Role of Globin Moiety in the Autoxidation Reaction of Oxymyoglobin: Effect of 8 M Urea

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ABSTRACT It is in the ferrous form that myoglobin or hemoglobin can bind molecular oxygen reversibly and carry out its function. To understand the possible role of the globin moiety in stabilizing the FeO_2 bond in these proteins, we examined the autoxidation rate of bovine heart oxymyoglobin (MbO_2) to its ferric met-form (metMb) in the presence of 8 M urea at 25°C and found that the rate was markedly enhanced above the normal autoxidation in buffer alone over the whole range of pH 5–13. Taking into account the concomitant process of unfolding of the protein in 8 M urea, we then formulated a kinetic procedure to estimate the autoxidation rate of the unfolded form of MbO_2 that might appear transiently in the possible pathway of denaturation. As a result, the fully denatured MbO_2 was disclosed to be extremely susceptible to autoxidation with an almost constant rate over a wide range of pH 5–11. At pH 8.5, for instance, its rate was nearly 1000 times higher than the corresponding value of native MbO_2. These findings lead us to conclude that the unfolding of the globin moiety allows much 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, therefore, the protein matrix can be depicted as a breakwater of the FeO_2 bonding against protic, aqueous solvents.

INTRODUCTION

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 mostly 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 (Jones et al., 1979). In native proteins, too, the oxygenated form of myoglobin (Mb) or hemoglobin (Hb) is known to be converted easily to the ferric(III) met-form, which cannot be oxygenated and is therefore physiologically inactive, with generation of the superoxide anion (Misra and Fridovich, 1972; Wever et al., 1973; Gotoh and Shikama, 1976; Wazawa et al., 1992). 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 (Shikama, 1988).

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(III), met-species. Therefore, it must be linked to the integrity of the conformation of the globin moiety so that it can protect the FeO_2 bonding against the autoxidation reaction in protic, aqueous media and at physiological temperatures.

In this paper, we examine the rate of autoxidation of oxymyoglobin (MbO_2) to metmyoglobin (metMb), over a wide range of pH in the presence of 8 M urea. Taking into account the concomitant process of unfolding of the protein, we have formulated a kinetic procedure to evaluate the autoxidation rate of the unfolded form of MbO_2 that might occur transiently in due course of the urea effect. Such an examination should facilitate a fuller understanding of the role of the globin moiety in stabilizing the FeO_2 bonding in the Mb or Hb molecule.

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MATERIALS AND METHODS

Chemicals

Sephadex G-50 (fine) was a product of Pharmacia (Uppsala, Sweden). DEAE-cellulose (DE-32) was purchased from Whatman. Urea (Baker analyzed reagent) was dissolved in water to form a saturated solution at 50°C, filtered, and crystallized in the presence of 20% methanol. The crystals were dried in vacuum over P_2O_5, and fresh urea solutions were prepared for each experiment. Guanidine hydrochloride (WakoPure Chemical, Osaka, Japan) was used without further purification. All other chemicals were of reagent grade from Wako, and solutions were made with deionized and glass-distilled water.

Oxymyoglobin preparation

Native MbO_2 was isolated directly from bovine heart muscle according to our standard procedure (Shikama and Sugawara, 1978; Suzuki et al., 1980). The essential step was the chromatographic separation of MbO_2 from metMb on a DEAE-cellulose column. The concentration of Mb was determined, after conversion into cyano-metMb, by using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 540 nm (Drabkin, 1950).

Autoxidation rate measurements

The rate of autoxidation of MbO_2 (25 μM) was measured in 0.05 M buffer over a wide range of pH (5–13) at 25°C, according to our standard procedure (Shikama and Matsuoka, 1986). In the presence of 8 M urea, the

following specifications were adopted. A 4.5-ml solution containing 8.9 M urea in an appropriate buffer was placed in a test tube and incubated in a water bath (Lauda circulator) maintained at 25 ± 0.1°C. The reaction was started by adding 0.5 ml of fresh MbO_2 solution (250 μM), and the tube was then sealed with a ground-glass stopper.

For spectrophotometry, the reaction mixture was quickly transferred to a quartz cell held at 25 ± 0.1°C, and the changes in the absorption spectrum from 450 to 650 nm were recorded on the same chart at measured intervals of time. For the final state of each run, the Mb was completely converted to the met-form by the addition of potassium ferricyanide.

The buffer systems used were acetate for pH 4.8–5.6, Mes for pH 5.2–7.1, Hepes for pH 6.7–7.9, Tris for pH 7.4–9.4, Taps for pH 8.2–10.8, Caps for pH 9.8–11.8, and phosphate (pK_3) for pH 10.8–13.1. The pH of the reaction mixture was checked carefully, before and after each run, with a Horiba pH meter (model F-22).

Spectroscopic measurements

Absorption spectra were recorded in a Hitachi two-wavelength double-beam spectrophotometer (model 557 or U-3210), and fluorescence measurements were carried out in a Hitachi (model MPF-4) fluorescence spectrophotometer, each being equipped with a thermostatically controlled cell holder.

Circular dichroism (CD) spectra were recorded in a Jasco spectropolarimeter (model J-20A or J-500) equipped with a thermostatically controlled cell holder. In the far ultraviolet zone, recordings were usually made with 10 μM Mb in a 2-mm cell and at the scale setting of 0.005°/cm on the chart. Temperature was controlled by a water bath (Lauda thermostat K2 or Tamson TC3) maintained at each required temperature to within ±0.1°C.

Curve fittings

The curve fittings were made by a least-squares method on a personal computer (NEC PC-9801) with graphic display, according to our previous specifications (Shikama and Sugawara, 1978; Shikama and Matsuoka, 1986).

RESULTS

Autoxidation of oxymyoglobin in the presence of 8 M urea

It is in the ferrous form that Mb can bind molecular oxygen reversibly and carry out its function.

$$Mb(II) + O_2 \rightleftharpoons MbO_2 \qquad (1)$$

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

$$MbO_2 \xrightarrow{k_A^n} metMb(III) + O_2^-, \qquad (2)$$

where k_A^n represents the first-order rate constant for the autoxidation reaction of MbO_2 in buffer alone, its magnitude being strongly dependent upon the pH of the solution as will be seen later in Fig. 3.

To understand the possible role of the globin moiety in stabilizing the FeO_2 bonding in myoglobin, it seemed of particular interest to examine the autoxidation rate of the unfolded or denatured form of MbO_2, if such a species could be produced, over a wide range of pH, and to compare its rate with that of the native protein.

For this purpose, the most direct way is to prepare a large amount of the metMb denatured completely in 8 M urea and to convert it into the oxygenated form by reduction with sodium hydrosulfite. As demonstrated in Fig. 1, however, it became evident that the oxygenated form can no longer be produced from metMb once it has been fully denatured in 8 M urea. This is true even if a very careful use of $Na_2S_2O_4$ was made for its reduction followed by aeration. In fact, the resulting product showed a spectrum quite similar to that of pyridine hemochromogen used as a reference. This is indicative of a nitrogenous residue (probably of histidine) being coordinated as the sixth ligand of the heme iron(III) of the denatured Mb. If guanidine hydrochloride was used in place of urea, circumstances were the same.

Our alternative strategy to this problem was to investigate the autoxidation reaction of MbO_2 in the presence of 8 M

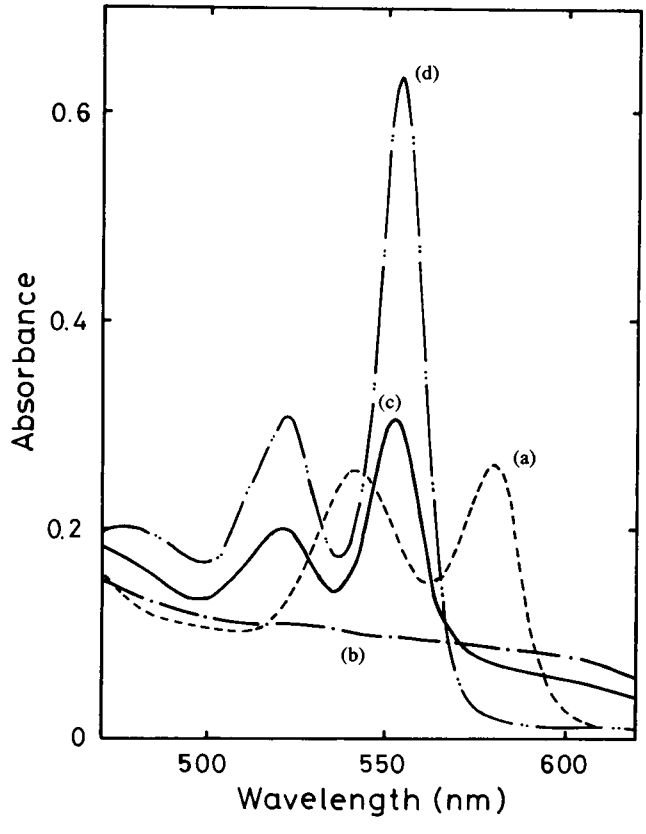

FIGURE 1 Spectral characterization of Mb derivatives in 8 M urea. For oxygenation of the fully denatured metMb (*spectrum b*) in 8 M urea, a minimal amount of $Na_2S_2O_4$ was employed for its reduction followed by aeration. The resultant spectrum (*c*) was not of the oxy-form (*a*) but corresponded exactly to that of the pyridine ferrohemochrome (*d*) that served as a reference. The Mb concentration was 18.3 μM in 0.05 M Taps buffer at pH 8.5, and 20% pyridine was used to convert it to hemichrome in 0.6 M NaOH. The fully denatured form was produced from metMb after incubation with 8 M urea for 24 hours.

urea, by taking into consideration the concomitant process of unfolding of the globin moiety as

$$MbO_2(N) \xrightarrow[\text{autoxidation}]{K_A} \text{metMb(D)}, \quad (3)$$

where N denotes the native form and D is the denatured form for each species.

Fig. 2 shows such an example for the spectral changes with time when fresh MbO_2 was oxidized in the presence of 8 M urea in 0.05 M Mes buffer at pH 6.0 and 25°C. The spectra were changed with a set of isosbestic points (at 526 and 594 nm) occurring to the final state of each run, which was identified to be a hemichrome as described already. This process of autoxidation was therefore followed by a plot of $-\ln\{[MbO_2]_t/[MbO_2]_0\}$ versus time t, where the ratio of MbO_2 concentration after time t to that at time $t = 0$ can be monitored by the absorbance ratio of $\{(A_t - A_\infty)/(A_0 - A_\infty)\}$ at 581 nm (α-peak of bovine heart MbO_2). At a given pH, the observed first-order rate constant, K_A in h^{-1}, for the autoxidation of MbO_2 in the presence of 8 M urea was thus determined from the slope of each straight line, although a sluggish phase sometimes appeared at the initial stage of the run just after the protein was mixed with the denaturant. We have also confirmed that the rate is independent of the initial concentrations of MbO_2 insofar as examined from 13 to 36 μM.

If the values of K_A are plotted against the pH of the solution, we can obtain, for the first time, a profile of the stability of MbO_2 in 8 M urea. Fig. 3 shows such a profile for bovine heart MbO_2 over a wide range of pH (5–13) in 0.05 M buffer at 25°C. When compared with the values of k_A^n for the normal autoxidation in buffer alone, it is quite clear that the protein in 8 M urea becomes extremely susceptible to autoxidation over the whole range of pH studied. At pH 7.5, for instance, its rate is more than 35 times higher than that in buffer alone.

It should be noted here that in the presence of guanidine hydrochloride MbO_2 was also oxidized very rapidly. At concentrations higher than 3.5 M, however, another oxidation reaction was accelerated in such a manner that no maximal velocity was attained despite sufficient denaturant being present to allow complete disorganization of the Mb molecule. The final product of this oxidation was also unusual. We failed to identify it as a hemichrome as is in the case of 8 M urea, but the possibility of a μ-oxo dimer cannot

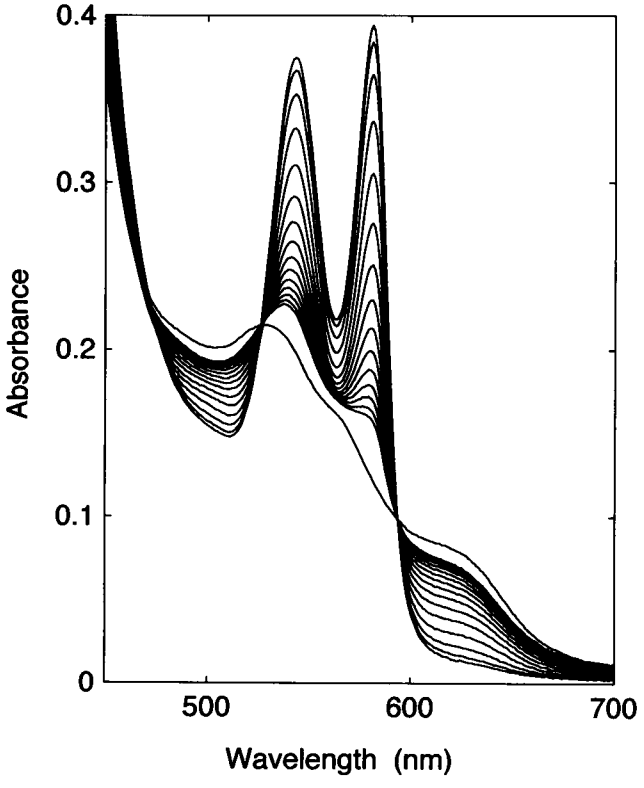

FIGURE 2 Spectral changes with time for the autoxidation of bovine MbO_2 in the presence of 8 M urea. Scans were made at 10-min intervals after fresh MbO_2 (25 μM) was mixed with 8 M urea in 0.05 M Mes buffer, pH 6.0, at 25°C. The reaction was followed at 581 nm by a single first-order rate constant of $K_A = 9.10 \times 10^{-1}\ h^{-1}$. The final spectrum was not of the usual acidic metMb but for a hemichrome as described in Fig. 1.

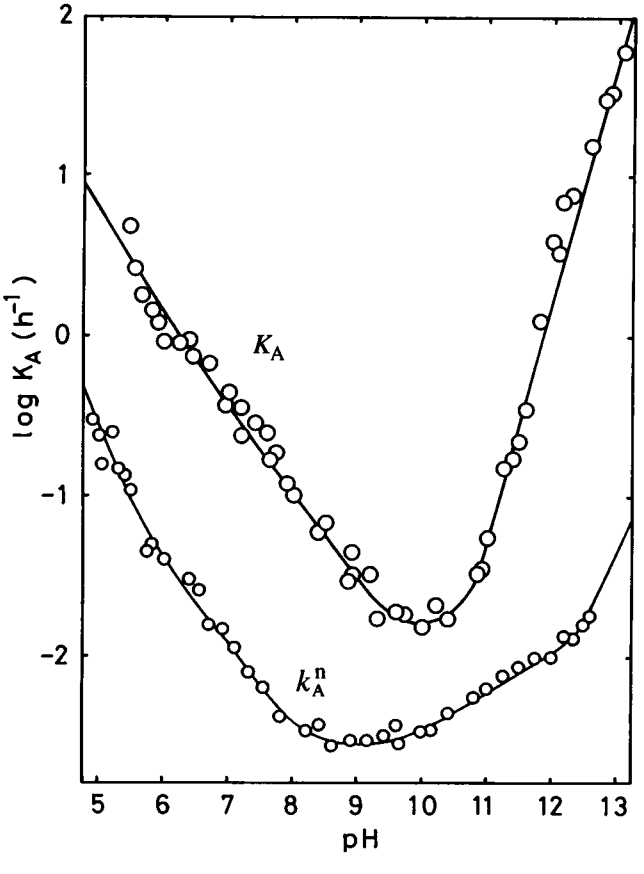

FIGURE 3 The pH dependence for the autoxidation of bovine MbO_2 in the presence of 8 M urea in 0.05 M buffer at 25°C. The logarithmic values of the observed first-order rate constant, K_A in h^{-1}, for the autoxidation reaction in 8 M urea are plotted against the pH of the solution. For comparison, the values of k_A^n (h^{-1}) for the normal autoxidation in buffer alone are also shown from the previous paper (Sugawara and Shikama, 1980). MbO_2 concentration was 25 μM.

be ruled out completely (Shikama, 1988; Kaino, 1989). This is in sharp contrast to urea effects on the autoxidation rate that was fully saturated at 8 M.

Unfolding of oxymyoglobin in the presence of 8 M urea

To understand such a marked increase in the autoxidation rate over a wide range of pH, we should take into account the concomitant process of unfolding of MbO_2 in 8 M urea. Denaturation of Mb, exclusively in its ferric met-form, has long been studied by quite a number of authors using various methods, as a function of pH and of denaturant concentration. As a result, the occurrence of partially unfolded forms was reported at low denaturant concentrations, as well as in due course of the unfolding pathway. Such intermediate forms have been characterized in terms of the free energy changes of unfolding, and discussed in relation to a three-domain structure of the Mb molecule, encoded by three exons separated by two introns in globin genes (Gō, 1981; Bismuto et al., 1983; Irace et al., 1986). Recent interests are much more focused on the molten globule intermediate that will appear transiently at the beginning of the refolding process of apomyoglobin on a time scale of less than 1 s (Hughson et al., 1990; Jennings and Wright, 1993; Barrick et al., 1994).

At an early stage of our experiments, the denaturation process of MbO_2 in 8 M urea was examined by fluorescence measurements. Upon excitation at 280 nm, changes in the emission spectra were recorded with time, and a marked increase in the intensity centered at 346 nm was subjected to a first-order plot. As demonstrated in Fig. 4, this is a wavelength characteristic of tryptophanyl residues exposed to aqueous solvent from a nonpolar protein matrix (Sugawara, 1981). Over a wide range of pH at 25°C, the resultant rate constants were found to be more than 10 times higher than the corresponding rate constants for the autoxidation in 8 M urea. This implies that the denatured form of MbO_2 could live for a fairly long time in 8 M urea and be resistant against the rapid autoxidation.

This paradox came from the fact that our fluorescence measurement can reflect only an early, local unfolding of Mb on its NH_2-terminal side so as to allow exposure of the two tryptophan residues at positions 7 and 14 to the surrounding solvent. In the native protein, these residues lie close to the heme moiety, which is known to cause the quenching of tryptophan fluorescence by an energy transfer mechanism (Förster, 1959; Postnikova and Yamakova, 1991). Our result was consistent with the work done by Fronticelli et al. (1989) on an initial event in the denaturation of apomyoglobin.

We have finally employed CD measurements in monitoring the overall denaturation process of MbO_2 in 8 M urea. For bovine heart Mb, the value of the mean residue molar ellipticity at 222 nm, $[\theta]_{222}^{MRW}$, was calculated to be $-27{,}000 \pm 500°$ cm^2dmol^{-1} for both native MbO_2 and metMb, and no significant change was observed in its value over the pH range of 4.8 to 12.6 in 0.05 M buffers. When the protein was denatured completely in 8 M urea, the CD magnitude at 222 nm decreased markedly to $-1{,}400 \pm 500°$ cm^2dmol^{-1} regardless of the pH of the solution.

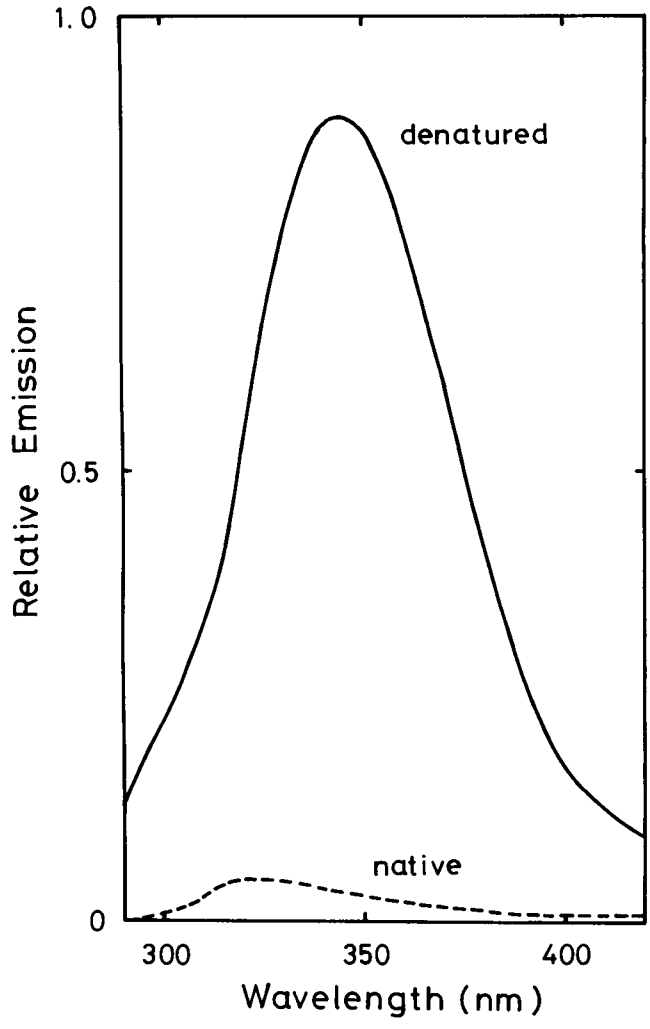

FIGURE 4 Fluorescence emission spectra of native and denatured forms of bovine Mb. Both MbO_2 and metMb showed the same emission spectra with a maximum centered at 320 nm in 0.05 M Tris-HCl buffer, pH 7.4, at 25°C. The denaturation with 8 M urea produces a marked increase in the intensity, showing a slight shift of the emission maximum to 346 nm, a wavelength characteristic of tryptophanyl residues exposed to aqueous solvent from a nonpolar protein matrix. Excitation was at 280 nm. The incubation with 8 M urea was made for 24 hours. Mb concentration was 7 μM.

In the following reaction of MbO_2 in the presence of 8 M urea,

$$MbO_2(N) \xrightarrow[\text{denaturation}]{K_D} metMb(D), \qquad (4)$$

the overall unfolding of Mb was therefore monitored by the CD magnitude at 222 nm, and a ratio of $-\ln\{(CD_t - CD_\infty)/(CD_0 - CD_\infty)\}$ was then plotted against the reaction time, t. Fig. 5 shows such a plot for the urea-induced

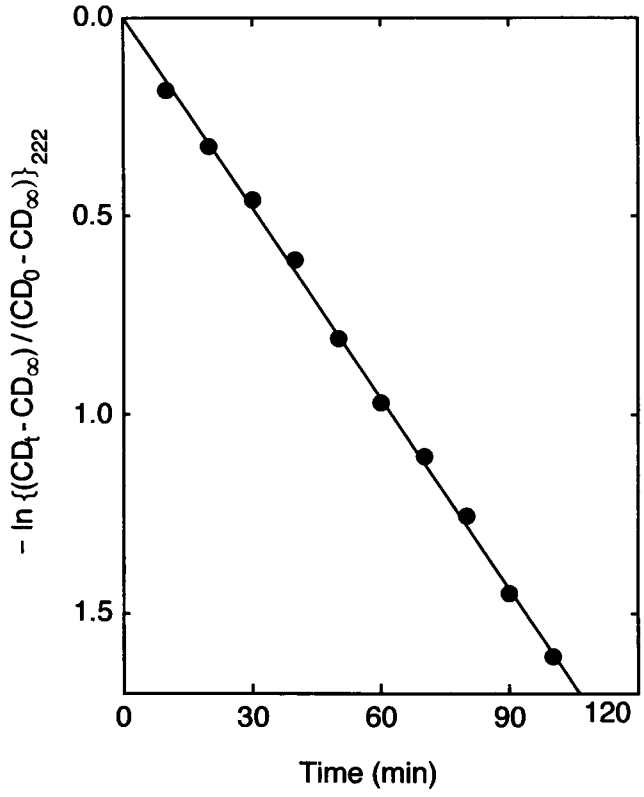

FIGURE 5 A first-order plot for the denaturation of bovine MbO_2 with 8 M urea in 0.05 M Mes buffer, pH 6.4, at 25°C. The unfolding process was monitored by the CD magnitude at 222 nm and described by a single rate constant of K_D = 0.96 h^{-1} at pH 6.4 and 25°C. Mb concentration was 10 μM.

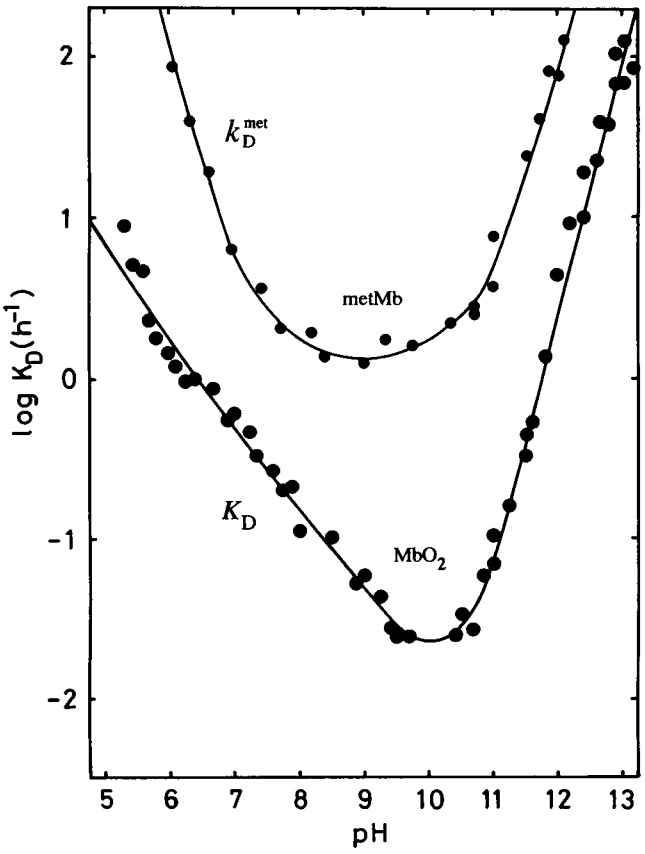

FIGURE 6 The pH profiles for the denaturation of bovine MbO_2 and metMb with 8 M urea in 0.05 M buffer at 25°C. The logarithmic values of the observed rate constant, K_D in h^{-1}, for the denaturation of MbO_2 are plotted against the pH of the solution, with those of k_D^{met} (h^{-1}) for that of metMb. Mb concentration was 10 μM.

unfolding of MbO_2 in 0.05 M Mes buffer, pH 6.4, at 25°C. At a given pH, the observed first-order rate constant, K_D in h^{-1}, was determined from the slope of each straight line. On a very large time scale of hours, it was quite clear that a simple two-state transition model is adequate to describe the unfolding kinetics of Mb, its single rate constant being independent of the initial concentrations of MbO_2 insofar as examined from 5 to 28 μM.

If the values of K_D are plotted against the pH of the solution, we can obtain a profile for the denaturation rate of MbO_2 in 8 M urea at 25°C as shown in Fig. 6. As a result, the unfolding rate of K_D was always higher than the corresponding autoxidation rate of K_A over the whole range of pH studied but to a very small extent so as to provide almost the same pH dependence as for K_A in Fig. 3. This indicates that the unfolding of MbO_2 is the first step to cause a marked increase in the autoxidation rate in the presence of 8 M urea.

At this point, it seemed of interest to examine the stability property of metMb in 8 M urea as a function of pH.

$$\text{metMb(N)} \xrightarrow{k_D^{met}} \text{metMb(D)} \qquad (5)$$

As is also clear in Fig. 6, the met-form of myoglobin was found to be much more susceptible to urea denaturation, providing a somewhat different pH profile. This suggests that the axial ligand as well as the oxidation state of the heme ion can affect profoundly the stability properties of the Mb molecule (McLendon and Sandberg, 1978).

Complete kinetic formulation for the reaction of MbO_2 in 8 M urea

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

$$\begin{array}{ccc} MbO_2(N) & \xrightarrow{k_D^{oxy}} & MbO_2(D) \\ {\scriptstyle k_A^n}\downarrow & & \downarrow{\scriptstyle k_A^d} \\ \text{metMb(N)} & \xrightarrow{k_D^{met}} & \text{metMb(D)} \end{array} \qquad (6)$$

In this scheme, a denatured form of MbO_2, which is fully unfolded as for the globin moiety 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 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 constants, k_A^n and k_D^{met}, were already defined by Eqs. 2 and 5, respectively, and the latter has been determined independently by the denaturation of metMb in 8 M urea. The rate constant k_A^n, on the other hand, might well be replaced by the autoxidation rate of MbO_2 in buffer alone, as the solvent effect of urea was negligibly small on the nucleophilic displacement of O_2^- from MbO_2 (Kaino, 1989), the mechanism proposed for the autoxidation reaction of MbO_2 to metMb (Satoh and Shikama, 1981).

Our primary concern is, therefore, to estimate the value of k_A^d from the consecutive reaction of Eq. 6 and to compare it with that of k_A^n over a wide range of pH at 25°C. In such a urea-induced transition of native MbO_2, we have followed up the two concurrent reactions. One is the overall denaturation process with the rate constant of K_D as defined in Eq. 4. The other is the overall autoxidation process with the rate constant of K_A given by Eq. 3. These rate constants can also be expressed, on rearrangement of Eqs. A11 and A12 in the Appendix, by the following relationships.

$$e^{-K_D t} = \frac{k_D^{met} - k_D^{oxy}}{k_D^{met} - k_D^{oxy} - k_A^n} e^{-(k_D^{oxy} + k_A^n)t} \times \left\{1 - \frac{k_A^n}{k_D^{met} - k_D^{oxy}} e^{-(k_D^{met} - k_D^{oxy} - k_A^n)t}\right\} \quad (7)$$

and

$$e^{-K_A t} = \frac{k_A^d - k_A^n}{k_A^d - k_D^{oxy} - k_A^n} e^{-(k_D^{oxy} + k_A^n)t} \times \left\{1 - \frac{k_D^{oxy}}{k_A^d - k_A^n} e^{-(k_A^d - k_D^{oxy} - k_A^n)t}\right\} \quad (8)$$

In the present case where $k_D^{met} \gg k_D^{oxy}$ and $k_D^{met} \gg k_A^n$, Eq. 7 approximates to

$$K_D t = (k_D^{oxy} + k_A^n)t \quad (9)$$

or

$$k_D^{oxy} = K_D - k_A^n. \quad (10)$$

Furthermore, the valid suppositions of $k_A^d \gg k_A^n$ and $k_A^d > k_D^{oxy}$, i.e., that a denatured form of MbO_2 will be oxidized much more quickly than native MbO_2, but its unfolding process is rate-limiting, lead to the following reduction of Eq. 8.

$$K_A t = K_D t - \ln \frac{k_A^d}{k_A^d - k_D^{oxy}} \quad (11)$$

In a more practical form (with relation to Figs. 3 and 6), it may be written as:

$$K_D t - K_A t = \ln \frac{k_A^d}{k_A^d - k_D^{oxy}} \quad (12)$$

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 unit of time the values of k_D^{oxy} from Eq. 10 and then k_A^d from Eq. 12.

In this respect, we have measured more than 70 points for each of K_A and K_D over the wide range of pH 5–13, and some of the results are shown in Figs. 3 and 6, respectively. The lines were drawn in by hand. It is thus clear that the two rate constants are strongly dependent upon the pH of the solution, and that the value of K_D is slightly but always higher than that of K_A if the pH is the same. From the graphs, however, it is not easy to see whether K_D and K_A provide a significant difference that is required to solve Eq. 12 for k_A^d. In Table 1, therefore, we have presented numerical values of K_D and K_A and also the processes to obtain the k_A^d values at several pH and 25°C.

In this way the rate constant k_A^d, with which we are primarily concerned, was finally determined as a function of pH (Fig. 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

TABLE 1 Kinetic parameters for describing the autoxidation and denaturation of MbO_2 in 8 M urea at 25°C

pH	K_D (h^{-1})	K_A (h^{-1})	k_A^n (h^{-1})	k_D^{met} (h^{-1})	k_D^{oxy} (h^{-1})	k_A^d (h^{-1})
5.6	4.59	2.40	7.40×10^{-2}	1.51×10^{2}	4.52	5.10
6.0	1.46	9.10×10^{-1}	4.20×10^{-2}	6.61×10^{1}	1.42	3.35
6.7	9.00×10^{-1}	6.50×10^{-1}	1.98×10^{-2}	1.58×10^{1}	8.80×10^{-1}	3.98
7.5	3.30×10^{-1}	2.54×10^{-1}	6.90×10^{-3}	3.24	3.23×10^{-1}	4.41
8.5	1.05×10^{-1}	6.84×10^{-2}	3.10×10^{-3}	1.48	1.02×10^{-1}	2.84
9.0	6.10×10^{-2}	4.44×10^{-2}	2.90×10^{-3}	1.48	5.81×10^{-2}	3.53
9.5	2.52×10^{-2}	2.00×10^{-2}	3.10×10^{-3}	1.48	2.21×10^{-2}	4.26
10.3	2.57×10^{-2}	2.12×10^{-2}	4.30×10^{-3}	2.00	2.14×10^{-2}	4.77
11.5	3.40×10^{-1}	2.20×10^{-1}	7.60×10^{-3}	3.55×10^{1}	3.32×10^{-1}	2.94
12.0	4.59	3.96	1.12×10^{-2}	1.32×10^{2}	4.58	9.80
12.6	2.10×10^{1}	1.60×10^{1}	2.00×10^{-2}	3.80×10^{2}	2.10×10^{1}	2.11×10^{1}

For the evaluation of k_D^{oxy} and k_A^d, see Eqs. 10 and 12 in the text. The errors contained in the rate constants of K_D and K_A were both within the range of 5%. Each of the errors at a given pH has effects as a matter of course on the determination of k_A^d, but its deviation falls completely within the range of data points shown in Fig. 7, as a function of pH.

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 buffer alone. Furthermore, its pH dependence is also unusual with an almost constant rate over a wide range, from pH 5 to 11. On the other hand, the values of k_D^{oxy} (not shown here) had the same pH dependence as K_D, as expected from Eq. 10 because of $K_D \gg k_A^n$.

DISCUSSION

Recent kinetic and thermodynamic studies of the stability of native MbO_2 have revealed that the autoxidation reaction is not a simple, dissociative loss of O_2^- from MbO_2 but is due to a nucleophilic displacement of O_2^- from MbO_2 by a water molecule or a hydroxyl ion that can enter the heme pocket from the surrounding solvent. The iron is thus converted to the ferric met-form, and the water molecule or the hydroxyl ion remains bound to the Fe(III) at the sixth coordinate position to form aqua- or hydroxide-metMb, respectively (Satoh and Shikama, 1981; Shikama, 1984, 1985, 1988). It has also been shown that the reductive displacement of the bound dioxygen as O_2^- by H_2O can proceed without any protonation, but the rate is enormously enhanced by a proton-assisted process. In this proton catalysis, the distal (E7) histidine, which forms a hydrogen bond to the bound dioxygen (Phillips and Schoenborn, 1981), appears to facilitate the effective movement of a catalytic proton from the solvent to the bound dioxygen via its imidazole ring by a proton relay mechanism (Shikama, 1985, 1988; Shikama and Matsuoka, 1986, 1994).

Even the complicated pH profile for the autoxidation rate can thereby be explained primarily in terms of the following three types of displacement process (Shikama, 1988):

$$\mathrm{Mb(II)(O_2) + H_2O \xrightarrow{k_0} Mb(III)(OH_2) + O_2^-} \quad (13)$$

$$\mathrm{Mb(II)(O_2) + H_2O + H^+ \xrightarrow{k_H} Mb(III)(OH_2) + HO_2} \quad (14)$$

$$\mathrm{Mb(II)(O_2) + OH^- \xrightarrow{k_{OH}} Mb(III)(OH^-) + O_2^-} \quad (15)$$

The contribution of these elementary processes to the observed or overall autoxidation rate, k_{obs}, can vary with the concentrations of H^+ or OH^- ions and with the dissociation states of the group(s) involved. Consequently, the stability of MbO_2 shows a very strong pH dependence having a parabolic part. To know definitely the kinetic and thermodynamic parameters contributing to each k_{obs} versus 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.

As for the pH dependence of the autoxidation rate of bovine heart MbO_2 (shown in Fig. 3 as well as Fig. 7), it has already been analyzed completely in terms of an "acid-catalyzed three-state model" (Shikama and Sugawara, 1978; Sugawara and Shikama, 1980). In this model, we assumed that two kinds of dissociable groups, AH with pK_1 and BH with pK_2, are involved in the reaction as

$$\begin{array}{ccccc} \mathrm{MbO_2(AH, BH)} & \overset{K_1}{\rightleftharpoons} & \mathrm{MbO_2\,(A^-, BH)} & \overset{K_2}{\rightleftharpoons} & \mathrm{MbO_2(A^-, B^-)} \\ k_0^A \downarrow\ k_H^A \downarrow & & k_0^B \downarrow\ k_H^B \downarrow\ k_{OH}^B \downarrow & & k_0^C \downarrow\ k_{OH}^C \downarrow \\ \text{metMb} & & \text{metMb} & & \text{metMb} \end{array} . \quad (16)$$

For the mechanism delineated in Eq. 16, the observed rate constant, k_{obs} (corresponding to k_A^n) in Eq. 2, can be reduced to

$$\begin{aligned} k_{obs}\,(\equiv k_A^n) = {} & \{k_0^A[H_2O] + k_H^A[H_2O][H^+]\}(\alpha) \\ & + \{k_0^B[H_2O] + k_H^B[H_2O][H^+] + k_{OH}^B[OH^-]\}(\beta) \\ & + \{k_0^C[H_2O] + k_{OH}^C[OH^-]\}(\gamma)\,, \end{aligned} \quad (17)$$

where

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

$$\beta = \frac{K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (18)$$

and

$$\gamma = (1 - \alpha - \beta) = \frac{K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2}\,.$$

By iterative least-squares procedures inserting various values for K_1 and K_2, the adjustable parameters in Eq. 18, the best fit to the experimental values of k_{obs} was obtained as a function of pH (see Fig. 7). In this way, the rate constants of the elementary processes involved were 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. 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 were assigned to the distal histidine (His-64) and Tyr-103, respectively (Sugawara and Shikama, 1980).

In sharp contrast to native MbO_2, its unfolded form can be oxidized very rapidly but at an almost constant rate over a wide range of pH 5–11, as is clear from Fig. 7. We have therefore established the best fit to the values of k_A^d as a function of pH, by the mechanism

$$k_A^d = k_0^d\,[H_2O] + k_{OH}^d[OH^-], \quad (19)$$

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 its

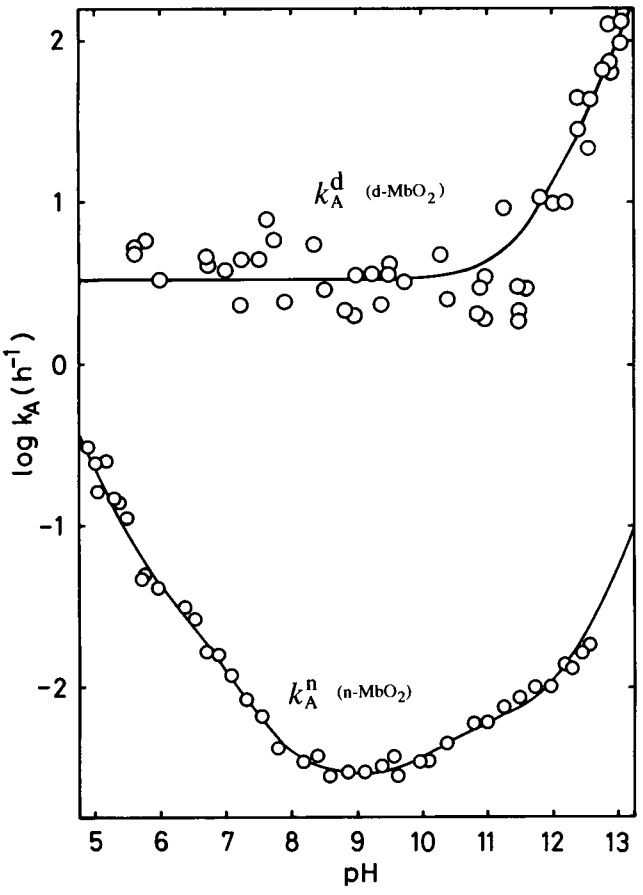

FIGURE 7 Comparison of the autoxidation rate between the native and the denatured form of bovine MbO_2 as a function of pH at 25°C. The logarithmic values of the rate constant, k_A^d in h^{-1}, evaluated for the autoxidation of unfolded MbO_2 are plotted against the pH of the solution, with those of k_A^n (h^{-1}) for native MbO_2 in buffer alone. See text for details.

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 native MbO_2, involving the distal histidine as its catalytic residue. 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. These findings lead us to conclude that the unfolding of the heme pocket allows a much easier attack of the solvent water molecule or hydroxyl ion on the FeO_2 bonding.

As already described, 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 (Tsubamoto et al., 1990), 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-limiting 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 of the ferric iron of the fully denatured Mb.

In vacua, the FeO_2 bond in Mb or Hb 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 (Shikama, 1985, 1990). In aqueous media, however, 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. Mb and Hb 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^+, as illustrated in Fig. 8.

In a protein-free system, Kao and Wang (1965) first studied the oxidation of dipyridine-ferrohemochrome by molecular oxygen using the stopped-flow technique. In aqueous solutions, the main path was interpreted by the mechanism that 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^-. Unfortunately, the rate constant for

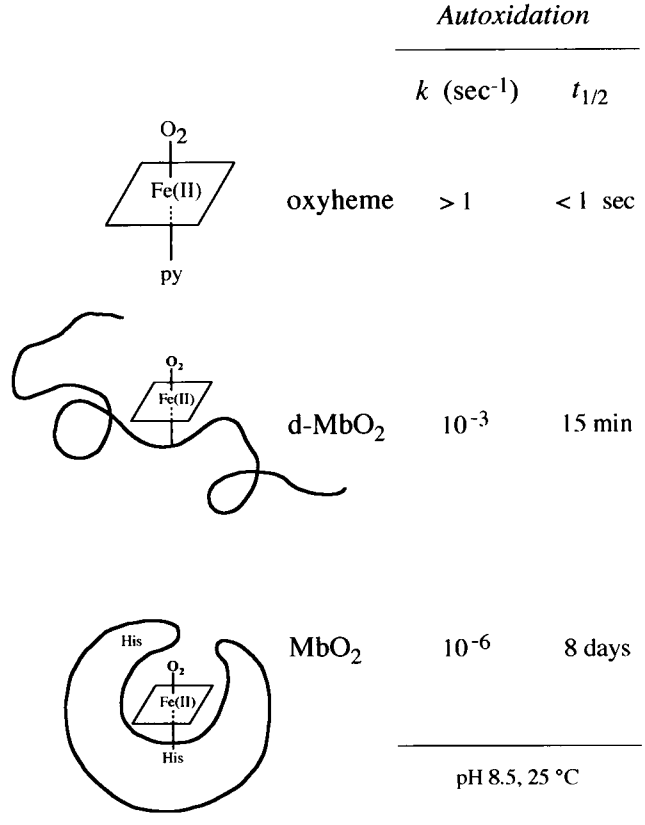

FIGURE 8 Role of the globin moiety in stabilizing the FeO_2 bonding in Mb. Mb has evolved with a globin moiety that can protect the FeO_2 center from easy access of a water molecule including its conjugate ions OH^- and H^+. In fact, the polypeptide matrix can play a considerable role in stabilizing the oxyheme, but the integrity of the native protein architecture is essential to obstruct access of a water molecule to the FeO_2 center. In a sense, the globin moiety of Mb can act as a breakwater in aqueous media.

this oxidation reaction could not be obtained as an explicit value, as the concentration term of pyridine was always involved in its rate equation in a somewhat complicated manner. By numerical calculations, however, it follows that the autoxidation of oxyheme can proceed with the rate constant of much higher than 1 s^{-1} in 0.1 M Tris-HCl buffer, pH 8.5, at 25°C.

If such an oxyheme is placed in a protein matrix, it would be protected against a nucleophilic attack of the solvent water molecule or hydroxyl ion so as to reduce its autoxidation rate by a factor of more than 10^3. This is just our present case with denatured MbO_2 in 8 M urea. Furthermore, when an oxyheme is embedded in the native protein architecture, it acquires a remarkable stability against the autoxidation reaction, but it is also true that Mb or Hb has still not attained maximal ability to block entering water molecules from the FeO_2 center. Nevertheless, we can conclude from the present study that the profound stability of the FeO_2 bonding in these molecules depends upon the integrity of the conformation of the globin moiety so that it can act as a breakwater in aqueous media.

APPENDIX

For the elementary processes involved in Eq. 6, we may write the following rate equations:

$$\frac{d[MbO_2(N)]}{dt} = -(k_D^{oxy} + k_A^n)[MbO_2(N)] \quad (A1)$$

$$\frac{d[MbO_2(D)]}{dt} = k_D^{oxy}[MbO_2(N)] - k_A^d[MbO_2(D)] \quad (A2)$$

$$\frac{d[metMb(N)]}{dt} = k_A^n[MbO_2(N)] - k_D^{met}[metMb(N)] \quad (A3)$$

$$\frac{d[metMb(D)]}{dt} = k_D^{met}[metMb(N)] + k_A^d[MbO_2(D)] \quad (A4)$$

By solving these differential equations for the concentration of each species at time t, we obtain, respectively,

$$[MbO_2(N)]_t = [MbO_2(N)]_0\, e^{-(k_D^{oxy}+k_A^n)t} \quad (A5)$$

$$[MbO_2(D)]_t = [MbO_2(N)]_0 \frac{k_D^{oxy}}{k_A^d - k_D^{oxy} - k_A^n} \times (e^{-(k_D^{oxy}+k_A^n)t} - e^{-k_A^d t}) \quad (A6)$$

$$[metMb(N)]_t = [MbO_2(N)]_0 \frac{k_A^n}{k_D^{met} - k_D^{oxy} - k_A^n} \times (e^{-(k_D^{oxy}+k_A^n)t} - e^{-k_D^{met} t}) \quad (A7)$$

$$[metMb(D)]_t = [MbO_2(N)]_0 - [MbO_2(N)]_t - [MbO_2(D)]_t - [metMb(N)]_t, \quad (A8)$$

where $[MbO_2(N)]_0$ represents the concentration of native MbO_2 at time zero.

When fresh MbO_2 is placed in 8 M urea at a given pH, we can observe only the following two reactions. One is the overall denaturation of the Mb molecule with the rate constant of K_D defined in Eq. 4. This process may be expressed for the total concentration of the native protein at time t by

$$[\text{native form}]_t = [MbO_2(N)]_0\, e^{-K_D t}. \quad (A9)$$

Insofar as examined by the CD magnitude at 222 nm, we cannot differentiate the oxy-form from the met-form in their native state as

$$[\text{native form}]_t \equiv \{[MbO_2(N)]_t + [metMb(N)]_t\}. \quad (A10)$$

By substituting Eqs. A5 and A7 into Eq. A10, it follows that

$$[MbO_2(N)]_0\, e^{-K_D t} = \{[MbO_2(N)]_t + [metMb(N)]_t\} = [MbO_2(N)]_0 \times \left\{e^{-(k_D^{oxy}+k_A^n)t} + \frac{k_A^n}{k_D^{met} - k_D^{oxy} - k_A^n}(e^{-(k_D^{oxy}+k_A^n)t} - e^{-k_D^{met} t})\right\}. \quad (A11)$$

The other concomitant process is for the autoxidation of MbO_2 in 8 M urea. 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 native MbO_2 in their spectra. This supposition is quite reasonable, as we have confirmed that cyano-metMb, for instance, shows no spectral difference between the native form in buffer alone and the completely denatured form in 8 M urea (Sugawara, 1981). For the total concentration of the oxygenated form at time t, therefore, the following expression may be valid with use of the rate constant of K_A defined in Eq. 3:

$$[MbO_2(N)]_0\, e^{-K_A t} = \{[MbO_2(N)]_t + [MbO_2(D)]_t\} = [MbO_2(N)]_0 \times \left\{e^{-(k_D^{oxy}+k_A^n)t} + \frac{k_D^{oxy}}{k_A^d - k_D^{oxy} - k_A^n}(e^{-(k_D^{oxy}+k_A^n)t} - e^{-k_A^d t})\right\}. \quad (A12)$$

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