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ARTICLE *in* BIOPHYSICAL JOURNAL · SEPTEMBER 1992

Impact Factor: 3.97 · DOI: 10.1016/S0006-3495(92)81608-9 · Source: PubMed

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Hydrogen peroxide plays a key role in the oxidation reaction of myoglobin by molecular oxygen

A computer simulation

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ABSTRACT The stability properties of the iron(II)-dioxygen bond in myoglobin and hemoglobin are of particular importance, because both proteins are oxidized easily to the ferric met-form, which cannot be oxygenated and is therefore physiologically inactive. In this paper, we have formulated all the possible pathways leading to the oxidation of myoglobin to metmyoglobin with each required rate constant in 0.1 M buffer (pH 7.0) at 25°C, and have set up six rate equations for the elementary processes going on in a simultaneous way. By using the Runge-Kutta method to solve these differential equations, the concentration progress curves were then displayed for all the reactive species involved.

In this complex reaction, the primary event was the autoxidation of MbO_2 to metMb with generation of the superoxide anion, this anion being converted immediately and almost completely into H_2O_2 by the spontaneous dismutation. Under air-saturated conditions (P_{O_2} = 150 Torr), the H_2O_2 produced was decomposed mostly by the metMb resulting from the autoxidation of MbO_2. At lower pressures of O_2, however, H_2O_2 can act as the most potent oxidant of the deoxyMb, which increases with decreasing O_2 pressures, so that there appeared a well defined maximum rate in the formation of metMb at ~5 Torr of oxygen. Such examinations with the aid of a computer provide us, for the first time, with a full picture of the oxidation reaction of myoglobin as a function of oxygen pressures. These results also seem to be of primary importance from a point of view of clinical biochemistry of the oxygen supply, as well as of pathophysiology of ischemia, in red muscles such as cardiac and skeletal muscle tissues.

INTRODUCTION

During reversible oxygen binding, myoglobin and hemoglobin undergo a slow, but considerable oxidation to the ferric met-form, which cannot be oxygenated and is therefore physiologically inactive. The mechanistic details of this autoxidation reaction, which are of clinical, as well as of chemical importance, have been investigated by a number of authors, but still remain unclear for a full understanding of the overall stoichiometry.

Since the early work of Brooks (1931, 1935) on HbO_2 and that of George and Stratmann (1952, 1954) on MbO_2, it has long been observed that even at a constant pH, the rate of the oxidation increases with decreasing partial pressure of O_2 and shows a well defined maximum value at approximately the pressure required for each half-saturation (P_{50}). Several proposals have therefore been made concerning the mechanism of this oxidation reaction, and these have recently been reviewed from a thermodynamic viewpoint (Shikama, 1984, 1990).

Along with the early work, Brown and Mebine (1969) and Wallace et al. (1982), among others, also agreed that the first step in autoxidation of MbO_2 and HbO_2 was the dissociation of the oxygen ligand, followed by the oxidation of the deoxy species by free O_2 to produce metMb or metHb and the superoxide anion. In the case of myoglobin, therefore, the reaction was written as

$$Mb(II)(O_2) \rightleftharpoons Mb(II) + O_2$$

$$Mb(II) + O_2 \overset{H^+}{\rightharpoonup} metMb(III) + O_2^-.$$

In this scheme, however, the differences in the deoxy species, which allow them to react with oxygen in one instance to become oxygenated and in another instance to become oxidized, were completely unknown (Snyder, 1963). Also, it should be noted here that free dioxygen is a poor electron acceptor with a lower redox potential, $\epsilon_0'(O_2/O_2^-) = -0.33$ V, than those, $\epsilon_0' = +0.046$ V for the Mb(III)/Mb(II) couple (Taylor and Morgan, 1942) and $\epsilon_0' = +0.150$ V for the Hb(III)/Hb(II) system (Antonini et al., 1964), and that such a one-electron transfer from the iron(II) to free O_2 cannot occur spontaneously (Shikama, 1990). In addition, the involvement of H^+ was not clear.

Recent kinetic and thermodynamic studies of the stability of native oxymyoglobin have revealed that the superoxide formation is not due to a simple, dissociative loss of O_2^- from MbO_2, but is due to a nucleophilic displacement of O_2^- from MbO_2 by a water molecule or a hydroxyl ion that can enter the heme pocket from the surrounding solvent, so that the iron is converted to the ferric met-form (Satoh and Shikama, 1981; Shikama, 1984, 1985). The reductive displacement of the bound dioxygen as O_2^- by H_2O can proceed without any pro-

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Abbreviations used in this paper: Mb, myoglobin; MbO_2, oxymyoglobin; metMb, metmyoglobin; Mb(II), ferrous myoglobin; Mb(III), ferric myoglobin; Mb(IV), ferryl myoglobin; Hb, hemoglobin; HbO_2, oxyhemoglobin; metHb, methemoglobin.

0006-3495/92/08/544/07 $2.00

tonation. Nevertheless, the rate is enormously enhanced by a proton-assisted process which involves the distal histidine (E7) as its catalytic residue by a proton-relay mechanism (Shikama and Matsuoka, 1986; Shikama, 1988). The autoxidation reaction of MbO_2 to metMb can therefore be explained by the following three types of displacement processes:

$$Mb(II)(O_2) + H_2O \rightarrow Mb(III)(OH_2) + O_2^-$$
$$Mb(II)(O_2) + H_2O + H^+ \rightarrow Mb(III)(OH_2) + HO_2$$
$$Mb(II)(O_2) + OH^- \rightarrow Mb(III)(OH^-) + O_2^-.$$

The extent of the contribution of these elementary processes to the observed or overall autoxidation rate can vary with the concentration of H^+ or OH^- ions. Consequently, the stability of MbO_2 shows a very complicated pH dependence having a parabolic part (Shikama, 1988).

Unfortunately, it seemed that there was no provision in this scheme for the inverse dependence of the autoxidation rate upon oxygen pressure. In this respect, however, it is of great interest to note that hydrogen peroxide can oxidize deoxyMb more than 100 times more easily than oxyMb (Yusa and Shikama, 1987). Since H_2O_2 is produced by dismutation of the superoxide anion generated from the autoxidation of the oxy-form, it must act as at least one of the potent oxidants of the deoxy-form that increases with decreasing O_2 pressures (Tajima and Shikama, 1987).

In this paper, we present a complete kinetic formulation for the autoxidation of MbO_2 to metMb, including several types of the subsequent reactions of myoglobin with H_2O_2. In dealing with this complex reaction in a quantitative way, we have carried out a detailed set of numerical analyses by solving the rate equations derived from each elementary processes involved. Such a computer simulation may provide us, for the first time, with a full picture of the oxidation reaction of myoglobin and hemoglobin by molecular oxygen.

DESCRIPTION OF REACTION PATHWAYS WITH REQUIRED RATE CONSTANTS

It is in the ferrous form that myoglobin and hemoglobin can bind molecular oxygen reversibly and carry out their functions. For oxygen binding to myoglobin, therefore, we may write the equation:

$$Mb(II) + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} MbO_2. \quad (1)$$

In neutral pH range and at 25°C, we adopt here the values of $k_{on} = 1.64 \times 10^7\ s^{-1}\ M^{-1}$ and $k_{off} = 19\ s^{-1}$ for sperm whale myoglobin having the oxygen dissociation constant of $K_D = 1.15 \times 10^{-6}$ M. These were calculated from the literature values given at 20°C, by using the corresponding activation energies (Antonini and Brunori, 1971).

Under air-saturated conditions, however, the oxygenated form of Mb or Hb is considerably oxidized to the ferric met-form with generation of the superoxide anion (Gotoh and Shikama, 1976),

$$MbO_2 \xrightarrow{k_A} Mb(III) + O_2^-, \quad (2)$$

where k_A represents the first-order rate constant for the autoxidation reaction of MbO_2, its magnitude being strongly dependent upon the pH of the solution. At pH 7.0, for instance, we observed the value of $k_A = 8.1 \times 10^{-3}\ h^{-1}$ for sperm whale MbO_2 in 0.1 M phosphate buffer at 25°C (Shikama and Matsuoka, 1986).

The superoxide anion generated above can easily be converted into hydrogen peroxide with a high rate constant (for instance, $k_d = 7.2 \times 10^8\ h^{-1}\ M^{-1}$ in 0.1 mM phosphate buffer at pH 7.4 and 24°C in the absence of heme compounds [Rabani and Nielsen, 1969; Fridovich, 1975]), by the following spontaneous dismutation:

$$2O_2^- + 2H^+ \xrightarrow{k_d} H_2O_2 + O_2. \quad (3)$$

Recently, Yusa and Shikama (1987) have found that hydrogen peroxide can induce very rapid oxidation of MbO_2 to metMb. Kinetic and spectrophotometric analyses have revealed that this oxidation proceeds through the formation of ferryl-Mb(IV) from deoxy-Mb(II), which is in equilibrium with MbO_2, by a two-equivalent oxidation with H_2O_2. Once the ferryl species is formed, it reacts rapidly with another deoxy-Mb(II) in a bimolecular fashion so as to yield 2 mol of metMb(III). The overall reaction may be written, therefore, as

$$Mb(II) + H_2O_2 \xrightarrow{k_\phi} Mb(IV) + 2OH^-$$
$$Mb(IV) + Mb(II) \xrightarrow{fast} 2Mb(III)$$
$$\text{Sum: } 2Mb(II) + H_2O_2 \rightarrow 2Mb(III) + 2OH^-. \quad (4)$$

In this coupled reaction, the rate-determining step was the oxidation of the deoxy species with H_2O_2, its rate constant being estimated to be on the order of $k_\phi = 1.3 \times 10^7\ h^{-1}\ M^{-1}$ within equimolar amounts of H_2O_2 to 50 μM deoxyMb in 0.1 M phosphate buffer at pH 7.0 and 25°C (Yusa and Shikama, 1987).

On the other hand, we have also found that H_2O_2 produced from the dismutation of O_2^- can be eliminated or decomposed mostly by the metMb resulting from the normal autoxidation reaction of MbO_2, via the cyclic formation of the ferryl species as

$$Mb(III) + H_2O_2 \xrightarrow{k_f} {}^*Mb(IV){=}O + H_2O \quad (5)$$

and

$${}^*Mb(IV){=}O \xrightarrow{k_r} Mb(III), \quad (6)$$

where the values of $k_f = 1.6 \times 10^6\ h^{-1}\ M^{-1}$ (within fivefold molar excess of H_2O_2 to 50 μM metMb) and $k_r = 4.0 \times 10^{-1}\ h^{-1}$ were obtained in 0.1 M phosphate buffer at pH 7.0 and 25°C (Tajima and Shikama, 1987, 1992). As to the reaction of metMb with H_2O_2, intensive studies have recently been made by several authors to elucidate the structure of ferryl myoglobin having an oxene ligand (Sitter et al., 1985; Chance et al., 1986) and also a protein radical centered on a tyrosine residue (Tew and Ortiz de Montellano, 1988; Davies, 1991), represented by *Mb(IV) =O in this paper. However, the molecular mechanism for the revert reaction (or auto-reduction) of ferryl Mb to the ferric met-form in Eq. 6 remains still open to future study including the possible involvement of H^+, OH^-, and H_2O with a stoichiometric balance (Uyeda and Peisach, 1981).

At this point, it should be noted that the values of the on-rate and off-rate constants of Eq. 1 are very high as compared with the other rate constants involved in the subsequent reactions of myoglobin. It is therefore concluded that the reversible oxygen binding to myoglobin can always proceed very quickly to an equilibrium extent. This conclusion

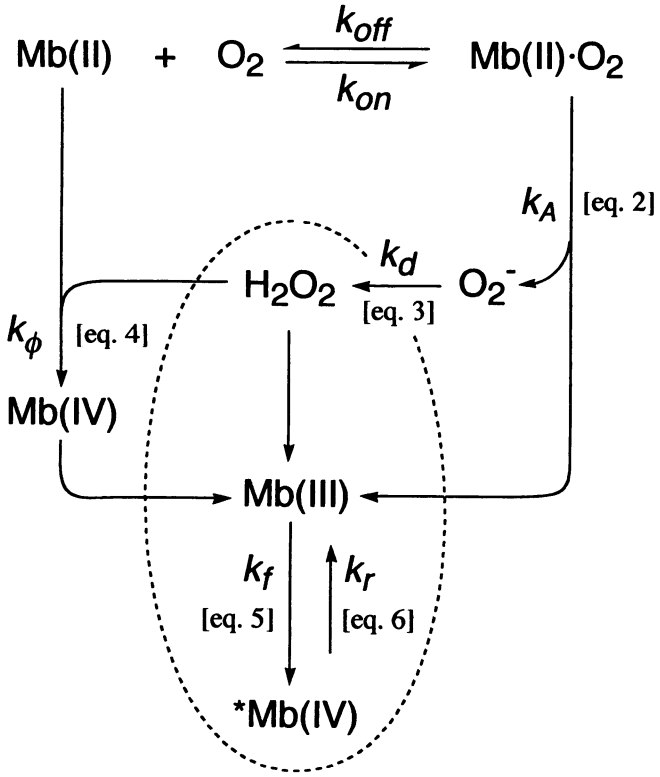

FIGURE 1 A schematic representation of the possible pathways for the oxidation reaction of myoglobin to metmyoglobin by molecular oxygen. Encircled by broken line is a system operating for the decomposition of H_2O_2 in the normal autoxidation reaction of MbO_2. Stoichiometric balance is not shown here (see text).

would be valid at any time during the course of the oxidation reaction of myoglobin to metmyoglobin.

CALCULATIONS AND DISCUSSION

Fig. 1 illustrates in a very schematic way all the possible pathways leading to the oxidation of myoglobin to metmyoglobin by molecular oxygen. The ferryl species produced, not from deoxyMb but from metMb, marked here with an asterisk above the letter of Mb(IV). It is quite clear that H_2O_2, which is formed by dismutation of the superoxide anion generated from the autoxidation of MbO_2, plays a key role in this complicated reaction.

Since the deoxy-form is the most preferred target for H_2O_2, the amount of deoxyMb that is in equilibrium with MbO_2 would become an important factor for the overall stoichiometry of myoglobin oxidation. Under air-saturated conditions (P_{O_2} = 150 Torr), the molar fraction of the deoxy-form is only ~0.45%, judging from the oxygen dissociation constant (K_D). In this case, the H_2O_2 would be eliminated or decomposed mostly, if not completely, by the metMb resulting from the normal autoxidation reaction of MbO_2, via the cyclic formation of the ferryl species. With decreasing partial pressure of O_2, on the other hand, the amount of deoxyMb increases rapidly and H_2O_2 would react with it.

In order to confirm these predictions more quantitatively, we have set up six rate equations in a simultaneous way (see Eqs. 7–10, 15, and 16 in the Appendix), and have carried out numerical analyses for the oxidation reaction of myoglobin to its met-form with the aid of a computer (IBM 3081-KX6, VM/SP CMS). To solve an initial-value problem for the differential equations, the Runge-Kutta integration method was employed from a program library (DIVPRK in MATH/LIBRARY, IMSL). The concentration progress curves for each reactive species were then plotted at intervals of 0.01 h over a period of 100 h. In order to display the early stage (<10 h) of the reaction in great detail, the step size of 1.56×10^{-4} h (0.56 s) was employed for integration, because the same results were obtained for a much smaller step size.

Fig. 2 is a typical computer representation for the autoxidation reaction of MbO_2 to metMb in 0.1 M buffer, pH 7.0, at 25°C. The reaction was started with myoglobin of 5.0×10^{-5} M, the same concentration that we have usually used for the autoxidation rate measurements. Under air-saturated conditions of P_{O_2} = 150 Torr, almost all the myoglobin exists in the oxy-form, so

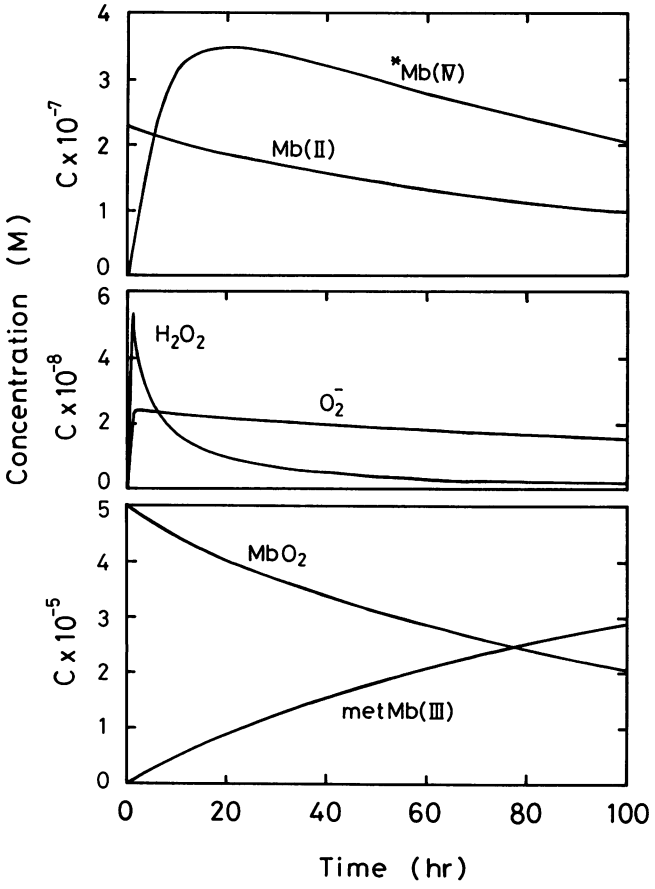

FIGURE 2 The concentration progress curves for each species involved in the autoxidation reaction of MbO_2 to metMb at 25°C in 0.1 M buffer, pH 7.0. The simulation was started with use of 50 μM myoglobin under air-saturated conditions.

that even in the initial concentration, the deoxy-species was found only of an order of 2.25×10^{-7} M.

When MbO_2 is oxidized to metMb, the stoichiometric amount of O_2^- should be generated. By the very rapid, spontaneous dismutation to H_2O_2, however, the superoxide level was found to fall immediately into an extremely low concentration on the order of 10^{-8} M. Surprisingly, the resulting H_2O_2 level was also found in the same concentration range as that of O_2^-, although a small but sharp accumulation of H_2O_2 (less than 6×10^{-8} M at the highest) appeared at the very early stage (within 1 h) of the reaction. These calculations are quite in agreement with our experimental observations: H_2O_2 produced is eliminated mostly, if not completely, by the metMb resulting from the normal autoxidation reaction of MbO_2, through the cyclic formation of the ferryl species (Tajima and Shikama, 1987, 1992).

At lower pressures of O_2, different pathways may occur in the oxidation reaction of myoglobin to metmyoglobin. Fig. 3 shows such a detailed set of calculations at three different pressures of O_2. Under air-saturated conditions (P_{O_2} = 150 Torr), it is quite clear that the rate of metmyoglobin formation can be explained almost completely by the normal autoxidation of $k_A[MbO_2]$, indicating that most of the H_2O_2 produced was eliminated by the metMb resulting from MbO_2. However, the extent of the contribution of the other term, $k_\phi[Mb(II)][H_2O_2]$, to the overall formation of metMb increased with decreasing partial pressure of O_2. At P_{O_2} = 0.68 Torr, for instance, almost half of the metMb formation came from the oxidation of deoxyMb with H_2O_2, indicating that most of the H_2O_2 produced was used up to yield two equivalents of metMb from deoxyMb as described in Eq. 4. This is mainly due to a large increase in the equilibrium concentration of the deoxy species. Furthermore, it seemed that the rate of formation of metMb increases with decreasing partial pressure of O_2. We have therefore made a first-order plot for the overall oxidation of myoglobin, from the ferrous state as a sum of MbO_2 and deoxyMb to the ferric met-form, by the following definition:

the rate of metMb formation

$$\equiv k_{met}\{[Mb(II)\cdot O_2] + [Mb(II)]\}.$$

The rate constant of k_{met} (h^{-1}) was then determined from the slope of each line at each given value of O_2 by a least-squares fitting.

Fig. 4 shows such a computer representation for the oxidation rate of myoglobin to metmyoglobin as a function of P_{O_2}. There appeared a well defined maximum rate at a partial pressure of $O_2 \sim 5$ Torr. Increase of the O_2 pressure above ~40 Torr was found to have little effect on the oxidation rate, its magnitude at these pressures becoming closer to a constant value of $k_A = 8.1 \times 10^{-3}$ h^{-1} under air-saturated conditions. This result is in good accord with the actual experimental data on the oxidation of equine myoglobin (George and Stratmann, 1952); the protein showed a maximum oxidation rate at the partial pressure of $O_2 \sim 2$ Torr in 0.6 M phosphate buffer, pH 5.69 at 30°C. At this stage our calculations would be satisfactory, because the rate constants used here have been derived from other studies in which sections of the oxidation mechanism have been isolated for study.

Other interesting aspects of this O_2-dependence curve would be demonstrated by changing the values of two relevant parameters. As shown in Fig. 4, the maximum rate of oxidation was found when a set values of $k_f = 3.0 \times 10^6$ h^{-1} M^{-1} and $k_\phi = 6.0 \times 10^7$ h^{-1} M^{-1} were employed, both being several times higher than the experimentally measured ones. On the other hand, if we assume both of the values to be zero, there should occur no reaction of myoglobin with H_2O_2. In this case, the rate of $k_A[MbO_2]$ would only be responsible for the formation of metmyoglobin, and the O_2-dependence curve becomes simply hyperbolic, with no detectable maximum rate of oxidation.

From these numerical examinations assisted with a computer, we can conclude unequivocally that H_2O_2, which is produced from the dismutation of O_2^-, plays a crucial role in the oxidation reaction of myoglobin to its met-form at lower pressures of O_2. These results also lead us to a new view that a good supply of oxygen provides a rather important defense against the oxidation of myoglobin with hydrogen peroxide, one of the most potent oxidants found in situ. This view seems to be of clinical importance in the oxygen supply to red muscles, because ischemia is known to cause abrupt cell destruction in cardiac and skeletal muscle tissues (Levine et al., 1971; Kagen et al., 1975).

APPENDIX

For numerical analysis, we may write the following rate equations for each elementary step involved in the oxidation reaction of myoglobin.

$$\frac{d}{dt}[O_2^-] = k_A[MbO_2] - k_d[O_2^-]^2 \quad (7)$$

$$\frac{d}{dt}[H_2O_2] = \frac{1}{2}k_d[O_2^-]^2 - k_f[H_2O_2][Mb(III)] - k_\phi[H_2O_2][Mb(II)] \quad (8)$$

$$\frac{d}{dt}[Mb(III)] = k_A[MbO_2] + 2k_\phi[H_2O_2][Mb(II)] - k_f[H_2O_2][Mb(III)] + k_r[*Mb(IV)] \quad (9)$$

and

$$\frac{d}{dt}[*Mb(IV)] = k_f[H_2O_2][Mb(III)] - k_r[*Mb(IV)]. \quad (10)$$

Since the total concentration of Mb is given in practice by

$$[Mb]_0 = [MbO_2] + [Mb(II)] + [Mb(III)] + [*Mb(IV)], \quad (11)$$

the following relationships should always be valid at any constant value of the partial pressure of O_2:

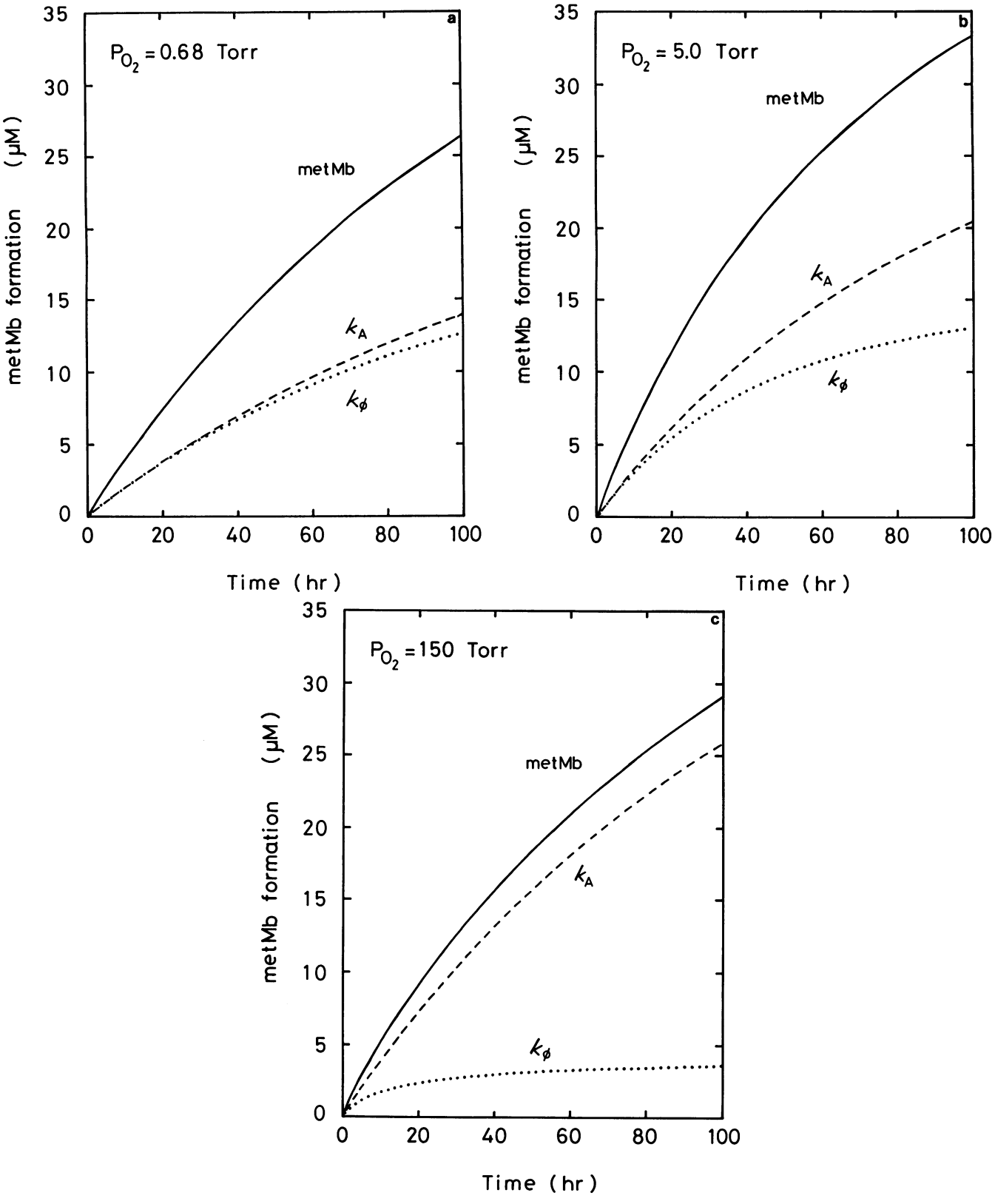

FIGURE 3 Different profiles for the formation of metmyoglobin at three different partial pressures of oxygen. The rate was broken down into two components of $k_A[MbO_2]$ and $k_\phi[H_2O_2][Mb(II)]$ at 25°C in 0.1 M buffer, pH 7.0. (*a*) P_{O_2} = 0.68 Torr; (*b*) P_{O_2} = 5.0 Torr; (*c*) P_{O_2} = 150 Torr.

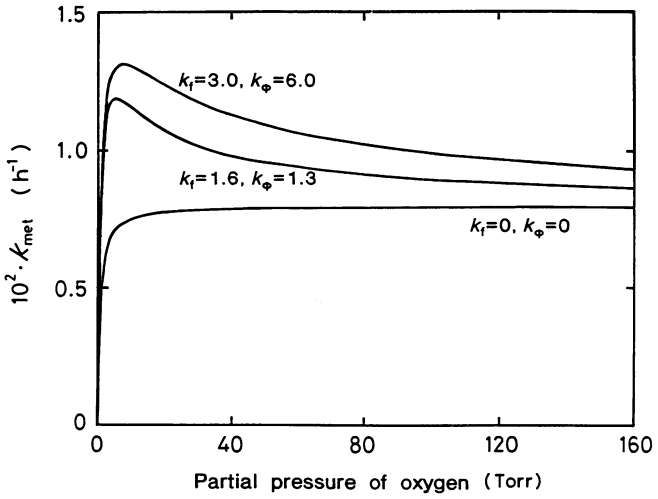

FIGURE 4 Plots for the oxidation rate of myoglobin to metmyoglobin as a function of partial pressures of oxygen. The simulation was carried out using three different set values of the rate constants: (*a*) $k_f = 3.0 \times 10^6\ h^{-1}\ M^{-1}$ and $k_\phi = 6.0 \times 10^7\ h^{-1}\ M^{-1}$; (*b*) $k_f = 1.6 \times 10^6\ h^{-1}\ M^{-1}$ and $k_\phi = 1.3 \times 10^7\ h^{-1}\ M^{-1}$; (*c*) $k_f = 0$ and $k_\phi = 0$.

$$[MbO_2] = \{[Mb]_0 - [Mb(III)] - [*Mb(IV)]\}(\alpha) \quad (12)$$

and

$$[Mb(II)] = \{[Mb]_0 - [Mb(III)] - [*Mb(IV)]\}(1 - \alpha), \quad (13)$$

where

$$\alpha = \frac{[MbO_2]}{[MbO_2] + [Mb(II)]} = \frac{[O_2]}{[O_2] + K_D}. \quad (14)$$

We may therefore differentiate Eqs. 12 and 13 to obtain the following two rate equations:

$$-\frac{d}{dt}[MbO_2] = \left\{\frac{d}{dt}[Mb(III)] + \frac{d}{dt}[*Mb(IV)]\right\}(\alpha)$$
$$= \{k_A[MbO_2] + 2k_\phi[H_2O_2][Mb(II)]\}(\alpha) \quad (15)$$

and

$$-\frac{d}{dt}[Mb(II)] = \left\{\frac{d}{dt}[Mb(III)] + \frac{d}{dt}[*Mb(IV)]\right\}(1 - \alpha)$$
$$= \{k_A[MbO_2] + 2k_\phi[H_2O_2][Mb(II)]\}(1 - \alpha). \quad (16)$$

For the conversion of the value of P_{O_2} (Torr) into the molar concentration of O_2 in solution, we used the following equation (Antonini and Brunori, 1971):

$$[O_2] = s \times P_{O_2},$$

where

$$s = 1.69 \times 10^{-6}\ M/Torr\ at\ 25°C.$$

The following values were also used for the rate constants and oxygen dissociation constant required in our calculation in 0.1 M buffer, pH 7 at 25°C:

$$k_A = 8.1 \times 10^{-3}\ h^{-1}, \quad k_d = 7.2 \times 10^8\ h^{-1}\ M^{-1},$$
$$k_\phi = 1.3 \times 10^7\ h^{-1}\ M^{-1},$$
$$k_f = 1.6 \times 10^6\ h^{-1}\ M^{-1}, \quad k_r = 4.0 \times 10^{-1}\ h^{-1}, \quad \text{and}$$
$$K_D = 1.15 \times 10^{-6}\ M.$$

We are grateful to Dr. Shigeo Takei (Education Center for Information Processing, Tohoku University, Sendai) for his valuable suggestions on the computer analysis.

This work was partly supported by a grant from the Ministry of Education, Science and Culture of Japan (04454022).

Received for publication 8 November 1991 and in final form 3 April 1992.

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