Oxymyoglobin (MbO₂) is oxidized easily to metmyoglobin (metMb) with generation of the superoxide anion, which can be converted by the spontaneous dismutation into H₂O₂, this being also a potent oxidant of MbO₂. In the presence of sodium azide in stoichiometric amounts, however, the rate of autoxidation of MbO₂ increased rapidly with increasing concentration of the anion, but soon reached a saturating level, the extent of which was about twice that of the normal autoxidation in buffer alone. Quantitative analysis has revealed that this enhancement is not due to the nucleophilic displacement of O₂⁻ from MbO₂ by the anion (Sato, Y., and Shikama, K. (1981) J. Biol. Chem. 256, 10272–10275), but is due to the additional oxidation of MbO₂ by H₂O₂ freed from the metMb being occupied by the anion at the sixth coordination position.

Based on these novel results and stoichiometric considerations, it is possible to propose a new view that H₂O₂ produced from O₂⁻ can be eliminated or decomposed mostly, if not completely, by the metMb resulting from the normal autoxidation reaction of MbO₂, presumably via the formation of the ferryl species.

In cardiac and skeletal muscle tissues, myoglobin plays an essential role in maintaining aerobic metabolism, both as an oxygen store and as an entity facilitating oxygen diffusion (1–3). With generation of the superoxide anion, however, MbO₂⁺ is easily oxidized to metMb, which cannot be oxygenated and is therefore physiologically inactive (4): MbO₂⁺ → metMb + O₂⁻. Recent kinetic and thermodynamic studies of this autoxidation reaction have revealed that its superoxide formation is not due to a simple, dissociative loss of O₂⁻ from MbO₂, but is due to a nucleophilic displacement of O₂⁻ from MbO₂ by a water molecule or a hydroxyl ion that can enter the heme pocket from the surrounding solvent (5–8).

On the other hand, the superoxide anion produced above can easily be converted into hydrogen peroxide by the following spontaneous dismutation with a high rate constant, for instance, of 2 × 10⁶ M⁻¹ s⁻¹ at pH 7.0 (9), and the resultant H₂O₂ may also turn to react with another MbO₂ to give metMb (10, 11): 2O₂⁻ + 2H⁺ → H₂O₂ + O₂. Several authors have therefore implicated involvement of subsequent side reactions of O₂⁻ and H₂O₂ with MbO₂ in myoglobin autoxidation, and the inhibitory effects of the presence of superoxide dismutase or/catalase were thus examined on the rate of autoxidation of MbO₂. However, the magnitude of this enzymatic suppression was smaller than the theoretically expected one and even varied considerably from literature to literature (4, 10, 12, 13). Thus, a full understanding of the overall stoichiometry for the autoxidation of MbO₂, which is of clinical as well as chemical interest, has not yet been achieved.

In this paper, we have studied the effect of sodium azide on the rate of autoxidation of MbO₂ since this anion in stoichiometric amounts reacts with metMb to form its azide complex so that one can expel the metMb in its reactivity from possible participation in the overall reaction of myoglobin autoxidation. Based on the novel findings, we then propose a minimal mechanism that H₂O₂ produced from the dismutation of O₂⁻ can be eliminated or decomposed mostly, if not completely, by the metMb resulting from the normal autoxidation reaction, presumably via the cyclic formation of the ferryl species.

MATERIALS AND METHODS

Chemicals—Sodium azide was of reagent grade from Merck, and used without further purification. All other chemicals were of reagent grade from Wako Pure Chemical, Osaka, and solutions were made with deionized and glass-distilled water.

Oxymyoglobin Preparation—Native oxymyoglobin was isolated directly from bovine heart muscle according to our standard procedure (14, 15). The essential step was the chromatographic separation of oxymyoglobin from metmyoglobin on a DEAE-cellulose column (Whatman, DE32). The concentration of myoglobin was determined, after conversion into cyanometmyoglobin, by using an extinction coefficient of 11.3 mM⁻¹ cm⁻¹ at 540 nm (16).

Catalase Preparation—Bovine liver catalase was prepared according to the method of Kitagawa and Shirakawa (17) and further recrystallized three times. The concentration of catalase was determined by using the absorption coefficient of 324 mM⁻¹ cm⁻¹ at 405 nm (18).

Oxidation Rate Measurements—In the presence of azide in a stoichiometric amount, the rate of autoxidation of MbO₂ (50 μM) was measured in 0.1 M buffer at 25 °C according to the following specifications. A 4-mL solution containing a given concentration of the salt in 0.1 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 1 mL of fresh MbO₂ 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 absorption spectrum from 550 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 metMb by the addition of potassium ferricyanide.

The buffers used were acetate and phosphate; the pH of the reaction mixture was checked carefully with a Hitachi-Horiba pH meter (Model F-755 II) before and after each run.

Spectrophotometric Measurements—Absorption spectra were recorded 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 K2) maintained to within ±0.1 °C.

Curve Fittings—The curve fittings were made by a least squares
RESULTS AND DISCUSSION

As suggested in the analogous case of hemoglobin autoxidation (10, 13), both the dismutation of superoxide anion and its direct electron donation to the coordinated dioxygen of MbO₂ may produce H₂O₂. However, since the dismutation of O₂ competes probably with its reaction with MbO₂, the following minimum mechanism has been proposed for an overall stoichiometry of the autoxidation of MbO₂ (12).

\[ 2\text{MbO}_2 \rightarrow 2\text{metMb} + 2\text{O}_2^- \]  

(1)

\[ 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_3 \]  

(2)

\[ 2\text{metMb} \rightarrow \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{metMb} + 2\text{H}_2\text{O} + 2\text{O}_2 \]  

(3)

\[ 4\text{MbO}_2 + 4\text{H}^+ \rightarrow 4\text{metMb} + 2\text{H}_2\text{O} + 3\text{O}_2 \]  

(4)

This mechanism certainly agrees with results of Brown and Mebine (19) that show 3/4 mol of O₂ is released during the autoxidation of 1 mol of MbO₂. It is also suggested by this mechanism that the rate of oxidation of MbO₂ should be decreased theoretically by 50% if it is measured in the presence of catalase.

Therefore, we have re-examined the inhibitory effect of catalase very carefully, but no reproducible result was obtained. From experiment to experiment by the addition of 0.1 μM catalase to 50 μM fresh MbO₂ solution in 0.1 M buffer, pH 7.0, at 25 °C, the observed retardation varied largely in its extent from only 6 to 22%, some being almost within the experimental deviations that appeared in the normal autoxidation rate. These observations seemed to indicate strongly that most, if not all, of the H₂O₂ produced by Reaction 2 may be eliminated by a path quite different from Reaction 3. Instead of using catalase, therefore, it seemed very interesting to probe the autoxidation reaction with azide, which may convert it to a catalytically inactive form.

In the presence of sodium azide in stoichiometric amounts, it is true that the rate of autoxidation of MbO₂ increased with increasing concentration of the salt. Fig. 1 shows examples of the spectral changes with time when fresh MbO₂ was oxidized in the presence of sodium azide in 0.1 M phosphate buffer, pH 7.2, at 25 °C. The final spectrum confirms that it is a mixture of metMb and its azide complex, the fraction of the latter species increasing with increasing concentration of the added azide, but each to an equilibrium extent.

This process of oxidation of MbO₂ with the isosbestic points occurring to the final state of each run, was therefore followed by a plot of \(-\ln([\text{MbO}_2]/[\text{MbO}_2])\) versus time \(t\), where the ratio of MbO₂ concentration after time \(t\) to that at time \(t = 0\) can be monitored by the absorbance ratio of \((A_0 - A_t)/(A_0 - A_{\infty})\) at 581 nm. Fig. 2 gives such a plot for the autoxidation of MbO₂ in the presence of sodium azide in 0.1 M phosphate buffer, pH 7.2, at 25 °C, and the observed first-order rate constant, \(k_{obs}\) in h⁻¹, was determined from the slope of each straight line.

As plotted in Fig. 3, this rate constant for the autoxidation of MbO₂ increased rapidly, over the normal autoxidation with \(k_0\) (i.e. the intercept on the ordinate) in buffer alone, with increasing concentration of the added N₃, but soon reached a saturating level, the extent of which seemed to be about double that of \(k_0\).

Here, if we assume that the formation of the metMb-azide complex may affect the autoxidation rate of MbO₂ in some manner and if we introduce the law of mass action to this binding process involved, our kinetic result may be written as

\[ k_{obs} = k_0 + k_{obs}/f_{max} \times [\text{N}_3]/1 + K_b [\text{N}_3] \]  

(5)

where \(k_0\) is the rate constant for the normal autoxidation of MbO₂ in buffer alone, \(f_{max}\) represents the maximal fraction of enhancement in the autoxidation rate by the presence of azide anion, and \(K_b\) is the binding constant for the reaction: \(\text{metMb} + \text{N}_3 \rightleftharpoons \text{metMb\cdotN}_3\).

Equation 5 can readily be rearranged to the linear forms that provide a convenient graphical method for determining the values of \(f_{max}\) and \(K_b\). By taking the reciprocal, for an example, a Lineweaver-Burk type form of Equation 5 can be obtained as

\[ \frac{k_0}{\Delta k_{obs}} = \frac{1}{f_{max} \cdot K_b} \times \frac{[\text{N}_3]}{1} \]  

(6)

where \(\Delta k_{obs} = k_{obs} - k_0\). Obviously a graph of \(k_0/\Delta k_{obs}\) versus \(1/[\text{N}_3]\) was linear with a slope of \(1/(f_{max} \cdot K_b)\) and an intercept of \(1/f_{max}\) on the ordinate axis, as shown in Fig. 4.

For further refinement of the values of \(f_{max}\) and \(K_b\) obtained above, iterative curve-fitting procedures were also carried out by a least squares method based on Equation 5 until the best
Autoxidation of MbO₂

Fig. 2. First-order plots for the autoxidation of MbO₂ in the presence of sodium azide in 0.1 M phosphate buffer, pH 7.2, at 25 °C. The concentration of MbO₂ was monitored by the absorbance changes at 581 nm. Conditions: 50 μM MbO₂ in buffer alone (O), and in the presence of 0.04 mM N₃⁻ (△), 0.22 mM N₃⁻ (△), and 1.50 mM N₃⁻ (●).

Fig. 3. A plot of kₐ,oₐ as a function of concentrations of the added azide anion for the autoxidation of MbO₂ in 0.1 M phosphate buffer, pH 7.2, at 25 °C. The computed curve (—) was obtained by least squares fitting to the experimental data (O), based on Equation 5 with the parameters of fₘₐₓ = 0.86 (±0.02) and pKₐ = -4.12 (±0.03). MbO₂ concentration: 50 μM.

Fig. 4. A Lineweaver-Burk reciprocal plot of kₐ,oₐ/Δkₐ,oₐ versus 1/[N₃⁻] for determination of the parameters of fₖ,max and Kₐₙ. See Equation 6 in the text.

Fig. 5. Plots of kₐ,oₐ as a function of concentrations of the added azide anion for the autoxidation of MbO₂ in 0.1 M acetate buffer, pH 5.0, at 25 °C. a, the observed rate constants (O) are plotted with a theoretical line (—) of kₐ = 2.6 × 10⁻² h⁻¹ mM⁻¹ for the displacing oxidation of MbO₂ by N₃⁻ at pH 5.0. See the text. b, the observed rate constants were corrected for the displacing oxidation involved, and analyzed by a least squares fitting, based on Equation 5. The computed curve (—) was obtained with the resulting parameters of fₘₐₓ = 1.00 (±0.03) and pKₐ = -4.37 (±0.03). Conditions: 50 μM MbO₂.

fit to the experimental values of kₐ,oₐ was obtained as a function of [N₃⁻] over the whole range examined in Fig. 3. In this way, the values of fₖₘₐₓ = 0.86 (±0.02) and pKₐ = -4.12 (±0.03) were established in 0.1 M phosphate buffer, pH 7.2, at 25 °C. The pKₐ value assigned for our enhancement reaction is in good accord with the literature value of pKₐ = -4.16 being obtained by Goldsack et al. (20) from the direct binding of N₃⁻ to metMb.

Here it should be clearly mentioned that the enhancement observed in the autoxidation rate by the presence of azide anion is not due to the nucleophilic displacement of O₂⁻ from MbO₂ by the anion leading to the formation of the metMb-anion complex. For the displacement oxidation of MbO₂, it has been shown in our previous paper (5) that its rate is linearly dependent upon the concentrations of the added anion, with an order of kₐ = 3.0 × 10⁻¹ h⁻¹ mM⁻¹ for N₃⁻ at pH 7.2 and 25 °C. Judging from the concentration range up to 2 mM used in the present study, therefore, it was quite apparent that the contribution of this displacing oxidation by N₃⁻ is negligibly small in its extent toward the value of kₐ,oₐ.

In the acidic range, on the other hand, the rate constant for this displacing oxidation is known to become much higher, so that its contribution to kₐ,oₐ may not be neglected completely. Fig. 5 demonstrates such a case in 0.1 M acetate buffer, pH 5.0, at 25 °C. The graph of kₐ,oₐ versus [N₃⁻] was corrected for the displacing oxidation by the anion on the basis of its rate constant of kₐ = 2.6 × 10⁻² h⁻¹ mM⁻¹ at pH 5.0 (5), and the values of fₖₘₐₓ = 1.00 (±0.03) and pKₐ = -4.37 (±0.03) were also established by the curve-fitting procedures. The resultant pKₐ was also in good accord with the literature
Autoxidation of MbO₂

These results strongly indicate that in the presence of N₃⁻ the metMb formed from MbO₂ reacts immediately with the anion binding at the sixth coordination position, so that the H₂O₂ produced from Reaction 2 may be freed to cause additional oxidation of MbO₂ to metMb, according to Reaction 3, with a fractional enhancement of fₓₓ in terms of the autoxidation rate. In the normal autoxidation reaction, therefore, most, if not all, of the H₂O₂ produced must be eliminated from the reaction system by the presence of the metMb formed from MbO₂ at the same time.

Based on these novel results and stoichiometric considerations, it is possible to propose the following minimum overall mechanism for the autoxidation of MbO₂ under air-saturated conditions.

\[
\begin{align*}
4\text{MbO}_2 & \rightarrow 4\text{metMb} + 4\text{O}_2^- \\
\text{O}_2^- + 4\text{H}^+ & \rightarrow 2\text{H}_2\text{O}_2 + 2\text{O}_2 \\
2\text{H}_2\text{O}_2 & \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\end{align*}
\]

Of course, this alternative mechanism also agrees with the previous findings of Brown and Mebine (19) that there is a net evolution of ¾ mol of O₂/mol of MbO₂ oxidized. On the other hand, little is known of the molecular mechanism for the decomposition of H₂O₂ by metMb, although the reaction of metMb with H₂O₂ has long been investigated by a number of authors (21-24). Relevant to the present problem, we are now studying in detail repeated cycles of the formation of ferryl-Mb and its decay to metMb in the presence of H₂O₂.

In conclusion, we have probed the autoxidation reaction of MbO₂ with azide anion and have found two mechanisms, both being essential for a full understanding of the overall stoichiometry of the reaction. From the previous study with the use of excess amounts of various anions including N₃⁻, it has been shown that the generation of O₂⁻ from MbO₂ is not due to a spontaneous dissociation but is due to a nucleophilic displacement of O₂⁻ from MbO₂, and that both the entering water molecule and the hydroxyl ion can react with MbO₂ as the most common nucleophiles in vivo (5). From the present study with the use of stoichiometric amounts of N₃⁻ it has also been shown that H₂O₂ produced from the dismutation of O₂ can be eliminated or decomposed mostly, if not completely, by the metMb resulting from the normal autoxidation of MbO₂. This conclusion can be reinforced more strongly by demonstrating that catalase prevents the enhancement effect of N₃⁻ on the rate of autoxidation of MbO₂. In this case, of course, it would be necessary to use the azide-insensitive, manganese-containing non-heme catalase of Lactobacillus plantarum (25).

REFERENCES