Haemichrome Formation from Haemoglobin Subunits by Hydrogen Peroxide

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The effect of H_2O_2 on ferrous human haemoglobin subunits (α_{SH} -, β_{SH} -, α_{PMB} - and β_{PMB} -chains) was studied. These chains were easily transformed to haemichrome by the addition of H_2O_2 or H_2O_2 -generating systems, including glucose oxidase (EC 1.1.3.4) and xanthine oxidase (EC 1.2.3.2), and this was ascertained by e.p.r. measurements and by absorption spectra. The changes in these haemoglobin subunits were not inhibited by superoxide dismutase (EC 1.15.1.1), but were decreased by catalase (EC 1.11.1.6). The rate of oxidation of α_{PMB} -chains was higher than that of α_{SH} -chains, and the rate of oxidation of β_{PMB} -chains was higher than that of β_{SH} -chains. Haemichrome was demonstrated to be formed directly from these ferrous chains by the attack by H_2O_2 , and this process did not involve formation of methaemoglobin. On the basis of these findings the kinetics of the reaction between the haemoglobin subunits and H_2O_2 was studied, and the pathological significance of H_2O_2 in disorders of erythrocytes such as thalassaemia was discussed.

Though haemichrome has been considered an intermediate step in the denaturation of haemoglobin, the detailed mechanism for the formation of this oxidized derivative of haemoglobin is still to be elucidated. Haemichrome is characterized by a specific absorption spectrum and considered to be dissociated to free subunits in which the haem group is bonded directly to the distal histidine residue E7 (Rachmilewitz & White, 1973).

Rachmilewitz *et al.* (1971) suggested that when haemoglobin tetramer is dissociated into free subunits such as α - and β -chains, they are more susceptible to haemichrome formation than is the haemoglobin tetramer itself, though a direct oxidant of haemoglobin in the erythrocytes was not identified.

Brunori *et al.* (1975) mentioned the possibility that superoxide and peroxide exert an influence on haemichrome formation, based on the studies of the autooxidation of α - and β -chains of haemoglobin. Winterbourn *et al.* (1976), however, indicated that the participation of H_2O_2 and superoxide in haemichrome formation might be negligible. In spite of these studies, direct evidence for haemichrome formation by physiologically produced substance is lacking.

Abbreviations used: α_{PMB} - and β_{PMB} -chains, ferrous α - and β -chains with thiol groups modified with *p*-chloromercuribenzoate; α_{SH} - and β_{SH} -chains, ferrous α - and β -chains with free thiol groups.

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In this study, we investigated haemichrome formation by examining the effect of H_2O_2 on the α - and β -chains of haemoglobin. As a result it became clear that haemoglobin, when dissociated into its subunits, is easily transformed into haemichrome by a small amount of H_2O_2 . Furthermore haemoglobin subunits were transformed directly to haemichrome by oxidation with H_2O_2 and this process did not involve methaemoglobin formation. Our results support the view that H_2O_2 is a predominant participant in the oxidation of haemoglobin to form haemichrome in intact erythrocytes.

Experimental

Materials

CM-cellulose and DEAE-Sephadex were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden), glucose oxidase (EC 1.1.3.4) from Kyowa Hakko Co. (Tokyo, Japan) and horseradish peroxidase (EC 1.11.1.7) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Xanthine oxidase (EC 1.2.3.2) and catalase (EC 1.11.1.6) were purchased from Boehringer Mannheim (Mannheim, West Germany) and superoxide dismutase (EC 1.15.1.1) was from Sigma. Dianisidine, xanthine and *p*-chloromercuribenzoate were from Wako (Tokyo, Japan) and Tris was from Sigma.

Preparation of haemoglobin solution

Fresh heparinized blood was centrifuged at 3000 rev./min for 10 min. After removal of serum and buffy coats, erythrocytes were washed three times with 0.9% NaCl. The erythrocytes were haemolysed by the addition of water and the stroma were removed by centrifugation at 10000 rev./min for 15 min. The haemoglobin solution thus obtained was applied to a column (2.4 cm $\times$ 60 cm) of DEAE-Sephadex, which was equilibrated with 50 mM-Tris/HCl buffer (pH 8.0), to remove catalase and superoxide dismutase by the method by Huisman & Dozy (1965). This enzyme-free haemoglobin was used for the preparation of α_{PMB}^- , β_{PMB}^- , α_{SH}^- and β_{SH}^- -chains. The concentration of haemoglobin subunits was determined at 540 nm as cyanmethaemoglobin by using a millimolar absorption coefficient $\epsilon_{\text{mM}} = 11.0 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ per haem (Van Assendelft & Zijlstra, 1975).

Preparation of haemoglobin subunits

The α_{PMB}^- and β_{PMB}^- -chains were obtained after treatment of haemoglobin with *p*-chloromercuribenzoate as described by Bucci & Fronticelli (1965) and Geraci *et al.* (1969). The α_{PMB}^- and β_{PMB}^- -chains were collected from the fractions eluted through CM-cellulose, and the α_{SH}^- and β_{SH}^- -chains were obtained by removal of *p*-chloromercuribenzoate from the α_{PMB}^- and β_{PMB}^- -chains through thiolated Sephadex G-25 (Tyuma *et al.*, 1966).

Determination of H_2O_2 concentration and the amounts of H_2O_2 generated by glucose oxidase and xanthine oxidase systems

The concentration of H_2O_2 was determined from the A_{240} by using a millimolar absorption coefficient $\epsilon_{\text{mM}} = 40 \text{ cm}^{-1} \cdot \text{mM}^{-1}$. The rate of H_2O_2 generation by glucose oxidase systems was determined by using the peroxidation of dianisidine, and that by xanthine oxidase was measured by the increase in A_{240} (Bergmeyer *et al.*, 1974a,b).

Measurement of the oxidation of haemoglobin subunits by H_2O_2 or H_2O_2 -generating systems

Each 0.2 ml of solution of the haemoglobin subunits (free from catalase and superoxide dismutase), such as α_{SH}^- , β_{SH}^- , α_{PMB}^- and β_{PMB}^- -chains (42.7, 42, 37.8 and 37.3 μM in haem respectively) was mixed with 1.3 ml of 0.2 M-potassium phosphate buffer (pH 7.0). Changes in the absorption of the mixture were measured at 578 nm, or between 500 and 650 nm, or between 240 and 300 nm at 25°C after addition of H_2O_2 to a final concentration of 120 μM or after H_2O_2 generation with glucose oxidase or xanthine oxidase (H_2O_2 -generating rates by these enzymes were 15 and 30 $\mu\text{M}/\text{min}$ respectively).

Measurement of e.p.r. spectra

The e.p.r. spectra of the H_2O_2 -oxidized α_{SH}^- (653 μM) and β_{SH}^- (733 μM) subunits at 123°K were recorded on a JEOL JES-PE-3X X-band spectrometer equipped with a 100 kHz field-modulation unit.

Results and Discussion

Oxidation of haemoglobin subunits by H_2O_2

Fig. 1 shows the changes in A_{578} of α_{SH}^- , α_{PMB}^- , β_{SH}^- and β_{PMB}^- -chains when these haemoglobin subunits were exposed to H_2O_2 . After the addition of H_2O_2 to these chains, a rapid decrease in the A_{578} was observed within 12 min at 25°C, and determined to be first-order (Figs. 1a–1d respectively). The extent of the initial rate of oxidation was in the sequence $\beta_{\text{PMB}}^- > \alpha_{\text{PMB}}^- > \alpha_{\text{SH}}^- > \beta_{\text{SH}}^-$ -chains. This tendency toward haemichrome formation is also indicated by Rachmilewitz *et al.* (1971). These changes in the absorbance were completely inhibited in the presence of catalase (broken lines).

Absorption spectra of the H_2O_2 -oxidized haemoglobin subunits

The spectra changes for the α_{SH}^- and β_{SH}^- -chains in the course of the reaction are shown in Figs. 2(a) and 2(b) respectively. In both cases, isosbestic points were obtained at 526 and 587 nm, though the absorption

Fig. 1. Effect of H_2O_2 on haemoglobin subunits. The experiments were carried out in the presence or absence of catalase. The changes in the A_{578} of the α_{SH}^- (a; 42.7 μM), α_{PMB}^- (b; 37.8 μM), β_{SH}^- (c; 42 μM) and β_{PMB}^- (d; 37.8 μM) chains were measured after the addition of 120 μM - H_2O_2 . The broken lines show the experiment with catalase.

Fig. 2. Absorption spectra of the H₂O₂-oxidized haemoglobin subunits

The reaction was initiated by addition of 120 μ M-H₂O₂ to α_{SH} -(a) and β_{SH} -(b) chains (42.7 and 42 μ M respectively) and the intermediate absorption changes for 12 min were measured between 500 and 650 nm.

spectra moved upward gradually out of the isosbestic points after the reaction finished. By the addition of dithionite, the final absorption spectra were changed to those that are typically observed for haemochrome (not shown in the Figure). These results suggest that haemichrome is directly formed by H₂O₂ oxidation of these ferrous chains.

The same results were also observed with the α_{PMB} - and β_{PMB} -chains. These absorption spectra were in good accordance with those reported by Rachmilewitz (1969) and Rachmilewitz & White (1973), who observed the haemichrome spectra of α_{SH} - and β_{SH} -chains prepared with methaemoglobin-forming reagents, such as ferricyanide and nitrite.

Since the spectral perturbation in the u.v. regions is indicated in a haemichrome compound (Rachmilewitz, 1969), we studied the absorption changes in α_{SH} - and β_{SH} -chains in the u.v. regions between 240 and 300 nm (Fig. 3). As a result, a decrease in the A_{264} was typically observed in both α_{SH} - and β_{SH} -chains. As the A_{264} is largely due to tyrosine and tryptophan residues, it is likely that the oxidation of these amino acid residues by H₂O₂,

which occurs concomitantly after the addition of the oxidant, is involved in the process of haemichrome formation.

E.p.r. spectra of the H₂O₂-oxidized haemoglobin subunits

To ascertain whether or not the oxidized compound that was derived from haemoglobin subunits is a haemichrome, e.p.r. spectra were measured. Fig. 4 shows the e.p.r. spectra of the H₂O₂-oxidized haemoglobin subunits (α_{SH} - and β_{SH} -chains). Each spectrum showed a typical low spin ($g = 2.45$, 2.29 and 2.05), which is specific for a haemichrome (Rachmilewitz *et al.*, 1969, 1971). These results also support the view that the compound produced by H₂O₂ is a haemichrome. The sharp signal near $g = 2$, which was observed with H₂O₂-oxidized α_{SH} -chains extensively and with β_{SH} -chains slightly, is probably due to free radicals produced by H₂O₂, though this was not studied in detail in the present work. Knowles *et al.* (1969) showed that the signal produced by the superoxide radical appears near $g = 2$.

Effect of different concentrations of H₂O₂ on oxidation of α_{SH} - and β_{SH} -chains

To see the reaction mode of the haemoglobin subunits with H₂O₂, the effect of different concentrations of H₂O₂ was studied. When the concentration of H₂O₂ was changed from 120 to 1200 μ M, the initial rates for the α_{SH} - and β_{SH} -chains were dependent on H₂O₂ concentration (Fig. 5). The reaction proceeded as a bimolecular process with respect to these chains and H₂O₂, and the rate constants were calculated to be 15 M⁻¹·s⁻¹ (α_{SH}) and 6.4 M⁻¹·s⁻¹ (β_{SH}).

Effect of H₂O₂-generating systems on oxidation of the haemoglobin subunits

Since both glucose oxidase and xanthine oxidase systems are known to produce H₂O₂, the effect of these H₂O₂-generating systems on the oxidation of haemoglobin subunits (α_{SH} and β_{SH}) was studied (Figs. 6 and 7).

Fig. 6 shows the effect of H₂O₂ produced by glucose oxidase or xanthine oxidase systems on the oxidation of the α_{SH} -chains. The decrease in the A_{578} was observed in the presence of these enzyme systems. The absorption spectra of the oxidized α_{SH} -chains were in good accordance with those produced by H₂O₂ addition, which shows that the compound formed is a haemichrome.

In the presence of catalase, the changes in the absorption of the α_{SH} -chains were largely inhibited (about 60%), though not so completely as was seen

Fig. 3. U.v.-absorption spectra of the H_2O_2 -oxidized haemoglobin subunits

The absorption changes after 12 min were measured between 240 and 300 nm after the addition of $120 \mu\text{M}$ - H_2O_2 to $42.7 \mu\text{M}$ - α_{SH} - (a) and $42 \mu\text{M}$ - β_{SH} - (b) chains.

after addition of authentic H_2O_2 . This result further confirms our finding that the haemichrome was produced from the α_{SH} -chains by the direct attack of H_2O_2 . If superoxide participates in haemichrome formation, it is expected that the oxidation rate of α_{SH} -chains might be decreased by superoxide dismutase in xanthine oxidase systems, which are known to generate superoxide as well as H_2O_2 . However, as shown in Fig. 6, the oxidation rate was

instead activated by superoxide dismutase in the xanthine oxidase systems. This activation is probably due to the dismutation of superoxide to H_2O_2 . This result suggests that the contribution of superoxide to haemichrome formation from the α -chains is small, which is in good agreement with the views reported by Winterbourn *et al.* (1976).

Similar results were also obtained with the β_{SH} -chains (Fig. 7). When H_2O_2 was generated in the

Fig. 4. E.p.r. spectra of the H₂O₂-oxidized haemoglobin subunits

The e.p.r. spectra of the H₂O₂-oxidized α_{SH} - (653 μ M) and β_{SH} - (733 μ M) chains were measured at a magnetic field between 250 and 350 mT at 123 K. The final concentration of H₂O₂ used for oxidation of the chains was 1.3 mM.

Fig. 5. Oxidation rates of α_{SH} - and β_{SH} -chains by H₂O₂ in the presence of various concentrations of H₂O₂

The oxidation rate of α_{SH} - ($\circ$) and β_{SH} - ($\bullet$) chains (42.7 and 42.0 μ M respectively) was measured by the decrease in A₅₇₈ in the presence of various concentrations of H₂O₂ (160–1200 μ M).

Fig. 6. Effect of H₂O₂-generating systems on oxidation of α_{SH} -chains

The changes in A₅₇₈ of the α_{SH} -chains were measured after addition of glucose oxidase (a) or xanthine oxidase (b). The effect of catalase and superoxide dismutase (SOD) was also studied in these systems. H₂O₂ was generated by glucose oxidase at 15 μ M/min and by xanthine oxidase at 30 μ M/min.

Fig. 7. Effect of H₂O₂-generating systems of oxidation of β_{SH} -chains

Experimental conditions and procedures are the same as those described in Fig. 6, except that β_{SH} -chains were studied.

reaction mixture by glucose oxidase or xanthine oxidase, oxidation of the β_{SH} -chains was observed and the oxidized compound was confirmed to be a haemichrome from the characteristic spectra.

The β_{SH} -chains were oxidized more slowly than α_{SH} -chains by the addition of these enzymes. The inhibition of the oxidation of β_{SH} -chains by catalase was 60%. However, superoxide dismutase slightly increased the rate of haemichrome formation in xanthine oxidase systems.

Mechanism of haemichrome formation and considerations of some pathological conditions

It seems to be generally accepted that haemichrome is formed after the formation of methaemoglobin (Rachmilewitz, 1969; Winterbourn *et al.*, 1976). However, our results shown in Figs. 2(a) and 2(b) strongly suggest that haemichrome was directly formed from the ferrous chains, because the isosbestic points were obtained in the course of the reaction. To see further whether haemichrome is derived from direct oxidation of ferrous chains or by way of methaemoglobin chains, we studied haemichrome formation by H_2O_2 in the presence of large amounts of cyanide, which is expected to inhibit the reaction to haemichrome by forming H_2O_2 -insensitive cyanide-methaemoglobin chain complex if methaemoglobin chains were formed as intermediates during the oxidation of the chains by H_2O_2 since the rate of cyanide binding is much higher than that of oxidation of haemoglobin subunits by H_2O_2 [the reaction rate constant of cyanide binding is estimated about $160M^{-1}\cdot s^{-1}$ (Antonini & Brunori, 1971) and that of α - and β -chains with H_2O_2 is $15M^{-1}\cdot s^{-1}$ and $6.4M^{-1}\cdot s^{-1}$ respectively (present work)].

As a result, haemichrome was also formed in the presence of cyanide (100-fold excess over haem) and the rate of formation was not altered compared with that in the absence of cyanide (results not shown). This result supports the views that haemichrome is directly formed by H_2O_2 oxidation of the ferrous chains and that this process does not involve formation of methaemoglobin.

With regard to haemoglobin tetramer (free from catalase and superoxide dismutase), methaemoglobin formation caused by the presence of H_2O_2 was clearly shown by Eyer *et al.* (1975). This suggests that the association of the α - and β -chains to form tetramers serves to protect against the attack of oxidants such as H_2O_2 . From these results, the following scheme may be produced (abbreviation: Hb, haemoglobin):

Thus a small amount of H_2O_2 which was exogenously added or generated by the enzyme systems can easily attack the α - and β -chains.

Furthermore the inhibitory effect of catalase on haemichrome formation is incomplete when H_2O_2 is generated by the enzyme systems. Considering these results and the fact that H_2O_2 is a physiologically produced substance, it is possible that in the erythrocytes containing excess haemoglobin subunits, the haemichrome formation may be accelerated. For example, in thalassaemic erythrocytes, which are known to contain excess of either α - or β -chains, the accumulation of haemichrome has been indicated (Rachmilewitz *et al.*, 1969). Furthermore Rachmilewitz & White (1973) and Winterbourn & Carrell (1974) observed haemichrome formation during the oxidation of abnormal haemoglobin Köln with facilitated dissociation into its subunits. The accumulation of haemichrome in the erythrocytes from the patient with haemoglobin Köln was also observed by Rachmilewitz & White (1973). The present results therefore strongly suggest that the persistent attack of H_2O_2 on haemoglobin, especially its subunits, may be the cause of its denaturation by forming haemichrome in intact erythrocytes.

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