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Kinetic Studies of the Reduction of Hemoglobin and Myoglobin by Chromium(II)

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Abstract: Kinetic studies of the reduction of methemoglobin and metmyoglobin by chromium(II) have been performed, giving rate constants of 3.6×10^4 (pH 6.00, $\mu = 0.167$, 30.5 °C) and 5.9×10^3 M^{-1} s^{-1} (pH 6.00, $\mu = 0.167$, 30.5 °C), respectively. The reduction of hemoglobin in the presence of thiocyanate gave a third-order rate constant of 4.0×10^6 M^{-2} s^{-1} (pH 6.00, $\mu = 0.167$, 30.5 °C). The pseudo-first-order rate plots for hemoglobin were linear and showed no evidence for cooperativity. The kinetic products in the absence of thiocyanate contained protein-bound Cr(III) with a Cr(III)/protein ratio of 1.0 for myoglobin and 4.0 for hemoglobin. Kinetic products retained their respective CO and O_2 binding capacity and showed no spectral changes from their Cr(III)-free analogues. The results of the kinetic and product studies suggest possible pathways for electron transfer in this system which lend support to pathways previously deduced for electron transfer in the reduction of ferricytochrome *c*.

This paper is a continuation of our research of electron-transfer reactions in metalloproteins and metalloporphyrins.[1] The kinetics and product study of the reduction of metmyoglobin and methemoglobin with chromous are presented in this paper. Although these heme proteins are primarily reversible oxygen carriers without being involved in redox chemistry in their primary biological function and despite the elaborate safeguards to maintain the hemoglobin in the Fe(II) state, autoxidation does indeed take place. The biological oxidant, or oxidants for the unwanted reaction, is still unknown.

The amount of methemoglobin in cells is the result of the two opposing processes—a continuing oxidation of hemoglobin balanced by the reduction process. The erythrocyte requires some reductive system to keep the hemoglobin sufficiently reduced to function properly as an oxygen carrier. Such enzyme systems have been discovered and studied and these methemoglobin and metmyoglobin reductases are enzymes that keep the iron in the hemoglobin and myoglobin in the divalent state.[2-12]

This paper uses a model system to gain insight into the physiological reduction of methemoglobin and metmyoglobin. Chromium(III) is substitutionally inert;[13] thus, the product of the reaction between metmyoglobin and chromous ions yields a product which is relatively stable and amenable to chemical structural studies. Recent kinetic and mechanistic studies[14-16] of the chromous ion reduction of ferricytochrome *c* have suggested that electron transfer may proceed via the heme crevice. Chromium(III) is bound to the cytochrome molecule[15] in the area of the heme crevices, and electron transfer involving tyrosine-67 was suggested.[16] This paper reports data indicating the probable location of the inert Cr(III) in the Cr(III)-myoglobin complex and, with kinetic data, considers mechanisms of electron transfer in hemoglobin and myoglobin and the relationship of these mechanisms to those suggested for the chromous ion reduction of ferricytochrome *c*.

Experimental Section

Sigma equine skeletal muscle myoglobin (type I) and Miles horse hemoglobin, twice crystallized, were used without further purification.

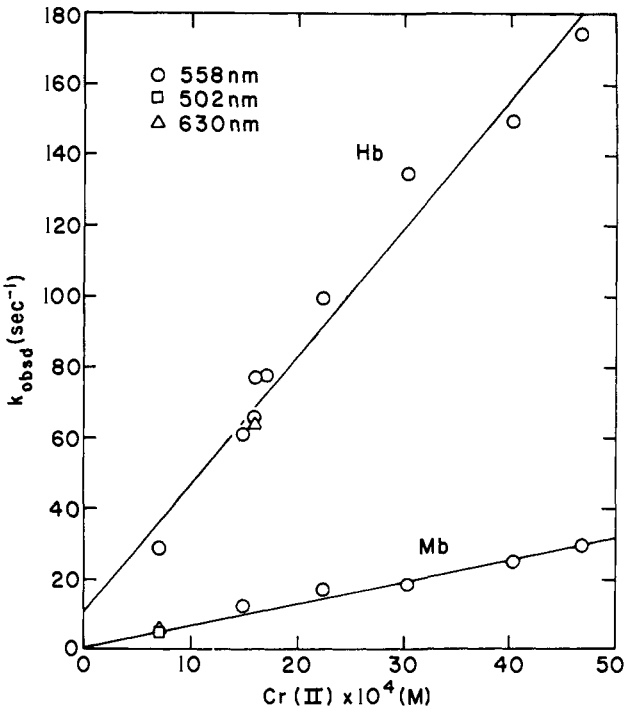

Figure 1. Plot of k_{obsd} vs. [Cr(II)] for the reactions of hemoglobin (Hb) and myoglobin (Mb) with chromium(II): 30.5 °C; pH 6.00; μ = 0.167.

The proteins were checked for complete oxidation prior to reduction and found to be completely in the oxidized form. Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone was obtained from Worthington Biochemical Co. Aminopeptidase M was from Henly and Co. of New York, N.Y. Sephadex G-25 superfine was from Pharmacia. Dowex 50W-X8 resin was from Bio-Rad, Richmond, Calif. ICN, Irvine, Calif., was the source of the ^{51}Cr(III) used to generate ^{51}Cr(II). The ^{51}Cr(III) was obtained in a solution of 0.5 N HCl and was diluted with 0.025 N HCl 500-fold before use. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities.

Chromous solutions for kinetic runs were prepared by deoxygenating either 0.10 M $CrCl_3 \cdot 6H_2O$ or 0.10 M $Cr(ClO_4)_3 \cdot 6H_2O$[17] solutions for 0.5 h with nitrogen gas, followed by the addition of Zn(Hg). Using standard syringe techniques, an aliquot of this was then injected into a previously degassed solution of sodium cacodylate–cacodylic acid buffer of the required ionic strength and pH. The concentration of the chromous ion in the reducing solution for both kinetic and product study measurements was determined by the change in absorbance of a deoxygenated permanganate solution with and without added chromous ion [λ max = 545 nm (ϵ 2340)].[14a]

Protein solutions for kinetic runs were prepared within 2 h of use by degassing the appropriate cacodylate buffer solution followed by the addition of the protein under constant nitrogen flow. Protein solutions were stored under constant nitrogen pressure in serum-capped flasks. The buffer concentrations for kinetic runs at μ = 0.167 and μ = 1.00 M were 0.073 and 0.050 M, respectively, in cacodylate anion. Buffered solutions in the range pH 5.5–7.0 (Mb) and pH 5.5–6.0 (Hb) were used for kinetic measurements.

Rate measurements were performed using a Durrum D-110 stopped-flow spectrophotometer, and absorbance–time curves were recorded using a Tektronix RM 564 oscilloscope. Pseudo-first-order conditions were used in all kinetic experiments, 100- to 500-fold excesses of reducing agent typically being present. Protein concentrations were ca. 1×10^{-5} M. Kinetic runs were performed at 630, 502, and 558 nm, where the disappearance of the ferri (630, 502) and production of the ferro (558) forms of the proteins could be monitored. Observed first-order rate constants were obtained by taking raw data from oscilloscope photographs and using a PDP-11 computer for data analysis. The program used a least-squares approach on plots of ln $|A_t - A_\infty|$ vs. time: A_∞ was varied to maximize the linear correlation coefficient. Correlation coefficients <0.999 were rejected as were runs requiring more than a 5% change from the measured A_∞ values on oscilloscope photographs.

Table I. Rate Data for the Reaction of Methemoglobin with Chromium(II) at 30.5 °C[a]

pH[b]	10^4[Cr^{II}], M	k_{obsd}, s^{-1}	pH[b]	10^4[Cr^{II}], M	k_{obsd}, s^{-1}
6.00	7.0	29	6.00	30.3	134
6.00	14.8	61	6.00	40.3	150
6.00	15.9	63[b]	6.00	46.7	175
6.00	15.9	65	5.74[d]	16.6	68
6.00	15.9	77	5.71[d]	15.6	61
6.00	16.9	77	5.48[d]	17.1	63
6.00	22.3	99			

[a] Measurements were made at 558 nm and an ionic strength of 0.167 maintained with sodium chloride; the hemoglobin was about 1×10^{-5} M. [b] This is the initial pH. The final pH of stopped-flow reactions was determined to be 5.96 ± 0.04. [c] 630 nm. [d] pH final. All initial and final pH values agreed to within ±0.04 pH units.

Visible–UV spectra were recorded with a Beckman Acta III spectrophotometer. A Beckman Century S.S. pH meter was used to take pH measurements. Stopped-flow products were equilibrated with O_2 and CO by gentle agitation of the solutions with the respective gases blowing over the surface.

Cr(III)/protein ratios for stopped-flow hemoglobin products reduced at pH 6.00 were determined by adding a slight stoichiometric excess of $K_3Fe(CN)_6$ to the reduced protein solution, centrifuging at 5000 rpm for 20 min at 20 °C, and dialyzing using the hollow fiber Dow Beaker Dialyser (Bio-Fiber 80) obtained from Bio-Rad Laboratories. Protein solutions of 50–100 ml were placed outside the fibers and dialyzed against 0.10 M NaCl, which was passed through the fibers at a flow rate of 50 ml/min. The dialysis was initiated 30 min after the reduction of the protein with chromous. Fractions of approximately 7 ml were collected every 30 min for 5 h. After ~50 ml had been collected, 20 ml of 0.10 M NaCl was added to the remainder of the protein solution inside the beaker to keep the fibers covered with liquid. The chromium content of each fraction was determined by the diphenylcarbazide method after oxidation of the chromium to chromium(VI) with $KMnO_4$.[23] The protein samples (5.00 ml) were wet-ashed first (three times) with concentrated reagent-grade HNO_3. The heme content of 1.00-ml samples was determined by the pyridine–hemochrome method[19,20] using A_{557}/A_{540} = 3.46 as an index of degradation and contamination by impurities with nonspecific absorption.[20] Cr(III)/protein ratios for stopped-flow myoglobin products reduced at pH 6.00 were determined by first converting the products to the CO form, followed by cation-exchange chromatography on Dowex-50W-X8. The analyses were then performed as previously described for hemoglobin, using A_{557}/A_{540} = 3.47 as the criterion for an acceptable test.[21,22]

For hemoglobin the second- and third-order rate constants reported are calculated for the heme monomer concentration rather than for the tetramer. Had the latter method been used, rate constants one-quarter those reported would have been obtained.

Results

I. Chromium(II) Concentration Dependence. Observed rate constants for the reduction of methemoglobin and metmyoglobin by chromous were obtained at 558 nm, 30.5 °C, and μ = 0.167 maintained with sodium chloride. All solutions contained 0.073 M sodium cacodylate buffer, pH 6.00. The first-order dependence of observed rate constants on the Cr(II) concentration is illustrated in Figure 1. The least-squares slopes of the data of Figure 1 give second-order rate constants k_1 = $(3.6 \pm 0.2) \times 10^4$ M^{-1} s^{-1} and $k_2 = (5.9 \pm 0.4) \times 10^3$ M^{-1} s^{-1} for the reduction of horse hemoglobin and equine skeletal muscle myoglobin, respectively. Table I reports the observed pseudo-first-order rate constants for the reactions of methemoglobin with chromium(II) at various pH values in cacodylate buffers. Table II reports the analogous data for myoglobin. Under these conditions, the rate laws obtained are

$$\frac{-d[HbFe(III)]}{dt} = k_1[Cr(II)][HbFe(III)] \qquad (1)$$

Table II. Rate Data for the Reaction of Metmyoglobin with Chromium(II) at 30.5 °C[a]

pH[b]	$10^4[Cr^{II}]$, M	k_{obsd}, s^{-1}	pH[e]	$10^4[Cr^{II}]$, M	k_{obsd}, s^{-1}
6.00	7.0	4.9[c]	6.64	16.6	14.8
6.00	7.0	5.3[d]	6.50	14.5	12.9
6.00	14.8	11.9	6.46	15.8	13.5
6.00	22.3	16.9	6.18	15.8	13.1
6.00	30.3	18.1	6.16	16.6	14.0
6.00	40.3	25.0	5.74	16.6	10.8
6.00	46.7	29.4	5.54	17.1	9.6
6.92[c]	8.8	8.3			

[a] Measurements were made at 558 nm and an ionic strength of 0.167 maintained with sodium chloride; the myoglobin was about 1×10^{-5} M. [b] Initial pH. The final pH for pH_i 6.00 reactions was 5.96 ± 0.04. [c] 502 nm. [d] 630 nm. [e] pH final.

and

$$\frac{-d[MbFe(III)]}{dt} = k_2[Cr(II)][MbFe(III)] \quad (2)$$

where the four iron(III) sites in hemoglobin are assumed to react essentially independently of one another.

II. pH Dependence. The effect of pH on the rate of reduction was evaluated at 558 nm, 0.073 M cacodylate, and $\mu = 0.167$, maintained with sodium chloride. It can be seen from Table I that over the limited pH range considered, the second-order rate constant is relatively invariant within experimental error for hemoglobin.[24a] An attempt was made to extend the pH range considered, but protein precipitation from an unknown cause precluded this.

The effect of pH on the rate of reduction for myoglobin was evaluated under the same conditions as for hemoglobin, except for the pH range considered. As can be seen from Table II, the rate is seen to increase with increasing pH.[24b] An attempt was made to extend the pH range below pH 5.5, but protein precipitation again precluded this.

III. Effect of Added Thiocyanate on the Reduction of Hemoglobin. The effect of added thiocyanate on the reduction of hemoglobin was monitored at 558 nm, 30.5 °C, pH 6.00, and $\mu = 0.167$. Thiocyanate was added only to the chromium(II) solution prior to reduction, replacing some of the sodium chloride. The buffer was 0.073 M cacodylate. The chromium(II) was kept constant at 16.9×10^{-4} M. The thiocyanate concentration was kept low enough to prevent any appreciable binding to the iron(III) sites prior to reduction. Gibson et al.[25] have reported binding constants of thiocyanate to human adult methemoglobin. They report binding rates (k on) of 300 M^{-1} s^{-1} for the α chains and 2700 M^{-1} s^{-1} for the β chains at pH 6.05. The binding was also determined to be a function of pH. Although horse methemoglobin was used in this study, we have assumed that thiocyanate binding rates for this protein are not more than two times those reported for human adult methemoglobin. We feel that this is a relatively safe assumption, since this is the case for azide.[25,26] The value of k on β for thiocyanate binding to human adult methemoglobin at pH 6.00 was calculated to be 2800 M^{-1} s^{-1}. Doubling this, we obtain an assumed k on β of 5600 M^{-1} s^{-1} for the horse protein. For 9.00×10^{-3} M thiocyanate, the binding $t_{1/2}$ is then calculated to be 13.8 ms. The observed reduction $t_{1/2}$ for 9.00×10^{-3} M thiocyanate as seen from Table III was 4.16 ms. Thus, the reduction is occurring before any appreciable thiocyanate binding to the axial positions on the iron(III) sites. The data of Table III are plotted in Figure 2. As can be seen, the data give a linear relationship between the second-order rate constant and thiocyanate concentration. The errors in this plot are large because the rates of the reactions were approaching the limit of our instrument. The slope of the line gives a third-order rate constant of $k_3 = (4.0 \pm 0.7) \times 10^6$ $M^{-2}s^{-1}$. The rate law obtained is

$$\frac{-d[HbFe(III)]}{dt} = k_3[Cr(II)][SCN^-][HbFe(III)] \quad (3)$$

where the four iron(III) sites are again assumed to react essentially independently of one another.

IV. Rate of Reduction of Myoglobin at $\mu = 1.00$. The rate of reduction of myoglobin was monitored at 558 nm, 30.5 °C, pH 6.00, and $\mu = 1.00$ maintained with sodium chloride. The buffer was 0.050 M cacodylate. The data are presented in Table IV. The second-order rate constant calculated from the

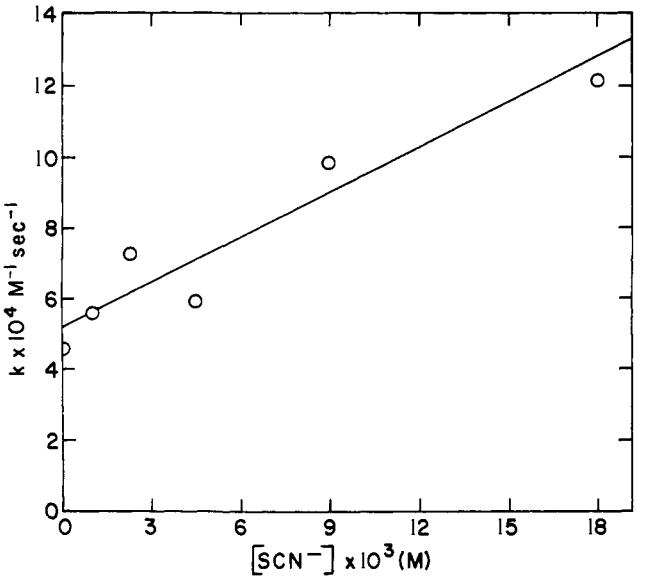

Figure 2. Plot of k vs. $[SCN^-]$ for the reaction of hemoglobin with chromium(II) in the presence of SCN^-: 30.5 °C; pH 6.00; $\mu = 0.167$.

Table III. Rate Data for the Reaction of Methemoglobin with Chromium(II) in the Presence of Thiocyanate[a]

$10^3[SCN^-]$, M	$10^4[Cr^{II}]$, M	k_{obsd}, s^{-1}
0.00	16.9	77
1.00	16.9	94
2.25	16.9	123
4.50	16.9	100
9.00	16.9	167
18.00	16.9	205

[a] 30.5 °C, pH_i 6.00, 558 nm, $\mu = 0.167$, maintained with sodium chloride. pH_f 6.00.

Table IV. Rate Data for the Reaction of Metmyoglobin with Chromium(II) at $\mu = 1.00$[a]

pH[b]	$10^4[Cr^{II}]$, M	k_{obsd}, s^{-1}
5.99	6.1	11
5.99	17.6	33
5.99	23.1	56
5.99	32.7	90
5.99	40.7	104
5.99	16.6	34
5.99	15.0	38
5.99	22.5	55

[a] 30.5 °C, 558 nm, 0.050 cacodylate buffer, ionic strength maintained with sodium chloride. [b] Initial pH. pH_f 5.98 ± 0.05.

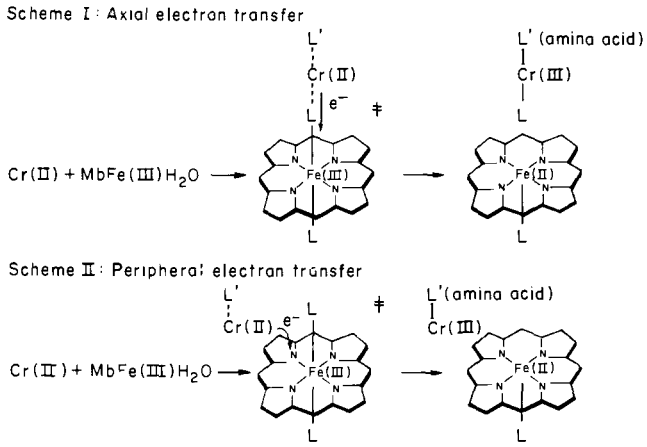

Figure 3. The major electron-transfer pathways in the chromium(II) reduction of metmyoglobin.

slope is $k = 2.9 \pm 0.2 \times 10^4\ M^{-1}\ s^{-1}$. No change in rate was found when the solution used to generate chromium(II) was changed from 0.10 M $CrCl_3 \cdot 6H_2O$ to 0.10 M $Cr(ClO_4)_3 \cdot 6H_2O$.

V. Stopped-Flow Products. The deoxy spectra of both hemoglobin and myoglobin stopped-flow products were determined and found to be identical with their Cr(III)-free analogues under all conditions used in this study.[27-29] Both proteins retained their O_2 and CO binding capacities and gave spectra for these forms identical with their respective Cr(III)-free counterparts. Stopped-flow hemoglobin products reduced at pH 6.00 were oxidized with ferricyanide and dialyzed against 0.10 M sodium chloride. Chromium/heme ratios were determined as a function of dialysis time. After 2.5 h, this ratio was determined to be 1.05. Ratios determined every 30 min for the following 2.5 h of dialysis were 1.05 (3 h); 0.98 (3.5 h); 1.27 (4 h); 1.24 (4.5 h); and 1.09 (5 h). The oxidized hemoglobin from the dialysis beaker showed no spectral differences from that of the original sample.

Stopped-flow myoglobin products reduced at pH 6.00 were converted to the CO form and passed through a cation-exchange column to remove excess chromium. Chromium/heme ratios were determined for the effluent protein fractions. This ratio was found to be 0.97 ± 0.06.

Discussion

The fact that the reactions of hemoglobin presented herein give linear plots, first order in hemoglobin, is entirely consistent with current information concerning the reduction mechanism of hemoglobin. The oxidation-reduction reaction of the half-cell couple

$$\text{methemoglobin} + e^- \rightleftharpoons \text{deoxyhemoglobin}$$
$$E_{1/2} = 0.15\ \text{V}$$

exhibits pH-dependent cooperativity.[22,30] For this reaction at pH 6, the Hill coefficient, a measure of heme-heme cooperativity, is equal to 1.1. This coefficient changes with pH, becoming 2.7 at pH 9. The pH dependence of the Hill coefficient has been explained on the basis of the spin states of the oxidized coordinated species involved.[31] The conclusion was reached that changes in protein-porphyrin nonbonded interactions accompany spin-state transitions of the iron.[32,33] Thus the oxidized low-spin six-coordinated and the reduced high-spin five-coordinated hemoglobin species are the only ones associated with cooperative subunit interaction. At pH 6, the iron(III) sites in hemoglobin are all high-spin six-coordinate species, in which a water molecule occupies the sixth coordinative position. Therefore, no cooperativity is to be expected.

The reduction of human methemoglobin at pH 6.5 by the iron(II) complex of *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetate ($CDTA^{4-}$) was also seen to be first order in hemoglobin when the reductant was present in large excess.[34]

The reductions of hemoglobin and myoglobin by chromium(II) follow simple second-order kinetics with no intermediates such as aquo complexes of the reduced forms being observed. A complete explanation for the relative rates of reduction of methemoglobin and metmyoglobin reported herein cannot be given at present. The relative order of reactivity reported here has also been obtained using hydrated electrons as the reducing species for human methemoglobin and horse metmyoglobin.[35] The reduction rate of the former using e_{aq}^- was found to be $5.8 \times 10^{10}\ M^{-1}\ s^{-1}$ at pH 7.3 and that of the latter protein $4.3 \times 10^{10}\ M^{-1}\ s^{-1}$ at pH 6.7. Thus hemoglobin was reduced 1.35 times faster than myoglobin by e_{aq}^-. For the e_{aq}^- work, the second-order rate constant reported for hemoglobin was calculated on the tetramer base. Dividing our second-order rate constant for the reduction of hemoglobin by 4, we obtain a second-order rate constant of $(9.0 \pm 0.5) \times 10^3\ M^{-1}\ s^{-1}$ based upon the concentration of the tetramer. Comparing this similarly to our rate for the reduction of myoglobin of $(5.9 \pm 0.4) \times 10^3\ M^{-1}\ s^{-1}$ gives a rate ratio of 1.53. The relative rates seen here may represent an effect of the potentials for the two proteins. For equine skeletal muscle myoglobin the $E_{1/2}$ is 0.060 V while that for horse hemoglobin is 0.150 V.[36]

In the study of the effect of pH on the rate of reduction of metmyoglobin, the rate was found to increase by a factor of only 1.5-2 from pH 5.5 to 7.0. The opposite trend has been observed using NADH as the reductant.[5] The rate expression for the reduction of horse metmyoglobin with hydrated electrons was found to have a component with a pH dependence suggestive of a titration curve. The rate decreased with increasing pH and a pK near 9 was determined.[35] It was concluded that the acid-alkaline transition for iron(III) was responsible, and a half-time of about 200 μs (pH 7.3) was found for the ejection of the axial water at low pH or of OH^- at high pH. The pK of the axial water for horse aquometmyoglobin is 8.93 at $\mu = 0.1$.[22] We found no evidence that leads us to implicate the ionization of the axial water or an amino acid side chain as a contributing factor to the rate vs. pH data obtained. Acid-base titrations of a number of proteins have indicated that the imidazole side chain of histidine ionizes with a pK between 6.4 and 6.9, and no other side chain is expected to have a pK value falling between 6 and 7.[37] Thus, we do not have any evidence supporting the participation of the distal imidazole from His-64 in the pH effect observed. Our observations may represent an effect of the reductant or a small medium effect and are not interpretable here.

The pH-rate profile for the reduction of methemoglobin shows no appreciable changes in rate over the narrow range studied, pH 5.5-6.0. Since we were unable to extend the pH range in this study, an interpretation of the pH-rate profile cannot be given.

Thiocyanate was added to Cr(II) solutions prior to reduction of methemoglobin in an attempt to ascertain whether or not a similar dependence would obtain as compared to previous work using Cr(II) with thiocyanate in the reduction of ferricytochrome *c*.[14b,38] A similar SCN^- dependence does occur. Thiocyanate catalyzes the reduction of methemoglobin by Cr(II). Since rate constant arguments show that the thiocyanate is not bonded to the iron(III) sites prior to reduction, we believe the mechanism here involves a remote or peripheral attack of the reductant complex on an electron-transfer site remote from the iron(III), possibly the exposed edge of the porphyrin ring system.

The third-order rates involving the SCN^- catalysis for this

reaction for ferricytochrome *c* were reported as 3.7×10^5 M^{-2} s^{-1} (pH 6.1, μ = 1.0 M chloride), $(1.1 \pm 0.2) \times 10^6$ M^{-2} s^{-1} ($[H^+]$ = 0.006-0.05 M, μ = 1.0 M chloride) and $(2.3 \pm 0.4) \times 10^6$ M^{-2} s^{-1} ($[H^+]$ = 0.006 − 0.05 M, μ = 0.1 M chloride).[14b,38] Ferricytochrome *c* in acidic media has at least one site on the iron free.[38] The rate obtained at μ = 0.1 M chloride is more suitable for comparison to our rate obtained, $(4.0 \pm 0.7) \times 10^6$ M^{-2} s^{-1}, since the media and iron(III) sites for the two systems are as similar as they can be under these conditions. The agreement between the two rates is seen to be very good, but we offer no detailed mechanistic explanation for the similarity of the two rates.

The kinetic products (pH 6.00, μ = 0.167, 30.5 °C) in the absence of thiocyanate contained protein-bound Cr(III) with Cr(III)/protein ratios of 1.0 for myoglobin and 4.0 for hemoglobin. Chromium(III)-myoglobin products reduced with a stoichiometric amount of chromium(II) contained 0.8-1.0 bound Cr(III) per molecule. Since the chromium-protein complexes remained intact through extensive dialysis (Hb, Mb), cation-exchange chromatography (Mb), and various electrophoretic and column chromatographic methods (Mb), we conclude that the Cr(III) in all cases is covalently bonded to the protein. Because the CO and O_2 binding capacities are retained for both hemoglobin and myoglobin after being reduced by chromous, the axial positions on the iron sites must not be hindered to a great extent by the covalently bound chromium(III). A pathway must also remain open for the expulsion of the axial water following reduction—the "distal" imidazole (64 His E7 side chain) is thought to be important in this context.[39] The rotation of histidine E7 around its C_α-C_β bond or a breathing of helix E have been suggested as one of the conformational changes required for the coordination or loss of any ligand from the heme pocket.[40]

Conclusion

The two principal possible paths of electron transfer for heme-containing proteins with small molecule reducing agents such as chromous are shown in Figure 3. In Figure 3 Scheme I demonstrates an axial pathway for electron transfer with the electron traversing a pathway which includes the iron fifth and sixth ligands while Scheme II is a peripheral electron-transfer pathway with the electron going to the iron via the porphyrin π cloud. Both the kinetic studies and preliminary product studies[41] of the reduction of metmyoglobin and methemoglobin lead to the mechanism as being via the peripheral pathway. Previous studies on other redox systems such as cytochrome *c*[16] and free metalloporphyrins[42] also have reached the conclusion of a peripheral pathway of the electron transfer.

Acknowledgments. This work was partially supported by the National Science Foundation.

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