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Changes in intermediate haemoglobins during autoxidation of haemoglobin

Akio TOMODA,* Yoshimasa YONEYAMA* and Akira TSUJI†
**Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa 920, Japan, and*
†Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan

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The time course of haemoglobin autoxidation was studied under various conditions at 37°C, and the changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during the reaction were analysed by isoelectric focusing on Ampholine/polyacrylamide-gel plates. Under various conditions (10mM-phosphate buffer, 10mM-phosphate buffer with 0.1M-phosphate buffer, 10mM-phosphate buffer with 0.1M-NaCl, and 10mM-phosphate buffer with 0.5mM-inositol hexaphosphate; pH range 6.6–7.8 in each case), the intermediate haemoglobins were found to be present as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids from their characteristic positions on electrophoresis. Oxyhaemoglobin changed consecutively to $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$, which were further oxidized to methaemoglobin, and the amounts of $(\alpha^{3+}\beta^{2+})_2$ were greater than those of $(\alpha^{2+}\beta^{3+})_2$ during the reaction. The modes of the quantitative changes in oxyhaemoglobin, intermediate haemoglobins, and methaemoglobin were very similar in all the media used except for the inositol hexaphosphate addition. In the presence of inositol hexaphosphate, the autoxidation rates were considerably accelerated, and the modes of the changes in the haemoglobin derivatives were also considerably altered; the effects of this organic phosphate were maximal at acidic pH and minimal at alkaline pH. It was concluded that haemoglobin autoxidation proceeds by first-order kinetics through two paths:

$$\text{Oxyhaemoglobin} \xrightarrow{k_{+1}} (\alpha^{2+}\beta^{3+})_2 \xrightarrow{k_{+2}} \text{methaemoglobin},$$

and

$$\text{Oxyhaemoglobin} \xrightarrow{k_{+3}} (\alpha^{3+}\beta^{2+})_2 \xrightarrow{k_{+4}} \text{methaemoglobin}$$

The reaction rate constants (k_{+1}–k_{+4}) best fitting all experimental values obtained by the isoelectric-focusing analysis were evaluated. By using these values, the mechanism of haemoglobin autoxidation is discussed.

Although the autoxidation of haemoglobin has been extensively investigated (Brooks, 1935; Kikuchi *et al.*, 1955; Mansouri & Winterhalter, 1973), an understanding of the reaction mechanism has not yet been reached. Kikuchi *et al.*, (1955) suggested that H_2O_2 and some free radicals play an important role in the autoxidation of haemoglobin. Later, Misra & Fridovich (1972) demonstrated that superoxide radical ($O_2^{-\cdot}$) is released during the autoxidation of shark haemoglobin. Furthermore, Winterbourn *et al.* (1976) showed that $O_2^{-\cdot}$ and H_2O_2 are involved in the autoxidation of haemoglobin, and they inferred that the reaction proceeds as follows:

$$Hb(O_2)_4 \rightarrow \text{metHb} + 4O_2^{-\cdot} \quad (1)$$

$$Hb(O_2)_4 + 4O_2^{-\cdot} + 8H^+ \rightarrow \text{metHb} + 4O_2 + 4H_2O_2 \quad (2)$$

$$2Hb(O_2)_4 + 4H_2O_2 + 8H^+ \rightarrow 2\text{metHb} + 8O_2 + 8H_2O \quad (3)$$

Totally, $Hb(O_2)_4 + 4H^+ \rightarrow \text{metHb} + 3O_2 + 2H_2O$ (4)

On the other hand, Mansouri & Winterhalter (1973) suggested from their study on haemoglobin autoxidation that intermediate haemoglobins such as

Abbreviations used: P_6-inositol, inositol hexaphosphate; $Hb(O_2)_4$, oxygenated haemoglobin tetramer; $IntHb(O_2)_2$, oxygenated intermediate haemoglobins including $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$; metHb, methaemoglobin tetramer.

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$(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ will be produced during the reaction, because there were many differences in the autoxidation rates between α- and β-chains in the haemoglobin tetramer. However, detailed studies on these intermediate haemoglobins were not performed. We recently showed by isoelectric focusing that intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids were formed during the chemical oxidation of haemoglobin with some oxidants (Tomoda & Yoneyama, 1979; Tomoda *et al.*, 1980, 1981*a*).

The present paper describes our study on the time course of haemoglobin autoxidation under various conditions and analysis of the quantitative changes in oxyhaemoglobin, intermediate haemoglobins, and methaemoglobin under various conditions by using the isoelectric-focusing technique. By using this method, the course of haemoglobin autoxidation and mechanism of the reaction have been extensively explored.

Experimental

Materials

DEAE-cellulose DE-32 and Sephadex G-25 (Coarse grade) were purchased from Whatman (Maidstone, Kent, U.K.) and Pharmacia Fine Chemicals (Uppsala, Sweden) respectively. Ampholine/polyacrylamide-gel plates (pH 3.5–9.5) were purchased from LKB Produkter (Bromma 1, Sweden).

Methods

Freshly obtained human blood samples (collected with the informed consent of donors) were centrifuged at 3000 *g* for 10 min. After removal of supernatant and buffy coats, the erythrocytes were washed three times with ice-cold 0.9% NaCl solution by centrifugation at 3000 *g* for 10 min. Then the erythrocytes were haemolysed by the addition of 5 vol. of ice-cold distilled water, and further centrifuged at 10000 *g* for 20 min. A 5 ml portion of the supernatant was passed through a column (3 cm × 2 cm long) of DE-32 cellulose that had been previously equilibrated with 5 mM-potassium phosphate buffer, pH 7.0. The sample proved to be free of catalase (EC 1.11.1.6) and superoxide dismutase (EC 1.15.1.1). The haemoglobin solution was further passed through a column (2 cm × 30 cm long) of Sephadex G-25 previously equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. The effluent was used for the experiments after adjustment of the solution to pH 6.6, 7.0, 7.4 and 7.8 with 10 mM-K_2HPO_4 or -KH_2PO_4 solutions.

After the addition of final concentrations of 0.1 M-potassium phosphate (pH 6.6, 7.0, 7.4 and 7.8), or 0.1 M-NaCl, and 0.5 mM-P_6-inositol to the haemoglobin solutions, the pH of the haemoglobin solutions was re-adjusted at 37°C with 10 mM-K_2HPO_4 or -KH_2PO_4 solutions. Then the solutions (380 μM in haem) were incubated at 37°C for 56 h in small glass tubes. Samples were removed at constant intervals for analysis and were applied on Ampholine/polyacrylamide-gel plates. Isoelectric focusing was performed at 4°C as described previously (Tomoda *et al.*, 1978). After electrophoresis, the gel plate was fixed (Tomoda *et al.*, 1978) and was gel-scanned at 630 nm by a Shimazu dual-wavelength t.l.c. scanner (LS-900). The percentages of oxyhaemoglobin, intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$, and methaemoglobin were determined by cutting out and weighing the chart paper. The observed values were inserted into the differential equations below.

Simulation of the gel-scanning pattern of haemoglobin autoxidation

The fractions of oxyhaemoglobin, intermediate haemoglobins and methaemoglobin as a function of time were fitted by using eqns. (1)–(4) derived from Scheme 1 through non-linear regression analysis (NONLIN program; Metzler, 1969). The digital computer FACOM M-160 was used at the Data-Processing Centre, Kanazawa University.

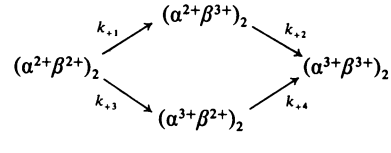
$$f_1 = e^{-(k_{+1}+k_{+3})t} \quad (1)$$

$$f_2 = \frac{k_{+1}}{k_{+1}+k_{+3}-k_{+2}} \cdot [e^{-k_{+2}t} - e^{-(k_{+1}+k_{+3})t}] \quad (2)$$

$$f_3 = \frac{k_3}{k_{+1}+k_{+3}-k_{+4}} \cdot [e^{-k_{+4}t} - e^{-(k_{+1}+k_{+3})t}] \quad (3)$$

$$f_4 = 1.0-(f_1+f_2+f_3) \quad (4)$$

where f_1, f_2 and f_3 are the fractions of total haem at time t of $(\alpha^{2+}\beta^{2+})_2$, $(\alpha^{2+}\beta^{3+})_2$, $(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{3+}\beta^{3+})_2$ respectively. The differential equations for four species of haem molecules can be solved by using appropriate Laplace-transformation techniques to yield eqns. (1)–(3) for the fractions of oxyhaemoglobin and intermediate haemoglobins of total haem. The mass balance produces eqn. (4) for the fraction of methaemoglobin.



Scheme 1. *Autoxidation of oxyhaemoglobin*

Results

Isoelectric-focusing patterns of partially autoxidized haemoglobin solutions

Fig. 1 shows the isoelectric-focusing pattern of the partially autoxidized haemoglobin solutions (10mM-phosphate buffer with or without P_6-inositol, pH 6.6–7.8) at 24h on Ampholine/polyacrylamide-gel plates. The results indicate that the two intermediate haemoglobins are formed in addition to methaemoglobin during the autoxidation of haemoglobin, though the distribution of these haemoglobin derivatives is different under the conditions studied. The intermediate haemoglobins were identified as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids from their characteristic positions on electrophoresis (Tomoda *et al.*, 1978). The electrophoretic patterns show that the rates of autoxidation of haemoglobin are faster at acidic pH values and are accelerated in the presence of P_6-inositol.

The samples at pH 7.0 with or without P_6-inositol were further gel-scanned at 630nm (Figs. 2*a* and 2*b*). As a consequence it was found that the samples without P_6-inositol are composed of 38.7% oxyhaemoglobin, 23.8% $(\alpha^{2+}\beta^{3+})_2$, 29.1% $(\alpha^{3+}\beta^{2+})_2$ and 8.4% methaemoglobin. In the case with P_6-inositol, the solutions were found to be composed of 13.2% oxyhaemoglobin, 18.0% $(\alpha^{2+}\beta^{3+})_2$, 37.5% $(\alpha^{3+}\beta^{2+})_2$, and 31.3% methaemoglobin. These results demonstrate that: (1) haemoglobin is autoxidized to methaemoglobin through two intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$; (2) P_6-inositol modifies the oxidation process of oxyhaemoglobin and intermediate haemoglobins sig-

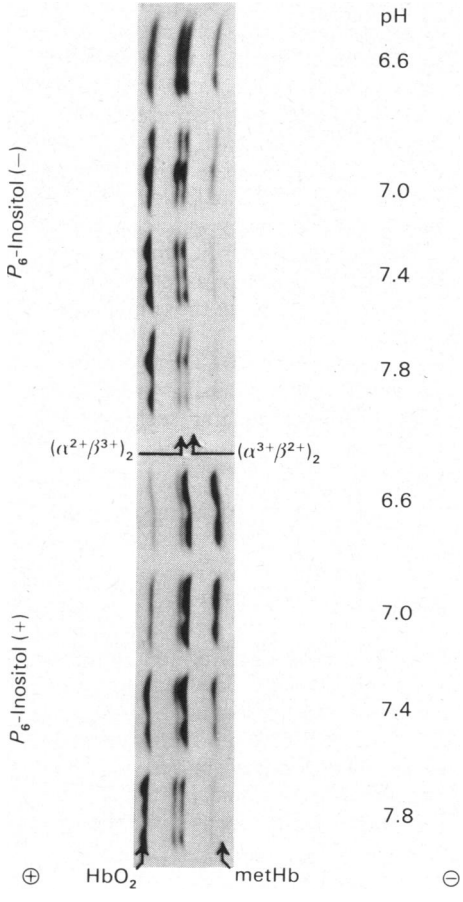


Fig. 1. *Isoelectric-focusing pattern for partially autoxidized haemoglobin at various pH values*
The samples at 24h obtained from the partially autoxidized haemoglobin solutions (10mM-phosphate buffer, pH 6.6–7.8, in the presence or absence of P_6-inositol) were applied to an Ampholine/polyacrylamide gel plate (pH 3.5–9.5). The isoelectric focusing was performed at 4°C for 1.5h.

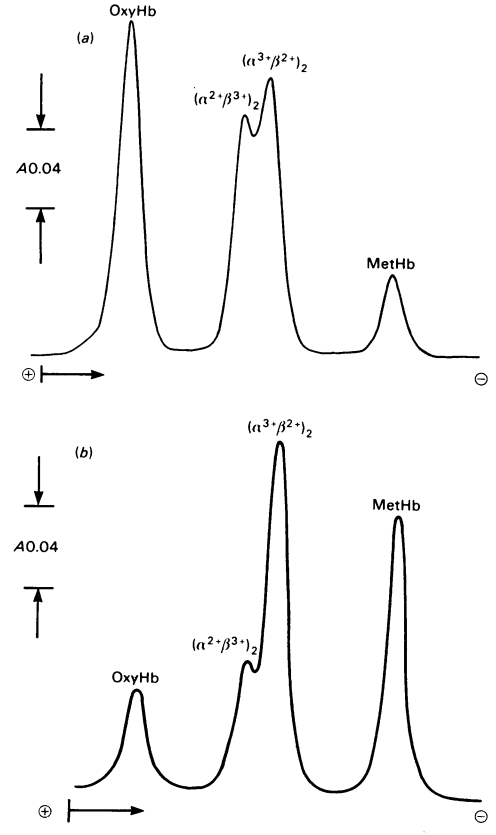


Fig. 2. *Gel-scanning pattern of partially autoxidized haemoglobin*
The samples at pH 7.0 in Fig. 1 were gel-scanned at 630nm. (*a*) Without P_6-inositol; (*b*) with P_6-inositol.

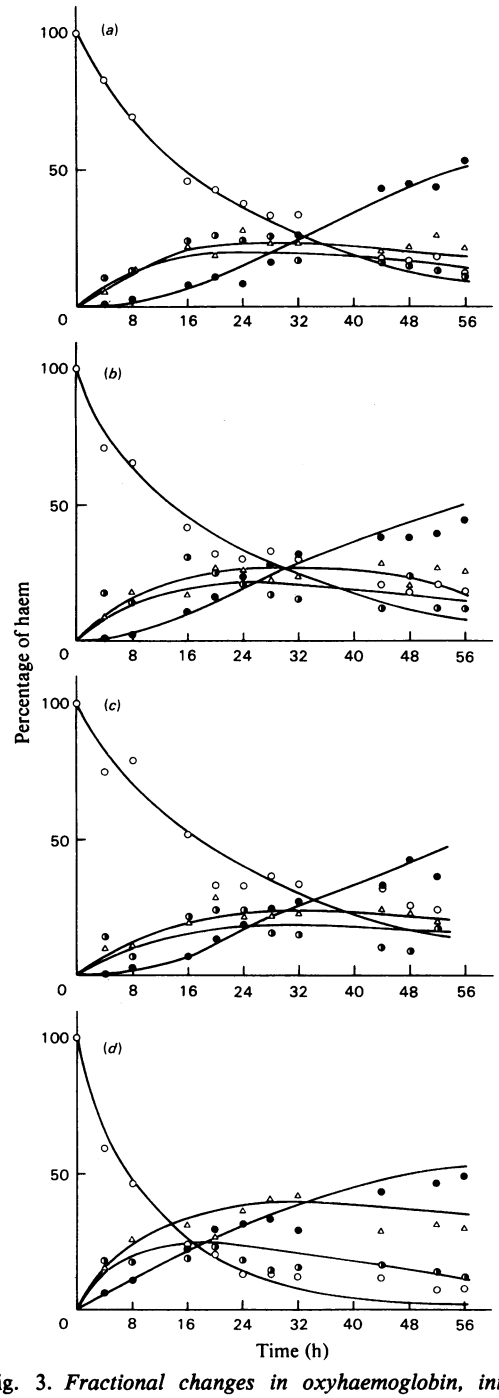


Fig. 3. *Fractional changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during the autoxidation of haemoglobin under various conditions at pH 7.0*

After isoelectric focusing of the samples (10mM-phosphate buffer, pH 7.0, with or without 0.1M-phosphate buffer, 0.1M-NaCl and 0.5mM-P_6-inositol), which were obtained at constant intervals (0–56h), were gel-scanned at 630nm. Then the fractions of oxyhaemoglobin, $(\alpha^{2+}\beta^{3+})_2$, $(\alpha^{3+}\beta^{2+})_2$ and methaemoglobin were estimated. ○, Oxyhaemoglobin; ◑, $(\alpha^{2+}\beta^{3+})_2$; △, $(\alpha^{3+}\beta^{2+})_2$; ●, methaemoglobin. (*a*) 10mM-Phosphate buffer alone; (*b*) 10mM-phosphate buffer + 0.1M-phosphate buffer; (*c*) 10mM-phosphate buffer + 0.1M-NaCl; (*d*) 10mM-phosphate buffer + 0.5mM-P_6-inositol.

nificantly, and this is mentioned in detail in the Discussion section.

Fractional changes in oxyhaemoglobin, $(\alpha^{2+}\beta^{3+})_2$, $(\alpha^{3+}\beta^{2+})_2$ and methaemoglobin during autoxidation of haemoglobin

The electrophoretic patterns of the sample obtained at constant intervals were gel-scanned, and the fractions of oxyhaemoglobin, intermediate haemoglobins and methaemoglobin were determined as described above. Figs. 3*a*–3*d* show the quantitative changes in oxyhaemoglobin, $(\alpha^{2+}\beta^{3+})_2$, $(\alpha^{3+}\beta^{2+})_2$ and methaemoglobin during the autoxidation of haemoglobin at pH 7.0 in the various solutions (10mM-phosphate buffer alone, 10mM-phosphate buffer with 0.1M-phosphate buffer, 10mM-phosphate buffer with 0.1M-NaCl and 10mM-phosphate buffer with 0.5mM-P_6-inositol). The concentration of each of the components, oxyhaemoglobin, $(\alpha^{2+}\beta^{3+})_2$, $(\alpha^{3+}\beta^{2+})_2$ and methaemoglobin, changed with time in all cases (Figs. 3*a*–3*d*). Except in the presence of P_6-inositol, the fractional changes with time were almost the same (Figs. 3*a*–3*c*). However, the extents of change considerably altered in the presence of P_6-inositol, i.e., the autoxidation rates were considerably accelerated and the changes in haemoglobin derivatives became rapid (Fig. 3*d*). In all cases the amounts of $(\alpha^{3+}\beta^{2+})_2$ were greater than those of $(\alpha^{2+}\beta^{3+})_2$ during the reaction.

By the non-linear curve-fitting analysis of each plot described above by using the equations mentioned in the Experimental section (f_1–f_4), it is possible to estimate the reaction rate constants for each phase:

$$\text{Oxyhaemoglobin} \xrightarrow{k_{+1}} (\alpha^{2+}\beta^{3+})_2 \xrightarrow{k_{+2}} \text{methaemoglobin}$$

and

$$\text{Oxyhaemoglobin} \xrightarrow{k_{+3}} (\alpha^{3+}\beta^{2+})_2 \xrightarrow{k_{+4}} \text{methaemoglobin}$$

under various conditions at pH 7.0. The reaction rate constants thus obtained are listed in Table 1. The reaction rate constants k_{+1}–k_{+4} were very similar when measured in 10mM-phosphate buffer alone, 10mM-phosphate buffer with 0.1M-phosphate buffer, or 10mM-phosphate buffer with 0.1M-NaCl. In these cases, k_{+3} was somewhat greater than

Table 1. *Rate constants estimated by non-linear least-squares analysis for the autoxidation of haemoglobin under various conditions at pH 7.0*

The values represent the apparent first-order rate constants ± S.D. The rate constants k_{+1}, k_{+2}, k_{+3}, k_{+4} best fitting to all experimental points were obtained by use of NONLIN computer program (Metzler, 1969) and eqns. (1)–(4) in the Experimental section.

Rate constant	Conditions	$10^2 \times$ Rate constant (h^{-1})			
		10 mM-P_i	10 mM-P_i + 0.1 M-P_i	10 mM-P_i + 0.1 M-NaCl	10 mM-P_i + 0.5 mM-P_6-inositol
k_{+1}		1.88 ± 0.11	1.96 ± 0.18	1.61 ± 0.16	3.76 ± 0.34
k_{+2}		2.56 ± 0.11	2.30 ± 0.15	2.58 ± 0.14	3.42 ± 0.31
k_{+3}		2.22 ± 0.11	2.38 ± 0.17	2.08 ± 0.17	3.92 ± 0.21
k_{+4}		2.79 ± 0.09	2.05 ± 0.18	2.59 ± 0.15	0.73 ± 0.11

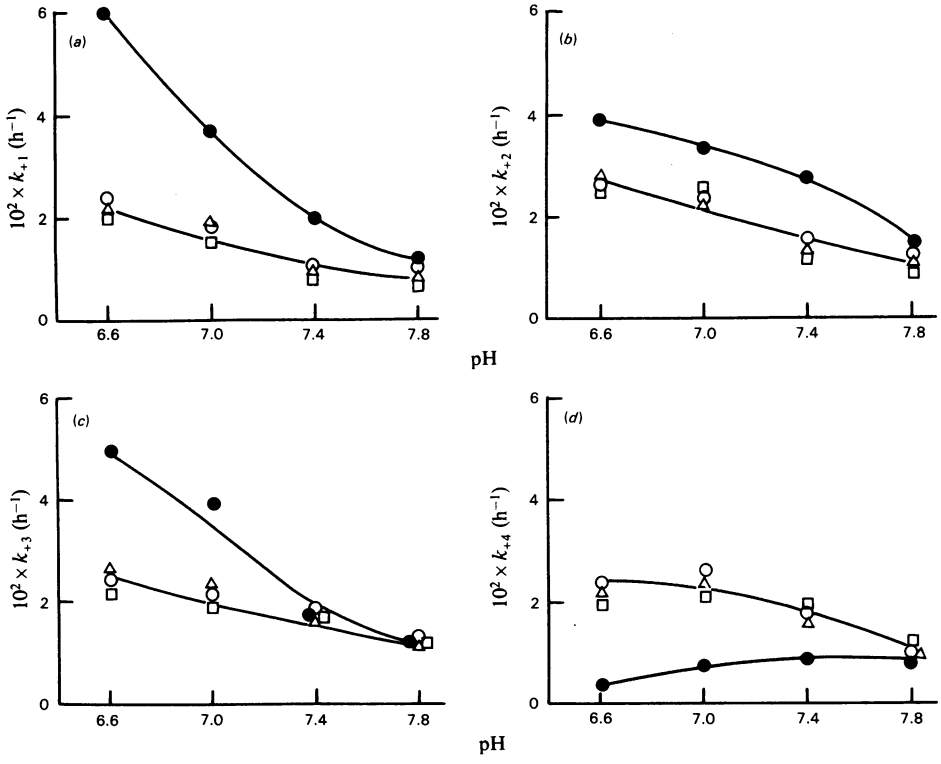


Fig. 4. *pH profiles of apparent first-order rate constants under various conditions*

The reaction rate constants obtained under various conditions (10 mM-phosphate buffer alone, 10 mM-phosphate buffer with 0.1 M-phosphate buffer, 10 mM-phosphate buffer with 0.1 M-NaCl, and 10 mM-phosphate buffer with 0.5 mM-P_6-inositol; pH range, 6.6–7.8) were plotted against pH. (*a*) pH profiles of k_{+1}; (*b*) pH profiles of k_{+2}; (*c*) pH profiles of k_{+3}; (*d*) pH profiles of k_{+4}. ○, 10 mM-Phosphate buffer alone; △, 10 mM-phosphate buffer with 0.1 M-inorganic phosphate; □, 10 mM-phosphate buffer with 0.1 M-NaCl; ●, 10 mM-phosphate buffer with 0.5 mM-P_6-inositol.

k_{+1}. In the presence of P_6-inositol, k_{+1}, k_{+2} and k_{+3} were increased as much as about 2-fold respectively, whereas k_{+4} was decreased.

Further, the quantitative changes in these haemoglobin derivatives during the autoxidation of haemoglobin were investigated under these conditions at various pH values (6.6–7.8) (results not shown). From the results, the reaction rate constants (k_{+1}–k_{+4}) under the various conditions were estimated. The pH profiles of the values are shown in Figs. 4(*a*)–4(*d*). As a result, it was found that these reaction rate constants were pH-dependent, i.e., the values were increased at acidic pH values. k_{+1}, k_{+2} and k_{+3} especially were much increased by the

presence of P_6-inositol at acidic pH values. The effects of P_6-inositol almost disappeared at pH 7.8. The reaction rate constant k_{+4}, however, was significantly decreased in the presence of P_6-inositol at acidic pH values.

Discussion

It has been suggested by Kikuchi *et al.* (1955) that H_2O_2 and some free radicals are involved in the autoxidation reaction of haemoglobin. They showed that 3 M-O_2 is released from 1 M-haemoglobin tetramer (4 M-haem) during the autoxidation reaction of haemoglobin. This stoichiometry is also demonstrated for the autoxidation of myoglobin by Brown & Mebine (1969).

Winterbourn *et al.* (1976) showed that $O_2^{-\cdot}$ and H_2O_2 are involved in the autoxidation mechanism of haemoglobin, and they suggested the chain reactions given in the introduction as a probable process for the autoxidation reaction. These equations are consistent with those proposed by Kikuchi *et al.* (1955). They also indicated that the dismutation of $O_2^{-\cdot}$ can compete with its reaction with $Hb(O_2)_4$, and results in the same stoichiometry:

$$Hb(O_2)_4 \longrightarrow metHb + 4O_2^{-\cdot} \quad (1')$$
$$4O_2^{-\cdot} + 4H^+ \longrightarrow 2H_2O_2 + 2O_2 \quad (2')$$
$$Hb(O_2)_4 + 2H_2O_2 + 4H^+ \longrightarrow metHb + 4O_2 + 4H_2O \quad (3')$$
$$\text{Totally, } 2Hb(O_2)_4 + 8H^+ \longrightarrow 2metHb + 6O_2 + 4H_2O \quad (4')$$
$$[=Hb(O_2)_4 + 4H^+ \longrightarrow metHb + 3O_2 + 2H_2O]$$

These chain reactions indicate that the autoxidation of haemoglobin will conform to the autocatalytic (or sigmoidal) mechanism that we have already shown for nitrite oxidation of haemoglobin (Tomoda *et al.*, 1981*a*). However, it has been shown that the autoxidation of haemoglobin proceeds by first-order kinetics (Mansouri & Winterhalter, 1973). This discrepancy may be explained by the fact that the eqns. (1) and (1′) are slow compared with the reactions shown in eqns. (2), (3) and (2′), (3′). A steady-state assumption for the reactions shown in eqns. (2), (3) and (2′), (3′) may be valid, because the reaction of haemoglobin with H_2O_2 (Eyer *et al.*, 1975) and $O_2^{-\cdot}$ (Sutton *et al.*, 1976) and the dismutation of $O_2^{-\cdot}$ itself (Behar *et al.*, 1970) are very rapid compared with eqns. (1) and (1′). Therefore it is possible to say that the overall reaction is strictly dependent on the reactions shown in eqns. (1) or (1′):

$$Hb(O_2)_4 \longrightarrow metHb + 4O_2^{-\cdot},$$

which will probably proceed by first-order kinetics. This view has been extensively discussed previously in connection with myoglobin autoxidation (Tomoda *et al.*, 1981*b*).

Since the autoxidation of haemoglobin proceeds by way of two intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ (Figs. 1 and 3), the reaction process may be visualized as shown in Scheme 1 above. From these results, the actual process of haemoglobin will be expressed as:

$$Hb(O_2)_4 \longrightarrow IntHb(O_2)_2 + 2O_2^{-\cdot} \quad (I)$$
$$Hb(O_2)_4 + 2O_2^{-\cdot} + 4H^+ \longrightarrow IntHb(O_2)_2 + 2O_2 + 2H_2O_2 \quad (II)$$
$$2Hb(O_2)_4 + 2H_2O_2 + 4H^+ \longrightarrow 2IntHb(O_2)_2 + 4O_2 + 4H_2O \quad (III)$$
$$IntHb(O_2)_2 \longrightarrow metHb + 2O_2^{-\cdot} \quad (IV)$$
$$IntHb(O_2)_2 + 2O_2^{-\cdot} + 4H^+ \longrightarrow metHb + 2O_2 + 2H_2O_2 \quad (V)$$
$$2IntHb(O_2)_2 + 2H_2O_2 + 4H^+ \longrightarrow 2metHb + 4O_2 + 4H_2O \quad (VI)$$
$$4Hb(O_2)_4 + 16H^+ \longrightarrow 4metHb + 12O_2 + 8H_2O \quad (VII)$$
$$[=Hb(O_2)_4 + 4H^+ \longrightarrow metHb + 3O_2 + 2H_2O]$$

where $IntHb(O_2)_2$ shows the oxygenated intermediate haemoglobins, including $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$. Eqns. (II) and (V) can be replaced with the dismutation of $O_2^{-}\cdot$, as mentioned above.

By comparing the apparent first-order reaction rate constants under various conditions, the mechanism of haemoglobin autoxidation can be deduced. As shown in Table 1 and Figs. 4(*a*)–4(*d*), we determined k_{+1}–k_{+4} at various pH values under various conditions (10mM-phosphate buffer alone, 10mM-phosphate buffer with 0.1M-phosphate buffer, 10mM-phosphate buffer with 0.1M-NaCl, and 10mM-phosphate buffer with 0.5mM-P_6-inositol). These results suggest the following.

(1) The changes in ionic strength do not affect the autoxidation of haemoglobin, though we showed previously that the chemical oxidation of haemoglobin with ferricyanide is much influenced by the changes in ionic strength (Tomoda & Yoneyama, 1979) (Table 1 and Figs. 4*a*–4*d*).

(2) Mansouri & Winterhalter (1973) reported that the autoxidation rates of α- and β-chains in the oxyhaemoglobin tetramer differ from the study of autoxidation of haemoglobin followed by rapid chain separation with *p*-chloromercuribenzoate. This method is based on the estimation of the ratio of the isolated ferrous and ferric haemoglobin subunits. From this result they concluded that the α-chains are more susceptible to autoxidation than the β-chains in oxyhaemoglobulin tetramer. The present result (Table 1) supports this view, because k_{+3}, the autoxidation rate constant of α-chains in oxyhaemoglobin tetramer, is larger, under various conditions, than k_{+1}, the autoxidation rate constant of β-chains.

(3) Mansouri & Winterhalter (1974) indicated that the α-chains become much more susceptible to autoxidation in the presence of organic phosphates such as 2,3-diphosphoglycerate. Present results are consistent with this finding, though the autoxidation rates of both α- and β-chains were accelerated as much as about 2-fold in the presence of P_6-inositol compared with the rates in the absence of the organic phosphate (Table 1).

(4) The accumulation of $(\alpha^{3+}\beta^{2+})_2$ during the autoxidation of haemoglobin in the presence of P_6-inositol (Fig. 3*d*) is due to both the increase of k_{+3} and the decrease of k_{+4} in the presence of the organic phosphate.

(5) The pH-dependence of the autoxidation reaction of haemoglobin is well known (Kikuchi *et al.*, 1955; Mansouri & Winterhalter, 1973).

This is reflected to the pH profiles of the reaction rate constants k_{+1}–k_{+4} in Figs. 4(*a*)–4(*d*), i.e., these values were maximal at acidic pH values when P_6-inositol is absent. In the presence of P_6-inositol, k_{+1}–k_{+3} were increased at acidic values pH, whereas k_{+4} was decreased significantly. It may be reasonable that the effects of the P_6-inositol on autoxidation are emphasized at acidic pH values, since the binding of the organic phosphate to haemoglobin is greater at acidic pH values.

The effects of P_6-inositol on the conformational changes in oxyhaemoglobin are in debate, though this organic phosphate binds to β-chains of haemoglobin (Arnone & Perutz, 1974). Since the addition of P_6-inositol to fully O_2-liganded haemoglobin produces u.v. and visible spectroscopic changes that are very similar to those observed when isolated unliganded α- and β-subunits combine to form haemoglobin, Adams & Schuster (1974) indicated that the changes in tertiary and quaternary structure of oxyhaemoglobin occurred by the binding of P_6-inositol to the protein. This change in quaternary structure corresponds to the R→T transition of haemoglobin, where R is defined as the oxy form of haemoglobin, and T as the deoxy form of haemoglobin (Perutz, 1970). But Perutz and his colleauges (Perutz *et al.*, 1974; Perutz, 1975) proposed that P_6-inositol combines with oxyhaemoglobin and somehow modifies the R-structure of the protein, probably including changes in the β-chains, because the organic phosphate did not produce the negative peak at 287nm in the c.d. spectrum that is characteristic of deoxyhaemoglobin. We previously suggested that the structural changes in β-chains induced by P_6-inositol are transferred to α-chains (Tomoda *et al.*, 1980) and that the changes in quaternary structure of oxyhaemoglobin from the R- to the T-state may be induced by the binding of the organic phosphate. Present results support this view, because both k_{+1} and k_{+3} are increased in the presence of P_6-inositol. This result suggests that the changes in tertiary structure of β-chains induced by the binding of P_6-inositol are transferred to the α-chains, and thereby the susceptibility of α-chains to autoxidation is increased.

From the effects of P_6-inositol on k_{+2} and k_{+4}, the changes in structure of $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ can be deduced. Since k_{+2}, the reaction rate constant of $(\alpha^{2+}\beta^{3+})_2$ was increased in the presence of P_6-inositol (Table 1), it is likely that the changes in tertiary structure of β methaemoglobin chains in $(\alpha^{2+}\beta^{3+})_2$ induced by the organic phosphate are transferred to the partner α oxyhaemoglobin chains. This suggests that the quaternary structure of $(\alpha^{2+}\beta^{3+})_2$ is changed by the binding of P_6-inositol to the protein. This view is consistent with the report of Cassoly (1976) that P_6-inositol induces the structural changes in $(\alpha^{CO}\beta^{3+})_2$. On the other hand, it is possible to say at least that the tertiary structure of β-chains in $(\alpha^{3+}\beta^{2+})_2$ may be changed by the binding of P_6-inositol, since k_{+4}, the reaction rate constant of $(\alpha^{3+}\beta^{2+})_2$, is significantly decreased in the presence of the organic phosphate. This result is consistent

with our previous views that P_6-inositol changes both the tertiary and quaternary structures of $(\alpha^{3+}\beta^{2+})_2$ (Tomoda *et al.*, 1979).

We previously showed that for the chemical oxidation of haemoglobin with ferricyanide (Tomoda & Yoneyama, 1979), nitrite (Tomoda *et al.*, 1981*a*) and ferricytochrome *c* (Tomoda *et al.*, 1980), the β-chains of haemoglobin tetramer are more susceptible to oxidation than the α-chains. This was confirmed in the oxidation of haemoglobin with cupric ion (Winterbourn & Carrell, 1977; Brittain, 1980). We also showed that, in the presence of P_6-inositol, the susceptibility of the β-chains of haemoglobin tetramer to chemical oxidation with ferricyanide (Tomoda & Yoneyama, 1979) and ferricytochrome *c* (Tomoda *et al.*, 1980) is much increased. In contrast, the autoxidation rates of the α-chains ($=k_{+3}$ in Table 1) are larger than the β-chains ($=k_{+1}$ in Table 1) in haemoglobin tetramer under the conditions explored in the present work (with or without P_6-inositol). The rates of autoxidation of both α- and β-chains were significantly increased in the presence of P_6-inositol. The present results therefore show that the mechanism of haemoglobin autoxidation is different from that of chemical oxidation of haemoglobin. This view is in good agreement with that of Wallace *et al.* (1978).

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