Copper and the Oxidation of Hemoglobin: A Comparison of Horse and Human Hemoglobins†

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ABSTRACT: Oxidation studies of hemoglobin by Cu(II) indicate that for horse hemoglobin, up to a Cu(II)/heme molar ratio of 0.5, all of the Cu(II) added is used to rapidly oxidize the heme. On the other hand, most of the Cu(II) added to human hemoglobin at low Cu(II)/heme molar ratios is unable to oxidize the heme. Only at Cu(II)/heme molar ratios >0.5 does the amount of oxidation per added Cu(II) approach that of horse hemoglobin. At the same time, binding studies indicate that human hemoglobin has an additional binding site involving one copper for every two hemes, which has a higher copper affinity than the single horse hemoglobin binding site. The Cu(II) oxidation of human hemoglobin is explained utilizing this additional binding site by a mechanism where a transfer of electrons cannot occur between the heme and the Cu(II) bound to the high affinity human binding site. The electron transfer must involve the Cu(II) bound to the lower affinity human hemoglobin binding site, which is similar to the only horse hemoglobin site. The involvement of β-2 histidine in the binding of this additional copper is indicated by a comparison of the amino acid sequences of various hemoglobins which possess the additional site, with the amino acid sequences of hemoglobins which do not possess the additional site. Zn(II), Hg(II), and *N*-ethylmaleimide (NEM) are found to decrease the Cu(II) oxidation of hemoglobin. The sulfhydryl reagents, Hg(II) and NEM, produce a very dramatic decrease in the rate of oxidation, which can only be explained by an effect on the rate for the actual transfer of electrons between the Cu(II) and the Fe(II). The effect of Zn(II) is much smaller and can, for the most part, be explained by the increased oxygen affinity, which affects the ligand dissociation process that must precede the electron transfer process.

The reversible oxygenation of hemoglobin involves the binding of oxygen to the divalent ferroporphyrin (protoheme) prosthetic group. Therefore, the effective transport of oxygen requires that the oxidation of hemoglobin be minimized, and it becomes important to understand the various mechanisms by which hemoglobin can be oxidized.

It has been shown that Cu(II) enhances the rate of oxidation of frog (Salvati et al., 1969), human (Bemski et al., 1969), and horse (Rifkind, 1974) hemoglobins. In fact, it has been suggested, partly on the basis of the catalytic oxidation of horse hemoglobin at trace concentrations of Cu(II), that Cu(II) may be involved in the oxidation of hemoglobin within the erythrocyte (Rifkind 1974).

Mechanism I has been proposed for the oxidation of horse HbO_2[1] by Cu(II) (Rifkind, 1974). $(HbO_2)_4$ is the fully oxygenated hemoglobin, which is the dominant species present at atmospheric oxygen pressures, and $(HbO_2)_3Hb$ is hemoglobin with one oxygen molecule dissociated. The dissociation of the oxygen from the heme prior to oxidation (step 2) was indicated by the finding that the initial rates of oxidation followed the order Hb > HbO_2 ≫ HbCO. The regeneration of Cu(II) (step 4) explains the catalytic oxidation by low Cu(II) concentrations. The rate constant, k_4, for this process is, however, much smaller than the rate constants for steps 1–3. Therefore, a biphasic oxidation curve is obtained in the presence of an excess of HbO_2. The initial rapid phase utilizes all the added Cu(II) and is over within 3 min. The oxidation then continues at a rate which is limited by the slow step 4 and which is more than an order of magnitude slower than the initial rapid phase of oxidation.

In the absence of oxygen, step 4 does not take place and only one phase of oxidation is observed. In the absence of any ligand, e.g., CO or O_2, Cu(II) binds directly to Hb and no step 2 involving the dissociation of the ligand is required prior to oxidation.

In this paper, we have investigated the various processes involved in the oxidation by looking at various different hemoglobins and by the addition of various substances which interact with hemoglobin. Oxidation and binding studies on human, rabbit, sheep, bovine, and horse hemoglobins, demonstrate that human and rabbit hemoglobins have an additional binding process. The effects of Mn(II), Zn(II), Hg(II), and NEM on the Cu(II) oxidation are interpreted in terms of alterations in the ligand dissociation and electron transfer processes (steps 2 and 3) of Mechanism I.

Experimental Section

Hemoglobin. Horse blood was defibrinated pooled blood obtained from Bioquest. Human blood was obtained from a local blood bank or from the participants of the longitudinal program at the Gerontology Research Center.

The red blood cells were separated from whole blood by centrifugation. The cells were washed several times by suspending them in 0.9% NaCl and recentrifuging them. The cells were them hemolyzed in 2 volumes of distilled water at 4 °C, and separated from the cell membranes by high-speed centrifugation (Rifkind, 1974). With human blood it was found that complete removal of the membranes was facilitated by freezing and thawing the hemolysate prior to centrifugation

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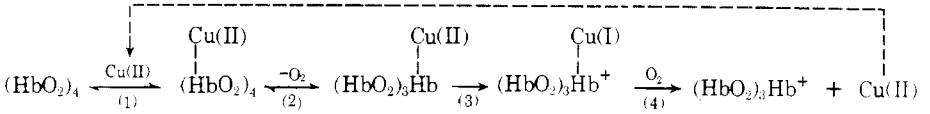
[1] Abbreviations used are: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carboxyhemoglobin; BATH, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

MECHANISM 1.



(Beutler, 1971). The low-molecular-weight components of the erythrocytes (Rifkind, 1972) were removed by dialysis and gel filtration on Sephadex G-25 (Rifkind, 1972) eluted at 4 °C with 0.01 M Trizma–0.1 M NaCl, pH 7.4, buffer. The hemoglobin was stored either as HbO_2 or, in order to minimize oxidation (Rifkind, 1974), as HbCO.

For hemoglobin stored as HbCO, HbO_2 was prepared by passing oxygen over a rotating sample of HbCO for 2 h at 4 °C. Hb was prepared by passing nitrogen over a rotating sample of HbO_2 for 2 h at 4 °C. The conversion to Hb was considered to be complete when the ratio of the absorbance of the solution at 555 nm divided by the absorbance at 540 nm was greater than 1.24.

Preparation of NEM–Hemoglobin. The hemoglobin was incubated overnight with a small excess of NEM. The unreacted NEM was removed by gel filtration. 4,4′-Dithiodipyridine was then used to check for unreacted sulfhydryl groups (Ampulski et al., 1969). The reactions with NEM were found to have blocked >98% of the free SH groups.

Oxidation of Hemoglobin. The desired amount of aqueous copper(II) chloride solution was added to the hemoglobin sample. The oxidation was monitored by following the changes in the spectrum of hemoglobin. The percent oxidation of hemoglobin was calculated from the observed change in absorbance at a given wavelength (577 or 630 nm for HbO_2, 569 nm for HbCO, and 555 nm for Hb) and from the change in absorbance at that wavelength calculated for complete conversion of methemoglobin (Van Assendelft, 1970). The rapid phases of the oxidation were followed by placing a sample in the spectrophotometer with the wavelength set at a certain wavelength and the chart moving at a set speed. A stop watch was started to coincide with one of the markings on the paper of the recorder. Without stopping the recorder, the Cu(II) was added to the hemoglobin sample, at a predetermined time, mixed by inversion, and replaced in the spectrophotometer. In this manner, readings could be obtained within 15 s of the time when the Cu(II) was added to the hemoglobin.

Experiments involving HbO_2 were carried out in a cell open to the atmosphere. Experiments with HbCO and Hb were carried out in a specially designed tonometer consisting of a 50-ml round-bottomed flask with a standard 1-cm quartz cell attached to the bottom and a small side arm attached near the neck. This was attached with standard tapered glass joints to a rotatory evaporator that, in turn, was connected to a gas source. The HbCO experiments were performed at a CO pressure of 1 atm and the Hb experiments were performed in an atmosphere of nitrogen. A rubber-septum cap placed on the side arm excluded atmospheric oxygen while allowing for the introduction of Cu(II) solutions into the hemoglobin samples.

Determination of Cu(I). It was possible to directly determine Cu(I) in the presence of Cu(II) under the conditions used in our oxidation studies. This was accomplished by using (BATH) 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (Blair and Diehl, 1961), which is a disulfonic acid water-soluble derivative of bathocuproine (Borchardt and Butler, 1957). This reagent reacts specifically with Cu(I) producing a yellow complex with a molar absorptivity of 12 250

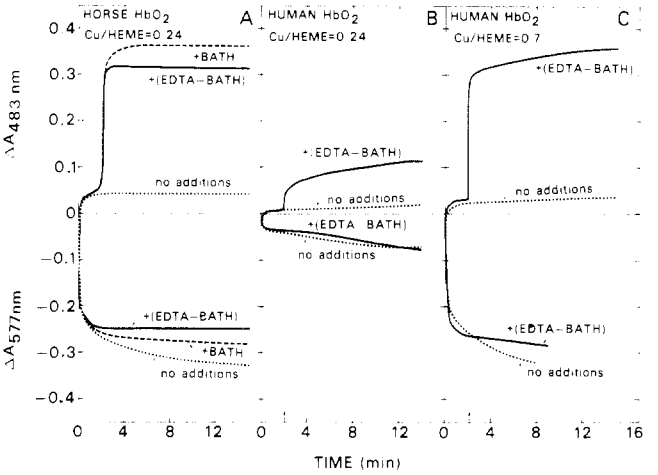


FIGURE 1: Comparison of the Cu(II) oxidation at 25 °C in 0.01 M Trizma–0.1 M NaCl, pH 7.4, of 1×10^{-4} M horse and human hemoglobin chromatographed on Sephadex G-25. The absorbance changes are monitored at 577 and 483 nm. The concentration of Cu(I) present 2 min after the addition of Cu(II) was determined by adding BATH to the cuvette at that time. The total volume prior to the addition of BATH was 2.83 ml. (A) Horse hemoglobin, Cu(II)/heme molar ratio = 0.24; (···), no additions at 2 min; (- - -), addition of 20 λ of 0.05 M BATH at 2 min; (—), addition of 20 λ of 0.05 M BATH plus 0.01 M EDTA at 2 min. (B) Human hemoglobin, Cu(II)/heme molar ratio = 0.24; (···), no additions at 2 min; (—), addition of 20 λ of 0.05 M BATH + 0.01 M EDTA at 2 min. (C) Human hemoglobin, Cu(II)/heme molar ratio = 0.7; (···), no additions at 2 min; (—), addition of 20 λ of 0.05 M BATH plus 0.01 M EDTA at 2 min.

at 483 nm. Hemoglobin has a relatively low molar absorptivity at this wavelength. Furthermore, 483 nm is close to the isosbestic point between HbO_2 and methemoglobin (Van Assendelft, 1970), and there is, therefore, a relatively small change in the absorbance at 483 nm when HbO_2 is oxidized (Figure 1).

BATH was added to hemoglobin solutions before the addition of Cu(II) or at a predetermined time after the addition of Cu(II). The formation of Cu(I) was then followed at 483 nm. The Cu(I) present at the time of addition of BATH was found to react within 15 s. By using a Model 2416 wavelength programmer attachment to a Model 2400 Gilford spectrophotometer, it was possible to follow the oxidation of HbO_2 at 577 nm and the formation of Cu(I) at 483 nm simultaneously.

Electron Spin Resonance (ESR) Measurements. The electron spin resonance spectra were taken on a modified Varian E-4 ESR spectrometer operating at 8.9 GHz, at 27 K.

Both horse and human HbCO were mixed with the desired amount of a $CuCl_2$ solution, and then transferred to a 5-mm quartz ESR tube. The sample was slowly cooled to 27 K. The spectra were calibrated by diphenylpicrylhydrazyl (DPPH) as an external standard.

Binding of Metal Ions to Hemoglobin. The binding was determined by equilibrium dialysis using the precedures previously described (Rifkind, 1974). The total concentration of each metal ion inside and outside of the dialysis bags was de-

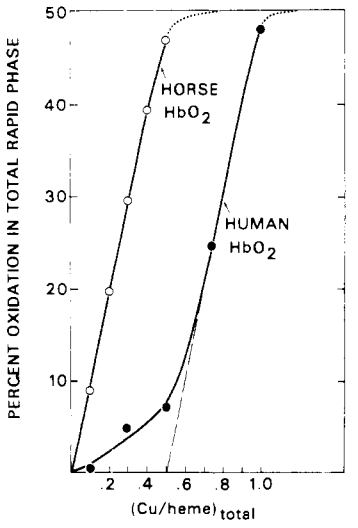


FIGURE 2: The percent oxidation at the end of the rapid phase of oxidation for human (●) and horse (○) HbO_2 at various Cu(II)/heme molar ratios. The hemoglobin was chromatographed on Sephadex G-25. Oxidation was performed in 0.01 M Trizma–0.1 M NaCl, pH 7.4, at 25 °C. The heme concentration was 1×10^{-4} M.

termined with a Perkin-Elmer Model 306 atomic absorption spectrophotometer. For samples with a very low total copper concentration an HGA-2100 Graphite Furnace attachment was used, while for the remainder of the samples the flame attachment was used. The apparent concentration of free metal ion was set equal to the total outside concentration. The concentration of metal ion bound to hemoglobin was calculated from the difference between the total metal ion concentration inside and outside the dialysis bags.

General. All glassware was cleaned with nitric acid, followed by exhaustive rinsing in deionized or distilled water, in order to eliminate trace metal contaminants on the glassware (Rifkind, 1974). All chromatographic columns were washed with a sample of EDTA before introducing hemoglobin samples (Rifkind, 1974). Unless specified otherwise, all experiments were performed in pH 7.4 0.01 M Trizma–0.1 M NaCl. BATH was obtained from G. Frederick Smith, and 4,4′-dithiodipyridine was obtained from Aldrich. All other chemicals were reagent grade and were used without further purification.

Results

Spectrophotometric Cu(II)-Induced Oxidation

The Oxidation of Various Types of Hemoglobin by Cu(II). All of the Cu(II) added to an excess of horse HbO_2 is stoichiometrically used to rapidly oxidize the heme within a period of 3 min (Rifkind 1974). We have used BATH to demonstrate that for horse hemoglobin this rapid oxidation of the heme coincides with the reduction to Cu(I) of all the Cu(II) added.

Figure 1 compares the effect of Cu(II) on horse and human HbO_2. These results at 577 nm indicate that horse hemoglobin is much more readily oxidized than human hemoglobin at a Cu(II)/heme molar ratio of 0.24 (Figure 1A, B); i.e., a large fraction of the added Cu(II) does not rapidly oxidize human HbO_2. In fact, a Cu(II)/heme molar ratio of 0.7 is necessary to oxidize human hemoglobin to the extent that a Cu(II)/heme molar ratio of 0.24 oxidizes horse hemoglobin (Figure 1A, C).

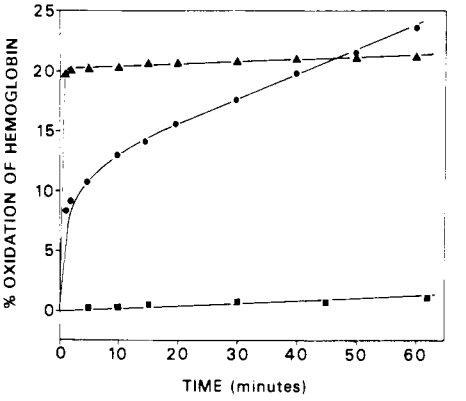


FIGURE 3: The oxidation of various human hemoglobin derivatives by copper(II) at a Cu(II)/heme molar ratio of 0.5. Hemoglobin forms used were deoxyhemoglobin under an N_2 atmosphere (▲), oxyhyemoglobin at atmospheric O_2 pressure (●), and carboxyhemoglobin at 1 atmosphere CO (■). The oxidation was performed in 0.01 M Trizma–0.1 M NaCl, pH 7.4, at 25 °C. The heme concentrations were $5.7\text{–}6.0 \times 10^{-5}$ M. The hemoglobin was chromatographed on Sephadex G-25.

In these experiments, a combination of EDTA[2] and BATH was added 2 min after the Cu(II) in order to determine how much Cu(I) is present at that time. It is found (Figure 1) that the amount of Cu(I) present after 2 min is correlated with the amount of oxidation that has taken place and not the amount of Cu(II) added; therefore, in both cases, one Cu(II) is reduced for each heme oxidized (Mechanism I) and the failure of all the Cu(II) to oxidize human hemoglobin is not due to an alternative process which rapidly reduces most of the Cu(II) to Cu(I).

The results of Figure 1 indicate that human HbO_2 is less susceptible to oxidation by low concentrations of Cu(II) than horse HbO_2. This phenomenon was studied at a series of Cu(II)/heme molar ratios. At each molar ratio, the percent hemoglobin oxidized in the initial rapid phase of oxidation was determined by extrapolating the percent oxidation in the slow phase of oxidation to zero time. These results are shown in Figure 2.

It is found that for horse HbO_2 the percent of hemoglobin rapidly oxidized corresponds to the rapid oxidation of HbO_2 by essentially all of the Cu(II) added to the hemoglobin up to a Cu(II)/heme molar ratio of 0.5. Higher Cu(II)/heme molar ratios precipitate horse hemoglobin. For human HbO_2-Cu(II)/heme molar ratios between 0.5 and 1.0 do not precipitate HbO_2, and are necessary to produce an increase in the percent hemoglobin rapidly oxidized per increment of added Cu(II) comparable to that found for horse HbO_2 at Cu(II)/heme molar ratios <0.5.

Figure 3 demonstrates that for human hemoglobin, as previously found for horse hemoglobin (Rifkind, 1974), the initial rate of oxidation follows the order $Hb > HbO_2 \gg HbCO$. Similar studies at Cu(II)/heme molar ratios of 1.0, 1.25, and 1.52 show the same relative rates of oxidation. The results at Cu(II)/heme molar ratios >1.0 indicate that Cu(II) only oxidizes 50% of the hemes of human hemoglobin, as previously found for horse hemoglobin (Rifkind, 1974). At the Cu(II)/heme molar ratios of 1.25 and 1.52, some hemoglobin precipitation is observed after 30 min when the oxidation has already plateaued off.

[2] The EDTA was added to chelate the Cu(II) and thereby stop, or at least slow down, the oxidation of hemoglobin and the formation of Cu(I). In this manner, a reliable measure of the Cu(I) present at the time of addition of BATH is obtained.

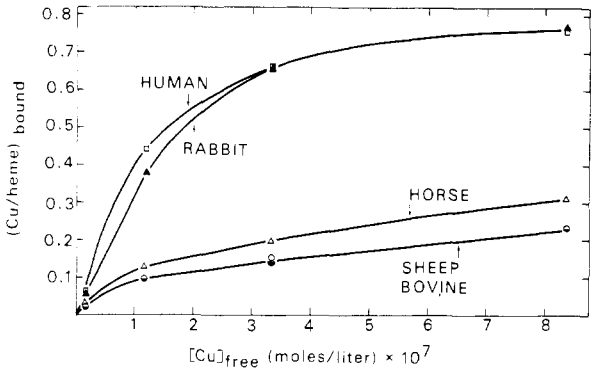


FIGURE 4: Binding of copper to 1×10^{-4} M hemoglobin in 0.01 M Bistris, pH 7.0, at 2 °C: (□), Human; (▲), rabbit; (△), horse; (○), sheep; (●), bovine.

In addition to the differences in the initial rate of oxidation, Figure 3 demonstrates two other differences between the Cu(II) oxidation of Hb and HbO_2. Twenty percent of the Hb is oxidized in the initial rapid phase, while only 12% of the HbO_2 is oxidized in the initial rapid phase. Nevertheless, both of these amounts correspond to considerably less than the 50% oxidation, which would be found with horse hemoglobin at a Cu(II)/heme molar ratio of 0.5 (Rifkind, 1974; Figure 2). The second difference is in the rate for the second slow phase of oxidation, which is much slower for Hb than HbO_2.

We have also investigated the Cu(II)-induced oxidation of bovine, sheep, rabbit, and human A_2 hemoglobins. Extensive oxidation of bovine and sheep hemoglobins is produced by low Cu(II)/heme molar ratios in the same manner as observed for horse hemoglobin. However, human A_2 hemoglobin, as well as rabbit hemoglobin, require Cu(II)/heme molar ratios >0.5 in order to produce extensive oxidation; i.e., they behave like human hemoglobin (Figures 1 and 2).

The Effect of Other Substances on the Oxidation of Hemoglobin by Cu(II). In addition to studies on the differences between various hemoglobins, we have investigated the oxidation in the presence of various other small substances which can interact with hemoglobin.

Even a 20-fold excess of Mn(II), which has a relatively low affinity for hemoglobin (Chang et al., 1975), has no effect on the Cu(II) induced oxidation.

Zn(II), which has a much higher affinity for hemoglobin (Oelshlegel et al., 1974), produces a significant decrease in the Cu(II)-induced oxidation of both human and horse hemoglobin. The major effect of Zn(II) on human HbO_2 is a decrease in the amount of hemoglobin in the rapid phase of oxidation. At a Cu(II)/heme molar ratio of 0.5, a concentration of Zn(II) equal to that of Cu(II) produces a four-to-fivefold decrease in the amount of hemoglobin in the rapid phase of oxidation (Figures 2 and 3). However, Zn(II) does not affect the amount of horse hemoglobin oxidized in the rapid phase of oxidation but does decrease the apparent rate at which the hemoglobin is oxidized.

Hg(II), which has a high affinity for hemoglobin (Riggs, 1960), decreases the Cu(II) oxidation of hemoglobin. A concentration of Hg(II) equal to that of Cu(II) eliminates the rapid oxidation of HbO_2. The residual slow rate is actually slower than the rate for the oxidation of HbCO by Cu(II) (Figure 3).

The highest affinity Hg(II) binding site involves the β-cysteine-93 residue (Riggs, 1960; Perutz et al., 1960). In order to ascertain that binding to this site is responsible for the reduced rate of oxidation, we have also investigated the Cu(II) oxidation of horse HbO_2 reacted with NEM. This reagent reacts covalently with the β-93 sulfhydryls (Riggs, 1961; Perutz et al., 1969), and does not react with any other sites unless a very large excess of NEM is present for a long period of time (Guidotti and Konigsberg, 1964). Oxidation studies with NEM-HbO_2 are similar to the results with equal concentrations of Hg(II) and Cu(II); i.e., the hemoglobin is oxidized very slowly.

Binding Studies

Figure 4 shows the binding of copper to horse, bovine, sheep, rabbit, and human HbO_2.[3] It is noted that the various hemoglobins fall into two distinct groups. Human and rabbit hemoglobins have a copper affinity which is approximately an order of magnitude higher than horse, bovine, and sheep hemoglobins. Furthermore, human and rabbit hemoglobins bind more than 1 copper for every 2 hemes in the concentration range where horse, bovine, and sheep hemoglobins bind only 1 copper for every 2 hemes.

The effect of the reaction with NEM on the binding of copper to horse and human hemoglobin was investigated in pH 7.4 0.01 M Trizma–0.1 M NaCl. The reaction with NEM produces a small 20% decrease in the affinity of horse hemoglobin for copper. The binding of copper to human hemoglobin is not affected by NEM below a ratio of 0.5 for Cu_{bound}/heme. However, at higher levels of bound copper, the reaction with NEM produces a decrease in the amount of copper bound, which is actually somewhat greater than that found with horse hemoglobin.

The binding of copper and zinc to hemoglobin was studied by varying the total concentration of 1 metal ion while the total concentration of the other metal ion was kept constant. In the absence of copper, the zinc affinity for human hemoglobin is similar to the zinc affinity for horse hemoglobin, which is similar to the horse hemoglobin copper affinity (Figure 4). The results with horse hemoglobin in the presence of both zinc and copper indicate that these two metal ions do not compete for the same binding site. The results with human hemoglobin in the presence of both zinc and copper are complicated by the binding of more than 1 copper for every 2 hemes. However, they seem to suggest that copper and zinc compete for the same site under certain conditions.

Electron Spin Resonance Spectra of Human and Horse Hemoglobins with Cu(II)

A portion of the ESR spectra of horse and human HbCO at a Cu(II)/heme molar ratio of 0.3 is shown in Figure 5. It is observed that the ESR spectrum for horse hemoglobin displays in this region a single major band with a slight shoulder. For human hemoglobin, this resonance is split into two bands, with the low-field band having superimposed on it a well-resolved superhyperfine structure. For both hemoglobins, three lower intensity bands due to hyperfine interactions are observed at lower field. The human HbCO spectrum is very similar to that observed for human hemoglobin by Bemski et al. (1969), who have interpreted the superhyperfine structure in terms of Cu(II) binding to 4 equivalent nitrogen atoms.

[3] In the period of time necessary to reach equilibrium, HbO_2 is significantly oxidized to methemoglobin, even at 2 °C. Therefore, the reported binding curves of Cu(II) to HbO_2 (Figure 4) are actually for the binding of copper, which may contain some Cu(I) (see above), to a mixture of HbO_2 and methemoglobin. However, binding studies of Cu(II) to horse HbCO, which becomes oxidized only very slowly (Rifkind, 1974), and to horse methemoglobin indicate that oxidation has a relatively negligible effect on the affinity of hemoglobin for Cu(II).

Discussion

A High Affinity Cu(II) Binding Site in Human Hemoglobin Not Involved in Oxidation. The mechanism previously proposed for the oxidation of horse hemoglobin by Cu(II) (Mechanism I) cannot explain the fact that only part of the Cu(II) added is utilized to rapidly oxidize human hemoglobin (Figures 1-3).

Various experiments have been performed in order to determine how the mechanism for Cu(II) oxidation is altered in human hemoglobin. The Cu(I) determinations shown in Figure 1 demonstrate that there is no alternative pathway for the reduction of Cu(II) which competes with the oxidation process. A reversible electron transfer process (step 3) with k_{-3} actually greater than k_3 could explain the results at any single Cu(II)/heme molar ratio. However, the data of Figure 2, which show an upward curvature in the amount of oxidation produced at higher Cu(II)/heme molar ratios, can only be explained by more than one binding process. The binding data of Figure 4 demonstrate the presence of such an additional binding process in human hemoglobin. A stoichiometry of 1 copper for every 2 hemes for the additional human hemoglobin binding process is suggested by the magnitude of the difference in $(Cu/heme)_{bound}$ for horse and human hemoglobin, as well as the extrapolation of the steep part of the human HbO_2 curve in Figure 2 to a Cu(II)/heme molar ratio of 0.5.

The binding results (Figure 4) with and without NEM, as well as the ESR results (Figure 5), indicate that the highest affinity human hemoglobin binding site is distinctly different from the horse hemoglobin binding site. It has a higher Cu(II) affinity, is not affected at all by NEM, and produces a Cu(II) ESR spectrum with a distinctive superhyperfine structure. The same binding results, as well as the reported (Bemski et al., 1969) ESR results of human hemoglobin at Cu(II)/heme molar ratio >0.5, suggest that the lower affinity human hemoglobin binding site is actually similar to the horse hemoglobin binding site.

The oxidation results can be explained by a requirement that both Cu(II) binding sites be occupied in order for the heme to be oxidized in human hemoglobin, or by the additional binding site being located in a position unsuitable for a transfer of electrons from the heme, but competing with the oxidation site for the available Cu(II). However, the similarity between the horse hemoglobin Cu(II) binding site, which produces oxidation without an additional Cu(II), and the human hemoglobin low-affinity Cu(II) binding site support the latter alternative. A mechanism where only 1 Cu(II) is involved in the human hemoglobin oxidation is also supported by the finding that only one Cu(II) is reduced for every heme oxidized (Figure 1) and the proposed location of the human hemoglobin high-affinity Cu(II) binding site far away from the heme (see below). We, therefore, propose Mechanism II for the oxidation of oxyhemoglobin by Cu(II).

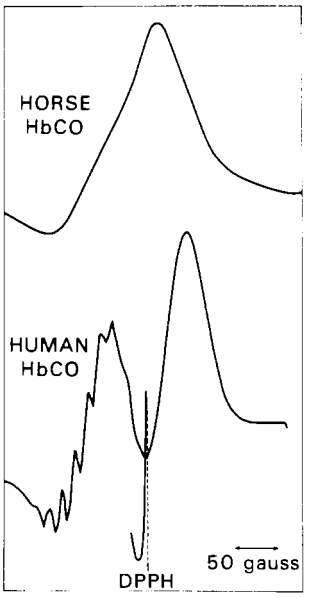


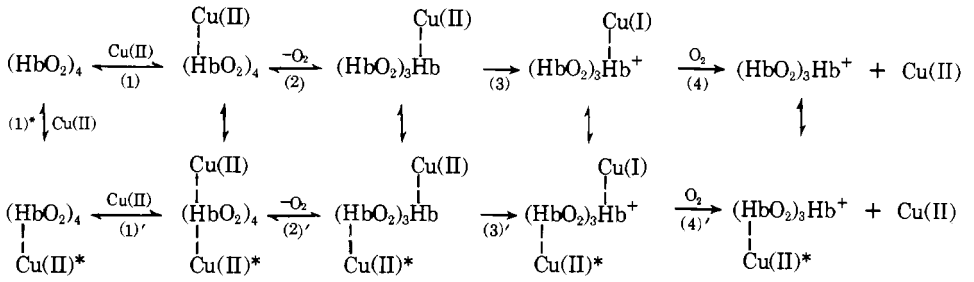
FIGURE 5: Electron spin resonance spectra of horse and human HbCO chromatographed on Sephadex G-25 in 0.01 M Trizma-0.1 M NaCl, pH 7.4, at 27 K. Frequency, 8.9 GHz; microwave power, 4 mW. The external standard DPPH has a g value equal to 2.0063.

Even though this mechanism requires only one Cu(II) for the oxidation, the binding of the second Cu(II) can affect the rate constants for the various steps involved in the oxidation. However, in order to simplify our discussion of this mechanism, we will assume that the rate constants for steps 1-4 are identical to those for steps 1′-4′.

The equilibrium constant for step 1*, K_1*, is the equilibrium constant for the Cu(II) binding site from which Cu(II) is unable to oxidize the heme (the K_1* site). For horse hemoglobin, K_1* → 0 and the oxidation proceeds via the upper pathway; i.e., Mechanism II becomes identical to Mechanism I. For human hemoglobin, K_1* is the equilibrium constant for the binding of Cu(II) to the highest affinity binding site (Figure 4). This constant is approximately an order of magnitude greater than K_1, the equilibrium constant for step 1, i.e., the equilibrium constant for the Cu(II) binding site which results in oxidation (the K_1 site).

Our kinetic data also permit us to place certain limits on some of the rate constants involved in binding. The dramatic decrease in the rate of oxidation when O_2 is replaced by CO indicates that k_2 (the rate constant for the dissociation of the first CO molecule from HbCO, which is in the range of 0.2-0.3 s^{-1} (Holland, 1970), must be much smaller than k_1, the forward binding constant to the K_1 site. The rate of oxidation of human Hb after the initial rapid rate is extremely slow (1%/h) even though 60% of the added copper is still present in the Cu(II) form (Figure 3) presumably bound to the K_1* site. This

MECHANISM II.



finding requires that the off constant from the K_1* site, k_{-1}* $\ll k_1$, k_2, k_3, and even k_4.

At Cu(II)/heme molar ratios <0.5, both sites are free to compete for the same Cu(II). However, most of the Cu(II) will bind to the higher affinity K_1* site and, therefore, only a small fraction of the Cu(II) added is used to rapidly oxidize hemoglobin (Figures 1-2), which takes place via the upper pathway (Mechanism II). At Cu(II)/heme molar ratios >0.5, the K_1* site is already saturated and most of the oxidation (Figures 1 and 2) takes place via the lower pathway (Mechanism II), with Cu(II) bound to both sites.

The actual amount of rapid oxidation at any Cu(II)/heme molar ratio will be determined by the relative values of the equilibrium constants K_1 and K_1* only if all the forward and reverse rate constants for binding are rapid relative to the ligand dissociation (step 2) and electron transfer (step 3) processes (k_1, k_{-1}, k_1*, k_{-1}* $\gg k_2$, k_{-2}, k_3). If this condition is not satisfied, some of the Cu(II) may be used to oxidize hemoglobin before the equilibrium distribution of Cu(II) between both binding sites is achieved. The amount of rapid oxidation at any Cu(II)/heme molar ratio will then lie between the limit predicted by the relative values of the forward binding rate constants (k_1 and k_1*), and the limit predicted by the relative values of the equilibrium constants for binding (K_1 and K_1*). Under these conditions, the amount of rapid oxidation will be affected by the ligand dissociation process (step 2) and/or the electron transfer process (step 3), unless they are much more rapid than the forward binding processes.

These various possibilities must be considered in any attempt to interpret differences in the amount of rapid oxidation found for HbO_2 and Hb (Figure 3). These results might suggest an increased affinity of Cu(II) for the K_1* site relative to the K_1 site in HbO_2. However, the results can also be explained by the required ligand dissociation process in HbO_2, which lowers the concentration of the unliganded hemes and, therefore, the overall rate of oxidation. Such a decrease in the rate of oxidation is actually suggested by the differences in the initial rates of oxidation (Figure 3).

The Effect of Other Substances on Cu(II) Oxidation of Hemoglobin. The comparison between human and horse hemoglobin (Figures 1-2) indicates how alteration of the Cu(II) binding process (step 1) changes the kinetics for the Cu(II) oxidation of hemoglobin. The comparison between HbCO and HbO_2 (Figure 3) indicates how changing step 2, the ligand dissociation process (Gibson and Roughton, 1955; 1957), can alter the kinetics for the oxidation.

Various steps in the oxidation mechanism (Mechanisms I and II) can also be altered by adding various other substances which interact with hemoglobin. The interaction of hemoglobin with Zn(II), Hg(II), and NEM was shown to decrease the Cu(II) oxidation of horse and human hemoglobins.

Our competitive binding studies indicate that, even though Zn(II) has a high affinity for hemoglobin, the addition of Zn(II) does not affect the binding of Cu(II) to horse hemoglobin. Therefore, at least for horse hemoglobin, the effect of Zn(II) on the Cu(II) oxidation must involve the oxygen dissociation process (step 2) and/or the electron transfer process (step 3).

Preliminary oxygenation studies indicate that Zn(II) produces an increase in the oxygen affinity of horse hemoglobin which is at least as large as the reported increase for human hemoglobin (Oelshlegel et al., 1974). An increase in the oxygen affinity is likely to reduce the equilibrium constant, k_2/k_{-2}, for the dissociation of the first oxygen molecule from HbO_2. Such a decrease would decrease the concentration of unliganded hemes and, thereby, the rate of oxidation (Mechanisms I and II). Therefore, the observed effect of Zn(II) on the Cu(II) oxidation of horse hemoglobin can be completely explained by a change in the ligand dissociation process (step 2), although an effect on the electron transfer process (step 3) can not be ruled out.

The interpretation of the effect of Zn(II) on the Cu(II) oxidation of human hemoglobin is somewhat more ambiguous, and very likely involves an effect on several steps of the oxidation (Mechanism II). On one hand the 1.3-fold increase in the oxygen affinity (Oelshlegel et al., 1974) would probably not produce a large enough effect on the ligand dissociation process (step 2) to explain the four to fivefold decrease in the amount of hemoglobin rapidly oxidized. On the other hand, the competitive binding results do not rule out an effect on the binding process (step 1).

The effect of blocking the β-cysteine-93 residue by Hg(II) and NEM on the Cu(II) oxidation of horse and human hemoglobins is much more dramatic than the effect of Zn(II). Such a decrease in the rate of oxidation could only be completely explained in terms of the binding process (step 1) if blocking the β-cysteine-93 residue essentially eliminates the K_1 binding site. Binding studies with NEM-hemoglobins, however, indicate that blocking the β-cysteine-93 residue produces only a small decrease in the affinity of Cu(II) for the K_1 site. Such a decrease can only account for a small fraction of the decrease in the rate of Cu(II) oxidation.

Blocking the β-93 sulfhydryl groups of hemoglobin is known to produce an increase in the oxygen affinity of hemoglobin (Taylor et al., 1966). However, this increase is similar in magnitude to the increase in the oxygen affinity produced by Zn(II), and could not produce the observed dramatic decrease in the rate of Cu(II) oxidation.

Therefore, the effect of sulfhydryl reagents on Cu(II) oxidation most likely involves a decrease in k_3, the rate constant for the electron transfer process (step 3, Mechanisms I and II). Such a dramatic effect on k_3 suggests that the free sulfhydryl may be involved in the electron transfer process. This conclusion is also supported by preliminary experiments on the Cu(II) oxidation of horse Hb reacted with NEM. In this case, there is no ligand dissociation step and reaction of the β-cysteine-93 residues with NEM still produces a dramatic decrease in the rate of Cu(II) oxidation.

Possible Location of the Cu(II) Binding Sites. The High Affinity K_1* Site Not Involved in the Oxidation. The finding that this site is only present in certain hemoglobins suggests that it may be possible to determine the location of this site by comparing the amino acid sequence (Dayhoff, 1969) of the various hemoglobins studied. Unfortunately, there are a large number of amino acid differences in the primary structure of the various hemoglobins; e.g., in comparing horse and human hemoglobin, there are 18 differences in the α chain and 43 differences in the β chain. Among these differences are a number of amino acids which could be involved in a high affinity binding site specific for human and rabbit hemoglobins. However, histidine is the preferred amino acid which is usually involved in Cu(II) binding to proteins. Binding to histidine is also supported by the ESR superhyperfine structure implicating nitrogenous ligands. Among the changes in the amino acid sequence of horse and human hemoglobins, the only histidines present in human and rabbit hemoglobins and absent in horse, sheep, and bovine hemoglobins are β-2, which is glutamine in horse hemoglobin, and β-116, which is arginine in horse, sheep, and bovine hemoglobins. In sheep and bovine hemoglobins, β-2 is leucine. Both of these positions are far

removed from the heme (Perutz, 1970) and, therefore, at sites which could not be expected to be involved in oxidation of the heme.

The involvement of β-116 in the high affinity K_1* binding site is ruled out by binding and oxidation studies on human A_2 hemoglobin. This hemoglobin has an amino acid sequence very similar to human hemoglobin A (the major component of human hemoglobin), except for an arginine residue at position β-116 (Dayhoff, 1969). Nevertheless, the oxidation studies at various Cu(II)/heme molar ratios and the binding studies indicate that this hemoglobin behaves like human and rabbit hemoglobins; i.e., it still has the high affinity K_1* Cu(II) binding site not involved in oxidation. It is, therefore, very likely that β-histidine-2 is an integral part of the K_1* binding site.

The K_1 Binding Site Involved in Oxidation. The observation that this site involves the binding of 1 Cu(II) to every 2 hemes and that only 50% of the hemes are oxidized indicates that the binding site is only on 1 type of subunit. By separating Cu(II)-oxidized human hemoglobin into subunits, Nagel et al. (1970) show that the β subunits are involved.

The effect of reagents, which block the β-cysteine-93 residue, on the oxidation and binding enables us to at least delineate the region of the β subunit where the K_1 binding site is located.

It is not uncommon for the binding of one substance to hemoglobin to affect the binding of a second substance even if it binds to a site far removed from the site of the first substance (Monod et al., 1965). Such heterotropic interactions must, however, be mediated by conformational changes extending over large portions of the protein. X-ray studies on NEM-hemoglobin indicate that the reaction of HbO_2 with NEM produces no detectable conformational changes outside the immediate vicinity of the cysteine residue (Perutz et al., 1969). Therefore, the K_1 Cu(II) binding site is most probably located on the proximal side of the heme in the region of the β-cysteine-93 residue. The relatively small 20% decrease in the Cu(II) binding constant of horse NEM-hemoglobin suggests that, at least for horse hemoglobin, the β-cysteine-93 residue is not an integral part of the K_1 binding site. There are, however, a number of other amino acid residues which can bind metal ions located in this region of the molecule.

Acknowledgments

The authors express their appreciation to Dr. Hideo Kon and Mitsuo Sato for help with the ESR experiments, and to Drs. Rufus Lumry and Helen Gootsmith for the A_2 hemoglobin. We also thank Drs. G. L. Eichhorn and J. P. Froehlich for their interest and advice, and are grateful to Ms. Jane Heim and Mr. Devereaux Sterrette for their valuable technical assistance.

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