Internal Electron Transfer between Hemes and Cu(II) Bound at Cysteine β93 Promotes Methemoglobin Reduction by Carbon Monoxide*

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Previous studies showed that CO/H2O oxidation provides electrons to drive the reduction of oxidized hemoglobin (metHb). We report here that Cu(II) addition accelerates the rate of metHb β chain reduction by CO by a factor of about 1000. A mechanism whereby electron transfer occurs via an internal pathway coupling CO/H2O oxidation to Fe(III) and Cu(II) reduction is suggested by the observation that the copper-induced rate enhancement is inhibited by blocking Cys-β93 with N-ethylmaleimide. Furthermore, this internal electron transfer pathway is more readily established at low Cu(II) concentrations in Hb Deer Lodge (β2His → Arg) and other species lacking His-β2 than in Hb A0. This difference is consistent with preferential binding of Cu(II) in Hb A0 to a high affinity site involving His-β2, which is ineffective in promoting electron exchange between Cu(II) and the β heme iron. Effective electron transfer is thus affected by Hb type but is not governed by the R ↔ T conformational equilibrium. The β hemes in Cu(II)-metHb are reduced under CO at rates close to those observed for cytochrome c oxidase, where heme and copper are present together in the oxygen-binding site and where internal electron transfer also occurs.

Hemoglobin (Hb) functions as an oxygen carrier only when reduced and is so maintained in vivo through enzymatic NADPH-driven reduction reactions (1–4). It has been suggested that low levels of copper, normally present in vivo, promote Hb oxidation and that copper-induced oxidation contributes to loss of reducing potential in the red cell and eventual red cell deterioration. Copper is toxic at elevated levels, and the mechanism of toxicity involves increased rates of Hb oxidation with adverse side reactions such as increased cell membrane oxidation (1, 2, 5, 6).

We owe much of our understanding of copper-induced oxidation of Hb to the work of Winterbourn and Rikkind and coworkers (1, 5), who showed that copper-induced oxidation of oxyHb occurs through an internal electron transfer pathway between the iron atoms of the β chain hemes and copper bound at or near the β93 sulphydryls. They found that Cu(II) binding to Hb facilitates the oxidation process through this internal pathway only when the amount of Cu(II) exceeds that bound at unreactive high affinity sites, which in Hb A0 includes the β2 histidine residues. As a consequence, the oxidative reaction proceeds less readily at low copper concentrations in Hb A0 than in the human Hb variant Hb Deer Lodge (β2His → Arg) and in Hbs of species that lack His-β2 (1). Hb Deer Lodge has higher oxygen affinity and increased anion sensitivity and is more readily oxidized than Hb A0 (7, 8). We have used Hb Deer Lodge and Hb A0 in the present studies in which we show that CO/H2O-driven electron transfer can bring about a reduction of the β chain hemes that utilizes this internal electron transfer pathway.

Previous studies carried out in our laboratories have shown that Fe3+ in isolated heme (hemin), in Hb, in cytochrome c oxidase and in several other oxygen-binding proteins can become reduced when maintained under an atmosphere of CO, with the half-cell reaction given in Eq. 1 driving the overall process (9).

\[ \text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2e^- \] (Eq. 1)

In the following we refer to metal reduction coupled to this half-reaction as CO/H2O-driven reduction. The overall free-energy change is clearly dependent upon the identity and environment of the metals reduced. The process of reduction under CO was called “auto-reduction” by previous workers (10–14) who noted that cytochrome c oxidase, with two constituent electron-accepting metal centers in the oxygen-binding site, becomes reduced under CO. Heme α3 in the oxygen-binding site of the oxidase is typically reduced in less than an hour, with a much slower (overnight) incubation required to bring about reduction of heme α. The details of the reduction of cytochrome c oxidase are not well understood, and there are clearly competing processes that have hindered elucidation of the mechanism (15).

We now report that Cu(II) addition to metHb dramatically increases the rate of heme reduction under CO. We show that Cu(II) is reduced to Cu(I) as the reaction proceeds and that the
enhanced rate of reduction is inhibited by sulphydryl reagents such as N-ethylmaleimide. The data indicate the existence of an internal pathway that is used for electron exchange between the heme iron and the bound Cu(II).

**EXPERIMENTAL PROCEDURES**

Samples of native adult human hemoglobin (Hb A0) and Hb Deer Lodge were prepared by the ammonium sulfate method, chromatographically purified, and stripped of organic phosphate cofactors as described previously (7). Horse Hb was prepared similarly but without chromatographic purification. The samples were typically dialyzed against 0.05 M Tris, pH 8.3 with selection as desired. Concentrations and compositions of samples were determined spectrophotometrically, using Hewlett-Packard 8451 and 8453 Diode Array spectro-photometers for routine measurements and a Perkin-Elmer A6 dual beam-scanning spectrophotometer for higher resolution work. Samples were usually measured in 1-cm path length cuvettes, but cells with 2-mm path lengths were used to obtain spectral data on samples whose optical density would have gone beyond the linear response range of these instruments. Oxidized (met)Hb samples were made by treatment with potassium ferricyanide at 1.2-fold excess over heme. Sequential Sephadex G-25 chromatographies (high salt then no salt) were used to remove free and bound ferricyanide and ferrocyanide from the metHb samples of oxidized Hb (metHb), deoxyHb, carboxyHb, and metHb (oxyHb), carbon monoxideHb (CO-Hb), and hemichrome were determined by spectral analysis. Samples that contained any detectable hemichrome were discarded. The stock Hb solutions, typically 1–2 mM in heme units (iron-porphyrin units), were stored in liquid nitrogen prior to use. Sodium dithionite at 2 mg/ml was used as a reductant to obtain spectra of the fully reduced proteins. Hb with only the β chain hemes oxidized was prepared by addition of a 5-fold excess of CuSO4 over oxyHb. It has been shown (2, 16, 17) that under these conditions the β chain hemes are selectively oxidized, whereas the α chain hemes remain in the reduced, ferrous state.

Isolation of α and β chains of Hb A0 was accomplished by use of a modified version of established methods in which para-hydroxymercuribenzoate binds to SH groups and encourages dissociation of the Hb tetramer (18). Our modifications consisted of carrying out the separations at 4 °C, using chromatographies with CMC cellulose (for β chains) and DEAE cellulose (for α chains) that effectively separate the chains. Regeneration of the SH groups of the β chains was done by treatment with β-mercaptoethanol to reduce SH groups, followed by chromatography through DEAE-cellulose to remove para-hydroxymercuribenzoate.

Tetramers of β chains form spontaneously after SH groups are regenerated.

The free SH groups of Hb (at position β83) were blocked by treating Hb A0 with N-ethylmaleimide (NEM) at a protein-to-reagent ratio of 1:3. The reaction was carried out at 37 °C for 1 h with the reactants in 0.05 M bis-Tris, pH 7.2, followed by Sephadex G-25 chromatography to separate the Hb from the low molecular weight reagent.

Carboxypeptidase A-digested Hb A0 (HbCPA) was prepared by treating the CO derivative of Hb A0 with carboxypeptidase A (Sigma type 1, diisopropyl fluorophosphate) at an enzyme-to-protein ratio of 1:50. The mixture was incubated at 37 °C for 2 h and then dialyzed at 4 °C against 0.05 M Tris buffer, pH 8.3. This enzymatic digestion under the conditions employed removes the C-terminal His and Tyr of the β chains as verified by electrospray ionization mass spectrometry.

Cu(II)-metHb complexes were made by adding CuSO4 to metHb at indicated ratios of copper to heme. Hb samples in some cases were treated with 5-fold excess of Cu(II) and then stripped of unbound or loosely bound copper by passage through a Chelex resin column. In other experiments, the copper-Hb complexes were made by addition of copper at the desired ratio of copper to heme without subsequent Chelex treatment. Elemental analysis of samples by atomic absorption spec-
copy (Perkin-Elmer 5000) following copper treatment validated the treatment. Elemental analysis of samples by atomic absorption spec-
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**RESULTS**

Visible Spectroscopy of the CO/H2O-driven Reduction of Cu(II)-MetHb Complexes—Heme reduction brought about by incubation under CO allows for binding of CO, so that time courses show a disappearance of the spectral features of metHb and an appearance of the features of CO-Hb. There are generally good isosbestic points for the transition, with the exception of experimental conditions described later. Cu(II) addition significantly enhances the rate of metHb reduction, with enhanced rates evident only for the β chains of the Hb tetramer.

Specificity for β chains in Cu(II)-enhanced rates of reduction is supported by the fact that there are similar reaction kinetics for half-reduction of a Cu(II)-metHb complex, α(III)β(III)4, and for full reduction of a half-metHb preparation, α(III)β(III)2, with the same ratio of Cu(II) to heme. Fig. 1 shows the spectral changes in these two samples brought about by incubation under CO. Good isosbestic points between the met and CO derivatives are observed in both cases. The fully oxidized Hb becomes half-reduced in about 10 h, with a half-time of β chain reduction of about 4.5 h. For the copper-Hb complex with only the β chains oxidized, the half-time for reduction is also about 4.5 h. We conclude that it is the reduction of the β chain hemes that is appreciably increased in rate by copper addition and that the oxidation state of the partner chains is not a major determinant of the reduction rate.

Isolated β chains were prepared as described under “Experimental Procedures.” After regeneration of SH groups, the β chains spontaneously form tetramers. The β tetramers do not show an increase in the rate of CO/H2O-driven reduction when observed at a copper:heme ratio of 0.5 (Table I). However, Cu(II)-enhanced rates of reduction are observed when the copper:heme ratio is raised to 2. Rates of reduction under CO at this Cu(II) level are appreciably enhanced over controls. Rate estimates at this Cu(II) level are not given in Table I because of uncertainties associated with some sample precipitation during these experiments. The requirement for higher Cu(II) levels in β4 tetramers can be explained by the fact that each β chain has a high affinity copper-binding site at the β2 position that would be expected to be ineffective in electron transfer. The failure to see copper-enhanced rates at ratios below 1 copper per heme is thus an indication that Cu(II) has limited access to effective electron transfer sites in the β4 tetramers.

We conducted an additional experiment to determine if the noncoordinated high copper-binding sites for reduction of β4 tetramers was due to some artificial modification of the β4 chains during the isolation procedure. We found that the β chains do become reduced under CO at a copper:heme ratio of 0.5 when present in half-met α(III)β(III)2 tetramers that were made by 1:1 mixing of the isolated β chains with reduced α chains. This experiment with reconstituted half-met tetramers...
Copper-enhanced Reduction of MetHb under CO

Fig. 1. Spectral changes during copper-enhanced CO/H_2O-driven reduction. The spectral changes shown were brought about by incubation of Cu(II)-treated Hb under 1 atm CO at 25 °C in 0.05 M Tris-HCl at pH 8.3. The Hb samples were 15 μM in heme and had Cu(II)-to-heme ratios of 0.5. A, MetHb A_0 reduction proceeds “rapidly” only to the half-reduced condition, as shown by spectra taken at 2-h intervals. B, full reduction of half-met (α(II)β(III)_2) Hb A_0 is shown by spectra taken at 3-h intervals. See text for details.

gave results similar to those shown in Fig. 1B. This confirmed that the isolated β chains were selectively reduced under CO after copper addition.

The effects of varied levels of Cu(II) on the time courses of β heme reduction under CO were explored. As shown in Fig. 2, with no copper present there is very little reduction of metHb A_0 or metHb Deer Lodge after a 24-h incubation under CO (data shown only for Hb A_0, open circles). The small extent of reduction of the copper-free metHb A_0 control is consistent with our previous work that established the half-time for metHb A_0 reduction under CO to be about 1000 h at 1 atm pressure. When the process is carried out with a copper:heme ratio of 0.25, the process is only slightly increased in rate for Hb A_0, but appreciably increased in rate for Hb Deer Lodge (β2His → Arg). The CO/H_2O-driven reduction of metHb A_0 occurs more quickly at elevated Cu(II) levels. As shown, at a copper:heme ratio of 0.7, approximately one-half of metHb A_0 becomes reduced at a copper-enhanced rate, attributable to preferential reduction of the β chains (see above). The remainder of the reaction is ascribed to the reduction of the α chains. This much slower phase is less sensitive to copper addition. No efforts were made to investigate copper-Hb interactions at ratios above 1.0 copper:heme, since ratios greater than 0.5:heme can result in gradual protein precipitation (1) and disulfide formation (2).

The time courses of heme reduction observed over a 24-h period were generally well fit by assuming that they are composed of two exponential phases. Apparent rate constants for the two phases, obtained under varied conditions, are given in Table I. The apparent first-order rate constant of the first phase (k_1) reflects the Cu(II)-sensitive rate of β chain reduction. The apparent first-order rate constants for the second phase (k_2) are like those of the copper-free controls.

The copper:heme ratios indicated in Table I were established by Cu(II) addition to thoroughly deoxygenated metHb samples (exceptions are indicated). Multiple stages of sequential deaeration and purging with argon or nitrogen ensured that no oxygen was present in the samples before CO incubation was begun, generally about 20 min after copper addition. That reactive oxygen species are not involved in these reactions was verified by observing similar time courses of CO/H_2O-driven reduction in the presence of added catalase and/or superoxide dismutase. Greatly enhanced rates of heme reduction for Cu(II)-metHb complexes under CO relative to samples without copper were observed in the Soret region with dilute samples (about 10 μM in heme) and in the 500–650-nm wavelength region with much more concentrated samples (about 500 μM in heme) in 2-mm path length cells. The phenomena observed are not artifacts of dissociation, as verified by observing similar behavior over a wide protein concentration range and use of high pressure liquid chromatography that showed no experimentally detectable subunit dissociation in the high concentration samples.

At lower levels of copper (≤0.25 copper:heme), the rate and extent of reduction of metHb Deer Lodge (closed squares in Fig. 2) are clearly more affected by copper addition than reduction of metHb A_0 (closed circles). Horse Hb, also lacking His-β2 residues, behaves like Hb Deer Lodge in having an appreciable copper-enhanced phase of CO/H_2O-driven reduction at 0.25 copper:heme (Fig. 2 and Table I). These results mirror the EPR results presented in the next section. The structural explanation for the difference in copper sensitivity exhibited by metHb A_0, horse metHb, and metHb Deer Lodge is that the His-β2 residues of Hb A_0 are key residues in high affinity copper-binding sites that are ineffective in electron exchange with the heme.

Although there are theoretically two high affinity copper-binding sites per tetramer (0.5 copper per heme) in Hb A_0, significant enhancement of its reduction rate under CO occurs at Cu(II) levels above 0.25 copper:heme, indicating a distribution of copper to the sites responsible for CO/H_2O-driven reduction before all “high affinity” sites are filled. A similar copper concentration dependence was reported by Louro and co-workers (19) in their studies of copper-induced oxidation of the β chains of Hb.

Sample treatment can alter the time courses of CO-driven reduction by altering the distribution or access of copper to the “effective” copper-binding sites. As illustrated in Fig. 3, significant inhibition of the copper effect occurs when copper-binding...
Copper-enhanced Reduction of MetHb under CO

| Heme protein Fe(III) source | Experimental conditions | Ratio of copper:heme | Apparent rate constants (1/s) | k<sub>1</sub> | % fast | k<sub>2</sub> |
|-----------------------------|-------------------------|---------------------|-----------------------------|-----------|-------|---------|
| HbA<sub>a</sub>             | Control                 | 0                   | 0.003                       |           |       |         |
| α2β2, β2α                    | Control                 | 0.45<sup>a</sup>    | 0.26                        | 38        | 0.009 |         |
| α2β2, β2α                    | Control                 | 0.5<sup>a,b</sup>   | 0.28                        | 49        | 0.007 |         |
| HbA<sub>b</sub>             | Control                 | 0.25                | <5                          | 0.001     |       |         |
| β of HbA<sub>b</sub>         | Control, 20 °C          | 0                   | 0.005                       |           |       |         |
| NEM of HbA<sub>b</sub>       | Control                 | 0.5                 | 0.009                       |           |       |         |
| 20 °C                       | Control                 | 0.5                 | <5                          | 0.009     |       |         |
| + IHP, 32 °C                | Control                 | 0.5                 | 0.006                       |           |       |         |
| CPA of HbA<sub>b</sub>       | Control                 | 0.5                 | <5                          | 0.016     |       |         |
| + IHP                       | 0.5                     | 0.13                | 17                          | 0.008     |       |         |
| Hb Deer Lodge               | Control                 | 0.25                | 0.61                        | 15        | 0.005 |         |
| Horse Hb                    | Control                 | 0.3                 | 0.16                        | 31        | 0.003 |         |

<sup>a</sup> Chelex treated after 5:1 copper.
<sup>b</sup> Data from Fig. 1.
<sup>c</sup> See text.

Sites near the β83 sulfhydryl groups are blocked by treatment with N-ethylmaleimide (NEM). NEM treatment also results in a slight increase in the rate of reduction of the copper-free control. Variable time courses, presumably reflecting variable distribution of Cu(II) among the binding sites, can also be achieved by variations in sample handling, as illustrated by the two lower (faster) time courses of Fig. 3 where samples gave significantly different time courses of reduction as a consequence of different sample handling procedures.

The rates and time courses (within experimental variability) of CO/H<sub>2</sub>O-driven reduction are unchanged when Cu(II)-metHb complexes are incubated under progressively lower CO levels over a range of solution concentrations from 1 to 0.05 mM CO. Within this range of CO concentrations, good isosbestic points are typically observed between the met and CO derivatives of Hb, and the decreases in absorbance at 406 nm (metHb disappearance) and the increases in absorbance at 419 nm (CO-Hb appearance) have the same time dependence. The CO concentration independence and the wavelength independence of the process together indicate that the Fe(III) (met) and CO-Hb forms are the only significantly populated species and that something other than CO concentration is rate-limiting in the transition toward CO-Hb. Intermediate species are, however, apparent when incubation is carried out at low CO levels and IHP is present. Under progressively lower CO concentrations (<0.25 mM) in the presence of IHP, the time courses observed at 406 and 419 nm become increasingly distinct, and the isosbestic point between met and CO-Hb is not maintained.

We examined the CO/H<sub>2</sub>O-driven reduction of Cu(II) complexes of metHb A<sub>a</sub> under a number of experimental conditions, with results summarized in Table I. Many of our experiments were performed at relatively low protein concentration (10–15 μM in heme) where the concentration of dimeric forms of metHb would be appreciable. Remarkably, there are no systematic differences in reduction rates when the heme concentration is varied over the range of 10 to 500 μM. This is evidence that the internal electron transfer responsible for heme reduction is governed by tertiary rather than quaternary conformational states. This conclusion was also supported by studies reported below, using T-state stabilization by IHP and R-state stabilization by digestion with carboxypeptidase A.

Strong allosteric effectors such as inositol hexaphosphate (IHP) can shift R-state metHb toward the T-state (20). IHP addition results in an approximately 2-fold decrease in the half-time for the rapid (β chain) phase of reduction at pH 8.3. This suggested that copper-enhanced reduction of metHb might be slower in its T-state conformation. We then looked for possible pH effects on the IHP-induced changes in rate, since IHP stabilization of the T-state would be expected to be more evident at low pH. However, the rate of reduction in the presence of IHP is appreciably faster at pH 7 than at pH 8.3. The pH shift alone caused only a minor alteration in reduction rate. Lower rates of reduction would have occurred at pH 7 if the IHP effect was due to a T-state shift. The altered efficacy of Cu(II) in the presence of IHP thus does not follow the pattern expected for R ↔ T shifts of quaternary conformation.

We further examined the possible effect of Hb conformation on the reductive process by monitoring the CO/H<sub>2</sub>O-driven reduction of metHb after modification by digestion with carboxypeptidase A (HbCPA). This digestion removes the C-terminal His and Tyr residues of the β chains, strongly stabilizes the R-state conformation, and inhibits the IHP-induced shift toward the T-state (21, 22). HbCPA controls behaved like the undigested protein, so that in the absence of copper metHbCPA became reduced under CO with an apparent rate constant similar to that of metHb A<sub>a</sub> (k<sub>2</sub> = 0.0061 s<sup>-1</sup>). In the presence of Cu(II) at 0.5 copper:heme, half-reduction of metHbCPA was enhanced in rate over that of the copper-free controls. The rate enhancement was relatively small, so that β chains became reduced at a rate about half that characteristic of the undigested protein. Constraining the protein to the R-state by this modification thus caused a decreased rate of reduction. Addition of IHP increased the rate to somewhat greater than observed for undigested protein in the absence of IHP, with a half-time for reduction of the fast phase of about 1 h. The rate increase brought about by IHP on the reduction of metHbCPA is in marked contrast to results obtained with metHb A<sub>a</sub>, where the reductive process is slowed by the presence of IHP (see Table I). We conclude from these comparative studies that the rate of reduction is governed by tertiary effects on the internal electron transfer pathway and not by the pattern of T ↔ R shifts of quaternary conformation that govern oxygen binding.

The rate of the reductive process is clearly affected by the level of Cu(II) that is bound at a site that is effective in internal electron transfer. In our studies with metHbCPA, we noted that the magnitude of the fast phase of reduction is not as great as that for metHb A<sub>a</sub> at a comparable copper:heme ratio. The reduced magnitude of the fast phase of reduction is probably due to sequestration of copper in non-reactive sites. The magnitude of the fast phase of reduction after addition of the allosteric effector IHP is not reduced, suggesting that IHP binding alters the copper distribution toward sites that are effective in electron transfer.

Visible spectroscopy using the Cu(I)-dependent absorption changes of bathocuproin at 483 nm (23) demonstrated that...
FIG. 2. Effect of Cu(II) on time courses of CO/H₂O-driven reduction of metHb A₀ and metHb Deer Lodge. The fraction of oxidized Hb at the indicated times was calculated independently from data collected at two wavelengths (such as 406 and 419 nm for measurements in the Soret region) and then averaged. The optical path lengths of cuvettes were selected to allow for readings in the linear range of spectrophotometers used. Samples were in 0.01 or 0.05 M Tris-HCl at pH 8.3 and were incubated under 1 atm CO at 25 °C. Open circles, copper-free metHb A₀; closed circles, metHb A₀ (0.17 mM in heme) with a ratio of 0.25 copper to heme; closed squares, metHb Deer Lodge (0.51 mM in heme) with a ratio of 0.25 copper to heme; closed diamonds, metHb A₀ (0.02 mM in heme) with a ratio of 0.38 copper to heme; closed triangles, metHb A₀ (0.02 mM in heme) with a ratio of 0.74 copper to heme.

FIG. 3. Time courses of CO/H₂O-driven reduction of Cu(II)-metHb. Hb samples were incubated under 1 atm CO in 0.05 M Tris-HCl buffer, pH 8.3, at 25 °C under varied experimental conditions. Open triangles, copper-free metHb A₀ treated with NEM; closed triangles, NEM-treated metHb A₀ after addition of 0.5 copper to heme; closed circles, metHb A₀ with a ratio of 0.6 copper to heme (established by exposure of metHb to Cu(II) at a ratio of 5 coppers to heme, followed by Chelex treatment prior to incubation under CO); closed diamonds, metHb A₀ with a ratio of 0.38 copper to heme (established by direct Cu(II) addition to deoxygenated metHb prior to CO incubation).
significant amounts of Cu(II) are reduced to Cu(I) when protein-free CuSO₄ is incubated under CO. EPR measurements described below confirmed that incubation of Cu(II) under CO led to its reduction. Since Cu(I) is a potential heme reductant, we investigated the possibility that addition of Cu(I) would bring about the reduction of \( b \) chain hemes in Hb. For this study thoroughly degassed solutions of Cu(I) as CuCl at a ratio of 0.5 copper:heme were added to deoxygenated metHb in tonometers. No appreciable spectral shifts were observed. We then added 1 atm CO. There was no spectral indication of heme reduction after 24 h (data not shown), indicating that under these conditions, free Cu(I) does not directly contribute to reduction of metHb. Exposure to air gave Cu(II)-metHb complexes equivalent to ones prepared by our normal procedures that were susceptible to CO/H₂O-driven reduction.

EPR Studies of the CO/H₂O-driven Reduction of Cu(II)-MetHb Complexes—Experiments described here demonstrate the crucial role of copper in the mechanism of reduction of Cu(II)-metHb complexes. As reported below, both copper and heme reduction occur to an appreciable extent when Cu(II)-metHb complexes are incubated under CO. We also found that maintaining various preparations of aqueous Cu(II) (in the absence of protein) overnight under 1 atm CO leads to the disappearance of at least 20% of the initial Cu(II) EPR signal as a result of CO/H₂O-driven Cu(II) reduction. Thus, Cu(II) in the absence of heme is susceptible to reduction under CO.

The EPR spectrum of human metHb \( A_0 \) at pH 8.3 typically contains two distinct sets of signals, as shown in Fig. 4A, that arise from high spin ferric hemes (\( g = 5.9 \) and 2.0) and low spin ferric hemes (\( g_{xy} = 2.59, 2.18, \) and 1.83). These two kinds of signals correspond to situations in which the sixth ligand to iron is H₂O and OH⁻ respectively (24, 25).

EPR signals, attributable to Cu(II) in a Cu(II)-metHb complex, appear following the addition of 0.5 eq (per heme) of CuSO₄ to anaerobic solutions of metHb, as shown in Fig. 4B. These signals were first observed by Bemski and co-workers (26, 27) and have since been confirmed by several other groups for the complex of Cu(II) with Hb \( A_0 \). Within our experimental uncertainty, the EPR signals we observed after Cu(II) addition to oxidized Hb \( A_0 \) are indistinguishable from those previously reported where Cu(II) was added to the deoxy derivative. Fig. 5A, showing enlarged details of Fig. 4B, illustrates the 9-line superhyperfine pattern indicative of square-planar coordination of the Cu(II) by four electronically equivalent nitrogen ligands (27, 28). As reported previously by Antholine et al. (29), this pattern is the spectral signature of the high affinity Cu(II) binding site (site 3 in Antholine’s terminology), located near the N terminus and His-b₂ of each \( b \) chain. Not all Cu(II) sites are occupied at a ratio of copper:heme of 0.5:1, and most of the Cu(II) binds preferentially to the high affinity site. Consequently the distinctive signature of Cu(II) at the lower affinity site or near the Cys-b93 (site 2 in Antholine’s terminology) is not readily observed in the spectrum. Fig. 5B shows that this superhyperfine pattern is absent in Hb Deer Lodge which lacks His-b₂.

We fortuitously prepared an uncomplexed metHb sample that contained no measurable low spin component. By using this sample as an integration standard for the \( g = 6 \) region, we
determined that the heme groups of Fig. 4A were at least 50% high spin. Upon complexation with Cu(II) (Fig. 4B), the heme groups were 30% (±10%) high spin. Apparently all the added Cu(II) contributed to the intensities of resulting spectra so that there was no clear evidence for coupling between the ferric hemes and Cu(II), although we would not have been able to detect low levels (10%, or less) of Cu(I) or anti-ferromagnetically coupled (and thus EPR silent) species.

Fig. 4C shows that there is no significant change in the EPR spectrum if the Cu(II)-metHb A0 complex is incubated at room temperature under 1 atm CO for 1 h prior to freezing in the EPR tube. However, if the CO incubation is continued overnight, some heme and copper reduction takes place as shown in Fig. 4D. In this case about 20% of the low spin heme signal and about 20% of the Cu(II) signal disappears (cf. Fig. 4, C and D) as a result of reduction. This result is consistent with spectral changes indicative of heme reduction observed by visible spectroscopy (see previous section).

Fig. 6A shows that the EPR spectrum of metHb Deer Lodge prepared at pH 8.3 contains the same high spin and low spin ferric heme signals as found for metHb A0, but the ratio of these two components differs in the two hemolysins. Typical Hb Deer Lodge samples contained only 15% (±5%) of the high spin component. Addition of 0.5 eq (per heme) of CuSO4 leads to the spectrum of Fig. 6B. Due to the overlapping low spin ferric heme signals in the present spectra, we cannot determine the EPR parameters for the Cu(II) signal in the Hb Deer Lodge complex with high precision, but we note that, within our experimental uncertainty, they are indistinguishable from those previously reported for a “low affinity” Cu(II)-binding site in deoxhemoglobin A0 that is associated with the Cys-$\beta$3 residue, located less than 10 Å from the $\beta$ chain heme (30). It is this copper-binding site that has been associated with internal electron transfer in copper-induced oxidation of heme (19).

Moreover, the Cu(II)-metHb complex of Hb Deer Lodge does not show the 9-line superhyperfine pattern associated with Cu(II) binding at or near the His-β2, indicating that this site is absent (Fig. 5B). The Cu(II) complexes of other Hbs without a His-β2, such as horse hemoglobin (1) and cat hemoglobin (30), have also been shown to give EPR spectra that lack the 9-line superhyperfine pattern.

Incubation of the Cu(II)-metHb Deer Lodge complex under CO leads to strikingly different results compared with the Cu(II)-metHb A0 complex. After 1 h there is 20–30% reduction of the hemes and 30–40% reduction of the copper, as shown in Fig. 6C. After 17 h, some 60–70% of both hemes and copper have been reduced as shown in Fig. 6D. The structural explanation for the greater extent of reduction in this case is that copper is more available at the effective site for electron transfer in Hb Deer Lodge, since its β2His → Arg substitution eliminates the high affinity copper-binding site found in Hb A0.

This result of heme and copper reduction mirrors previous reports on heme oxidation (1) where more copper is required for oxidation of the β chains in Hb A0 than in Hbs like Hb Deer Lodge that lack His-β2 residues.

The site of copper binding clearly determines its ability to participate in the CO/H2O-driven reduction reaction, as evidenced by the much slower rate of reduction of Hb A0 relative to Hb Deer Lodge ($\beta$2His → Arg) at low copper levels (Figs. 4 and 6). Copper is clearly a partner in CO/H2O-driven reduction of the Cu(II)-metHb complexes since both heme and copper reduction occurs for Cu(II)-metHb complexes incubated under CO.

**DISCUSSION**

We previously demonstrated that a reaction similar to the water-gas shift reaction, driven by the oxidation of CO/H2O to CO2 + 2H+ + 2e− (Equation 1), can drive the reduction of a number of heme proteins, with free energy changes dependent on the type of heme protein and the experimental conditions (9). The results presented here show that Cu(II) addition enhances the rate of reduction of the β chains of metHb under CO, implicating β heme and Cu(II) as acceptors of the two electrons generated by Equation 1. The EPR measurements show reduction of Fe(III) and Cu(II) when metHb is incubated under CO in the presence of Cu(II). The β chain hemes in Cu(II)-metHb assemblies exhibit rates of CO/H2O-driven reduction that are roughly 1000-fold faster than those for Hb in the absence of Cu(II). The fastest β chain reductions we observed have half-times of about 1 h, only slightly less rapid than previously reported for the CO/H2O-driven reduction of heme α and copper of the oxygen-binding site of cytochrome c oxidase (9).

Clearly, the heme and copper together enhance the rate of CO/H2O oxidation that drives the reductive process in Cu(II)-metHb complexes. The rate enhancement makes use of an internal pathway of electronic communication in the Cu(II)-metHb complexes that is functionally similar to but structurally distinct from that of heme α and copper in cytochrome c oxidase. The differences are evident in the EPR spectra arising from these two systems, where the Cu(II) in the binuclear site of the oxidase, but not in the Cu(II)-metHb complexes, is EPR silent (31) due to an anti-ferromagnetic exchange interaction with heme α (Ref. 32 and references therein).

Scheme A and Scheme B as described below summarize two reaction sequences that are consistent with the available data. They are not mutually exclusive and differ primarily in how CO...
oxidation is coupled to the electron transfer reaction.

Scheme A invokes the generation of an intermediate as a result of CO attack on a metal hydroxide. This process limits the reaction rate at low CO concentration (less than 0.05 mM) and achieves saturation and becomes CO independent above 0.05 mM. Subsequently, as CO is released, an intermediate species is formed with two-electron reducing potential that is equivalent to a transient species with the reducing potential of a metal hydride (shown in quotes). A subsequent internal electron transfer process is postulated to be the rate-limiting step at high CO pressure, supported by the observed Cu(II) dependence of the reaction rate. In Hb, Fe(III) would serve as M1 and Cu(II) bound at or near Cys-93 would serve as M2. Cu(II) bound at or near Cys-93 in Hb is less likely to serve as M1 because this less hydrophobic environment would be less favorable for generation and distribution of electrons from the postulated intermediate to heme in the active site. The presence of a second electron acceptor, M2, in electronic communication with M1 is ultimately necessary for facile reduction of both metals and release of protons.

Under some conditions the partial oxidation of Fe(III) by Cu(II) may also play a role in utilizing the electrons provided by CO/H2O oxidation (Scheme B). Since Cu(II) can oxidize the heme Fe(III) of the chains of Hb (1), it is possible that the internal electron exchange pathway can allow for some population of a Fe(IV)-Cu(I) redox pair.

\[
\begin{align*}
\text{Fe(III)} + \text{Cu(II)} & \quad \leftrightarrow \quad \text{Fe(IV)} + \text{Cu(I)} \\
\text{CO/H}_2\text{O} + \text{Fe(IV)} & \quad \rightarrow \quad \text{CO}_2 + \text{Fe(III)} + 2\text{H}^+ \\
2\text{CO} + \text{Fe(II)} + \text{Cu(I)} & \quad \rightarrow \quad \text{Fe(II)}\text{CO} + \text{Cu(I)}\text{CO}
\end{align*}
\]

**Scheme B**

The lack of accurate redox potentials for these redox pairs as they exist in the protein prevents us from calculating the extent of such a process. However, as indicated by our results, as much as 10% of metHb is converted to a quickly CO-reducible form of Hb after 30 min of exposure to Cu(II) at a copper:heme ratio of 0.7. The absorption spectrum of this altered Hb form resembles that of ferriyl Hb. Moreover, we found that the treatment of Hb with H2O2, known to promote ferriyl Hb production, generates a state that is also readily reducible by CO, similar to the quickly CO-reducible fraction observed for Cu(II)-treated metHb. Creation of an oxyferriyl heme intermediate is known to occur in peroxidase-catalyzed reactions, and is likely to occur in Hb when it mimics such chemistry (33). Furthermore, under conditions where the CO/H2O-driven reduction reaction does not show good isosbestic points between metHb and CO-bound forms of Hb, the inferred intermediates in the spectral transition have features that appear similar to those published for ferryl Hb (33).

This report deals exclusively with the Cu(II)-induced enhancement of the reduction of metHb under CO that is NEM-sensitive and utilizes an internal electron transfer pathway. Comparative studies revealed that the rate and extent of reduction of the Cu(II)-metHb complexes under CO are limited by the availability of Cu(II) at a site that allows for electron exchange with the β chain hemes. The sulphydryl group at Cys-93 is implicated in the process since Cu(II)-induced rate increases are inhibited when this residue is blocked by treatment with NEM. The β chain hemes are separated from Cys-93 by only a single residue, the proximal histidine. Therefore, this histidine can be presumed to be part of the pathway between iron and copper bound at or near Cys-93. It may constitute the entire pathway, since an effective electron transfer pathway would be created if Cu(II) bound at or near the Cys-93 rearranged its binding site by displacing the N2 proton of the imidazolate of the proximal histidine. This rearrangement would create an imidazolate bridge between the heme iron and the bound copper and provide a short internal pathway for electron transfer. Our data are unable to support or refute an imidazolate bridge as the sole member of an internal electron transfer pathway.

We tested the hypothesis that metHb reduction proceeds via CO reduction of Cu(II), followed by Cu(I) coordination of the sulphydryl of Cys-93, followed by electron transfer to the heme group. However, experiments conducted with Cu(I) addition to metHb (with 0.5 Cu(I)/heme) showed no detectable heme reduction before or after incubation under 1 atm CO. It is possible that strong Cu(I) binding to Cys-93 prevents Cu(I) from occupying the site where electron transfer is effective. There is some experimental basis for this possibility, since it has been shown that when Cu(II) is added to oxyHb, the Cu(II) changes its position with respect to Cys-93 prior to Cu(II)-induced oxidation of the β chains (30). The alternative explanation is that Cu(I) does not promote heme reduction because of unfavorable energetics.

The rate of the process whereby Cu(II)-metHb becomes reduced when incubated under CO is affected by Hb type, by digestion with carboxypeptidase A, by IHP binding, and by reaction temperature. We investigated the possibility that these variations were due to alterations in allosteric equilibrium between quaternary conformations of high and low oxygen affinity. Although unable to bind oxygen, metHb is also switchable between these conformational states (20, 24). The rates observed for CO/H2O-driven reduction of the Cu(II)-metHb complexes did not, however, correlate with alterations in the R ↔ T equilibrium. Furthermore, the conformational equilibrium is very sensitive to pH, but the CO/H2O-driven reduction of Cu(II)-metHb complexes shows no appreciable pH sensitivity. No systematic changes were noted between experiments conducted at high concentration (500 μM in heme) where the percentage of dimers was confirmed to be vanishingly small and low concentration (10 μM in heme) where the percentage of dimers was appreciable. We conclude that while the process is sensitive to protein conformation, the quaternary R ↔ T equilibrium is not the primary determining factor that establishes the rate or extent of this reductive process. The internal pathway may, however, be altered by structural changes induced by IHP binding, such as the movement of the A helices of the β chains toward the center of the molecule (34).

There is an increasing awareness that novel internal electron exchange pathways in proteins can be of physiological significance. Copper toxicity associated with Hb oxidation (1)
and CO/H₂O-driven reduction of metHb demonstrated in this paper are cases in point. We speculate that CO and NO may have parallel biological functions that are dependent upon internal electron exchange pathways in proteins. The pathway shown to be utilized in CO/H₂O-driven reduction of metHb may also be involved in NO-driven reduction of metHb. We have shown, for example, that the rate of NO-driven reduction of metHb is, like CO/H₂O-driven reduction, dependent on the nature of the Hb being reduced and, also as shown for CO/H₂O-driven reduction, the rate of the process is independent of NO concentration over a wide range (35).

The importance of SH groups at β93 is indicated by the fact that they are highly conserved in mammalian Hbs. Our studies emphasize the role of these SH groups in the internal electron exchange pathway that links heme and SH groups and can lead to regeneration of active (Fe(II)) heme. In earlier studies, it was suggested that these SH groups are protective against Hb oxidation (1–3). Other studies have shown that the interactions of NO with these SH groups are critical for control of blood pressure and that formation of SNO-Hb is dependent upon the concentration over a wide range (35).

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