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Reviews

Nature of the FeO_2 bonding in myoglobin: An overview from physical to clinical biochemistry

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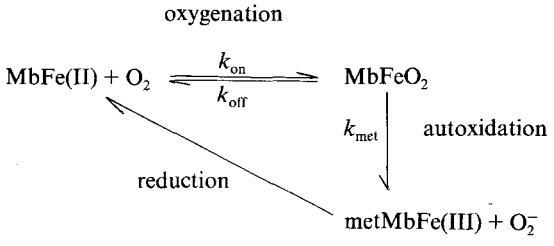
Summary. The iron(II)-dioxygen bond in myoglobin and hemoglobin is a subject of wide interest. Studies range from examinations of physical-chemical properties dependent on electronic structure, to investigations of stability as a function of oxygen supply. Stability properties are of particular importance in vivo, since the oxygenated form is known to be oxidized easily to the ferric form, which cannot be oxygenated and is therefore physiologically inactive.

Kinetic and thermodynamic studies of the stability of native oxymyoglobin have revealed a new feature in FeO_2 bonding. In vivo, the iron center is always subject to a nucleophilic attack of the water molecule or hydroxyl ion, which can enter the heme pocket from the surrounding solvent, and thereby irreversibly displace the bound dioxygen from MbO_2 in the form of O_2^- so that the iron is converted to the ferric form. A free energy diagram for the potential reactions of FeO_2 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.

Key words. Myoglobin; FeO_2 bonding; oxymyoglobin; free energy diagram.

Introduction: Dynamics of FeO_2 bonding in vivo

In red muscles, such as cardiac and skeletal, myoglobin plays an essential role in maintaining aerobic metabolism, both as an oxygen store and as an entity facilitating oxygen diffusion[15,29,31]. It is in the ferrous form that myoglobin can bind molecular oxygen reversibly and carry out its functions. From known changes in valency of the heme iron, one can write the cycle of myoglobin function as follows:



(1)

The process of oxygen binding to myoglobin has recently been analyzed by Frauenfelder and co-workers assuming multiple barriers encountered by the oxygen molecule as it comes from the solvent and approaches the heme iron[1,3]. During reversible oxygen binding, on the other hand, the oxygenated myoglobin is known to be oxidized easily to the ferric met-form with generation of the superoxide anion[6,7]. The rate of this autoxidation reaction depends strongly on the pH of the solution. At 35 °C, for instance, Sugawara and Shikama[25] showed that the half-life for conversion of bovine heart MbO_2 to metMb was 3.3 days at pH 9.1, but that it became 11 h at pH 7.0 and even less than 30 min at pH 5.0 in 0.1 M buffer. The metMb thus formed cannot be oxygenated and is therefore physiologically inactive.

However, Hagler et al. have found that muscle tissues contain an NADH-dependent enzyme that can reduce metMb to deoxy ferrous myoglobin and thus prevent the continued accumulation of metMb in situ[9]. In fact, it is a matter of our experience that the metMb content in myoglobin extracts from various muscle tissues is commonly about 40% or less. This cyclic reduction of metMb is, therefore, a basis for the continuity of myoglobin function in vivo.

Geometry and electronic structure of the FeO_2 bonding

In consequence of the wide interest in the magnetism, spectrum and reactivity of the FeO_2 center in hemoglobin and myoglobin, many proposals have been made concerning its electronic structure. Nevertheless, the following 2 are of primary importance.

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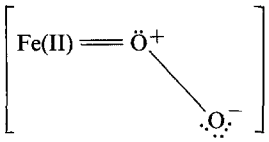
In Pauling's formulation



the iron atom is shown 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[16].

In Weiss' ionic description

$[Fe(III) \cdot O_2^-]$

an electron transfer from iron to dioxygen creates a fully developed Fe(III)-superoxide ion pair[30]. The resulting unpaired spins resident on Fe(III) and O_2^- are assumed to couple to give rise to a diamagnetic species[32]. Phillips and Schoenborn have recently carried out X-ray and neutron diffraction studies of the structure of sperm whale oxymyoglobin, and revealed that the bound dioxygen is indeed bent, held end-on and stabilized by a hydrogen bond to the distal (E 7) histidine. The detailed geometry is shown in figure 1, where the Fe–O–O bond angle is 121°, the O^1–O^2---H angle is 96°, and the proton bonded to N^δ projects into the solvent surrounding the molecule[17,18]. This structure, however, does not resolve the controversy between proponents of an ionic description and of a covalent description for the FeO_2 bonding, although it does rule out the cyclic formulation (Fe<O/O>) proposed by Griffith[8].

Other lines of physical and chemical evidence indicate that when the deoxy-form combines with molecular oxygen, a partial, not full, transfer of an electron occurs from the ferrous iron to the antibonding π^* orbitals of dioxygen. For instance, Caughey et al.[14] observed that the O–O stretching frequency of dioxygen decreases from its 1555 cm^{-1} in the gas phase to 1107 cm^{-1} in HbO_2 and to 1103 cm^{-1} in MbO_2. They concluded that these shifts and associated intensity changes provide clear evidence for such electron transfer to the dioxygen bound to the heme iron, in a bent, end-on geometry[4]. In Mössbauer spectroscopy, Lang and Marshall also showed that the isomer shift (δ) of HbO_2, a measure of electron density at the iron nucleus, is much closer to that for the ferric form than to that for the deoxy ferrous form, and that the quadrupole splitting (ΔE_Q), a measure of charge asymmetry in the d-electron orbitals in particular, corresponds to a strong covalent character in the bond between the iron and dioxygen[12]. All of these recent measurements 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[4]:

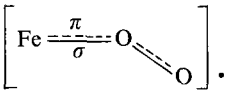


Figure 2 gives a simplified molecular orbital representation of the FeO_2 bonding, by Jones et al.[10] with some modifications. When deoxymyoglobin in a high-spin ferrous state (S = 2) combines with dioxygen in a triplet ground state (S = 1), σ-donation primarily arises from the overlap of an antibonding π^* orbital of dioxygen with the d_{z^2} orbital of iron. A partial transfer of an electron also occurs simultaneously from a t_{2g} orbital of iron to another π^* orbital of dioxygen so as to place oxymyoglobin in a diamagnetic low-spin state (S = 0). On the other hand, it is well known that the oxygenated form of myoglobin or hemoglobin is easily oxidized to

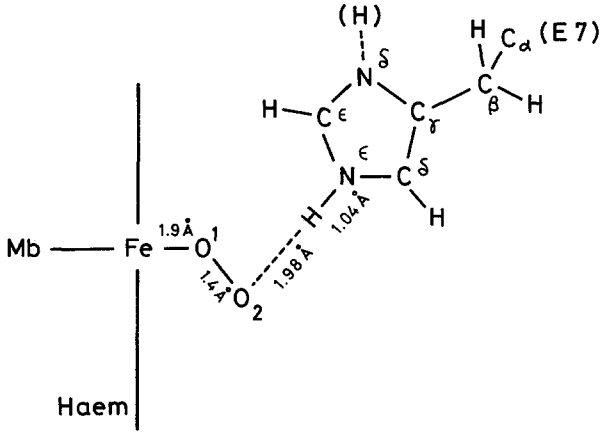


Figure 1. Geometry of the FeO_2 bond in sperm whale oxymyoglobin[17,18].

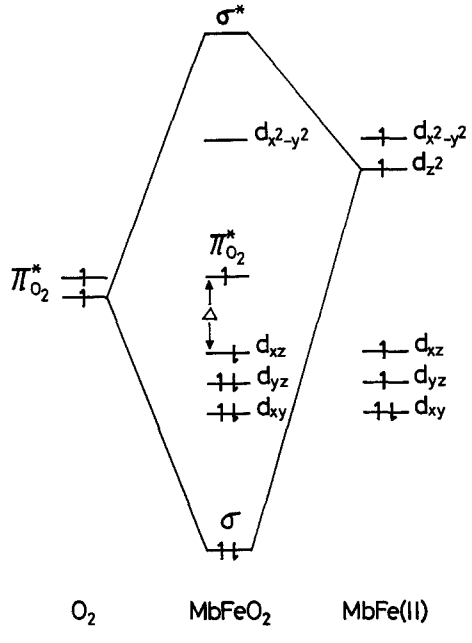


Figure 2. A simplified MO scheme for FeO_2 bonding in myoglobin, showing both σ and π interactions between iron and coordinated dioxygen. The unpaired electron spin density on the dioxygen moiety increases when the energy separation, Δ, is smaller than the electron-pairing energy[10].

the ferric form. Nevertheless, the mechanistic details of this process have been quite unclear despite the fact that the factors influencing the rate of autoxidation have long been studied by a number of authors. Using native oxymyoglobin isolated directly from muscle tissues[22, 27, 33], we have carried out kinetic and thermodynamic studies of its stability that have recently revealed other interesting features of the FeO_2 bonding. These new properties of MbO_2 are of primary importance for a full understanding, not only of the nature of the FeO_2 bonding, but also of the clinical biochemistry of the oxygen supply from blood capillaries to mitochondria in red muscles, where ischemia causes abrupt cell destruction in cardiac and skeletal tissue.

Stability properties of the FeO_2 bonding

Oxymyoglobin is oxidized easily and irreversibly in vitro to metmyoglobin, with the generation of the superoxide anion;

$$MbO_2 \xrightarrow{k_{met}} metMb + O_2^- \qquad (2)$$

where k_{met} represents the first-order rate constant observed at a given pH[6]. The generation of the superoxide anion has been demonstrated by Gotoh and Shikama[7] testing the inhibitory effect of superoxide dismutase on the reduction of cytochrome c or on the oxidation of epinephrin coupled with the autoxidation of MbO_2.

Therefore, the rate of autoxidation is given by

$$\frac{-d\,[MbO_2]}{dt} = k_{met} \cdot [MbO_2] \cdot \qquad (3)$$

If the values of k_{met} are plotted against the pH of the solution, a profile of the stability of oxymyoglobin can be obtained. Figure 3 shows such a profile for bovine heart MbO_2 in 0.1 M buffer at 25 °C[21]. This graph indicates that the rate of autoxidation increases rapidly with increasing hydrogen ion concentration, that a rate minimum appears at pH 9 and that a small increase sets in at the higher values of pH.

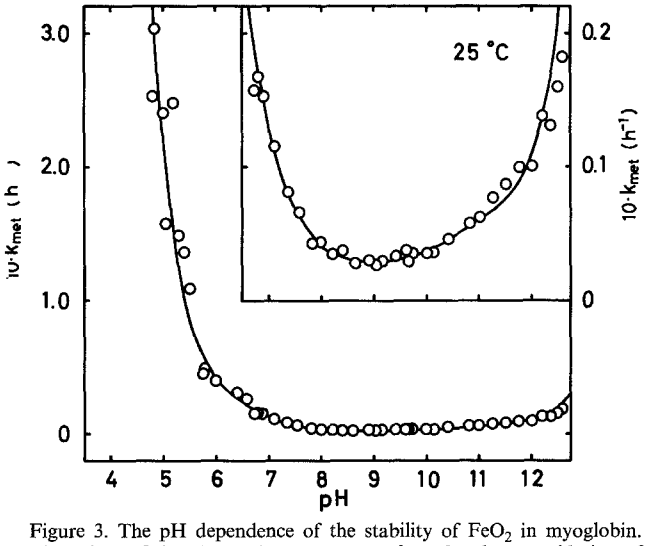


Figure 3. The pH dependence of the stability of FeO_2 in myoglobin. The values of the observed rate constant, k_{met}, for the autoxidation of bovine heart MbO_2 are plotted against the pH of the solution in 0.1 M buffer at 25 °C. The computed curve (–) was obtained from Eq. (5) by a least-squares fitting to the experimental data (○) over the whole range of pH 4.8–12.6. The expanded scale is used above pH 6.5. MbO_2 concentration, 50 μM[21, 25].

In order to unravel the kinetic and thermodynamic parameters contributing to the k_{met} versus pH profile, Shikama and Sugawara[21] have proposed some mechanistic models for the autoxidation reaction. The rate equations derived therefrom have been used for fitting experimental data according to their procedures using a digital computer (ACOS 900 system, Tohoku University, Sendai). They have concluded that the complicated pH-profile for the autoxidation rate of bovine heart MbO_2 can be best explained by an 'acid-catalyzed three-states model'[21, 25]. In this scheme it is assumed that 2 different kinds of dissociable groups, AH with pK_1 and BH with pK_2, are involved in the reaction. Also it is assumed that there are 3 forms of MbO_2, represented by A, B, and C, at molar fractions of α, β, and $(1\text{-}\alpha\text{-}\beta)$ respectively, which are in equilibrium with each other but which differ in dissociation states for the groups AH and BH (see Eq. (4)). 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 6th coordination position to form aqua- or hydroxide-metMb, respectively. Both of these met-species have already been established definitely by Stryer et al. from X-ray analysis[24]. The reaction scheme may be written, therefore, as

$$\begin{array}{ccccc} MbO_2(AH,BH) & \overset{K_1}{\rightleftharpoons} & MbO_2(A^-,BH) & \overset{K_2}{\rightleftharpoons} & MbO_2(A^-,B^-) \\ \downarrow k_0^A \quad \downarrow k_H^A & & \downarrow k_0^B \quad \downarrow k_H^B \quad \downarrow k_{OH}^B & & \downarrow k_0^C \quad \downarrow k_{OH}^C \\ metMb & & metMb & & metMb \end{array} \qquad (4)$$

where for each form of MbO_2 k_0 is the rate constant for the 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. (4) the observed rate constant, k_{met} in Eq. (3), can finally be reduced to

$$\begin{aligned} k_{met} = {} & (k_0^A \cdot [H_2O] + k_H^A \cdot [H_2O] \cdot [H^+])\,(\alpha) \\ & + (k_0^B \cdot [H_2O] + k_H^B \cdot [H_2O] \cdot [H^+] + k_{OH}^B \cdot [OH^-])\,(\beta) \\ & + (k_0^C \cdot [H_2O] + k_{OH}^C \cdot [OH^-])\,(1 - \alpha - \beta) \end{aligned} \qquad (5)$$

where

$$\alpha = \frac{[H^+]^2}{[H^+]^2 + K_1 \cdot [H^+] + K_1 \cdot K_2}$$

$$\beta = \frac{K_1 \cdot [H^+]}{[H^+]^2 + K_1 \cdot [H^+] + K_1 \cdot K_2} \qquad (6)$$

and

$$(1 - \alpha - \beta) = \frac{K_1 \cdot K_2}{[H^+]^2 + K_1 \cdot [H^+] + K_1 \cdot K_2}.$$

By iterative least-square procedures inserting various values for K_1 and K_2, the adjustable parameters in Eq.

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(6), the best fit to the experimental values of k_{met} was obtained as a function of pH as shown in figure 3. In this way the rate constants of the elementary processes involved in the autoxidation reaction of MbO_2 were also established as follows: $k_0^A = 0.79 \times 10^{-4}$ $h^{-1}M^{-1}$, $k_H^A = 0.34 \times 10^3$ $h^{-1}M^{-2}$, $k_0^B = 0.47 \times 10^{-4}$ $h^{-1}M^{-1}$, $k_H^B = 0.25 \times 10^4$ $h^{-1}M^{-2}$, $k_{OH}^B = 0.18 \times 10^2$ $h^{-1}M^{-1}$, $k_0^C = 0.31 \times 10^{-4}$ $h^{-1}M^{-1}$, and $k_{OH}^C = 0.50$ $h^{-1}M^{-1}$ in 0.1 M buffer at 25 °C[25]. From the best values found for pK_1 (= 6.7) and pK_2 (= 10.4), the most probable candidates for the dissociable groups AH and BH (see Eq. (4)) are histidyl and tyrosyl residues respectively. This identification was confirmed by a thermodynamic characterization for the groups[25].

In the table a complete set of the 3 types of rate constants involved in the autoxidation reaction in the neutral pH range, k_0^B, k_H^B, and k_{OH}^B, is given under the more general nomenclature of k_{H_2O}, $k_{H_2O}^H$, and k_{OH} respectively, since these are the pertinent ones for the present purpose. It is apparent that the proton-catalyzed process with the rate constant $k_{H_2O}^H$ promotes the autoxidation reaction of MbO_2 above the spontaneous process in water with the rate constant k_{H_2O}. In fact, the catalytic proton enhances the rate by a factor of 5×10^7/mol. From the value of $k_{H_2O}^H = 0.25 \times 10^4$ $h^{-1}M^{-2}$ one can also calculate a half-life ($t_{1/2}$) of 18 msec for autoxidation of MbO_2 placed in 1 M H^+ aqueous solution at 25 °C. These estimations reveal that the proton-catalyzed autoxidation of MbO_2 is inherently a fairly fast reaction, but in practice the extremely low concentration of H^+ ion at physiological pH (corresponding to 10^{-7} M H^+) reduces the rate to a negligible magnitude.

In this proton catalysis, the imidazole ring of the distal histidine (the dissociable group AH with pK_1) appears to participate by a proton-relay mechanism in facilitating the effective movement of a catalytic proton from the solvent to the bound dioxygen[25,26]. As is evident in figure 1, when a catalytic proton from the solvent protonates transiently the remote nitrogen (N^δ) of the distal histidine at neutral pH, the opposite nitrogen (N^ϵ) releases its proton so that it moves closer to the bound polarized dioxygen. Such an activated state serves to facilitate a full charge transfer from Fe(II) to O_2. This transfer facilitates displacement of O_2^- as the hydroperoxyl radical HO_2, which departs and, since its pK_a is 4.8, then dissociates into the superoxide anion and a catalytic proton[5].

Although water molecules can participate in 2 types of paths, i.e. that with and the other without proton-catalysis, each elementary process in Eq. (5) involved in the autoxidation reaction of MbO_2 can be written as a displacement of the bound dioxygen, as superoxide anion, by the entering ligand, H_2O or OH^-:

$$Fe(II)(O_2) + H_2O \xrightarrow{k_{H_2O}} Fe(III)(OH_2) + O_2^-$$

$$Fe(II)(O_2) + OH^- \xrightarrow{k_{OH}} Fe(III)(OH^-) + O_2^-$$

To elucidate further the molecular mechanism of these substitution reactions leading to metmyoglobin formation in vivo, Satoh and Shikama have studied the oxidation of MbO_2 induced by excess anion[19]. The anions examined were SCN^-, F^-, OCN^-, N_3^- and CN^-, whose nucleophilicity differs from H_2O and OH^-. In each case, the observed oxidation rate was linearly dependent upon the concentration of an added anion. A Brønsted plot for the series showed that the rates correlated with the pK_a of the conjugate acid, a measure of the nucleophilicity of the anion. 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 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 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 Eq. (5) for the autoxidation reaction of MbO_2 under physiological conditions can therefore be viewed in the general form of Eq. (7), as a S_N2 mechanism,

$$\overset{\delta+\quad\delta-}{MbFe(II)O_2} + N^- \xrightarrow{k_N} \left[MbFe\begin{matrix} \diagup N^- \\ \diagdown O_2 \end{matrix} \right] \longrightarrow metMbFe(III) \cdot N^- + O_2^- \qquad (7)$$

where k_N represents the rate constant for the anion-induced oxidation of MbO_2, and N^- can be SCN^-, F^-, OCN^-, N_3^-, or CN^-, and, in vivo, H_2O or OH^-.

This nucleophilic displacement can proceed without any protonation. Nevertheless, the rate is enormously enhanced, by the factor of more than 10^6/mol, with proton assistance[19]. Our kinetic analysis of the stability properties of native MbO_2, therefore, reveals a new feature of the FeO_2 bonding. The iron center is very susceptible to attacking nucleophiles; only in their presence is a full charge transfer from the Fe to O_2 produced.

Kinetic and thermodynamic parameters for reversible oxygen-binding to myoglobin and for irreversible autoxidation of oxymyoglobin under air-saturated conditions in the neutral pH range at 25 °C[2,25]

Reaction	Rate constant (units)	k	$\Delta G^{0\ddagger}$ (kcal·mol^{-1}) (at specified state)
Oxygen-binding[a]	k_{on} (sec^{-1} M^{-1})	1.64×10^7	12.5 (in 0.25 mM dissolved O_2)
	k_{off} (sec^{-1})	19	15.6
Autoxidation[b]	k_{H_2O} (h^{-1} M^{-1})	0.47×10^{-4}	25.7 (in aqueous solution)[c]
	$k_{H_2O}^H$ (h^{-1} M^{-2})	0.25×10^4	15.2 (in 1 M H^+ aqueous solution)
	k_{OH} (h^{-1} M^{-1})	0.18×10^2	20.5 (in 1 M OH^- solution)

[a] See Eq. (1). [b] The rate constants correspond to k_0^B, k_H^B, and k_{OH}^B, respectively, in Eq. (5). See the text. [c] The concentration of H_2O is taken as 55.5 M.

Free energy diagram for reactivity of the FeO_2 bonding

The table summarizes the kinetic and thermodynamic parameters for reversible oxygen-binding to myoglobin

and for irreversible autoxidation of oxymyoglobin in the neutral pH range. The values of the on-rate and off-rate constants for the oxygen binding at 25 °C were calculated from the literature values at 20 °C using the corresponding activation energies listed by Antonini and Brunori[2]. The free energy change for the formation of the activated complex of each elementary process was also calculated in each specified state from the corresponding rate constant k_r by the relation

$$k_r = (kT/h)\exp(-\Delta G^{0*}/RT)$$

where R represents the gas constant, k the Boltzmann constant, h the Planck constant, and T the absolute temperature.

The free energy diagram for the reactions of myoglobin with oxygen is presented in figure 4 with the FeO_2 chosen as the reference state.

It is apparent that the reaction of myoglobin with molecular oxygen 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 MbO_2 is stabilized at the bottom of a deep ravine between 2 energy barriers. To release the bound oxygen, 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.

In the neutral pH range, MbO_2 is 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 oxygen-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; molecular oxygen is a poor one-electron acceptor with a lower redox potential, E_0' $(O_2/O_2^-) = -0.27 \sim -0.33$ V[20], than that, $E_0' = +0.046$ V, for the metMb-Mb system[28]. The high activation energy may also be due partly to the low nucleophilicity of water and to the difficulty in access to the hydrophobic heme pocket[19,23].

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.

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/mol at 25 °C. From the thermodynamic analysis Sugawara and Shikama have shown that the catalytic proton decreases ΔH^{o*}, and also increases ΔS^{o*}, thereby lowering the free energy barrier ΔG^{o*} for the formation of the activated complex by the order of magnitude of more than 10 kcal/mol[25]. It thus becomes evident that the 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 the cardiac and the skeletal.

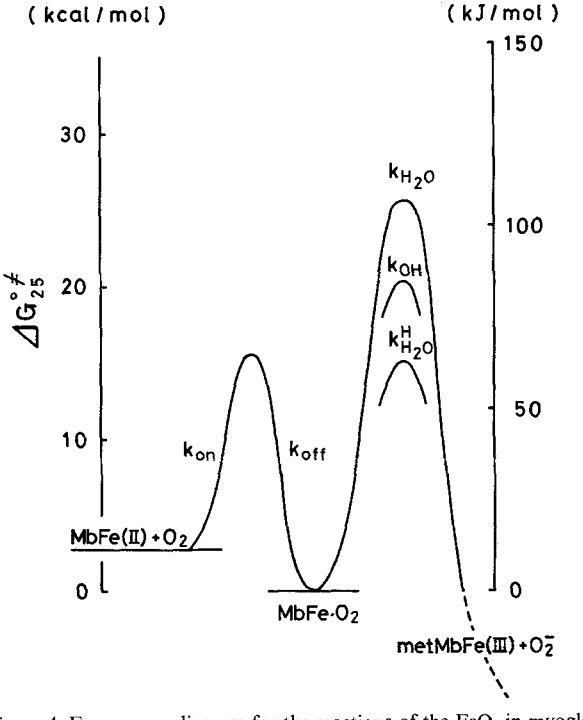

Figure 4. Free energy diagram for the reactions of the FeO_2 in myoglobin under air-saturated conditions at 25 °C.

Conclusion

From the free energy diagram for the reactivity of myoglobin with dioxygen, it becomes clear that the FeO_2 bonding is inherently stable if placed in vacuo since the completion of one-electron transfer from the Fe(II) to O_2 is an energetically unfavorable process. However, in vivo, the FeO_2 center is always subject to nucleophilic attack of an entering water molecule, with and without proton-catalysis, and to the attack of an entering hydroxide anion. These can cause irreversible oxidation of MbO_2 to metMb with the generation of the superoxide anion. Myoglobin has thus evolved with a globin moiety that can provide in aqueous media the delicate balance of kinetic and thermodynamic factors necessary to stabilize reversible oxygen-binding as opposed to irreversible autoxidation induced by the water molecule and its conjugate anionic species[23,26].

The stabilization of 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 autoxidation of oxymyoglobin has been shown to be a nucleophilic displacement accompanied by one-electron transfer to the bound dioxygen, this reaction, in its molecular nature,

can be viewed as a meeting point of the stabilization and the activation of molecular oxygen performed by hemeproteins. It may therefore provide us with an insight into general principles governing molecular oxygen enzymology including oxygenases and oxidases[11,13].

Abbreviations. Mb, myoglobin; MbO_2, oxymyoglobin; metMb, metmyoglobin; Hb, hemoglobin; HbO_2, oxyhemoglobin.

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