Involvement of superoxide anion in the reaction mechanism of haemoglobin oxidation by nitrite

Akio TOMODA,* 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

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The sigmoidal time course of haemoglobin oxidation by nitrite, involving an initial slow reaction accompanied by a subsequent rapid reaction, was extensively explored. The initial slow reaction was much prolonged by the addition of superoxide dismutase to the reaction mixture. On the other hand, in the presence of superoxide anion generated by xanthine oxidase systems, the slow phase disappeared and the reaction changed to first-order kinetics. The oxidation of intermediate haemoglobins [defined as haemoglobin tetramer in which different chains (α - or β -) are in the ferric state and in the ferrous state] such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ also proceeded in a sigmoidal manner. Similar effects of superoxide anion on these reactions were observed. Since the intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ were found to be produced by the oxidation of haemoglobin by nitrite, the changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during the reaction were followed by isoelectric-focusing electrophoresis. The amounts of $(\alpha^{2+}\beta^{3+})_2$ were larger than those of $(\alpha^{3+}\beta^{2+})_2$ at the initial stages of the reaction, suggesting that there is a functional difference between α - and β -chains in the oxyhaemoglobin tetramer. On the basis of these results, a reaction model of the haemoglobin oxidation by nitrite was tentatively proposed. The changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin were well fitted to the simulation curves generated from the reaction model. Details of the derivation of the equations used for kinetic analysis have been deposited as Supplement SUP 50112 (5 pages) with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K. from whom copies may be obtained on the terms indicated in *Biochem. J.* (1978) 169, 5.

The oxidation of haemoglobin by nitrite has been recognized as an autocatalytic reaction (Martin & Huisman, 1963; Smith & Beutler, 1966; Jung & Kahl, 1967; Hayashi *et al.*, 1968). Jung & Remmer (1949) proposed that the overall reaction proceeds as follows:

In spite of these studies, the true explanation for the autocatalytic reaction is still to be clarified. Jung & Remmer (1949) indicated that the addition of methaemoglobin to the reaction mixture did not

change the reaction mode between haemoglobin and nitrite. This result suggests that some product other than haemoglobin derivatives such as methaemoglobin operates in the catalysis of the reaction.

Since nitrite is known as a univalent oxygen reductant, as is dithionite (Creutz & Sutin, 1974; Lemberg & Legge, 1949), it is expected that superoxide anion is produced during the reaction as a result of univalent reduction by nitrite of oxygen bound to the haemoglobin molecule. From this viewpoint, we investigated whether superoxide is involved in haemoglobin oxidation by nitrite or not.

It was demonstrated that superoxide participates in the reaction, i.e. the lag phase of the reaction was shortened in the presence of superoxide anion and prolonged in the presence of superoxide dismutase as a scavenger of the anion. Furthermore, we found by using isoelectric-focusing electrophoresis that the intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and

Abbreviations used: Bistris, 2-[bis-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; $\text{Hb}(\text{O}_2)_4$, oxygenated haemoglobin tetramer; $\text{IntHb}(\text{O}_2)_2$, oxygenated intermediate haemoglobins including $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$; MetHb, methaemoglobin tetramer.

$(\alpha^{3+}\beta^{2+})_2$ valency hybrids are formed during the reaction.

Taking account of these results, we propose a series of reaction processes that can explain both the autocatalytic mechanism of haemoglobin oxidation by nitrite and the hitherto reported results.

Experimental

Chemicals

Catalase (EC 1.11.1.6) and xanthine oxidase (EC 1.2.3.2) were purchased from Boehringer Mannheim (Mannheim, West Germany), and superoxide dismutase (EC 1.15.1.1) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dowex 1 X8 was obtained from Wako Chemical Co. (Tokyo, Japan) and DE-32 DEAE-cellulose from Whatman (Maidstone, Kent, U.K.). Sephadex G-25 (fine grade) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Ampholine plate gel (pH 3.5–9.5) was obtained from LKB Produkter (Uppsala, Sweden). Bistris was purchased from Sigma, and sodium nitrite and xanthine were from Wako.

Preparation of haemoglobin

Freshly obtained erythrocytes were haemolysed with 5 vol. of ice-cold distilled water and centrifuged at 12000g for 20 min. A 5 ml sample of haemolysate was applied on a Sephadex G-25 column (2.5 cm × 30 cm) previously equilibrated with 50 mM-Bistris/HCl buffer, pH 7.0 or 7.4, containing 0.1 M-NaCl, and organic phosphates such as 2,3-bisphosphoglycerate and ATP were removed from haemoglobin solutions. The effluents are referred to below as 'organic-phosphate-free haemoglobin'.

To obtain the catalase- and superoxide dismutase-free haemoglobin solutions, a 5 ml sample of the haemolysate was passed through a DEAE-cellulose column (3 cm × 7 cm) previously equilibrated with 2 mM-potassium phosphate buffer, pH 7.0. The effluents were then passed through a Sephadex G-25 column (2.5 cm × 30 cm) previously equilibrated with 50 mM-Bistris/HCl buffer, pH 7.0, containing 0.1 M-NaCl, and is referred to below as 'partially purified haemoglobin'. These haemoglobin solutions were diluted appropriately with 50 mM-Bistris/HCl buffer, pH 7.0 or 7.4, containing 0.1 M-NaCl before use.

Optical measurement of haemoglobin oxidation by nitrite

The optical changes during haemoglobin oxidation by nitrite was pursued at 630 nm with a Hitachi 124 spectrophotometer (slit width 2 cm) after addition of nitrite in haemoglobin solutions (pH 7.0) containing the organic-phosphate-free haemoglobin or partially purified haemoglobin. For the experiments involving addition of superoxide

dismutase, catalase and xanthine oxidase systems, the detailed conditions are mentioned in the appropriate Figure legends.

Purification of $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ haemoglobins

The intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ were purified as mentioned in the Appendix. The intermediate haemoglobins were passed through a Sephadex G-25 (fine grade) column (1 cm × 20 cm) previously equilibrated with 50 mM-Bistris/HCl buffer, pH 7.0, containing 0.1 M-NaCl, and used for the experiment of nitrite oxidation.

Analysis of changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin by isoelectric focusing on Ampholine plate gel

The oxidation of organic-phosphate-free haemoglobin by nitrite was performed by pH 7.0 and 7.4 at 25°C, and the changes in oxyhaemoglobin, intermediate haemoglobins, such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$, and methaemoglobin were analysed by isoelectric-focusing electrophoresis.

(a) *Oxidation of haemoglobin by nitrite at pH 7.0.* An 80 µl portion of 60 mM-sodium nitrite, which was freshly prepared, was added to 8 ml oxyhaemoglobin solutions (pH 7.0) containing 200 µM-haem. The reaction was performed at 25°C in a small glass tube. Samples (0.5 ml) were taken out at intervals and were passed immediately through a Dowex 1 X8 (neutralized) column (1 cm × 1 cm). By this procedure, nitrite in the mixture was removed. The effluents were applied on Ampholine plate gel, and the isoelectric focusing was performed as described previously (Tomoda *et al.*, 1978, 1979). The percentages of oxyhaemoglobin, intermediate haemoglobins and methaemoglobin to total haemoglobin were estimated by cutting out and weighing the chart paper after gel-scanning of the fixed plate gel (Tomoda *et al.*, 1978, 1979).

(b) *Oxidation of haemoglobin by nitrite at pH 7.4.* The oxidation of haemoglobin by nitrite was performed under various conditions at pH 7.4 at 25°C, because the rate of oxidation is lower at this pH than at pH 7.0.

(1) Effects of different concentrations of nitrite. An 80 µl portion of sodium nitrite solution (100 mM or 200 mM) was added to 8 ml oxyhaemoglobin solutions containing 125 µM-haem, and the reactions were performed in a small glass tube.

(2) Effects of different concentrations of oxyhaemoglobin. An 80 µl portion of sodium nitrite solution (200 mM) was added to 8 ml of oxyhaemoglobin solution containing 125 µM-, 192 µM- or 412 µM-haem. The reactions were performed at pH 7.4 at 25°C in a small glass tube.

Samples (0.5 ml) were taken out at intervals

during the reaction and passed immediately through a Dowex 1 X8 column as mentioned above. The effluents were analysed by isoelectric-focusing electrophoresis, and the percentages of oxyhaemoglobin, intermediate haemoglobins, including $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$, and methaemoglobin were estimated.

Kinetic treatment of the experimental values obtained by isoelectric-focusing electrophoresis

The experimental values of the samples at pH 7.4, obtained by isoelectric-focusing electrophoresis, were applied to the equations as follows, and were treated by non-linear regression analysis (NONLIN program; Metzler, 1969) (the computer analysis was done with a FACOM M-160 digital computer of the Data Processing Center, Kanazawa University):

$$\frac{df_1}{dt} = -k'_1 \left(1 + \frac{f_1 + \gamma f_2}{f_1 + \alpha f_2} \beta' \right) f_1 \quad (\text{A})$$

$$\begin{aligned} \frac{df_2}{dt} = & k'_1 \left(1 + \frac{f_1 + \gamma f_2}{f_1 + \alpha f_2} \beta' \right) f_1 \\ & - k'_1 \left(\gamma + \frac{\alpha(f_1 + \gamma f_2)}{f_1 + \alpha f_2} \beta' \right) f_2 \end{aligned} \quad (\text{B})$$

$$\frac{df_3}{dt} = k'_1 \left(\gamma + \frac{\alpha(f_1 + \gamma f_2)}{f_1 + \alpha f_2} \beta' \right) f_2 \quad (\text{C})$$

where

$$\alpha = \frac{k_5}{k_3}, \quad \beta' = \frac{k'_2}{k_6}, \quad \gamma = \frac{k'_4}{k'_1} \left(= \frac{k_4}{k_1} \right)$$

The details for the derivation of these equations are described in Supplement SUP 50112.

The values for the fractions of oxyhaemoglobin (f_1), intermediate haemoglobins (f_2) and methaemoglobin (f_3), which were obtained experimentally as a function of time, were applied to the differential equations (A)–(C), and were treated by non-linear regression analysis (NONLIN program; Metzler, 1969). To use this program, the initial estimate of the rate constant, k'_1 , and the three relative rates of α , β' and γ are required. The values of these initial estimates appear to be quite critical to the outcome of the NONLIN program for the use of the differential equations. A computerized Runge–Kutta method was used to obtain these four initial estimates. Trial and error values for the four values defined in eqns. (A)–(C) were used until the Runge–Kutta estimation of the fractions f_1 – f_3 at any time approximately agreed with the fractions found experimentally, as shown in Figs. 5(a) and 5(b). With the initial estimates thus evaluated, k'_1 and the values of α , β' and γ were determined.

Results

Effect of superoxide dismutase and catalase on haemoglobin oxidation by nitrite

Fig. 1(a) shows the changes in absorbance at 630 nm during haemoglobin oxidation by nitrite in the presence and in the absence of enzymes such as superoxide dismutase and catalase. In the absence of the enzymes (control), the reaction of haemoglobin oxidation by nitrite proceeded sigmoidally with an initial slow reaction and a following rapid reaction. The addition of superoxide dismutase prolonged the slow reaction and accelerated the rapid reaction. However, the addition of catalase slightly altered the mode of the reaction. In the presence of both

Fig. 1. Effect of superoxide dismutase and catalase on haemoglobin oxidation by nitrite

Methaemoglobin formation by nitrite was followed by the changes in absorbance at 630 nm in the presence of 29 units of superoxide dismutase and 1300 units of catalase. The concentrations of partially purified haemoglobin and nitrite used are $120 \mu\text{M}$ (haem) and $670 \mu\text{M}$ respectively. (a) Effects of superoxide dismutase and catalase on haemoglobin oxidation by nitrite. (b) Effect of different concentrations of superoxide dismutase on haemoglobin oxidation by nitrite. The half-time ($=t_1$) of the reaction was plotted against the concentrations of superoxide dismutase added.

Fig. 2. Effect of superoxide anion on haemoglobin oxidation by nitrite

The generation of superoxide was done by a superoxide-generating system containing 1 mM-xanthine and 0.04 unit of xanthine oxidase. The oxidation of organic-phosphate-free haemoglobin (125 μ M-haem) and intermediate haemoglobins containing both $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ (135 μ M-haem) by nitrite (1 mM) was followed by the changes in absorbance at 630 nm. (a) Oxidation of haemoglobin; the broken line shows the experiment of complete systems minus nitrite. (b) Oxidation of intermediate haemoglobins.

catalase and superoxide dismutase, the initial lag phase was more prolonged than that with the single addition of superoxide dismutase. The rapid phase, however, was not affected.

Fig. 1(b) shows the effect of different concentrations of superoxide dismutase on haemoglobin oxidation by nitrite. It was found that the oxidation rate ($=t_{\frac{1}{2}}$) was inhibited to an extent dependent on the amounts of the enzyme added.

These results suggest that: (1) the appearance of the rapid phase is dependent on the accumulation of superoxide anion, which was produced during the lag-phase reaction; (2) the acceleration of the rapid phase reaction in the presence of superoxide dismutase is probably due to the direct oxidation of haemoglobin by hydrogen peroxide, which was generated by the dismutation of superoxide anion. This view is supported by the result of further addition of catalase in the reaction mixture.

Effect of superoxide anion on haemoglobin oxidation by nitrite

To test whether superoxide anion is involved in haemoglobin oxidation by nitrite or not, the effects of superoxide anion, generated by xanthine oxidase systems, on nitrite oxidation of oxyhaemoglobin and intermediate haemoglobins containing $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ were examined (Figs. 2a and 2b). In the presence of superoxide anion the initial lag phase observed in the nitrite oxidation of oxyhaemoglobin disappeared and the reaction proceeded nearly by first-order kinetics (Fig. 2a). In this case the effect of direct oxidation of haemoglobin by xanthine oxidase systems was excluded, because the oxidation rate was low in the medium containing haemoglobin,

Fig. 3. Effect of superoxide-generating systems on the reaction rate constants of haemoglobin oxidation by nitrite

The experimental conditions are the same as mentioned in the legend to Fig. 2. The first-order rate constants (min^{-1}) of haemoglobin oxidation by nitrite were obtained in the presence of various concentrations of xanthine oxidase.

xanthine and xanthine oxidase without nitrite. The oxidation of intermediate haemoglobins proceeded sigmoidally, and changed to a first-order kinetics in the presence of superoxide anion (Fig. 2b).

Fig. 3 shows the first-order reaction rate constants obtained for the nitrite oxidation of oxyhaemoglobin under the same conditions but with the concentrations of xanthine oxidase varied. The reaction rate constant was tightly dependent on the

Fig. 4. Changes in fractions of oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during nitrite oxidation of oxyhaemoglobin

Haem contents (%) of each of the components such as oxyhaemoglobin ($\alpha^{2+}\beta^{3+}$)₂, ($\alpha^{3+}\beta^{2+}$)₂ and methaemoglobin, which were analysed by the isoelectric focusing on Ampholine plate gel, were plotted for time. The broken line shows the changes in intermediate haemoglobins involving ($\alpha^{2+}\beta^{3+}$)₂ and ($\alpha^{3+}\beta^{2+}$)₂. O, Oxyhaemoglobin; ●, ($\alpha^{2+}\beta^{3+}$)₂; △, ($\alpha^{3+}\beta^{2+}$)₂; ●, methaemoglobin.

concentration of xanthine oxidase. These results mean that the second rapid phase is dependent on the concentrations of superoxide anion generated during the initial lag reaction, and that superoxide anion is playing a key role in the second burst reaction.

Analysis of changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during haemoglobin oxidation by nitrite

To follow the quantitative changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during the oxidation of haemoglobin by nitrite, isoelectric-focusing electrophoresis of the samples obtained at intervals during the reaction of pH 7.0 was performed. The values at each time for oxyhaemoglobin, intermediate haemoglobins and methaemoglobin are shown in Fig. 4. The oxyhaemoglobin decreased in a sigmoidal manner, and changed into intermediate haemoglobins. With respect to the changes in intermediate haemoglobins (broken lines), a bell-shaped curve was obtained. Methaemoglobin increased in a sigmoidal manner. The amounts of ($\alpha^{2+}\beta^{3+}$)₂ and ($\alpha^{3+}\beta^{2+}$)₂ were different from each other during the reaction period, i.e. the amounts of ($\alpha^{2+}\beta^{3+}$)₂ were greater than those of ($\alpha^{3+}\beta^{2+}$)₂ at the initial stages of the reaction.

Kinetics of haemoglobin oxidation by nitrite

Since the oxidation rate of haemoglobin by nitrite is lower at pH 7.4 than at pH 7.0, we analysed the

changes in these haemoglobin derivatives during the reaction at pH 7.4 by using isoelectric focusing, and explored whether the reaction models proposed in the Discussion section may be kinetically plausible.

Fig. 5(a) shows the time-fraction profiles for oxyhaemoglobin, intermediate haemoglobins including ($\alpha^{2+}\beta^{3+}$)₂ and ($\alpha^{3+}\beta^{2+}$)₂ and methaemoglobin at different haemoglobin concentrations (125 μ M-, 192 μ M- and 412 μ M-haem) and constant nitrite concentration (2 mM). In spite of the changes in concentrations of haemoglobin, the time-fraction profiles for these haemoglobin derivatives fitted well on one single curve.

The changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during the reaction were studied at 125 μ M-haem and 1 mM-nitrite (Fig. 5b). In this case the overall reactions were greatly retarded compared with the case for Fig. 5(a). The time-fraction profiles for these haemoglobin derivatives fitted well on a single curve.

Discussion

The participation of NO_3^- and NO in the nitrite oxidation of haemoglobin is repeatedly indicated in the literature (Jung & Remmer, 1949; Kiese, 1974, p. 73). The present results further demonstrated that superoxide anion is involved in the reaction process and that intermediate haemoglobins are produced during the reaction. Therefore these factors and autocatalytic behaviour of the reaction may be essential to consideration of the mechanism of

Fig. 5. Effect of different concentrations of haemoglobin and nitrite on the changes in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin

(a) Oxidation of haemoglobin by nitrite (2 mM) was studied at various concentrations of oxyhaemoglobin (125 μ M-, 192 μ M- and 412 μ M-haem) at pH 7.4. The fractional changes (%) in oxyhaemoglobin, intermediate haemoglobins such as $(\alpha^2\beta^3)_2$ and $(\alpha^3\beta^2)_2$ and methaemoglobin during the reactions were analysed by isoelectric-focusing electrophoresis. The continuous lines show the curves generated from eqns. (I)–(VI) of Supplement SUP 50112 by using the rate constants mentioned in the Discussion section. Circles show the observed points of oxyhaemoglobin (○, 125 μ M-haem; ●, 192 μ M-haem; ●, 412 μ M-haem). Squares show the observed points of intermediate haemoglobins [$(\alpha^2\beta^3)_2 + (\alpha^3\beta^2)_2$] (□, 125 μ M-haem; ■, 192 μ M-haem; ■, 412 μ M-haem). Triangles show the observed points of methaemoglobin (△, 125 μ M-haem; ▲, 192 μ M-haem; ▲, 412 μ M-haem). (b) Oxidation of haemoglobin by nitrite was studied at pH 7.4, and the fractional changes (%) in oxyhaemoglobin, intermediate haemoglobins and methaemoglobin during the reactions were analysed by isoelectric-focusing electrophoresis. Nitrite and oxyhaemoglobin concentrations were 1 mM and 125 μ M haem respectively. The continuous lines show the curves generated from eqns. (I)–(VI) in Supplement SUP 50112 by using the rate constants mentioned in the Discussion section. ●, Oxyhaemoglobin; □, intermediate haemoglobins [$(\alpha^2\beta^3)_2 + (\alpha^3\beta^2)_2$]; △, methaemoglobin.

haemoglobin oxidation by nitrite. Taking account of all this evidence, we considered the following

equations will satisfy a series of the reaction during the oxidation of haemoglobin by nitrite:

Also:

Eqns. (7) and (8) together give:

The lag phase of haemoglobin oxidation by nitrite is prolonged by superoxide dismutase and shortened by superoxide anion (Figs. 1–3). These results support the view that superoxide anion, which might be generated during the initial lag reactions (1) and (4), is playing a key role in the subsequent rapid reaction (2),(5) and (3),(6). The production of superoxide during the reaction may be considered reasonable, because nitrite is a one-electron reductant.

Only the oxygenated form of haemoglobin is utilized as a substrate of nitrite oxidation (Wallace & Caughey, 1975; Wallace *et al.*, 1978). This fact is important in consideration of the mechanism of haemoglobin oxidation by nitrite, because the participation of deoxyhaemoglobin, which reacts promptly with the oxidants such as ferricyanide regardless of a minor distribution in oxyhaemoglobin mixture, can be excluded, and thereby the oxidation steps of haemoglobin were simplified as eqns. (1)–(6). Therefore oxyhaemoglobin and intermediate haemoglobins are described in $\text{Hb(O}_2)_4$ and $\text{IntHb(O}_2)_2$ respectively in the equations.

Though direct evidence for eqn. (2) and (5) is not available, it would be expected that NO_2^- is reduced by $\text{O}_2^{\cdot-}$ to produce NO, because the redox potentials of NO_2^- and $\text{O}_2^{\cdot-}$ are 0.99 and -0.33 V respectively. [The reduction of ferricyanide, the redox potential of which is 0.36 V, occurs in the presence of $\text{O}_2^{\cdot-}$ with $k = 270 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Zehavi & Rabani, 1972).] The fact that NO is produced during the oxidation of oxyhaemoglobin by nitrite is probably explained by this reaction (Rein *et al.*, 1964).

It is well known that NO reacts with oxyhaemoglobin very rapidly and that methaemoglobin is produced in consequence (Kiese, 1974, p. 73). Kiese (1974, p. 63) showed that the addition of small amounts of NO to the reaction mixture of haemoglobin with nitrite accelerated the formation of methaemoglobin and caused the corresponding formation of nitrite at early stages of the reaction. These results support the views that the third

equations, including reactions (3) and (6), are reasonable.

Thus it is possible to say that autocatalytic reaction of haemoglobin oxidation by nitrite is mediated by NO, generated by the reduction of nitrite with superoxide anion produced by the reaction in the eqns. (1) and (4). A series of eqns. (1)–(6) can be taken for an autocatalytic reaction from the kinetic viewpoint, as mentioned below.

Summing up eqns. (1)–(6), and taking account of eqn. (8), the overall reaction of haemoglobin oxidation by nitrite is as described by eqn. (9). This equation was completely consistent with the results obtained by Kosaka *et al.* (1979). This equation means that 1 mol of oxygen will be produced by oxidation of 1 mol of oxyhaemoglobin (4 mol of haem) by nitrite, and that 1 mol of oxyhaemoglobin is oxidized by 4 mol of nitrite to produce 1 mol of methaemoglobin and 4 mol of nitrate. The stoichiometry of the reaction was clearly determined by Kosaka *et al.* (1979). It is indicated that nitrite oxidation of haemoglobin is promoted by acidification of the reaction mixture and decelerated by alkalization (Jung & Kahl, 1969). The above equation explains this effect of pH on haemoglobin oxidation by nitrite.

With regard to eqn. (8), Heppel & Porterfield (1949) showed that nitrite was oxidized to nitrate by H_2O_2 in the presence of catalase and they attributed this phenomenon to the peroxidase activity of the enzyme. It is probable that methaemoglobin, which was produced during the oxidation of haemoglobin by nitrite, catalyses the reaction:

since methaemoglobin is known to have a peroxidatic activity as well as catalase (Keilin & Hartree, 1951).

Eqns. (1)–(8) were kinetically treated as shown in the Experimental section and Supplement SUP 50112. If the time–fraction profiles for oxyhaemoglobin, intermediate haemoglobins and methaemo-

globin satisfy eqns. (A)–(C) given in the Experimental section, the process of oxidation of haemoglobin by nitrite in accordance with eqns. (1)–(8) will be supported. Assuming that the steady state in nitric oxide and superoxide concentrations is valid, it is expected from eqns. (A)–(C) of the Experimental section that there should be no change in time-fraction profiles for all species, in spite of the changes in the initial oxyhaemoglobin concentrations under constant nitrite ion concentrations, although changes in nitrite ion concentrations significantly influence these profiles. These predictions were substantiated, by using the isoelectric-focusing technique to follow the reactions of oxyhaemoglobin (125 μ M-, 192 μ M- and 492 μ M-haem) with 2 mM-nitrite, and the reactions of oxyhaemoglobin (125 μ M-haem) with nitrite at different concentrations (1.0 and 2 mM) (Figs. 5a and 5b). The continuous lines, which were generated by using eqns. (A)–(C) with the NONLIN fitted parameters, were reasonably close to the experimental points in all reactions under various conditions. These results support the view that the oxidation of haemoglobin by nitrite proceeds according to the mechanism proposed in the present paper.

The reaction rate constant k'_1 thus obtained from the computer analysis was dependent on the concentrations of nitrite ($0.93 \times 10^{-3} \text{ min}^{-1}$ and $4.85 \times 10^{-3} \text{ min}^{-1}$ for 1 mM- and 2 mM-nitrite respectively), and independent of the concentrations of haemoglobin ($4.85 \times 10^{-3} \text{ min}^{-1}$ for each of 125 μ M-, 192 μ M- and 412 μ M-haem). This result explains the fact that the initial lag phase of the reaction is not influenced by changes in the concentration of haemoglobin, but is influenced by changes in the concentration of nitrite (Kakizaki *et al.*, 1964). When the apparent first-order rate constants k'_1 were divided by $[\text{NO}_2^-]^2$, a constant value of $1.09 \times 10^{-3} \text{ M}^{-2} \cdot \text{min}^{-1}$ was obtained, to give the third-order rate constant, k_1 . This result, together with constant γ value of 16.3, supports a mechanism [shown in eqns. (I)–(VI) in Supplement SUP 50112] in which the reaction of oxyhaemoglobin or intermediate haemoglobins with nitrite proceeds by second-order kinetics with respect to nitrite. Furthermore it is possible to say that the reaction of oxyhaemoglobin or intermediate haemoglobins with nitrite [eqns. (I)–(VI) in Supplement SUP 50112] proceeds by first-order kinetics with respect to the proteins, because k_1 was not altered in spite of the differences of the concentrations of haemoglobin (125 μ M-, 192 μ M- and 412 μ M-haem).

With regard to the rates of disappearance of superoxide anion from the reaction medium, the results suggest that the reactivity of superoxide with NO_2^- is much larger than with $\text{O}_2^{\cdot -}$ itself in the medium containing haemoglobin, because the values of β ($=k_2/k_6$), which are obtained by the

division of β' by $[\text{NO}_2^-]^2$, are $3.08 \times 10^6 \text{ M}^{-2}$. This result means that the contribution of the disproportionation of superoxide anion to the overall reaction for haemoglobin oxidation by nitrite is not large. Therefore we did not add the reaction:

in the process of oxidation of haemoglobin by nitrite shown by eqns. (1)–(9). Thus the prediction that the oxidation of haemoglobin by nitrite proceeds according to reactions (1)–(9) was also supported by the kinetic studies.

Since the absorption spectra during haemoglobin oxidation by nitrite have excellent isosbestic points (Kosaka *et al.*, 1979), it is apt to be considered that oxyhaemoglobin is directly oxidized to methaemoglobin by the oxidant without formation of intermediate haemoglobins. However, the present results clearly show that intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ are produced during haemoglobin oxidation by nitrite. This means that the presence of isosbestic points is not necessarily good evidence for lack of production of intermediate haemoglobins. Furthermore, the present results show that there is a functional difference between α - and β -chains in oxyhaemoglobin tetramer in the reaction with nitrite, because β -chains are more susceptible to oxidation by nitrite (Fig. 4). This fact is well consistent with the results reported by Olson *et al.* (1971) that there are functional differences between α - and β -chains in oxygen dissociation from oxyhaemoglobin tetramer. From the present results, the following course of the nitrite oxidation of haemoglobin can be visualized:

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APPENDIX

Characterization of intermediate haemoglobins produced during haemoglobin oxidation by nitrite

The haemoglobin solutions that were partially oxidized by nitrite were subjected to the analysis of intermediate haemoglobins by preparative isoelectric focusing. Four main zones appeared, including oxyhaemoglobin, intermediate haemoglobins and methaemoglobin in the gel bed. The intermediate haemoglobins collected from the gel bed were found to be $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids from the absorption spectra and inositol hexaphosphate-induced difference spectra.

Though it was shown that intermediate haemoglobins such as IB_I [or $(\alpha^{2+}\beta^{3+})_2$] and IB_{II} [or $(\alpha^{3+}\beta^{2+})_2$] were produced by the partial oxidation of haemoglobin with ferricyanide by using isoelectric-focusing electrophoresis (Bunn & Drysdale, 1971; Carrel *et al.*, 1978; Tomoda & Yoneyama, 1979), the intermediate haemoglobins produced during the oxidation of haemoglobin by nitrite were not sufficiently investigated except for the report by Chanutin & Curnish (1966). We previously showed that preparative isoelectric focusing is very useful for preparing the intermediate haemoglobins such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids produced during methaemoglobin reduction by NADH-cytochrome *b*₅ reductase (Tomoda *et al.*, 1979). In the present work the separation of intermediate haemoglobins produced by the partial oxidation of haemoglobin with nitrite was performed by using preparative isoelectric focusing. Two intermediate haemoglobins obtained by this procedure were characterized as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ valency hybrids from the optical experiments.

Experimental procedure

Materials

The buffers used were 50 mM-Bistris/HCl buffer, pH 7.0, containing 0.1 M-NaCl and 10 mM-potassium phosphate buffer, pH 7.0.

Preparation of organic-phosphate-free haemoglobin

Solutions of haemoglobin were obtained as described previously (Tomoda *et al.*, 1978), and were passed through a column of Sephadex G-25 (fine grade) previously equilibrated with 50 mM-Bistris/HCl buffer, pH 7.0, containing 0.1 M-NaCl.

Partial oxidation of haemoglobin

The oxidation of organic-phosphate-free haemoglobin was initiated by addition of 40 μ l of nitrite (100 mM) to 4 ml of haemoglobin solutions (480 μ M-haem), and the reaction was performed at 25°C for 3 min. The partially oxidized haemoglobin solutions were promptly passed through a column of Sephadex G-25 (coarse grade) equilibrated with 10 mM-

potassium phosphate buffer, pH 7.0, at 4°C. Samples (4 ml) of the effluents were subjected to preparative isoelectric-focusing electrophoresis.

Preparative isoelectric-focusing electrophoresis

The samples obtained by the procedure stated above were mixed with Sephadex G-75 (superfine grade) gel containing carrier Ampholite (pH 6.0–8.0), and the preparative isoelectric focusing was performed at 4°C for 17 h (settled at initial current 6 mA, final voltage 1200 V and constant 6 W). After performance of the electrophoresis, the two intermediate zones were collected from the gel bed, and applied on a Sephadex G-25 (fine grade) column (1 cm × 20 cm) equilibrated with 50 mM-Bis-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl. Each of the samples was subjected to the optical measurements.

Identification of intermediate haemoglobins

(a) *Absorption spectra of IB_I and IB_{II} (intermediate bands I and II).* Absorption spectra of intermediate haemoglobins collected from the intermediary zones (IB_I and IB_{II} show the second and third zones from the anode respectively) were measured between 350 and 650 nm under aerobic and anaerobic conditions with a Hitachi 124 spectrophotometer (slit width 2 nm). The experiment under anaerobic conditions was conducted after replacement of air with Q gas (helium/isobutane, 99.05:0.95) in Thunberg-type quartz cells.

(b) *Difference spectra of IB_I and IB_{II} induced by inositol hexaphosphate.* The difference spectra between the original solutions containing intermediate haemoglobins (IB_I or IB_{II}) and the solutions containing the intermediate haemoglobins (IB_I or IB_{II}) to which inositol hexaphosphate (inositol hexaphosphate/haem molar ratio 10:1) had been added were measured between 500 and 650 nm with a Hitachi 124 spectrophotometer (slit width 2 nm).

Results and discussion

Separation of intermediate haemoglobins by preparative isoelectric focusing

Fig. 1 shows the fractionation of the solutions of the partially oxidized haemoglobin with nitrite by preparative isoelectric focusing. The result was the appearance of four zones including oxyhaemoglobin, intermediate haemoglobins (IB_I and IB_{II}) and methaemoglobin. The intermediate zones were collected from the gel bed and the optical measurements were made as follows. The contents of haemoglobin IB_I were higher than those of haemoglobin IB_{II}.

Identification of intermediate haemoglobins

The absorption spectra of intermediate haemoglobins such as IB_I and IB_{II} are shown in Figs. 2(a)

Fig. 1. Profiles of preparative isoelectric focusing of partially oxidized haemoglobin

Oxyhaemoglobin was partially oxidized by nitrite for 5 min, and the preparative isoelectric focusing of the sample was performed at 4°C for 17 h.

Fig. 2. Absorption spectra of intermediate haemoglobins

Absorption spectra of intermediate haemoglobins, collected from the zones shown in Fig. 1, were measured between 350 and 650 nm under aerobic (—) and anaerobic (---) conditions. The experiment under anaerobic conditions was performed after replacement of air with Q gas (helium/isobutane, 99.05:0.95) in a Thunberg-type quartz cell. The left-hand scale shows a millimolar absorption coefficient at the regions between 350 and 450 nm, and the right-hand scale shows a millimolar absorption coefficient between 450 and 650 nm. (a) Intermediate haemoglobin collected from the second zone from anode (IB_I); (b) intermediate haemoglobin collected from the third zone from anode (IB_{II}).

Fig. 3. Inositol hexaphosphate-induced difference spectra of intermediate haemoglobins

The difference spectra between the original solutions containing intermediate haemoglobin (IB_I or IB_{II} in Fig. 1) and the solutions containing the intermediate haemoglobins (IB_I or IB_{II}) to which inositol hexaphosphate had been added were measured between 500 and 650 nm. (a) Intermediate haemoglobin (IB_I); (b) intermediate haemoglobin (IB_{II}).

and 2(b) respectively. The absorption spectra of haemoglobin IB_I under aerobic and anaerobic conditions (350–650 nm) were in good agreement with those of the reconstituted $(\alpha^2\beta^3)_2$ valency hybrids, the detailed characterization of which is mentioned in a previous paper (Tomoda *et al.*, 1978). The absorption spectra of haemoglobin IB_{II} under aerobic and anaerobic conditions were in good

agreement with those of the reconstituted $(\alpha^3\beta^2)_2$ valency hybrid (Tomoda *et al.*, 1978).

Furthermore, the inositol hexaphosphate-induced difference spectra of haemoglobins IB_I and IB_{II} are shown in Figs. 3(a) and 3(b). The results showed clearly that haemoglobin IB_I is $(\alpha^2\beta^3)_2$ and that haemoglobin IB_{II} is $(\alpha^3\beta^2)_2$ by comparing the inositol hexaphosphate-induced difference spectra with those of the reconstituted $(\alpha^2\beta^3)_2$ and $(\alpha^3\beta^2)_2$ valency hybrids (Tomoda *et al.*, 1978).

We previously showed that two intermediate haemoglobins such as $(\alpha^2\beta^3)_2$ and $(\alpha^3\beta^2)_2$ valency hybrids were produced during methaemoglobin reduction by NADH-cytochrome *b*₅ reductase (Tomoda *et al.*, 1979), and that only one intermediate haemoglobin such as $(\alpha^3\beta^2)_2$ was produced during methaemoglobin reduction by ascorbic acid (Tomoda *et al.*, 1978), by isoelectric-focusing electrophoresis. Present results show that two intermediate haemoglobins appear when oxyhaemoglobin is oxidized by nitrite, and these haemoglobin derivatives can be identified as $(\alpha^2\beta^3)_2$ and $(\alpha^3\beta^2)_2$ valency hybrids from the characteristic absorption spectra and inositol hexaphosphate-induced difference spectra (Figs. 1–3).

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