Reaction of Dioxygen with Cytochrome c Oxidase Reduced to Different Degrees: Indications of a Transient Dioxygen Complex with Copper-B†

Mikael Oliveberg and Bo G. Malmström*

Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, S-412 96 Göteborg, Sweden

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ABSTRACT: The reactions of the fully reduced, three-electron-reduced, and mixed-valence cytochrome oxidase with molecular oxygen have been followed in flow-flash experiments, starting from the CO complexes, at 445 and 830 nm at pH 7.4 and 25 °C. With the fully reduced and the three-electron-reduced enzyme, four kinetic phases with rate constants in the range from 1×10^5 to 10^3 s^{-1} can be observed. The initial fast phase is associated with an absorbance increase at 830 nm. This is followed by an absorbance decrease (2.8×10^4 s^{-1}), the amplitude of which increases with the degree of reduction of the oxidase. The third phase (6×10^3 s^{-1}) displays the largest absorbance change at both wavelengths in the fully reduced enzyme and is not seen in the mixed-valence oxidase at 830 nm; a change with opposite sign but with a similar rate constant is found at 445 nm in this enzyme form. The slowest phase (10^3 s^{-1}) is also largest in the fully reduced oxidase and not seen in the mixed-valence enzyme. It is suggested that O_2 initially binds to reduced Cu_B and is then transferred to cytochrome a_3 before electron transfer from cytochrome a/Cu_A takes place. The fast oxidation of cytochrome a seen with the fully reduced enzyme is suggested not to occur during natural turnover. A reaction cycle for the complete turnover of the enzyme is presented. In this cycle, the oxidase oscillates between electron input and output states of the proton pump, characterized by cytochrome a having a high and a low reduction potential, respectively.

Cytochrome oxidase is a key enzyme in mitochondrial energy transduction. It catalyzes the oxidation of ferrocytochrome c by molecular oxygen and couples this reaction to the translocation of protons across the inner mitochondrial membrane. The reaction of the fully reduced and mixed-valence oxidase with dioxygen has repeatedly been studied spectrophotometrically by the flow-flash technique [for reviews, see Hill et al. (1986) and Oliveberg et al. (1989)]. An oxygen adduct of reduced cytochrome a_3 is first formed, followed by oxidation of the binuclear site with the formation of peroxide and, in the case of the fully reduced enzyme, subsequent electron transfer from reduced cytochrome a and Cu_A.

In this paper, we describe an extension of previous spectrophotometric flow-flash investigations. It includes not only the fully reduced and mixed-valence oxidase but also the three-electron-reduced enzyme. The use of an improved detection system in the near-infrared region (Hoganson et al., 1991; Oliveberg & Malmström, 1991) led to the discovery that the initial bimolecular reaction with molecular oxygen is associated with a small absorbance increase at 830 nm. It is suggested that this stems from a transient binding of O_2 to reduced Cu_B, in analogy with the initial event in CO recombination with the reduced binuclear site (Woodruff et al., 1991). The subsequent reoxidation of Cu_A, seen as a large absorbance increase at 830 nm, is preceded by a rapid absorbance decrease, the amplitude of which increases with the degree of reduction of the enzyme. This reaction phase is proposed to reflect a transfer of O_2 from Cu_B to cytochrome a_3. At 445 nm, a slow fourth reaction phase was observed in the fully reduced enzyme, in agreement with previous results (Oliveberg et al., 1989).

Since this paper constitutes the final contribution of a series of studies from this laboratory dealing with internal electron transfer and dioxygen chemistry in cytochrome oxidase [see Oliveberg and Malmström (1991) for references], we conclude it with the presentation of an extensive reaction scheme. This represents an attempt to rationalize not only our own observations but also a wealth of experimental information in the literature on the kinetics of electron transfer, oxygen reduction, and proton translocation.

Materials and Methods

Cytochrome oxidase was prepared essentially by the method of Brandt et al. (1989), which yields homogeneous "fast" enzyme as defined by Baker et al. (1987). Sample preparations, the excitation laser and observation equipment, the photolysis method, and the curve-fitting procedure were exactly as described by Oliveberg and Malmström (1991). All experiments were carried out in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)[1] buffer, pH 7.4, containing 0.167 M K_2SO_4 and 0.5% Tween 80, and at 25 °C.

The rate constants estimated by curve fitting to sequential exponential phases are rather uncertain, particularly when the constants differ by less than a factor of 10, in which case the error may be as large as a factor of 2.

Results

Oxygen Reaction Followed at 830 nm. The absorbance changes at 830 nm following photolysis of the CO compounds of cytochrome oxidase reduced to different degrees are shown in Figure 1. The initial phase (F_1) has a pseudo-first-order rate constant of about 1×10^5 s^{-1}. With the oxygen concentration used (approximately 1 mM), this corresponds to a second-order rate constant of 10^8 M^{-1} s^{-1}, in agreement with the results of Greenwood and Gibson (1967).

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[1] Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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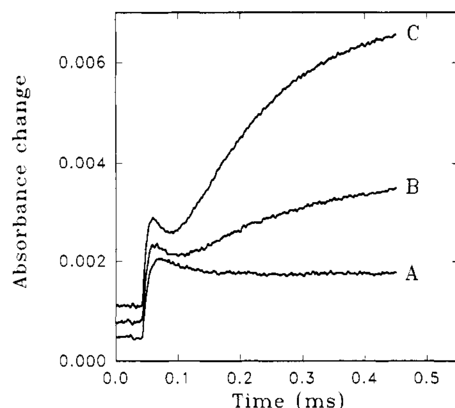

FIGURE 1: Time course of the absorbance changes at 830 nm following flash photolysis in the presence of O_2 (1 mM) of carboxycytochrome oxidase (4 μM) reduced to different degrees: (A) mixed-valence; (B) three-electron-reduced; (C) fully reduced.

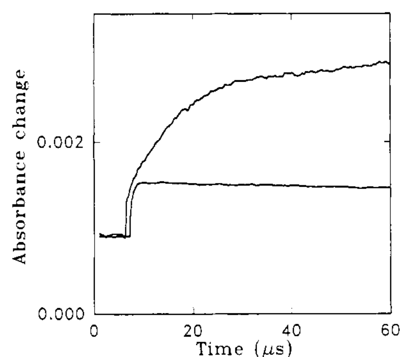

FIGURE 2: Initial absorbance changes at 830 nm following flash photolysis of mixed-valence carboxycytochrome oxidase (4 μM) in the absence (bottom) and presence (top) of O_2 (0.5 mM).

Table I: Absorbance Changes at 830 nm following Flash Photolysis of Carboxycytochrome Oxidase (4 μM)

	absorbance change × 10^3		
enzyme form	1st phase	2nd phase	3rd phase
mixed-valence	1.6	−0.9	
3-e⁻-reduced	1.9	−2.3	2.7
fully reduced	1.7	−4.4	8.0

A difficulty with the experiment in Figure 1 is that F_1 is so rapid that it is not resolved from the step in absorbance corresponding to the photodissociation of CO. Therefore, the experiment was repeated with 0 and 0.5 mM oxygen, as shown in Figure 2. Here the photodissociation is clearly separated from the oxygen reaction, which now has an apparent rate constant of 5×10^4 s^{-1}, demonstrating that F_1 really represents a bimolecular reaction with dioxygen. In Table I, the amplitudes for F_1 in Figure 1 have been corrected for the absorbance change in the photodissociation step.

The rate constant for the second phase (F_2), which involves an absorbance decrease, is 2.8×10^4 s^{-1}. Its amplitude increases with an initial degree of reduction of the enzyme (Table I), but it is noticeable that it is seen even in the mixed-valence enzyme.

In the third phase (F_3), there is an absorbance increase, which is largest in the fully reduced oxidase and not seen in the mixed-valence enzyme (Table I). F_3 has a rate constant of 6000 s^{-1}. This is the same phase for which a rate constant of 10 000 s^{-1} was estimated earlier with a less sensitive detecting device (Oliveberg et al., 1989).

The 830-nm laser used for the analysis light in these experiments was not stable enough to record accurately events with rate constants below 2000 s^{-1}. Earlier experiments with conventional light sources have, however, shown that F_3 is followed by a phase (F_4) in which there is a further absorption increase at 830 nm with a rate constant of 1000 s^{-1} (Hill et al., 1986).

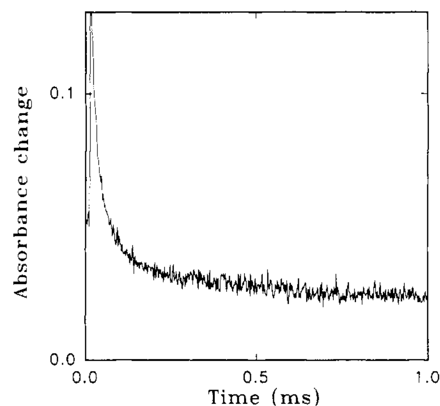

FIGURE 3: Time course of the absorbance changes at 445 nm following flash photolysis of three-electron-reduced carboxycytochrome oxidase (1 μM) in the presence of O_2 (1 mM).

Oxygen Reaction Followed at 445 nm. Four phases in the oxygen reaction of the fully reduced enzyme, with the same rate constants as derived from the 830-nm experiments, can also be observed at 445 nm (Hill et al., 1986; Oliveberg et al., 1989). Figure 3 shows the reaction trace at 445 nm starting with the three-electron-reduced enzyme. Curve fitting to this trace is rather uncertain, since it consists of at least five exponential components with the same sign. Therefore, it has been analyzed by simulations with the use of rate constants determined with the mixed-valence or fully reduced enzyme.

The amplitude of the absorbance change at 445 nm in F_1 is independent of the initial degree of reduction of the enzyme. The next phase (F_2) can be observed as an absorbance decrease in the fully reduced and three-electron-reduced oxidase but not in the mixed-valence enzyme. The absorbance increase in F_3 is seen in the fully reduced enzyme but cannot be resolved in the three-electron-reduced enzyme. A phase with a similar rate constant, but with opposite sign, can be observed with the mixed-valence oxidase. The final slow absorbance decrease in F_4 can be measured in the fully reduced and, to a smaller extent, in the three-electron-reduced oxidase, but it is not seen with the mixed-valence enzyme.

Discussion

The initial rapid phase (F_1) has earlier been seen at 445 nm (Oliveberg et al., 1989), but here it is observed for the first time at 830 nm (Figures 1 and 2). An absorbance increase at this wavelength is generally ascribed to the oxidation of Cu_A, but a contribution to the total absorption of 10–15% from other components cannot be excluded (Greenwood et al., 1974; Beinert et al., 1980). Because F_1 is observed also in the mixed-valence oxidase (Table I), it cannot represent partial oxidation of Cu_A. An obvious possibility is that cytochrome a_3 has a contribution at 830 nm, as suggested by Greenwood et al. (1974), particularly since F_1 can also be seen at 445 nm. Iron porphyrin bands in this region are, however, generally weak (Makinen & Churg, 1983). An alternative would be that the initial binding of O_2 involves reduced Cu_B, as illustrated in Figure 4. This scheme is formulated in analogy with the sequence of events suggested for CO binding by Woodruff et al. (1991). Karlin et al. (1991) have recently demonstrated that a mononuclear Cu^I–O_2 adduct has an absorption band in the near-infrared with an extinction coefficient of 1000 M^{-1} cm^{-1} and a stronger band at 420 nm. Thus, the suggestion

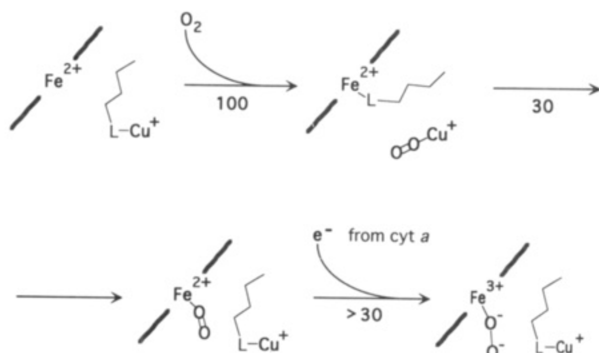

FIGURE 4: Model for the initial events in the reaction of O_2 with the reduced binuclear site in cytochrome oxidase. The model is based on the mechanism for CO binding to this site, proposed by Woodruff et al. (1991).

for the F_1 phase in Figure 4 is a real possibility. It may be noted that Blackmore et al. (1991) have recently provided evidence that the initial oxygen adduct does not involve cytochrome a_3.

Phase F_2, with a rate constant of 30 000 s^{-1}, is not related to the back-flow of electrons earlier observed anaerobically (Oliveberg & Malmström, 1991), since this is not expected to occur in the presence of oxygen with the binuclear center in a high-potential state. We have previously assigned this phase to the formation of the peroxide intermediate (Oliveberg et al., 1989). Hill and Greenwood (1983) have suggested that this occurs with a rate constant of 6000 s^{-1} in the mixed-valence state. It is, however, possible that F_2 could represent peroxide formation also in this enzyme form, which would thus occur with the same rate as in the fully reduced oxidase. The lower rate, seen only as a change in heme absorption, must then correspond to a structural change.

The introduction of the Cu_B adduct suggests another possibility, illustrated in Figure 4, and further developed in the following. Here F_2 represents a shift of bound O_2 from Cu_B to cytochrome a_3. Again following Woodruff et al. (1991), this shift is proposed to involve a ligand exchange between Cu_B and cytochrome a_3. The absorbance change in F_2 cannot involve Cu_A. First, it is seen in the mixed-valence state, albeit with a smaller amplitude than in the more reduced forms (Table I). Second, it would represent reduction of Cu_A, which can hardly occur in the fully reduced enzyme. Thus, the reaction in F_2 is likely to be a structural rearrangement in the binuclear site, as suggested. The fact that it is not seen at 445 nm may be related to the rate with which Cu_B is oxidized in the different enzyme forms, as discussed later in connection with a detailed reaction scheme.

Han et al. (1990) have concluded from resonance Raman measurements, starting from the fully reduced enzyme, that the primary oxygen intermediate decays synchronously with cytochrome *a* oxidation with a rate constant of 30 000 s^{-1}. The rate constant for electron transfer from cytochrome *a* to the binuclear site has, however, been shown to be 2×10^5 s^{-1} (Oliveberg & Malmström, 1991), so that it is probable that the rate constant of 30 000 s^{-1} is determined by the structural change, the actual electron transfer being faster, as shown in Figure 4. It is natural that the amplitude of F_2 is different in the more reduced states (Table I), since the structural rearrangement is coupled to oxidation of Cu_B in the mixed-valence enzyme but not in the other forms.

The fully reduced enzyme is an artificial species, not expected to be formed in a normal turnover situation, because in turnover the peroxide is likely to be formed before cytochrome *a* is re-reduced (Malmström & Andréasson, 1985).

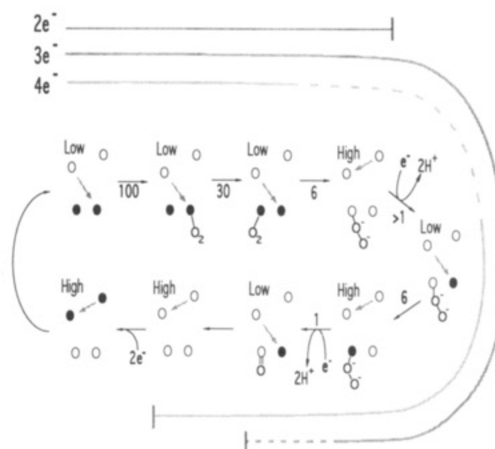

FIGURE 5: Turnover cycle for cytochrome oxidase as a redox-linked proton pump. The small circles represent the four redox centers: cytochrome *a* and Cu_A (top) and cytochrome a_3 and Cu_B (bottom). Open and filled circles denote oxidized and reduced centers, respectively. The designations "high" and "low" refer to the reduction potentials of cytochrome *a*. Arrows indicate the equilibrium directions for electron transfers between individual centers. Not to make the cycle too large, several steps have been combined, e.g., electron entry from cytochrome *c* and subsequent internal electron transfer. The parts of the cycle which are expected to be observed in the three enzyme forms studied here are indicated. The dashed line shows reactions that are short-circuited in the four-electron-reduced enzyme, whereas in the three-electron-reduced form it indicates that the final state is different compared to the other forms. For further explanations, see the text.

Thus, the state relevant to turnover should be the mixed-valence state. As first suggested by Hill and Greenwood (1983), the formation of the peroxide in the mixed-valence state is slow (6000 s^{-1}) compared to that seen with the fully reduced enzyme. We propose that this is related to the function of cytochrome oxidase as a redox-linked proton pump. Such pumps must exist in at least two different conformations, representing separate input and output states for electrons and protons (DeVault, 1971). In Figure 5, we present a reaction cycle for the electron-transfer and proton translocation steps in cytochrome oxidase turnover, incorporating this principle, and consistent with a good deal of experimental information. In the figure, we indicate which transient experiments give relevant information for the different steps in the turnover cycle. Thus, the mixed-valence oxidase gives valid results for the steps leading to the peroxide, whereas the fully reduced enzyme reaches this intermediate too rapidly, for reasons already discussed. On the other hand, the fully reduced enzyme reproduces most steps in the remaining cycle. The three-electron-reduced enzyme includes all the steps of the cycle except those triggered by the last electron. A combination of results from all three enzyme forms, together with stopped-flow measurements of the input of the first two electrons, consequently makes it possible to construct the whole cycle.

In the scheme in Figure 5, it is proposed that the redox interactions between the sites in cytochrome oxidase (Blair et al., 1986) are directly related to the input and output states for electrons. Thus, cytochrome *a* has a low reduction potential in the output state and a high one in the input state. The oxidation state of Cu_B is assumed to determine these states, as first suggested by Wikström (1988). When Cu_B is oxidized, cytochrome *a* has a high potential, whereas the potential is low when Cu_B is reduced. Electrons from cytochrome *c* enter the oxidase via Cu_A (Pan et al., 1991), which is in rapid redox equilibrium with cytochrome *a* (Morgan et al., 1989; Oliveberg & Malmström, 1991). Cu_A also serves as an electron buffer

and can be reduced in all states.

Starting from the mixed-valence state in the upper left-hand corner of Figure 5, the first two reactions are the same as described in Figure 4. Here there is no electron on cytochrome *a*, however, to keep Cu_B reduced (Figure 4), so the peroxide formation is coupled to the output–input transition triggered by the oxidation of Cu_B with a rate constant of 6000 s^{-1}. This corresponds to phase F_3, which was first discovered by Brunori and Gibson (1983), who, however, did not have any data at 830 nm. Our results (Figure 1) show that F_3 involves partial oxidation of Cu_A, caused by cytochrome *a* going from the low- to the high-potential state. This cannot occur in the mixed-valence state, in which both cytochrome *a* and Cu_A are oxidized.

In the next step, cytochrome *c* donates one electron to Cu_A/cytochrome *a*. As this is transferred to Cu_B, the enzyme returns to the electron output state at a rate which must be >1000 s^{-1}, since otherwise a plateau in the 445-nm trace would be seen also with the three-electron-reduced enzyme. The assignment is also consistent with the fact that this phase is not seen in the mixed-valence oxidase. In this step, two protons are picked up. They are then released as the oxidase returns to the input state on formation of the three-electron-reduced intermediate, here depicted as the peroxide intermediate with cytochrome a_3 reduced (Blair et al., 1985). Now this sequence is repeated on donation of the fourth electron from cytochrome *c*. The O–O bond is suggested to be broken in the 1000-s^{-1} reaction, since this step is the slowest one and the one with the largest temperature dependence (Oliveberg et al., 1989).

In experiments with the fully reduced enzyme in phospholipid vesicles, proton release to the outside has been observed in the step from the three-electron-reduced to the oxidized enzyme but not in the transition from peroxide to the three-electron-reduced intermediate (Nilsson et al., 1990). The reason may be that in this case there is still an electron on cytochrome a/Cu_A, when three electrons have been donated to the binuclear site, and that this reduces Cu_B before the enzyme has gone from the low- to the high-potential state of cytochrome *a*. No protons are released in the final high–low-potential transition at the bottom of Figure 5 (Wikström, 1989). This could be related to the two-electron requirement for the initial reduction of the binuclear site (Antonini et al., 1970) and to the electron-buffer function of Cu_A. Thus, Cu_B is only very transiently oxidized during the transfer of the first two electrons, so that the oxidase does not have time to undergo the input–output transitions.

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Registry No. O_2, 7782-44-7; Cu, 7440-50-8; cytochrome oxidase, 9001-16-5; cytochrome a_3, 72841-18-0.

REFERENCES

Antonini, E., Brunori, M., Greenwood, C., & Malmström, B. G. (1970) *Nature 228*, 936–937.

Baker, G. M., Noguchi, M., & Palmer, G. (1987) *J. Biol. Chem. 262*, 595–604.

Beinert, H., Shaw, R. W., Hansen, R. E., & Hartzell, C. R. (1980) *Biochim. Biophys. Acta 591*, 458–470.

Blackmore, R. S., Greenwood, C., & Gibson, Q. H. (1991) *J. Biol. Chem. 266*, 19245–19249.

Blair, D. F., Witt, S. N., & Chan, S. I. (1985) *J. Am. Chem. Soc. 107*, 7389–7399.

Blair, D. F., Ellis, W. R., Jr., Wang, H., Gray, H. B., & Chan, S. I. (1986) *J. Biol. Chem. 261*, 11524–11537.

Brandt, U., Schägger, H., & Von Jagow, G. (1989) *Eur. J. Biochem. 182*, 705–711.

Brunori, M., & Gibson, Q. H. (1983) *EMBO J. 2*, 2025–2026.

DeVault, D. C. (1971) *Biochim. Biophys. Acta 225*, 193–199.

Greenwood, C., & Gibson, Q. H. (1967) *J. Biol. Chem. 242*, 1782–1787.

Greenwood, C., Wilson, M. T., & Brunori, M. (1974) *Biochem. J. 137*, 205–215.

Han, S., Ching, Y.-C., & Rousseau, D. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8408–8412.

Hill, B. C., & Greenwood, C. (1983) *Biochem. J. 215*, 659–667.

Hill, B. C., Greenwood, C., & Nicholls, P. (1986) *Biochim. Biophys. Acta 853*, 91–113.

Hoganson, C. W., Casey, P. A., & Hansson, Ö. (1991) *Biochim. Biophys. Acta 1057*, 399–406.

Karlin, K. D., Wei, N., Jung, B., Kaderli, S., & Zuberbühler, A. D. (1991) *J. Am. Chem. Soc. 113*, 5868–5870.

Makinen, M. W., & Churg, A. K. (1983) in *Iron Porphyrins, Part I* (Lever, A. B. P., & Gray, H. B., Eds.) pp 141–235, Addison-Wesley Publishing Co., Reading, MA.

Malmström, B. G., & Andréasson, L.-E. (1985) *J. Inorg. Biochem. 23*, 233–242.

Morgan, J. E., Li, P. M., Jang, D.-J., El-Sayed, M. A., & Chan, S. I. (1989) *Biochemistry 28*, 6975–6983.

Nilsson, T., Hallén, S., & Oliveberg, M. (1990) *FEBS Lett. 260*, 45–47.

Oliveberg, M., & Malmström, B. G. (1991) *Biochemistry 30*, 7053–7057.

Oliveberg, M., Brzezinski, P., & Malmström, B. G. (1989) *Biochim. Biophys. Acta 977*, 322–328.

Pan, L.-P., Hazzard, J. T., Lin, J., Tollin, G., & Chan, S. I. (1991) *J. Am. Chem. Soc. 113*, 5908–5910.

Wikström, M. (1988) in *Cytochrome Oxidase* (Brunori, M., & Chance, B., Eds.) pp 199–206, New York Academy of Sciences, New York.

Wikström, M. (1989) *Nature 338*, 776–778.

Woodruff, W. H., Einarsdóttir, O., Dyer, R. B., Bagley, K. A., Palmer, G., Atherton, S. J., Goldbeck, R. A., Dawes, T. D., & Kliger, D. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2588–2592.