Kinetic analysis of myoglobin autoxidation by isoelectric-focusing electrophoresis

Akio TOMODA,* Takenori TAKIZAWA,* Akira TSUJI† and Yoshimasa YONEYAMA*
*Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920, Japan, and
†Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Ishikawa 920, Japan*
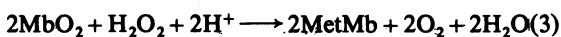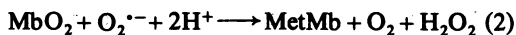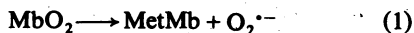
The autoxidation of horse myoglobin was studied in the presence or absence of catalase (EC 1.11.1.6) and/or superoxide dismutase (EC 1.15.1.1) at various pH values (6.6–7.8). Changes in the percentages of oxymyoglobin and metmyoglobin during the reaction were analysed by means of isoelectric focusing on Ampholine gel plates. Oxymyoglobin was decreased in a first-order manner, with an accompanying increase in metmyoglobin, under the various conditions studied. The observed reaction rate constants obtained under these conditions were pH-dependent; however, they were also greatly affected by the presence of the enzymes. The pH-dependence of the overall reaction was explained by the acid–base three-state model of myoglobin proposed by Shikama & Sugawara [(1978) *Eur. J. Biochem.* **91**, 407–413]. The reaction process of myoglobin autoxidation was explained by the model suggested by Winterbourn, McGrath & Carrell [(1976) *Biochem. J.* **155**, 493–502], indicating that superoxide anion and hydrogen peroxide are involved in the reaction mechanism.

Oxymyoglobin is converted into metmyoglobin spontaneously in the presence of air (George & Stratmann, 1954; Brown & Mebine, 1969; Gotoh & Shikama, 1974). However, the mechanism of this autoxidation remains to be completely clarified. Brown & Mebine (1969) demonstrated that $\frac{3}{4}$ molecule of O_2 is released during the autoxidation of 1 molecule of oxymyoglobin:

$$MbO_2 + H^+ \longrightarrow MetMb + \tfrac{3}{4}O_2 + \tfrac{1}{2}H_2O$$

This unusual number for O_2 was also indicated for haemoglobin autoxidation by Kikuchi *et al.* (1955), who suggested the involvement of H_2O_2 and free radicals in the reaction mechanism. Winterbourn *et al.* (1976) studied the effects of the superoxide anion $O_2^{\cdot-}$ on the autoxidation of haemoglobin, and proposed a plausible process for haemoglobin and myoglobin, considering the following overall stoicheiometry:

$$MbO_2 \longrightarrow MetMb + O_2^{\cdot-} \qquad (1)$$

$$MbO_2 + O_2^{\cdot-} + 2H^+ \longrightarrow MetMb + O_2 + H_2O_2 \quad (2)$$

$$2MbO_2 + H_2O_2 + 2H^+ \longrightarrow 2MetMb + 2O_2 + 2H_2O \quad (3)$$

In summary:

$$MbO_2 + H^+ \longrightarrow MetMb + \tfrac{3}{4}O_2 + \tfrac{1}{2}H_2O \qquad (4)$$

Abbreviations used (in equations and Figures): MbO_2, oxymyoglobin; MetMb, metmyoglobin.

This scheme suggests that $O_2^{\cdot-}$ is generated during the reaction, and that $O_2^{\cdot-}$ and H_2O_2 are involved in the reaction mechanism. Gotoh & Shikama (1976) showed that $O_2^{\cdot-}$ is generated during the autoxidation of myoglobin, and also showed that eqn. (1) takes place. Shikama & Sugawara (1978) proposed that the reaction proceeds by first-order in accordance with eqn. (1) stated above and the equation:

$$O_2^{\cdot-} + H^+ \longrightarrow \tfrac{1}{2}H_2O + \tfrac{3}{4}O_2$$
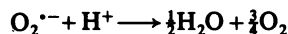
and that the two dissociation groups of myoglobin molecule are involved in the autoxidation reaction. However, the fact that the autoxidation of myoglobin was significantly suppressed in the presence of catalase (Gotoh & Shikama, 1976) cannot be explained by this model alone. In view of this fact, the models proposed by Winterbourn *et al.* (1976) seem more likely to be correct, because the involvement of H_2O_2 and $O_2^{\cdot-}$ is taken into account in the reaction mechanism.

In the present study we investigated the changes in the percentages of oxymyoglobin and metmyoglobin during the autoxidation of myoglobin under various conditions by using isoelectric-focusing electrophoresis. On the basis of the results, we tried to clarify the mechanism of myoglobin autoxidation, and compared our results with the reaction models already proposed.

Experimental

Horse myoglobin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in distilled water. The insoluble materials were removed by centrifugation at $10000g$ for 15 min at 4°C. The supernatant was applied, after adjustment of pH to 6.8 with 200 mM-KH_2PO_4, on a column (2 cm × 5 cm long) of CM-Sephadex C-50 (Seikagaku Kogyo, Tokyo, Japan) previously equilibrated with 10 mM-potassium phosphate buffer, pH 6.8. Metmyoglobin was partially purified by a pH gradient prepared from 100 ml of 10 mM-potassium phosphate buffer, pH 6.8, and 100 ml of 10 mM-K_2HPO_4. The partially purified metmyoglobin was converted into ferrous

myoglobin by the addition of a small excess of dithionite at 4°C. The solution was then passed through a column (2 cm × 25 cm long) of Sephadex G-25 (coarse grade; Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 50 mM-potassium phosphate buffer, pH 6.6, 7.0, 7.4 and 7.8, at 4°C. The effluents were used for the autoxidation experiment in the presence and in the absence of catalase (EC 1.11.1.6) (Boehringer Mannheim, Mannheim, West Germany) and superoxide dismutase (EC 1.15.1.1) (Sigma Chemical Co.) at 37°C.

Samples were removed from a small glass tube at intervals for analysis (every 2 h) and applied on an Ampholine gel plate (pH 3.5–9.5; LKB, Uppsala, Sweden). Isoelectric focusing was performed at 4°C for 1.5 h. Then the gel was fixed, and the percentages of oxymyoglobin and metmyoglobin during the autoxidation of myoglobin were measured as mentioned previously (Tomoda et al., 1979).

Results

Electrophoretic pattern of partially autoxidized myoglobin on Ampholine gel plate

The isoelectric-focusing pattern of the myoglobin solutions that were partially autoxidized during 4 h incubation at pH 7.0 at 37°C is shown in Fig. 1(a) along with those of authentic oxymyoglobin and
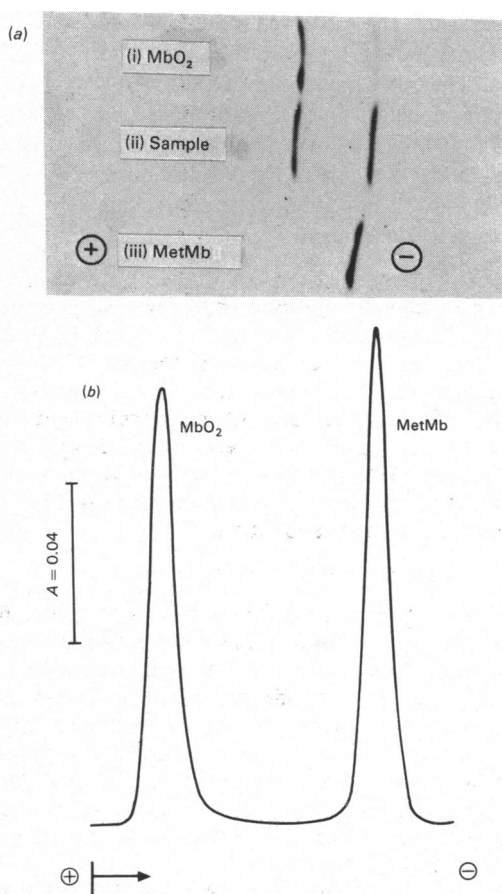

Fig. 1. Isoelectric-focusing pattern of partially autoxidized myoglobin

For full details see the text. (a) Protein band pattern of partially autoxidized myoglobin (ii) compared with that of authentic oxymyoglobin (i) and metmyoglobin (iii). (b) Gel-scanning pattern of partially autoxidized myoglobin performed at 630 nm on a Gilford 2400-S spectrometer with a gel scanner.
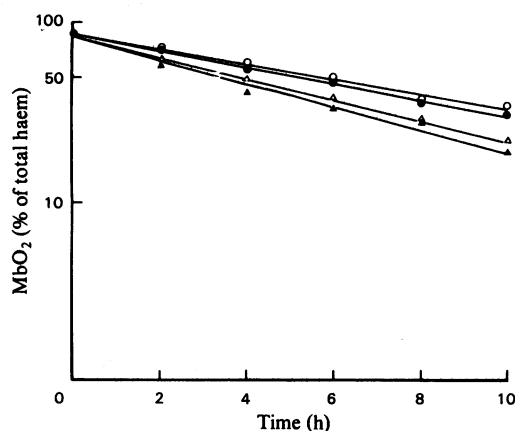

Fig. 2. Effects of superoxide dismutase and catalase on autoxidation of myoglobin at pH 7.0

For full details see the text. The autoxidation of myoglobin was studied at pH 7.0 at 37°C in the presence and in the absence of superoxide dismutase (29 units) and catalase (1300 units). ▲, Control; △, superoxide dismutase present; ●, catalase present; ○, superoxide dismutase + catalase present. The percentage fractions of oxymyoglobin during the autoxidation of myoglobin were determined by analysis by isoelectric-focusing electrophoresis.
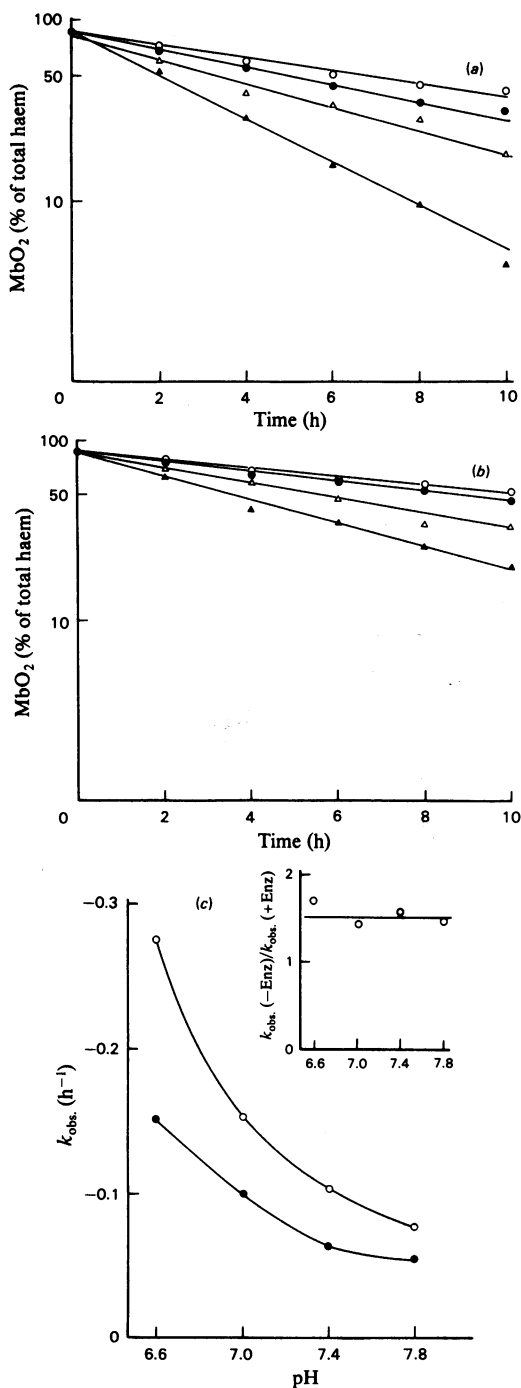
Fig. 3. *Effects of superoxide dismutase and catalase on autoxidation of myoglobin at various pH values*

For full details see the text. (*a*) The autoxidation of myoglobin was studied at pH 6.6 (▲), pH 7.0 (△), pH 7.4 (●) and pH 7.8 (○) at 37°C without addition of enzymes. (*b*) The autoxidation was studied at pH 6.6 (▲), pH 7.0 (△), pH 7.4 (●) and pH 7.8 (○) at 37°C in the presence of both superoxide dismutase (29 units) and catalase (1300 units). (*c*)

metmyoglobin. Oxymyoglobin and metmyoglobin in the solutions were separated from each other by this method. The electrophoretic pattern was further analysed by gel scanning as shown in Fig. 1(*b*). The percentages of oxymyoglobin and metmyoglobin compared with total haem in this sample were found to be 44.2% and 55.8% respectively.

Fractional changes in the percentage of oxymyoglobin during autoxidation of myoglobin under various conditions

Fig. 2 shows the fractional changes in the percentage of oxymyoglobin during the autoxidation reaction in the presence and in the absence of catalase, and of superoxide dismutase at pH 7.0, as shown by analysis by isoelectric-focusing electrophoresis. The oxymyoglobin decrease was first-order, but, however, was affected by the presence of the enzymes. From the results, the observed first-order reaction rate constants ($k_{obs.}$) were obtained. In the absence of superoxide dismutase and catalase $k_{obs.}$ was $-0.153\,h^{-1}$. In the presence of catalase $k_{obs.}$ was decreased by 31% ($k_{obs.} = -0.105\,h^{-1}$). The reaction rate constant in the presence of both superoxide dismutase and catalase was decreased by 35% ($k_{obs.} = -0.100\,h^{-1}$). The addition of superoxide dismutase alone decreased the rate constants by 10% ($k_{obs.} = -0.137\,h^{-1}$).

The effect of superoxide dismutase and catalase on myoglobin autoxidation was investigated at various pH values (6.6–7.8) (Figs. 3*a* and 3*b*). As shown in Fig. 3(*a*), the autoxidation of myoglobin was dependent on pH, i.e. the reaction rates were accelerated in the acidic regions in the absence of both catalase and superoxide dismutase. This tendency was observed in the presence of both catalase and superoxide dismutase at pH regions studied, though the reaction rates were much decreased by the enzymes (Fig. 3*b*).
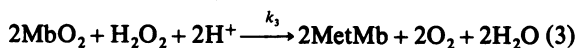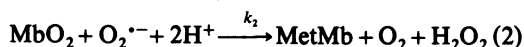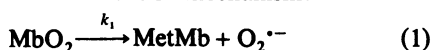
Fig. 3(*c*) shows the pH profiles of the observed first-order rate constants obtained from Figs. 3(*a*) and 3(*b*). The observed first-order rate constants were closedly correlated to the changes in pH in spite of the presence or absence of the enzymes. However, the ratio of $k_{obs.}$ (−Enz) to $k_{obs.}$ (+Enz) (about 1.6) was not altered by the changes in pH (inset of Fig. 3*c*).

Discussion

We showed that oxymyoglobin is autoxidized to metmyoglobin directly and that the reaction pro-

The observed first-order rate constants obtained from (*a*) (○) and (*b*) (●) were plotted against pH of the solution. The inset shows the ratio of $k_{obs.}$ (−Enz) (i.e. observed first-order rate constants in the absence of superoxide dismutase and catalase) to $k_{obs.}$ (+Enz) (i.e. observed rate constants in the presence of the enzymes) at various pH values.

ceeds as first-order, by using the isoelectric-focusing technique (Figs. 1–3). By comparison of the observed first-order reaction rate constants obtained under various conditions (Figs. 2 and 3), it is possible to deduce the mechanism of the autoxidation of myoglobin. Winterbourn *et al.* (1976) suggested that the following processes for myoglobin autoxidation are probable from their results on haemoglobin autoxidation, because $O_2^{\cdot-}$ and H_2O_2 were involved in the reaction mechanism:
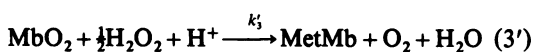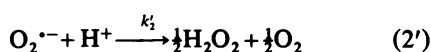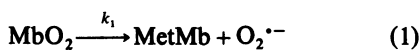
$$MbO_2 \xrightarrow{k_1} MetMb + O_2^{\cdot-} \qquad (1)$$

$$MbO_2 + O_2^{\cdot-} + 2H^+ \xrightarrow{k_2} MetMb + O_2 + H_2O_2 \quad (2)$$

$$2MbO_2 + H_2O_2 + 2H^+ \xrightarrow{k_3} 2MetMb + 2O_2 + 2H_2O \quad (3)$$

Sum:

$$4MbO_2 + 4H^+ \xrightarrow{k_4} 4MetMb + 3O_2 + 2H_2O \quad (4)$$

The stoicheiometry of eqn. (4) was shown experimentally by Brown & Mebine (1969). By this mechanism the oxidation rates of oxymyoglobin should theoretically decrease by 50% in the presence of catalase, by 25% in the presence of superoxide dismutase and by 75% in the presence of both enzymes.

It is probable that the dismutation of superoxide anion competes with its reaction with MbO_2, and results in the same stoicheiometry, i.e.:

$$MbO_2 \xrightarrow{k_1} MetMb + O_2^{\cdot-} \qquad (1)$$

$$O_2^{\cdot-} + H^+ \xrightarrow{k_2'} \tfrac{1}{2}H_2O_2 + \tfrac{1}{2}O_2 \qquad (2')$$

$$MbO_2 + \tfrac{1}{2}H_2O_2 + H^+ \xrightarrow{k_3'} MetMb + O_2 + H_2O \quad (3')$$

Sum:

$$2MbO_2 + 2H^+ \xrightarrow{k_4'} 2MetMb + 1\tfrac{1}{2}O_2 + H_2O \quad (4')$$

By this mechanism, the oxidation rates of myoglobin should theoretically decrease by 50% in the presence of catalase alone and of both catalase and superoxide dismutase. Our estimation at pH 7.0 (31% by catalase, 35% by superoxide dismutase and catalase, and 10% by superoxide dismutase) may be reasonable, though somewhat smaller than the expected values. Present results greatly support the views that $O_2^{\cdot-}$ and H_2O_2 participate in the autoxidation reaction of myoglobin and that the reaction will, in consequence, proceed according to the chain reactions (1)–(3) and (1)–(3').

The chain reactions such as eqns. (1)–(3) and (1)–(3') imply, from the kinetic viewpoint, that the autoxidation of myoglobin will proceed sigmoidally, as was demonstrated in the nitrite oxidation of haemoglobin (Tomoda *et al.*, 1981). In spite of this

expectation, the reaction proceeded as first-order in the experiment. This discrepancy may be explained by the kinetics shown below.

From eqns. (1), (2') and (3'), the following rate equations for each reaction species would be written:

$$\frac{d[MbO_2]}{dt} = -k_1[MbO_2] - k_3'[MbO_2][H_2O_2]^{\frac{1}{2}}[H^+] \quad (I)$$

$$\frac{d[O_2^{\cdot-}]}{dt} = k_1[MbO_2] - k_2'[O_2^{\cdot-}][H^+] \quad (II)$$

$$\frac{d[H_2O_2]}{dt} = k_2'[O_2^{\cdot-}][H^+]$$
$$- k_3'[MbO_2][H_2O_2]^{\frac{1}{2}}[H^+] \quad (III)$$

Since eqns. (2') and (3') are extremely fast compared with eqn. (1), the steady-state assumption for $O_2^{\cdot-}$ and H_2O_2 may be valid to produce:

$$\frac{d[O_2^{\cdot-}]}{dt} = 0 \quad \text{and} \quad \frac{d[H_2O_2]}{dt} = 0$$

Therefore:

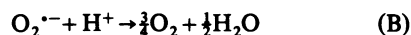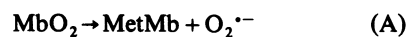$$[O_2^{\cdot-}] = \frac{k_1[MbO_2]}{k_2'[H^+]} \quad (IV)$$

$$[H_2O_2]^{\frac{1}{2}} = \frac{k_2'[O_2^{\cdot-}]}{k_3'[MbO_2]}$$
$$= \frac{k_1}{k_3'[H^+]} \quad (V)$$

$$\frac{d[MbO_2]}{dt} = -2k_1[MbO_2] \quad (VI)$$

Eqn. (VI) means that the reaction proceeds as first-order apparently, though the autoxidation reaction of myoglobin should essentially conform to the sigmoidal kinetics.

Furthermore, eqn. (VI) means that the overall rates of myoglobin autoxidation are not influenced by changes in pH. However, our results in Figs. 3(*a*) and 3(*b*) that the overall reaction rates are dependent on pH are inconsistent with this expectation. Why did this pH-dependence appear in the experiment? This may be explained by the results obtained by Shikama & Sugawara (1978) for whale myoglobin autoxidation. They considered that the reaction will proceed as:

$$MbO_2 \rightarrow MetMb + O_2^{\cdot-} \qquad (A)$$

$$O_2^{\cdot-} + H^+ \rightarrow \tfrac{3}{4}O_2 + \tfrac{1}{2}H_2O \qquad (B)$$

Sum:

$$MbO_2 + H^+ \rightarrow MetMb + \tfrac{3}{4}O_2 + \tfrac{1}{2}H_2O$$

In order to explain the pH-dependence of myoglobin autoxidation by a scheme including eqn. (A), they introduced the acid–base three-state model of myoglobin, indicating that three species of myoglobin, H^+-dissociated, OH^--dissociated and undissociated, whose percentage fractions vary according to the pH, are in equilibrium with each other, and are autoxidized separately. This view was tested experimentally by them, and is just applicable to the eqn. (1) in the present paper. Since autoxidation rates were dependent on pH in the presence of superoxide dismutase and catalase, where eqns. (2') and (3') are suppressed (Fig. 3c), this finding is probably due to the fact that the eqn. (1) will proceed according to the acid–base three-state model. If the pH-dependence of the overall autoxidation of myoglobin is due to only the acid–base three-state model of eqn. (1), it should be satisfied that the ratio of $k_{obs.}$ (−Enz) [which includes eqns. (1), (2') and (3')] to $k_{obs.}$ (+Enz) [which includes only eqn. (1)] will approximate to 2 in spite of the changes in pH. This was clarified by the results shown in the inset of Fig. 3(c), though the ratio of $k_{obs.}$ (−Enz) to $k_{obs.}$ (+Enz) was somewhat smaller than expected.

With regard to eqns. (1)–(3), the same conclusion was obtained, because the oxidation rate of myoglobin was kinetically expressed as:

$$\frac{d[MbO_2]}{dt} = -3k_1[MbO_2]$$

In this case, the value of the $k_{obs.}$ (−Enz)/$k_{obs.}$ (+Enz) ratio is expected to be 3 theoretically. Therefore our results in the inset of Fig. 3(c) support the view that eqns. (1)–(3') will be more probable than eqns. (1)–(3).

Summing up the results stated above, the autoxidation of myoglobin will proceed according to the chain reactions shown by eqns. (1), (2') and (3'), where $O_2^{\cdot-}$ and H_2O_2 are involved. The pH-dependence of the reaction may be explained by the acid–base three-state model proposed by Shikama & Sugawara (1978).

References

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

George, P. & Stratmann, C. J. (1954) *Biochem. J.* **57**, 568–573

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

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

Kikuchi, G., Shukuya, R., Suzuki, M. & Nakamura, C. (1955) *J. Biochem. (Tokyo)* **42**, 3–20

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

Tomoda, A., Imoto, M., Hirano, M. & Yoneyama, Y. (1979) *Biochem. J.* **181**, 505–507

Tomoda, A., Tsuji, A. & Yoneyama, Y. (1981) *Biochem. J.* **193**, 169–179

Winterbourn, C. C., McGrath, B. M. & Carrell, R. (1976) *Biochem. J.* **155**, 493–502