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Mechanism of Autocatalytic Oxidation of Oxyhemoglobin by Nitrite

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Oxidation of oxyhemoglobin by nitrite is characterized by the presence of a lag phase followed by autocatalysis. The stoichiometry of the overall reaction is described by the following equation: $4HbO_2 + 4NO_2^- + 4H^+ = 4Hb^+ + 4NO_3^- + O_2 + 2H_2O$ (Hb denotes hemoglobin monomer). During the oxidation, we detected a free radical at $g = 2.005$, which is very similar to the methemoglobin free radical generated by the reaction with hydrogen peroxide. Nitrosylhemoglobin was not detected. The oxidation was delayed by the addition of KCN or catalase, but was not modified by superoxide dismutase in phosphate buffer. In bistris buffer, however, superoxide dimutase markedly prolonged the lag phase. The results suggest that during the oxidation, the methemoglobin peroxide compound is generated and converts nitrite into nitrogen dioxide by its peroxidatic activity. Nitrogen dioxide oxidizes oxyhemoglobin to methemoglobin and nitrite, yielding the autocatalytic phase.

Introduction

Nitrogen dioxide (NO_2) and nitric oxide (NO) are the major hazardous substances among many oxides of nitrogen in the air. Biological effects of NO_2 have been studied extensively and reviewed recently (*1–3*), however, the effects of NO are still uncertain. It is well known that NO binds to hemoglobin (Hb) with an extremely high affinity, approximately 1000 times that of CO. NO exposure produced not only nitrosylhemoglobin but also a significant amount of methemoglobin in mice (*4*). Oxidation of nitrosylhemoglobin by oxygen may be responsible for methemoglobin production. Alternatively, NO dissolved in blood will be converted to nitrite, which is known to be a potent methemoglobin former. In contrast, only nitrosylhemoglobin was detected in mice exposed to NO_2 (*4*). *In vitro* exposure of red cell suspensions to NO_2, however, converted oxyhemoglobin to methemoglobin in a mole-for-mole ratio to NO_2 consumed (*5*). Oral administration of $NaNO_2$ induced nitrosylhemoglobinemia, as well as methemoglobinemia, in rats (*6*). These conflicting findings clearly indicate that methemoglobin production by nitrite is one of the key reactions to be elucidated for understanding the health effects of NO and NO_2.

Although it has long been known that the reaction of oxyhemoglobin and nitrite proceeds autocatalytically (*7–13*), the detailed mechanism of this reaction has not been clarified. The reaction proceeds linearly, with much retardation in the presence of inositol hexaphosphate, while the oxidation of oxyhemoglobin by ferricyanide caused the reaction to occur 10 times faster than did the addition of inositol hexaphosphate (*9,13*). The major difference between the two methemoglobin formers is that nitrite is a one-electron reducing agent, while ferricyanide is an oxidizing agent. Castro et al. (*14*) have shown that human oxyhemoglobin is converted to methemoglobin by a wide array of organic and inorganic reductants, including arylhydrazines and sodium dithionite.

This article is a brief review of our previous investigations dealing with the stoichiometry of the reaction of nitrite and oxyhemoglobin (*11*), the detection and property of the methemoglobin free radical produced during the reactions (*12*), and the role of superoxide anion in the reaction (*13*).

Materials and Methods

Human adult hemoglobin from fresh blood was freed of superoxide dismutase and catalase by a CM-Sephadex column (*15*) and stripped of phosphates by the Dintzis column method (*16*). Methemoglobin was prepared by treating oxyhemoglobin with 5 equivalents of ferricyanide at 37°C for 30 min and passing the mixture through a Sephadex G-25 column. *N,N*-bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane (bistris), superoxide dismutase, and catalase were from Sigma Chemical Co. and used without further purification. Nitrite was measured by the method of Wegner (*17*), where the sample was quenched by the addition of protein-precipitation agent kept at 0°C to stop the oxidation rapidly. The concentration of nitrate was determined

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continuously by an Orion model 93-07 nitrate ion electrode.

Spectrophotometric measurements were carried out on a Hitachi 124 recording spectrophotometer with cell compartments thermostatically controlled at 25°C. The formation of methemoglobin was monitored by measuring absorbance at 577 or 630 nm. The formation of the methemoglobin peroxide compound was measured at 630 nm. ESR data were taken on a Varian model E-12 X-band ESR spectrometer with 100-kHz field modulation at 77 K.

Results

Although nitrite has long been used as a methemoglobin former, the stoichiometry of the reaction has been controversial. Meier (*18*), Betke et al. (*19*), and Kakizaki et al. (*20*) proposed a metheme/NO_2^- molar ratio of unity, whereas Greenberg et al. (*21*) and Jung and Remmer (*7*) reported a ratio of 2. No or little data are available for the reaction products, e.g., NO_3^- and O_2. We measured the time course of the change in the concentration of NO_2^-, NO_3^-, O_2, and methemoglobin during the oxidation reaction. As depicted in Figure 1, the rate of NO_2^- consumption and NO_3^- production exactly coincides with the rate of methemoglobin formation, and 1 mole of NO_2^- is consumed and 1 mole of NO_3^- produced to yield one equivalent ferriheme. The separate determination of the amount of oxygen evolved disclosed that 0.25 mole of O_2 is produced to yield one equivalent ferriheme. Thus, the stoichiometry for the overall reaction can be described as follows:

$$4HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb^+ + 4NO_3^- + O_2 + 2H_2O$$

During the oxidation, we detected a radical by ESR technique at $g = 2.005$, the concentration of which reached a maximum before the autocatalysis began. As shown in Figure 2*A*, the shape of the ESR signal is asymmetric, with shoulders. The signal is indistinguishable from that produced by the addition of $^{15}NO_2^-$ (Fig. 2*B*), suggesting that a nitrogen atom of nitrite or nitrate is not involved in the radical. The signal was not saturated fully by increasing the microwave power, indicating that that the radical is derived from hemoglobin. The radical is very similar to the methemoglobin free radical generated on mixing the protein and hydrogen peroxide (Fig. 2*C*), not only in its asymmetric shape but also in *g*-value, peak-to-peak width, saturation behavior, and pH dependence of the shape. Furthermore, the oxidation of oxyhemoglobin by nitrite was inhibited by the addition of catalase or KCN and was accelerated by the addition of hydrogen peroxide (*12*), suggesting that the methemoglobin free radical plays a key role in the autocatalysis. Nitrosylhemoglobin was not detected during the oxidation.

Unexpectedly, in bistris buffer the oxidation was markedly delayed in contrast with the oxidation in phosphate buffer. As shown in Figure 3, nearly 900 μM nitrite was required in 50 mM bistris buffer to obtain a similar reaction rate as in 50 mM phosphate buffer with 180 μM nitrite. The addition of 2640 units superoxide dismutase scarcely inhibited the oxidation in phosphate buffer, whereas only 2 units of the enzyme significantly inhibited the oxidation in bistris buffer. The results suggest that bistris salts (or contaminating agents in the salts) react with the methemoglobin free radical, resulting in the inhibition of the oxidation and the production of a radical from bistris. Superoxide can be gen-

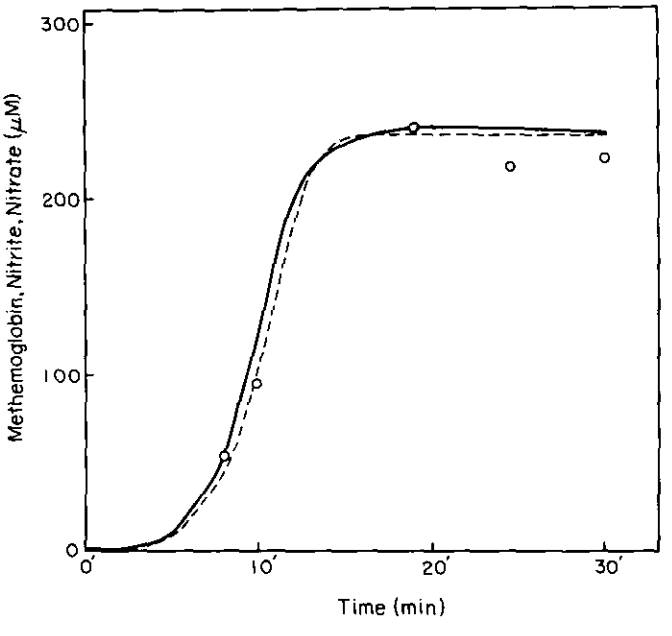

FIGURE 1. Time courses of the oxidation of oxyhemoglobin by nitrite in 50 mM phosphate buffer at pH 7.4 and 25°C. (———) methemoglobin formed; (– – –) nitrate produced; (○) nitrite consumed.

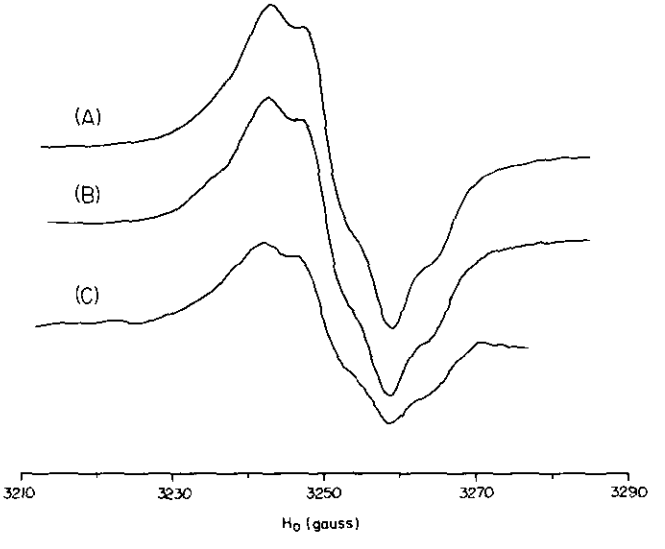

FIGURE 2. Comparison of three ESR signals in 50 mM phosphate buffer. (*A*) Generated during the oxidation of 1.8 mM oxyhemoglobin by 1.8 mM ^{14}N-nitrite at pH 7.4; (*B*) generated during the oxidation of 0.9 mM hydrogen peroxide and 0.9 mM methemoglobin at pH 7.0. Microwave power is 10 mW. Modulation width: (*A*), (*B*) 2.5 G; (*C*) 1.6 G.

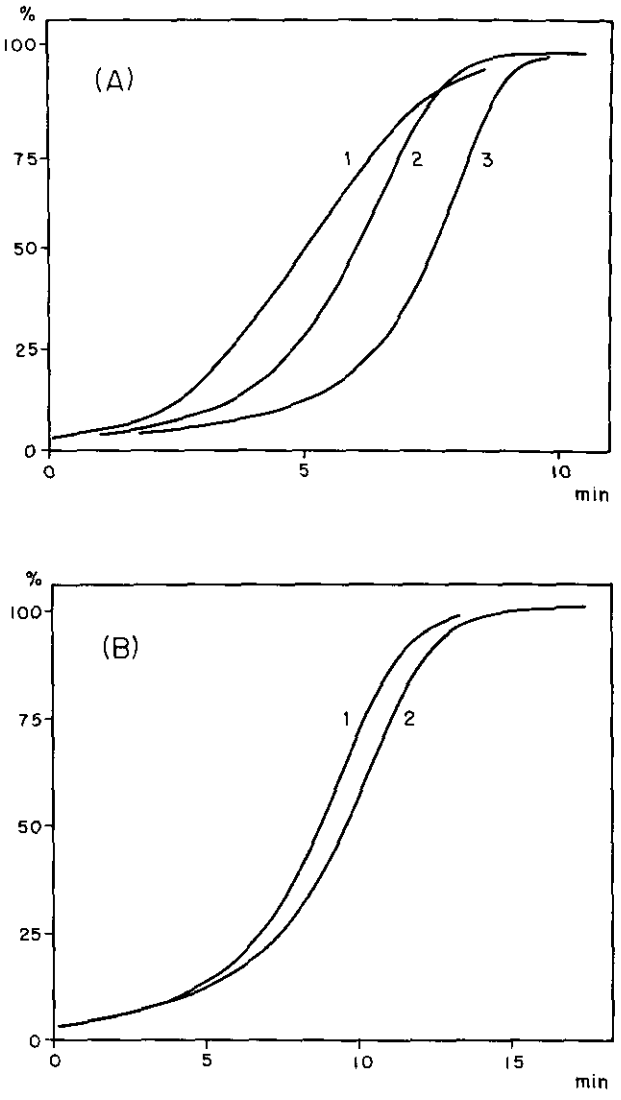

FIGURE 3. Comparison of the effects of superoxide dismutase on the oxidation of 120 μM oxyhemoglobin by nitrite in bistris buffer and in phosphate buffer at pH 7.0 and 25°C. (*A*) By 900 μM nitrite in 50 mM bistris buffer. Superoxide dismutase concentrations (units) are: (1) 0; (2) 2; (3) 230; (*B*) by 180 μM nitrite in 50 mM phosphate buffer. Superoxide dismutase concentrations are: (1) 0; (2) 2640.

erated by the reduction of molecular oxygen by the bistris radical.

Discussion

The involvement of superoxide anion in the oxidation of oxyhemoglobin by nitrite has not been certain. We detected no inhibition of the oxidation by the addition of 2640 units superoxide dismutase in phosphate buffer, whereas in bistris buffer the oxidation was significantly inhibited by 2 units of superoxide dismutase. In addition, the oxidation was markedly delayed in bistris buffer (Fig. 3). In phosphate buffer and in Tris buffer, the rate of oxidation was independent of the concentration of the buffer salts, whereas in bistris buffer, the rate decreased with increasing bistris concentration. The inhibition of oxidation may be due to contaminating agents in bistris salts because of the high concentration of salts required for the significant inhibition. We have shown that the methemoglobin peroxide complex is decomposed by the addition of bistris, and a free radical is produced (*13*). The radical reacts with dioxygen to yield superoxide anion, as addition to superoxide dismutase inhibited the decomposition of the methemoglobin peroxide compound by bistris salts (*13*). It is worth mentioning that the reaction of the radicals of paraquat or quinones and oxygen produces superoxide anion in a similar manner (*22*).

The chemiluminescence of luminol during the oxidation of oxyhemoglobin by nitrite was reported to be inhibited by superoxide dismutase (*23*). However, this does not directly mean that superoxide anion is produced by the oxidation because superoxide is a reaction product between oxygen and luminol free radical generated from luminol oxidation (*24*). The chemiluminescence of luminol can be evoked by ferricyanide, persulfate, hypochlorite, or by peroxidation using peroxidase as well (*24,25*).

These results support our proposed mechanism (*12*) that the electron from nitrite and an electron from heme iron are taken up by the bound dioxygen to yield hydrogen peroxide, methemoglobin, and nitrogen dioxide (NO_2):

$$HbO_2 + NO_2^- + 2H^+ \rightarrow Hb^{3+} + NO_2 + H_2O_2 \quad (1)$$

According to Wallace et al. (*26*), the oxidation of hemoglobin under the stimulation of anions is very slow because of the thermodynamic unfavorability of dioxygen as a one-electron acceptor. If a second electron could be made available to the bound dioxygen through addition of an external electron donor, then the reduction of dioxygen to peroxide might occur quite rapidly. An external electron donor (e.g., nitrite > hydroquinone > phenol > resorcinol > ascorbate > salicylate) provides an electron and reduces the bound dioxygen to peroxide. Subsequently, the peroxide may be displaced by water, the hemoglobin is left in the aquomet (oxidized) form, and peroxide appears in the solution.

The reaction of methemoglobin (Hb^{3+}) and hydrogen peroxide is known to produce a spectrophotometrically detectable red compound (peroxide compound, ferrylhemoglobin, Hb^{4+}) (*27*), and a free radical intermediate (methemoglobin free radical, $Hb^{\cdot 4+}$) has been assumed to be a precursor of the peroxide compound (*28*):

$$Hb^{3+} + H_2O_2 \rightarrow Hb^{\cdot 4+} + H_2O \quad (2)$$

Equations 3 and 4 are proposed as a likely mechanism for the autocatalytic phase in the reaction of oxyhemoglobin and nitrite (*12*).

$$Hb^{\cdot 4+} + NO_2^- \rightarrow Hb^{4+} + NO_2 \quad (3)$$

$$Hb^{4+} + NO_2^- + H^+ \rightarrow Hb^{3+} + H_2O + NO_2 \quad (4)$$

Nitrogen dioxide or its dimer oxidizes oxyhemoglobin to methemoglobin, resulting in autocatalysis. Nitrate

formation is expected to occur from dimerization of nitrogen dioxide. T-conformation of oxyhemoglobin induced by inositol hexaphosphate seems to decelerate the rate of reaction 1 because reactions 2, 3, and 4 were not inhibited by the phosphate (*13*).

We had detected neither superoxide nor hydroxyl radicals in the oxidation of oxyhemoglobin by nitrite (*12*). George and Irvine proposed that homolysis of hydrogen peroxide by myoglobin yields the hydroxyl radical and an Fe(IV)=O complex (*29*), or that heterolysis yields an Fe(V)=O complex that is reduced so rapidly to the Fe(IV)=O species by the protein that it cannot be detected (*30*). The two mechanisms proposed for formation of the hemoglobin or myoglobin Fe(IV)=O species predict the formation of transient protein radicals that are very similar to what we have found during the oxidation of hemoglobin by nitrite. Montellano and Catalano (*31*) showed that the peroxide-dependent oxidation of styrene catalyzed by methemoglobin does not involve the superoxide or hydroxyl radicals and suggest hydrogen peroxide-dependent formation of a protein-peroxy radical that oxidizes styrene. Howell and Wyngaarden (*32*) showed that urate was oxidized by hydrogen peroxide only in the presence of hematin or methemoglobin and interpreted their results to mean that hydroxyl radicals were not involved in the initial oxidation of urate. Ames et al. (*33*) also showed by experiments on urate oxidation that oxoheme is different in its properties from free hydroxyl radicals, and those authors state that although the role of hemoglobin is that of an oxygen carrier, it can be oxidized to a nonspecific oxidase that may be deleterious for the organism because it is a catalyst for initiating lipid peroxidation. Smith and Nunn (*34*) reported that uric acid and ribosyluric acid inhibited the oxidation of oxyhemoglobin by nitrite. Because both the acids do not react with hydrogen peroxide unless the hemoglobin is present (*32*), the results support our view that the methemoglobin peroxide compound is involved in the oxidation of oxyhemoglobin by nitrite.

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