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Kinetics and Mechanisms of Metal Reduction by Hemoglobin. 1. Reduction of Iron(III) Complexes

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The kinetics of the reduction of Fe(III) complexes by deoxyhemoglobin present in equilibrium with oxyhemoglobin can be described by two possible electron-transfer mechanisms. The first is a simple outer-sphere mechanism over the exposed heme edge. The second is a site-specific mechanism that requires metal–chelate binding to the protein to form a ternary complex prior to electron transfer. The reduction potential, stability constant, and the nature of the electron-transfer orbitals of the metal–chelate influence the rate and mechanism of reduction. The reduction of iron(III)–nitrilotriacetate (NTA) by hemoglobin involves the site-specific mechanism. Ni^{II}·NTA and Zn^{II}·NTA compete with Fe^{III}·NTA for the binding site. Ionic strength and pH studies indicate that this site is positively charged. The reaction follows second-order kinetics with $k = 3.7\ M^{-1}\ s^{-1}$ (pH 7.0, $\mu = 0.14$ M, 37 °C). The electron-transfer rate constant is $6.5\ s^{-1}$. The equilibrium constant for the metal–chelate–hemoglobin complex is $3.6 \times 10^2\ M^{-1}$.

The study of electron-transfer reactions between metals and heme proteins has an extensive history.[1-3] The present state of the field of long-range electron transfer in heme proteins has been intensively reviewed by Mayo et al.[4] The biological importance of these reactions in iron metabolism has only recently been recognized.[5] Under aerobic conditions, Fe(III) is the predominant form of unassimilated iron. However, essential biochemical functions require the reduced state of the metal, Fe(II), including the synthesis of heme[6] and non-heme iron–sulfur proteins[7] and the incorporation and mobilization of ferritin iron.[8]

In vitro studies on the release of ferritin iron have focused on the reduction of Fe(III) by intracellular reducing agents such as $FMNH_2$ and $FADH_2$, cysteine, glutathione, ascorbic acid, superoxide, and xanthine oxidase.[9,10] Of these reductants, only the reduced flavins mobilize significant amounts of ferritin iron; however, this system requires near-anaerobic conditions.

The role of hemoglobin (Hb) as an aerobic biological reductant of iron was suggested by Egyed et al.,[11] who observed that Hb mediated the transfer of Fe(III) from transferrin to 2,2′-bipyridine (bpy), an Fe(II) chelator. This hypothesis was later confirmed by using rabbit reticulocytes and erythrocytes where intracellular Fe(II) was maintained only in the presence of functional Hb.[12] Our laboratory has further characterized the redox reaction to involve deoxyHb in the generalized scheme of electron transfer[5]

$$HbO_2 \rightleftarrows Hb + O_2$$

$$Hb + Fe(III)\text{–chelate} \rightarrow MetHb + Fe(II)\text{–chelate}$$

The nature of the metal–chelate greatly influences the rate of reduction; ATP is the most effective biological Fe(III) chelator tested.

Myoglobin (Mb) and cytochrome b_5 also reduce metal complexes. The reduction kinetics of Fe(III) and Cu(II) complexes by Mb has recently been described.[13] As with Hb, deoxyMb is the redox-active species. Two outer-sphere electron-transfer mechanisms are involved in the reduction of metals by Mb: (1) a simple outer-sphere mechanism over the exposed heme edge for the reduction of Fe(III) and (2) a site-specific mechanism requiring binding of the metal to histidines prior to electron transfer for the reduction of Cu(II). The reduction potential and the stability constant of the metal–chelate determine the mechanism of reduction. In the site-specific mechanism, the dissociation of at least one of the metal ligands is required for binding to the protein; the formation constant of the ternary complex is inversely proportional to the stability constant. The simple outer-sphere mechanism is not influenced by the strength of the metal–ligand bond. In both mechanisms, the reduction potential of the ternary complex or the metal–chelate influences the rate of reduction. Rifkind et al.[14,15] demonstrated that the reduction of Cu(II) by Hb involves Cys β-93 in a site-specific mechanism.

This investigation further describes the kinetics and mechanisms of the aerobic reduction of Fe(III) chelates by Hb.

Materials and Methods

Reagents. Hb was isolated from fresh human red cells by toluene extraction[16] and dialyzed against *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer, pH 7.0, at 4 °C. All other reagents were purchased from Sigma. HEPES buffer contained 20 mM Na-HEPES and 140 mM NaCl, and the pH was adjusted to the desired value with solid $NaHCO_3$ or 1 N HCl. The ionic strength of the buffer was adjusted by the addition of NaCl.

Stock solutions of chelators [NTA, citrate, EDTA, 2,3-diphosphoglyceric acid (2,3-DPG), inorganic pyrophosphate (PP_i), and ATP] and metal salts [Fe(III), Cu(II), Mn(II), Ni(II), and Zn(II)] were prepared in distilled water. Working solutions of the metal–chelate, 5 mM, were prepared by adding the metal salt to the chelator under continuous stirring. The solution was brought to volume with distilled water. The pH of the Fe(III) complexes was adjusted to 7.0 with solid $NaHCO_3$ before bringing to volume. The pH of the other metal chelates was not adjusted due to the precipitation of concentrated solutions of the complexes at neutral pH. Fe(III) complexes with 2,3-DPG, PP_i, and ATP were prepared immediately prior to the experiment.

Sulfhydryl groups on Hb were modified with *N*-ethylmaleimide (NEM). A 10 mM stock solution of NEM was prepared in distilled water. NEM was added to an HbO_2 solution, pH 7.0, at the molar ratio 3:1 NEM:HbO_2 (4 hemes). The solution was incubated in the dark for 2 h at 37 °C. Unreacted NEM was removed on a Sephadex G-25 column (2 × 25 cm) equilibrated with HEPES buffer, pH 7.0. The most concentrated fractions of modified Hb were pooled and used in the experiments. At this low NEM:HbO_2 ratio, Cys β-93 is selectively modified.[17]

Measurements. A typical test solution contained 100 μM heme, 500 μM Fe(III)–chelate, and 2 mM bpy in HEPES buffer, pH 7.0. The

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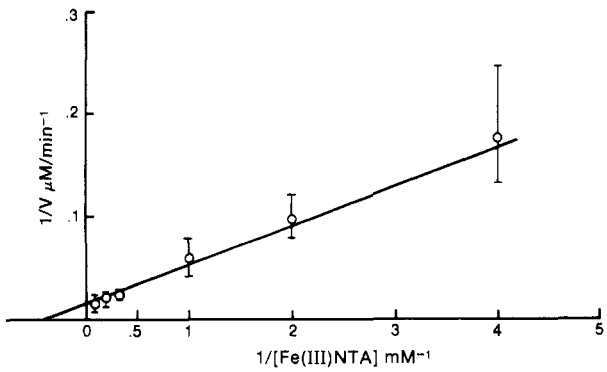

Figure 1. Double-reciprocal plot of Fe^{III}·NTA reduction. The kinetic parameters K_m and V_{max} were determined by Lineweaver–Burk analysis of the initial velocities of MetHb formation.

reaction was carried out at 37 ± 0.1 °C in a water bath. The Fe(III)–chelate and bpy in the buffer were preincubated at 37 °C for 10 min; the reaction was initiated by addition of Hb. Aliquots of test solutions, 1.0 mL, were added at specified time intervals to 0.5 mL of cold 7.5% trichloroacetic acid to precipitate the protein. The solution was centrifuged at 4 °C at 5000 rpm for 5 min, and the absorbance of the supernatant was measured at 520 nm, $\epsilon = 8600\ M^{-1}\ cm^{-1}$, to determine Fe^{II}·3bpy concentration. Alternatively, the MetHb concentration was measured by the absorbance of the test solution at 630 nm in a temperature-controlled (±0.1 °C) spectrophotometer. The spectral properties of MetHb are pH-dependent. The following extinction coefficients were used to quantitate MetHb concentration at 630 nm:

pH 6: $\epsilon = 3940\ M^{-1}\ cm^{-1}$

pH 7: $\epsilon = 3760\ M^{-1}\ cm^{-1}$

pH 8: $\epsilon = 2930\ M^{-1}\ cm^{-1}$

All spectrophotometric measurements were performed on a Hitachi 110A dual-beam UV–vis spectrophotometer.

The initial rates of MetHb formation were determined at Fe^{III}·NTA concentrations ranging from 50 μM to 20 mM. The MetHb concentration was measured at 10-s intervals for 2 min and at 30-s intervals for an additional 3 min. The plot of A_{630} vs time was linear over the initial 60 s and represents a pseudo-first-order reaction. The initial rate (velocity) of Fe^{III}·NTA reduction is reported in μM/min.

Results

Initial Rates of Fe^{III}·NTA Reduction. Preliminary studies on the initial rate of Fe^{III}·NTA reduction by Hb[18] revealed hyperbolic kinetics with maximum velocity achieved at 7 mM Fe^{III}·NTA. Detailed kinetic analysis of the initial reduction rates, based on five experiments, by the Lineweaver–Burk plot is presented in Figure 1. Linear regression analysis reveals a K_m value of 2.4 mM and a V_{max} value of 60 μM/min ($r = 0.943$). Saturation kinetics are characteristic of a site-specific outer-sphere mechanism. However, a simple outer-sphere mechanism can also display saturation, since at high Fe^{III}·NTA concentrations, the rate-limiting reaction is the formation of Hb.

Second-Order Rate Constant. The second-order rate constant (k_{obsd}) for the overall redox reaction was determined by using the initial velocity (v) data of MetHb formation in which

$$k_{obsd} = v/[HbO_2]_i[Fe^{III}\cdot NTA]_i$$

and $[HbO_2] = [heme]$. At Fe^{III}·NTA concentrations ranging from 50 to 250 μM, the initial second-order rate constant appears to be constant at 3.7 $M^{-1}\ s^{-1}$ (pH 7.0, $\mu = 0.14$ M, 37 °C) (Figure 2); above 250 μM, the rate constant decreased to 0.6 $M^{-1}\ s^{-1}$ at 15 mM Fe^{III}·NTA (data not shown). The rate constant for the reduction of Fe^{III}·NTA by deoxyHb (k_{Hb}) was calculated from

$$k_{Hb} = k_{obsd}K[O_2]$$

where K = the equilibrium constant for O_2 binding to Hb = 2.5

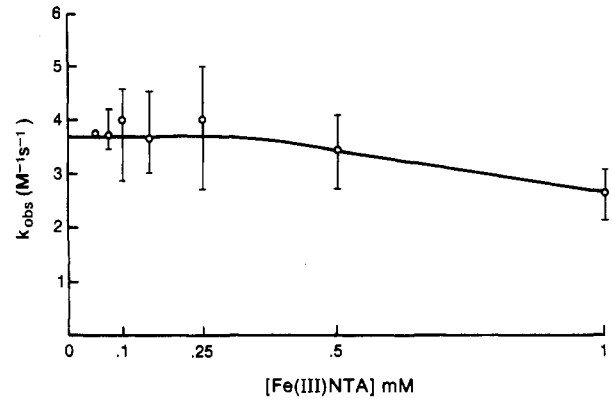

Figure 2. Second-order rate constant of Fe^{III}·NTA reduction. The second-order rate constant was determined from the initial velocities of MetHb formation at Fe^{III}·NTA concentrations ranging from 50 μM to 1 mM.

$\times 10^6\ M^{-1}$ [19] and $[O_2] = 235$ μM on the basis of the solubility of O_2(air) in Ringer's solution ($\mu = 0.154$) at 1 atm and 37 °C.[20] At lower Fe^{III}·NTA concentrations, $k_{Hb} = 2.2 \times 10^3\ M^{-1}\ s^{-1}$, indicating that deoxyHb is 3 orders of magnitude more reactive than HbO_2.

Derivation of the three outer-sphere mechanisms (two site-specific and one outer-sphere electron transfer) that may be involved in Fe^{III}·NTA reduction by Hb are presented in the Appendix. In all three mechanisms, k_{obsd} is dependent upon both the Fe^{III}·NTA and O_2 concentration. At low Fe^{III}·NTA concentrations, the O_2 term dominates, resulting in a constant k_{obsd} value; at higher concentrations, the Fe^{III}·NTA term dominates, resulting in a decrease in k_{obsd} with increasing Fe^{III}·NTA concentration (Figure 2). The kinetic data support both mechanisms (simple outer-sphere and site-specific electron transfer) in the reduction of Fe^{III}·NTA by Hb. Of the site-specific mechanisms, the formation of the ternary complex between the metal–chelate and HbO_2 prior to the release of O_2 is more likely due to equilibrium conditions that favor HbO_2 ($K_D = 4 \times 10^{-7}$ M and $k_4 = 10\ s^{-1}$; K_D and k_4 are the dissociation equilibrium constant and the dissociation rate constant for the release of the first O_2 from $Hb_4(O_2)_4$, respectively).[19]

Pseudo-First-Order Rate Constant. The pseudo-first-order rate constant of Fe^{III}·NTA reduction by Hb was determined at high Fe(III) concentrations where $V_{max} = 60$ μM/min $= 1 \times 10^{-6}$ M/s. Under these conditions, the formation of the protein–metal–chelate complex is not rate-limiting, which simplifies the site-specific mechanism

$$HbO_2\cdot Fe^{III}\cdot NTA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hb\cdot Fe^{III}\cdot NTA + O_2$$

$$Hb\cdot Fe^{III}\cdot NTA \xrightarrow{k_3} MetHb\cdot Fe^{II}\cdot NTA$$

Assuming steady-state conditions for deoxyHb

$$d[MetHb]/dt = \frac{k_1k_3[HbO_2\cdot Fe^{III}\cdot NTA]}{k_{-1}[O_2] + k_3}$$

$$k_{obsd} = \frac{k_1k_3}{k_{-1}[O_2] + k_3} \quad (1)$$

The values of k_1, k_{-1}, $[O_2]$, and k_{obsd} are

$$k_1 = 22\ s^{-1}$$ [18]

$$k_{-1} = 5.5 \times 10^7\ M^{-1}\ s^{-1}$$ [18]

$$[O_2] = 235\ \mu M$$

$$k_{obsd} = v/[HbO_2] = 1 \times 10^{-6}\ M\ s^{-1}/1 \times 10^{-4}\ M = 0.01\ s^{-1}$$

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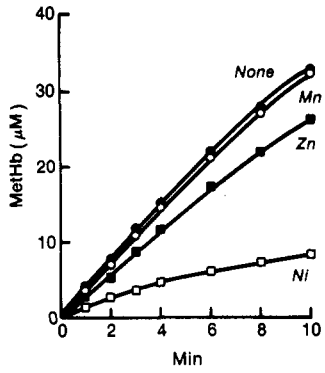

Figure 3. Effect of metals on Fe^{III}·NTA reduction. Concentrations were 100 μM heme, 100 μM Fe^{III}·NTA, and 1 mM Mn^{II}·NTA, Zn^{II}·NTA, or Ni^{II}·NTA in HEPES buffer, pH 7.0, at 37 °C.

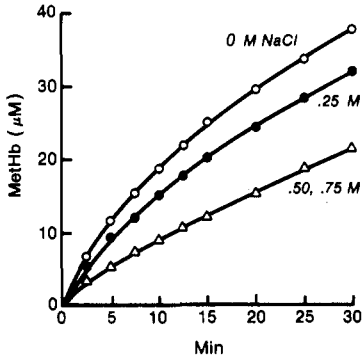

Figure 4. Effect of ionic strength on Fe^{III}·NTA reduction. The NaCl concentration of the HEPES buffer, pH 7.0, was varied to 0, 0.25, 0.50, and 0.75 M. Concentrations were 100 μM heme and 500 μM Fe^{III}·NTA at 25 °C.

When these values are substituted into (1), $k_{obsd} = 22k_3/1.3 \times 10^4 + k_3$. If $k_3 \gg 1.3 \times 10^4$, then the reaction rate is limited by the dissociation of O_2 from HbO_2, and $k_{obsd} = k_1 = 22\ s^{-1}$. If $k_3 \ll 1.3 \times 10^4$, then the electron-transfer step is rate-limiting, and $k_{obsd} = 22k_3/1.3 \times 10^4$ with the electron-transfer rate constant $k_3 = 6.5\ s^{-1}$. Since $k_{obsd} \ll k_1$ and k_3, the dissociation of O_2 from HbO_2 and the electron-transfer steps are not rate-limiting. Rather, the rapid formation of HbO_2 ($k_{-1} = 5.5 \times 10^7\ M^{-1}\ s^{-1}$) competes with the electron transfer step for deoxyHb, thereby limiting the reaction rate.

Ternary Complex Equilibrium Constant. The equilibrium constant for the ternary complex [HbO_2·Fe^{III}·NTA] was calculated from the derived rate expression

$$d[MetHb]/dt = \frac{k_1k_3K[HbO_2][Fe^{III}\cdot NTA]}{(k_{-1}[O_2] + k_3)(1 + K[Fe^{III}\cdot NTA])} \quad (2)$$

where

$$K = k_2/k_{-2} = \frac{[HbO_2\cdot Fe^{III}\cdot NTA]}{[HbO_2][Fe^{III}\cdot NTA]}$$

From the initial reduction velocity data, $dMetHb/dt = 1.68 \times 10^{-7}\ M\ s^{-1}$ at 500 μM Fe^{III}·NTA and 100 μM HbO_2. When the values are substituted into (2), the equilibrium constant $K = 3.6 \times 10^2\ M^{-1}$.

Effect of Metals on Fe(III) Reduction. Competition studies with redox-inactive metals were performed to further clarify the mechanism of electron transfer between Fe^{III}·NTA and Hb (Figure 3). Ni^{II}·NTA and, to a lesser extent, Zn^{II}·NTA inhibited Fe^{III}·NTA reduction, which supports a site-specific mechanism. Mn^{II}·NTA (1:2) had no effect on the rate of reduction.

Effect of Ionic Strength on Fe(III) Reduction. The role of charge–charge interactions between Fe^{III}·NTA and Hb was investigated by varying the ionic strength of the HEPES buffer from $\mu = 0$ to 0.75 M, pH 7.0 (Figure 4). The enhanced rate at low ionic strength suggests interaction between oppositely charged species. Fe(III) is an octahedrally coordinated metal. When chelated to NTA, Fe(III) is coordinated to one N and three carboxyl groups from NTA and two H_2O molecules. The pK_a's of the H_2O molecules are 5.0 and >8.0.[21] At pH 7, one H_2O is deprotonated to give [Fe^{III}·NTA·OH·H_2O]$^-$ with an overall negative charge. The increased rate of reduction at low salt concentrations indicates that the site(s) of Fe^{III}·NTA interaction with Hb is positively charged.

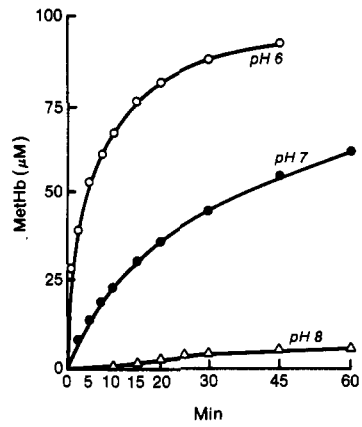

Figure 5. Effect of pH on Fe^{III}·NTA reduction. The reaction was measured at pH 6, 7, and 8 at 25 °C. Concentrations were 100 μM heme and 500 μM Fe^{III}·NTA.

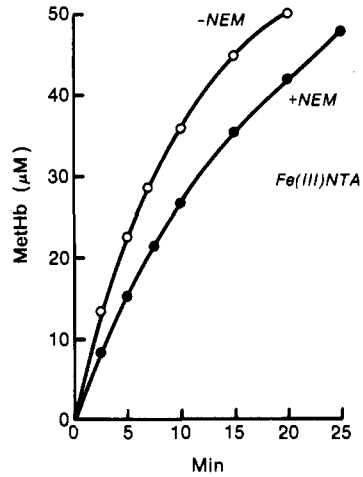

Figure 6. Effect of sulfhydryl-blocked Hb on Fe^{III}·NTA reduction. Concentrations were 100 μM normal or modified heme (3:4 NEM: hemes) and 100 μM Fe^{III}·NTA in HEPES buffer, pH 7.0, at 37 °C.

Effect of pH on Fe(III) Reduction. Further evidence for a positively charged binding site on Hb for Fe^{III}·NTA was seen in the effect of pH on the rate of reduction (Figure 5). The initial rates of Fe^{III}·NTA reduction at pH 6, 7, and 8 were 25, 3, and 0.23 μM/min, respectively, indicating approximately a 1 order of magnitude decrease in rate per unit increase. The enhancement at lower pH may result from the protonation of the metal binding site(s) on Hb, which favors charge interactions with Fe^{III}·NTA. It is also consistent with the increase in O_2 dissociation from Hb upon the binding of protons, the Bohr effect.

Effect of Sulfhydryl-Blocked Hb on Fe(III) Reduction. The reduction of Cu(II) by Hb has been demonstrated to involve Cys β-93 in a site-specific mechanism.[14,15] The role of Cys β-93 in metal reduction by Hb was tested by blocking the residue with NEM. The rate of Fe^{III}·NTA reduction was only slightly inhibited

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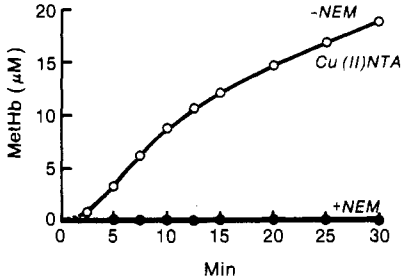

Figure 7. Effect of sulfhydryl-blocked Hb on Cu^{II}·NTA reduction. Concentrations were 100 μM normal or modified heme (3:4 NEM: hemes) and 100 μM Cu^{II}·NTA in HEPES buffer, pH 7.0, at 25 °C.

Table I. Effect of Chelation on Fe(III) Reduction[a]

chelator (Fe:chelator)	$FeII·(bpy)_3$/ μM	chelator (Fe:chelator)	$FeII·(bpy)_3$/ μM
NTA (1:1)	(100)	Citrate (1:20)	21
ATP (1:10)	110	DPG (1:10)	59
EDTA (1:1)	32	PP_i (1:10)	9

[a] Concentrations were 100 μM heme, 500 μM Fe^{III}·chelate, and 2 mM bpy in HEPES buffer, pH 7.0, at 37 °C.

Table II. Reduction Potentials and Stability Constants of Fe(III) Complexes

chelator (Fe:chelator)	redn potential		stability constant	
	$E°$/V	ref	log K	ref
CN (1:6)	+0.36	22	43.6	23
NTA (1:1)	+0.32[a]	23	15.9	23
citrate (1:1)	+0.35[a]	23	11.5	23
EDTA (1:1)	+0.12[a]	23	25.0	23
ATP (1:1)			6.6	24

[a] Calculated from the stability constants of the Fe(III) and Fe(II) complexes.

(Figure 6), whereas Cu^{II}·NTA reduction was completely inhibited (Figure 7). These results indicate that Cys β-93 is involved at the Cu(II) binding site but does not directly participate in Fe(III) reduction, although it may be in the general vicinity of interaction.

Effect of Chelators on Fe(III) Reduction. Various Fe(III) chelates (NTA, ATP, EDTA, CN, citrate, 2,3-DPG, and PP_i) were tested for their ability to act as iron sources for reduction (Table I). Of the biological chelators, only ATP was as effective as NTA. The rate of Fe^{III}·6CN reduction was very rapid with $t_{1/2}$ = 15 s at 25 °C compared to $t_{1/2}$ = 240 s at 37 °C for Fe^{III}·NTA (data not shown).

Correlation of Fe(III)–chelate reduction rates with their reduction potentials and stability constants (Table II) generally confirmed the hypothesis that a higher reduction potential and weaker metal–chelate binding promote the redox reaction through a specific binding site. For example, Fe^{III}·EDTA, with both a lower reduction potential and a higher stability constant than Fe^{III}·NTA, had a slower rate of reduction. The reduction potential of Fe^{III}·ATP has not been determined, but its weak binding (log K = 6.6) favors the formation of the ternary complex with the protein and, therefore, a moderate reduction rate.

Discussion

The reduction of Fe(III) complexes by Hb occurs by two mechanisms: (1) a simple outer-sphere mechanism over the exposed heme edge and (2) a site-specific mechanism requiring the formation of a ternary complex between the Fe(III)–chelate and protein. The reaction is influenced by the overall reduction potential and the stability constant of the metal–chelate. Of the biological chelators tested, ATP is the most effective in Fe(III) reduction.

The reduction of Fe^{III}·NTA by Hb occurs primarily through the site-specific mechanism as seen in metal competition studies, where the addition of a 10-fold excess of Ni^{II}·NTA inhibited Fe^{III}·NTA reduction by 75%. The decrease in reduction rate at increasing ionic strength and pH supports a positively charged binding site for Fe(III), possibly involving histidine(s).

The kinetics of Fe^{III}·NTA reduction by Hb follow a second-order rate law with k_{obsd} = 3.7 M^{-1} s^{-1} (pH 7.0, μ = 0.14 M, 37 °C). The electron-transfer rate constant is 6.5 s^{-1}, and the ternary complex (HbO_2·Fe^{III}·NTA) equilibrium constant is 3.6 × 10^2 M^{-1}. deoxyHb is the redox-active species. Under aerobic conditions, the rate of reduction is limited by the low value of the equilibrium dissociation constant for HbO_2 (K_D = 4 × 10^{-7} M), which favors HbO_2 formation.

Hb and Mb are similar proteins with respect to their structure and function. Both reversibly bind oxygen and participate in electron-transfer reactions. However, they differ in their redox mechanisms with metal complexes. The reduction of Fe(III) complexes by Mb occurs through the simple outer-sphere mechanism whereas Cu(II) complexes are reduced through the site-specific mechanism.[13] In contrast, Fe(III) complexes are reduced by Hb through the simple or site-specific mechanisms depending upon the stability constant of the chelate. As will be described in the following paper, the reduction of Cu(II) complexes by Hb occurs through both mechanisms, depending upon the protein subunit. The rate constant of Fe(III) reduction by Hb is 2 orders of magnitude less than that for reduction by Mb,[25-27] which is a consequence of their different reduction potentials (+0.17V for Hb vs +0.05V for Mb[28]) and the shorter separation distance between the metal–chelate and Mb in the simple outer-sphere mechanism.[29]

The well-characterized crystal structures of Hb and Mb have provided a convenient method for studying and understanding long-range electron-transfer reactions involved in biological processes such as oxidative metabolism. These reactions are also important in the aerobic reduction of metal ions required for many cellular functions. Although Hb and Mb are restricted to erythrocytes and muscle, respectively, cytochromes c and b_5, found in all aerobic cells, are also effective intracellular reductants of metals.[32]

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Appendix

Derivation of Rate Equations for Three Reduction Mechanisms. The k_{obsd} value for each mechanism was derived to determine which mechanism(s) is consistent with the experimental k_{obsd} value. The rate constants of the reaction steps reflect comparable reactions rather than the order of the steps:

k_1 = O_2 dissociation from Hb

k_2 = ternary complex formation (Hb·Fe^{III}·NTA)

k_3 = electron transfer

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(29) Long-range electron-transfer reactions (10–25 Å) at fixed distances have been the focus of recent investigations involving heme proteins.[4] The electron-transfer rate constant for Ru(II)–His 33–cytochrome c is 30 s^{-1},[30] and the rate constants for Ru–His 48–Mb are 0.019 and 0.041 s^{-1} for Ru(II) → Fe(III) and Fe(II) → Ru(III), respectively.[31] Our rate constant of 6.5 s^{-1} for the reduction of Fe^{III}·NTA by Hb indicates that the electron-transfer distance is in the same range (10–15 Å) as that examined in the ruthenated heme proteins.
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The first site-specific mechanism involves the dissociation of HbO_2 prior to Fe^{III}·NTA binding:

$$HbO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hb + O_2$$

$$Hb + Fe^{III}\cdot NTA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Hb\cdot Fe^{III}\cdot NTA$$

$$Hb\cdot Fe^{III}\cdot NTA \xrightarrow{k_3} MetHb\cdot Fe^{II}\cdot NTA$$

Assuming the deoxy forms of Hb (Hb and $Hb\cdot Fe^{III}$·NTA) are at steady-state conditions, the reaction rate is

$$d[MetHb]/dt = \frac{k_1k_2k_3[HbO_2][Fe^{III}\cdot NTA]}{(k_{-2} + k_3)(k_{-1}[O_2]) + k_2k_3[Fe^{III}\cdot NTA]}$$

with

$$k_{obsd} = \frac{k_1k_2k_3}{(k_{-2} + k_3)(k_{-1}[O_2]) + k_2k_3[Fe^{III}\cdot NTA]}$$

The second site-specific mechanism involves the binding of Fe^{III}·NTA to HbO_2 prior to the dissociation of O_2:

$$HbO_2 + Fe^{III}\cdot NTA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HbO_2\cdot Fe^{III}\cdot NTA$$

$$HbO_2\cdot Fe^{III}\cdot NTA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hb\cdot Fe^{III}\cdot NTA + O_2$$

$$Hb\cdot Fe^{III}\cdot NTA \xrightarrow{k_3} MetHb\cdot Fe^{II}\cdot NTA$$

Assuming equilibrium conditions for the ternary complex ($HbO_2\cdot Fe^{III}$·NTA) formation and steady-state conditions for the deoxyHb species ($Hb\cdot Fe^{III}$·NTA), the mechanism gives the reaction rate

$$d[MetHb]/dt = \frac{k_1k_2k_3[HbO_2]_t[Fe^{III}\cdot NTA]}{(k_{-1}[O_2] + k_3)(k_{-2} + k_2[Fe^{III}\cdot NTA])}$$

where

$$[HbO_2]_t = [HbO_2] + [HbO_2\cdot Fe^{III}\cdot NTA]$$

$$k_{obsd} = \frac{k_1k_2k_3}{(k_{-1}[O_2] + k_3)(k_{-2} + k_2[Fe^{III}\cdot NTA])}$$

A third possible mechanism of electron transfer involves a simple outer-sphere process with no metal binding to the protein:

$$HbO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hb + O_2$$

$$Hb + Fe^{III}\cdot NTA \xrightarrow{k_3} MetHb + Fe^{II}\cdot NTA$$

Again, assuming steady-state conditions for deoxyHb, the rate of MetHb formation is

$$d[MetHb]/dt = \frac{k_1k_3[HbO_2][Fe^{III}\cdot NTA]}{k_{-1}[O_2] + k_3[Fe^{III}\cdot NTA]}$$

with

$$k_{obsd} = \frac{k_1k_3}{k_{-1}[O_2] + k_3[Fe^{III}\cdot NTA]}$$

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Kinetics and Mechanisms of Metal Reduction by Hemoglobin. 2. Reduction of Copper(II) Complexes

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The kinetics of the reduction of Cu(II) complexes by hemoglobin (Hb) are consistent with two mechanisms: (1) a simple outer-sphere mechanism in which electron transfer occurs over the heme edge and (2) a site-specific mechanism requiring the formation of a metal–chelate–protein ternary complex prior to electron transfer. Both mechanisms are operative in Cu(II) reduction by the β subunits. The site-specific mechanism involves metal binding to Cys β-93. The α subunits reduce Cu(II) strictly through the simple outer-sphere mechanism. The extent of Cu(II) reduction by the α subunits is a function of the π overlap of the metal–chelate with the porphyrin ring. Copper(II)–bis(bathocuproine), with a favorable redox potential and orbital overlap, is reduced by both the α and β subunits. In contrast, aquocopper(II) and copper(II)–nitrilotriacetate (NTA) reduction is limited to the β subunits. The initial rate of Fe^{III}·NTA reduction by hemoglobin is enhanced by 50% upon addition of aquocopper(II). The K_m value for Fe^{III}·NTA decreases from 1.5 to 0.5 mM. The mechanism for this enhancement involves Cu(I) serving as an electron mediator for Fe(III) reduction.

The kinetics and mechanisms of Fe(III) and Cu(II) reduction by myoglobin (Mb),[1] hemoglobin (Hb),[18] and cytochrome b_5[2] have been recently reported by our laboratory to involve two outer-sphere mechanisms: (1) a site-specific mechanism that requires binding of the metal to the protein prior to electron transfer and (2) a simple outer-sphere mechanism in which electron transfer occurs over the exposed heme edge. The rate and mechanism of reduction are determined by the reduction potential and stability constant of the protein–metal–chelate ternary complex. The reduction of Fe(III) chelates by Mb and cytochrome b_5 occurs strictly through the simple outer-sphere mechanism. We have demonstrated that Hb reduces Fe(III) complexes through both mechanisms depending upon the stability constant and the nature of the electron-transfer orbitals of the metal–chelate. The reduction of Cu(II) complexes by Mb and cytochrome b_5 involves the site-specific mechanism. For Mb, histidines are involved at the binding site.

The outer-sphere oxidation of Hb by Cu(II) has been described by Rifkind et al.[3,4] to involve Cys β-93 through a site-specific mechanism. This residue is not directly involved in the Fe(III) binding site. In contrast to our findings with Fe(III), Rifkind found that only the β subunits are redox-active with Cu(II); oxidation of only half of the total hemes was observed. Also,

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