OXIDATION OF HAEMOGLOBIN BY P-QUINONE

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The oxidation mechanism of human haemoglobin by p-quinone was investigated in aerobic and anaerobic states. In anaerobic state, in which all haemoglobin molecules were in reduced form, the oxidation took place according to the following reaction formula,

In aerobic state, at the initial stage of the reaction the same reaction as that in anaerobic state may occur, but in the later stage many reactions, of which a further investigation is awaited, are supposed to occur simultaneously.

When aromatic nitro or amino compounds are induced into the body, these compounds are converted to iminoquinone and this iminoquinone is reduced to hydroxyaminobenzene by accepting two electrons from haemoglobin. This is a theory accepted by many investigators as the reason of methaemoglobinemia in the poisoning of aromatic nitro or amino compounds. But the mechanism of haemoglobin oxidation, even whether protein or haem moiety of haemoglobin molecule is first attacked by quinone, has not yet been cleared.

This paper describes the oxidation mechanism of haemoglobin by p-quinone.

MATERIALS AND METHODS

Oxyhaemoglobin solution. Crystallized oxyhaemoglobin was prepared from human blood by Suzuki's method.¹⁾ Before use crystallized oxyhaemoglobin obtained was washed with cold water and dissolved in deionized water.

p-Quinone solution. p-Quinone was purified by sublimation immediately before use and dissolved in deionized water.

Oxidation of oxyhaemoglobin by p-quinone. The reaction of oxyhaemoglobin to methaemoglobin was carried out by adding 1 ml of p-quinone solution to 2 ml of oxyhaemoglobin solution buffered with M/50-phosphate buffer and the amount of methaemoglobin formed was followed by recording the increase of optical density at 630 m μ using a Cary recording spectrophotometer model 14. The initial rate of methaemoglobin formation was determined from the average slope of the reaction and was expressed as $\nu\mu\text{M}$ of methaemoglobin formed per second.

Oxidation of haemoglobin under various partial pressures of oxygen. Two milliliters of buffered oxyhaemoglobin (pH 6.2, M/50-phosphate buffer) and 1 ml of water were put into an apparatus of Thunberg tube type (total capacity: 91.5 ml) equipped with an optical cuvette (light path: 10 mm). After substituting the air in the tube with nitrogen gas, an adequate amount of air was introduced in the tube and the equilibria between oxygen and haemoglobin was attained in a water bath of 19°C for 5 minutes. Then, 1 ml of p-quinone solution, which had the same partial pressure of oxygen as in the vessel by bubbling a mixed gas of air and nitrogen, was added into the vessel. The oxidation process of haemoglobin was followed as described above. Experimental conditions were 0.05 mM haemoglobin, 0.5 mM p-quinone, pH 6.2 and 19°C.

Determination of p-quinone. At adequate intervals of time after the beginning of the reaction, 1 ml of the reaction mixture was poured into 4 ml of cold 5 % trichloroacetic acid solution. Immediately after the precipitate was centrifuged off, the optical density of the clear supernatant solution at 420 m μ was measured. Six millimoles p-quinone solution and 0.05 mM haemoglobin solution were used as the standards after treated as above. The concentration of p-quinone was calculated from a standard curve obtained before the experiments.

RESULTS AND DISCUSSION

Effect of concentration of oxyhaemoglobin and p-quinone on the rate of methaemoglobin formation

Fig. 1. Oxidation of oxyhaemoglobin by p-quinone.

The oxidation process of oxyhaemoglobin by p-quinone was followed by measuring the increase of optical density ($\Delta \log I_0/I$) at 630 m μ . Concentration of p-quinone was 6.0 mM. Concentration of oxyhaemoglobin used was indicated in the figure. Experimental conditions: pH 6.2, 25°C.

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Fig. 2. Effect of concentration of oxyhaemoglobin on the oxidation rate.

The oxidation rate was determined from the average slope of the reaction curve in three seconds after the beginning of the reaction. Experimental conditions: p-quinone; 6.0 mM, pH 6.2, 25°C. The rate of methaemoglobin formation was determined from the amount of methaemoglobin formed in three seconds after the beginning of the reaction.

The rate of methaemoglobin formation at various concentration of oxyhaemoglobin and 6.0 mM p-quinone was measured. The results obtained at pH 6.2 and 25°C were shown in Fig. 1. As shown in the figure, the rate of methaemoglobin formation increased with increasing the concentration of oxyhaemoglobin. The relationship between the initial concentration of oxyhaemoglobin and the initial rate of methaemoglobin formation, $v \mu\text{M}/\text{second}$, was linear within the concentration range of oxyhaemoglobin used in this experiments as indicated in Fig. 2. The relationship between the concentration of oxyhaemoglobin and v , therefore, may be expressed as follows when the concentration of p-quinone used is constant, 6.0 mM,

$$v = k' [\text{oxyhaemoglobin}] = k [\text{p-quinone}] [\text{oxyhaemoglobin}],$$

$$k' = k [\text{p-quinone}]$$

The value k' was calculated to be 0.04/second from the data illustrated in Fig. 2, and k to be $6.7 \times 10^{-6} \mu\text{M}/\text{second}$.

As illustrated in Fig. 3, similar relationship was also observed between the concentration of p-quinone and v , that is, this relationship may be expressed as

$$v = k [\text{oxyhaemoglobin}] [\text{p-quinone}]$$

where the concentration of oxyhaemoglobin is constant and the value of k is $6 \times 10^{-6} \mu\text{M}/\text{second}$. From these experimental results, it was found that the oxidation of oxyhaemoglobin by p-quinone was a second order reaction with respect to the concentrations of oxyhaemoglobin and p-quinone. Therefore, the initial rate of the oxidation may be expressed as follows,

$$v = k [\text{oxyhaemoglobin}] [\text{p-quinone}]$$

k : velocity constant

and the value of k is $6 - 6.7 \times 10^{-6} \mu\text{M}/\text{second}$ at pH 6.2 and 19°C

Fig. 3. Effect of concentration of p-quinone on the oxidation rate.
Experimental conditions: oxyhaemoglobin; 0.05 mM, pH 6.2, 25°C.

Effect of pH on the initial rate of methaemoglobin formation

The effect of pH on the initial rate of methaemoglobin formation was examined at 14°C. The concentration of oxyhaemoglobin and p-quinone used in this experiment were 0.05 mM and 6.0 mM, respectively. The results obtained were shown in Fig. 4. As illustrated in the figure, the initial rate was nearly constant within a range from pH 5.5 to pH 6.5, and it decreased markedly above pH 6.5. This relationship between v and pH was quite similar as that between $pO_{2\frac{1}{2}}$ in oxygen equilibrium of haemoglobin and pH reported by Wyman *et al.*^{2,3)} The results obtained, therefore, may suggest that the oxidation rate has a close relation to the dissociations of amino acid residues of haemoglobin molecule, particularly those of imidazol radicals which are called as oxygen linked acid group.

Effect of fractional oxygenation of haemoglobin on the methaemoglobin formation

If the dissociation states of oxygen linked acid group have an influence on the rate of methaemoglobin formation by p-quinone as suggested in the relation of v and pH, the oxygenation of haemoglobin may have a great influence on the rate

Fig. 4. Effect of pH on the oxidation rate.
Experimental conditions: oxyhaemoglobin; 0.05 mM, p-quinone; 6.0 mM, 14°C.

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because the dissociation constants of the group shift by the oxygenation of haemoglobin. By this reason, the effect of oxygenation of haemoglobin at various oxygenated states on the rate of methaemoglobin formation was experimented using 0.05 mM haemoglobin and 0.5 mM p-quinone at pH 6.2 and 19°C. The concentration of p-quinone used in this experiment was about one-tenth of that indicated in Fig. 1, 3 or 4 because the rate of methaemoglobin formation at zero of oxygen partial pressure was about ten times of that at 150 mmHg of oxygen partial pressure. In Fig. 5, the initial rate of methaemoglobin formation was plotted against the fractional oxygenation of haemoglobin expressed as the ratio of oxyhaemoglobin concentration to total haemoglobin concentration (oxyhaemoglobin plus reduced haemoglobin). As seen in the figure, the rate decreased with the increase of fractional oxygenation of haemoglobin, namely, with increase of partial oxygen pressure. In the decreasing process of the rate mentioned above, stepwise reduction of the rate, reduction of four steps, was most interesting, that is, the reduction of the rate was observed at the fractional oxygenation of 0-3% (I), 25-40% (II), 70-80% (III) and 95-100% (IV). In these four steps, at the steps of (I) and (IV) there was scarcely increase of oxygenation, but significant increase of partial oxygen pressure, because the relation of fractional oxygenation and logarithms of partial oxygen pressure was already known to be represented as a sigmoid curve. The rapid reduction of the rate at the steps (I) and (IV), therefore, may be explained as due to a simple inhibitory effect of oxygen. But the reduction of the rate observed at the steps of (II) and (III) may be difficult to explain as a simple inhibition by oxygen, because if such inhibitory effect of oxygen takes place linear reduction of the rate, not a stepwise reduction, should

Fig. 5. Effect of fractional oxygenation of haemoglobin on the oxidation rate.

Abscissa: fractional oxygenation of haemoglobin represented as the ratio of [oxyhaemoglobin] to [oxyhaemoglobin + reduced haemoglobin]. Experimental conditions: haemoglobin; 0.05 mM, p-quinone; 0.5 mM, pH 6.2, 19°C.

be observed. As already reported by Hasegawa *et al.*,⁴⁾ oxygenation of four haems of haemoglobin molecule was assumed to be stepwise with the partial pressure of oxygen and, for example, when the value of fractional oxygenation of haemoglobin was 50 %, most of haemoglobin molecules were assumed to be exist at the state of half oxygenated which two haems of one haemoglobin molecule were at oxygenated state and the other two haems were at reduced state. The reduction of the rate at the steps of (II) and (III), therefore, may be interpreted as that states of oxygenation of haemoglobin molecule have a great influence on the oxidation of haemoglobin molecule by p-quinone. From these facts, it was considered that both of oxygen and states of haemoglobin molecule had effects on the oxidation of haemoglobin by p-quinone.

Amount of p-quinone consumed during the oxidation of haemoglobin

The amounts of p-quinone consumed during the oxidation of haemoglobin in anaerobic and in aerobic conditions were determined.

The experiments in anaerobic state were carried out using 0.05 mM haemoglobin and 0.05 or 0.10 mM p-quinone at pH 6.2 and 19°C. In Fig. 6, the relation of reaction time and the amount of p-quinone consumed were illustrated. When 0.05 mM p-quinone was used, a half of the total haemoglobin, 0.025 mM haemoglobin was converted to methaemoglobin. This conclusion was obtained by extrapolating the curve in the figure. When 0.10 mM p-quinone, which was twice of the concentration of haemoglobin, total haemoglobin was converted to methaemoglobin. From these results, it may be assumed that two molecules of p-quinone are required for the oxidation of one molecule of haemoglobin, namely, the overall reaction in anaerobic state may be expressed as follows,

Fig. 6. Oxidation of reduced haemoglobin by p-quinone in anaerobic state.

The concentration of haemoglobin used in this experiment was 0.05 mM. When total haemoglobin was completely oxidized to methaemoglobin, the increase of optical density at 630 mμ was found to be 0.48. p-Quinone concentrations used were indicated in the figure. Experimental conditions: pH 6.2, 19°C.

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where Hb and Hb(OH)₄ stand for reduced haemoglobin and methaemoglobin molecule, respectively.

Under aerobic condition, 4.8 mM p-quinone and 1.2 mM oxyhaemoglobin were mixed at pH 6.2 and 19°C. In Fig. 7, the amount of methaemoglobin formed and that of p-quinone consumed were shown with the reaction time. As may be seen from the figure, when the time was over 5 seconds, the rate of methaemoglobin formation was reduced significantly, while that of p-quinone consumed was not almost reduced. In Table 1, the ratio represented as [the amount of p-quinone consumed during the reaction time (t_2-t_1)]/[the amount of methaemoglobin

formed during the same reaction time] was shown at various t_1 and t_2 . As seen clearly from the Table, in the initial stage of the reaction the ratio value was about 2. This fact shows that two molecules of p-quinone are necessary for the oxidation of one haemoglobin molecule, and coincides well with the results obtained at anaerobic condition. The ratio value showed a rapid increase till 14 in 30 seconds after the beginning of the reaction,

Fig. 7. p-Quinone consumption with methaemoglobin formation in aerobic state.

Concentration of oxyhaemoglobin: 1.2 mM. Concentration of p-quinone: 4.8 mM. The amount of p-quinone consumed with methaemoglobin formation was calculated by subtracting the amount of remaining p-quinone, which was determined by measuring the optical density at 420 m μ immediately after deproteination of the reaction mixture with a cold trichloroacetic acid solution, from the total amount of p-quinone at the beginning of the reaction. Experimental conditions: pH 6.2, 19°C.

Table 1. Ratio of the amount of p-quinone consumed and the amount of methaemoglobin formed.

$t_1 \sim t_2$ (Seconds)	$[\text{p-Quinone}]_{t_2-t_1}$
	$[\text{Methaemoglobin}]_{t_2-t_1}$
0 ~ 5	2.0
5 ~ 10	2.5
10 ~ 20	12.0
20 ~ 30	14.0
30 ~ 45	11.0
45 ~ 60	10.0
60 ~ 90	9.8
90 ~ 120	8.0
120 ~ 150	8.0
150 ~ 180	8.0

then it reduced to a constant value, 8. These high ratio values seemed to suggest that methaemoglobin could consume p-quinone. Therefore, then p-quinone was mixed with methaemoglobin and the amount of p-quinone in this mixture was determined at adequate intervals of time. In this experiment methaemoglobin used was prepared as follows. Oxyhaemoglobin was mixed with enough amount of potassium ferricyanide, and after oxyhaemoglobin was perfectly oxidized to methaemoglobin this mixture solution was treated with column chromatography of G-25 Sephadex column. By this procedure, pure methaemoglobin solution which did not contain potassium ferro- and ferricyanide was obtained. The concentration of methaemoglobin and p-quinone used were 0.77 mM and 4.8 mM, respectively. The results were shown in Fig. 8. As indicated in the figure, the amount of p-quinone decreased within 30 seconds till about 30 % of the initial concentration of p-quinone added, and then recovered gradually to the initial level. The initial rapid decrease of p-quinone may be too difficult to explain, but a possible explanation may be given by introducing a modified state of methaemoglobin, although which has not yet been verified. The initial rapid decrease of p-quinone may be too difficult to explain even if the formation of modified methaemoglobin, perturbation of methaemoglobin is introduced in the consideration, because the action mechanism of perturbed haemoglobin on p-quinone has not yet been clarified. Anyway, the action of methaemoglobin on p-quinone observed in Fig. 8 may be enough to explain the rapid increase of the ratio, $[\text{p-quinone consumed}]/[\text{methaemoglobin formed}]$, and the constant ratio values observed in the later stage of the reaction shown in Table 1.

Fig. 8. Consumption of p-quinone by methaemoglobin.

p-Quinone consumption by methaemoglobin was observed. Methaemoglobin used in this experiment was prepared as follows. Oxyhaemoglobin was mixed with an enough amount of potassium ferricyanide, and after the oxidation of oxyhaemoglobin was completed ferricyanide and ferrocyanide were excluded from the methaemoglobin solution through a Sephadex column. Concentration of methaemoglobin: 0.77 mM. Concentration of p-quinone: 4.8 mM. Experimental conditions: pH 6.2, 19°C.

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Therefore, in anaerobic and in the initial stage at aerobic condition, the overall reaction may be expressed as follows,

In later stage of the reaction in the presence of oxygen, following reactions may proceed simultaneously, although the details has been obscure,

- (1) $\text{Hb(O}_2)_4 + 2 \text{ p-quinone} + 4\text{H}_2\text{O} \longrightarrow \text{Hb(OH)}_4 + 2 \text{ hydroquinone} + 4\text{O}_2$
- (2) p-quinone consumption by methaemoglobin formed
- (3) formation of perturbed oxyhaemoglobin by p-quinone
- (4) conversion of perturbed oxyhaemoglobin to methaemoglobin

where, it must be mentioned that during the oxidation process of oxyhaemoglobin there was no change of the amount of dissolved oxygen, which was measured using a Shimadzu oxygen meter of model OX-2, in the reaction medium, that is, all of oxygen molecules dissociated from oxyhaemoglobin by the oxidation were spent during the oxidation processes.

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