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A Flash-Photolysis Study of the Reactions of a caa_3-Type Cytochrome Oxidase with Dioxygen and Carbon Monoxide

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The time course of absorbance changes following flash photolysis of the fully-reduced carboxy-cytochrome oxidase from *Bacillus* PS3 in the presence of O_2 has been followed at 445, 550, 605, and 830 nm, and the results have been compared with the corresponding changes in bovine cytochrome oxidase. The PS3 enzyme has a covalently bound cytochrome *c* subunit and the fully-reduced species therefore accommodates five electrons instead of four as in the bovine enzyme. In the bovine enzyme, following CO dissociation, four phases were observed with time constants of about 10 μs, 30 μs, 100 μs, and 1 ms at 445 nm. The initial, 10-μs absorbance change at 445 nm is similar in the two enzymes. The subsequent phases involving heme *a* and Cu_A are not seen in the PS3 enzyme at 445 nm, because these redox centers are re-reduced by the covalently bound cytochrome *c*, as indicated by absorbance changes at 550 nm. A reaction scheme consistent with the experimental observations is presented. In addition, internal electron-transfer reactions in the absence of O_2 were studied following flash-induced CO dissociation from the mixed-valence enzyme. Comparisons of the CO recombination rates in the mixed-valence and fully-reduced oxidases indicate that more electrons were transferred from heme a_3 to *a* in PS3 oxidase compared to the bovine enzyme.

KEY WORDS: Oxygen intermediates; electron transfer; flow flash; *Bacillus* PS3.

INTRODUCTION

Cytochrome caa_3 is a terminal oxidase in the thermophilic bacteria, *Bacillus* PS3 (Sone and Yanagita, 1982) and *Thermus thermophilus* (Mather *et al.*, 1993). It is closely related to other heme-copper oxidases which are redox-linked proton pumps and have a bimetallic dioxygen-reducing site, heme a_3-Cu_B, as one of their characteristics (Fee *et al.*, 1993; Trumpower and Gennis, 1994). It has five redox-active metal sites: two copper sites, Cu_A and Cu_B, two heme groups, hemes *a* and a_3, and a covalently-bound cytochrome *c*, which accepts electrons from water-soluble electron donors (Nicholls and Sone, 1984).

In this paper, we describe a study of the kinetics of the reactions of cytochrome caa_3 with dioxygen and carbon monoxide using two different techniques and compare the kinetics with that of bovine cytochrome oxidase (cytochrome aa_3): (1) Internal electron-transfer reactions in the absence of O_2 were studied following photolysis of CO from two-electron reduced enzyme (mixed-valence). In this enzyme form CO stabilizes the electrons at the binuclear center. Photodissociation of CO results in a drop of the apparent reduction potential of heme a_3 and a rapid intramolecular electron redistribution among the redox sites. (2) The reaction between fully-reduced enzyme and O_2 was studied following photodissociation of CO after rapid mixing with an O_2-containing buffer (flow-flash technique).

The reaction between the fully-reduced bovine enzyme and O_2 has been studied extensively using

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the flow-flash technique, combined with a variety of different spectroscopic techniques (see Varotsis *et al.*, 1993; Babcock and Wikström, 1992). First a transient O_2 adduct is formed with heme a_3 in about 10 μs. This is followed by intramolecular electron transfer from heme *a* to the binuclear center with a time constant of about 30 μs and formation of a peroxy intermediate. Proton uptake and electron redistribution among the redox centers leads to formation of a ferryl intermediate in about 100 μs, followed by the delivery of the fourth electron to the binuclear center and additional proton uptake in about 1 ms.

With the PS3 cytochrome caa_3, an oxygenated form and a peroxy intermediate have been reported at low temperatures (−120 to −80°C) after flash photolysis of CO from fully-reduced enzyme in the presence of O_2 (Sone *et al.*, 1984).

Our results suggest that the reaction sequence in the reaction of reduced cytochrome caa_3 with O_2 is very similar to that observed with the bovine enzyme. However, unlike the bovine enzyme these reactions are accompanied by the oxidation of the covalently bound cytochrome *c* which partly re-reduces heme *a* and Cu_A during the time course of the reactions, as has been observed previously in the electrostatic cytochrome *c*–bovine cytochrome *c* oxidase complex (Hill, 1991).

Internal electron transfers in the absence of O_2 have been studied after flash photolysis of the mixed-valence CO complex of cytochrome oxidase. In the bovine enzyme electron transfer from heme a_3 to *a* with a time constant of 3 μs was followed by electron transfer from the two hemes to Cu_A with a time constant of about 35 μs (see, e.g., Ädelroth *et al.*, 1995; Verkhovsky *et al.*, 1992). In cytochrome caa_3 the electron transfer from heme a_3 to *a* was presumably faster than the time resolution of the experimental setup (~1 μs) and therefore only the 35-μs electron transfer was observed.

MATERIALS AND METHODS

Enzymes

Cytochrome caa_3 from *Bacillus* PS3 was prepared as described previously (Sone and Yanagita, 1982). Bovine cytochrome oxidase was purified as described in Brandt *et al.* (1989).

Flow-Flash Measurements

The stock of cytochrome oxidase was dissolved to ca. 5 μM in an airtight Thunberg cuvette with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% Triton X-100 and 5 μM phenazine methosulfate (PMS). The sample was made anaerobic by repeated evacuation and flushing with purified N_2. Then ascorbate was added anaerobically to a final concentration of 2 mM to reduce the enzyme, followed by replacements of N_2 by CO. The samples were transferred anaerobically to a drive syringe of an RX-1000 stopped flow device (Applied Photophysics, Leatherhead, UK). The enzyme solution and the oxygenated buffer were mixed in a ratio of 1:5. The concentrations of the enzyme and O_2 after the mixing were 0.5–1 μM and ~1 mM, respectively. The experimental setup for time-resolved measurements of absorbance changes has been described in detail in Hallén and Brzezinski (1994). All measurements were carried out at 21 ± 1°C. Time constants ($1/e$) were determined using the Igor software (Wave Metrics Inc.).

Flash-Photolysis Measurements

The stock caa_3 enzyme was dissolved to ca. 2 μM in an airtight Thunberg cuvette with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% Triton X-100. The cuvette was repeatedly evacuated and flushed with CO. It was kept at room temperature and the mixed-valence state, verified by its optical spectrum, was formed within 1–2 hours (Brzezinski and Malmström, 1985). The sample of bovine cytochrome oxidase was prepared in the same way as the caa_3 sample except that 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% (weight per volume) dodecyl maltoside was used. Measurements were carried out just after the mixed-valence state was confirmed by the optical difference spectrum in order to avoid heme *a* reduction. This heme was more easily reduced in the bacterial than in the bovine enzyme, indicating a higher reduction potential of heme *a* in the caa_3 enzyme than in the bovine enzyme.

RESULTS

The Reaction of the Fully-Reduced Enzymes with Oxygen

The time courses of the absorbance changes at 445 nm following flash photolysis of the fully-reduced

carboxy cytochrome oxidases in the presence of O_2 are shown in Fig. 1A. In both enzymes a rapid absorbance increase associated with CO dissociation was followed by a decrease in absorbance with time constants of ~10 μs, attributed to binding of O_2 to heme a_3, and ~30 μs, associated with oxidation of hemes a_3 and a and formation of the peroxy intermediate. In the caa_3 oxidase the amplitude of this decrease was smaller than in the bovine oxidase. Assuming the same absorption coefficients of heme a in the bovine and bacterial oxidases, this indicates that heme a was partially re-reduced from the covalently bound cytochrome c, as evidenced by an absorption decrease at 550 nm with a time constant of 30 μs (Fig. 1B). In the bovine enzyme the following increase in absorbance at 445 nm, with a time constant of ~100 μs, is attributed to formation of the ferryl intermediate and electron transfer from Cu_A to heme a. This phase has a significantly smaller amplitude at 445 nm in the caa_3 enzyme, which together with the absorbance decrease at 550 nm with the same time constant indicates the involvement of cytochrome c in this step.

The subsequent heme a oxidation with a time constant of ~1 ms, seen in the bovine enzyme both at 445 nm and 605 nm, is not observed in the bacterial oxidase at these wavelengths (Fig. 1A and C) and is replaced by a minor increase in absorbance at 605 nm with the same time constant. Instead, in the bacterial enzyme, but not in the bovine enzyme, an absorbance decrease with a time constant of ~1 ms (2/3 of the total amplitude) is observed at 550 nm, indicating oxidation of the covalently bound cytochrome c (Fig. 1B). Using an absorption coefficient of 21.2 $mM^{-1}cm^{-1}$ for the PS3 cytochrome c (Sone and Yanagita, 1982) the total concentration of oxidized cytochrome c was estimated to be about 0.5 μM, which is about the same as the total enzyme concentration.

At 830 nm (Fig. 1D) in the bovine enzyme, there are small contributions from CO dissociation and changes in the reduction levels of the hemes (Boelens *et al.*, 1982), but the main contribution is from the 100-μs oxidation of Cu_A (see above). An absorbance change with the same time constant is observed also in the caa_3 enzyme, but the amplitude is much smaller,

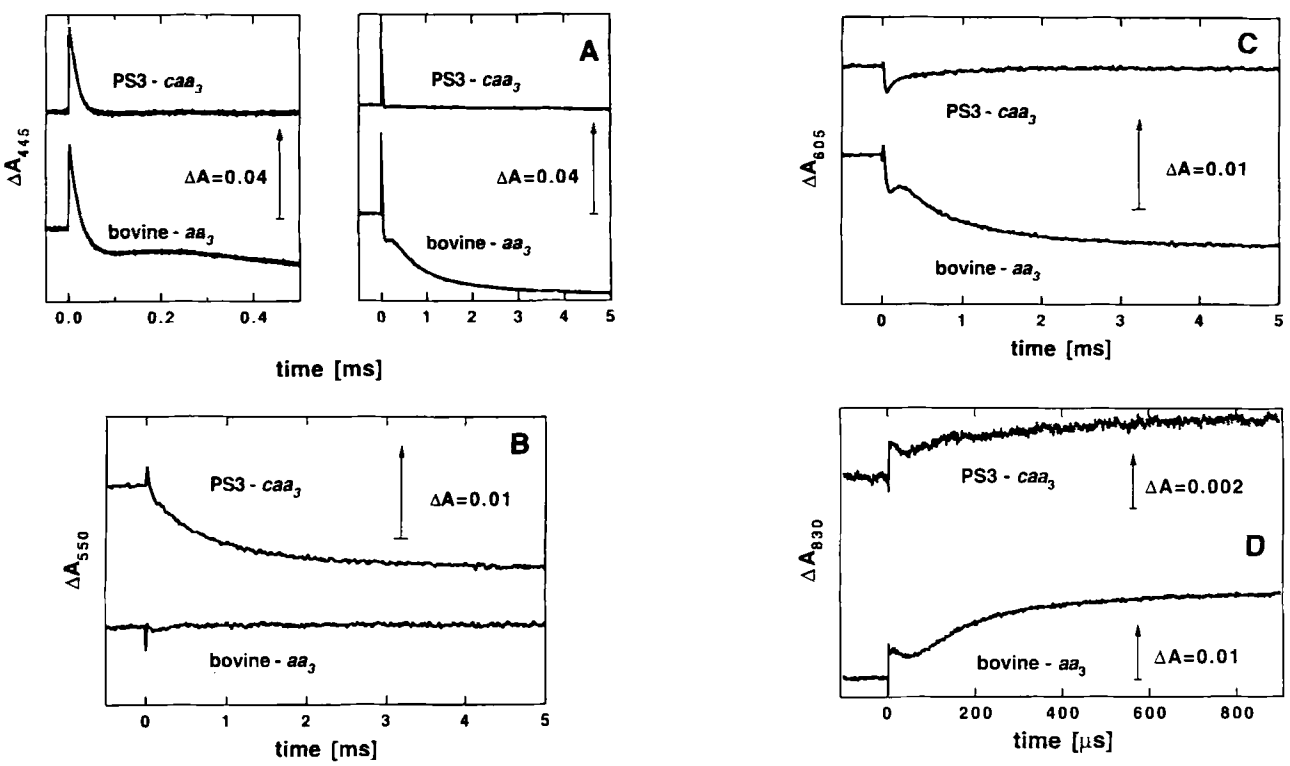

Fig. 1. Time course of the absorbance changes at 445 nm (A), 550 nm (B), 605 nm (C), and 830 nm (D) following flash photolysis of PS3 caa_3 and bovine fully-reduced carboxycytochrome oxidases in the presence of O_2. The concentrations of PS3 enzyme and oxygen after the mixing were about 0.5 μM and 1 mM, respectively. The concentration of bovine enzyme was 1.7 μM, but the ordinate is scaled to the CO photodissociation change in the PS3 enzyme at 445 nm, i.e., both traces in each panel correspond to the same concentration of photoactive enzyme. The traces shown are averages of 3–20 transients. Note the different time scales. The cuvette path length was 1 cm.

consistent with partial electron transfer from cytochrome *c* as discussed above.

A very slow absorbance decrease with a time constant of ≥20 ms at both 445 and 605 nm was observed with the caa_3 enzyme after completion of the O_2 reaction. This absorbance change is presumably associated with removal of the fifth electron remaining in the caa_3 enzyme after reduction of dioxygen (see Discussion).

Electron Transfer and CO Recombination in the Mixed-Valence Enzymes

Absorbance changes at 445 nm following flash photolysis of the CO complexes of the mixed-valence and fully-reduced enzymes are illustrated in Figs. 2 and 3. The rapid phase, with a time constant of 3 μs, found in the bovine enzyme is not seen in cytochrome caa_3 (Fig. 3). The second reaction phase in the bovine oxidase, on the other hand, is seen in the caa_3 case and has a time constant of 35 μs.

The ratio of the absorbance changes at 445 and 605 nm immediately following the CO dissociation from mixed-valence caa_3 (data not shown) was ~11, which is about half the value found in the fully-reduced enzyme (cf. Greenwood *et al.*, 1974). This suggests that heme a_3 is already oxidized at the shortest observation time, meaning that there is an unresolved electron transfer from heme a_3 to *a*. No absorbance change could be seen at 550 nm in mixed-valence caa_3, which indicates that the reduction potential of cytochrome *c* is significantly lower than those of heme *a* and Cu_A.

The extent of electron transfer was also estimated by a comparison of the CO recombination rates in the mixed-valence and fully-reduced enzymes (Fig. 2). Assuming all internal electron-transfer reactions in the oxidases to be much faster than the CO recombination, the observed recombination rate in the mixed-valence enzyme is given by (Verkhovsky *et al.*, 1992)

$$k_{mv} = k_{fr} \frac{[a_3^{2+}]}{[a_3^{2+}] + [a_3^{3+}]} \qquad (1)$$

where k_{mv} and k_{fr} are the CO recombination rates in the mixed-valence and fully-reduced enzymes, respectively. With the fully-reduced enzymes we found rates of 32 and 86 s^{-1} for the bacterial and bovine enzymes, respectively. Due to internal electron transfer from heme a_3 to *a* and Cu_A after CO dissociation the recombination rates were slower in the mixed valence forms, with rates of 18 and 60 s^{-1} with the bacterial and bovine enzymes, respectively. Applying Eq. (1) to

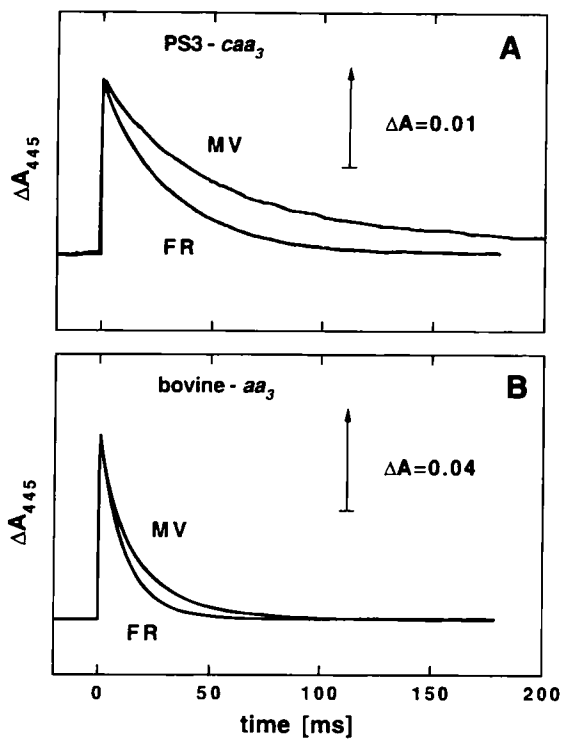

Fig. 2. Time course of the absorbance change at 445 nm following flash photolysis of mixed-valence (MV) and fully-reduced (FR) carboxycytochrome oxidases. (A) PS3 cytochrome oxidase (0.26 μM photoactive MV enzyme); (B) bovine cytochrome oxidase (1.1 μM photoactive MV enzyme). The FR ordinate scales have been scaled to the same CO-dissociation absorbance changes as in the MV samples. The traces shown are averages of 100 and 16 transients for PS3 and bovine enzymes, respectively.

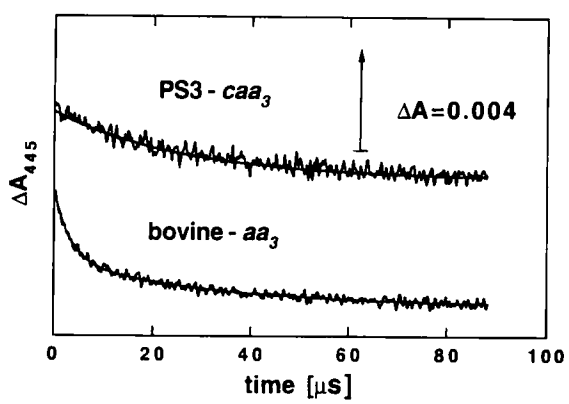

Fig. 3. Time courses of the absorbance changes at 445 nm following flash photolysis of mixed-valence CO complexes of PS3 and bovine cytochrome oxidases on a shorter time scale and on an expanded absorbance scale compared to those shown in Fig. 2.

these data we estimate that about 40% of heme a_3 has been oxidized in the caa_3 enzyme compared to about 30% in the bovine oxidase. This shows that the extent of electron transfer following CO dissociation was larger in the bacterial than in the bovine enzyme.

DISCUSSION

In Fig. 4 we present a scheme consistent with our experimental results for the reaction of fully-reduced cytochrome caa_3 with O_2. The oxygenated and peroxy intermediates of cytochrome caa_3 have been observed in low-temperature experiments (Sone *et al.*, 1984). The structures of the other intermediates in this scheme are based on resonance Raman measurements on the bovine enzyme (Varotsis *et al.*, 1993; Han *et al.*, 1990; Ogura *et al.*, 1993). Oxygen binds to reduced heme a_3 with a time constant of about 10 μs, followed by formation of the peroxide and ferryl ion intermediates with time constants of 30 and 100 μs, respectively (see Fig. 1C). The 445-nm data in Fig. 1A suggest that in the 30-μs step the fraction of oxidized heme *a* is immediately re-reduced by the covalently bound cytochrome *c*, possibly through Cu_A. This is consistent with the 550-nm data in Fig. 1B which shows that part of cytochrome *c* is oxidized on the same time scale.

The increase in absorbance at 605 nm with a time constant of about 100 μs (Fig. 1C) is partly associated with electron transfer from Cu_A to heme *a*, but also with formation of the "ferryl" species (Proshlyakov *et al.*, 1994). In the bovine enzyme this electron transfer takes place with a time constant of about 100 μs. This transfer is presumably rate limited by reactions at the binuclear center because the intrinsic electron-transfer between Cu_A and heme *a* is faster (see Fig. 3 and Morgan *et al.*, 1989; Verkhovsky *et al.*, 1992; Ädelroth *et al.*, 1995). The interaction between heme *a* and the binuclear center may be electrostatic in nature; proton uptake with a time constant of 100 μs at the binuclear center results in stabilization of the electron at heme *a* (Hallén and Brzezinski, 1994). In cytochrome caa_3 the reduction potential of heme *a* is higher and a partial re-reduction of this site is therefore faster (time constant 30 μs). Rapid electron transfer from the cytochrome *c* moiety of the PS3 enzyme to heme *a* has been reported (Nicholls and Sone, 1984).

The absorbance changes at 830 nm in the bacterial enzyme are rather small (Fig. 1D), which indicates that this site is re-reduced by the *c* cytochrome during the reaction sequence. Thus, electron transfer from

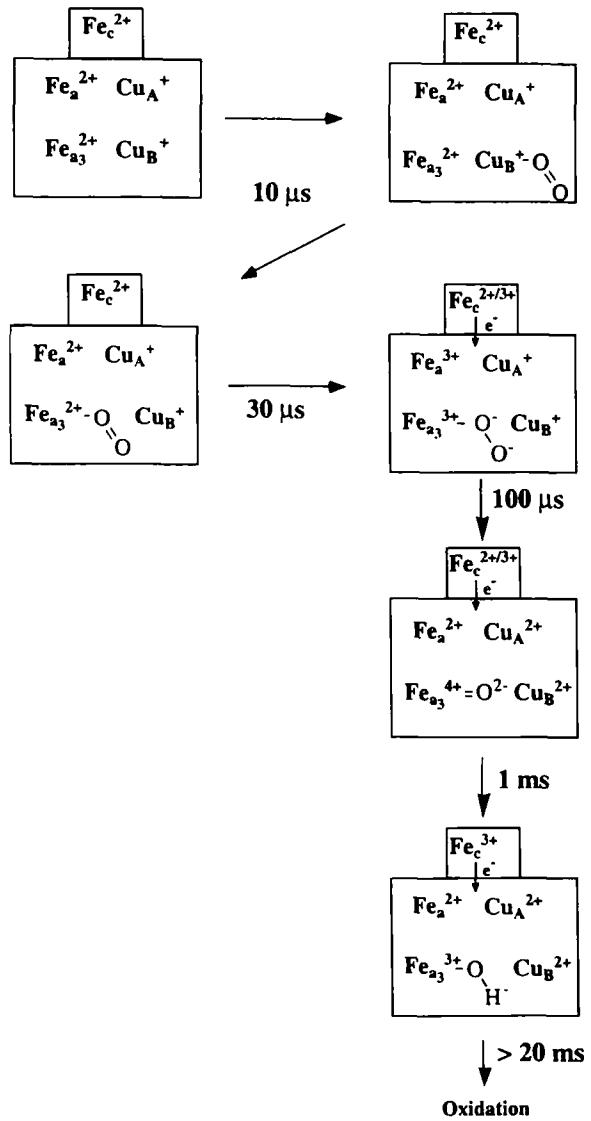

Fig. 4. Model for the sequence of the reaction between fully-reduced caa_3-type cytochrome oxidase and O_2. $Fe_c^{2+/3+}$ indicates a partially oxidized *c*-cytochrome. The fractional reduction of heme *a* and Cu_A during the 30 and 100 μs reactions is not shown (see text). After completion of the 1-ms reaction the electron resides primarily at heme *a*. For simplicity, proton uptake and release of water are not shown.

cytochrome *c* to Cu_A is rapid (<30 μs) as has been observed in the electrostatic complex of cytochrome *c* and the bovine enzyme (Pan *et al.*, 1993; Brzezinski *et al.*, 1995). This shows that in both systems Cu_A can be re-reduced on a time scale shorter than that of electron transfer from Cu_A.

In the bovine enzyme the last step (the 1-ms absorbance decrease) is partly associated with the transfer of the 4th electron to the binuclear center

from heme *a* (Fig. 1C). In the bacterial enzyme this decrease in absorbance (at 445 and 605 nm) is not observed and is replaced by an absorbance decrease at 550 nm, associated with oxidation of cytochrome *c*. This shows that either heme *a* is re-reduced rapidly by cytochrome *c* or the electron is transferred directly from cytochrome *c*, via Cu_A, to heme a_3. The latter transfer pathway has recently been discussed in terms of the three-dimensional *Paracoccus denitrificans* structure (Iwata *et al.* 1995) and may take place when the electron-transfer driving force for the reaction is large (Brzezinski, 1996). Oxidation of cytochrome *c* with a time constant of 1.8 ms was also observed in the electrostatic complex of cytochrome *c* with the bovine oxidase (Hill, 1991).

There is also a very slow oxidation of heme seen at both 445 and 605 nm. This is presumably because after the completion of the dioxygen reduction, there is still one electron in the enzyme. It resides mainly on heme *a*, which has a higher reduction potential than the covalently bound cytochrome *c*, as demonstrated by the reaction in the 1-ms phase (Figs. 1B and C). As has already been found with the bovine enzyme, such one-electron reduced oxidase does not react rapidly with O_2, since the reaction is limited by intermolecular electron transfer between the partially reduced molecules (Antonini *et al.*, 1970).

The 3-μs electron transfer from heme a_3 to *a* was not observed in the mixed-valence caa_3 enzyme and only the following electron transfer to Cu_A with a time constant of 35 μs was resolved. Assuming the same absorption coefficient for the heme a_3-to-*a* electron transfer as in the bovine enzyme and the same absorption coefficients for CO dissociation in the mixed-valence and fully-reduced forms, the difference in amplitudes of the absorbance changes immediately following the flash at 445 and 605 nm corresponds to about 15% unresolved electron transfer from heme a_3 to *a*. This is also consistent with the larger extent of oxidation of heme a_3 in the bacterial than in the bovine enzyme, as determined from the CO recombination kinetics [see Eq. (1)]. A rapid, unresolved electron transfer between heme o_3 and *b* has also been observed in cytochrome bo_3 from *E. coli* (Morgan *et al.*, 1993) and is also consistent with the estimation of the maximum possible rate of $2 \cdot 10^8\ s^{-1}$ between hemes *a* and a_3 in the bovine enzyme (when the driving force equals the reorganization energy; Brzezinski, 1996).

It has earlier been argued that there is a close relation between the intramolecular rate between hemes *a* and a_3 in the mixed-valence enzyme and during transfer of the third electron (30 μs phase) to the binuclear center during the reaction between the fully-reduced enzyme and O_2 (Verkhovsky *et al.*, 1994). In cytochrome caa_3 the intramolecular rate in the mixed-valence enzyme is presumably faster than in the bovine enzyme whereas the peroxy-intermediate is formed with the same rate, which suggests that the rate may instead be limited by an internal protonation reaction which stabilizes the peroxy intermediate (see, e.g., Varotsis *et al.*, 1993; Varotsis and Babcock, 1995).

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