Laine, A., Davril, M., & Hayem, A. (1984) *Eur. J. Biochem. 140*, 105-111.
Lennick, M., Brew, S. A., & Ingham, K. C. (1985) *Biochemistry 24*, 2561.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149-152.
McKay, E. J., Laurell, A., Martensson, U., & Sjoholm, A. G. (1981) *Mol. Immunol. 18*, 349-357.
McRae, B. J., Lin, T.-Y., & Powers, J. C. (1981) *J. Biol. Chem. 256*, 12362-12366.
Medicus, R. G., & Chapuis, R. M. (1980) *J. Immunol. 125*, 390-395.
Neurath, H. (1984) *Science (Washington, D.C.) 224*, 350-357.
Nilsson, T., & Wiman, B. (1982) *Biochim. Biophys. Acta 705*, 271-276.
Nilsson, T., & Wiman, B. (1983) *Eur. J. Biochem. 129*, 663-667.
Odermatt, E., Berger, H., & Sano, Y. (1981) *FEBS Lett. 131*, 283-285.
Ogamo, A., Matsuzaki, K., Uchiyama, H., & Nagasawa, K. (1982) *Carbohydr. Res. 105*, 69-85.
Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891-14895.
Petersen, L. C., & Clemmensen, I. (1981) *Biochem. J. 199*, 121-127.
Reboul, A., Arlaud, G. J., Sim, R. B., & Colomb, M. G. (1977) *FEBS Lett. 79*, 45-50.
Salvesen, G. S., Catanese, J. J., Kress, L. F., & Travis, J. (1985) *J. Biol. Chem. 260*, 2432-2436.
Sim, R. B., Porter, R. R., Reid, K. B. M., & Gigli, I. (1977) *Biochem. J. 163*, 219-227.
Sim, R. B., Reboul, A., Arlaud, G. J., Villiers, C. L., & Colomb, M. G. (1979) *FEBS Lett. 97*, 111-115.
Sim, R. B., Arlaud, G. J., & Colomb, M. G. (1980) *Biochim. Biophys. Acta 612*, 433-449.
Tenner, A. J., & Frank, M. M. (1986) *J. Immunol.* (in press).
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655-709.
Tschopp, J., Villager, W., Fuchs, H., Kilchherr, E., & Engel, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7014-7018.
Valet, G., & Cooper, N. (1974) *J. Immunol. 112*, 339-350.
van der Graaf, F., Koedam, J. A., Griffin, J. H., & Bouma, N. (1983) *Biochemistry 22*, 4860-4866.
Weiss, V., & Engel, J. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 295-301.
Ziccardi, R. J. (1981) *J. Immunol. 126*, 1769-1773.

Aplysia Oxymyoglobin with an Unusual Stability Property: Kinetic Analysis of the pH Dependence

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ABSTRACT: Native oxymyoglobin (MbO_2) was isolated directly from the radular muscle of *Aplysia kurodai* and was examined for its stability properties over the wide range of pH 4-13 in 0.1 M buffer at 25 °C. When compared with sperm whale MbO_2 as a reference, *Aplysia* MbO_2 is found to be extremely unstable with an unusual pH dependence for its autoxidation rate. Kinetic analysis has revealed that *Aplysia* MbO_2, lacking the distal histidine, does not show such a proton-catalyzed process that can play a dominant role in the autoxidation reaction of sperm whale MbO_2, involving the distal histidine as its catalytic residue. Rather, *Aplysia* MbO_2 contains two kinds of dissociable groups with pK_a = 4.2 and 6.1, respectively, both probably being carboxyl groups and both also being responsible for an increase in its autoxidation rate in the acidic pH range. Therefore, the extreme susceptibility of *Aplysia* MbO_2 to autoxidation comes mainly from the rate constant for a nucleophilic displacement of O_2^- from MbO_2 by an entering water molecule, with the iron ending up as the ferric form. Its value is found to be 100 times higher than the corresponding value for sperm whale MbO_2. In relation to structural evidence, these findings suggest that the heme pocket of *Aplysia* MbO_2 is open enough to allow easier attack of the solvent water molecule on the FeO_2 center.

Unlike mammalian myoglobins, *Aplysia* myoglobins contain only a single histidine residue, lacking the usual distal one (Tentori et al., 1973; Suzuki et al., 1981; Takagi et al., 1984), and the circular dichroism magnitude being about two-thirds that of sperm whale myoglobin (Shikama et al., 1982). Furthermore, the hydropathy profiles obtained from the amino acid sequence of *Aplysia* myoglobins are quite different from that of sperm whale myoglobin, especially on the distal side of the heme iron (Takagi et al., 1984).

We have recently succeeded in isolating native oxymyoglobin directly from the radular muscle of *Aplysia kurodai*, a common species around the Japanese coast, and have examined its spectral and stability properties. The absorption spectrum of *Aplysia* MbO_2[1] is very similar to those of mammalian oxymyoglobins. Its stability, however, is quite different from those of the mammalian oxymyoglobins, and *Aplysia* MbO_2 is found to be extremely susceptible to autoxidation (Shikama & Katagiri, 1984).

In this paper, we describe a mechanistic analysis of the unusual pH dependence for the stability properties of *Aplysia* MbO_2. This kinetic analysis seems to be of great interest if

[1] Abbreviations: MbO_2, oxymyoglobin; metMb, metmyoglobin; HbO_2, oxyhemoglobin; metHb, methemoglobin; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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compared with that of sperm whale MbO_2 as a reference and may provide a clue for elucidating the role of the distal histidine residue and the heme environments in myoglobin chemistry as well as in myoglobin evolution, since the kinetics of the autoxidation should reflect differences on the distal side of the heme.

Materials and Methods

Chemicals. Sephadex G-50 and G-75, and DEAE-Sephadex A-25 were products of Pharmacia. DEAE-cellulose (DE-32) and CM-cellulose (CM-32) were purchased from Whatman. MES, PIPES, TAPS, and CAPS for buffer systems and all other chemicals were of reagent grade from Wako Pure Chemical, Osaka; solutions were made with deionized and glass-distilled water.

Oxymyoglobin Preparations. Specimens of *A. kurodai* were collected around the Onagawa coast, Miyagi Prefecture, Japan. Native oxymyoglobin was isolated directly from the radular muscle and purified according to our previous method (Shikama & Katagiri, 1984). Fresh buccal masses, about 150 g being gathered from 25 heads, were homogenized in 3 volumes of cold distilled water containing 0.5 mM EDTA. After insoluble material had been removed by centrifugation, the extract was fractionated with ammonium sulfate between 70% and 100% saturation, the pH being kept at 8.0 by the addition of 1 M ammonium hydroxide. The myoglobin precipitate was centrifuged down, dissolved in a minimum volume of 5 mM Tris·HCl buffer (pH 8.0), and dialyzed against the same buffer containing 0.5 mM EDTA. After gel filtration on Sephadex G-75, the essential step was the chromatographic separation of MbO_2 from metMb on a DEAE-cellulose column that had been equilibrated with 5 mM Tris·HCl buffer (pH 8.0). The elution was carried out with a linear concentration gradient of Tris·HCl buffer from 5 to 50 mM at pH 7.5. The concentration of *Aplysia* myoglobin was determined, after conversion into cyanometmyoglobin, by using an extinction coefficient of 10.2 mM^{-1} cm^{-1} at 540 nm (Shikama et al., 1982).

For comparison, sperm whale oxymyoglobin was also prepared directly from skeletal muscle according to our standard procedure (Suzuki & Shikama, 1983). After being separated completely from hemoglobin by gel filtration on a Sephadex G-50 column, the myoglobin was applied to a DEAE-Sephadex A-25 column and developed into its polymorphic forms with 15 mM Tris·HCl buffer (pH 9.0). The major fraction, which was first eluted with 50 mM Tris·HCl buffer (pH 9.0), was dialyzed against 5 mM Tris·HCl (pH 7.0) and applied to a CM-cellulose column equilibrated with the same buffer, to separate the MbO_2 completely from the metMb with 15 mM Tris·HCl buffer (pH 7.5). The major MbO_2 thus obtained was placed in 10 mM Tris·HCl buffer (pH 9.0) and kept at low temperature (0–4 °C) before use.

Autoxidation Rate Measurements. The measurements were carried out in 0.1 M buffer at 25 °C over the pH ranges of 4–13 for *Aplysia* MbO_2 and of 5–13 for sperm whale MbO_2, according to our standard procedure. A 2-mL solution containing 0.2 M 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 2 mL of fresh oxymyoglobin solution (50 μM for *Aplysia* or 100 μM for sperm whale), 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 absorption spectrum from 450 to 650 nm were recorded on the same chart at measured intervals of time. For the final state of the runs, the myoglobin was completely converted to metmyoglobin by the addition of potassium ferricyanide. The rate constant observed at a given pH for the autoxidation of MbO_2 to metMb (k_{obsd}) was determined from the slope of each straight line in the first-order plot. The pH of the reaction mixture was carefully checked with a Hitachi-Horiba pH meter (Model F-7SSII).

The buffers used were acetate, MES, PIPES, TAPS, CAPS, and phosphate. In order to test for any specific effect of the anions of the buffers on the autoxidation, we checked the rate by varying the concentration of each buffer from 10 to 100 mM and by overlapping the pH regions with the different buffer systems used, but no practical change was observed in the autoxidation rate (Sugawara & Shikama, 1979). It was further proved by absorption spectra at 408 nm and by circular dichroism measurements at 222 nm that both myoglobins were not denatured during the rate determination over the pH range in which the autoxidation was being studied.

Spectrophotometric Measurements. Spectrophotometric measurements were carried out in a Hitachi two-wavelength double-beam spectrophotometer (Model 557) equipped with a thermostatically controlled cell holder. Temperature was controlled by a water bath (Lauda thermostat) maintained to within ±0.1 °C.

Curve Fittings. The curve fittings for a plot of k_{obsd} vs. pH were made by a least-squares method with the use of a digital computer (ACOS Series 77 NEAC System 1000, Tohoku University) and also on a microcomputer (NEC PC-9801F2) with graphic display (NEC PC-8853N), according to our previous specifications (Shikama & Sugawara, 1978).

Results

Complete Kinetic Description for the pH Dependence. Under air-saturated conditions, oxymyoglobin is oxidized easily to metmyoglobin with the generation of the superoxide anion as

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

where k_{obsd} represents the first-order rate constant observed at a given pH (Gotoh & Shikama, 1974, 1976; Shikama, 1984). Therefore, the rate of the autoxidation is given by

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

If the values of k_{obsd} are plotted against the pH of the solution, a profile of the stability of oxymyoglobin can be obtained. Figure 1 shows such a profile for *Aplysia* MbO_2 over the wide range of pH 4–13 in 0.1 M buffer at 25 °C. It is evident that, compared with sperm whale MbO_2 as a reference, *Aplysia* MbO_2 is extremely unstable over the whole range of pH studied and that its pH dependence of stability is also quite unusual.

Over the quite wide range of pH 7–12, *Aplysia* MbO_2 is oxidized to metMb with a constant value for k_{obsd} of 0.10 (±0.01) h^{-1}, which is, for instance, 100 times higher than k_{obsd} = 0.0011 h^{-1} for sperm whale MbO_2 at pH 9.0. In the acidic range of pH 4–7, on the other hand, the rate of autoxidation of *Aplysia* MbO_2 increases with increasing hydrogen ion concentration, but much less so than for sperm whale MbO_2: In a plot of log k_{obsd} vs. pH, sperm whale MbO_2 gave a slope very close to −1, but *Aplysia* MbO_2 showed a slope of less than −0.5 and even a saturating level in the most acidic range, as clearly demonstrated in Figure 2. All these properties of *Aplysia* MbO_2 are in sharp contrast to the stability property of sperm whale MbO_2, which depends heavily upon the pH of the solution. These suggest strongly that the mode of action of the proton in the autoxidation reaction is quite different in the case of *Aplysia* MbO_2, lacking the distal histidine, from

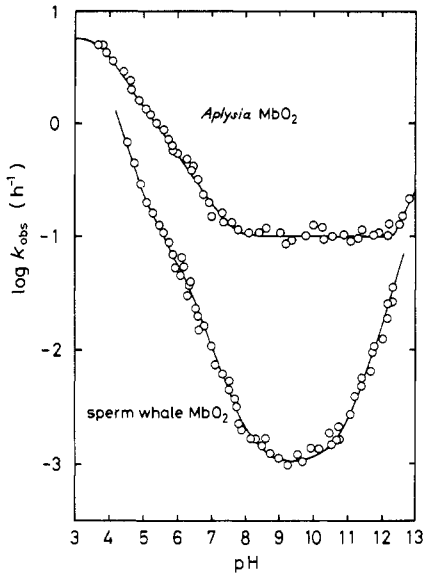

FIGURE 1: pH dependence of the stability properties of *Aplysia* MbO_2 and sperm whale MbO_2 in 0.1 M buffer at 25 °C. The logarithmic values of the observed rate constant, k_{obsd}, for the autoxidation reaction are plotted against the pH of the solution. The computed curve (—) was obtained by a least-squares fitting to the experimental data (O) over the whole range of pH studied, based on eq 4 for *Aplysia* and eq 10 for sperm whale, respectively. MbO_2 concentration: 25 μM for *A. kurodai*; 50 μM for sperm whale.

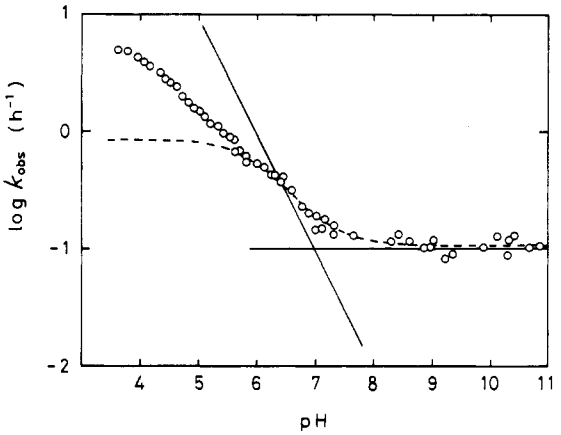

FIGURE 2: Plot of log k_{obsd} vs. pH for the autoxidation reaction of *Aplysia* MbO_2 with some theoretical lines. Two theoretical lines (—) having slopes of −1 and 0 with ascending pH are drawn to indicate an unusual mode of action of the proton in the reaction. A computed curve (- - -) involving a single dissociation process with $pK = 6.1$ was also insufficient to cover all the acidic part of the reaction. *A. kurodai* MbO_2 concentration: 25 μM in 0.1 M buffer at 25 °C.

sperm whale MbO_2, which involves the distal histidine that has been shown to participate in the proton-catalyzed process for the autoxidation of bovine heart MbO_2 (Sugawara & Shikama, 1980).

Recently it has also been shown that the autoxidation reaction is not a simple, dissociative loss of O_2^- from MbO_2 but is a nucleophilic displacement of O_2^- from MbO_2 by the entering water molecule or hydroxyl ion, involving three types of elementary processes (Sugawara & Shikama, 1980; Satoh & Shikama, 1981; Shikama, 1984):

$$MbFe(II)(O_2) + H_2O \xrightarrow{k_O} MbFe(III)(OH_2) + O_2^-$$

$$MbFe(II)(O_2) + H_2O + H^+ \xrightarrow{k_H} MbFe(III)(OH_2) + HO_2$$

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

According to these specifications, therefore, we proposed some mechanistic models for the autoxidation reaction of *Aplysia* MbO_2. The rate equations derived therefrom were tested for fitting experimental data with the use of a computer, and it was finally concluded that the unusual pH profile for the autoxidation rate of *Aplysia* MbO_2 can be best explained by a "three-state model".

In this scheme it is assumed that two 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 three forms of MbO_2, represented by A, B, and C, at molar fractions of α, β, and $1 - \alpha - \beta$, respectively, which are in equilibrium with each other but which differ in dissociation states for the groups AH and BH (see eq 3). These forms can be oxidized to metMb by displacement of O_2^- from MbO_2 by an entering water molecule, and at extremely high pH by an entering hydroxyl ion. The reaction scheme may be written, therefore, as

$$\begin{array}{ccccc} MbO_2(AH, BH) & \overset{K_1}{\rightleftharpoons} & MbO_2(A^-, BH) & \overset{K_2}{\rightleftharpoons} & MbO_2(A^-, B^-) \\ \downarrow k_O^A & & \downarrow k_O^B & & \downarrow k_O^C \quad \downarrow k_{OH}^C \\ \text{metMb} & & \text{metMb} & & \text{metMb} \end{array} \qquad (3)$$

where for each form of MbO_2 k_O is the rate constant for the displacement by H_2O and k_{OH} is the rate constant for the displacement by OH^-.

For the mechanism delineated in eq 3 the observed rate constant, k_{obsd} in eq 2, can finally be reduced to

$$k_{obsd} = \{k_O^A[H_2O]\}(\alpha) + \{k_O^B[H_2O]\}(\beta) + \{k_O^C[H_2O] + k_{OH}^C[OH^-]\}(1 - \alpha - \beta) \quad (4)$$

where

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

$$\beta = \frac{K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2} \qquad (5)$$

$$1 - \alpha - \beta = \frac{K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2}$$

By iterative least-squares procedures inserting various values for K_1 and K_2, the adjustable parameters in eq 5, the best fit to more than 70 experimental values of k_{obsd} was obtained as a function of pH, as shown in Figure 1. In this way the rate constants and the acid dissociation constants involved in the autoxidation reaction of *A. kurodai* MbO_2 were also established in 0.1 M buffer at 25 °C, as summarized in Table I. Here, it should be noted that there was no way to explain the pH dependence of k_{obsd} for *Aplysia* MbO_2 by a single dissociation process (Figure 2).

Analytical Characterization for the pH Dependence. Unfortunately, it seemed obscure from Figure 1 that two dissociation processes are involved in the autoxidation reaction of *A. kurodai* MbO_2. However, *Aplysia* MbO_2 is such a case that some analytical treatments could also be applied to confirm the validity of the kinetic and thermodynamic parameters resulting from the curve-fitting procedures.

If three forms of the MbO_2 are in equilibrium with each other as delineated in eq 3, the Henderson–Hasselbalch type equations must be given between the forms A and B involving the dissociable group AH with pK_1 and between the forms B and C involving the group BH with pK_2:

$$\log [(k_{obsd} - k_B)/(k_A - k_{obsd})] = -pH + pK_1 \quad (6)$$

and

$$\log [(k_{obsd} - k_C)/(k_B - k_{obsd})] = -pH + pK_2 \quad (7)$$

Table I: Rate Constants and Acid Dissociation Constants Obtained from the pH Dependence for the Autoxidation Reactions of *A. kurodai* MbO_2 and Sperm Whale MbO_2 in 0.1 M Buffer at 25 °C[a]

source	state of MbO_2	k_O (h^{-1} M^{-1})	k_H (h^{-1} M^{-2})	k_{OH} (h^{-1} M^{-1})	pK
Aplysia MbO_2	A(AH,BH)	0.11			
	$\rightleftharpoons K_1$				4.2
	B(A^-,BH)	0.13×10^{-1}			
	$\rightleftharpoons K_2$				6.1
	C(A^-,B^-)	0.18×10^{-2}		0.83	
sperm whale MbO_2	A(AH)	0.78×10^{-4} [b]	0.37×10^3		
	$\rightleftharpoons K_1$				6.2
	B(A^-)	0.18×10^{-4}	0.20×10^4	0.14×10	

[a] See eq 3 and 4 for *Aplysia* and eq 9 and 10 for sperm whale in the text. [b] The earlier estimation of k_O^A appears to have been in error by a factor of 2 on the low side (Suzuki & Shikama, 1983).

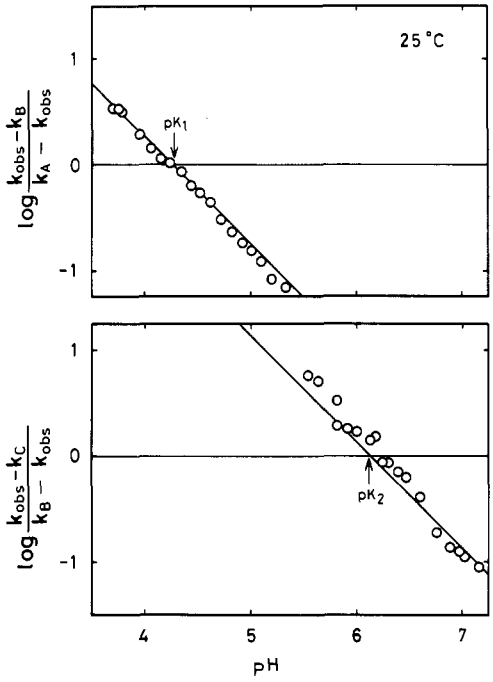

FIGURE 3: Henderson–Hasselbalch plot for the autoxidation of *Aplysia* MbO_2 in the acidic range of pH 4–7. The two dissociation processes were revealed with their corresponding values of pK_1 = 4.2 and pK_2 = 6.1 for *A. kurodai*, given by the midpoint of the two respective stages of the reaction in 0.1 M buffer at 25 °C.

where $k_A \equiv k_O^A[H_2O]$, $k_B \equiv k_O^B[H_2O]$, and $k_C \equiv k_O^C[H_2O]$, taken as $[H_2O]$ = 55.5 M. Therefore, in eq 6, for instance, if the kinetic values of log $[(k_{obsd} - k_B)/(k_A - k_{obsd})]$ are plotted against the corresponding values of pH, a straight line of slope −1 would be expected to obtain, with its midpoint giving a value referred to as pK_1 for the dissociable group AH involved.

Using the values already evaluated for k_O^A, k_O^B, and k_O^C, Figure 3 shows such a Henderson–Hasselbalch plot for the stability of *A. kurodai* MbO_2. It becomes thus unequivocal that there are involved two dissociation processes with pK_1 = 4.2 and pK_2 = 6.1 in the acidic range of pH 4–7. Also, it is clear that the proton participating in the pH dependence of the reaction is not the catalytic one but is the equilibrium proton that changes only the molar fractions of the three forms of the MbO_2 according to eq 5.

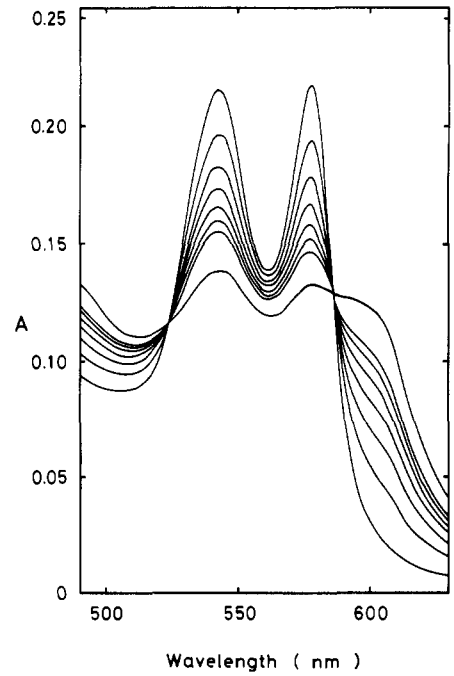

FIGURE 4: Spectral changes with time for the autoxidation of *Aplysia* MbO_2 with a constant rate over the range of pH 8–12. Scans were made at the following times: 0, 100, 300, 500, 700, 900, and 1100 min. The final spectrum was that of the hydroxide–metMb. *A. kurodai* MbO_2 concentration: 16 μM; k_{obsd} = 0.091 h^{-1} in 0.1 M buffer, pH 10.33 at 25 °C.

In the range where pH > pK_2, we might reduce eq 4 to the simple form:

$$k_{obsd} \approx k_O^C[H_2O] + k_{OH}^C[OH^-] \quad (8)$$

Over the quite wide range of pH 8–12, however, the rate of autoxidation of *Aplysia* MbO_2 was constant and independent of increasing OH^- concentration, although the final spectrum of the reaction was essentially that of the hydroxide–metMb (Figure 4). These conflicting observations led us readily to conclude that *Aplysia* MbO_2 was oxidized mostly to aqua-metMb with a constant rate of $k_O^C[H_2O]$, since this term for the nucleophilic displacement by H_2O dominated over the other term of $k_{OH}^C[OH^-]$ in this pH range; the aqua-metMb thus formed was then converted instantaneously to its hydroxide form by the acid–alkaline transition with pK_a = 8.3 at 25 °C (Katagiri, 1983). In the extremely basic range, the values of $k_{OH}^C[OH^-]$ turn to rise higher above that of $k_O^C[H_2O]$, and so there does appear a small increase in its autoxidation rate as already seen in Figure 1.

From the pK_a values given in Table I, and also taking into account the fact that *Aplysia* myoglobins contain only a single histidine residue corresponding to the heme-binding proximal one (Takagi et al., 1984), the most probable candidates for the groups AH and BH may both be carboxyl groups. Although this identification should further be confirmed by other lines of evidence, it is clear from Table I that the rate of autoxidation of *Aplysia* MbO_2 increases about 10 times each in every stage of protonation for whatever the respective groups involved.

Kinetic Comparison with Sperm Whale Oxymyoglobin. The unusual stability property of *Aplysia* MbO_2 would become much more distinct if compared with the stability of sperm whale MbO_2. In our preliminary examination, it was assumed that a single dissociable group, AH with pK_1, is involved in the autoxidation reaction of sperm whale MbO_2 (Suzuki & Shikama, 1983). Consequently, there are two forms of the MbO_2, represented by A and B, at molar fractions of α and

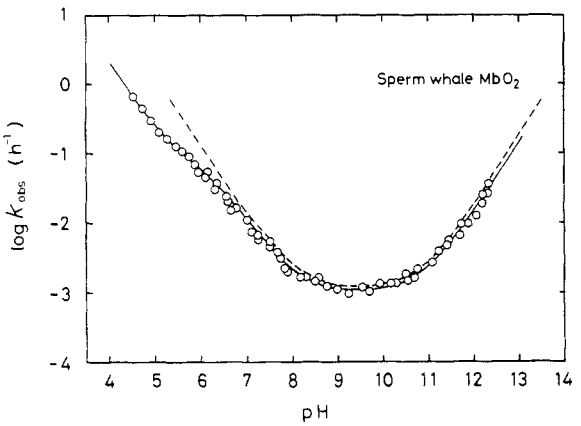

FIGURE 5: log k_{obsd} vs. pH profile for the autoxidation of sperm whale MbO_2 in 0.1 M buffer at 25 °C. Since its parabolic component (---) was manifested by the term $k^B = k_O^B[H_2O] + k_H^B[H_2O][H^+] + k_{OH}^B[OH^-]$ in eq 10, the rate constants for the B state were first established so as to fit most of the basic range.

β, respectively, which are in equilibrium with each other but which differ in dissociation state for the group AH. The autoxidation reaction of sperm whale MbO_2 may therefore be written as

$$\begin{array}{ccc} MbO_2(AH) & \overset{K_1}{\rightleftharpoons} & MbO_2(A^-) \\ \downarrow k_O^A \quad \downarrow k_H^A & & \downarrow k_O^B \quad \downarrow k_H^B \quad \downarrow k_{OH}^B \\ \text{metMb} & & \text{metMb} \end{array} \qquad (9)$$

where for each form of MbO_2 k_O 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 this reaction, the observed rate constant, k_{obsd} in eq 2, was therefore given by

$$\begin{aligned} k_{obsd} &= \{k^A\}(\alpha) + \{k^B\}(\beta) = \\ &\{k_O^A[H_2O] + k_H^A[H_2O][H^+]\}(\alpha) + \\ &\{k_O^B[H_2O] + k_H^B[H_2O][H^+] + k_{OH}^B[OH^-]\}(\beta) \end{aligned} \qquad (10)$$

where

$$\begin{aligned} \alpha &= [H^+]/\{[H^+] + K_1\} \\ \beta &= 1 - \alpha = K_1/\{[H^+] + K_1\} \end{aligned} \qquad (11)$$

We have measured the values of k_{obsd} at some 85 different values of pH over the range 5–13 in 0.1 M buffer at 25 °C and then carried out evaluation of the kinetic and thermodynamic parameters involved in the autoxidation reaction of sperm whale MbO_2.

From the basic range, where the molar fraction of β approaches unity, the values of k_O^B, k_H^B, and k_{OH}^B were first established so as best to cover a parabolic part of the k_{obsd}–pH profile, as illustrated in Figure 5. After these parameters had been fixed, iterative least-squares procedures inserting various values for K_1, the adjustable parameter in eq 11, were carried out so as to obtain the best fit to the experimental values of k_{obsd} as a function of pH over the whole range 5–13. In this way, the conjugate values of k_O^A and k_H^A, as well as the value of pK_1, were established at the minimum of the sum of the squared residuals, as demonstrated in Figure 6. Here, it should be noted that if a proper value were not inserted for pK_1, the rate constant k_O^A results in even a negative value.

Table I also summarizes the rate constants and the acid dissociation constant involved in the stability properties of

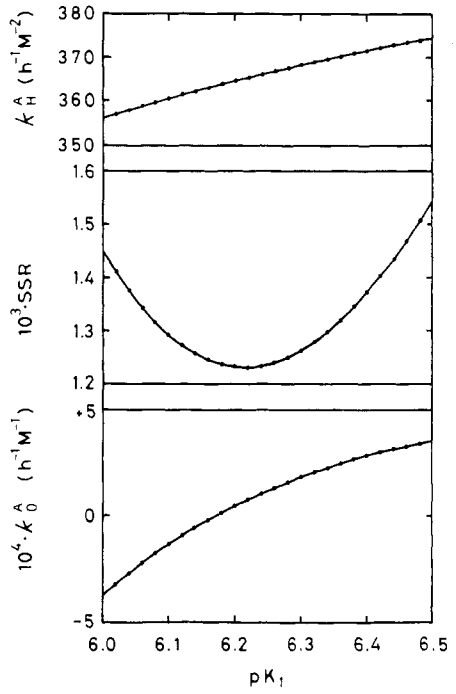

FIGURE 6: Graph of results of iterative least-squares procedures to find the best values of k_O^A, k_H^A, and pK_1 corresponding to a minimum of the sum of the squared residuals (SSR). After k_O^B, k_H^B, and k_{OH}^B in eq 10 were fixed, iterative least-squares procedures inserting various values for K_1 into eq 11 were carried out. The resulting values of k_O^A, k_H^A, and the corresponding sum of the squared residuals are shown as a function of pK_1 inserted.

sperm whale MbO_2 in 0.1 M buffer at 25 °C. These results clearly indicate that the proton-catalyzed displacement processes with the rate constants k_H^A and k_H^B are mainly responsible for promoting the autoxidation reaction of the MbO_2 above the spontaneous displacement processes by H_2O with the rate constants k_O^A and k_O^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.

In this proton catalysis, the distal histidine (the dissociable group AH with $pK_1 = 6.2$), which forms a hydrogen bond to the bound dioxygen (Phillips & Schoenborn, 1981), appears to participate in facilitating the effective movement of a catalytic proton from the solvent to the bound dioxygen via its imidazole ring by a proton-relay mechanism (Sugawara & Shikama, 1980; Suzuki & Shikama, 1983; Shikama, 1984). This proton transfer can lead to a favorable displacement of O_2^- as the hydroperoxyl radical HO_2, which departs and, since its pK_a is 4.8 (Fridovich, 1978), then dissociates into the superoxide anion and a catalytic proton again.

In Table I, one of the most remarkable features is that *Aplysia* MbO_2, lacking the distal histidine, does not show such a catalytic term k_H that can play a dominant role in the stability properties of sperm whale MbO_2. This finding leads us to a general conclusion that the proton catalysis appearing in the autoxidation reaction of MbO_2 can be caused by the distal histidine residue. It is therefore evident that the extreme susceptibility of *Aplysia* MbO_2 to autoxidation comes, not from the proton catalysis, but mainly from the rate constant k_O^C, its value being 100 times higher than the corresponding one for sperm whale MbO_2. A high value of k_O^C implies that the heme pocket of *Aplysia* MbO_2 is open enough to allow easier attack of the solvent water molecule on the FeO_2 center, with a consequent very rapid formation of metMb (Shikama et al., 1982). This may be due partly to lack of the distal histidine residue that is in a location where it can act just like a gate

to the heme pocket (Tucker et al., 1978). The hydropathy profiles also reveal another interesting feature relevant to our interpretation: whereas sperm whale myoglobin shows a most strong hydrophobic lobe on the distal side of the heme iron, *Aplysia* myoglobin does not show such a hydrophobic character on its corresponding E-helix region (Takagi et al., 1984).

Discussion

In characterizing the stability properties of oxymyoglobins from different origins, here we have been primarily concerned with the pH dependence of their autoxidation rate under air-saturated conditions. Since pH is the most crucial factor influencing the autoxidation reaction of MbO_2, the effect of pH has long been investigated by a number of authors, but no mechanistic clue to the reaction has been found from those studies in the very limited range of pH 5–7 (Brown & Mebine, 1969).

Another important factor influencing the rate of autoxidation of MbO_2 as well as HbO_2 may be the oxygen pressure. It has also long been observed that the autoxidation rate increases with decreasing partial pressure of O_2, although the extent of this effect is very small as compared with the enormous effect of pH that we have dealt with here.

Taking various influencing factors into account, several proposals have therefore been made concerning the mechanism of this autoxidation reaction, and these are recently reviewed from a thermodynamic viewpoint (Shikama, 1984). Along with the early work, Wallace et al. (1982), among others, agree that the first step in autoxidation of HbO_2 is the dissociation of the oxygen ligand, followed by the oxidation of the deoxy species by free O_2 to produce metHb and O_2^-. Further, they presented the idea that anion binding to the deoxy species mediates one-electron transfer from iron(II) to free O_2 through porphyrin or aromatic amino acid residues of the protein and that under physiological conditions one of the most potent anions on this oxidation is Cl^- (Wallace et al., 1974, 1982).

In the case of MbO_2, however, chloride anion showed no appreciable amount of enhancement in metMb formation, even if the concentrations were examined from 0.1 to 0.5 M over the whole range of pH studied (Satoh & Shikama, 1981). For the inverse dependence of the autoxidation rate upon O_2 pressure, therefore, it is very interesting to note that H_2O_2, which may be produced by dismutation of the superoxide anion generated from autoxidation of oxy form in eq 1, can oxidize deoxyMb more than 100 times more easily than can oxyMb (Yusa, 1984). Accordingly, it must act as at least one of the potent oxidants of the deoxy form that increases with decreasing O_2 pressures. This idea seems to be attractive for another possible interpretation of the O_2 dependence and remains open to our future study.

Brown and Mebine (1969) and Wallace et al. (1982) also agree with the involvement of a proton in the autoxidation reactions of MbO_2 and HbO_2, but their explanations are still qualitative in nature. Their rate equations are insufficient to analyze and interpret such complicated and different types of the pH dependence over the *wide* range of pH 5–13 as shown in Figure 1.

Although other mechanisms can not be ruled out, it is of great interest to see whether our proposal can be applied to the stability properties of other types of myoglobins from other sources of evolutional interest and also to see whether it can be applied to those of abnormal human hemoglobins with substitutions on the distal side such as Zurich[HisE7(63)β→Arg] and Sydney[ValE11(67)β→Ala] (Tucker et al., 1978). These examinations seem no doubt to contribute to a full understanding of the role of the distal histidine residue and heme environments in myoglobin–hemoglobin chemistry and in their evolution.

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References

Brown, W. D., & Mebine, L. B. (1969) *J. Biol. Chem. 244*, 6696–6701.

Fridovich, I. (1975) *Annu. Rev. Biochem. 44*, 147–159.

Gotoh, T., & Shikama, K. (1974) *Arch. Biochem. Biophys. 163*, 476–481.

Gotoh, T., & Shikama, K. (1976) *J. Biochem. (Tokyo) 80*, 397–399.

Katagiri, T. (1983) M.Sci. Thesis, Tohoku University, Sendai, Japan.

Phillips, S. E. V., & Schoenborn, B. P. (1981) *Nature (London) 292*, 81–82.

Satoh, Y., & Shikama, K. (1981) *J. Biol. Chem. 256*, 10272–10275.

Shikama, K. (1984) *Biochem. J. 223*, 279–280.

Shikama, K., & Sugawara, Y. (1978) *Eur. J. Biochem. 91*, 407–413.

Shikama, K., & Katagiri, T. (1984) *J. Mol. Biol. 174*, 697–704.

Shikama, K., Suzuki, T., Sugawara, Y., Katagiri, T., Takagi, T., & Hatano, M. (1982) *Biochim. Biophys. Acta 701*, 138–141.

Sugawara, Y., & Shikama, K. (1979) *Sci. Rep. Tohoku Univ., Ser. 4: 37*, 253–262.

Sugawara, Y., & Shikama, K. (1980) *Eur. J. Biochem. 110*, 241–246.

Suzuki, T., & Shikama, K. (1983) *Arch. Biochem. Biophys. 224*, 695–699.

Suzuki, T., Takagi, T., & Shikama, K. (1981) *Biochim. Biophys. Acta 669*, 79–83.

Takagi, T., Iida, S., Matsuoka, A., & Shikama, K. (1984) *J. Mol. Biol. 180*, 1179–1184.

Tentori, L., Vivaldi, G., Carta, S., Marinucci, M., Massa, A., Antonini, E., & Brunori, M. (1973) *Int. J. Pept. Protein Res. 5*, 187–200.

Tucker, P. W., Phillips, S. E. V., Perutz, M. F., Houtchens, R., & Caughey, W. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1076–1080.

Wallace, W. J., Maxwell, J. C., & Caughey, W. S. (1974) *FEBS Lett. 43*, 33–36.

Wallace, W. J., Houtchens, R. A., Maxwell, J. C., & Caughey, W. S. (1982) *J. Biol. Chem. 257*, 4966–4977.

Yusa, K. (1984) M.Sci. Thesis, Tohoku University, Sendai, Japan.