The Molecular Mechanism of Autoxidation for Myoglobin and Hemoglobin: A Venerable Puzzle

Keiji Shikama

Biological Institute, Graduate School of Science, Tohoku University, Sendai 980-8578, Japan

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Contents

Keiji Shikama was born in Yamagata, Japan, in 1936. He received his B.S. in 1958, and his D.Sci. degree in 1964 from Tohoku University at Sendai. His thesis work was concerned with the effect of freezing and thawing on the stability properties of proteins and nucleic acids. The results, which appeared in *Nature* in 1961 and 1965, have provided a fundamental basis for preserving biological materials at low temperatures below − 80 °C. From 1965 to 1967, he worked with I. M. Klotz on anion binding by macromolecules at the Department of Chemistry, Northwestern University (Evanston, IL) as a Fulbright exchange scholar. After returning to Sendai, he joined again the Biological Institute, Faculty of Science, Tohoku University where he is now a professor of Biochemistry and Molecular Biology. During 1980s his research moved on to study the hemoprotein reaction with molecular oxygen, and the hemoglobin-like proteins from lower organisms, such as those of insect larva, sea mollusk, protozoa, and yeast, in terms of the structure, function, and molecular evolution.

I. Introduction

The transport and storage of molecular oxygen by hemoglobin (Hb) and by myoglobin (Mb) are essential to life. The iron(II)−dioxygen bond in these heme proteins plays a vital role in their physiology. Studies of these proteins range from examinations of physical−chemical properties dependent on electronic structure, to investigations of stability as a function of oxygen supply.[1] Among these, stability properties are of particular importance in vivo. Like all known dioxygen carriers synthesized so far with transition metals, the oxygenated forms of myoglobin and hemoglobin are oxidized easily to their ferric met forms, which cannot be oxygenated and are therefore physiologically inactive. The mechanistic details of this autoxidation reaction, which are of clinical, as well as of chemical, interest, have long been investigated by a number of authors, but a full understanding of the reaction has not been reached. The issue "Metal−Dioxygen Complexes" (*Chem. Rev.* **1994**, *94*, No. 3, edited by I. M. Klotz and D. M. Kurtz) devotes much attention to this venerable puzzle.

It is well-known that simple iron(II) complexes are oxidized very quickly and irreversibly in the presence of dioxygen, within seconds at ambient temperature. The rate-determining step of these autoxidation reactions in nonaqueous solvents is second order in iron(II) and first order in dioxygen. This observation was made by Cohen and Caughey[2] for free ferrous porphyrins and by Hammond and Wu[3] for ferrous salts. A possible mechanism has been proposed, therefore, on the basis of the dimeric nature of the autoxidation products[2−8] and involving the following steps:

$$LFe^{II} + O_2 \rightleftharpoons LFe^{II}(O_2) \quad (1)$$

$$LFe^{II}(O_2) + Fe^{II}L \rightharpoonup LFe^{III}-O_2-Fe^{III}L \quad (2)$$

$$LFe^{III}-O_2-Fe^{III}L \rightharpoonup 2LFe^{IV}=O \quad (3)$$

$$LFe^{IV}=O + Fe^{II}L \rightharpoonup LFe^{III}-O-Fe^{III}L \quad (4)$$

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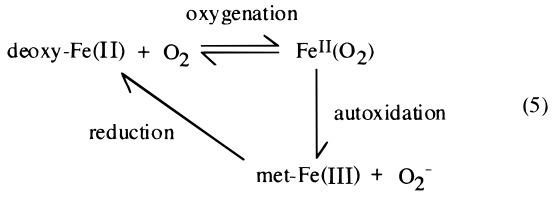
When L represents an axial ligand such as pyridine, an initial 1:1 binding of dioxygen by iron(II) is followed by a bimolecular redox process, which leads to the formation of a dioxygen-bridged diiron complex. This μ-peroxo dimer can also be converted, presumably via the formation of a ferryl intermediate Fe^{IV}=O, to the μ-oxo dimer.[4] At this point, however, it should be remarked that the oxidation of iron(II) porphyrins by dioxygen is not always the same in its molecular mechanism, but depends on the reaction conditions including the solvent systems used and the concentrations of the axial base and dissolved O_2. We shall return to this problem in a latter section

For native proteins, it is quite clear that such a μ-peroxo-bridged autoxidation mechanism cannot be appropriate, for the protein matrix surrounding each heme moiety prevents the close approximation of two irons. Therefore an alternative mechanism(s) is needed to explain the autoxidation of myoglobin and hemoglobin by molecular oxygen.[9] Recently, Busch et al. have studied the autoxidation of iron(II) cyclidenes as a sterically hindered pentacoordinate model heme, and implicated an outer-sphere electron transfer between the iron(II) species and the unbound dioxygen molecule.[10,11] However, the relevance of this mechanism to the native proteins is not yet clear.

In this paper, attempts are made to compare the autoxidation reaction of myoglobin and hemoglobin with that for synthetic iron(II) complexes, with special focus on an outer-sphere or an inner-sphere electron transfer mechanism. At the same time, various mechanisms proposed so far for the autoxidation reaction will be examined individually in order to understand the complicated stability properties of these oxygen-carrying heme proteins.

II. Dynamics of the FeO_2 Bonding of Myoglobin and Hemoglobin in Vivo

It is in the ferrous form that myoglobin (Mb) or hemoglobin (Hb) can bind molecular oxygen reversibly and carry out its physiological function. From known changes in valency of the heme iron, one can write the functional cycle as follows:



(5)

During reversible oxygen binding, the oxygenated form of myoglobin or hemoglobin is known to be oxidized easily to the ferric met form with generation of the superoxide anion. The rate of this autoxidation reaction depends strongly upon the pH of the solution. At 35 °C, for instance, the half-life for conversion of bovine heart oxymyoglobin (MbO_2) to metmyoglobin (metMb) was found to be 3.3 days at pH 9, but it became 11 h at pH 7, and less than 30 min at pH 5 in 0.1 M buffer.[1,12] For tetrameric oxyhemoglobin (HbO_2) the situation is essentially the same, although human HbA is a little more resistant to autoxidation with the α and β chains being nonequivalent in their oxidation rates in acidic pH range.[13,14] At any rate, the metmyoglobin or methemoglobin thus produced cannot be oxygenated and is therefore physiologically inactive.

In muscle tissues and red blood cells, however, an NADH-cytochrome b_5 oxidoreductase is present which can reduce metMb or metHb to the respective ferrous deoxy species and thus prevent the continued accumulation of the ferric met form in situ. The oxidoreductase is called metmyoglobin reductase[15] and methemoglobin reductase,[16] respectively. The cyclic reduction of the met form by these enzymes is a basis for the continuity of myoglobin and hemoglobin functions in vivo. Nevertheless, it is also true that the autoxidation reaction is inevitable in nature for all oxygen-binding heme proteins, as well as for all synthetic dioxygen carriers.[11,17] In fact, it is a matter of our experience that the metMb content in myoglobin extracts from various muscle tissues is commonly about 40%, while the metHb content of freshly drawn blood is usually maintained within 1−2% but by a strong reduction environment.

In preparation for examining details, one should be aware of some general features characteristic of the autoxidation reaction of myoglobin and hemoglobin molecules. Some important aspects are the following: (1) The rate is dependent upon the partial pressure of dioxygen (P_{O_2}), its effect being small but peculiar.[18,19] (2) The rate shows a much more profound pH dependence, not only in the acidic range but also on the alkaline side.[20,21] (3) The rate is substantially different from protein to protein. In the autoxidation reaction of myoglobins in 0.1 M phosphate buffer at pH 7.2 and 25 °C, for instance, the first-order rate constant is 0.50×10^{-2} h^{-1} for sperm whale MbO_2 (with a half-life period of $t_{1/2} = 138.6$ h), 0.72×10^{-2} h^{-1} ($t_{1/2} = 96.2$ h) for bovine heart MbO_2, 0.83×10^{-2} h^{-1} ($t_{1/2} = 83.5$ h) for human psoas MbO_2, 0.31×10^{-1} h^{-1} ($t_{1/2} = 22.3$ h) for *Paramecium* MbO_2, and 0.11 h^{-1} ($t_{1/2} = 6.3$ h) for *Aplysia* MbO_2.[22] (4) The rate of oxidation for the heme iron(II) is enormously reduced if it is embedded in a native protein matrix, by a factor of more than 10^6 in the case of the myoglobin molecule.[23]

Our next step is therefore to see what kind of molecular mechanisms are consistent with these kinetic observations.

III. Dioxygen Pressure Dependence of the Autoxidation Rate: An Outer-Sphere Electron Transfer Mechanism

Since the early work of Brooks[18,24] on HbO_2 and of George and Stratmann[19,25] on MbO_2, it has long been observed that the autoxidation rates of these proteins increase first with dioxygen pressure, pass through a maximum, and then show a 2−3-fold decrease with further increases in partial pressure of O_2.[26,27,28] Figure 1 shows such a typical P_{O_2}-dependence curve for the autoxidation rate of horse heart myoglobin in 0.6 M phosphate buffer, pH 5.69, at 30 °C. The data points of the first-order rate constant (k_{obs}) at different O_2 pressures were taken from George and

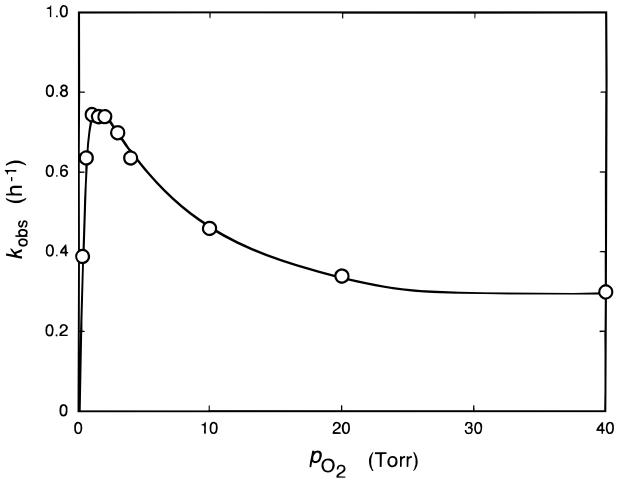


Figure 1. Dioxygen pressure dependence for the autoxidation rate of horse heart myoglobin. The first-order rate constant (k_{obs}) was observed in 0.6 M phosphate buffer of pH 5.69 at 30 °C. The data points were taken from George and Stratmann (ref 19).

Stratmann.[19] There appeared a well-defined maximum value at a partial pressure of $O_2 \approx 2$ Torr. A similar dependence curve was also reported for the autoxidation rate of bovine hemoglobin, but in this case a rate maximum was found at a partial pressure of $O_2 \approx 20$ Torr.[18] In each case, the oxygen pressure for the maximum rate corresponds closely to that required to half saturate the protein with O_2, that is to say, P_{50}. The curves indicate that although oxidation of Mb or Hb does occur only in the presence of O_2, high O_2 pressures seem to protect each protein from autoxidation.

Along with the early investigations, Brown and Mebine,[26] among others, proposed that the first step in autoxidation of MbO_2 or HbO_2 is the dissociation of the dioxygen ligand followed by the oxidation of the deoxy species by free O_2 to produce metMb or metHb and the superoxide anion. For myoglobin, one may write the autoxidation reaction as

$$MbFe^{II}(O_2) \rightleftharpoons MbFe^{II} + O_2 \quad (6)$$

$$MbFe^{II} + O_2 \rightharpoonup metMbFe^{III} + O_2^- \quad (7)$$

In this way, enhancements in the oxidation rate of MbO_2 or HbO_2 at lower oxygen pressures have been attributed to the increased concentration of the deoxy species. In fact, the unliganded deoxy-form of Mb or Hb is certainly the preferred target for many kinds of oxidants, such as ferricyanide,[29] ferricytochrome *c*,[30] and ferricytochrome b_5.[31] Our kinetic study on the oxidation of sperm whale MbO_2 with H_2O_2 has also revealed that H_2O_2 can oxidize the deoxy species more than 100 times more readily than it can oxidize oxy-Mb, and that once the deoxy form reacts with H_2O_2, MbO_2 quickly dissociates into another deoxy species to an equilibrium concentration.[32]

With O_2 as the oxidant, however, things are not so simple. To explain the inverse O_2 dependence of the autoxidation rate, the participation of deoxy species in the reaction seems to be essential both for myoglobin and hemoglobin molecules. Even then, the differences in the deoxy species which allow them to react with dioxygen in one instance to become oxygenated (eq 6) and in another instance to become oxidized (eq 7) are completely unknown. In this regard, Caughey and his associates suggested that anion binding to the deoxy species mediates one-electron transfer from iron(II) to free O_2, probably through the porphyrin or aromatic amino acid residues of the protein.[27] For Hb oxidation, the anion-mediated electron transfer mechanism thus may be represented by the following equations:

$$HbFe^{II}(O_2) \rightleftharpoons HbFe^{II} + O_2 \quad (8)$$

$$HbFe^{II} + L^- \rightleftharpoons HbFe^{II}(L^-) \quad (9)$$

$$HbFe^{II}(L^-) + O_2 \rightharpoonup HbFe^{III}(L^-) + O_2^- \quad (10)$$

In this mechanism, dioxygen binding in eq 8 and electron transfer in eq 10 are competing reactions. As Caughey appropriately pointed out, there is no evidence that anions ever bind to the iron of reduced hemoglobin or myoglobin. Nevertheless, he favored the possibility that entry of the facilitating anion (L^-) into the heme pocket (eq 9) produces, by its nucleophilicity, sufficient labilization of the iron valence electron so that it can be taken off by free O_2 at an electron transfer site at a distance from the heme iron(II).[27] Under physiological conditions for the oxidation of hemoglobin, one of the most potent anions was assumed to be Cl^- ion.[27,33,34] More recently, Olson and co-workers have favored the view that in the oxidation of myoglobin at low oxygen pressures a bimolecular reaction occurs between O_2 and the deoxy species, with a weakly coordinated water molecule serving as ligand to stabilize iron(III).[28] At any rate, all these mechanisms invoke an outer-sphere electron transfer from the deoxy species to the unbound, free dioxygen. However, Caughey could not rule out completely the displacement of O_2^- (or HO_2) from HbO_2 by a nucleophile (L^-), especially at high oxygen pressures.[27] The nucleophilic displacement pathway will be treated fully in the next section.

As for an outer-sphere electron transfer mechanism, several lines of evidence had already been provided from studies of the oxidation of dipyridine-ferrohemochromes by molecular oxygen. In aqueous solvent containing an excess amount of pyridine under air-saturated conditions, Kao and Wang[35] proposed that the major path of the reaction is an inner-sphere mechanism in which an oxygen molecule replaces one of the pyridine molecules in dipyridine-ferrohemochrome to form an oxyheme, which then undergoes decomposition to ferrihemochrome and O_2^-. But a very small portion of the rate was assigned to an outer-sphere mechanism. However, at high pyridine concentrations in nonaqueous solvents, Chu et al.[36] found no spectral evidence for oxygenation of the iron nor of dissociation of an axial ligand, and they concluded therefore that hexacoordinate low-spin iron(II) atoms in porphyrins are oxidized with O_2 by an outer-sphere electron transfer

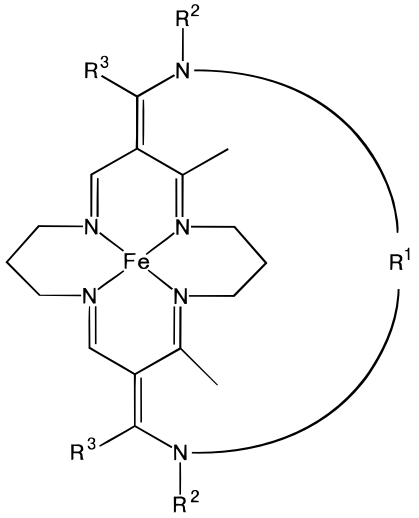


Figure 2. A structural representation of the iron(II) cyclidene complexes $[Fe^{II}(R^1,R^2,R^3)Cl]^+$. The bridge R^1 is a polymethylene group whose length can be varied by the number n in $(CH_2)_n$, while R^2 and R^3 represent the terminal substituents.

mechanism, probably through the porphyrin ring with proton assistance:

$$LFe^{II}L + O_2 + H^+ \rightharpoonup LFe^{III}L + HO_2 \quad (11)$$

More recently, Busch and his associates have studied the autoxidation rate of iron(II) cyclidene complexes as a function of dioxygen pressure, and implicated the outer-sphere electron transfer mechanism in the reaction.[10,11]

In the structure depicted in Figure 2, the bridge R^1 is a polymethylene group whose length can be changed from $(CH_2)_3$ to $(CH_2)_8$, so that the size of the dioxygen binding cavity can be increased. On the other hand, R^2 and R^3 represent the terminal substituents such as those of methyl (Me) and phenyl (Ph) groups. The cyclidene complexes may be thus abbreviated simply by a chemical formula, $[Fe^{II}((CH_2)_n,R^2,R^3)Cl]^+$, with many structural variations. In a trimethylene (C3)-bridged cyclidene $[Fe^{II}((CH_2)_3,Me,Me)Cl]^+$, no binding affinity was observed for either O_2 or CO. In other words, it follows that these ligands cannot enter the cavity of the C3-bridged species. Nevertheless, this iron(II) complex was oxidized very rapidly, with the rate constant showing a simple first-order dependence on the dioxygen pressure. At P_{O_2} =101 Torr, for instance, the observed rate constant (k_{obs}) was 0.32×10^{-4} s^{-1} even at − 25 °C, its value corresponding to a half-life of $t_{1/2}$ = 3.6 min for the conversion of iron(II) to iron(III) by the unliganded, free dioxygen. In a tetramethylene (C4)-bridged cyclidene $[Fe^{II}((CH_2)_4,Me,Ph)Cl]^+$, the autoxidation rate was found to increase with increasing O_2 pressure, but the rate showed a saturated behavior at high dioxygen pressures. When the group R^1 was lengthened to C4, a dioxygen molecule could enter the cavity, so that the O_2 binding competed with an electron-transfer process that caused the very rapid oxidation for the C3 complex. On the basis of these results, Busch and his associates have concluded that the molecule of O_2 that oxidizes the iron(II) species is not bound and proposed an outer-sphere electron transfer mechanism in the autoxidation reaction of iron(II) cyclidenes. They also suggested the same mechanism to explain the similar O_2-dependence curves for the autoxidation of myoglobin and hemoglobin molecules.[10,11] All their experiments were carried out in CH_3OH containing 1 M 1-methylimidazole and 0.05 M TBAT (tetrabutylammonium tetrafluoroborate). In the autoxidation reaction of single-face-hindered iron(II) complexes, however, it is quite difficult to distinguish electron transfer to dioxygen by an inner-sphere path in contrast to an outer-sphere one because of the lability of the axial base, even if it is present in high concentration in the solvent.[17]

Table 1. Comparison of Autoxidation Rates between Native Oxymyoglobins and Iron(II) Cyclidene under Air-Saturated Conditions

	k_{obs} (h^{-1})	
source	25 °C[a]	0 °C[b]
bovine MbO_2	0.72×10^{-2}	0.12×10^{-3} ($t_{1/2}$ = 240 day)
Aplysia MbO_2	0.11	0.15×10^{-2} ($t_{1/2}$ = 19 day)
iron(II) cyclidene[c] $[Fe^{II}((CH_2)_4,Me, Ph)Cl]^+$		0.25×10^2 ($t_{1/2}$ = 1.6 min)

[a] The observed first-order rate constant was determined in 0.1 M phosphate buffer at pH 7.2 for the native proteins. [b] Calculated using the activation energies of E_A = 26.5 kcal mol^{-1} for bovine heart MbO_2,[81] and of E_A = 27.8 kcal mol^{-1} for *Aplysia* MbO_2,[82] in the neutral pH range. [c] At 0 °C, the value of $k_{obs} = 7.0 \times 10^{-3}$ s^{-1} was reported at P_{O_2} = 101 Torr in MeOH/1 M 1-methylimidazole/0.05 M tetrabutylammonium tetrafluoroborate by Dickerson et al.[10]

At this point, it is interesting to compare the autoxidation rate of a tetramethylene (C4)-bridged iron(II) cyclidene with those of bovine heart and *Aplysia* (sea hare) oxymyoglobins under air-saturated conditions (See Table 1). The autoxidation rates for the proteins at 0 °C are calculated from the values at 25 °C using the known activation energies. It is evident that the C4-bridged complex undergoes extremely rapid autoxidation, 10^4-10^5 times faster than the native proteins. In our experience, cytochrome *c*, for instance, is resistant to autoxidation. Its crystals can be kept in the reduced form a couple of years in air-saturated buffers. If an outer-sphere electron transfer occurred to the free, dissolved O_2, the heme iron(II) of cyt *c* should have been oxidized within this time.

These arguments suggest that the molecular mechanism of aqueous autoxidation of native heme proteins is quite different from that of the nonaqueous autoxidation of simple iron(II) complexes. Even in air-saturated buffers, both myoglobin and hemoglobin show very strong pH dependencies of the autoxidation rate, not only in the acidic range but on the alkaline side as well. Moreover, each of the dependence curves differs significantly from protein to protein of different origin. Consequently, it is difficult to explain the various types of complicated pH profiles by a simple event consisting of an outer-sphere electron transfer between free O_2 and the iron(II) species that do not contain bound O_2. Recently, Abugo and Rifkind[37] have studied the autoxidation

of human hemoglobin as a function of oxygen pressure, and shown that the outer-sphere, bimolecular oxidation of deoxygenated chains by free oxygen can only account for a minor part of the observed autoxidation enhancement at intermediate oxygen pressures. Tsuruga and Shikama[14] have also found that human HbO_2 shows a biphasic autoxidation reaction in the pH range from neutral to acidic and that the autoxidation rate of the fast component (due to the α chain) is enhanced markedly by dissociation of tetramers into $\alpha\beta$ dimers. However, this result cannot be attributed to the increased concentration of the deoxygenated species, since the $\alpha\beta$ dimer is known to have a higher oxygen affinity with fewer deoxygenated subunits. They also showed that the addition of 2,3-diphosphoglyceric acid offers no significant effect on the increment of the autoxidation rate of HbO_2. DPG is an allosteric effector to stabilize the deoxy conformation of HbA. At any rate, the oxidation of iron(II) with free O_2 via an outer-sphere mechanism seems to be unlikely for the native proteins.

IV. Effects of pH on the Autoxidation Rate: An Inner-Sphere Electron Transfer Mechanism

A. Unimolecular, Spontaneous Dissociation of O_2^- from the FeO_2 Center in Mb and Hb

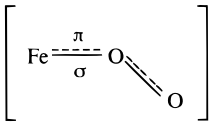
Weiss[38] proposed that the iron-dioxygen bond in HbO_2 or MbO_2 could be described as a fully developed Fe^{III}−superoxide ion pair created by an electron transfer from iron(II) to bound dioxygen. The resulting unpaired spins resident on Fe(III) and O_2^- are assumed to couple to give rise to a diamagnetic species. Therefore, it has long been suggested that a superoxide anion may be split off directly from the iron during autoxidation of HbO_2 or MbO_2 to its ferric met form, i.e. that the autoxidation is essentially the direct dissociation of O_2^-:

$$MbFe^{III}(O_2^-) \rightharpoonup metMbFe^{III} + O_2^- \qquad (12)$$

In fact, generation of O_2^- has been demonstrated during autoxidation of shark HbO_2,[39] bovine HbO_2,[40] isolated α and β chains of human HbO_2,[41] and bovine MbO_2.[42] As a direct electron acceptor from iron(II), the coordinated O_2 would be much more acceptable than the freely dissolved O_2 in solution. Nevertheless, this scheme seems to be too simple to provide any basis for interpretation of the complicated pH- or O_2-dependence of the autoxidation rate.

In contrast to Weiss' ionic description, Pauling[43] favored a covalent formulation that the iron atom is in a double bond with the nearby oxygen atom and the Fe−O−O bond angle is about 120°. The bound dioxygen becomes polarized and the iron atom retains the ferrous low-spin state, in harmony with its observed diamagnetism.

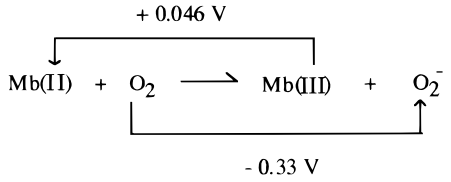
Other lines of physical and chemical evidence, such as those from Mössbauer spectroscopy[44] and infrared measurement,[45] clearly indicate that the conversion of myoglobin and hemoglobin into their oxygenated forms is associated with a profound electronic rearrangement, which may be described by strong π-donation (charge transfer) from Fe(II) to O_2 and synergistic σ-donation (coordination) from O_2 to Fe, resulting in strong covalent bonding between the iron and the bent, end-on dioxygen.[1,17,46] This partial, not full, charge transfer description may be represented by



(13)

Certainly, dioxygen is a very strong oxidizing agent. For O_2 at 1 atm, pH 7 and 25 °C, its midpoint oxidation−reduction potential is + 0.81 V for the complete, four-equivalent reduction to water, showing a total free energy change of −74.7 kcal mol^{-1} (− 312 kJ mol^{-1}). Nevertheless, the addition of the first electron to O_2 is an unfavorable, uphill process with a low redox potential of $\epsilon^{\circ\prime}(O_2/O_2^-) = -0.33$ V.[47] All the subsequent steps to water are downhill. In this sense, molecular oxygen is rather a poor one-electron acceptor. This thermodynamic barrier to the first step, therefore, seems to be the crucial ridge located between the stabilization and the activation of dioxygen bound to the hemoproteins.[48]

Using the value of +0.046 V for the redox potential of the Mb^{III}/Mb^{II} couple,[49] we may write the primary step for the autoxidation reaction of MbO_2 as



(14)

In this scheme, the reaction from left to right is associated with a change in redox potential ($\Delta\epsilon^{\circ\prime}$) of − 0.37 V, which corresponds to a positive free energy change of + 8.5 kcal mol^{-1} (+ 35.6 kJ mol^{-1}). Therefore, a considerable energy barrier accompanies the reduction of O_2 to O_2^- by deoxy-Mb, so this one-electron transfer cannot occur spontaneously. The same conclusion can be reached for the reaction of deoxy-Hb with O_2, since an oxidation−reduction potential of + 0.150 V has been reported for human Hb at pH 7 and 30 °C.[50] In this case the free energy change for the formation of O_2^- by Hb(II) is calculated to be +11.0 kcal mol^{-1} (+ 46.1 kJ mol^{-1}).

In many respects, the spontaneous, unimolecular dissociation of O_2^- from the FeO_2 center is an energetically unfavorable process, so that there must be involved some specific mechanism that makes it possible to produce O_2^- so readily from both MbO_2 and HbO_2 in aqueous solvent.

B. Various Types of pH Profiles for the Autoxidation Rate of Oxymyoglobins: A Comparative Study

Among the factors influencing the rate of autoxidation of MbO_2 or HbO_2, the effect of pH has been widely investigated by a number of authors.[24−27,33] All their observations clearly showed that the rate of

autoxidation increases with increasing hydrogen ion concentration, but no mechanistic clue to the reaction was obtained from such studies in the limited range of pH 5−7.

Using bovine heart MbO_2, Shikama and Sugawara[51] were the first to investigate the pH-dependence for the autoxidation rate over the wide range of pH 4−13 in 0.1 M buffer at 25 °C. Since then, comparative studies have been carried out with some refinements and controls for a variety of oxymyoglobins isolated directly from various sources; these include sperm whale skeletal muscle,[52] chicken gizzard smooth muscle,[53] bigeye tuna fish,[54] the radular muscle of sea hare *Aplysia kurodai*,[52,55] and ciliated protozoa *Paramecium caudatum*[22] and *Tetrahymena pyriformis*.[56] Along with this line, tetrameric human HbO_2 has recently been examined for the nonequivalent autoxidation rates of the α and β chains as a function of pH.[14] These results clearly indicate that the pH-dependence curves for autoxidation vary among these proteins. Nevertheless, it is true that the rate increases markedly, not only with increasing hydrogen ion concentration but also with increasing hydroxyl ion concentration. Consequently, a simple examination of the autoxidation rate at a single pH or within a limited range of pH is not adequate for a full understanding of the aqueous autoxidation of these heme proteins.

Under air-saturated conditions, oxymyoglobin is oxidized easily to its ferric met form with generation of the superoxide anion as

$$MbO_2 \xrightarrow{k_{obs}} \text{metMb} + O_2^- \qquad (15)$$

where k_{obs} represents the first-order rate constant observed at a given pH.[42] Therefore, the rate of the autoxidation reaction is given by

$$\frac{-\mathrm{d}[MbO_2]}{\mathrm{d}t} = k_{obs}[MbO_2] \qquad (16)$$

This process was followed by a plot of experimental data as $-\ln([MbO_2]_t/[MbO_2]_0)$ vs time t, where the ratio of MbO_2 concentration after time t to that at time $t = 0$ can be monitored by the absorbance change, for instance, at α-peak of the protein (581 nm in the case of bovine heart MbO_2). From the slope of each straight line, the observed first-order rate constant, k_{obs} in h^{-1}, was determined.

As shown in Figure 3, on the other hand, the oxygenated form of human HbA showed a biphasic autoxidation reaction that can be described by first-order kinetics containing two rate constants as follows:

$$\frac{[HbO_2]_t}{[HbO_2]_0} = P\exp(-k_f t) + (1-P)\exp(-k_s t) \qquad (17)$$

where k_f and k_s represent the rate constants for the initial fast autoxidation and for the second slow autoxidation respectively, and P is the molar fraction of the rapidly reacting hemes. The value of k_f is due to the α-chain, while k_s is for the β-chain of tetrameric HbO_2. This nonequivalence of the α- and β-chains

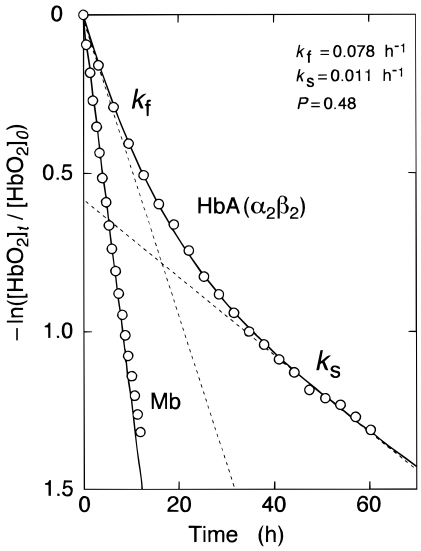


Figure 3. First-order plots for the autoxidation of human HbO_2 and horse heart MbO_2 in 0.1 M Mes buffer pH 6.5 at 35 °C. The rate measurements were carried out with 8 μM HbO_2 and 32 μM MbO_2 in the presence of 1 mM EDTA. The computed curve (—) for HbA was obtained by a nonlinear least-squares fitting to the experimental points (○), based on eq 17. The parameters thus obtained were k_f = 0.078 (±0.007) h^{-1}, k_s = 0.011 (±0.002) h^{-1} and P = 0.48 (±0.04). As for MbO_2, the reaction was described completely by a single rate constant of 0.122 h^{-1}. The rate constant of k_f is due to autoxidation of the α-chain, while k_s is for the β-chain of tetrameric HbO_2. Redrawn from Tsuruga and Shikama (ref 14).

in the autoxidation rate becomes much more remarkable in the acidic pH range. At pH values higher than 8.5, however, the autoxidation of human HbO_2 was monophasic.[14]

If the values of k_{obs} in eq 16 are plotted against the pH of the solution, a profile of the stability of oxymyoglobin can be obtained. Figure 4 shows two such pH profiles, for sperm whale MbO_2 and *Aplysia* (sea hare) MbO_2 over the wide range of pH 4−13 in 0.1 M buffer at 25 °C.[20,52] This graph indicates that the rate of autoxidation of sperm whale MbO_2 increases rapidly with increasing hydrogen ion concentration, that a minimum rate appears at pH 9.2 and that a further increase occurs at higher values of pH. In sharp contrast to this, *Aplysia* MbO_2 is oxidized to metMb at an almost constant rate over a wide range of pH 7−12, its value being 100 times higher than that of sperm whale MbO_2 at pH 9.0. In the acidic range of pH 7−4, however, the rate of autoxidation of *Aplysia* MbO_2 also increases with increasing hydrogen ion concentration but much less so than for sperm whale MbO_2, and shows rather a saturation behavior. This suggests strongly that the mode of action of the proton is quite different between sperm whale and *Aplysia* oxymyoglobins. The former protein has a value close to −1 for the slope of $\log(k_{obs})$ vs pH, as is clear in Figure 4.

For a very long time, much attention has been directed to the dioxygen pressure dependence of the autoxidation rate both for hemoglobin and myoglobin, mainly to their peculiar curves. However, it should be remarked that enhancements in the oxidation rate at lower oxygen pressures are only two or three times

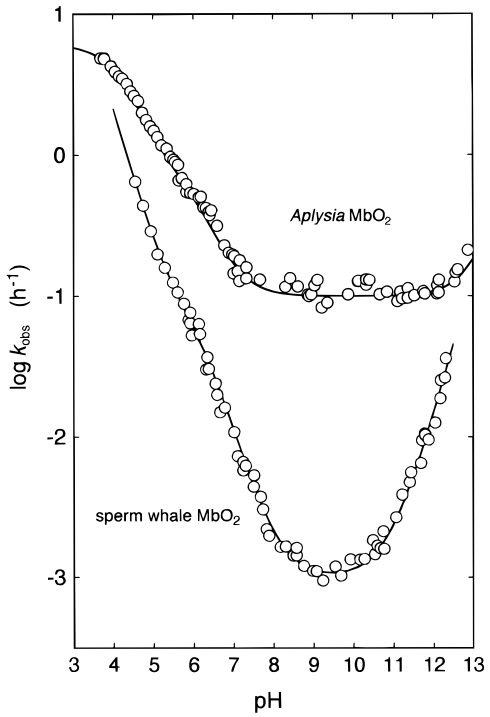


Figure 4. pH dependence of the stability of sperm whale MbO_2 and *Aplysia* MbO_2 in 0.1 M buffer at 25 °C. The logarithmic values of the observed first-order rate constant, k_{obs}, for the autoxidation reaction are plotted against the pH of the solution. The computed curve (—) was obtained by a least-squares fitting to the experimental points (○) over the whole range of pH studied, based on eq 19 for sperm whale MbO_2 and eq 22 for *Aplysia* MbO_2, respectively. MbO_2 concentration: 25 μM for *Aplysia*; 50 μM for sperm whale. Redrawn from Shikama and Matsuoka (ref 20).

as much as the autoxidation rate under air-saturated conditions. On the contrary, varying the pH of the solution produces a much more profound effect on the autoxidation reaction of these heme proteins. Both H^+ and OH^- ions can enhance the rate more than 100 times above the minimum rate appearing around pH 8−9, with the proteins showing no significant change in their circular dichroism magnitude at 222 nm over the whole range of pH studied. Moreover, the rate differs from protein to protein, by a factor of more than 100 times in some cases, at a given pH in air-saturated buffers. To understand such a remarkable pH effect on the autoxidation rate of MbO_2 and HbO_2 is, therefore, of primary importance from a basic chemical, as well as from a clinical, viewpoint.

C. A Complete Kinetic Formulation for the pH Dependence

In the autoxidation reaction, pH can affect the rate in many ways. To elucidate the kinetic and thermodynamic parameters contributing to each k_{obs} vs pH profile, we have proposed some mechanistic models for each case. The rate equations derived therefrom were tested for their fit to the experimental data with the aid of a computer. The pH dependence of the autoxidation rate of sperm whale MbO_2, as a reference, has already been analyzed completely in terms of an "acid-catalyzed two-state model".[52] In this model, it is assumed that a single, dissociable group, AH with pK_1, is involved in the reaction. Consequently, there are two forms of the MbO_2, represented by A and B, at molar fractions of α and β respectively, which are in equilibrium with each other but which differ in dissociation state for the group AH. These forms can be oxidized to metMb by displacement of O_2^- from MbO_2 by an entering water molecule or hydroxyl ion. The iron is thus converted to the ferric form, and the water molecule or the hydroxyl ion remains bound to the Fe(III) at the sixth coordinate position to form aquo- or hydroxy-metMb, respectively. The existence of both of these met species has been established definitively from X-ray analysis.[57]

The reaction scheme may, therefore, be written as

$$\begin{array}{ccc} MbO_2(AH) & \overset{K_1}{\rightleftharpoons} & MbO_2(A^-) \\ \downarrow k_0^A \quad \downarrow k_H^A & & \downarrow k_0^B \quad \downarrow k_H^B \quad \downarrow k_{OH}^B \\ \text{metMb} & & \text{metMb} \end{array} \qquad (18)$$

where, for each form of MbO_2, k_0 is the rate constant for the spontaneous displacement by H_2O, k_H is the rate constant for the proton-catalyzed displacement by H_2O, and k_{OH} is the rate constant for the displacement by OH^-. For the mechanism delineated in eq 18, the observed rate constant, k_{obs} in eq 16, can be reduced to

$$k_{obs} = \{k_0^A[H_2O] + k_H^A[H_2O][H^+]\}(\alpha) + \{k_0^B[H_2O] + k_H^B[H_2O][H^+] + k_{OH}^B[OH^-]\}(\beta) \quad (19)$$

where

$$\alpha = \frac{[H^+]}{[H^+] + K_1}$$

and

$$\beta = (1 - \alpha) = \frac{K_1}{[H^+] + K_1} \qquad (20)$$

By iterative least-squares procedures inserting various values for K_1, the adjustable parameters in eq 20, the best fit to the experimental values of k_{obs} was obtained as a function of pH (Figure 4). In this way, the rate constants and the acid dissociation constant involved in the autoxidation reaction of sperm whale MbO_2 were established in 0.1 M buffer at 25 °C and are given in Table 2.

It is thus evident that the proton-catalyzed processes with the rate constants k_H^A and k_H^B promote the autoxidation reaction of MbO_2 above the spontaneous processes in water with the rate constants k_0^A and k_0^B. In fact, the catalytic proton enhances the rate enormously, by a factor of 4.7×10^6 mol^{-1} for state A and by 1.1×10^8 mol^{-1} for state B. In this proton catalysis, the distal histidine (the dissociable group AH with pK_1= 6.2), which forms a hydrogen bond to the bound dioxygen,[58] appears to facilitate the effective movement of a catalytic proton from the

Table 2. Rate Constants and Acid Dissociation Constants Obtained from the pH Dependence Curves for the Autoxidation Rate of Sperm Whale and *Aplysia* Oxymyoglobins in 0.1 M Buffer at 25 °C[a]

source	state of MbO_2	k_0 (h^{-1} M^{-1})	k_H (h^{-1} M^{-2})	k_{OH} (h^{-1} M^{-1})	pK
sperm whale MbO_2	A (AH)	0.78×10^{-4}	0.37×10^{3}	–	
	$\downarrow\uparrow K_1$				6.2
	B (A^-)	0.18×10^{-4}	0.20×10^{4}	0.14×10	
Aplysia kurodai MbO_2	A (AH,BH)	0.11	–	–	
	$\downarrow\uparrow K_1$				4.3
	B (A^-,BH)	0.13×10^{-1}	–	–	
	$\downarrow\uparrow K_2$				6.1
	C(A^-,B^-)	0.18×10^{-2}	–	0.83	

[a] Taken from Shikama and Matsuoka.[20,52]

solvent to the bound, polarized dioxygen via its imidazole ring by a proton-relay mechanism.[1,12,20,52,59] A random and undirected access of a proton to the bound dioxygen cannot yield such an enzyme-like, catalytic effect on the acidic autoxidation of MbO_2.

On the other hand, the unusual pH profile for the autoxidation rate of *Aplysia* MbO_2 (shown also in Figure 4) can best be explained in terms of a "three-state model".[20,52] In this model, we assumed that two dissociable groups, AH with pK_1 and BH with pK_2, are involved in the reaction. Using the rate constants defined above, therefore, the autoxidation reaction of *Aplysia* MbO_2 may by written as

$$\begin{array}{ccccc} MbO_2(AH,BH) & \overset{K_1}{\rightleftharpoons} & MbO_2(A^-,BH) & \overset{K_2}{\rightleftharpoons} & MbO_2(A^-,B^-) \\ \downarrow k_0^A & & \downarrow k_0^B & & \downarrow k_0^C \quad \downarrow k_{OH}^C \\ \text{metMb} & & \text{metMb} & & \text{metMb} \end{array} \quad (21)$$

For this reaction, the observed rate constant, k_{obs} in eq 16, is given by

$$k_{obs} = \{k_0^A[H_2O]\}(\alpha) + \{k_0^B[H_2O]\}(\beta) + \{k_0^C[H_2O] + k_{OH}^C[OH^-]\}(\gamma) \quad (22)$$

where

$$\alpha = \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1K_2}$$

$$\beta = \frac{K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2}$$

and

$$\gamma = \frac{K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (23)$$

By using the same procedures as for sperm whale myoglobin, the rate constants and the acid dissociation constants involved in the autoxidation reaction of *Aplysia* MbO_2 were obtained in 0.1 M buffer at 25 °C,[52] and are summarized also in Table 2.

As a result, it becomes evident that the extreme susceptibility of *Aplysia* MbO_2 to autoxidation in the neutral pH range comes mainly from a large value of k_0^C, the rate constant for the spontaneous displacement by H_2O. In fact, its value is 100 times higher than the corresponding one for sperm whale MbO_2. With regard to the structural evidence, a high value of k_0^C implies that the heme pocket of *Aplysia* myoglobin is sufficiently open to allow easier attack of the solvent water molecule on the FeO_2 center, with a consequent very rapid formation of metMb.[20]

Due to lack of the distal histidine residue, *Aplysia* MbO_2 did not show any proton-catalyzed process having the term of $k_H[H_2O][H^+]$, such as the one that can play a dominant role in the autoxidation reaction of mammalian oxymyoglobins. Instead, *Aplysia* MbO_2 contains two types of carboxyl groups which are responsible for the small increase in the autoxidation rate appearing in the acidic pH range; one is a heme propionate with pK_a = 6.1 and the other a protein residue (probably of Glu-94) with pK_a = 4.3.[55,60] In addition, the lack of a distal histidine has a profound effect on the spectrum of *Aplysia* myoglobin in the acidic met form. For instance, its Soret peak is considerably blue-shifted and accompanied by a marked decrease in intensity, probably due to a broadening of the spectrum. This is just the reverse of what is observed in sperm whale myoglobin, and is attributed to the concomitant removal of a water molecule which is usually ligated at the sixth coordinate position of the ferric heme iron, by the formation of a hydrogen bond to the distal histidine residue.[61,62] All these kinetic and spectral features are unique for myoglobins lacking the usual distal histidine residue.[20,63]

The effects of the distal residues on the autoxidation rate have recently been examined systematically by Olson and co-workers using site-directed mutagenesis of sperm whale and pig myoglobins.[28,64] They showed that mutation of the distal (E7) histidine residue at position 64 causes dramatic increases in the autoxidation rate in a range of pH 5−9, with the trend H64V > H64G > H64L ≫ H64Q > H64 (wild-type) in 0.1 M buffer at 37 °C. However, when the absolute values are normalized to pH 7, the relative effects of pH were roughly the same for all the proteins examined. Since the similar pH dependence was observed independent of the structure of the distal amino acid residues, Brantley et al.[28] concluded that a proton-relay mechanism involving the distal histidine was not necessary and that the protonated form of the iron−O_2 complex, namely $Fe^{II}(O_2H^+)$, was the common species capable of autoxidizing. Consequently, the increase in the autoxidation rate with increasing hydrogen ion concentration could be interpreted as due to the equilibrium protonation of the $Fe^{II}(O_2)$ complex to form the acidified $Fe^{II}(O_2H^+)$ species which then rapidly dissociates into Fe(III) and HO_2. Their proposal is principally based on Weiss' view,[38] indicating that under air-saturated conditions the autoxidation of MbO_2 is essentially the direct, spontaneous dissociation of O_2^-. In their scheme, the distal histidine is serving rather to protect the $Fe^{II}(O_2)$ complex from its acidification by forming a hydrogen bond to the bound dioxygen. When the hydrogen bond is lost, therefore, the inert species could easily be converted into the protonated

form, resulting in a very rapid formation of met species.[28]

However, their examinations of the autoxidation rate were made only at several points within a limited range of pH, so that the proposed mechanism is not adequate for a full understanding of various types of the complicated pH dependences found in native oxymyoglobins from various sources. In Figure 4, for example, sperm whale MbO_2 is more than 100 times more resistant to autoxidation, if compared with *Aplysia* MbO_2 having the distal Val. Such a comparison would certainly lead us to conclude that the distal histidine inhibits autoxidation by blocking entering water molecules from the FeO_2 center. This is true in neutral pH range. Nevertheless, it is also true that the rate of autoxidation of sperm whale MbO_2 increases markedly, not only with increasing hydrogen ion concentration but also with increasing hydroxyl ion concentration as well. In the acidic pH range, the rate is enhanced so abruptly that it could be reaching a level equal with that of *Aplysia* MbO_2, showing a saturation behavior in a sharp contrast to sperm whale MbO_2. To see these problems more clearly, therefore, detailed pH-dependence studies will be needed for the mutant proteins such as H64V, H64G, H64L, and H64Q, in comparison with their corresponding myoglobins in nature.

At any rate, it is quite clear that all these complicated pH profiles for the autoxidation rate of MbO_2 or HbO_2 cannot be attributed to changes in the equilibrium concentration of the deoxygenated species, which has been frequently assumed to be oxidized with free O_2.

D. Bimolecular Displacement of O_2^- from the FeO_2 Center by Nucleophiles: An S_N2 Mechanism

A mechanistic clue was first obtained by Caughey and co-workers for the autoxidation of oxyhemoglobin. They observed that its rate was enhanced in the presence of excess amounts of anion such as those of N_3^-, SCN^-, OCN^-, F^-, and Cl^-, and suggested that the reaction proceeds by way of a proton-assisted nucleophilic displacement of O_2^- from HbO_2 by the anions. Under physiological conditions, one of the most potent nucleophiles was thus considered to be chloride anion for the formation of metHb in red blood cells.[33,34]

As compared with the normal autoxidation in buffer alone, Satoh and Shikama[65] have studied extensively the anion-induced oxidation of bovine heart MbO_2 leading to the formation of the corresponding metMb−anion complex. The anions examined were SCN^-, F^-, OCN^-, N_3^-, and CN^- at various concentrations from 0.1 to 0.5 M in 0.1 M buffer at 25 °C. In each case, the rate of the induced oxidation was linearly dependent upon the concentration of the added anion, $[X^-]$, at a given pH. For each salt, the rate was also examined at some 30 different values of pH, and the resulting pH profile has revealed that the reaction involves two types of path, i.e. one with, and the other without, proton assistance. In the presence of anion X^-, the observed oxidation rate, k_X, was given by

$$k_x = k_0 + k_D[X^-] + k_D^H[X^-][H^+] \qquad (24)$$

where k_0 represents the rate constant for the normal autoxidation of MbO_2 in buffer alone (in h^{-1}), while k_D is the rate constant for the displacement by the anion without proton assistance (in h^{-1} M^{-1}), and k_D^H is the rate constant for the proton-assisted displacement by the anion (in h^{-1} M^{-2}).

Table 3. Rate Constants for the Oxidation of Bovine Heart MbO_2 by Displacing Anions with and without Proton Assistance in 0.1 M Buffer at 25 °C[a]

nucleophile	pK_a	log k_D (h^{-1} M^{-1})	log k_D^H (h^{-1} M^{-2})
H_2O	−1.74	−4.32	3.40
SCN^-	0.85	−2.00	6.44
F^-	3.17	−1.60	5.44
OCN^-	3.92	−1.26	5.11
N_3^-	4.72	−1.30	6.60
CN^-	9.40	−0.55	11.00
OH^-	15.7	1.25	

[a] Taken from Shikama.[59]

Table 3 summarizes the rate constants (in logarithmic units) for the oxidation of bovine heart MbO_2 by displacing anions with and without proton assistance in 0.1 M buffer at 25 °C. For comparison, the corresponding kinetic paramerters are also listed for the normal autoxidation of the MbO_2 in 0.1 M buffer alone at 25 °C: $k_{H_2O} = 0.47 \times 10^{-4}$ h^{-1} M^{-1} and $k_{OH} = 0.18 \times 10^2$ h^{-1} M^{-1} for the oxidation by H_2O and OH^- respectively, and $k_{H_2O}^H = 0.25 \times 10^4$ h^{-1} M^{-2} for the proton-assisted oxidation by H_2O.[65]

For nucleophilic attack on the same atomic center, Edwards[66] has suggested two components, one related to classical basicity (H) and the other related to polarizability (P) of the nucleophiles (N):

$$\log (k_N / k_{H_2O}) = \alpha P + \beta H$$

where H is a function of basicity toward a proton and is expressed by

$$H = pK_a + 1.74$$

For the displacing oxidation of bovine heart MbO_2 by an added anion, therefore, we may plot the logarithmic value of the rate constant, $\log(k_D)$ or $\log(k_D^H)$, against the pK_a of the conjugate acid of the anion. This Brønsted plot for the series showed that the rates correlate with the pK_a value, a measure of the nucleophilicity of the anion, in a quite acceptable way, despite the fact that the anions examined are of significantly different size and chemical structure and also that an attack of anion on the FeO_2 center is not the same as an attack of the anion on a proton to give its conjugate acid.[59,65]

These results clearly indicate that the mechanism of autoxidation is not a dissociative or S_N1 process which involves the spontaneous, ionic loss of O_2^- from MbO_2 and the subsequent formation of the corresponding metMb-anion complex. Rather, the oxidation of MbO_2 proceeds by way of a nucleophilic attack of anions at the iron(II) center leading to a reductive

displacement of the bound dioxygen as O_2^- and the conversion of the iron to the ferric state. Satoh and Shikama[65] also concluded that as the most common nucleophiles in vivo both H_2O and OH^- can react with native MbO_2. The elementary processes involved in the autoxidation reaction of MbO_2 under physiological conditions can thus be viewed as a S_N2 mechanism by the general form:

$$\overset{\delta+\ \ \delta-}{\mathrm{MbFe(II)(O_2)}} + \mathrm{X^-} \longrightarrow \left[\mathrm{MbFe}\begin{matrix}\mathrm{X^-}\\ \mathrm{O_2}\end{matrix}\right] \longrightarrow \mathrm{MbFe(III)(X^-)} + \mathrm{O_2^-} \qquad (25)$$

where X^- can be SCN^-, F^-, OCN^-, N_3^-, or CN^-, and in vivo, H_2O or OH^-. This octahedral, nucleophilic displacement can proceed without any protonation. Nevertheless, it is true that the rate is enormously accelerated, by a factor of more than 10^6 mol^{-1}, with proton assistance (Table 3).

For metHb formation in normal human erythrocytes, it was frequently assumed that chloride anion is one of the most potent displacing ligands. However, we found that Cl^- ion produced no appreciable amount of enhancement in metMb formation at concentrations from 0.1 to 0.5 M over the whole range of pH studied.[65] In the case of tetrameric HbO_2, Tsuruga and Shikama[14] have recently disclosed that allosteric binding of Cl^- ion can induce the β chain to perform a conformational change so as to cause a considerable increase in the autoxidation rate. In this case, chloride anion does not act as a nucleophile to the FeO_2 center, because the effect of KCl shows a distinct saturation behavior.

V. Subsequent Side Reactions in the Autoxidation of Oxymyoglobin: An Overall Stoichiometry

Whether the mechanism is an inner-sphere or outer-sphere electron transfer, it is quite difficult to explain the autoxidation behavior of myoglobin or hemoglobin by a simple event since several types of subsequent reactions of these proteins with H_2O_2 are involved. In fact, hydrogen peroxide is produced by dismutation of the superoxide anion generated from the autoxidation of MbO_2 and HbO_2, and it must act as one of the most potent oxidants for their deoxy forms, which increase with decreasing O_2 pressures. Figure 5 illustrates in a very schematic way all the possible pathways leading to the oxidation of myoglobin to met myoglobin by molecular oxygen. It is thus possible to develop a complete kinetic formulation for the autoxidation reaction of MbO_2, if each elementary process is provided with each required rate constant in 0.1 M buffer (pH 7.0) at 25 °C.

For oxygen binding to myoglobin, one may write the equation:

$$\mathrm{Mb^{II}} + \mathrm{O_2} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{MbO_2} \qquad (26)$$

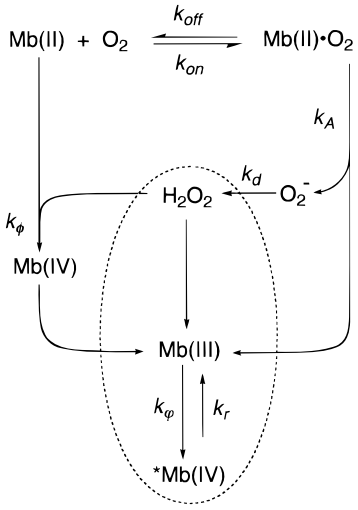


Figure 5. A schematic representation for the possible pathways leading to the oxidation of myoglobin (or hemoglobin) to its ferric met form by molecular oxygen. Encircled by broken line is a system operating for the decomposition of H_2O_2 in the normal autoxidation reaction of MbO_2. The ferryl species produced, not from deoxy-Mb(II) but from metMb(III), is marked here with an asterisk, *Mb(IV). Redrawn from Wazawa et al. (ref 76).

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 $K_{dis} = 1.15 \times 10^{-6}$ M.[1,67]

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[42] as

$$\mathrm{MbO_2} \xrightarrow{k_A} \mathrm{Mb^{III}} + \mathrm{O_2^-} \qquad (27)$$

where k_A represents the first-order rate constant for the autoxidation reaction of MbO_2, its magnitude depending strongly upon the pH of the solution as described already. 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.[52]

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 [68,69]) by the following spontaneous dismutation:

$$2\mathrm{O_2^-} + 2\mathrm{H^+} \xrightarrow{k_d} \mathrm{H_2O_2} + \mathrm{O_2} \qquad (28)$$

Yusa and Shikama[32] 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 2 equiv 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

$$Mb^{II} + H_2O_2 \xrightarrow{k_\phi} Mb^{IV} + 2OH^-$$

$$Mb^{IV} + Mb^{II} \xrightarrow{fast} 2Mb^{III}$$

$$\text{Sum:}\quad 2Mb^{II} + H_2O_2 \rightharpoonup 2Mb^{III} + 2OH^- \qquad (29)$$

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} in 0.1 M phosphate buffer at pH 7.0 and 25 °C.[32]

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

$$Mb^{III} + H_2O_2 \xrightarrow{k_\varphi} {}^*Mb^{IV}{=}O + H_2O \qquad (30)$$

and

$${}^*Mb^{IV}{=}O \xrightarrow{k_r} Mb^{III} \qquad (31)$$

where the values of $k_\varphi = 1.6 \times 10^6$ h^{-1} M^{-1} 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.[70,71] As for 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 and also a protein radical centered on a tyrosine or a tryptophan residue,[72−75] which is represented by $^*Mb^{IV}{=}O$ in this section. However, the molecular mechanism for the revert reaction (or self-reduction) of ferryl Mb to the ferric met form in eq 31 remains still open to further study.[71]

At this point, it should be noticed that the values of the on-rate (k_{on}) and off-rate (k_{off}) constants of eq 26 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 equilibrium. This conclusion would be valid at any time during the course of the oxidation reaction of myoglobin to metmyoglobin.

In the complex reaction delineated in Figure 5, the amount of deoxyMb, which is in equilibrium with MbO_2, would become an important factor for the overall stoichiometry of myoglobin oxidation, since the deoxy form is the preferred target for H_2O_2. Under air-saturated conditions ($P_{O_2} = 150$ Torr), the molar fraction of the deoxy form of sperm whale myoglobin is only ~0.45%, calculated from its oxygen dissociation constant (K_{dis}). Under these conditions, the H_2O_2 would be rapidly removed by the metMb resulting from the normal autoxidation 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.

To confirm these predictions more quantitatively, we have set up six rate equations for the simultaneous elementary processes and have carried out numerical analyses with the aid of a computer (IBM 3081-KX6). By using the Runge−Kutta method to solve these differential equations, the concentration progress curves for all the reactive species involved were displayed over a period of 100 h at intervals of 0.01 h (36 s). For the very early stage of the reaction, a much smaller step size of 1.56×10^{-4} h (0.56 s) was employed for integration.[76]

As a result, the rate of metmyoglobin formation under air-saturated conditions 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 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, suggesting that most of the H_2O_2 produced was used up to yield 2 equiv of metMb from deoxyMb as described in eq 29. This is mainly due to a large increase in the equilibrium concentration of the deoxy species. Furthermore, the rate of formation of metMb seemed to increase with decreasing partial pressure of O_2. As a function of O_2 pressure, therefore, we have computed the overall oxidation rate of myoglobin, from the ferrous state as a sum of MbO_2 and deoxyMb to the ferric met form, by the following definition:

$$\text{the rate of metMb formation} = k_{met}\{[MbO_2] + [Mb^{II}]\}$$

where k_{met} is the apparent first-order rate constant (in h^{-1}) deduced at a given value of O_2 pressure.

Figure 6 shows such a computer representation for the oxidation rate of myoglobin to metmyoglobin as a function of P_{O_2} in 0.1 M buffer (pH 7.0) at 25 °C. There appeared a well-defined maximum rate at a partial pressure of $O_2 \approx 5$ Torr. Increase of the O_2 pressure above ~40 Torr was found to have little effect on the oxidation rate, its magnitude being closer to a value of $k_A = 8.1 \times 10^{-3}$ h^{-1}, the rate constant observed for sperm whale MbO_2 in 0.1 M phosphate buffer at 25 °C under air-saturated condition. This computed result is in good accord with the actual experimental data by George and Stratmann[19] for the oxidation of horse heart myoglobin; the protein showed a maximum oxidation rate at the partial pressure of $O_2 \approx 2$ Torr in 0.6 M phosphate buffer, pH 5.69 at 30 °C, as already depicted in Figure 1.

The computer simulation made by Wazawa et al.[76] seems to be quite satisfactory, for the rate constants used in their calculation were derived from various experiments in which sections of the oxidation mechanism were isolated for study. Another interesting aspect of this O_2-dependence curve was demonstrated by changing the values of two relevant parameters. As shown in Figure 6, a maximum peak in the

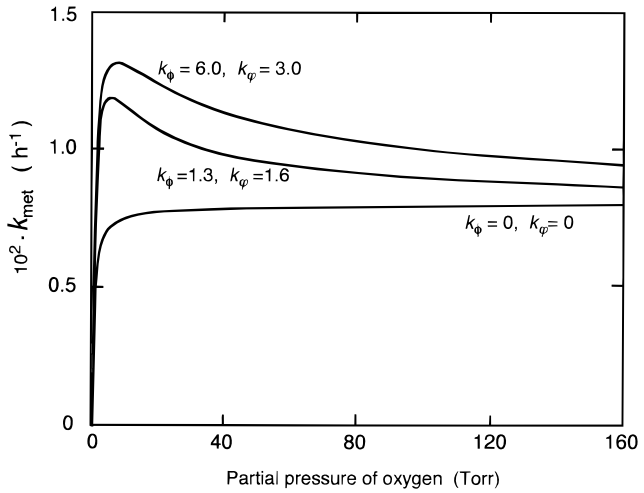


Figure 6. A computer representation for the oxidation rate of myoglobin to metmyoglobin as a function of partial pressures of oxygen. The simulation was carried out using three different set values for the rate constants, k_ϕ and k_φ, in the complex reaction delineated in Figure 5. (a) $k_\phi = 6.0 \times 10^7$ h^{-1} M^{-1} and $k_\varphi = 3.0 \times 10^6$ h^{-1} M^{-1}; (b) $k_\phi = 1.3 \times 10^7$ h^{-1} M^{-1} and $k_\varphi = 1.6 \times 10^6$ h^{-1} M^{-1}; (c) $k_\phi = 0.0$ h^{-1} M^{-1} and $k_\varphi = 0.0$ h^{-1} M^{-1}. At lower pressures of O_2, the H_2O_2 produced by the spontaneous dismutation of O_2^-, can act as the most potent oxidant of the deoxyMb, which increases with decreasing O_2 pressure, so that a well-defined maximum rate appeared in the formation of metMb at $P_{O_2} \approx 5$ Torr (see text). Redrawn from Wazawa et al. (ref 76).

oxidation rate was found when values of $k_\phi = 6.0 \times 10^7$ h^{-1} M^{-1} and $k_\varphi = 3.0 \times 10^6$ 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, no reaction of myoglobin with H_2O_2 should occur. In this case, only the rate of $k_A[MbO_2]$ would be responsible for the metmyoglobin formation, and the O_2-dependence curve becomes simply hyperbolic, with no detectable maximum rate of oxidation.

In this connection, it should be noted that several authors have examined the inhibitory effect of the presence of superoxide dismutase and/or catalase on the autoxidation rate of MbO_2 or HbO_2. However, the magnitude of this enzymatic suppression was smaller than the theoretically expected one, and varied considerably in the literature.[28] In our experience, too, the retardation effect of catalase on the autoxidation rate of bovine heart MbO_2 varied from 6 to 22% despite the presence of sufficient amount of the enzyme. This is mainly due to a very large value of K_m ($\approx$ 25 mM) of catalase for H_2O_2,[77] so that both of the deoxy and met forms of myoglobin can also react with H_2O_2 preferably. As a result, the effect of additional catalase would become always partial.[32,70,71]

At any rate, these computer-assisted numerical examinations provide us, for the first time, with a full picture of the oxidation behavior of myoglobin molecule as a function of oxygen pressure, and have disclosed that H_2O_2, which is produced from the dismutation of O_2^-, plays a crucial role in the oxidation of myoglobin at lower pressures of O_2. These results lead us to the finding that a good supply of dioxygen provides a rather important defense against the oxidation of myoglobin or hemoglobin with H_2O_2, one of the most potent oxidants in situ. This finding would seem 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.[78,79]

VI. Role of the Globin Moiety in Stabilizing the FeO_2 Bonding in Myoglobin

The reversible and stable binding of molecular oxygen to iron(II) is not a simple process. In a protein-free system, the small heme complexes are oxidized very rapidly and irreversibly by O_2, although a new class of porphyrins has been synthesized by the introduction of certain steric restraints to prevent the formation of an oxygen-bridged dimer, as a way of providing a protective enclosure for the bound dioxygen.[17,80] In native proteins, too, the oxygenated form of myoglobin or hemoglobin is converted easily to the ferric met form in protic, aqueous media and at physiological temperatures. Nevertheless, the relative stability of the oxygenated forms is the basis for the Mb and Hb functions in vivo, and differentiates these naturally occurring oxygen carriers from simple ferrous complexes.[59]

On the other hand, this functional stability of Mb or Hb is known to be lost easily on denaturation, with a consequent very rapid formation of the ferric species; therefore, it must be linked to the integrity of the conformation of these native proteins. In this regard, a view has been frequently expressed that the globin moiety of hemoglobin or myoglobin is merely a kind of steric hindrance to prevent dimerization or the formation of a bridge, by isolating each heme iron in a separate pocket. This is apparently based on the analogy of the nonaqueous autoxidation of small heme complexes (see eqs 1−4). In the absence of such a dimeric species, however, the oxygenated form of myoglobin or hemoglobin is subjected to aqueous autoxidation at a considerable rate, as already described.

Moreover, despite the same heme iron complex, the rate of autoxidation is substantially different from protein to protein, by a factor of more than 10^2 in the case of myoglobin molecules. In the molecular mechanism of autoxidation for myoglobin and hemoglobin, therefore, one of the crucial problems to be solved is the possible role of the globin moiety in stabilizing the FeO_2 bonding. In this regard, Sugawara et al.[23] have recently examined the autoxidation rate of bovine heart oxymyoglobin in the presence of 8 M urea and found that the rate is markedly enhanced above the normal autoxidation in buffer alone, over the whole range of pH studied from 5 to 13. Taking into account the concomitant process of unfolding of the protein in 8 M urea, they formulated a kinetic procedure to estimate the autoxidation rate of the unfolded form of MbO_2 that might occur transiently in due course of the urea effect.[23]

In the presence of 8 M urea, the autoxidation reaction of MbO_2 to metMb may be delineated by the

following possible pathways:

$$\begin{array}{ccc} MbO_2\,(N) & \xrightarrow{k_D^{oxy}} & MbO_2\,(D) \\ \downarrow k_A^n & & \downarrow k_A^d \\ metMb\,(N) & \xrightarrow{k_D^{met}} & metMb\,(D) \end{array} \qquad (32)$$

where N in parentheses denotes the native form and D is the denatured form for each species. In this scheme, a denatured form of MbO_2, which is fully unfolded but is still unoxidized, is assumed to occur transiently in the kinetic pathway leading to the formation of a completely disorganized met species. Therefore, k_D^{oxy} represents the rate constant for the unfolding of native MbO_2 to its denatured form, and k_A^d is the rate constant for the subsequent autoxidation reaction of denatured MbO_2. The other rate constant k_A^n might well be replaced by the autoxidation rate of native MbO_2 in buffer alone, as the solvent effect of urea was negligibly small on the nucleophilic displacement of O_2^- from MbO_2. The rate constant k_D^{met}, on the other hand, can be determined independently by the denaturation of metMb in 8 M urea. Our primary concern is, therefore, to estimate the value of k_A^d from the consecutive reactions of eq 32, and to compare it with that of k_A^n over a wide range of pH at 25 °C.

When fresh MbO_2 is placed in 8 M urea at a given pH, we can observe two concurrent reactions. One is the overall autoxidation reaction of MbO_2 described by the following equation:

$$MbO_2\,(N) \xrightarrow[\text{autoxidation}]{K_A} metMb\,(D) \qquad (33)$$

In this reaction, the spectra were changed from MbO_2 to its ferric form, showing a set of isosbestic points. Therefore, even if a denatured form of MbO_2, which may be fully unfolded but still unoxidized, appears in the kinetic pathway, we cannot differentiate it from the native form of MbO_2 in their spectra. This process of autoxidation was thus followed at 581 nm (α-peak of bovine heart MbO_2) by a single first-order rate constant of K_A (in h^{-1}) at a given pH. The resultant K_A values were strongly pH-dependent, and much higher than the corresponding values of k_A^n for the normal autoxidation in buffer alone, over the pH range of 5−13, at 25 °C.

The other is the overall denaturation process defined by the equation:

$$MbO_2\,(N) \xrightarrow[\text{denaturation}]{K_D} metMb\,(D) \qquad (34)$$

The overall unfolding of myoglobin molecule was monitored by circular dichroism (CD) at 222 nm and could be described adequately by a single rate constant of K_D (in h^{-1}) at a given pH. This urea-induced unfolding rate was always higher slightly than the corresponding autoxidation rate (K_A) over the whole range of pH studied, and showed almost the same pH dependence as for K_A. This indicates that the unfolding of MbO_2 is the first step causing a marked increase in the autoxidation rate in the presence of 8 M urea.

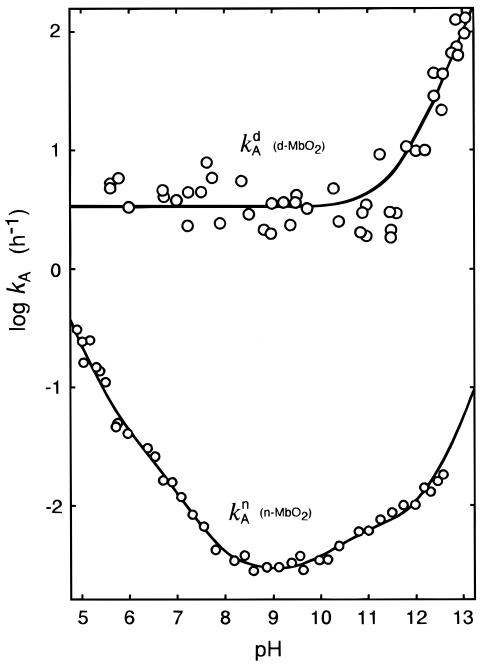


Figure 7. Comparison of the autoxidation rate between the native and the denatured form of bovine heart MbO_2 as a function of pH at 25 °C. The logarithmic values of the rate constant, k_A^d (in h^{-1}), evaluated for the autoxidation of unfolded MbO_2 (in 8 M urea) are plotted against the pH of the solution; those of k_A^n (in h^{-1}) are for native MbO_2 in buffer alone. Redrawn from Sugawara et al. (ref 23).

By solving the differential equations for the concentration change of each of the species involved in eq 32, they obtained finally the following relationships based on the appropriate suppositions:[23]

$$k_D^{oxy} = K_D - k_A^n \qquad (35)$$

and

$$K_D t - K_A t = \ln \frac{k_A^d}{k_A^d - k_D^{oxy}} \qquad (36)$$

The latter indicates that the difference between the values of K_D and K_A at a given pH holds a constant value at any time. Using the measured quantities of K_D, k_A^n and K_A, therefore, we can calculate easily at a given time the values of k_D^{oxy} from eq 35 and then k_A^d from eq 36.

In this way, the rate constant k_A^d was determined as a function of pH as shown in Figure 7. When compared with native MbO_2 in buffer alone, the fully denatured form of MbO_2 was found to be extremely susceptible to autoxidation over the whole range of pH studied. At pH 8.5 and 25 °C, for instance, its rate was nearly 1000 times higher than the corresponding value of k_A^n in 0.05 M buffer. Furthermore, its pH dependence is also unusual with an almost constant rate over a wide range of pH 5 to 11. Sugawara et al. [23] have therefore established the

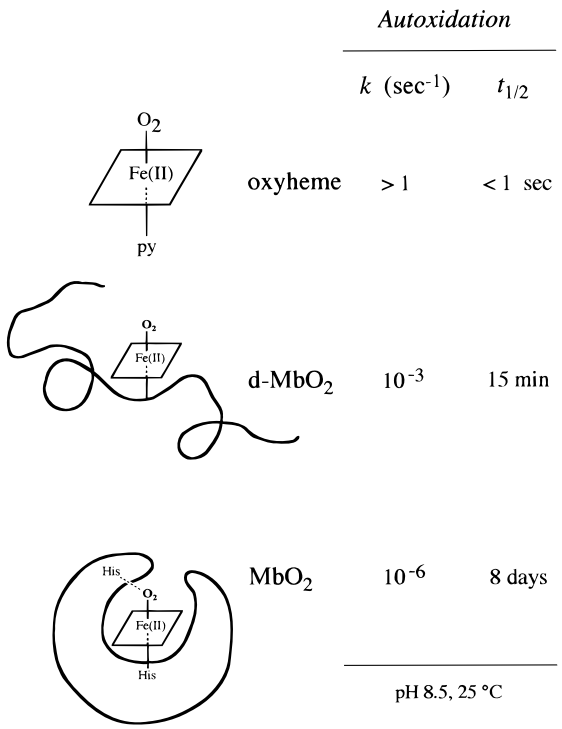


Figure 8. Role of the globin moiety in stabilizing the FeO_2 bonding in myoglobin. Mb has evolved with a globin moiety that can protect the FeO_2 center from easy access of a water molecule and its conjugate ions (OH^- and H^+). The polypeptide matrix can play a considerable role in stabilizing the oxygenated heme, but the integrity of the native protein architecture is essential to obstruct access of a water molecule to the FeO_2 center. In the molecular evolution from simple ferrous complexes to myoglobin and hemoglobin molecules, therefore, the protein matrix can be depicted as a breakwater of the FeO_2 bonding against protic, aqueous solvents. Redrawn from Sugawara et al. (ref 23).

best fit to the values of k_A^d as a function of pH, by the mechanism

$$k_A^d = k_0^d[H_2O] + k_{OH}^d[OH^-] \qquad (37)$$

where $k_0^d = 0.61 \times 10^{-1}$ h^{-1} M^{-1} and $k_{OH}^d = 0.91 \times 10^3$ h^{-1} M^{-1} in 0.05 M buffer at 25 °C. In this kinetic formulation, one of the most remarkable features is that the unfolded protein has lost completely the proton-catalyzed process ($k_H[H_2O][H^+]$), the one that plays a dominant role in the autoxidation reaction of native MbO_2, involving the distal histidine as its catalytic residue (see eq 19). Therefore, the extreme susceptibility of denatured MbO_2 to autoxidation comes, not from the proton catalysis, but mainly as a result of large values of k_0^d and k_{OH}^d, both being nearly 1000 times higher than the corresponding values of the native protein (see Table 2 for sperm whale MbO_2).

It should be noted here that in the presence of 8 M urea, MbO_2 was oxidized into a hemichrome with no detectable intermediate spectra of aqua- or hydroxide-metMb. On the basis of our previous work,[22] this can be explained as follows. The nucleophilic displacement of O_2^- from the unfolded form of MbO_2 by an entering water molecule or hydroxyl ion is the rate-determining step, and the subsequent conversion of the resultant met form into a hemichrome must proceed very quickly, with a nitrogenous residue (probably of histidine) being coordinated as the sixth ligand to the ferric iron of the fully denatured myoglobin molecule.

At any rate, these findings lead us to conclude that the unfolding of the globin moiety allows a much more easier attack of the solvent water molecule or hydroxyl ion on the FeO_2 center and causes a very rapid formation of the ferric met species by the nucleophilic displacement mechanism. In the molecular evolution from simple ferrous complexes to myoglobin and hemoglobin molecules, as illustrated in Figure 8, the protein matrix can be depicted as a breakwater of the FeO_2 bonding against protic, aqueous solvent.[23,83]

VII. Conclusion

The stabilization of molecular oxygen for transport and storage and the activation of oxygen for use in terminal oxidation are reciprocal but essential functions that support living organisms on the earth. Since the primary process for the autoxidation reaction of MbO_2 or HbO_2 is a nucleophilic displacement following one-electron transfer from iron(II) to the bound dioxygen, this reaction, at a molecular level, may be viewed as a meeting point of the stabilization and the activation of molecular oxygen performed by hemoproteins.[48]

Figure 9 illustrates the free-energy diagram for the reactions of myoglobin with dioxygen, when the FeO_2 form is chosen as the reference state. It is apparent that the reaction of myoglobin with O_2 proceeds by way of a considerable energy barrier for the formation of the activated complex. This reflects a profound change in the electronic configuration produced both on the iron and the dioxygen to give rise to the observed diamagnetism of MbO_2. The resulting

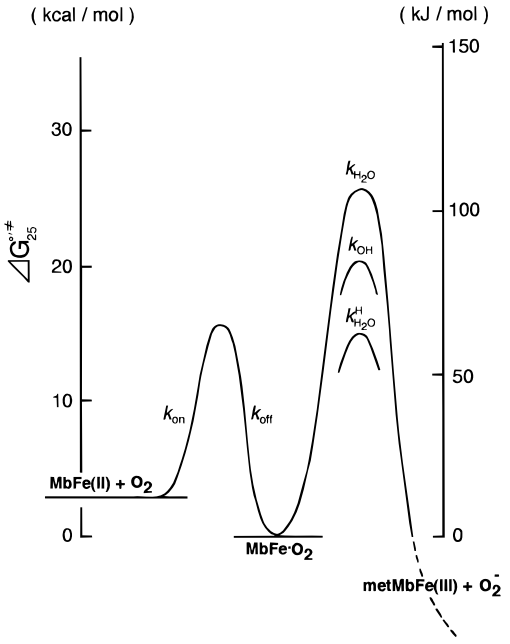


Figure 9. Free-energy diagram for the reactions of myoglobin with molecular oxygen under air-saturated conditions at 25 °C. The FeO_2 form is chosen as the reference state (see text). Redrawn from Shikama (ref 1).

MbO_2 is stabilized at the bottom of a deep ravine between two energy barriers. To release the bound dioxygen, therefore, MbO_2 must go back across a barrier somewhat higher than that for the on-rate process, during which step the electronic configuration is rearranged back to its initial state for separated Fe(II) and O_2.

From Figure 9, it is also clear that the FeO_2 bonding in Mb or Hb, if placed in vacuo, is inherently stable and so unlikely to dissociate O_2^- spontaneously. O_2 is a rather poor one-electron acceptor, so a considerable thermodynamic barrier exists for such an electron transfer from the Fe(II) to O_2. In aqueous media, however, things are quite different. In the neutral pH range, MbO_2 seems to be still protected against irreversible autoxidation primarily by a high energy barrier of approximately 26 kcal mol^{-1} (107 kJ mol^{-1}) for the formation of the activated complex with an entering water molecule. This barrier is more than 1.5 times higher than that for the off-rate process, and thus provides myoglobin with a strong bias for reversible O_2 binding as opposed to irreversible autoxidation. Its height may reflect partly the unfavorable free energy for one-electron reduction of O_2 by Mb. That free energy change is approximately +8 kcal as already described. The high activation energy may also be due partly to the low nucleophilicity of water molecule and to the difficulty of it in access to the hydrophobic heme pocket.

In the higher pH range, hydroxide anion, with a nucleophilicity much stronger than that of H_2O, increases in concentration, and it can displace O_2^- from MbO_2 more easily than the water molecule can, because it lowers the free energy barrier by approximately 5 kcal mol^{-1}.

In the acidic pH range, the autoxidation of MbO_2 increases very rapidly with increasing hydrogen ion concentration. In fact, the proton enhances the rate constant by a factor of 5×10^7 per mole at 25 °C. From thermodynamic analysis, Sugawara and Shikama[12] have shown that the catalytic proton decreases $\Delta H^{\circ\ddagger}$ and also increases $\Delta S^{\circ\ddagger}$, thereby lowering the free energy barrier $\Delta G^{\circ\ddagger}$ for the formation of the activated complex by an order of magnitude of more than 10 kcal mol^{-1}. It thus becomes evident that the free energy barrier for the autoxidation of MbO_2 to metMb could be reduced with increasing hydrogen ion concentration to a value comparable to that for the off-rate process of MbO_2. This means that in the lower pH range, MbO_2 is always subject to a very rapid autoxidation reaction during the reversible oxygen-binding process. Acidosis must therefore have a serious effect on the oxygen supply to red muscles such as those of cardiac and skeletal tissues.

The free heme iron(II) in aqueous solution at room-temperature autoxidizes within seconds. Once it is protected by the protein matrix, however, the autoxidation rate slows down to hours or days. Myoglobin and hemoglobin have thus evolved with a globin moiety that can protect the FeO_2 center from easy access of a water molecule including its conjugate ions OH^- and H^+. A free-energy diagram for the potential reactions of FeO_2 also visualizes myoglobin as a molecular structure that can provide in solution the delicate balance of kinetic and thermodynamic factors necessary to stabilize reversible oxygenation, as opposed to irreversible autoxidation to metmyoglobin.

VIII. Acknowledgments

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