The Reaction of Ferrous Ascaris Perienteric Fluid Hemoglobin with Hydrogen Peroxide*

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SUMMARY

Hydrogen peroxide reacts with ferrous Ascaris hemoglobin and converts it to oxyhemoglobin in a sequence of two reactions. The first is the reaction of ferrous Ascaris hemoglobin with hydrogen peroxide to form a stable product; the second is the reaction of this product with hydrogen peroxide to form oxyhemoglobin.

Both reactions follow second order kinetics, since they are first order with respect to each of the reactants. They proceed without detectable intermediates, and, therefore, appear to be single step, two electron oxidations.

The stable product of the reaction of ferrous Ascaris hemoglobin with hydrogen peroxide is believed to be at the formal oxidation state IV. The optical spectrum of this product strongly suggests that the heme iron atom is best described as low spin ferric heme iron ($d^4$).

The hemoglobin found in the perienteric fluid of the parasitic nematode, Ascaris lumbricoides, is a protein of molecular weight 328,000. It is made up of eight subunits, each of molecular weight 40,600 and each bearing a single protoporphyrin IX heme (1). The kinetics (2, 3) and equilibria (2, 4) of the reactions of Ascaris hemoglobin with gaseous ligands are well established. Each heme reacts independently with oxygen or carbon monoxide without significant heme-heme interaction (4). An outstanding property of the protein is its extraordinary affinity for oxygen ($p_50 = 0.001$ to 0.004 mm Hg) (2, 4), which is a consequence of the very slow rate of oxygen dissociation ($k_1 = 190$ s $^{-1}$ at 20°C) (2). We take advantage of this latter property in the experiments to be described. Oxyhemoglobin once formed in a reaction, remains as a stable product for a time long enough to complete the experiment, even though the oxygen concentration may be vanishingly small.

The reactions of ferrous hemeproteins with oxidizing agents are of interest and have been studied very little. We report here for the first time a study of the reaction of a ferrous hemoglobin with the oxidant, hydrogen peroxide. The products are stable and may be isolated. Recently, Noble and Gibson (5) have reported that hydrogen peroxide reacts with ferrous horseradish peroxidase to convert it to oxyperoxidase in a sequence of two reactions, each of which appears to be a single step, 2-electron oxidation. The first is the reaction of ferrous peroxidase with H$_2$O$_2$ to form compound II, a derivative of horseradish peroxidase at the formal oxidation state IV. The second is the reaction of compound II with H$_2$O$_2$ to form oxyperoxidase.

We here report results to show that an analogous sequence of reactions takes place when ferrous Ascaris perienteric fluid hemoglobin is mixed with H$_2$O$_2$. The first product in this instance is a stable compound, presumed to be at the formal oxidation state IV, and characterized by its optical spectrum. This product, in turn, reacts with further H$_2$O$_2$ to form oxy Ascaris hemoglobin.

A preliminary account of this work has been published (6).

MATERIALS

Ascaris Perienteric Fluid Hemoglobin—This was prepared by a modification of the methods of Davenport (2) and Wittenberg et al. (7). All operations were at 4°C. The ammonium sulfate-purified fraction was further fractionated on a column of Sephadex, and the electrophoretic separation used previously was omitted. The ammonium sulfate precipitate, prepared from 1 liter of perienteric fluid, was dissolved in a minimum volume of 0.05 M potassium phosphate buffer, pH 7.0, and applied to a column of Sephadex G-100, 5 × 90 cm. The column was eluted with the same buffer. The high molecular weight hemoglobin fraction which was eluted first sometimes was partially resolved into two peaks conceivably corresponding to octamer and tetramer of the subunit. No attempt was made to collect these separately since we could detect no differences in chemical properties of appropriate portions of the eluate. A smaller hemoglobin fraction appearing in the eluate approximately in the position expected for a protein of molecular weight 40,000 was not used in this study. Final purification was by chromatography on a column of DEAE-Sephadex, 5.0 × 9.5 cm, which had been equilibrated with 0.12 M potassium phosphate buffer, pH 7.0. The column was developed with a gradient of concentration from 0.12 to 0.25 M potassium phosphate buffer, pH 7.0 (700 ml of 0.12 M; 700 ml of 0.25 M). The ratio of absorbances of the

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purified protein at 410 nm to 280 nm was 1.50 which compares favorably with the ratio exhibited by material prepared by the earlier procedure.

_Ferroxy Ascaris Hemoglobin—_This was prepared by titration of AeschHbO₂ with a solution of dithionite (1 g of sodium dithionite per liter of oxygen-free 1 mM NaOH) under anaerobic conditions. The reaction is complex. The end point was taken as the appearance of the spectrum of ferrous AeschHb, unchanged by further additions of dithionite. Dithionite was usually added to no more than 5% excess.

_Hydrogen Peroxide Solutions—_These were prepared by diluting a stock 30% hydrogen peroxide solution into anaerobic buffer containing 1 mM EDTA. No attempt was made to deoxygenate the stock H₂O₂ solutions as the ratio of H₂O₂ to O₂ in such a solution should be greater than 10⁴.

**Methods**

**Reaction Kinetics—** A Gibson-Milnes stopped flow apparatus (8) with a 2-cm light path in the observation cell was used for all kinetic measurements. Wave lengths frequently used were 560 nm and 340 nm for the rapid and slow reactions, respectively.

**Kinetic Difference Spectra—** These express the change in millimolar extinction coefficient observed during the course of the kinetic process as a function of the wave length of the observation light.

**Static Spectra—** These were determined with a Cary model 11 recording spectrophotometer equipped with a cuvette holder which could be cooled to 4°C. Low temperature spectra were determined as described previously (9), except that the reference cuvette contained buffer at 77°C.

**Spectra of Ascaris Hemoglobin—** These are taken from Wittenberg et al. (7). All concentrations are expressed per mole of heme.

**Rapid Mixing—** Solutions for static spectrophotometry were prepared by rapid mixing. Solutions, delivered from syringes, were mixed by being forced through a mixing chamber similar to that used in the stopped-flow apparatus at a flow rate of approximately 10 ml per s. The temperature was 0°C.

**Experimental Conditions—** All kinetic experiments were carried out at 20°C. Buffers were: 0.05 M potassium phosphate buffer at pH 6.0 and pH 7.0; 0.05 M sodium pyrophosphate buffer (Na₂HPO₄ brought to pH with HCl) at pH 6.0, pH 6.5, pH 7.0, pH 7.5, and pH 9.2; 0.05 M sodium borate, pH 9.2. All solutions contained 1 mM EDTA.

**Formal Oxidation State**

The concept of formal oxidation state was introduced (10) in order to consider the structure of the entire complex comprising the heme and its associated ligands among which sharing of electrons may occur. The oxidation state of a complex is defined as the number of reducing equivalents required to convert the complex, in a hypothetical reaction, to metallic iron plus simple compounds such as water. Thus simple ferrous salts are at oxidation state II, and simple ferric salts are at oxidation state III. Ferrous hemoglobin, ferric hemoglobin, and oxyhemoglobin are, respectively, at the oxidation states II, III, and VI.

Myoglobin at the formal oxidation state IV has been named "ferryl myoglobin" (11). However, since this name implies a particular structure, which is not proved, we prefer to name the products by their parentage and formal oxidation state. For instance myoglobin IV and Ascaris hemoglobin IV.

**Results**

_Kinetics of Reaction of Ferrous Ascaris Hemoglobin with H₂O₂—_ Two kinetic events are seen in the reaction of ferrous AeschHb with H₂O₂. A relatively rapid event is followed by a very much slower subsequent event. Under favorable conditions both events may be observed following a single mixing. For the most part, however, it was found convenient to follow the reactions separately using 0.5 mM H₂O₂ to follow the more rapid reaction and 5 mM H₂O₂ to follow the slower reaction.

The more rapid reaction obeyed first order kinetics with respect to each reactant. A single homogeneous reaction was seen. Since H₂O₂ was present in 25-fold excess, the reaction was treated as pseudo first order and ln (ΔO.D.₄/ΔO.D.₃) was plotted against time. Fig. 1 shows that this plot is linear, indicating a first order dependence of the reaction rate on ferrous AeschHb concentration. The rate of reaction was measured as a function of the H₂O₂ concentration. Fig. 2 shows the linear relation that was found, indicating a first order dependence on H₂O₂ concentration. The second order rate constant did not vary significantly over the range of wave lengths used to construct the kinetic difference spectrum of Fig. 5.

The slower reaction was studied using a 10-fold greater concentration of H₂O₂. At this H₂O₂ concentration the more rapid reaction was too fast to be observed. A single homogeneous reaction was seen. Fig. 3 is a pseudo-first order plot of the time course of the reaction. Fig. 4 presents the rate as a function of H₂O₂ concentration. The linearity of both of these plots establishes that the reaction is first order both with respect to AeschHb-IV concentration and with respect to H₂O₂ concentration. The second order rate constant for this reaction was independent of wave length of the observation light at the wave lengths used to construct the kinetic difference spectrum of Fig. 6.

The second order rate constant for the more rapid kinetic

![Fig. 1 (left). In (ΔO.D.₄/ΔO.D.₃) is plotted against time for the conversion of ferrous AeschHb to AeschHb-IV under conditions where H₂O₂ is present in a 25-fold excess. The initial ferrohemoglobin concentration was 0.0255 mM, and that of H₂O₂ was 0.5 mM; 0.05 M sodium pyrophosphate buffer, pH 7.0.](http://www.jbc.org/content/250/12/4009)
event is relatively independent of pH (Table I). The slower kinetic event is about 100-fold slower than the rapid event. The second order rate constant for this process decreases markedly with increasing pH (Table I).

**Optical Spectrum of Product of More Rapid Reaction**—To discover the optical spectrum of the rapidly formed product, kinetic difference spectra were constructed for the rapid and slow kinetic events (Figs. 5 and 6). The kinetic difference spectrum of the rapid event combined with the static spectrum of ferrous AscHb, and the kinetic difference spectrum of the slow event combined with the static spectrum of AscHbO2 each generate the direct spectrum of the first-formed product, that is the substance formed in the rapid event and consumed in the slow (Figs. 5 and 6). The wave length maxima are approximately 542 nm (ε_{max} ~ 10.5) and 576 nm (ε_{max} ~ 8.5). These spectra, within experimental error, are indistinguishable from that of the stable product described below, evidence that the stable product is the rapidly formed product.

A number of comments may be made about the validity of this spectrum. The direct spectra of Figs. 5 and 6, generated from two independent sets of data, are very similar. This indicates that only one intermediate product is seen on the time scale of these experiments. In a separate experiment the kinetic difference spectra and reconstructed direct spectra for both reactions were repeated working at a more alkaline pH: pH 8.5 in 0.05 m potassium phosphate buffer. These did not differ from those done at pH 7.0 and reported in extenso here. This indicates that the nature of the product is independent of pH in this range. The optical density increments occurring in the conversion of the first product to AscHbO2 were the same in three separate kinetic difference spectra constructed at pH 6.5, pH 7.0, and pH 8.5. This finding helps to dispel any residual doubt that the spectrum reconstructed for the rapidly formed product might actually have been that of oxyhemoglobin.

**Optical Spectra of Ascaris Hemoglobin IV**—Relying on the experience gained in kinetic experiments, it was possible to devise conditions under which AscHb-IV was generated with minimal side reactions. Solutions of ferrous AscHb (0.091 mM) in 0.05 m pyrophosphate buffer, pH 6.5 or pH 8.5, containing 1 mM EDTA, were mixed rapidly, at 0°, with equal volumes of anaerobic solutions of H2O2 (10 mM) in the same buffer. At this temperature the rate of the reaction of AscHb-IV with H2O2 is reduced to the point where this reaction does not interfere. The spectrum of the product was the same at the two pHs. As a control, portions of the same solutions of ferrous AscHb were mixed rapidly with equal volumes of oxygen-equilibrated buffer to generate solutions of AscHbO2 of exactly the same concentration.

Ascaris hemoglobin IV was found to be relatively stable. At pH 6.5 a small spectral change, which could be interpreted as
the formation of AscHbO₂ became evident in solutions standing at room temperature for about 20 min. At pH 8.5, where the reaction of AscHb-IV with H₂O₂ is much slower, a very small spectral change was seen after 50 min with no suggestion of the formation of AscHbO₂.

The spectrum of the product, AscHb-IV, is presented in Figs. 7 and 8, and the spectral constants are collected in Table II. The spectra of AscHbO₂ prepared from the selfsame solutions of ferrous AscHb are presented for comparison. The outstanding finding is that the wave length maxima are the same as those of the oxyhemoglobin, although the molar extinctions are less. Also noteworthy is the finding that the spectra were invariant with pH in the range studied.

Other physical properties of hemeproteins, of which one is electron paramagnetic resonance, are often best studied at low temperature. Optical spectra are a most sensitive indicator of the configuration of the heme and its associated ligands and may be used to show that the species predominating at low temperature and probed by other physical techniques is the same as the species studied in solutions at 0°. For this reason the optical spectrum of AscHb-IV was determined at the temperature of liquid nitrogen.

**Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spectral constants</th>
<th>213°K</th>
<th>77°K</th>
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<tr>
<td></td>
<td>Wave length maximum</td>
<td>ε₉₃₅</td>
<td>Wave length maximum</td>
</tr>
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<td>Oxyhemoglobin isolated as such*</td>
<td>412</td>
<td>190.5</td>
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<td></td>
<td>544</td>
<td>12.3</td>
<td>544</td>
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<tr>
<td></td>
<td>576.5</td>
<td>10.4</td>
<td>576.5</td>
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<tr>
<td>Oxyhemoglobin generated from deoxyhemoglobin</td>
<td>412</td>
<td>110</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>544</td>
<td>12.3</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td>575</td>
<td>10.4</td>
<td>575</td>
</tr>
<tr>
<td>Homoglobin IV generated from deoxyhemoglobin</td>
<td>411</td>
<td>97.5</td>
<td>411</td>
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<tr>
<td></td>
<td>542</td>
<td>10.6</td>
<td>542</td>
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<tr>
<td></td>
<td>576</td>
<td>8.7</td>
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</table>

* From Wittenberg, Okazaki, and Wittenberg (7).
Ferrous Ascaris hemoglobin and hydrogen peroxide react to generate a stable product. Only small amounts of the side products ferric AscHb and AscHbO₃ contaminate the product, and the formation of the latter is minimal at slightly alkaline pH. The simplest interpretation of the data is to assume that first-formed product is at the formal oxidation state IV; in which case it may be named Ascaris hemoglobin IV. The kinetics of the formation and subsequent reaction of this product are consistent with the assigned oxidation state. Furthermore there is a strong analogy between the reactions of Ascaris hemoglobin with hydrogen peroxide and those of horseradish peroxidase. The first-formed product of the latter reaction is identified by its optical spectrum as compound II of horseradish peroxidase; the first-formed product of the reaction of ferrous Ascaris hemoglobin with H₂O₂ is identified by its optical spectrum as compound II of horseradish peroxidase. The wave length maxima of the optical spectra of AscHb-IV and AscHbO₃ are sensibly the same, both at ice and liquid nitrogen temperature. Optical spectra are most sensitive indicators of the electronic structure of the heme iron atom and its adjacent ligand atoms in hemeproteins. Elsewhere (10, 12) it has been argued that if a series of hemeprotein derivatives have sensibly identical optical spectra, the state of the heme iron must be the same in all members of that series, and that if the state of the heme iron could be known independently for any one member, it would be known for all. The heme iron atom of oxyhemoglobin is believed to be low spin ferric heme iron (heme d₅) (12, 13). This consideration suggests most strongly that the heme iron atom of AscHb-IV is likewise low spin ferric heme iron (heme d₅).

The reaction of ferrous AscHb with H₂O₂ to form AscHb-IV is first order with respect to both reactants. The simplest interpretation is that a single molecule of ferrous AscHb and a single molecule of H₂O₂ are involved. If so, all of the oxidizing equivalents must remain with the hemoglobin molecule. The reaction may be regarded as a two electron oxidation.

The reaction of AscHb-IV with H₂O₂ is also first order with respect to both reactants. The formal oxidation state of the product, AscHbO₃, is VI. If a single molecule of each reactant is involved in the reaction, all of the oxidizing equivalents must be retained in the oxyhemoglobin formed. This reaction also may be regarded as a two electron oxidation.

Kinetic measurements cannot establish a reaction stoichiometry, nor can they prove the absence of reaction intermediates. Nevertheless the data clearly show that H₂O₂ converts ferrous Ascaris hemoglobin to oxyhemoglobin, with the intermediate formation of a product provisionally identified as AscHb-IV. The simplest mechanism consistent with the data is that both of these reactions are single step, 2-electron oxidations, each involving a single molecule of hydrogen peroxide.

REFERENCES


The Reaction of Ferrous Ascaris Perienteric Fluid Hemoglobin with Hydrogen Peroxide
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