Oxidation of (Horse) Hemoglobin by Copper: An Intermediate Detected by Electron Spin Resonance†

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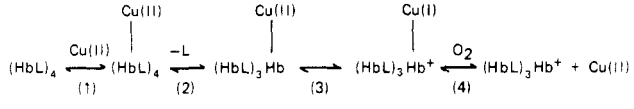
ABSTRACT: The oxidation of horse hemoglobin by Cu(II) has been followed by the changes in the electron spin resonance spectra of copper. By stopped-flow and freeze-quenching techniques, it is shown that the second-order rate constant for the binding of Cu(II) to hemoglobin is $>5 \times 10^5$ mol^{-1} s^{-1} and the apparent first-order rate for the reduction of Cu(II) to Cu(I) is 0.051 s^{-1}. It is also shown that the binding of Cu(II) to hemoglobin is followed by an alteration of the Cu(II) spectrum, decreasing the g values. This process has an apparent rate constant of 17 s^{-1} and presumably involves a conformational change in the region of the copper binding site. It is also shown that this conformational change is apparently necessary for Cu(II) to oxidize hemoglobin.

It has previously been reported that Cu(II) rapidly oxidizes horse hemoglobin (Rifkind, 1974). A mechanism has been proposed involving the binding of Cu(II) to hemoglobin (step 1), the dissociation of the Fe(II) ligand (step 2), and the transfer of electrons between Cu(II) and Fe(II) (step 3), which is followed in the presence of oxygen by the slow regeneration of Cu(II) (step 4).

mechanism I



Step 1 was proposed on the basis of the high equilibrium affinity of Cu(II) for hemoglobin. Step 2 was proposed on the basis of the relative rates of oxidation given by the order Hb[1] > HbO_2 > HbCO. Step 3 was proposed by the formation of Cu(I) as indicated by the reaction with the Cu(I)-specific reagent 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (Rifkind et al., 1976b). Step 4 was proposed on the basis of the catalytic effect at low concentrations of Cu(II) in the presence of oxygen.

Changes in the visible absorption spectra were used to monitor the oxidation in these previous studies. Absorption changes have also been used to monitor Cu(II) oxidation of other hemoglobins (Salvati et al., 1969; Rifkind et al., 1976b; Winterbourn & Carrell, 1977).

The electron spin resonance (ESR) spectrum of Cu(II) bound to hemoglobin has previously been reported (Bemski et al., 1969; Nagel et al., 1970; Rifkind et al., 1976b). While the reduction of some of the Cu(II) to Cu(I) has been observed, no attempt has been made to follow the time course of the oxidation by ESR. This technique is particularly valuable because of the ability not only to distinguish between paramagnetic Cu(II) and diamagnetic Cu(I) but also to detect changes in coordinated Cu(II).

By following the oxidation reaction by ESR spectroscopy, it has been possible to estimate rate constants for some of the individual steps in the oxidation reaction and to detect a previously unidentified intermediate step in the oxidation of hemoglobin by Cu(II). This intermediate is thought to involve a conformational change of the hemoglobin molecule which must take place prior to the oxidation of hemoglobin.

Experimental Section

Hemoglobin. Defibrinated pooled horse blood was obtained from Bioquest. The red cells were separated from whole blood by centrifugation and washed several times by suspending them in 0.9% NaCl and recentrifuging them. The cells were then hemolyzed in 2 volumes of distilled water at 4 °C and separated from the cell membranes by high-speed centrifugation (Rifkind et al., 1976b).

The low molecular weight components were removed by extensive dialysis against 0.9% NaCl followed by dialysis against water to remove the NaCl. The hemoglobin was then stored at −60 °C until used. The freezing and thawing procedure frequently precipitated additional membranous material (Beutler, 1971), which was removed by centrifugation before the hemoglobin was used. Buffers were found to interfere with ESR measurements because they form Cu(II) complexes with their own ESR spectrum. For that reason no buffers were used. The pH for each experiment was determined by measuring the pH of a 1:1 mixture of the particular Cu(II) and hemoglobin solutions. In all cases the pH was between 6 and 7. It was found that this range of pH does not alter any of the conclusions reached in this paper. All other chemicals were reagent grade and were used without further purification.

Electron Spin Resonance (ESR). A Jeolco JES-ME ESR spectrometer was used for all measurements. A Mn^{2+} sample in MgO was used as an external standard in order to calculate g values. Low-temperature studies were performed by using a Jeolco JES-VT 3A temperature controller, where the flow of cold nitrogen gas from a Dewar is regulated to maintain a set sample temperature.

A Wilmad quartz liquid flow mixing chamber and reaction cell were used for the stopped-flow measurements. The two entry ports of this cell were connected with stainless steel tubing to two syringes which were driven by nitrogen gas. A mixer is contained in this cell just below the flat observation tube. A long tygon tube was connected to the exit port at the top of the cell. A flow rate in the range of 8–9 mL/s was

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[1] Abbreviations used: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carboxyhemoglobin; ESR, electron spin resonance; MalNEt *N*-ethylmaleimide; MalNEt–HbO_2, oxyhemoglobin with β-93 sulfhydryls reacted with *N*-ethylmaleimide; Zn–HbO_2, oxyhemoglobin with bound Zn(II).

generally used. At this rate mixing was adequate and no cavitation was detected.

Since the volume between the mixer and the region of observation for the reaction cell used is less than 0.05 mL, the dead time was in the range of 5-6 ms. A large enough sample was used for each determination to produce a stable signal while the sample was still flowing. However, vibrations of the sample tube due to stopping of the flow could not always be completely damped out. In those cases some uncertainty has to be considered for the data obtained during the first few milliseconds subsequent to stopping the flow.

The kinetics were followed in the manual sweep mode with the signal fed directly to the recorder of the ESR spectrometer in the Y-T mode. The early stages of the reaction were followed by bypassing the recorder with the signal fed directly to a Nicolet 1072 averager, which was triggered by a microswitch on the syringe drive. The signal stored in the averager was then plotted on the recorder at a slower rate.

We have adapted previously reported procedures (Bray, 1961; Bray & Petterson, 1961; Ballou, 1971) for the freeze-quenching of our samples. The drive system and mixing chamber of the quenching apparatus of Froehlich et al. (1976) were used with a Lucite exit nozzle attached to the end of the stainless steel capillary. The nozzle consisted of four 0.008-in. holes. This size was found by Ballou (1971) to provide optimum quenching. Four holes were necessary to provide an adequate flow through the mixers of this quenching apparatus. Tubes with a funnel attachment at the top similar to the demountable ESR tubes of Ballou (1971) were used. These were filled with isopentane and kept at ca. −100 °C with a liquid N_2–ethanol bath.

The samples were sprayed directly into the ESR tubes and then packed to the bottom. After being packed, samples were stored in either liquid N_2 (77 K), liquid N_2–ethanol (−100 °C), or a freezer at −60 °C. In order to maintain similar packing properties, we maintained a constant flow rate through the quenching apparatus and the reaction time was changed by varying the volume of the stainless steel capillary between the mixer and the nozzle.

While the volume of the capillary and the flow rate could be determined quite accurately with reaction times as short as 2.5 ms, at least 10 ms must be added to this reaction time to account for the quenching time (Bray, 1961; Ballou, 1971). In addition to a possible variability in this quenching time, the packing of the sample is not always uniform. This second factor can produce an uncertainty in the absolute intensity of the observed ESR spectrum, complicating the analysis of kinetic data. Therefore, freeze-quenching does not furnish as reliable kinetic data as stopped-flow. We have, however, used quenching to investigate the entire spectrum at various stages of the chemical reaction. For this purpose it is much more advantageous than stopped-flow, which looks at one point of the spectrum at a time.

Results

Stopped-Flow ESR. Cu(I) is diamagnetic and has no ESR spectrum. Aqueous $CuCl_2$ at room temperature has a single ESR band with $g = 2.22$. Cu(II) bound to hemoglobin at both room temperature and in frozen solutions has a completely different ESR spectrum with $g_\perp = 2.06$ and $g_\parallel \simeq 2.2$–2.3 (see below). Because of this change in spectrum at room temperature, stopped-flow ESR can be used to follow the various steps in the oxidation of hemoglobin by Cu(II) (mechanism I).

Figure 1 shows the results of mixing copper with horse hemoglobin when the intensity of the Cu(II)–hemoglobin signal is monitored in the region of the large $g_\perp$ band. In this figure it is shown that by using ESR, the interaction of Cu(II) with horse hemoglobin can clearly be separated into three separate processes. The first process corresponds to the binding of Cu(II) to hemoglobin (step 1 of mechanism I). The second process corresponds to the reduction of Cu(II) to diamagnetic Cu(I). In mechanism I this coincides with the oxidation of the heme (step 3) and corresponds to the change in visible absorption. The third process corresponds to the reoxidation of Cu(I) back to Cu(II), which also binds to oxidized hemoglobin (Rifkind, 1974). This will coincide with step 4 if there is no additional Fe(II)–hemoglobin for Cu(II) to oxidize. In the presence of an excess of hemoglobin, the slow rate for the increase in the Cu(II)–hemoglobin signal observed in the ESR spectrum is dependent on the amount of Fe(II)–hemoglobin and the relative rates for the reduction and oxidation of copper (steps 3 and 4 of mechanism I).

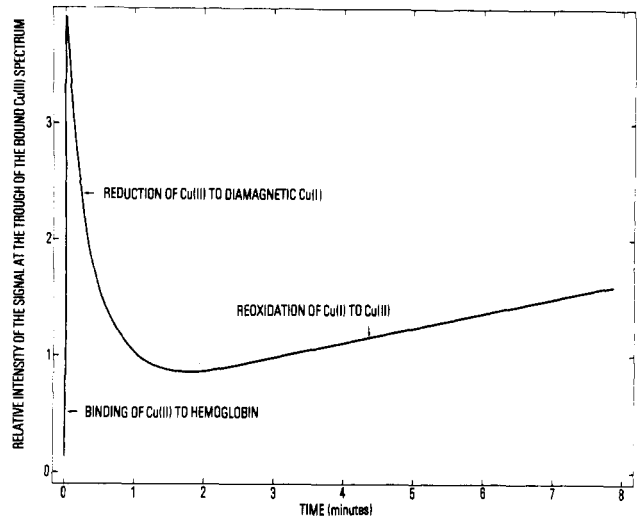


FIGURE 1: Change in the ESR signal as a function of time at the trough of the $g_\perp$ band of Cu(II) bound to hemoglobin. $CuCl_2$ and horse hemoglobin are mixed at room temperature directly in the ESR cavity to a final concentration of 5×10^{-4} M Cu(II) and 1.9×10^{-3} M heme.

The change in the ESR signal on a more expanded time scale was followed by monitoring the change both in the region of the free copper band (Figure 2A) as well as in the region of the $g_\perp$ Cu(II)–hemoglobin band (Figure 2B). These results show that the entire free copper signal disappears within the dead time of the apparatus, which is <10 ms, while less than 80% of the intensity of the Cu(II)–hemoglobin band is reached within this time.

The failure to observe any free Cu(II) within the dead time of the stopped-flow indicates that the second-order rate constant for the binding of Cu(II) to hemoglobin is $>5 \times 10^5$ mol^{-1} s^{-1}. The reduction of Cu(II) to Cu(I) (Figure 1), which coincides with the oxidation of the heme (mechanism I), has an apparent first-order rate constant of 0.051 s^{-1} (Figure 3A). The continued change in the Cu(II)–hemoglobin signal after the flow stops (Figure 2) has an apparent first-order rate constant of 17 s^{-1} (Figure 3B).

Freeze-Quenching. Figure 4 shows that, at the same concentrations used for the stopped-flow experiments, the ESR spectrum of a solution frozen immediately after mixing $CuCl_2$ with hemoglobin (the time of reaction is equal to 2.5 ms plus the quenching time, i.e., $\simeq$13 ms) indicates the presence of a single Cu(II)-bound species (spectrum A) with $g_\parallel = 2.28$ and $g_\perp = 2.06$ and no detectable free Cu(II). The very rapid binding of Cu(II) to hemoglobin was further demonstrated by the fact that no free Cu(II) was detected even for a 10-fold dilution of both Cu(II) and hemoglobin. For longer reaction

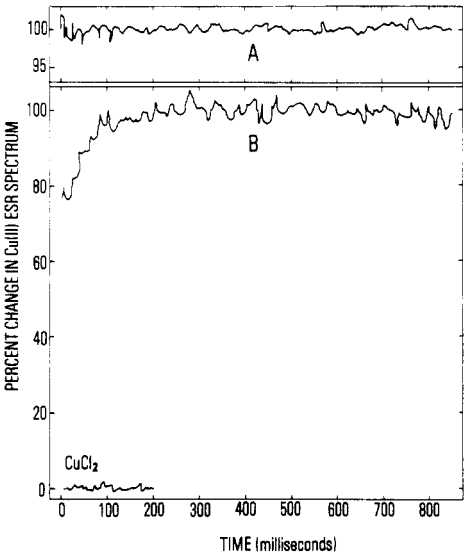


FIGURE 2: Comparison of the time course for the disappearance of the free Cu(II) band ($g = 2.22$) and for the formation of the major Cu(II)–hemoglobin band ($g_\perp = 2.06$). $CuCl_2$ and horse hemoglobin are mixed at room temperature in the ESR cavity to a final concentration of 5×10^{-4} M Cu(II) and 1.9×10^{-3} M heme. (A) The instrument is set at the peak of the $CuCl_2$ signal, and the disappearance of this signal is followed. Zero percent corresponds to the Cu(II) signal from 5×10^{-4} M $CuCl_2$, and 100% corresponds to the low-intensity signal observed in this region of the spectrum for Cu(II)–hemoglobin. (B) The instrument is set at the trough of the Cu(II)–hemoglobin signal (same position as for Figure 1), and the percent formation of this signal is followed. Zero percent corresponds to the low intensity of 5×10^{-4} M $CuCl_2$ in this region of the spectrum, and 100% corresponds to the maximum intensity observed after mixing Cu(II) and hemoglobin.

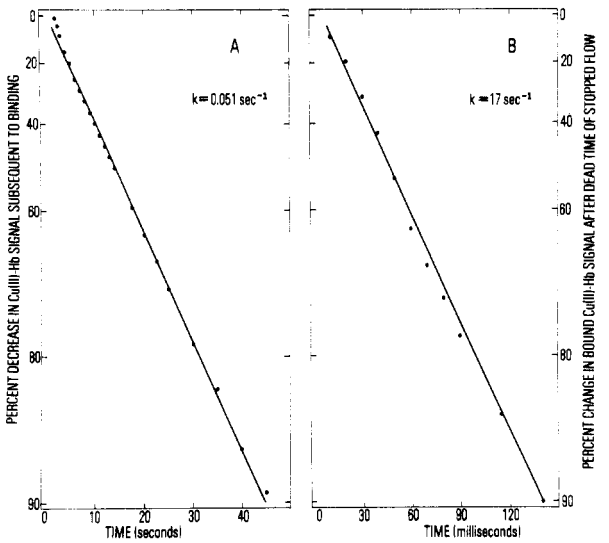


FIGURE 3: Semilogarithmic plots for the changes in the ESR spectra as a function of time. (A) Reduction of Cu(II) to Cu(I). Zero percent corresponds to the maximum Cu(II)–hemoglobin signal observed (Figure 2B), and 100% corresponds to the plateau obtained 2 min after the $CuCl_2$ and hemoglobin are mixed (Figure 1). (B) Increase in the signal of the bound Cu(II)–hemoglobin signal after the entire free Cu(II) signal has disappeared (Figure 2B). Zero percent corresponds to the intensity of the Cu(II)–hemoglobin signal immediately after the dead time of the stopped-flow apparatus, and 100% corresponds to the maximum intensity of the Cu(II)–hemoglobin signal.

times (spectrum B is for a reaction time of 5 s) a mixture of two Cu(II)-bound species is detected, with a major portion of the Cu(II) converted to a form with $g_\parallel = 2.21$. It is difficult to resolve separate $g_\perp$ values for the two species, although it is clear that $g_\perp$ also decreases for this second species. These

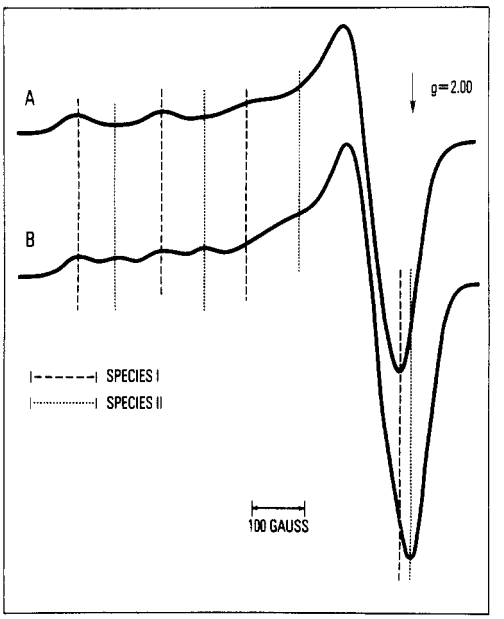


FIGURE 4: Electron spin resonance spectra of Cu(II) bound to horse hemoglobin at −140 °C. The concentrations of $CuCl_2$ and hemoglobin are the same as those used for the stopped-flow experiments (Figures 1 and 2). (A) Spectrum obtained when the solution is frozen immediately (<20 ms) after Cu(II) and hemoglobin are mixed. (B) Spectrum obtained when the solution is frozen 5 s after Cu(II) and hemoglobin are mixed. The dashed lines show the bands observed in spectrum A. The dotted lines show the additional bands observed in spectrum B.

two species also have slightly different values for the copper hyperfine splitting constant $A_\parallel^{Cu}$, with $A_\parallel^{Cu}$ for the initial species = 0.0173 cm^{-1} and $A_\parallel^{Cu}$ for the second species = 0.018 cm^{-1}.

The time course for the transformation from the first species to the second is shown in Figure 5A, where just the two lowest field hyperfine peaks are shown. It is during this time period that the entire spectrum changes from spectrum A to spectrum B in Figure 4.

Effect of Various Substances Which Decrease the Rate of Cu(II) Oxidation. We have previously found three ways to decrease the rate of Cu(II) oxidation (Rifkind, 1974; Rifkind et al., 1976b). Replacing oxygen with carbon monoxide decreases the rate of oxidation by several orders of magnitude. The reaction of the β-93 sulfhydryl groups by reagents such as MalNEt has an equally dramatic effect on the rate of oxidation. The addition of Zn(II) produces a relatively small but significant decrease in the rate of oxidation.

Stopped-flow studies with HbCO, MalNEt–HbO_2, and Zn–HbO_2 indicate that the binding of Cu(II) (step 1, mechanism I) is still very rapid. For HbCO and MalNEt–HbO_2 no reduction of Cu(II) to Cu(I) is detected, presumably because the reduction of Cu(II) (step 3, mechanism I) is actually slower than the reoxidation of Cu(I) to Cu(II) (step 4, mechanism I).[2] For Zn–HbO_2 the reduction is somewhat slower than that for HbO_2 (Figure 4A).

The early stages of the oxidation in the presence of CO, MalNEt, and zinc were also studied by freeze-quenching (Figure 6). These results show that the species I → II transformation of Cu(II)–hemoglobin occurs with HbCO to the same extent and over the same time course as for HbO_2 (Figure 5A). On the other hand, the addition of zinc and the reaction with MalNEt have a very dramatic effect on this

[2] The HbCO experiments were done in the presence of oxygen where reoxidation of Cu(I) can take place.

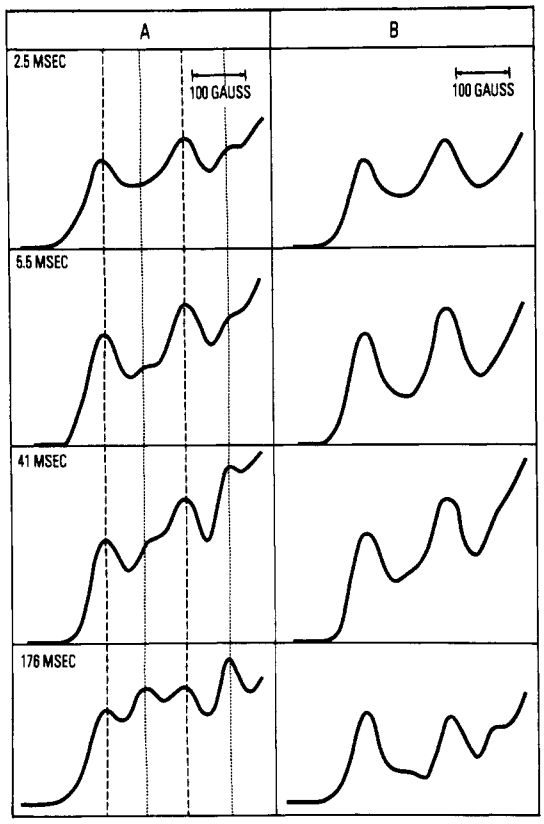


FIGURE 5: Changes in the region of the first two low-field hyperfine bands for Cu(II)–hemoglobin at −50 °C. The conditions are the same as those of Figure 4. (A) Low-field hyperfine bands for $CuCl_2$ mixed with hemoglobin and sprayed into isopentane at ca. −100 °C at different times subsequent to the mixing. The times given on the figure do not include ~10-ms quenching time. The dashed lines indicate the peaks in the 2.5-ms spectrum. The dotted lines show the additional bands which appear at later times. (B) Effect on the ESR spectra of raising the temperature to −10 °C. The spectra in part B are for the same samples as in part A, except that the temperature was raised to −10 °C and then lowered back to −50 °C.

transformation. With zinc only a relatively small amount of species II is observed. The rate for the transformation and/or the equilibrium constant for the formation of the second species must be much slower. When hemoglobin reacted with MalNEt, only species I is detected.

Stability of the Cu(II)–Hemoglobin ESR Spectrum at Different Temperatures. The samples of Cu(II) plus hemoglobin were frozen rapidly at ca. −100 °C. We found that the particular Cu(II)–hemoglobin spectrum obtained (Figure 4) was stable in the frozen solution at temperatures below −50 °C with little or no change in the spectrum even after storage for several weeks. As shown in Figure 1, above freezing the intensity of the ESR spectrum changes rapidly as the Cu(II) is reduced and then slowly reoxidized. Freeze-quenching studies show that during the time that the Cu(II) is being reduced only small changes occur in the relative amounts of the two Cu(II)–hemoglobin species.

We have also investigated the effect of varying the temperature of frozen solutions of HbO_2 plus copper between −50 and −10 °C. Slowly raising the temperature from −50 °C gradually decreases the intensity of the entire spectrum. This decrease was reversible on recooling and can be attributed to the usual temperature dependence of Cu(II) ESR spectra. At some point between −20 and −10 °C, relatively larger changes were detected which were found to be irreversible. Figure 5B shows the effect of heating such solutions to −10 °C and then recooling them to −50 °C. It can be seen that this treatment has essentially no effect on the amount of species I, but

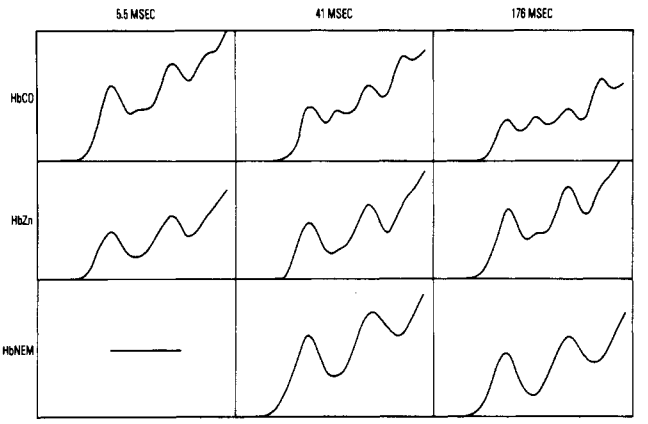


FIGURE 6: Changes in the low-field hyperfine bands for different hemoglobin preparations. The different times correspond to the time lapsed between the mixing of $CuCl_2$ and hemoglobin and the spraying of the mixture into isopentane at ca. −100 °C. They do not include the ~10-ms quenching time. (HbCO) Carboxyhemoglobin; (HbZn) oxyhemoglobin in the presence of 10^{-3} M $ZnCl_2$; (HbNEM) oxyhemoglobin with the β-93 sulfhydryl groups reacted with MalNEt.

dramatically decreases the amount of species II. This decrease in species II coincides with an increase in the amount of oxidation and does not occur when no oxidation takes place, e.g., with HbCO.

Discussion

Rate Constants for the Various Steps in the Oxidation Reaction. The ESR stopped-flow results indicate a second-order rate constant for the binding of Cu(II) to hemoglobin (step 1 of mechanism I) which is greater than 5×10^5 mol^{-1} s^{-1} (Figure 2).

This rapid binding of Cu(II) to hemoglobin is consistent with the inability to detect free Cu(II) by freeze-quenching. It is also consistent with the previously reported concentration-dependent oxidation studies of human hemoglobin (Rifkind et al., 1976a), which show that the rate of oxidation is independent of the copper and hemoglobin concentration; i.e., binding is not rate limiting, until the heme and copper concentrations are reduced to ~10^{-6} M, where the binding process does become rate limiting.

The stopped-flow data of Figure 1 enable us to estimate an apparent first-order rate constant for the reduction of Cu(II) to Cu(I) of 0.051 s^{-1} (Figure 3A). This apparent rate measures the transfer of electrons between Cu(II) and Fe(II) (step 3 of mechanism I). The intrinsic rate constant for this step is actually much greater because of the role of the ligand dissocation process (step 2 of mechanism I).

An estimate of the intrinsic constant can be made from the known rate constants for the binding of oxygen; k_2, the first-order rate constant for the dissociation of the fourth oxygen molecule, is ≃200 s^{-1} (Holland, 1969), and at atmospheric oxygen pressure (Roughton et al., 1955; Roughton & Lyster, 1961) the second order k_{-2} is even faster. The ligand dissociation process can therefore be considered a rapid equilibrium with the apparent rate for the electron transfer process limited by the equilibrium concentration of $Hb_4(O_2)_3$. Since more than 99% of the hemoglobin is saturated with oxygen, the intrinsic k_3 is ~2 orders of magnitude faster than the apparent constant, or ~5 s^{-1}.

The Two Cu(II)–Hemoglobin Species and Oxidation. By investigating the ESR spectra of the bound copper (Figures 2, 4, and 5), it is apparent that the initial very rapid binding of Cu(II) to hemoglobin is followed by an alteration in the coordination sphere of Cu(II) which changes its ESR spectrum. This second species must correspond to a preferred

Cu(II) binding site which requires some modification of the hemoglobin conformation at least in the region of the Cu(II) binding site. The decrease in g values is indicative of more covalent Cu(II) coordination. The conformational change must therefore result in either a stronger band with some of the Cu(II) ligands or perhaps a replacement of certain of the Cu(II) ligands by other ligands. More covalent binding for the second species is also consistent with the previously reported 20% decrease in the affinity of Cu(II) to horse hemoglobin when the β-93 sulfhydryl groups are blocked by MalNEt (Rifkind et al., 1976b), and only species I is detected (Figure 6).

The results shown in Figures 5 and 6 indicate that the change in the Cu(II) coordination, i.e., the change in the hemoglobin conformation in the region of the Cu(II) binding site, is necessary for the oxidation of hemoglobin by Cu(II).

In Figure 6 we investigate the effect on the species I → II transformation of slowing down the oxidation of hemoglobin by replacing oxygen with carbon monoxide, binding zinc to hemoglobin, and reacting the β-93 sulfhydryl group with MalNEt. In all three cases all of the Cu(II) is still rapidly bound to hemoglobin. It is possible that the rate constant for binding Cu(II) is altered, but this constant is still too rapid to explain the decrease in the rate of oxidation.

For HbCO it is known that the rate constant for the dissociation of the fourth ligand, k_2, is $\sim 0.2\ s^{-1}$, which is 3 orders of magnitude slower than that for oxygen (Holland, 1969, 1970). This rate constant is slower than the intrinsic k_3 of $\sim 5\ s^{-1}$ (mechanism I) and becomes the rate-limiting step, which results in a much slower rate of oxidation. Even though very little oxidation takes place, the transformation from the Cu(II)–hemoglobin species I to species II occurs just as readily and in the same time span (Figures 5 and 6). This change in conformation can therefore take place without oxidation of the heme.

The binding of zinc to hemoglobin produces an increase in the oxygen affinity (Oelshlegel et al., 1974; Rifkind & Heim, 1977) which could produce the relatively small decrease in the rate of oxidation by decreasing the equilibrium constant k_2/k_{-2} for the dissociation of the fourth ligand from hemoglobin. However, an alternative explanation can be suggested by the smaller amounts of species II found with zinc–hemoglobin (Figure 6). The importance of species II for the oxidation is strengthened by the results with MalNEt–hemoglobin. Here a very drastic reduction in the oxidation is obtained. The residual slow rate of oxidation (Rifkind et al., 1976b) can actually be attributed to autoxidation and may have nothing to do with the binding of Cu(II). This dramatic effect cannot be explained by the relatively small increase in oxygen affinity (Taylor et al., 1966). The failure to detect any species II thus suggests that the conformational change involved in going from species I to II may be necessary for the oxidation of hemoglobin by Cu(II).

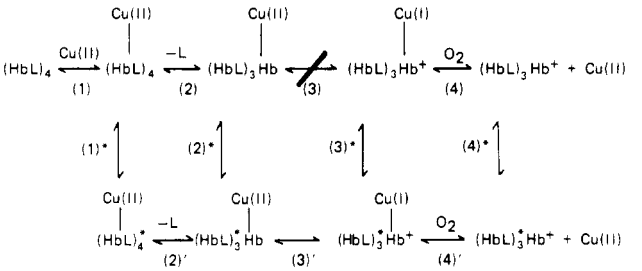
This hypothesis is apparently confirmed by the temperature studies shown in Figure 5. By raising the temperature for frozen solutions to the region of −10 °C and the recooling the samples to −50 °C, the Cu(II) signal associated with species II disappears while no change is observed in the Cu(II) signal associated with species I.

At −10 °C the electron-transfer process is no longer quenched. The conformational change associated with the interconversion from species I → II apparently involves a greater molecular rearrangement and is still quenched in these frozen solutions. The reduction of Cu(II) associated with species II but not Cu(II) associated with species I indicates that the conformational change greatly facilitates the electron-transfer process. This step may, therefore, be a necessary intermediate for the oxidation of hemoglobin, explaining the reduction of the rate of oxidation in the presence of zinc and MalNEt.

The necessary requirement for this conformational transition is also consistent with the reported 65-ms lag in the oxidation reaction (Rifkind, 1978). This lag is too large to be explained by the binding process, but is consistent with the time required for the interconversion from species I → II (Figures 2 and 5).

On the basis of these results, mechanism II can be proposed for the oxidation of hemoglobin by Cu(II), where the binding of Cu(II) and the conformational change are necessary for the oxidation to occur. The occurrence of the conformational change is indicated by an asterisk in mechanism II.

mechanism II



The dramatic effect of MalNEt on the oxidation and the conformational change suggests that copper bound as species II may be located relatively close to this sulfhydryl group, and is perhaps even coordinated to this group. The electron-transfer process may then proceed via the sulfhydryl, a pathway previously suggested as being involved in the oxidation and reduction of the heme (Holmquist & Vinograd, 1963; Rifkind, 1972). The absence of this sulfhydryl on the α chains would then explain why only the β chains are oxidized by copper (Nagel et al., 1970; Rifkind et al., 1976b; Winterbourn & Carrell, 1977).

Acknowledgments

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References

Ballou, D. P. (1971) Ph.D. Dissertation, University of Michigan.

Bemski, G., Arends, T., & Blanc, G. (1969) *Biochem. Biophys. Res. Commun. 35*, 599.

Beutler, E. (1971) in *Red Cell Metabolism—A Manual of Biochemical Methods*, p 12, Grune and Stratton, New York.

Bray, R. C. (1961) *Biochem. J. 81*, 189.

Bray, R. C., & Pettersson, R. (1961) *Biochem. J. 81*, 194.

Froehlich, J. P., Sullivan, J. V., & Berger, R. L. (1976) *Anal. Biochem. 73*, 331.

Holland, R. A. B. (1969) *Respir. Physiol. 7*, 30.

Holland, R. A. B. (1970) *Ann. N.Y. Acad. Sci. 174*, 154.

Holmquist, W. R., & Vinograd, J. R. (1963) *Biochem. Biophys. Acta 69*, 337.

Nagel, R. L., Bemski, G., & Pincus, P. (1970) *Arch. Biochem. Biophys. 137*, 428.

Oelshlegel, F. J., Brewer, G. J., Knutsen, C., Prasad, A. S., & Schoomaker, E. B. (1974) *Arch. Biochem. Biophys. 163*, 742.

Rifkind, J. M. (1972) *Biochim. Biophys. Acta 273*, 30.

Rifkind, J. M. (1974) *Biochemistry 13*, 2474.
Rifkind, J. M. (1978) Abstracts of Papers, 176th National Meeting of the American Chemical Society, Miami Beach, FL, BIOL-037.
Rifkind, J. M., & Heim, J. M. (1977) *Biochemistry 16*, 4438.
Rifkind, J. M., Heim, J. M., Chiang, S., & Li, N. C. (1976a) Abstracts of Papers, 172nd National Meeting of the American Chemical Society, San Francisco, CA, BIOL-069.
Rifkind, J. M., Lauer, L. D., Chiang, S. C., & Li, N. C. (1976b) *Biochemistry 15*, 5337.
Roughton, F. J. W., & Lyster, R. L. J. (1961) *Hvalradets Skr. No. 48*, 191.
Roughton, F. J. W., Otis, A. B., & Lyster, R. L. J. (1955) *Proc. R. Soc. London, Ser. B 144*, 29.
Salvati, A. M., Ambrogioni, M. T., & Tentori, L. (1969) *Ital. J. Biochem. 18*, 1.
Taylor, J. F., Antonini, E., Brunori, M., & Wyman, J. (1966) *J. Biol. Chem. 241*, 241.
Winterbourn, C. C., & Carrell, R. W. (1977) *Biochem. J. 165*, 141.

Iron(III)–Phosphoprotein Chelates: Stoichiometric Equilibrium Constant for Interaction of Iron(III) and Phosphorylserine Residues of Phosvitin and Casein†

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Appendix: Equilibrium Equations for Iron(III) Chelates of (Ethylenedinitrilo)tetraacetate, Nitrilotriacetate, Citrate, and Phosphoprotein *O*-Phosphorylserines

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ABSTRACT: Estimates of the strength of iron binding to model phosphoproteins were obtained from equilibrium dialysis experiments. Iron-free phosvitin (chicken and frog) or α_{s1}-casein (cow) was dialyzed against the iron(III) chelates of nitrilotriacetate (NTA), (ethylenedinitrilo)tetraacetate (EDTA), or citrate. Protein-bound metal was measured at equilibrium; competition of chelator and phosphoprotein for iron(III) was determined by reference to comprehensive equilibrium equations presented in the Appendix. Analysis of the iron-binding data for phosvitin suggested that clusters of di-*O*-phosphorylserine residues (SerP·SerP) were the most probable iron-binding sites. A stoichiometric equilibrium constant of $10^{18.0}$ was calculated for the formation of the Fe^{3+}(SerP·SerP) chelate. When compared on the basis of phosphate content, casein bound iron more weakly than phosvitin. However, if the stoichiometric equilibrium constant for the formation of the casein Fe^{3+}(SerP·SerP) chelate ($10^{17.5}$) was adjusted to account for the fact that a smaller percentage of casein phosphoserines occurs in di-*O*-phosphorylserine clusters, the affinity of casein and phosvitin for iron was very similar. A theoretical comparison showed that the "strengths" of the ferric chelates can be ranked: EDTA > phosphoprotein di-*O*-phosphorylserine > citrate > NTA.

The binding of metal by phosphoproteins has great physiological and nutritional relevance. For example, the calcium–casein micelles of milk are important sources of calcium and phosphorus for children and adults, and the iron complex of phosvitin, the principal phosphoglycoprotein of egg yolk (Shainkin & Perlmann, 1971), is a rich source of dietary iron for many people, as well as an essential reservoir of this metal for embryonic development in the eggs of lower vertebrates (Clark, 1974). Physical–chemical studies of the iron–phosvitin complex have recently outlined the coordination requirements of bound iron(III). The structural model of the iron(III)–phosvitin complex proposed by Webb et al. (1973) and by Gray (1975) explains the high probability of iron binding by adjacent phosphorylserine residues, as well as the strong iron–iron interactions observed by measurements of magnetic susceptibility. We are now studying the ligand-exchange behavior of casein and phosvitin to provide biochemical explanations for observations that milk and egg yolk may inhibit the gastrointestinal absorption of dietary iron (Carmichael et al., 1975). A related aim of some nutritional importance is to explain and predict the large proportion of natural and supplemental iron that is found associated with the casein micelles of milk (Loh & Kaldor, 1973). A complete analysis of these questions requires an estimate of the strength of iron binding by phosphoproteins.

To determine optimal conditions for saturating phosphoproteins with iron(III) for physical–chemical studies, we considered four methods for the presentation of iron: (1) presentation of iron(II) under oxidizing conditions (Taborsky, 1963); (2) addition of simple iron(III) salts; (3) direct addition of iron(III) chelates to apoproteins (Webb et al., 1973); and (4) presentation of iron(III) chelates by equilibrium dialysis under conditions similar to those used by Aasa et al. (1963)

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