However, when these spectra are compared with the data presented here, two major differences are evident. First, the occupancy of both F and especially P was much lower in the work with the bovine enzyme as compared with the data presented here, where virtually all enzyme molecules can be converted to these intermediates. Secondly, it is probable that Orii and King (1976) did not actually observe P and F (which they called compounds I and II, respectively) as intermediates of the *forward* reaction with O_2. In their case, it is more likely that the enzyme was quickly oxidized to the "pulsed" state on addition of O_2 and then reacted secondarily with H_2O_2 to produce P and F in the fashion that is now well understood (see the introduction). The H_2O_2 may well have been formed from a reaction between O_2 and the excess reductant present in the enzyme samples.

The pH dependence of the rate of decay of P into F in the reaction of the three-electron-reduced enzyme with O_2 provides strong support for the proposal that this reaction is associated with proton uptake (Wikström, 1988; Oliveberg et al., 1991; Babcock & Wikström, 1992).

An interesting finding with cytochrome aa_3-600 is that the P states formed from the two- and three-electron-reduced enzymes differ somewhat in their spectroscopic appearance; the α-band absorption peak of the latter is blue-shifted by about 4 nm relative to the former (cf. Figures 3B and 6B). This may reflect a difference in protonation state of the bound peroxide and/or a difference in the redox state of Cu_B. In the P state obtained from the three-electron-reduced enzyme, the third electron is clearly not resident in the low-spin heme *a*, and must therefore reside either in Cu_B or in heme a_3, of which the former seems more likely. Blair et al. (1985) proposed a ferrous heme a_3–peroxy–Cu_B^{2+} intermediate to account for an EPR signal from Cu_B, which occurred prior to O–O bond scission and formation of the EPR-silent ferryl form. However, the decay properties of this species at low temperature were such that it would hardly be detectable under our present conditions (Blair et al., 1985). In agreement with this work (Blair et al., 1985), we have also failed to observe any EPR signal arising from the F state of cytochrome aa_3-600.

The primary unprotonated peroxy species is relatively stable in the absence of electron donors [see Babcock and Wikström (1992)]. Its further conversion in the catalytic cycle was suggested first to require reduction of Cu_B, after which protonation of the bound peroxide occurs, followed by O–O bond scission and formation of the F state. The transient formation of a 603-nm peroxy species from the three-electron-reduced enzyme, which is spectrally different from the peroxy state formed from the two-electron-reduced enzyme (599 nm), is consistent with this proposal. The 4-nm red shift might be attributed to protonation of the peroxide bound to the ferric heme a_3 iron, subsequent to reduction of Cu_B. Interestingly, Denis (1981) has described a strongly temperature-dependent 4-nm red shift of the absorption peak of "Compound C", generated from two-electron-reduced enzyme in bovine heart mitochondria. Denis (1981) ascribed this to a molecular reorganization rather than to electron transfer, and it could thus involve protonation. This phenomenon was observed in mitochondria, but not in the isolated enzyme (Denis, 1981). In view of the endogenous reductants present in mitochondrial preparations, this phenomenon might thus have been triggered by mobilization of a "third electron" to reduce Cu_B, as we suggest here for cytochrome aa_3-600.

Conclusions. The catalytic mechanism of O_2 reduction in the quinol-oxidizing cytochrome aa_3-600 from *B. subtilis* is similar to that of the cytochrome *c* oxidases. However, in cytochrome aa_3-600, peroxy (P) and ferryl (F) intermediates were detected at high occupancy in the reactions with O_2 of both the fully reduced and the two-electron-reduced enzyme. A relatively stable ferric–cupric peroxy intermediate (P) is formed when the two-electron-reduced enzyme reacts with O_2 at neutral pH. At low pH, this intermediate becomes unstable due to dissociation of peroxide from the site, and reduction of undissociated P to F by the released H_2O_2. P is also formed as an intermediate when the fully reduced enzyme reacts with O_2, but its spectral properties suggest that it may be protonated, relative to P formed from the two-electron-reduced enzyme. Conversion of P into F at neutral or alkaline pH is preceded by transfer of a third electron (from heme *a*) into the binuclear site, presumably to Cu_B. This is then followed by proton uptake into the site before scission of the O–O bond and formation of the F intermediate can occur. The F formed is stable in the absence of a fourth electron.

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