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REACTION MECHANISM OF ENZYME STUDIED BY PULSE RADIOLYSIS

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ABSTRACT

By virtue of its ability to generate hydrated electrons (e_{aq}^-) and various radicals as reductants, the pulse radiolysis technique has been employed for investigating the mechanism of action of peroxidase, cytochrome P-450, and cytochrome oxidase. The oxy forms of hemoproteins, such as myoglobin, peroxidase, and cytochrome P-450, were reduced by hydrated electrons to form the higher oxidation states of these hemoproteins. From these results, the reactive oxygen intermediate of cytochrome chrome P-450 is discussed. The reduction of cytochrome oxidase by the 1-methylnicotinamide radical was investigated. A decrease of the 830-nm band was detected due to the reduction of "visible" copper. After the first phase of the reduction of copper, the return of the 830-nm band corresponding to oxidation of copper was observed. Concomitantly, the absorption at 605 and 445 nm due to the reduction of heme *a* increased. This suggests that 1-methylnicotinamide radical reacts with the "visible" copper and subsequently flows to heme *a* by intramolecular migration.

INTRODUCTION

The steady-state kinetics for electron transfer reaction in biochemical systems have been studied extensively, but such studies supply only a limited amount of information concerning the mechanism of action of the enzyme.

A more appropriate way to clarify the individual steps of the reaction is to study transient kinetics. Among such studies, the rapid-mixing technique is widely used. The applicability of this method, however, is limited by mixing times of milliseconds. Such time is often too long for an accurate description of the events in biochemical redox processes.

On the other hand, by virtue of its ability to generate hydrated electrons (e_{aq}^-), pulse radiolysis is well suited to investigate a number of biochemical redox processes [1-3]. The e_{aq}^- reduces the redox proteins rapidly within the microsecond time scale. Thus, one might expect to observe the unstable intermediates after reduction and the subsequent reaction steps. This technique is also useful for investigating electron-transfer reactions in proteins containing multiple electron-accepting sites per molecule. In this system it can be anticipated that e_{aq}^- reacts with the primary redox site and subsequently moves to other electron-accepting sites by intramolecular migration until equilibrium is reached.

Typical examples are presented in this paper. As our first example, we describe the reaction of e_{aq}^- with the oxy form of hemoproteins such as oxymyoglobin, oxyperoxidase, and oxycytochrome P-450 in Reaction (1):

$$Fe^{2+}-O_2 + e_{aq}^- \longrightarrow \qquad (1)$$

As a second example, the reaction of 1-methylnicotinamide radical with cytochrome oxidase has been investigated. This enzyme contains four electron-accepting sites, two heme *a* and two copper atoms. One of the interesting questions to be answered is how the electrons are transferred within the enzyme during the catalytic cycle.

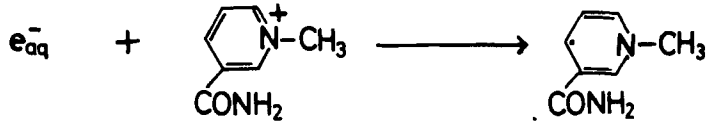
PRINCIPLE AND METHOD

Since the experiments in biochemical systems are carried out in aqueous solution, high-energy electrons interact exclusively with the water molecule. In these systems we observe the reaction of solute with the intermediates formed by the water molecule. These are e_{aq}^-, the hydroxyl radical, and the hydrogen atom. Among these species, the reaction of an individual free radical and a solute can be isolated under appropriate conditions. For example, in order to investigate the reaction of e_{aq}^- with a solute, we add 0.1 mol/L *t*-butyl alcohol to the solution. The OH radical reacts with *t*-butyl alcohol within 1 μs under the experimental conditions and yields an unreactive radical.

$$\cdot OH + CH_3-\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{\overset{|}{\underset{|}{C}}}}-OH \longrightarrow H_2O + \cdot CH_2-\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{\overset{|}{\underset{|}{C}}}}-OH. \qquad (2)$$

The production of hydrogen atoms is small compared with that of e_{aq}^-. The e_{aq}^- has a broad absorption with a peak near 700 nm and is the most potent reductant known ($E^0 \leqslant 2.67$ V) [14]. Therefore, the kinetics of e_{aq}^- as the reductant with a solute can be measured directly by optical absorption methods.

The e_{aq}^-, however, is nonspecific in its reaction, whereas various radicals, such as pyridinum radicals [5, 6] and $CO_2 \cdot^-$, are sometimes more selective in their reactions. In the presence of 1 mmol/L pyridinum compounds, all the primary radicals of water radiolysis are converted, within less than 1 μs, into pyridinum radicals via Reaction (3).



(3)

One-Electron Reduction in Oxy Form of Hemoproteins

Horseradish peroxidase (HRP), which contains iron(III) protoporphyrin, catalyzes the oxidation of a wide variety of hydrogen donors by H_2O_2. The widely accepted mechanism of HRP reaction in Eqs. (4)-(6) was derived principally from the work of Chance [8].

$$HRP + H_2O_2 \longrightarrow \text{Compound I}, \qquad (4)$$

$$\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + AH\cdot \qquad (5)$$

$$\text{Compound II} + AH_2 \longrightarrow HRP + AH\cdot \qquad (6)$$

$$2AH\cdot \longrightarrow A + AH_2. \qquad (7)$$

The AH_2 is a hydrogen donor, such as ascorbate, phenol, or aromatic amines. HRP is a unique enzyme in that it possesses five oxidation-reduction states of HRP. Figure 1 shows the relationships among the five oxidation-reduction states of HRP. In this scheme it has been thought that oxyperoxidase is at one equivalent oxidized state above Compound I [9]. The one-electron

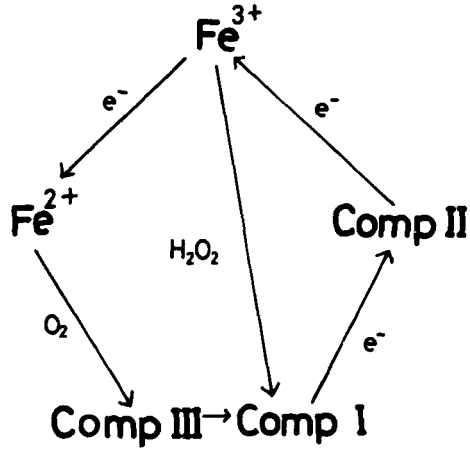


FIG. 1. The relationship of the five oxidation-reduction states of HRP.

reduction of oxiperoxidase to Compound I presumably occurs according to

$$Fe^{2+}\text{–}O_2 \longrightarrow \text{Compound I}, \tag{8}$$

where it has been proposed that Compound III is an oxy form structure like oxymyoglobin [10, 11] and Compound I is a ferryl π cation radical [12, 13]. Reaction (8), however, has not yet been observed directly. This difficulty stems from the fact that the reaction products, Compounds I and II, further react with the reducing agents and oxyperoxidase [9].

On the other hand, cytochrome P-450 is unique among oxygenases in its ability to cleave dioxygen into water and a single oxygen atom which can subsequently insert into hydrocarbon bonds. Among this enzyme's complexes, the reactive oxygen intermediate which is formed by one-electron reduction of the ferrous oxy form has received considerable attention. This intermediate can be written formally as a loss of water to yield $(FeO)^{3+}$, which is analogous in terms of redox stoichiometry to Compound I of HRP. This can be supported by the fact that organic hydroperoxide can replace NADPH and molecular oxygen in hydroxylation of various substrates [14, 15]. However, the structures which have been written for this intermediate are largely speculative, and the one-electron reduction of the ferrous oxy form of cytochrome P-450 has not yet been observed directly.

In this report we focus our attention on the reaction of e_{aq} with the oxy form of hemoproteins such as myoglobin, HRP, and cytochrome P-450 in an attempt to observe the one-electron-reduced state of the oxy form of hemoproteins. In the case of HRP and cytochrome P-450, the oxy form of artificial enzymes, in which the 2,4-diacetyldeuteroheme replaced the protohemin IX of native HRP and cytochrome P-450, were employed in these experiments. The oxy form of this enzyme is very stable and can be kept at room temperature.

We found that the oxy form of HRP was reduced by e_{aq}^- to form Compound I, which was demonstrated by the kinetic difference spectra. Similarly, oxymyoglobin was reduced by e_{aq}^- to form the hydrogen peroxide-induced compound, so-called "ferryl" myoglobin, not deoxy myoglobin or metmyoglobin, which in turn was established by the kinetic difference spectra. Both reactions were found to follow second-order kinetics with a rate constant of 4×10^{10} $L \cdot mol^{-1} \cdot s^{-1}$ at pH 7.4, without detection of intermediates. That is to say, e_{aq}^- apparently reduces the redox site of oxyheme in a direct reaction. These reactions, however, can be interpreted in terms of the following sequence events:

$$e_{aq}^- + Fe^{2+}\text{–}O_2 \longrightarrow Fe^{2+}\text{–}O_2^- \longrightarrow \text{product.} \qquad (9)$$

An initial step is the formation of Fe^{2+}–O_2^-, followed by an intramolecular electron transfer from heme iron to the ligand forming Fe^{3+}–O_2^{2-}. Subsequent rapid association of H^+ and loss of water would then lead to the observed final product. The rate constant k_{9b} would be greater than 10^7 s^{-1}. Thus the reaction of e_{aq}^- with oxyheme is the rate-limiting step in the overall process.

The oxy form of cytochrome P-450 was reduced by e_{aq}^- to form a product which exhibits absorption maximum at 470 and 370 nm. This product is considered to be a higher oxidation state of this enzyme, as observed in the case of HRP and myoglobin. However, the higher oxidation state of cytochrome P-450 has been difficult to observe. The reaction of peracid with purified liver microsomal cytochrome P-450 gives an intermediate with an absorption maximum at 421 nm [16]. The difference spectra obtained in our experiments are quite different in that absorption maxima are observed at 470 and 370 nm. Moreover, the spectrum is quite dissimilar from that observed in the spectra of Compound I of HRP or of chloroperoxidase [17]. Both of these peroxidases show a marked decrease in the Soret band with a broad absorbance band.

On the other hand, the spectrum of Compound II of chloroperoxidase exhibits an absorption band at 440 nm and a broad absorption shoulder with a

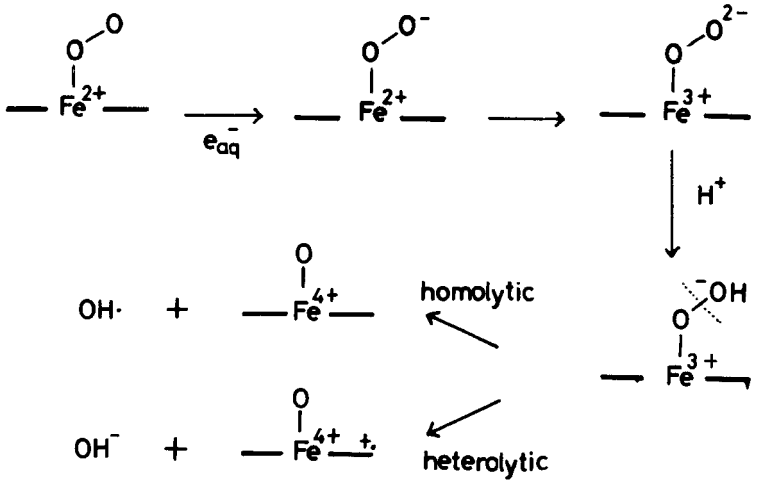


FIG. 2. Schematic presentation of one-electron reduction of the oxy form of hemoproteins.

maximum at about 380 nm [17]. This compares quite favorably to the difference spectrum observed for the one-electron reduced oxy form of diacetyl-deutero cytochrome P-450 when one takes into account the fact that the Soret band of this modified hemoprotein is expected to shift to a longer wavelength due to the electron-withdrawing effects of the acetyl groups. This finding is of interest in that chloroperoxidase, unlike other peroxidases, has other similarities to cytochrome P-450. For example, the absorption spectrum of the CO complex of ferrous chloroperoxidase exhibits a Soret band near 450 nm, a characteristic which is attributed to the evidence that chloroperoxidase has a sulfhydryl axial ligand, as does cytochrome P-450 [18]. The spectral similarity of the one-electron reduced form of the oxy form of cytochrome P-450 to that of Compound II of chloroperoxidase suggests that the active oxidizing form of cytochrome P-450 is a Compound II-like structure of chloroperoxidase.

From these results, the possible reaction mechanism for the formation of the higher oxidation states is considered as follows (Fig. 2). An initial step is the formation of Fe^{2+}–O_2^-, followed by an intramolecular electron transfer from heme iron to the ligand, forming Fe^{3+}–O_2^-. Subsequently, two reaction mechanisms can be considered. A heterolytic cleavage of the O–O bond occurs to form two equivalent oxidized states above the ferric form and H_2O. Alternatively, a homolytic cleavage of the O–O bond occurs to form one equivalent

oxidized state above the ferric enzyme and OH•. The oxidation states in the former and latter mechanisms correspond to Compounds I and II of HRP, respectively. In the case of myoglobin, the oxidation state which corresponds to Compound I of HRP was not detected. In the oxidation of ferric myoglobin by H_2O_2, the production of OH•, which is similar to the ferrous Fenton-like reaction, was suggested by George and Irvine [19]. We can propose that homolytic cleavage of the O—O bond occurs to form one equivalent oxidized state above metmyoglobin and OH•, unlike HRP. On the other hand, an important finding of the present work is that the active oxidizing form produced by the pulse radiolysis method is a Compound II-like structure of chloroperoxidase, not Compound I. This suggestion is compatible with a homolytic mechanism of the O—O bond cleavage, not with the heterolytic formation.

Cytochrome Oxidase

Figure 3 shows the time course of absorption change in the reaction of 1-methylnicotinamide radical with fully oxidized cytochrome oxidase. The absorption at 445 and 605 nm, a characteristic to ferrous heme *a*, increased. These absorption changes deviated from first-order kinetics and consisted of

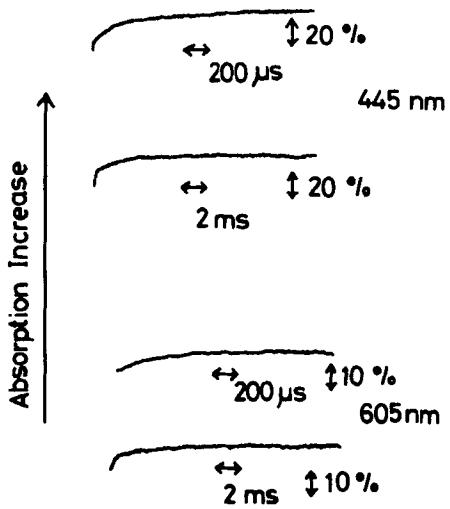


FIG. 3. Oscilloscope traces of the transmittance change in the reaction of cytochrome oxidase with the 1-methylnicotinamide radical.

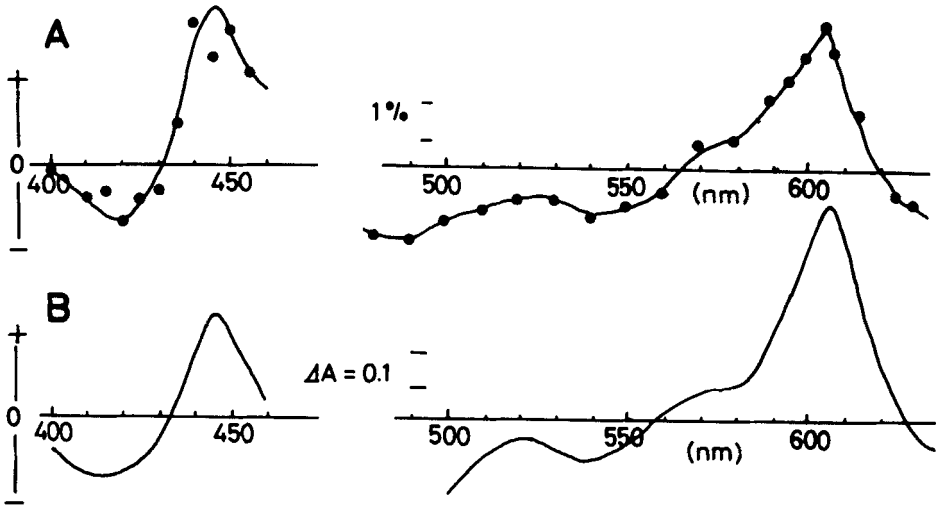


FIG. 4. (A) Kinetic difference spectrum of pulse radiolysis of cytochrome oxidase. The spectrum was taken 1 ms after pulse radiolysis. (B) Difference spectrum of fully oxidized minus fully reduced cytochrome oxidase.

fast and slow phases. Figure 4 shows the fully reduced minus fully oxidized cytochrome oxidase. This spectrum was obtained in reduction of fully oxidized cytochrome oxidase by dithionite. The kinetic difference spectrum has an absorption maximum at 605 nm and a broad peak around 540 nm and is similar to the difference spectrum of fully oxidized minus fully reduced cytochrome oxidase. Therefore, it is concluded that the 1-methylnicotinamide radical transfers an electron to ferric heme *a* of cytochrome oxidase.

At high cytochrome oxidase concentration (>100 μmol/L), the absorption at 830 nm decreased with a half time of 5 μs after pulse radiolysis, as shown in Fig. 5. The difference spectrum 10 μs after pulse is similar to the difference spectrum of fully oxidized minus fully reduced cytochrome oxidase in the infrared region of the spectrum. The infrared band at 830 nm is thought to be due to both copper and heme *a* [20]. In this time scale, however, the reduction of heme *a* at 445 and 605 nm was not observed. This suggests that rapid bleaching of the 830 nm band is due to the reduction of "visible" copper. This absorption change obeys pseudo-first-order kinetics, which is to be expected since the concentration ratio [cyt oxidase]/[1-methylnicotinamide radical] is greater than 10. The pseudo-first order of the decay of the 830-nm band in-

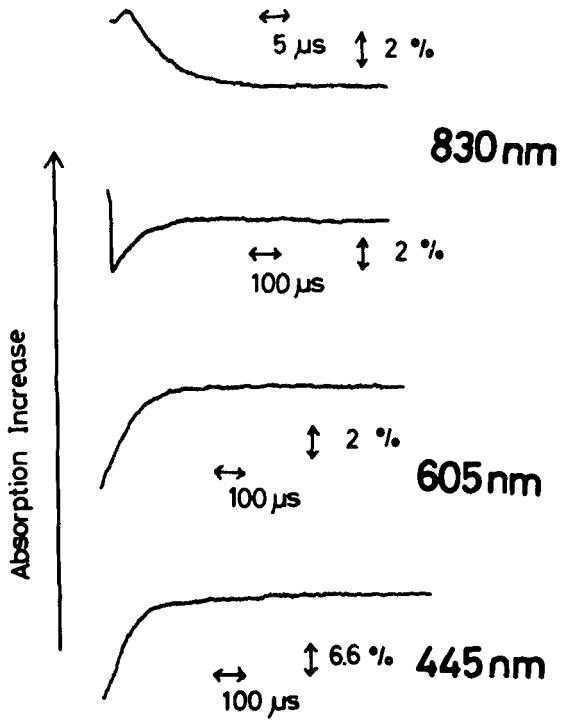


FIG. 5. Oscilloscope traces of the transmittance change in the reaction of a high concentration of cytochrome oxidase with the 1-methylnicotinamide radical.

creases with increasing concentration of cytochrome oxidase (Fig. 6). This suggests that the absorption change of Fig. 5 is followed by bimolecular reaction between copper of this enzyme and 1-methylnicotinamide radical. From the slope of Fig. 7(A), the second-order rate constant is 1.2×10^9 $L \cdot mol^{-1} \cdot s^{-1}$. After the first phase of the reduction of copper, the return of the 830-nm band corresponding to oxidation of copper was observed with a half time of 100 μs. Concomitantly, the absorption at 445 and 605 nm characteristic to the reduction of heme *a* increased, as shown in Fig. 5. Figure 7(B) shows the enzyme concentration dependence of the first-order rate constant obtained at 445, 605, and 830 nm. The rate constants are almost constant within experimental error. Therefore, it is concluded that the slow process is due to the intramolecular electron transfer from copper to heme *a* within the enzyme.

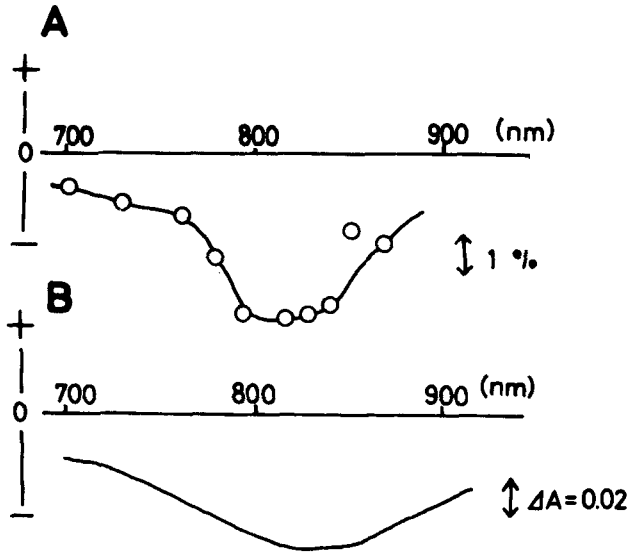


FIG. 6. (A) Kinetic difference spectrum of pulse radiolysis of cytochrome oxidase. The spectrum was taken 10 μs after pulse radiolysis. (B) Difference spectrum of fully oxidized minus fully reduced cytochrome oxidase.

The rapid absorption change with a rate of 1.4×10^4 s^{-1} observed at 830 nm and in the visible region is due to electron transfer from copper to heme *a* and can be summarized as in Fig. 8. In this scheme the absorption change in the heme region might be due to the reduction of heme *a*, not heme a_3. This is supported by the difference spectrum of Fig. 4, where heme *a* absorbs by 605 nm in the visible region [21]. In addition, it has been reported that the rate of reduction of heme a_3, which proceeds by intramolecular electron transfer from heme *a*, is very slow (0.3-0.6 s^{-1}) [22-24]. Therefore, 10 ms after pulse, the reduction of heme a_3 may not take place.

In the reaction of the reduced cytochrome C with cytochrome oxidase, heme *a* and copper are reduced simultaneously [22]. In this case it has been believed that all electrons enter the oxidase molecule heme *a*, and heme *a* rapidly hands on electrons to copper. However, in the present experiments we may discount the possibility that electrons are being transferred directly from the 1-methylnicotinamide radical to the heme *a*, as the rates measured

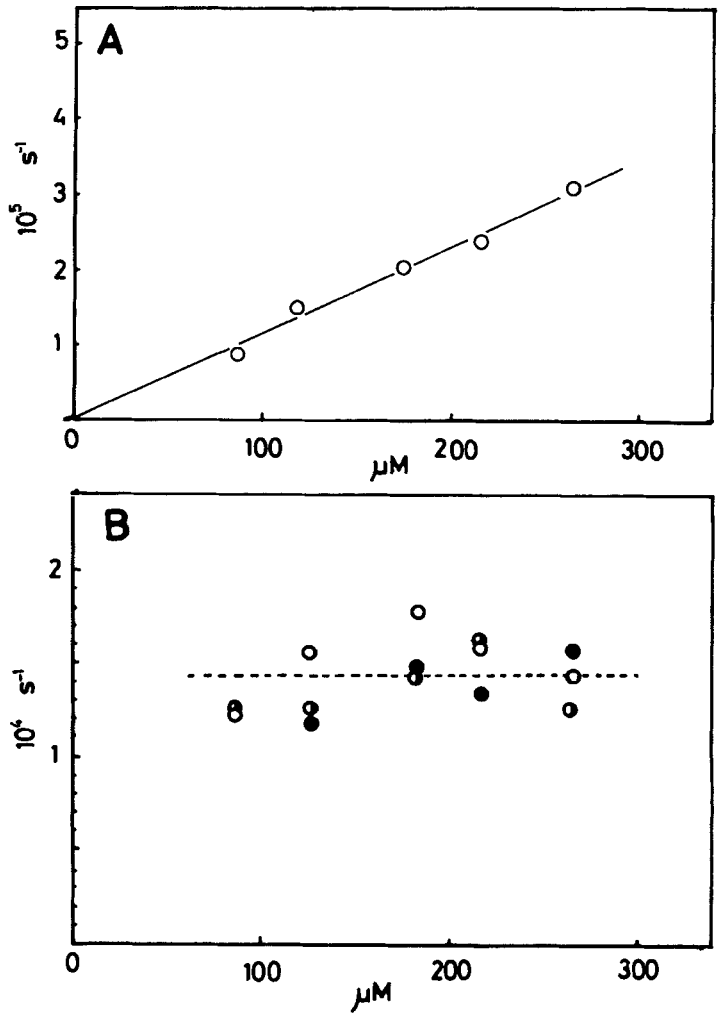


FIG. 7. (A) Concentration dependence of the first-order rate constants obtained at 830 nm. (B) Concentration dependence of the first-order rate constants obtained at 445 nm (●), 605 nm (○), and 830 nm (◑).

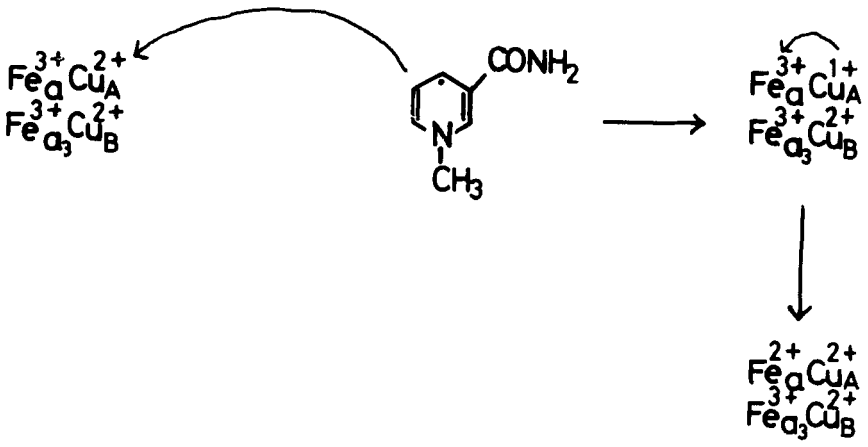


FIG. 8. Schematic presentation of one-electron reduction of cytochrome oxidase.

at 605 and 445 nm were independent of cytochrome oxidase concentration. Here, the present findings are incompatible with the proposal that the primary event in the cytochrome oxidase reaction is the transfer of an electron from reduced cytochrome C to heme *a*. The alternative is that the first acceptor of copper on the oxidase reacts with reduced cytochrome C at the rate measured for the cytochrome oxidation and subsequently passes on the electron to heme *a* at a much faster rate. Indeed, the rate of intramolecular electron transfer (1.4×10^4 s^{-1}) is much faster than the rate measured for cytochrome C oxidation. In this case, an electron apparently transfers from cytochrome C to heme *a*.

REFERENCES

[1] K. Kobayashi and K. Hayashi, in *Fast Methods in Physical Biochemistry and Cell Biology* (R. Sha'afi and R. Fernandez, eds.), Elsevier North-Holland Biomedical, New York, 1983, p. 87.

[2] K. Kobayashi, K. Hirota, H. Ohara, K. Hayashi, R. Miura, and T. Yamano, *Biochemistry, 22,* 2239 (1983).

[3] N. Shimizu, K. Kobayashi, and K. Hayashi, *J. Biol. Chem., 259,* 4414 (1984).

[4] M. S. Matheson and L. M. Dorfman, *Pulse Radiolysis,* M.I.T. Press, Cambridge, Massachusetts, 1969, p. 6.
[5] M. G. Simic and I. A. Taub, *Biophys. J., 24,* 285 (1978).
[6] P. Debey, E. J. Land, R. Santus, and A. J. Swallow, *Biochem. Biophys. Res. Commun., 86,* 953 (1979).
[7] H. Seki, Y. A. Ilan, and G. Stein, *Biochim. Biophys. Acta, 440,* 573 (1976).
[8] B. Chance, *J. Biol. Chem., 151,* 553 (1943).
[9] M. Tamura and I. Yamazaki, *J. Biochem., 71,* 311 (1972).
[10] K. Yokota and I. Yamazaki, *Biochem. Biophys. Res. Commun., 18,* 48 (1965).
[11] J. B. Wittenberg, R. W. Noble, B. A. Wittenberg, E. Antonini, M. Brunori, and J. Wyman, *J. Biol. Chem., 242,* 626 (1967).
[12] D. Dolphin, A. Forman, D. C. Borg, J. Fajer, and R. H. Felton, *Proc. Natl. Acad. Sci. U.S.A., 68,* 614 (1971).
[13] G. N. La Mar, J. S. de Ropp, K. M. Smith, and K. C. Langry, *J. Biol. Chem., 256,* 237 (1981).
[14] F. F. Kadlubar, K. C. Morton, and D. M. Ziegler, *Biochem. Biophys. Res. Commun., 54,* 1255 (1973).
[15] A. Berg, M. Ingelman-Sundberg, and J-A. Gustafsson, *J. Biol. Chem., 254,* 5264 (1979).
[16] R. C. Blake II and M. J. Coon, *Ibid., 255,* 4100 (1980).
[17] J. A. Thomas, D. R. Morris, and L. P. Hager, *Ibid., 245,* 3135 (1970).
[18] T. L. Poulos, B. C. Finzel, I. C. Gunsalus, G. C. Wagner, and J. Kraut, *Ibid., 260,* 16122 (1985).
[19] P. George and D. H. Irvine, *J. Colloid Sci., 11,* 327 (1956).
[20] C. Greenwood, M. T. Wilson, and M. Brunori, *Biochem. J., 137,* 205 (1974).
[21] S. Horie and M. Morrison, *J. Biol. Chem., 238,* 1855 (1963).
[22] O. H. Gibson, C. Greenwood, D. C. Wharton, and M. T. Wilson, *Biochem. J., 165,* 413 (1977).
[23] C. Greenwood, T. Brittain, M. Brunori, and M. T. Wilson, *Ibid., 165,* 413 (1977).
[24] M. T. Wilson, C. Greenwood, M. Brunori, and C. Antonini, *Ibid., 147,* 145 (1975).