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STABILITY PROPERTIES OF DIOXYGEN–IRON(II) PORPHYRINS: AN OVERVIEW FROM SIMPLE COMPLEXES TO MYOGLOBIN

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CONTENTS

ABBREVIATIONS

Mb	myoglobin
MbO_2	oxymyoglobin
metMb	metmyoglobin
Hb	hemoglobin
HbO_2	oxyhemoglobin

SUMMARY

The reversible and stable binding of molecular oxygen to iron(II) is not a simple process. Although it has been possible to synthesize a class of O_2-binding porphyrins by the introduction of certain steric restraints to prevent the formation of an oxygen-bridged dimer, small heme complexes are mostly oxidized very rapidly and irreversibly by O_2. In the cases of myoglobin and hemoglobin, each heme iron atom is embedded in its protein matrix so as to be unable to form such a dimer. Nevertheless, the oxygenated form is still oxidized to the ferric met-species, at a slow but considerable rate, with the generation of superoxide anion.

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Kinetic and thermodynamic studies of the stability of native oxymyoglobin have revealed that its autoxidation proceeds through a pathway quite different from that of the simple heme complexes. The FeO_2 center of myoglobin is always subject to nucleophilic attack by an entering water molecule with strong proton assistance from the distal histidine (E 7), which acts as a catalytic residue. This center is also open to the attack of an entering hydroxide anion. These reactions can cause irreversible displacement of the bound dioxygen from MbO_2 in the form of O_2^- so that the iron is converted to the ferric met-form. Myoglobin has thus evolved with a globin moiety that can protect the FeO_2 center from easy access of a water molecule and its conjugate anionic species.

These new features of the stability of MbO_2 are of primary importance, not only for a full understanding of the nature of FeO_2 bonding, but also for planning new molecular designs for synthetic oxygen carriers which may be able to function in aqueous solutions under physiological conditions.

A. INTRODUCTION

In supporting living organisms on the earth, heme proteins play an essential role both in stabilizing molecular oxygen for transport and storage and in activating oxygen for use in terminal oxidation. The former function is performed by hemoglobin and myoglobin, and the latter is carried out by enzymes such as cytochrome oxidase and cytochrome P-450 [1,2].

Dioxygen has a triplet ground state, $^3\Sigma_g^-$, whose biradical electronic configuration is described by the notation: $O_2(\sigma_g 1s)^2(\sigma_u^* 1s)^2(\sigma_g 2s)^2(\sigma_u^* 2s)^2(\sigma_g 2p_z)^2(\pi_u 2p_x)^2(\pi_u 2p_y)^2(\pi_g^* 2p_x)^1(\pi_g^* 2p_y)^1(\sigma_u^* 2p_z)^0$. The reversible and stable coordination of this strong oxidizing agent to iron(II) is not a simple process [3,4]. In a protein-free system the small heme compounds mostly react irreversibly with O_2 although it has been possible to synthesize a new class of porphyrins by the introduction of certain steric restraints to prevent the formation of an oxygen-bridged dimer [5]. In native proteins, too, it is well known that HbO_2 and MbO_2, during reversible oxygen-binding, still undergo a slow oxidation to the ferric met-form. Since the iron(III) form cannot be oxygenated and is therefore physiologically inactive, stability properties of the oxygenated forms are of particular importance in vivo. Nevertheless, the mechanistic details of this autoxidation process have remained unclear despite the fact that the factors influencing the rate of autoxidation have long been studied by a number of authors [6].

Recent kinetic and thermodynamic studies of the stability of native oxymyoglobin have revealed that the autoxidation of the protein in aqueous solvent proceeds through a pathway quite different to that of simple heme

complexes in aprotic, non-aqueous solvents [7–9]. This new insight into MbO_2 seems to be 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 in cardiac and skeletal tissues causes abrupt cell destruction [4]. This new insight may also provide a new molecular basis for designing synthetic oxygen carriers that are able to function in protic, aqueous solutions under physiological conditions.

B. AUTOXIDATION OF SIMPLE IRON(II)–PORPHYRIN COMPLEXES: DIMER FORMATION

It is well known that simple iron(II) complexes are oxidized very rapidly and irreversibly with molecular oxygen. The rate-determining step of these autoxidation reactions in non-aqueous solvents is second order in iron(II) and first order in dioxygen. This observation was made by Cohen and Caughey [10] for ferrous porphyrins and by Hammond and Wu [11] for ferrous salts. A possible mechanism has therefore been proposed based on the dimeric nature of the autoxidation products [10–15].

An initial 1 : 1 binding of dioxygen by iron(II) (step (a) below) is followed by a bimolecular redox process (step (b)), 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 $Fe^{IV}(O^{2-})$, to the μ-oxo dimer [16]. These bridged species could further be decomposed to iron(III) species by treatment with hydrogen chloride [13] or by the participation of a trace amount of water present in non-aqueous solvents [10].

$$Fe(II) + O_2 \overset{(a)}{\rightleftharpoons} Fe^{II}(O_2)$$

$$Fe^{II}(O_2) + Fe(II) \overset{(b)}{\rightarrow} Fe^{III}{-}O_2{-}Fe^{III}$$

$$Fe^{III}{-}O_2{-}Fe^{III} \rightarrow 2\ Fe^{IV}(O^{2-})$$

$$Fe^{IV}(O^{2-}) + Fe(II) \rightarrow Fe^{III}{-}O{-}Fe^{III} \rightarrow Fe(III)$$

If the second dimerization process (step (b)) were impeded or reduced in ome manner, it should be possible to obtain reversible behavior of the on(II) in oxygen binding (step (a)). This situation has been achieved by egant steric approaches using new porphyrins which have a protective closure for the bound dioxygen. These include Baldwin's "capped" rphyrin [13], Chang's "crowned" porphyrin [17], and Collman's "picket-ce" porphyrin [15,18] as illustrated in Fig. 1. The half-life of decomposi-n of these oxygen adducts to the ferric species was markedly increased

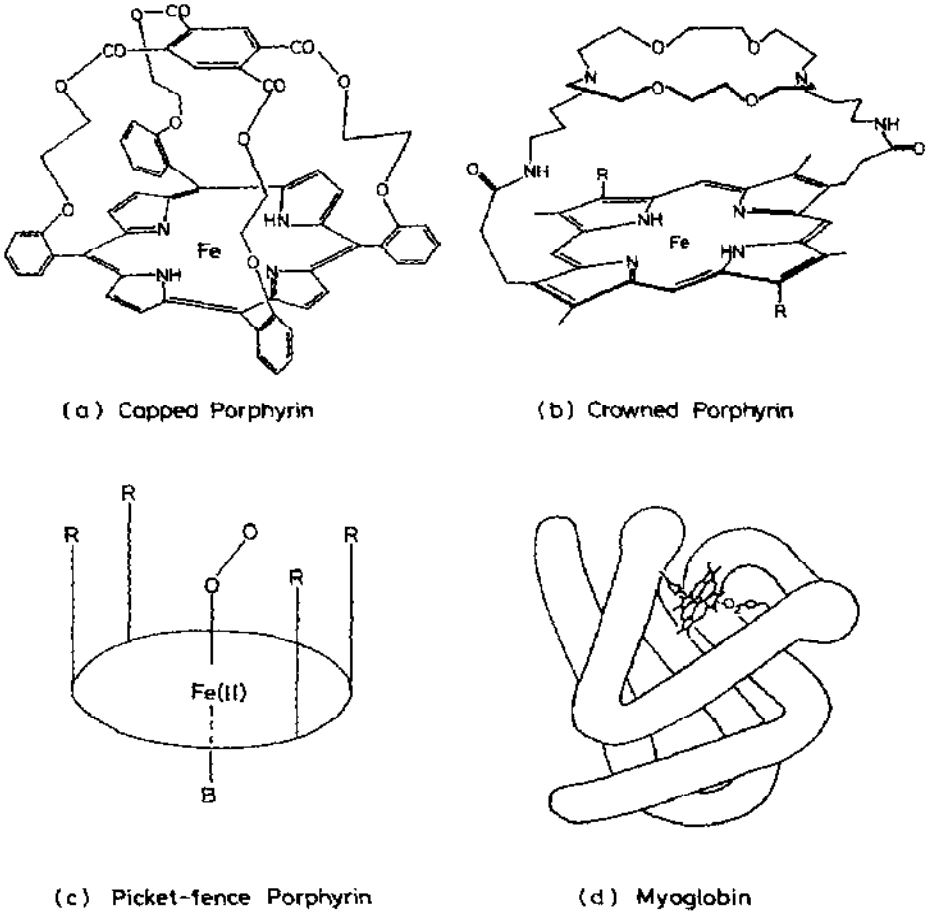

Fig. 1. Various types of steric hindrance to prevent the heme iron(II) from formin oxygen-bridged dimers. (a) Baldwin's "capped" porphyrin. The tetraphenylporphinatoiron(I is capped with a macrocycle having a polycarboxylated aromatic ring. The stability of th dioxygen adduct is largely dependent upon the nature and concentration of the coordinatir nitrogenous base at the unprotected side [13]. (b) Chang's "crowned" porphyrin. T porphinatoiron(II) is crowned with a macrocycle containing a crown ether; R is an n-hex group [17]. (c) Collman's "picket-fence" porphyrin. The porphinatoiron(II) is fenced in four pival-amido-phenyl (R) groups; the axial B is a base such as *N*-methylimidazole [15,1 (d) Myoglobin. The porphinatoiron(II) is embedded in the heme pocket or cleft of its glol moiety.

and reached an order of magnitude from minutes to hours in apr non-aqueous solvents and at ambient temperatures.

Low temperature can also reduce the rate of dimerization (step (I that even the simplest ferrous porphyrins, without any kind of hindrance to prevent dimerization, were found to be able to react r

with molecular oxygen. This significant disclosure was made by Basolo and co-workers using *meso*-tetraphenyl-porphyrin–iron(II) derivatives, Fe(TPP)$(B)_2$, in methylene chloride solution at $-79°C$

$$Fe(TPP)(B)_2 + O_2 \rightleftharpoons Fe(TPP)(B)(O_2) + B$$

where the apical ligand, B, included pyridine, 1-methylimidazole and piperidine. At this temperature, several oxygenation–deoxygenation cycles were possible with a minimum of irreversible oxidation occurring. At room temperature, however, the complexes were oxidized immediately with no detectable spectral evidence for the formation of the oxygen adducts [14,19].

These recent advances in the field of coordination chemistry seem to support the presumption that the reversible oxygen binding to the heme does not require the protein architecture [14,20], and 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 [21]. However, it should be pointed out that oxygen binding to the synthetic iron(II)–porphyrin complexes has generally been conducted in aprotic, non-aqueous solvents and at reduced temperatures. Even under such inert conditions, the half-life period attained by these oxygen adducts was no longer than minutes to hours.

On the other hand, hemoglobin and myoglobin can bind molecular oxygen reversibly and carry out their functions in protic, aqueous media and at physiological temperatures. Under these active conditions, however, their oxygenated forms are not completely stable, and are subject to autoxidation at a slow but considerable rate, even though each heme iron is embedded in its protein matrix and thus is unable to form an oxygen-bridged dimer. In the absence of the dimeric species found in simple ferrous porphyrins during autoxidation, therefore, we have in myoglobin an opportunity to examine the stability properties of the FeO_2 bond, a subject that has recently been studied most extensively.

C. STABILITY PROPERTIES OF NATIVE OXYMYOGLOBIN

(i) Oxymyoglobin preparation

Since there is a strong tendency for MbO_2 to become oxidized easily during isolation procedures, all early myoglobin preparations reported were obtained in the ferric met-form [22,23]. Accordingly, if needed, MbO_2 was produced from metMb by strong reduction with sodium hydrosulfite. However, there have been a number of reports that the chemically-generated MbO_2 showed various artifacts attributable to side reactions with this reducing agent [24–26]. Instead of preparing MbO_2 from metMb, therefore,

modern procedures for isolating native oxymyoglobin directly from muscle tissues all stem from the method of Shikama and co-workers for horse heart muscle [27]. This has been improved with some refinements and controls using bovine heart muscle [28], human psoas muscle [29], and sperm whale skeletal muscle [30]. In these procedures the essential step is the chromatographic separation of oxymyoglobin from metmyoglobin in the hemoglobin-free extract on a DEAE- or CM-cellulose column. Figure 2 shows a typical elution profile for bovine heart myoglobin on a DEAE-cellulose column. The absorbance ratio of 581 nm/593 nm, used for identification [31], changed from 1.0 for the first major peak to 3.5 for the second, corresponding to the presence of metMb and MbO_2, respectively. A satisfactory separation of these two species was readily achieved.

The spectroscopic parameters of the mammalian oxymyoglobins isolated so far are given in Table 1. The most remarkable feature of our preparations is that the absorbance ratio of the α- to β-maximum (α/β ratio) con-

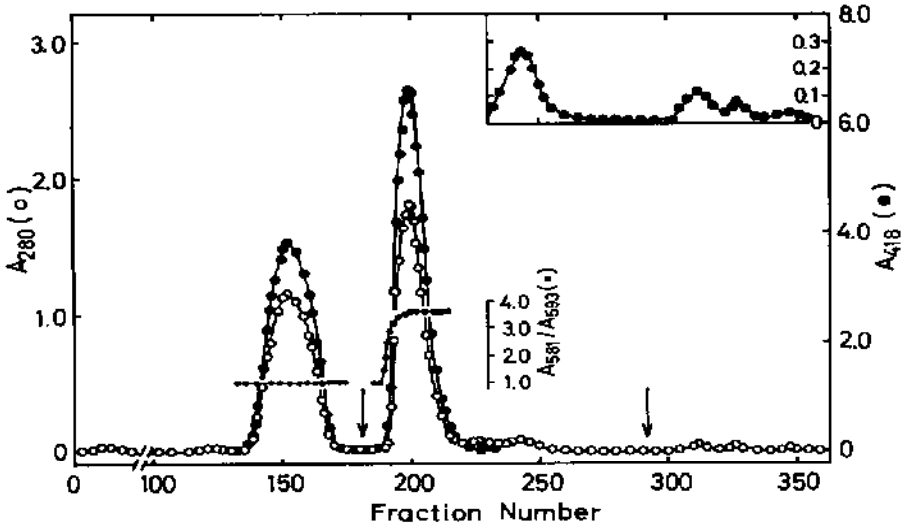

Fig. 2. DEAE-cellulose chromatography of bovine heart myoglobin. Myoglobin solution (250 ml, 500 mg), which was obtained by molecular sieve chromatography on Sephadex G-50, was applied to a DEAE-cellulose column (4.5 cm×15 cm) equilibrated with 5 mM Tris–HCl buffer (pH 8.4). The column was washed with a large volume of 15 mM Tris–HCl buffer (pH 8.0) at a flow-rate of 60 ml h^{-1} until the brown-colored band of metMb (the first major peak) was eluted out completely. At the point indicated by the first arrow, the buffer was changed to 30 mM Tris–HCl (pH 8.0) for elution of MbO_2 (the second major peak) at a flow-rate of 60 ml h^{-1}. The major component was followed by one minor component, and if the buffer was changed to 50 mM Tris–HCl (pH 8.0) at the point indicated by the second arrow, additional minor species came off. The protein and the heme protein levels were monitored by the absorbances at 280 nm (○) and at 418 nm (●), respectively. MbO_2 and metMb were identified by the absorbance ratio of 581 nm/593 nm (•). Fraction size, 20 ml [47].

TABLE 1

Spectroscopic parameters of mammalian oxymyoglobins at pH 8.0

Source	Absorption maximum (nm) (Extinction coefficient ($mM^{-1} cm^{-1}$))						Ref.
	α	β	γ	UV	α/β	γ/UV	
Sperm whale	581 (15.4)	543 (14.3)	418 (129)	280 (36.6)	1.07	3.52	30
Bovine	581 (15.5)	544 (14.5)	418 (134)	280 (36.4)	1.07	3.68	28
Horse	582 (15.3)	544 (14.3)	418 (133)	281 (36.3)	1.07	3.66	27
Human	582 (15.4)	544 (14.4)	418 (133)	280 (36.9)	1.07	3.60	29

sistently shows a high value of 1.07; the α-peak is always higher than the β-peak. The value of this ratio decreases from 1.07 with increasing extents of contamination of oxymyoglobin with metMb. The drop in ratio varies with pH since the spectrum of metMb changes markedly with the pH of the solution, for it reflects the transition of the aquo form to the hydroxide form, with a $pK_a \simeq 8.9$ [32]. For instance, when this ratio drops to less than 1.00, one must recognize that such MbO_2 preparations contain more than 30% metMb at pH 7.0, more than 50% at pH 9.2, and more than 70% at pH 11.0 [30]. This change reflects the rapid autoxidation of MbO_2 to metMb [33].

(ii) Primary step for the autoxidation reaction

Oxymyoglobin is oxidized to metmyoglobin, but in a way quite different from that of the simple ferrous complexes. Under air-saturated conditions, the primary step for the autoxidation reaction has been found to be first-order with respect to unoxidized MbO_2 with generation of the superoxide anion

$$MbO_2 \xrightarrow{k_{met}} metMb + O_2^- \quad (1)$$

where k_{met} represents the first-order rate constant observed spectrophotometrically at a given pH. The generation of the superoxide anion was demonstrated by Goton and Shikama [34] using the reduction of ferri-cytochrome c or the oxidation of epinephrin coupled with the autoxidation of MbO_2 and following the inhibitory effect of the presence of superoxide dismutase.

Therefore, the rate of autoxidation is given by

$$\frac{-d[MbO_2]}{dt} = k_{met}[MbO_2] \quad (2)$$

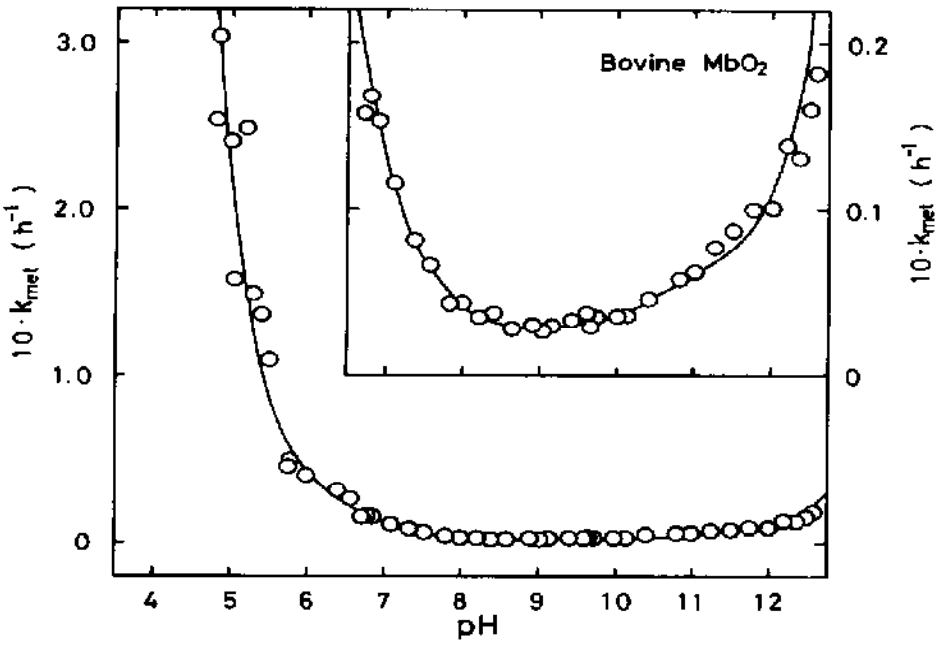

Fig. 3. The pH-dependence of the stability of native oxymyoglobin from bovine heart muscle. The values of the observed rate constant, k_{met}, for the autoxidation of MbO_2 to metMb are plotted against the pH of the solution in 0.1 M buffer at 25 °C. The expanded scale is used above pH 6.5. MbO_2 concentration, 50 μM. The computed curve (———) was obtained by a least-squares fitting to the experimental data (○) using the rate equation derived from an 'acid-catalyzed three-state model' [7]. Two different kinds of dissociable groups (AH with $pK_1 = 6.7$ and BH with $pK_2 = 10.4$) were found to be involved in the autoxidation reaction of bovine heart MbO_2. Redrawn from ref. 8.

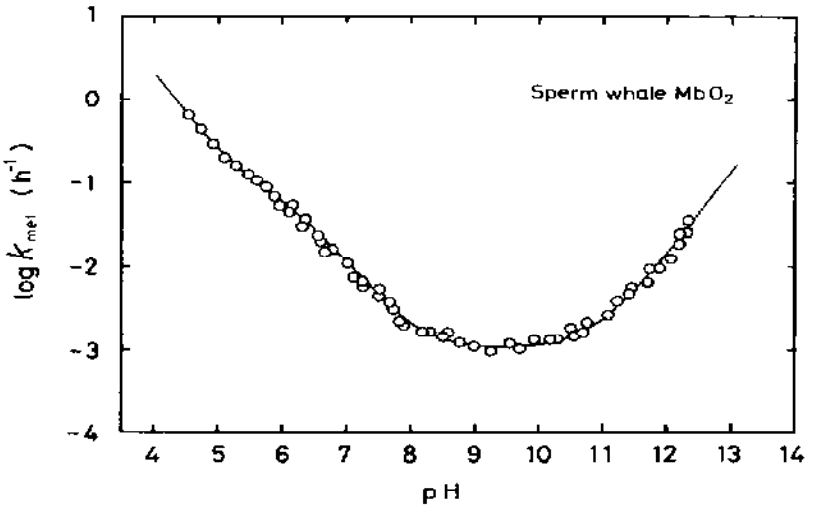

Fig. 4. A plot of log k_{met} versus pH for the autoxidation of sperm whale MbO_2 to metMb in 0.1 M buffer at 25 °C. The computed curve (———) was obtained by a least-squares fitting to the experimental data (○) over the whole range of pH 5 to 13 using eqn. (4) derived from an 'acid-catalyzed two-state model' (see text). MbO_2 concentration, 50 μM. Redrawn from ref. 35.

If the values of k_{met} are plotted against the pH of the solution, a profile of the stability of oxymyoglobin can be obtained. Figures 3 and 4 show such profiles for bovine heart MbO_2 and for sperm whale MbO_2, respectively, in 0.1 M buffer at 25 °C [7,35]. These graphs indicate that the rate of autoxidation increases rapidly with increasing hydrogen ion concentration, a rate minimum appears at about pH 9 and a small increase occurs at higher pH values. The half-life for conversion of sperm whale MbO_2 to metMb, at pH 9.5 for instance, was 35 days, but became 1.8 days at pH 7.0, and less than 3 h at pH 5.0 at 25 °C [30].

There is also a marked effect of temperature on the autoxidation rate, the activation energy being approximately 26.5 kcal mol^{-1} (110.8 kJ mol^{-1}) over a wide range of pH, from 5–10. Thus, the half-life for the autoxidation was greatly prolonged, for instance, for bovine heart MbO_2 at pH 9.0 from 7 days at 25 °C to more than 1 year at 0 °C [28].

(iii) A complete kinetic formulation for the pH-dependence

In the autoxidation reaction, pH can affect the rate in many ways. In order to unravel the kinetic and thermodynamic parameters contributing to the k_{met} versus pH profile, we have proposed some mechanistic models for the autoxidation reaction. The rate equations derived therefrom have been used for fitting experimental data according to a least-squares procedure using a digital computer [7,8,30].

In the case of sperm whale MbO_2, it is assumed that a single dissociable group, AH with pK_1, is involved in the autoxidation reaction. Consequently, there are two forms of 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 (see eqn. 3). 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 coordination position to form aquo- or hydroxy-metMb, respectively. The existence of both of these met-species has been established definitively by Stryer et al. [36] from X-ray analysis. The reaction scheme may, therefore, be written as

$$\begin{array}{ccc} MbO_2(AH) & \overset{K_1}{\rightleftharpoons} & MbO_2(A^-) \\ k_0^A \downarrow \quad k_H^A \downarrow & & k_0^B \downarrow \; k_H^B \downarrow \; k_{OH}^B \downarrow \\ \text{metMb} & & \text{metMb} \end{array} \qquad (3)$$

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 eqn. (3) the observed rate constant, k_{met} in eqn. (2), can be reduced to

$$k_{met} = \left(k_0^A[H_2O] + k_H^A[H_2O][H^+]\right)(\alpha) + \left(k_0^B[H_2O] + k_H^B[H_2O][H^+] + k_{OH}^B[OH^-]\right)(\beta) \quad (4)$$

where

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

and

$$\beta = (1 - \alpha) = \frac{K_1}{[H^+] + K_1} \quad (5)$$

By iterative least-square procedures inserting various values for K_1, the adjustable parameter in eqn. (5), the best fit to the experimental values of k_{met} was obtained as a function of pH (Fig. 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 [35].

It becomes apparent that the proton-catalyzed processes with the rate constants k_H^A and k_H^B facilitate the autoxidation reaction of MbO_2 far more than the spontaneous processes in water with the rate constants k_0^A and k_0^B. In fact, the catalytic proton enhances the rate by a factor of 4.7×10^6/mol for state A, and by a factor of 1.1×10^8/mol for state B. From the values of $k_H^A = 0.37 \times 10^3$ h^{-1} M^{-2} and $k_H^B = 0.20 \times 10^4$ h^{-1} M^{-2}, one can also calculate half-lives ($t_{1/2}$) of 121 and 22 ms, respectively, for these processes if oxymyoglobin is placed in 1 M H^+ aqueous solution at 25 °C. These estimations reveal that the proton-catalyzed autoxidation of MbO_2 is inher-

TABLE 2

Rate constants and acid dissociation constant obtained from simulating the pH-dependence for autoxidation of sperm whale MbO_2 in 0.1 M buffer at 25 °C by the use of eqn. (4) [35]

Two states of MbO_2	k_0 (h^{-1} M^{-1})	k_H (h^{-1} M^{-2})	k_{OH} (h^{-1} M^{-1})	pK_1
A (AH)	0.78×10^{-4}	0.37×10^3	–	
⇵ K_1				6.2
B(A^-)	0.18×10^{-4}	0.20×10^4	0.14×10	

ently a very fast reaction, but the extremely low concentration of H^+ ion at physiological pH (corresponding to 10^{-7} M H^+) reduces the rate to a negligible magnitude.

From the kinetic analysis of the stability properties of native oxymyoglobins, we can conclude that the autoxidation reaction of MbO_2 proceeds by way of the following three types of elementary processes [6]

$$Fe(II)(O_2) + H_2O \overset{k_{H_2O}}{\rightarrow} Fe(III)(OH_2) + O_2^- \quad (6)$$

$$Fe(II)(O_2) + H_2O + H^+ \overset{k^H_{H_2O}}{\rightarrow} Fe(III)(OH_2) + HO_2 \quad (7)$$

$$Fe(II)(O_2) + OH^- \overset{k_{OH}}{\rightarrow} Fe(III)(OH^-) + O_2^- \quad (8)$$

The extent of contribution of these elementary processes to the observed or overall autoxidation rate can vary with the concentrations of H^+ or OH^- ions and with the dissociation states of the group involved. Consequently the stability of MbO_2 fits a complicated pH profile.

(iv) Nature of the proton catalysis

From the value found for pK_1 (= 6.2) in sperm whale MbO_2, the most probable candidate for the dissociable group AH (see eqn. (3)) is histidyl residue. In the case of bovine heart MbO_2, this identification was further confirmed by a thermodynamic characterization for the group AH. The ionization parameters that were obtained from the effect of temperature on its pK_1 value were $\Delta G^0 = 9.3$ kcal mol^{-1} (38.9 kJ mol^{-1}), $\Delta H^0 = 8.1$ kcal mol^{-1} (33.9 kJ mol^{-1}), and $\Delta S^0 = -4.0$ cal mol^{-1} K^{-1} (-17 J mol^{-1} K^{-1}) in 0.1 M buffer at 25°C [8]; these are in good accord with the literature values for the histidine residue at 25°C and ionic strength $I = 0.15$ [37,38]. However, it is not easy to single out an histidine from the 12 or 13 such residues found in sperm whale or bovine myoglobin, respectively. Nevertheless, the distal histidine (E 7) at position 64 ought to be of primary importance, since it is the only one that is in a location where it can interact directly with the dioxygen bound to the heme iron(II). For the pK of the distal histidine of sperm whale myoglobin, it should be noted that the literature values differ, since various types of interactions with the heme make its direct determination and unambiguous assignment extremely difficult, especially in the case of the oxy-form [39]. Contrary to earlier conclusions that the E7 histidine titrated in the pH range of 5.5 [40], the most recent evidence from proton NMR measurements indicates that this

titrating group in the heme cavity of myoglobin is the histidine-FG3, whose imidazole ring lies parallel to and very close to the porphyrin ring [41].

In order to understand further the nature of the proton-catalyzed process involved, the effect of temperature on the autoxidation rate has been studied extensively with bovine heart MbO_2. In the neutral pH range, MbO_2 was found to be protected against spontaneous autoxidation (eqn. (6)) by a high energy barrier of approximately 26 kcal mol^{-1} (107 kJ mol^{-1}). However, the catalytic proton in eqn. (7) was found to lower the free energy barrier, by an order of magnitude of more than 10 kcal mol^{-1}, by decreasing $\Delta H^{0\neq}$ and also increasing $\Delta S^{0\neq}$ for the formation of an activated complex of MbO_2 with an entering water molecule [8].

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 [8,30]. Figure 5 illustrates the mechanistic path of the proton-catalyzed autoxidation process in a very schematic way.

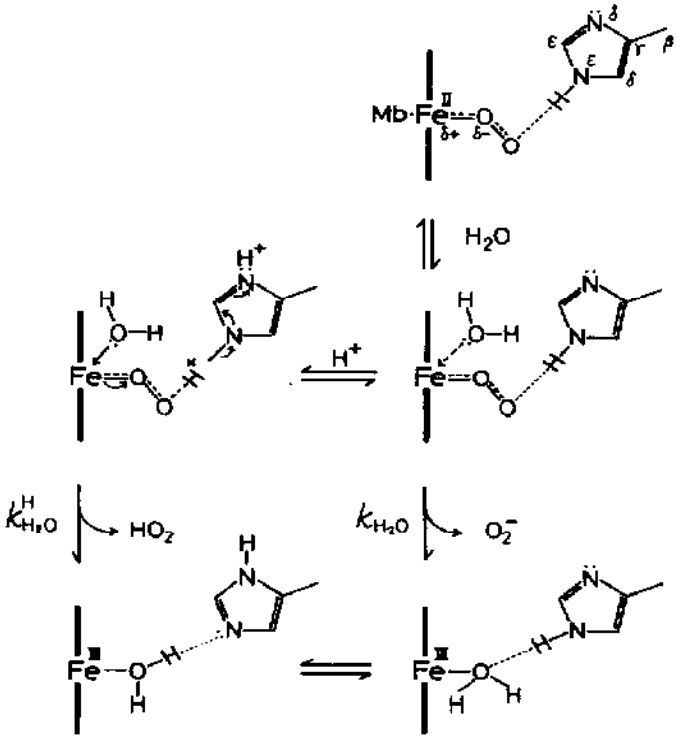

Fig. 5. Schematic representation of the spontaneous autoxidation process in water with the rate constant k_{H_2O} and the proton-catalyzed process with the rate constant $k^{H}_{H_2O}$ in neutral pH range. For bovine heart MbO_2, $k_{H_2O} = 0.47 \times 10^{-4}$ h^{-1} M^{-1} and $k^{H}_{H_2O} = 0.25 \times 10^{4}$ h^{-1} M^{-2} in 0.1 M buffer at 25 °C. The aquo-metMb formed by each process can be isomerized into the other.

The bound dioxygen is bent, held end-on and forms a hydrogen bond with the distal (E 7) histidine. Its detailed geometry has recently been established definitively from X-ray and neutron diffraction studies of the structure of sperm whale oxymyoglobin [42,43]. The bound dioxygen is so stabilized by a hydrogen bond to the distal histidine that an entering water molecule can displace O_2^- from MbO_2 only at the very slow rate. This is in accord with the low value of the rate constant k_{H_2O} for the spontaneous process in water. When a catalytic proton comes from the solvent and protonates transiently the remote nitrogen (N^δ) of the distal histidine at neutral pH, the opposite nitrogen (N^ϵ) releases its proton so that the latter moves closer to the bound, polarized dioxygen. Such an activated state serves to facilitate a full charge transfer from Fe(II) to O_2 produced by an entering water molecule. This transfer thus facilitates displacement of O_2^- as the hydroperoxyl radical HO_2 which departs and, since its pK_a is 4.8 [44], then dissociates into the superoxide anion and a catalytic proton. This mechanism leads to the proton-catalyzed process with the rate constant of $k^H_{H_2O}$, which thus promotes the autoxidation reaction of MbO_2 above the spontaneous process in water.

(v) A nucleophilic displacement of the bound dioxygen: a S_N2 mechanism

It is clear from eqns. (6–8) that each elementary process involved in the autoxidation reaction of MbO_2 is an octahedral substitution reaction of the bound dioxygen, as superoxide anion, by the entering ligand, which can be a water molecule or an hydroxyl ion, the former participating in two types of pathway, i.e. that with, and the other without, proton-catalysis. Since in aqueous solution the concentration of H_2O is always about 55.5 M, the effect of changes in ligand water concentration on the autoxidation rate cannot be determined. Therefore, the decision as to whether the reaction proceeds by way of a S_N1 dissociation of O_2^- from MbO_2 with the subsequent addition of H_2O, or by way of a S_N2 displacement of O_2^- from MbO_2 by H_2O must be made from other experimental evidence.

In order to elucidate the molecular mechanism of these substitution reactions leading to metmyoglobin formation in vivo, Satoh and Shikama [9] have studied the oxidation of MbO_2 induced by excess anion, as compared with the normal autoxidation in buffer alone, leading to the formation of the corresponding metMb–anion complex. The anions examined were SCN^-, F^-, OCN^-, N_3^-, and CN^-, whose nucleophilicity differs from H_2O and OH^-. For this anion-induced oxidation of MbO_2, two basic pathways appeared to be possible

$$\begin{array}{l} MbFe(II)(O_2) \rightarrow \quad MbFe(III) \quad + O_2^- \\ \qquad\qquad\qquad\quad \downarrow X^- \\ \qquad\qquad\quad MbFe(III)(X^-) \end{array} \qquad (9)$$

or

$$\overset{\delta+\quad\delta-}{\mathrm{MbFe(II)(O_2)}} + \mathrm{X^-} \rightarrow \left[\mathrm{MbFe}\begin{matrix}\mathrm{X^-}\\ \diagdown\\ \mathrm{O_2}\end{matrix}\right] \rightarrow \mathrm{MbFe(III)(X^-) + O_2^-} \qquad (10)$$

where X^- represents an anion. Equation (9) represents a S_N1-type process involving spontaneous loss of O_2^- from MbO_2 in the rate-determining step. The subsequent binding of X^- to metMb is known to be very rapid and to require only stoichiometric amounts of the anion. In this case, therefore, the oxidation of MbO_2 must proceed at a rate independent of the concentration and the nature of X^- examined. On the other hand, reaction (10) takes place by a S_N2-type process that involves the formation of an intermediate with X^- seeking a partial positive center on the Fe(II) and that requires a large excess of the anion. In this process, therefore, two reactants, MbO_2 and X^-, must be involved in the rate-limiting step.

The anion-induced oxidation of MbO_2 was therefore measured for each salt at various concentrations from 0.1 to 0.5 M and at some 30 different values of pH. In each case, the rate of the induced oxidation was linearly dependent upon the concentration of an added anion, $[X^-]$, at a given pH, and its pH-dependence revealed that the reaction involves two types of paths, i.e. one with, and the other without, proton assistance. In the presence of anion, the observed oxidation rate, k_{obs}, was therefore given by

$$k_{obs} = k_0 + k_D^H[X^-][H^+] + k_D[X^-] \qquad (11)$$

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

Table 3 summarizes the rate constants (in logarithmic units) for the oxidation of bovine MbO_2 by displacing anions with and without proton assistance in 0.1 M buffer at 25 °C. For comparison, the corresponding kinetic parameters are also listed for the normal autoxidation of MbO_2 in 0.1 M buffer at 25 °C: $k_{H_2O}^H = 0.25 \times 10^4\ h^{-1}\ M^{-2}$ for the proton-assisted oxidation by H_2O, and $k_{H_2O} = 0.47 \times 10^{-4}\ h^{-1}\ M^{-1}$ and $k_{OH} = 0.18 \times 10^2\ h^{-1}\ M^{-1}$ for the oxidation by H_2O and OH^-, respectively, without proton assistance [9].

For nucleophilic attack on the same atomic center, Edwards [45] has suggested two components, one related to classical basicity (H) and the other related to polarizability (P) of the nucleophiles (N)

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

TABLE 3

Rate constants for the oxidation of bovine MbO_2 by displacing anions with and without proton assistance in 0.1 M buffer at 25 °C [9]

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

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

$$H = pK_a + 1.74$$

In Fig. 6, the logarithm of the rate constant for the oxidation of MbO_2 by displacing anion, $\log k_D$, is plotted against the pK_a of the conjugate acid of

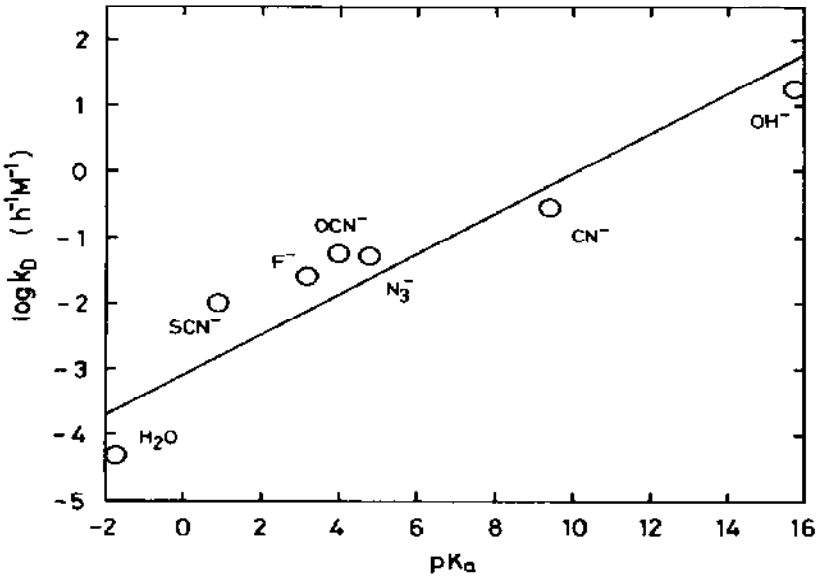

Fig. 6. Brønsted plot for the oxidation of MbO_2 by displacing nucleophilic anions in 0.1 M buffer at 25 °C. The logarithm of the rate constant for the displacing oxidation of bovine MbO_2 by an added anion, $\log k_D$, without proton assistance, is plotted against the pK_a of the conjugate acid of the anion. The corresponding kinetic parameters by H_2O and OH^- involved in the normal autoxidation reaction are also plotted for reference. MbO_2 concentration, 50 μM (see text). Taken from ref. 9.

the anion. This Brønsted plot for the series shows 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.

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 [9] 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 eqn. (4) for the autoxidation reaction of MbO_2 under physiological conditions can thus be viewed in the general form of eqn. (10) as a S_N2 mechanism, where X^- 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, it is true that the rate is enormously accelerated, by a factor of more than 10^6/mol in k_D^H, with proton assistance (Table 3).

Our kinetic analysis of the stability properties of native MbO_2 has thus revealed 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(II) to O_2 produced, to yield metmyoglobin.

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* Hydrogen peroxide and the anionic form (HOO^- with $pK_a = 11.6$) can also induce very rapid oxidation of MbO_2 to metMb, but its mechanism is quite different from the nucleophilic displacement. Recent kinetic studies have revealed that the process involves the primary formation of ferryl-Mb(IV) from deoxy-Mb(II), which comes from MbO_2 in equilibrium, by two-equivalent oxidation with HOOH. The ferryl species thus formed reacts then with another deoxy-Mb(II) to yield two moles of metMb(III) species [46]. The overall reaction scheme can be written, therefore, as

$2\ MbO_2 \rightleftharpoons 2\ Mb(II) + 2\ O_2$

$Mb(II) + HOOH \rightarrow \text{ferryl } Mb(IV) + 2\ OH^-$

$\text{ferryl } Mb(IV) + Mb(II) \rightarrow 2\ \text{metMb(III)}$

$2\ OH^- + 2\ H^+ \rightarrow 2\ H_2O$

or

$2\ MbO_2 + HOOH + 2\ H^+ \rightarrow 2\ \text{metMb} + 2\ O_2 + 2\ H_2O.$

D. CONCLUSION: A POSSIBLE ROLE OF THE PROTEIN MOIETY AS A BREAKWATER

The binding of dioxygen to the simple ferrous complexes has been studied mostly in aprotic, non-aqueous solvents and at reduced temperatures. Even under such inert conditions, these compounds are oxidized very rapidly and irreversibly with dioxygen, through the formation of an oxygen-bridged dimer. From this information, it is generally assumed that the protein moiety of myoglobin and hemoglobin provides a kind of steric hindrance to prevent the dimerization that leads to the oxidation. However, the oxygenated form of these native proteins, without any formation of such a dimer, still undergoes a slow autoxidation to the met-form, with the generation of superoxide anion.

Recent kinetic and thermodynamic studies have revealed that the mechanism of aqueous autoxidation of oxymyoglobin is quite different in its molecular nature from that of the non-aqueous autoxidation of simple ferrous complexes. The elementary processes involved in autoxidation of the native proteins can be viewed as a displacement of O_2^- from the FeO_2 center by a water molecule or an hydroxyl ion that can enter the heme environment from the surrounding solvent. 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 coordination position to form an aquo- or hydroxide-met species. It has also been shown that this reductive displacement of the bound dioxygen proceeds by way of a nucleophilic attack of the entering ligand on the iron center. This can proceed without any protonation, but the rate is enormously enhanced with proton assistance by a factor of more than 10^6/mol. In its proton catalysis, the distal histidine, which forms a hydrogen bond to the bound dioxygen, appears to participate by facilitating the effective movement of a catalytic proton from the solvent to the bound dioxygen via its imidazole ring by a proton-relay mechanism. This proton-catalysed nucleophilic displacement of O_2^- from the FeO_2 center by the entering water molecule, a S_N2-type process with proton assistance, can thus account for most of the autoxidation reaction occurring under physiological conditions.

In vacuo, the FeO_2 bond is inherently stable and so unlikely to dissociate O_2^- spontaneously. O_2 is rather a poor one-electron acceptor, so a considerable thermodynamic barrier exists for such electron transfer [4]. However, in vivo, it becomes evident that the FeO_2 bonding is always subject to the nucleophilic attack of an entering water molecule, with or without proton catalysis, and to the attack of an entering hydroxide anion. These can cause irreversible oxidation of the FeO_2 to met-species with generation of the superoxide anion. Myoglobin and hemoglobin have thus evolved with a

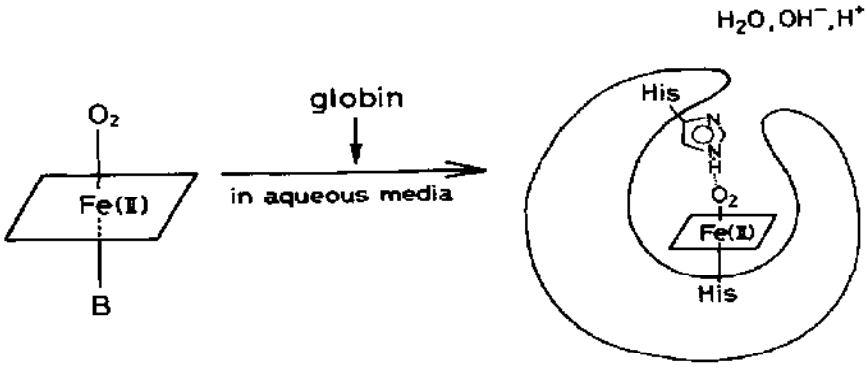

Fig. 7. From simple ferrous complexes to the proteins. Myoglobin has evolved with a globin moiety that can obstruct access of a water molecule (including its conjugate ions) to the FeO_2 center. The distal histidine is also proposed to participate, via its imidazole ring and a proton-relay mechanism, in controlling the effective movement of a catalytic proton from the solvent to the coordinated dioxygen. The distal histidine can also stabilize the dioxygen by hydrogen bond formation.

globin moiety that can protect the FeO_2 center from easy access of a water molecule including its conjugate ions OH^- and H^+, as is illustrated in Fig. 7. It also becomes evident that even in these native proteins the globin moiety has still not attained maximal ability to block entering water molecules from the FeO_2 center. Nevertheless, the relative stability of the oxygenated forms, in protic aqueous solution and at physiological temperature, provides the basis for the myoglobin and hemoglobin functions in vivo, and differentiates these naturally-occurring oxygen carriers from simple ferrous complexes. This functional stability of myoglobin or hemoglobin is known to be lost easily on denaturation. Therefore, it must be linked to the integrity of the conformation of the globin moiety so that it can act as a breakwater.

These new views on the nature of FeO_2 bonding and on a possible role of the globin moiety in the stability properties of oxymyoglobin may provide us with an insight into general principles governing the reactions between the iron(II) and molecular oxygen in biological oxidations that support living organisms on the earth. These views may also provide us with an insight into new molecular designs for synthesis of novel oxygen-carriers that are able to function under physiological conditions.

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