Interaction of Copper(II) with Hemoglobins in the Unliganded Conformation†

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ABSTRACT: The interaction of exogenous Cu(II) with stable T-state Ni(II)- and Cu(II)-reconstituted hemoglobins has been studied. The relative binding affinities for the two human hemoglobin Cu(II) binding sites are found to be reversed in these hemoglobins relative to native iron(II) hemoglobin A. Nickel hemoglobin, modified by *N*-ethylmaleimide (NEM), iodoacetamide, and carboxypeptidase A, is used to establish that the observed differences can be attributed to the protein quaternary conformation and not to the metal substitution. Magnetic interactions between the Cu(II) responsible for oxidation and the metal–heme center suggest that the Cu(II) is closer to the heme in T-state hemoglobin than R-state hemoglobin. This finding suggests a pathway for T-state heme oxidation which does not require the β-93 sulfhydryl group, consistent with rapid Cu(II) oxidation for NEM-reacted deoxyhemoglobin.

The reversible oxygenation of hemoglobin requires the reduced ferrous state of the prosthetic group, namely, the ferroporphyrin. Effective transport of oxygen thus requires that oxidation be minimized. It has been shown (Salvati et al., 1969; Bemski et al., 1969; Nagel et al., 1970; Rifkind, 1972, 1974; Rifkind et al., 1976; Brittain, 1980; Brittain & Ivanetich, 1980; Tabak & Louro, 1985) that copper(II) catalytically oxidizes the iron(II) site in a variety of hemoglobins. It is thus important to understand fully the mechanism of Cu(II) oxidation.

The binding of Cu(II) to horse hemoglobin (Rifkind, 1979, 1981) has been shown to involve an equilibrium between two low-affinity sites. The initial binding occurs in the region of the β-143 histidine near the surface of the β-chain. A subsequent conformational change results in an alteration of the Cu EPR signal and the Cu located closer to the heme. There is, however, no indication for any magnetic interaction between the Cu(II) and the Fe(II).

For human hemoglobin, two Cu(II) binding sites are found; a high-affinity or tight-binding site (A) and a low-affinity or weak-binding site (B).[1] This latter Cu(II) binding site in human hemoglobin is comparable in affinity to the site in horse hemoglobin, and electron transfer between this Cu(II) and the Fe(II) results in the oxidation of hemoglobin.

It appears that in human hemoglobin the high-affinity site involves cross-linking between the two β-chains (Tabak & Louro, 1985; Antholine et al., 1981). This cross-linking produces a perturbation of the low-affinity B site involved in Cu(II) oxidation. Antholine et al. (1984) have thus found in human hemoglobin a diminution of the Cu(II) and Fe(III) signals attributed to the close proximity of both paramagnetic centers.

All of these studies have been done with hemoglobin (met, carbonmonoxy, or oxy) in the liganded R conformation. A fuller understanding of the interaction of Cu(II) with hemoglobin and the oxidation mechanism requires comparative studies with hemoglobin in the unliganded T-state. Unfortunately, the rapid oxidation of deoxyhemoglobin by Cu(II) makes it impossible to perform these studies on deoxyhemoglobin.

Recent studies (Alston et al., 1984; Manoharan et al., 1986) on hemoglobins reconstituted with Ni(II) or Cu(II) at the heme center indicate that these metal-substituted hemoglobins do not bind oxygen or carbon monoxide and exist in a stable T-type conformation. We have therefore used these hemoglobins to study the interactions of exogenous Cu with hemoglobin in a T-type conformation as compared with earlier studies limited to R-state hemoglobin.

In this paper, we report that the high- and low-affinity sites of the reconstituted hemoglobins are reversed in comparison to human hemoglobin. It was also observed that after binding *N*-ethylmaleimide (NEM) or iodoacetamide (IAC) to the β-93 sulfhydryl group of the reconstituted hemoglobins, the relative Cu(II) affinity of the two sites became more similar, as expected for a shift toward the R-state conformation found when these sulfhydryl reagents react with deoxyhemoglobin. For CPA-treated NiHbA, shown to be in the R-state, the relative affinities for the Cu(II) binding sites are similar to those of native HbA-CO.

Strong magnetic interactions in T-state hemoglobin between the metal at the heme center and Cu(II) bound to site B as well as a particularly rapid rate of oxidation in deoxyhemoglobin suggests an altered mechanism for Cu(II) oxidation in the T-state.

Experimental Procedures

NiHbA and CuHbA. Human blood was obtained from a local blood bank, and horse blood was defibrinated pooled blood from Bioquest. The cells were 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. Complete removal of the membranes was facilitated by freezing and thawing the hemolysate prior to centrifugation. The low molecular weight components were removed by dialysis and gel filtration on Sephadex G-25 eluted at 4 °C with 0.01 M Trizma–0.1 M NaCl, pH 7.4, buffer.

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[1] Tight-binding and weak-binding sites referred to here are as identified in the reaction of Cu(II) with carboxy adult human hemoglobin (FeHbA·CO) by earlier EPR experiments (Rifkind et al., 1976).

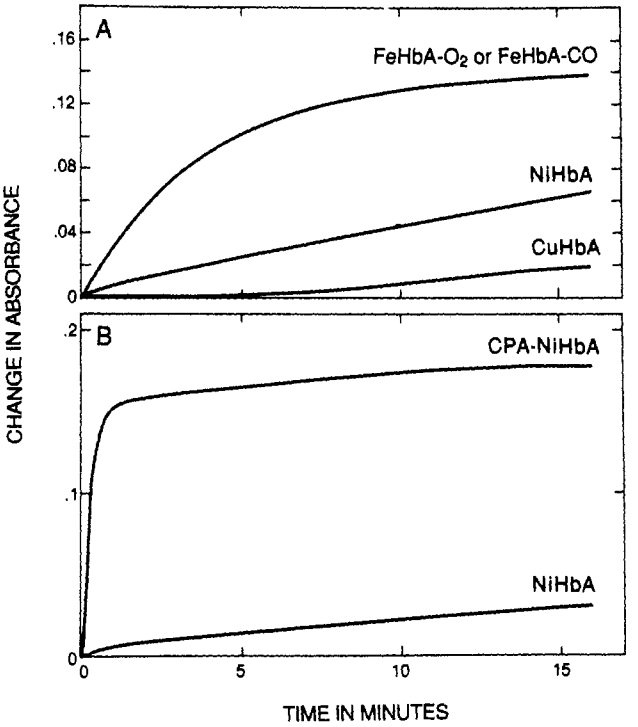

FIGURE 1: Comparative reactivity profile of -SH group in various heme proteins. The reagent is 4-PDS. Reaction performed at ambient temperature in 0.15 M NaCl.

CuHbA and NiHbA were prepared as reported earlier (Alston et al., 1984).

Most solutions of NiHbA and CuHbA used were of molarity (2–3) × 10^{-3} M. The exact amounts of nickel(II) and copper(II) present in the solutions of reconstituted hemoglobins were determined by atomic absorption. The exogenous Cu(II) was added in the form of a dilute solution of copper sulfate of proper molarity to NiHbA and CuHbA to make Cu(II) to heme molar ratios in the range of 0.1–2.0.

In certain experiments, the -SH groups of cysteine β-93 of the reconstituted hemoglobins were blocked with NEM or IAC before addition of Cu(II). This reaction was performed by overnight incubation with an excess of reagent. The unreacted NEM or IAC was removed by gel filtration. 4,4-Dithiodipyridine was then used to check for unreacted sulfhydryl groups (Ampulski et al., 1969). The reactions were found to have blocked >98% of the free -SH groups.

Des(His-β146,Tyr-β145)-NiHbA could not be prepared directly by reaction of carboxypeptidase A (CPA) with NiHbA. It was therefore necessary to first digest HbA-CO with CPA. The globin prepared in this way was reconstituted with Ni heme.

EPR spectra were measured within the temperature range of 10–300 K using a JEOL-JES Me-1 X-band EPR instrument with 100-kHz modulation and an Air Products cryogenic unit equipped with an Air Products temperature controller. DPPH was used as a g marker, and the magnetic field sweep was calibrated by using Mn^{2+} in SrO.

The reactivity of sulfhydryl groups was measured by following the time course for the reaction of 4,4-dithiopyridine (4-PDS) with various heme proteins. The increase in the intensity of the 324-nm band as a function of time was measured and plotted relative to the absorbance of the heme protein in the absence of 4-PDS (Ampulski et al., 1969).

The oxidation of hemoglobin was followed by monitoring the changes in the visible spectrum. For studies on deoxyhemoglobin, a Thunberg cell was used, and the changes were monitored at the 570.6 isosbestic point for oxyhemoglobin and deoxyhemoglobin.

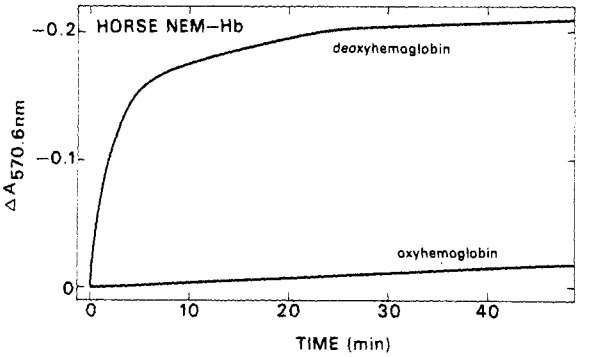

FIGURE 2: Cu(II) reactivity profile of NEM-treated oxy and deoxy horse hemoglobin.

RESULTS

Sulfhydryl Reactivity Experiment. The comparative reactivity profiles of the -SH group in various heme proteins are shown in Figure 1. The reactivity of NiHbA or CuHbA is extremely slow compared to that of FeHbA-O_2 or FeHbA-CO possessing the R structure (Figure 1A). These results are consistent with a T-type conformation for these metal-substituted proteins. The CPA-modified NiHbA (Figure 1B) has a reactivity even faster than R-state FeHbA-CO or FeHbA-O_2. This experiment shows that the T structure of NiHbA and CuHbA is transformed to the R structure on treatment with CPA.

Oxidation of Sulhydryl-Reacted Hemoglobins. Figure 2 shows the effect of reacting the β-93 sulfhydryl group with NEM on the copper oxidation of horse oxyhemoglobin and deoxyhemoglobin. The very slow rate for Cu(II) oxidation of HbO_2 when the β-93 sulfhydryl group is blocked has previously been reported. On the basis of these results, it has been proposed (Winterbourn & Carrell, 1977) that the sulfhydryl group may be one of the coordination sites for Cu(II) and is at least involved in the electron-transfer process (Rifkind et al., 1976; Rifkind, 1981).

As shown in Figure 2 for deoxyhemoglobin, a rapid rate of Cu(II) oxidation is observed even with the sulfhydryl blocked. This rate is comparable to the rate for HbO_2 with the sulfhydryl group free. These results suggest an altered mechanism for Cu(II) oxidation in T-state hemoglobin.

EPR Studies of Cu(II) Binding Site in NiHbA. The EPR spectra of Cu(II) added at different molar ratios to NiHbA were recorded at 12 K. The EPR spectrum (Figure 3) at a low Cu(II) to NiHbA molar ratio of 0.1 (Figure 3A) is similar to that of Cu(II) bound to the weak-binding sites (site B) in human FeHbA-CO (Rifkind et al., 1976). However, on further addition of Cu(II) (Figure 3B,C), new lines are detected corresponding to a second spectral component, which overlaps the one already present due to the first site. The general pattern of the EPR spectrum and the spin Hamiltonian parameters from the second site are comparable to those of the tight-binding Cu(II) sites observed in human FeHb-CO (Rifkind et al., 1976); even the ^{14}N hyperfine lines in the perpendicular part appear, but are less resolved due to the overlapping strong signals from the first binding site. The EPR spectra at three different concentrations of exogenous Cu(II) are shown in Figure 3, along with the computer simulations. The spin Hamiltonian parameters for both Cu(II) sites are given in Table I.

EPR Studies of Cu(II) Binding Sites in Modified NiHbA. The effect of blocking the -SH group at the β-93 position by

Table I: Summary of Spin Hamiltonian Parameters Obtained for the Exogenous Cu(II) Added to NiHbA, NEM-Treated NiHbA, and FeHbA-CO[a,b]

condition	Cu(II) with	site A				site B			
		$g_\parallel$	$A_\parallel$	$g_\perp$	$A_\perp$	$g_\parallel$	$A_\parallel$	$g_\perp$	$A_\perp$
1	NiHbA	2.237	219.3	2.05	16.0	2.322	149.9	2.055	
2	IAC–NiHbA	2.218	217.5	2.06	16.0	2.315	161.8	2.055	
3	NEM–NiHbA	2.217	217.0	2.07	16.0	2.314	162.0	2.055	
4	human FeHbA-CO	2.210	203.3	2.054	16.6	2.270	182.0	2.057	

[a] Site A is the first binding (or tight-binding site) in conditions 2, 3, and 4 while B is the first binding site in condition 1. [b] All A values in 10^{-4} cm^{-1}; errors in g values ±0.003; errors in $A_\parallel$ values ±1.5; errors in $A_\perp$ values ±1.0. A (^{14}N) in site B is 15.9×10^{-4} cm^{-1}. Estimated $A_\parallel$ (^{14}N) from line broadening is $\sim 13.4 \times 10^{-4}$ cm^{-1}.

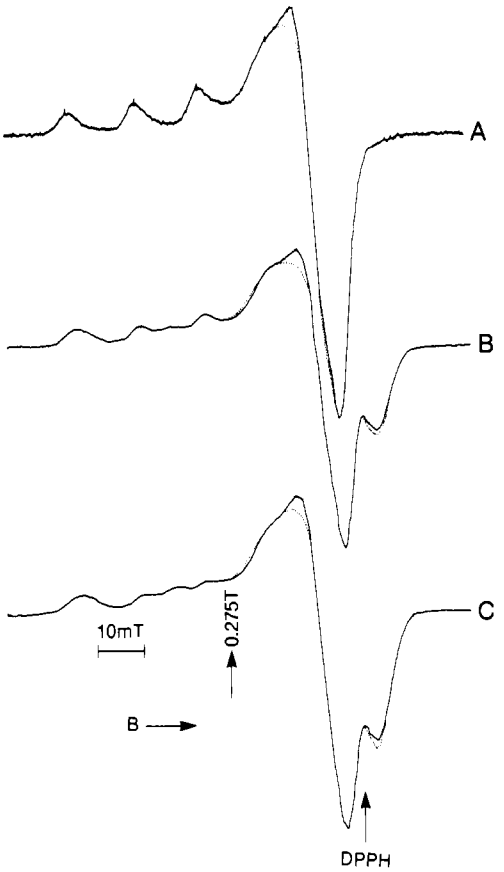

FIGURE 3: EPR spectra of Cu(II) added to NiHbA in the molar ratio (A) 0.1:1.0, (B) 0.6:1.0, and (C) 1.4:1.0. Spectrum B shows the partially resolved hyperfine lines due to $^{63,65}Cu$ and ^{14}N in the more intense part of the perpendicular component. The dotted lines represent the computer simulation using the spin Hamiltonian parameters and relative intensities found in Tables I and II.

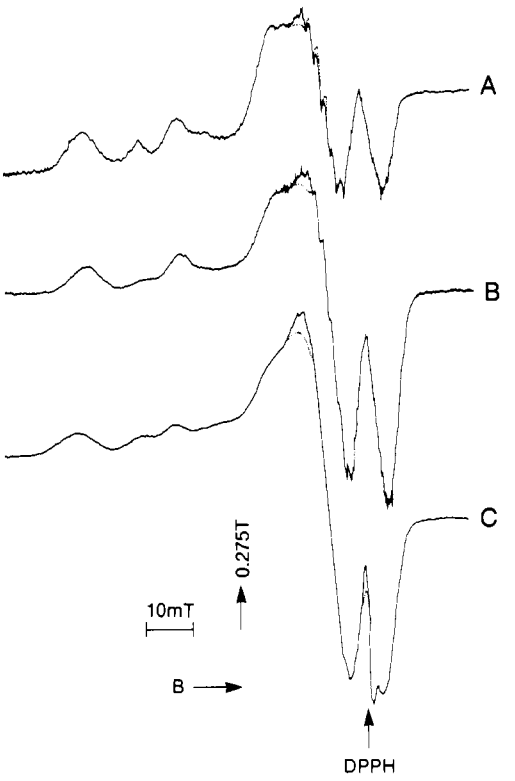

FIGURE 4: EPR spectra of NEM-reacted NiHbA in the molar ratios (A) 0.25:1.0, (B) 0.5:1.0, and (C) 1.0:1.0. The dotted lines represent the computer simulation using the spin Hamiltonian parameters and relative intensities reported in Tables I and II.

NEM on the Cu(II) EPR spectrum is shown in Figure 4. The initial addition of Cu(II) to NEM-reacted NiHbA produces a spectrum with a low $g_\parallel$ of 2.217 and a high $A_\parallel$ of 217×10^{-4} cm^{-1} (Table I) with the characteristic presence of well-resolved hyperfine lines due to four equivalent ^{14}N's and $^{63,65}Cu$ in the perpendicular component similar to that of Cu(II) added to site A of human FeHbA-CO. However, even the spectrum at a molar ratio of 0.25 has a contribution from a second component with a high $g_\parallel$ of 2.314 and a low $A_\parallel$ of 162×10^{-4} cm^{-1}. The spin Hamiltonian parameters for NEM-reacted NiHbA (Table I) are similar to those for NiHbA.

In Table II, the ratio of the simulated intensity for site A relative to site B is given for different hemoglobin molar ratios. The ratio of relative intensities (Table II), particularly at low Cu:hemoglobin molar ratios, provides a measure of the relative Cu(II) affinity for site A and site B. A comparison of FeHbA-CO, NiHbA, IAC–NiHbA, and NEM–NiHbA indicates a relationship between the relative affinity for both sites and the protein conformation, where site A has an appreciably higher affinity in the R-liganded state and site B has an appreciably higher affinity in the T-unliganded state.

Table II: Relative Intensities of the EPR Spectra (I_A/I_B) of Cu(II) at Binding Sites A and B at 12 K

Cu(II):Hb molar ratio	I_A/I_B for species			
	NiHbA	IAC–NiHbA	NEM–NiHbA	FeHbA-CO
0.2	≤0.05			
0.25	≤0.05	<0.1	1.8	19
0.4	<0.05			
0.5	*a*	0.54	1.4	5.8
0.6	0.42			
0.75	*a*	0.59	1.3	2.0
0.8	0.38			
1.0	*a*	0.55	1.0	~1.0

[a] No measurements at this molar ratio.

The destabilization of the T-state by the reaction of the sulfhydryl groups produces a similar affinity for both sites. Furthermore, NEM, which has a greater effect than IAC on the T-state as seen by the larger change in affinity and decrease in n values (Imai, 1973) for oxygen binding, produces a greater

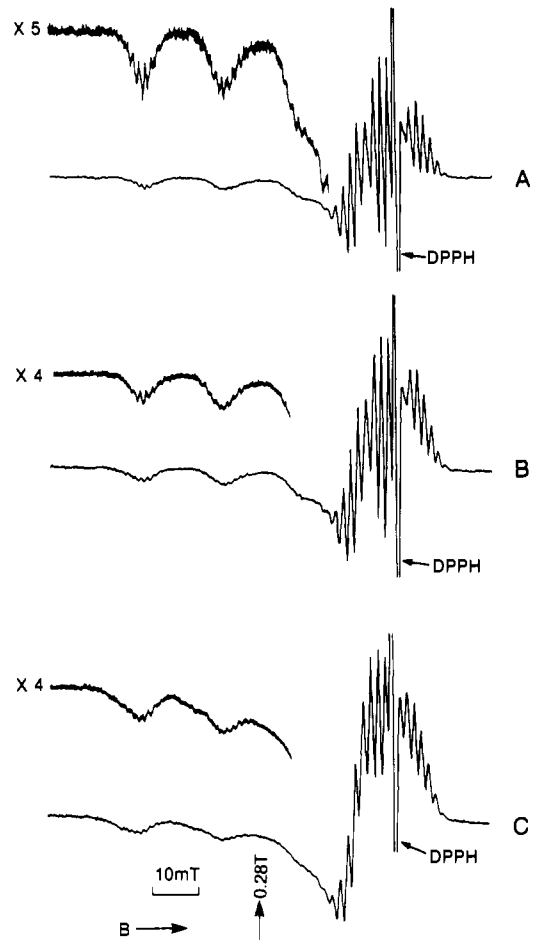

FIGURE 5: EPR spectra of Cu(II) added to CuHbA: (A) molar ratio 0.1:1.0, gain 1.0×10^2; (B) molar ratio 0.3:1, gain 1.4×10^2; (C) molar ratio 1.0:1.0, gain 1.4×10^2.

shift in the relative affinity for the NiHbA Cu binding sites toward those of HbA (Table II).

At higher Cu:hemoglobin molar ratios, as the higher affinity sites are saturated, the relative population of both sites becomes more nearly equivalent in all cases. For des(His-β146,Tyr-β145)-NiHbA, even at low Cu:hemoglobin molar ratios, the dominant component for Cu(II) is site A of NiHbA, with no evidence for any copper in site B. This hemoglobin, which is in an R-state (Figure 1), thus shows a relative copper affinity for site A and site B similar to FeHbA-CO, which is also in the R-state.

Interaction of Cu(II) with CuHbA. One of the most interesting results comes from the EPR study of the interaction of Cu(II) with reconstituted CuHbA. During the initial addition of copper(II) up to a molar ratio of 0.5, we observed (i) the absence of any new signal due to the exogenous Cu(II), (ii) a decrease in the intensity of the original EPR signals due to the CuHbA, and (iii) almost negligible broadening effect as can be seen by comparing Figure 5A and Figure 5B. However, when excess copper(II) is added ($\geq$0.5 molar ratio), one can observe the formation of the new peak due to additional Cu(II) binding site as well as the distortion and broadening of the signals due to CuHbA (Figure 5C). By comparing the expected signal intensity in the absence of magnetic interactions with the observed signal intensity in the 0–0.5 range of molar ratios (Figure 6), it is possible to demonstrate an exchange interaction between the heme CO and the exogenous Cu(II) bound to the initial Cu(II) site at low molar ratios. The magnitude of the exchange interaction is an indication of the close proximity of these two metal centers.

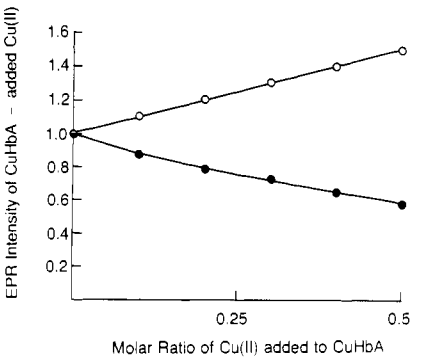

FIGURE 6: Plot of the integrated total EPR intensity (at 12 K) of CuHbA and exogenously added Cu(II) as a function of added Cu(II). (●) Indicates the observed intensities normalized to the intensity of the initial concentration of CuHbA while (○) represents the expected total intensities in the absence of any antiferromagnetic interaction between the heme copper and the copper(II) in site B.

Discussion

Effect of Hemoglobin Conformation on the Binding of Copper to Hemoglobin. (A) Site A. For both HbA-CO and met-HbA in the R-state, the highest affinity site corresponds to a site with four nitrogens (Bemski et al., 1969) involving the N-terminus and histidine β-2 (Rifkind et al., 1976; Taketa & Antholine, 1982). Figure 1 indicates that NiHbA and CuHbA, which do not bind O_2 or CO, have a conformation similar to T-state Hb as indicated by a slow rate for the reaction of the β-93 sulfhydryl group (McDonald & Noble, 1974). This conclusion is also consistent with the other studies based on CD spectroscopy and immunochemical reactions (Alston et al., 1984).

The EPR studies of exogenous Cu(II) bound to NiHb (Figure 3, Table II) indicate that the initial binding does not show nitrogen hyperfine and is much more similar to site B of HbA-CO. Only at higher Cu:heme molar ratios is a site with spin Hamiltonian parameters similar to those of site A occupied (Table I). Equilibrium dialysis studied by Rifkind and Heim (unpublished results) further indicates that the change in the relative populations of site A and site B is due to a decrease in the affinity for site A.

Recent evidence (Antholine et al., 1985; Tabak & Louro, 1985) indicates that in solution site A copper actually produces a cross-link between β-chains with some of the coordination sites on the other β-chain relatively close to the β-93 sulfhydryl group. Although this cross-link appears disrupted in the frozen state as indicated by a greater distance to the β-93 SH group, an EPR spectrum with similar spin Hamiltonian parameters and four nitrogenous ligands is retained. Presumably, nitrogenous ligands arising from the region of the β-93 sulfhydryl group are replaced by other nitrogenous ligands in the region of the β-chain amino terminus. Since X-ray data indicate that the crevice between the β-chains is greater in the T-state than the R-state, it is expected that the cross-link configuration would not be possible in the T-state. This alteration in the structure would be expected to lower the affinity of Cu(II) to site A. The spin Hamiltonian parameters for site A may still reflect the nearly equivalent nitrogens, analogous to that found in the frozen state result cited above.

NEM and IAC are known to shift the T-conformation toward the R-state. Thus, the oxygen affinity increases, and the Hill constant decreases with the effect of NEM greater than IAC (Imai, 1973). Consistent with this known shift in the T $\rightleftarrows$ R equilibrium, the relative Cu(II) affinities for site A and site B become more equivalent, with the effect of NEM greater than IAC (Table II).

This partial reversal as against the more complete reversal with CPA toward the binding characteristics in HbA supports the contention that the differences observed in NiHbA are primarily due to changes in the T to R conformational equilibrium and not due to local changes attributed to the substitution of Fe by Ni.

(*B*) *Site B.* Site B is the Cu(II) binding site associated with the oxidation of hemoglobin. Studies on horse hemoglobin in the R-state (met, CO, and O_2) indicate two associated Cu binding sites with Cu(II) initially bound to the surface near β-143 and a subsequent rearrangement which brings the Cu(II) close to the β-93 sulfhydryl (Rifkind, 1979). No direct magnetic interaction between Cu(II) and Fe(III) has been detected. The early studies on human hemoglobin in the R-state also gave no indication for magnetic interactions between the Cu(II) and the Fe(III) (Bemski et al., 1969; Nagel et al., 1970). Recent studies, however, suggest an interaction between the Cu(II) and the Fe(III), presumably associated with the Cu(II) cross-link made when Cu(II) binds to site A (Antholine et al., 1984).

For both horse and human hemoglobin, the drastic reduction in the rate of oxidation when the β-93 SH group is blocked has led to the proposal that the electron transfer occurs via the β-93 sulfhydryl group (Rifkind et al., 1976; Rifkind, 1981; Winterbourn & Carrell, 1977). However, for deoxyhemoglobin, oxidation is still a rapid process even with the β-93 sulfhydryl blocked (Figure 2). These results indicate that for the deoxy T-state the electron-transfer process does not proceed via the β-93 sulfhydryl and an alternative mechanism must be involved.

The interactions between the Cu(II) bound to site B and the heme Cu(II) in CuHb (Figures 5 and 6) suggest a strong exchange interaction with close proximity of the metal centers. This interaction is considerably greater than observed by Antholine et al. (1984) in the R-state. These metal centers are presumably as close to or closer to the heme metal center than the heme center to β-93 sulfhydryl distance. A direct transfer of electrons between metals or perhaps via the porphyrin moiety is therefore suggested.

Altered Spin Hamiltonian Parameters for NiHbA. The parameters given in Table I indicate small but noticeable g and A shifts of 0.052 and -32.1×10^{-4} cm^{-1} for site B Cu(II) (as compared to the values for the same site in FeHbA-CO), and smaller shifts of 0.027 and $+16 \times 10^{-4}$ cm^{-1} for site A. Although these shifts could be attributed to slightly altered binding sites in T-state NiHbA, they could also be attributed to an exchange interaction.

Visible spectroscopy (Manoharan et al., 1986a,b; Shibayama et al., 1986a,b), magnetic susceptibility, EPR (Kuppusamy et al., 1988), Raman (Shelnutt et al., 1986), and NMR (Shibayama et al., 1987) results indicate that NiHb is a mixture of 4-coordinated diamagnetic porphyrin $S = 0$ states and 5-coordinated paramagnetic porphyrin $S = 1$ states. Studies with Ni–Fe hybrids (Shibayama et al., 1986, 1987) indicate that in the tetrameric T-state the β-chains to which Cu(II) binds are in the paramagnetic $S = 1$ state. Exchange interactions between the $S = 1$ β(Ni) center and the $S = {}^1/_2$ Cu(II) binding site could produce the observed shift in the spin Hamiltonian parameters (Table I). Though the exchange between heme Cu(II) of CuHbA with $S_1 = {}^1/_2$ and site B Cu(II) with $S_2 = {}^1/_2$ will provide an $S = 0$ ground state and $S = 1$ excited state in an antiferromagnetic coupling interaction, the coupling between heme β(Ni) with $S_1 = 1$ and site B Cu(II) with $S_2 = {}^1/_2$ provides exchange-coupled states $S = {}^3/_2$ and ${}^1/_2$. This exchange interaction (Mehran et al., 1971) will act like a magnetic field on the β(Ni) ions. The $S = 1$ Ni(II) adjacent to Cu(II) will therefore be polarized, and this will then introduce a g shift of the Cu(II) in its EPR spectrum. Such exchange interaction is also noticeable at site A, but to a smaller degree because of a larger β(Ni)–Cu(II) distance.

Conclusion

By combining Cu(II) studies on deoxyhemoglobin and T-state NiHb and CuHb, it has been possible to delineate a more complete picture of the Cu(II) interaction with hemoglobin. The results indicate that the potential detrimental effects of Cu(II) oxidation are enhanced in the T-state, which is found in deoxyhemoglobin or at low oxygen pressure where the T-state is prevalent. This enhancement involves a more effective oxidation mechanism involving a direct Cu oxidation, not via the sulfhydryl. Furthermore, the protective effect due to the high-affinity A site which is not involved in oxidation is eliminated by a reversal of the relative affinities of site A and site B.

Both of these factors may lead to the enhancement of the oxidative stress on erythrocytes at low oxygen pressures.

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In Vitro Collagen Fibril Assembly: Thermodynamic Studies

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ABSTRACT: The in vitro fibril assembly of calf skin collagen was examined as a function of ionic strength and temperature. In a 0.03 M NaP_i, pH 7.0, buffer, fibril assembly required a minimum critical concentration of collagen. At nearly physiological ionic strengths and temperatures, the critical concentration was less than 1 μg/mL and required a very sensitive method for measurement. Raising the ionic strength of the buffer resulted first in higher and then lower critical concentrations. Raising the temperature led to lower critical concentrations. A van't Hoff plot of the fibril growth constant calculated from the critical concentration gave positive enthalpy changes and positive heat capacity changes which indicate that the fibril growth is driven by both hydrophobic and ionic inter-collagen interactions. Sedimentation equilibrium studies showed the collagen to be monomeric at subcritical concentrations. Differential scanning microcalorimetric studies showed only one very sharp heat absorption peak for the fibril assembly which coincided with the appearance of solution turbidity. Within experimental error, the enthalpy changes of the fibril assembly measured with the microcalorimeter were of the same magnitude as the van't Hoff enthalpy changes. These results are discussed in light of a cooperative nucleation–growth mechanism of collagen fibril assembly proposed earlier [Na, G. C., Butz, L. J., Bailey, D. G., & Carroll, R. J. (1986) *Biochemistry 25*, 958–966].

Type I collagen is synthesized inside fibroblast cells and secreted into extracellular space in the form of procollagen. Subsequent enzymatic cleavages of the N- and C-propeptides yield the collagen monomer which can then self-associate into fibrils to fulfill its biological functions (Miyahara et al., 1982, 1984; Kivirikko & Myllyla, 1984). Collagen fibrils, particularly those in the connective tissues of young animals, can be solubilized under a variety of buffer conditions. The solubilized collagen, when brought back to nearly physiological conditions, can be reconstituted into fibrils of the same morphology, indicating that the collagen molecule itself contains all the necessary structural information for the ordered fibril assembly [for a recent review, see Piez (1984)].

Numerous studies of collagen fibril assembly in vitro have been reported in the past 3 decades aimed at understanding the process of fibrillogenesis in the extracellular matrix. Major interest has been focused on the mechanism of initiation of the reaction, the control of the diameter and length of the fibrils, and the roles of the triple-helical domain and the short nonhelical telopeptides at the carboxy and amino terminals of the molecule. It has been known for decades that the assembly of collagen fibrils, when monitored by measuring the turbidity of the solution, shows two different phases (Bensusan & Hoyt, 1958; Gross & Kirk, 1958; Wood & Keech, 1960; Wood, 1960; Comper & Veis, 1977a,b; Williams et al., 1978; Gelman et al., 1979a). The reaction starts with a lag phase during which the solution does not display any turbidity. Unbanded microfibrils with diameters in the range of 3–15 nm have been observed by electron microscopy during this period (Veis et al., 1979; Gelman et al., 1979a; Na et al., 1986a,b). This is followed by a growth phase in which the turbidity of the solution increases in a sigmoidal manner. Electron microscopy of negatively stained samples taken in this phase showed the appearance of fibrils with periodic 67-nm striations. Such two-phase kinetics led to the suggestion of a cooperative nucleation–growth mechanism for the reaction (Wood & Keech, 1960; Wood, 1960; Cassel et al., 1962). A recent detailed analysis of the kinetic data confirmed that the fibril assembly can be adequately described by a cooperative nucleation–growth association with the initial formation of pentamers as the nucleation centers (Na et al., 1986b).

In a recent equilibrium study, we have shown the requirement of certain minimal concentrations of collagen, referred to as critical concentrations, for the in vitro fibril assembly in buffers containing glycerol (Na et al., 1986a). Furthermore, a velocity sedimentation study showed the collagen to be monomeric at subcritical concentrations. The observation of critical concentrations is consistent with the cooperative self-association mechanism derived from the kinetic data. The results also suggested that the fibril growth is very strong at nearly physiological conditions which rendered the critical concentrations too low to be detected by the method used in earlier studies (Williams et al., 1978; Gelman et al., 1979a,b). By adding glycerol, a fibril assembly inhibitor, to the buffer, the critical concentration of the reaction was brought to an easily measurable range (Na et al., 1986a).

In order to demonstrate the presence of critical concentrations for fibril assembly at nearly physiological conditions and to understand the thermodynamic properties of the reaction, we developed a more sensitive method of measuring the collagen concentration (Na, 1988). By use of this method, the critical concentrations of the fibril assembly reaction were determined as functions of ionic strength and temperature.

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