MECHANISM OF THE OXIDATION OF HEMOGLOBIN BY COPPER (II) COMPLEXES

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Abstract

An outer sphere electron transfer mechanism by which human hemoglobin reduces the complexes of copper(II) and, in turn, is oxidized to methemoglobin has been characterized. We have found that the rate of oxidation of hemoglobin is a function of pH, temperature, concentration of copper(II), and the environment of the hemoglobin. Prior to oxidation, copper(II) complex binds to specific sites on the surface of the protein by losing one or more of its ligands, forming a ternary complex. This process is followed by electron transfer between the Cu(II) and Fe(II) with the Cu(II)-deoxyhemoglobin being the active intermediate. The dominant factors which govern the rate of oxidation of hemoglobin by copper(II) complexes seem to be the stability constant of the Cu(II) complexes and the overall redox potential of the ternary complex.

INTRODUCTION

Hemoglobin, the oxygen carrying molecule in vertebrate erythrocyte, is a tetrameric protein made of two α and two β subunits. Each subunit consists of a heme group and a polypeptide chain folded around it. The heme consists of a protoporphyrin ring with an iron in the ferrous form at
its center. In deoxyhemoglobin the iron has a high spin state, located about 0.6 Angstrom above
the plane of the porphyrin, coordinated to four nitrogens of the porphyrin, and covalently
bonded to the nitrogen of a proximal histidine residue of the polypeptide chain. The sixth
coordination of the iron is vacant and available for oxygen binding(1).

A special characteristic of hemoglobin is its tendency to undergo conformational changes
during binding with oxygen or other ligands. Deoxyhemoglobin exists almost exclusively in the so
called T(Tense) configuration while fully oxygenated hemoglobin exists in the R(Relax)
configuration. When an oxygen binds with hemoglobin, the iron reverts to low spin state, shrinks
in size and moves down to the plane of the heme. This motion causes further displacement of
various amino acids, changing the area of contact between the unlike subunits. Further binding
of oxygen with the hemes produces enough strain to convert the protein structure from T to R
state at which the affinity of the hemoglobin for oxygen increases drastically.

The ability of hemoglobin to carry oxygen requires that iron be in the ferrous form. Once
hemoglobin is oxidized and is converted to methemoglobin, it is no longer capable of oxygen
binding. Many nucleophilic anions such as SCN⁻, F⁻, Cl⁻, and some heavy metals can cause the
conversion of ferrous to ferric (2). In fact, about 3% of the hemoglobin in human blood
undergoes oxidation on the daily basis, forming methemoglobin, which is converted to
hemoglobin by the enzymatic action in the erythrocyte.

A possible catalyst for the oxidation of hemoglobin is hypothesized to be copper(II). Copper
is the most abundant metal in human body after zinc and iron and although most heavy metals
increase the rate of oxidation of hemoglobin, copper(II) ranks first (3).

The mechanism of the oxidation of hemoglobin by copper(II) complexes follows an outer
sphere electron transfer process. Generally, two mechanisms for the electron transfer between a
protein and a metal complex is postulated, an outer sphere and an inner sphere electron transfer
mechanism. For the oxidation of hemoglobin by copper(II) complexes the inner sphere electron
transfer mechanism is not possible due to inaccessibility of the heme to the solvent and that the
heme crevice is too small to allow the relatively large copper(II) complex to form bonds with the
iron. According to outer sphere mechanism, the transfer of electron from hemoglobin to cupric
ion is accomplished by binding of the cupric ion to the surface of the protein, dissociation of the
ligand, and finally transfer of electron between the redox centers.

The site on the hemoglobin surface that the cupric ion binds to is the \( \beta \)-93 sulphydryl group. The binding studies by Bemski et al.\( (4) \) have shown several Cu(II) binding sites on the surface of the protein. Of these sites only the \( \beta \)-93 SH group seems to have a major role in the oxidation process. This site is in close proximity of the heme, and on blocking this site with N-ethylmaleimide the rate of oxidation decreases by about 98\% \( (5) \).

In the following, we have undertaken the study of oxidation of hemoglobin by Cu(II) complexes in neutral and basic medium and at various reaction conditions in order to explore the factors which govern the rate of oxidation, and from them to draw a qualitative picture for the mechanism of the oxidation of hemoglobin by copper(II) complexes.

**MATERIALS AND METHODS**

Hemoglobin solutions were obtained by purifying human blood and dialyzing them against buffers with various pH values. The purity of these samples were determined using the ratio of peaks at 576 and 540 nm. A value of 1.06 or higher for the ratio indicated the presence of minimal amount of methemoglobin in the samples. These samples were kept at 0°C prior to analyses.

**BUFFERS**

Phosphate buffers with pH values of 6, 7, and 8 were prepared by mixing appropriate amount of potassium dihydrogen phosphate and dipotassium hydrogen phosphate. Tris-HCl buffers with pH values of 7, 8, and 9 were prepared by mixing the appropriate volumes of 1.0 M HCl solution and 1.0 M tris(hydroxymethyl)aminomethane solution. The above buffers had an ionic strength of 0.1 \( (6) \).

**OXIDIZING AGENTS**

For most of the experimental work, copper(II) sulfate, \( 5\text{H}_2\text{O} \) was utilized as an oxidizing agent. Other copper(II) complexes were prepared by mixing a solution of the ligand with the concentrated solution of copper(II) chloride. The crystalized products were isolated, dried and
kept in a desicator prior to use. The hemocyanin from Keyhole Limpets was obtained from SIGMA.

REACTION CONDITION

For the experiments 3.0 ml of a 110μM oxyhemoglobin solution was transferred into the cuvette and mixed with 1.0 ml of the copper(II) complex with an appropriate concentration to produce the desired Cu(II)/heme concentration ratios. The oxidation of oxyhemoglobin was monitored by following the changes in the spectrum of the oxyhemoglobin on a Shimadzu UV-VIS double beam spectrophotometer, model 2100U.

The change in the concentration of oxyhemoglobin was calculated using the absorbance values at 576 nm for oxyhemoglobin with (Σ=16500 Cm⁻¹M⁻¹) and at 540 nm for methemoglobin (Σ=10500 Cm⁻¹M⁻¹). The desired temperatures were maintained automatically by circulating air through the cuvette chamber of the spectrophotometer. The exact reaction conditions are shown with each graph.

RESULTS AND DISCUSSION

Oxidation of oxyhemoglobin by copper(II) ions was studied in three different mediums of water, tris-HCL buffer, and phosphate buffer and at temperatures of 15, 20, and 25°C. The results suggested several factors were influential in determining the rate of oxidation of oxyhemoglobin. Below these factors are stated and discussed.

EFFECT OF MEDIUM

The environment of the oxyhemoglobin seems to be effective in the rate of oxidation (Table 1). Comparison of the data indicates that the rate of oxidation is the slowest for the phosphate buffer. Here, phosphate may act as chelating agent for the cupric ions. As a result, the number of cupric ions in the solution is reduced, and the rate of oxidation is decreased.

EFFECT OF pH

As the pH of the oxyhemoglobin solution increases, the rate of oxidation decreases. This
behavior may be the result of two different processes. First, pH may affect the redox potential of the copper(II) complex and/or oxyhemoglobin, leading to a slower rate of oxidation at higher pH values. Second, as the oxyhemoglobin solution becomes more basic, the hydroxide ions may provide the protein with an electron rich environment. As a result, negative charge density flows toward the heme, causing stability of the protein, and thus, slower rate of reaction.

Table 1. Half-life values for the oxidation of oxyhemoglobin by Cu(II) sulfate.

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>[Cu]: [Heme] Ratio</th>
<th>Half-life values, hour at 15°C</th>
<th>20°C</th>
<th>25°C</th>
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<td>15</td>
</tr>
<tr>
<td>Water</td>
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<td>3</td>
<td>1.6</td>
<td>1.5</td>
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Fig. 1. Effect of temperature on the rate of oxidation of oxyhemoglobin in Tris-HCl buffer by copper(II) sulfate at pH = 8 and Cu(II)/heme = 1:1
EFFECT OF TEMPERATURE

As the temperature of the oxyhemoglobin solution increases, the rate of oxidation increases as well (Figure 1). The Arrhenius energy of activation for the oxidation process was found to be 17KCal/mol at neutral pH value. The activation energy increases at higher pH values which indicates the greater stability of the system as stated above.

EFFECT OF Cu(II) CONCENTRATION

When Cu(II) ions are mixed with oxyhemoglobin solution, the concentration of oxyhemoglobin shows a reaction consisting of an exponential decay. At high concentration of cupric ion precipitation of the protein occurs shortly after mixing the reagents. Lower pH values and higher temperature speed the precipitation process.

The pattern of oxidation of oxyhemoglobin consists of a fast and a slow phase (Figure 2). Initially, the rate is fast, but after about 50% of the oxyhemoglobin is oxidized, that is, it is converted to methemoglobin, the rate of oxidation decreases substantially. The fast phase is believed to be due to the oxidation of \( \beta \) chain while the slow phase is due to the oxidation of the \( \alpha \) chain. However, before the \( \alpha \) chains are completely oxidized, the protein denatures and its precipitation takes place (7).

EFFECT OF IONIC STRENGTH

As the concentration of sodium acetate in oxyhemoglobin solution was increased, the rate of oxidation increased as well (Figure 3). Although the effect of the salt on the rate of oxidation was not significant, it indicated that the nature of interaction between the protein and the Cu(II) complex is electrostatic.

EFFECT OF Cu(II) COMPLEXES

We compared the rate of oxidation of oxyhemoglobin by Cu(II)(phen)\(_2\), Cu(II)(py)\(_4\), Cu(II)(EDA)\(_2\), Cu(II)citrate, and hemocyanin with that by Cu(II)sulfate (Figure 4). Our results indicated that the size of the Cu(II) complex is not a factor in the rate of oxidation of oxyhemoglobin. For instance, EDA and hemocyanin seem to oxidize hemoglobin at the same
Fig. 2. Effect of copper(II) concentration on the rate of oxidation of oxyhemoglobin in water at 20°C
Fig. 3. Effect of ionic strength on the rate of oxidation of oxyhemoglobin in water at 20°C.
Fig. 4. Effect of various copper(II) complexes on the rate of oxidation of oxyhemoglobin in water at 20°C.
rate, yet they are different in size. In addition, the ionic radii of the Cu(II)(py)₄ and the Cu(II) (phen)₂ complexes are nearly the same, yet they oxidize oxyhemoglobin at different rates. Therefore, we can disregard the size of the Cu(II) complex as a factor in the rate of oxidation.

It seems that the charge of the Cu(II) complex has no effect on the rate of oxidation of oxyhemoglobin. The Cu(II) complexes, which we have utilized, bear a dipositive charge yet the rate at which they oxidize oxyhemoglobin is different. Therefore, we conclude that the charge of the Cu(II) complex is not a factor in the rate of oxidation of oxyhemoglobin.

Our results indicated that a direct relationship exists between the rate of oxidation and the stability constant of the Cu(II) complexes (Figure 5). Since the rate of oxidation of oxyhemoglobin depends on how easily the Cu(II) complex loses its ligands, it is likely that the loss of ligand by Cu(II) complex is a necessary step in the oxidation process.

P. Hegescheuser et al. (8) have demonstrated that oxidation of myoglobin by Cu(II) complexes is accompanied by the formation of a ternary complex between the Cu(II) complex and the protein. Indeed, the same can be applied to hemoglobin considering our results. The Cu(II) complex loses one or more of its ligands and forms a bond with β-93 SH group. Then, the new complex, Cu(II)-oxyhemoglobin undergoes internal electron transfer.

Another factor which influences the rate of oxidation is the difference in the redox potential of the interacting species. Generally, copper ligands that contain nitrogen or oxygen have a low redox potential while those containing sulfur have a high redox potential. Here, the copper(II) complexes which we utilized have a low redox potential since they contain nitrogen or oxygen. However, these complexes must bind to the sulfur of the β-93 residue in order to follow with the oxidation process. As a result, their redox potential increases, and their oxidizing power becomes larger.

**MECHANISM OF THE OXIDATION**

From the results obtained we can now establish the mechanism of the oxidation of oxyhemoglobin by Cu(II) complexes. Prior to oxidation, the Cu(II) complex loses one or more of its ligands, forming a new complex with the protein (Eq. 1). As a result, certain conformational changes may take place in the protein that facilitates the release of oxygen (Eq. 2). Once oxygen
Fig. 5. Rate of oxidation of oxyhemoglobin by copper(II) complexes as a function of the stability constant.
is released, an electron is rapidly transferred between the redox centers (Eq. 3).

\[ \text{HbO}_2 + \text{Cu(II)} \text{ L}_m \iff \text{L}_m \text{ Cu(II)-HbO}_2 \]  \hspace{1cm} (1)

\[ \text{L}_m \text{ Cu(II)-HbO}_2 \iff \text{L}_m \text{ Cu(II)-Hb} + \text{O}_2 \]  \hspace{1cm} (2)

\[ \text{L}_m \text{ Cu(II)-Hb} \iff \text{L}_m \text{ Cu(I)-Hb}^+ \]  \hspace{1cm} (3)

Dissociation of oxygen may be facilitated by certain conformational changes. As mentioned earlier, oxyhemoglobin exists in the "relax" state. However, it is possible that when the Cu(II) complex binds with the protein, it may cause conformational changes within the protein opposite that of oxygen binding. That is, the area of contact between the subunits changes, the iron is pulled up above the plane of the porphyrin, and the protein reverts to the "tense" state. As a result, oxygen is dissociated from the protein much easier.

Some studies have suggested that during the oxyhemoglobin oxidation superoxide is formed which, in turn, reduces the Cu(II) complex (9). This finding seems unlikely considering our results and studies performed on myoglobin oxidation by Cu(II) complexes. Oxymyoglobin, which is similar to oxyhemoglobin in behavior, is oxidized by Cu(II) complexes without producing any superoxide but oxygen is simply dissociated (8). In addition, the temperature dependence of the oxyhemoglobin oxidation allowed us to calculate the Arhenious energy of activation. At neutral pH this value was 17 KCal/mol which is slightly higher than the energy required to break the Fe-O_2 bond. If superoxide were to form, it seems that the energy required to separate two charged species, namely superoxide and Fe(III), would be much higher. It is possible that the 17 KCal/mol is the sum of the energies for the dissociation of oxygen and the electron transfer itself.

The second equation is the rate determining step. The rate of oxidation of oxyhemoglobin is a function of oxygen concentration (10). As the concentration of oxygen increases, the rate of oxidation decreases. It is clear that an increase in the concentration of oxygen shifts the equilibrium to the left, thus decreasing the rate of oxidation. In addition, comparison of the rate of oxidation of deoxyhemoglobin, oxyhemoglobin, and carboxyhemoglobin had shown that the rate is fastest for the deoxyhemoglobin and the slowest for the carboxyhemoglobin (11). This behavior is consistent with the Fe-ligand bond energies. Fe-Co bond energy is the highest, thus the rate of oxidation of carboxyhemoglobin is the slowest. Therefore, dissociation of oxygen is
the rate determining step and takes place prior to the transfer of electron.

Following the dissociation of oxygen electron transfer takes place. Although the distance between the redox centers in the Hb-Cu(II) complex is about 10 Å, the electron has no difficulty passing through. The reason for this facile transfer of electron seems to be the pathway that lies between the redox centers. This pathway seems to be made up of an array of π-orbitals of the intervening aromatic residues. The importance of the aromatic residue in the long range electron transfer has been shown by Liang et al. (12). They showed that the rate of inter protein electron transfer was increased by about 10000 times when an aromatic residue was substituted in place of an aliphatic residue. The rate enhancement was interpreted to be a result of the effective mixing of the heme π electron system through the intervening aromatic residue. Indeed, such a pathway exists in hemoglobin as well. The overlap of the heme π electron system with the empty 3d-orbital of the sulfur through the intervening aromatic imidazol ring can provide an efficient pathway for the rapid transfer of electron in the Cu(II)-hemoglobin system.

CONCLUSION

The rate of oxidation of hemoglobin has been found to be inversely proportional to the pH and directly proportional to the temperature of the hemoglobin solution. It appears that the buffer systems are also effective in the rate of oxidation.

Furthermore, the rate of oxidation was found to be the function of Cu(II) concentration and on the type of Cu(II) complex. However, neither size nor the charge of the Cu(II) complex has any influence on the rate of oxidation. The mechanism of the oxidation of hemoglobin by Cu(II) complexes follows an outer sphere electron transfer process. Accordingly, Cu(II) complex loses one or more of its ligands and binds to β-93 sulfhydryl group prior to electron transfer and the dominant factors which determine the rate of oxidation of hemoglobin seem to be both the redox potential of the Cu(II)-hemoglobin complex and the stability constant of the Cu(II) complex.
REFERENCES


