The Reversible Reduction of Horse Metmyoglobin by the Iron(II) Complex of *trans*-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetate†

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ABSTRACT: The reduction of metmyoglobin by the iron(II) complex of *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetate ($FeCDTA^{2-}$) has been investigated. The equilibrium constant, measured spectrophotometrically, is 0.21 with a resulting reduction potential of 0.050 V for Mb^0. The rate constant for the reduction is 28 M^{-1} sec^{-1} with a $\Delta H^{\ddagger}$ of 13 kcal M^{-1} and $\Delta S^{\ddagger}$ of −11 eu. Both CN^- and OH^- inhibit the reduction because of the relatively low reactivity of cyanometmyoglobin (Mb^+CN^-) and ionized metmyoglobin (Mb^+OH^-). The rate constant for the reduction of Mb^+CN^- by $FeCDTA^{2-}$ is 4.0×10^{-2} M^{-1} sec^{-1} and that for reduction of Mb^+OH^- is 4.8 M^{-1} sec^{-1}. The nitric oxide complex of metmyoglobin is reduced with a rate constant of 10 M^{-1} sec^{-1}. The kinetics of oxidation of oxymyoglobin by $FeCDTA^-$ were studied. The data are consistent with a mechanism where oxidation takes place entirely through the deoxy form. A rate constant of 1.45×10^2 M^{-1} sec^{-1} was calculated for the oxidation of deoxymyoglobin by $FeCDTA^-$, in excellent agreement with that calculated from the equilibrium constant and rate constant for reduction. The above data are discussed in terms of a simple outer-sphere reduction reaction.

The kinetics and mechanism of redox reactions of heme proteins, especially cytochrome *c*, have been the subject of several recent investigations because of the importance of the heme proteins in biological electron transfer reactions. Fewer studies of redox reactions of the oxygen carriers, hemoglobin and myoglobin, have been undertaken in part because suitable reversible redox agents have not been available. Even though hemoglobin and myoglobin are not directly involved in electron transfer reactions, an understanding of the mechanisms of the redox reactions involving these proteins is important in the understanding of the overall picture of electron transfer processes in heme proteins. Oxidation of these proteins is a side reaction with the result that complex systems are required to maintain hemoglobin and myoglobin in the reduced state in vivo. In the case of hemoglobin, the oxidation reaction is of special importance since the tertiary structural changes which accompany the oxidation of deoxyhemoglobin are believed to be the same as those which accompany the oxygenation.

In this paper we report kinetic and equilibrium data for the reduction of Mb^+.[1] The purpose of this study was to characterize the oxidation–reduction reactions of myoglobin using a reactant which could be used to study both the oxidation (using the reactant in the reduced state) of Mb^0 and the reduction of Mb^+ (using the reactant in its reduced state). Further we wished to study the role of coordinated ligand on the kinetics of reduction of myoglobin as a potential model for cytochrome *c* where similar studies cannot be carried out because both ligand binding sites are occupied by groups from the protein.

To carry out this study $FeCDTA^{2-/-}$ has been used as the reductant/oxidant. Recently, $FeEDTA^{2-}$ has been used as a reducing agent for cytochrome *c* (Hodges et al., 1974; Kurihara and Sano, 1970). The reduction potential of 0.12 V (Kolthoff and Auerbach, 1952) is of the right order of magnitude for studies of myoglobin (E_0 = 0.06 V, Behlke and Scheler, 1962) and hemoglobin (E_0 = 0.16 V, Antonini et al., 1964). However, dimerization of the oxidized product, $FeEDTA^-$, at neutral and slightly basic pH's precludes its use as a reversible redox agent (Schugar et al., 1969; Wilkins and Yelin, 1969). $FeCDTA^{2-/-}$ was chosen because it has previously been shown that $FeCDTA^-$ has a much lower tendency to dimerize than $FeEDTA^-$ (Gustafson and Martell, 1963; Wilkins and Yelin, 1969). Furthermore, the reduction potential of $FeCDTA^{2-/-}$ has been assumed to be equal to that of $FeEDTA^{2-/-}$ (Wilkins and Yelin, 1968) because of the similarity in structure.

Experimental Section

Materials

Myoglobin from equine skeletal muscle (Sigma) was standardized as Mb^+ using an extinction coefficient of 1.88×10^5 at 408 nm (Scheler et al., 1957). Where necessary, myoglobin was reduced by $Na_2S_2O_4$, which was removed on a column of AG 501 mixed-bed resin (Bio-Rad) (Brown and Mebine, 1969). $FeCDTA^{2-}$ was prepared in situ as follows: $FeCl_2$ (standardized spectrophotometrically as the *o*-phenanthrolene complex (Brandt and Smith, 1949)), H_2CDTA^{2-} (Aldrich), and buffer were deoxygenated by bubbling prepurified N_2 through 125-ml erlenmeyer flasks equipped with serum caps near the bottom. H_2CDTA^{2-} (in slight excess) was added to the $FeCl_2$, and then buffer was added to the solution of $FeCDTA^{2-}$ complex using Hamilton gas-tight syringes. Mb^+ was deoxygenated by passing N_2 over the protein solution with stirring. Most experiments were carried out at pH 6.8 in 0.1 *M* phosphate buffer. All other chemicals were reagent grade.

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[1] Abbreviations used are: $CDTA^{4-}$, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetate; Mb^0, deoxymyoglobin; Mb^+, metmyoglobin; Mb^+OH^-, ionized metmyoglobin; Mb^+CN^-, cyanometmyoglobin; MbO_2, oxymyoglobin; Mb^+NO, nitric oxide complex of Mb^+.

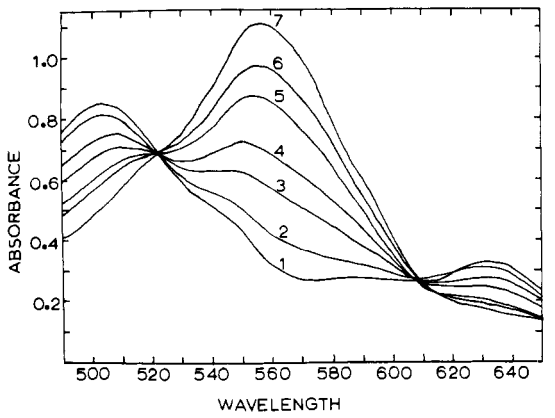

FIGURE 1: Selected spectra of myoglobin as a function of varying ratios of $[FeCDTA^{2-}]/[FeCDTA^{-}]$ (25°, 0.1 *M* phosphate buffer (pH 6.8)). The total myoglobin concentration was 8.5×10^{-5} *M* in a 1-cm cell. Spectrum 1 is that of Mb^{+} before reaction. Spectrum 7 is that of Mb^{0} produced by addition of a large excess (sufficient to cause 99% reaction) of $FeCDTA^{2-}$. Spectra 2–6 were produced by addition of the following ratios of $[FeCDTA^{2-}]/[FeCDTA^{-}]$: spectrum 2, 0.65; spectrum 3, 2.06; spectrum 4, 3.86; spectrum 5, 9.9; spectrum 6, 17.5.

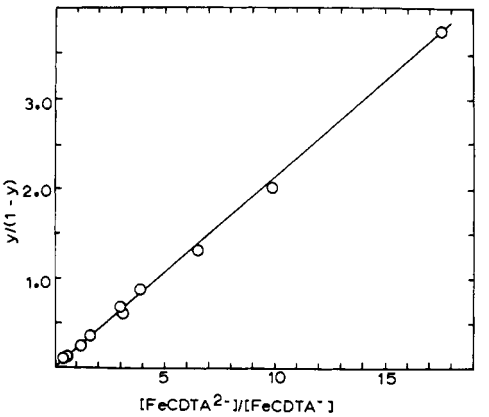

FIGURE 2: A plot of $y/(1 - y)$ as a function of $[FeCDTA^{2-}]/[FeCDTA^{-}]$, where y is equal to the extent of reaction determined at 560 nm. The conditions were the same as in Figure 1.

Methods

Equilibria. The equilibrium experiments were carried out by injecting appropriate amounts of reactants into a 1-cm cuvette previously flushed with N_2 and sealed with a serum cap. The reactions were followed until completion (usually less than 5 min for Mb^{+} and 2 hr for $Mb^{+}CN^{-}$) with the spectra recorded on a Beckman Acta CV spectrophotometer. The temperature was controlled to ±0.1° with a circulating water bath.

Kinetic Measurements. In all cases, rate measurements were carried out under pseudo-first-order conditions. Plots of $\log |A - A_\infty|$ as a function of time were made at appropriate wavelengths and values for the observed rate constant were determined. The data shown represent the average of at least three runs with an error of ±10%. Kinetic measurements of the reduction of Mb^{+} and $Mb^{+}OH^{-}$ by $FeCDTA^{2-}$ were carried out on an Aminco-Morrow stopped-flow apparatus. Mb^{+} (or $Mb^{+}OH^{-}$) and $FeCDTA^{2-}$ were deoxygenated as previously described and the solutions transferred to the stopped-flow via gas-tight syringes. The stopped-flow apparatus was flushed several times with the reactant solutions before measurements were made. In the other kinetic experiments, the reactions were slow enough to permit measurement of the rates on the spectrophotometer. In the experiments involving the oxidation of MbO_2 by $FeCDTA^{-}$, the O_2 concentration was varied by mixing O_2 and N_2 saturated water in the proper amounts. $Hb^{+}NO$ was prepared in similar fashion. The entire system was first deoxygenated to prevent formation of NO_2. In all cases the temperature was controlled to ±0.1° with a circulating water bath.

Determination of the Potential for FeCDTA. The redox potential of $FeCDTA^{2-/-}$ was determined under the conditions of our experiments (0.1 *M* phosphate buffer, pH 6.8, 25°) by titrating 0.02 *M* $FeCDTA^{-}$ (previously deoxygenated) with 0.02 *M* $FeCDTA^{2-}$ (prepared as described above) under N_2 and measuring the potential with a Radiometer pHM 26 pH meter equipped with platinum and saturated calomel electrodes. The temperature was controlled to ±0.1° with a circulating water bath. Based on six points over a wide range of concentrations, the reduction potential (neglecting activity coefficients[2]) of $FeCDTA^{2-/-}$ was found to be 0.090 ± 0.004 V.

Results

Equilibrium. Mb^{+} was reacted with mixtures containing varying ratios of $FeCDTA^{2-}$ to $FeCDTA^{-}$ and the resulting spectra were measured. Selected spectra, corrected for a small contribution (less than 7%) to the absorbance by $FeCDTA^{-}$, are shown in Figure 1. Reasonable isosbestic points were obtained at 522 and 608 nm. The small departures shown can be accounted for by errors in mixing the solutions *via* syringes and in errors in reading and correcting the spectra. The spectrum of the product is clearly that of deoxymyoglobin. The observation that reasonable isosbestic points are obtained shows lack of interference by O_2. The fact that $FeCDTA^{2-}$ can be slowly autoxidized helps in this respect. A plot of $y/(1 - y)$ (where y equals the fraction of Mb^{+} reacted) as a function of $[FeCDTA^{2-}]/[FeCDTA^{-}]$ is shown in Figure 2. Values for y were calculated at 560 nm where the spectral change is largest. Values for y calculated at 500 nm were in excellent agreement. From the slope of the graph, the equilibrium constant for the reduction of Mb^{+} by $FeCDTA^{2-}$ is equal to 0.21. Using the previously determined E_0 value of 0.090 V for the reduction potential of $FeCDTA^{2-/-}$, a value of 0.050 V is calculated for the reduction potential of Mb^{+}. This value is in excellent agreement with previously reported values for myoglobin which range from 0.047 to 0.06 V (Behlke and Scheler, 1962; Taylor and Morgan, 1942).

Kinetics. The kinetics of reduction of Mb^{+} by $FeCDTA^{2-}$ were determined under a large excess of $[FeCDTA^{2-}]$ in order to drive the reaction to completion and ensure pseudo-first-order conditions. The reaction was monitored at 435 nm on the stopped-flow apparatus. A plot of the observed rate constant, k_{obsd}, as a function of $[FeCDTA^{2-}]$ is shown in Figure 3. As observed, there are no deviations from second-order behavior even at high $[FeCDTA^{2-}]$. From the slope of the line, the rate constant for the reduction of Mb^{+} by $FeCDTA^{2-}$ is 28 M^{-1} sec^{-1}.

The activation parameters were determined by measuring the rate constant at five temperatures from 10 to 45°. For the purpose of comparing our results with those of Hod-

[2] Activity coefficients were neglected because of the difficulty in their determination at the high ionic strength employed here. In any case they cancel out in the determination of the reduction potential of Mb^{+}.

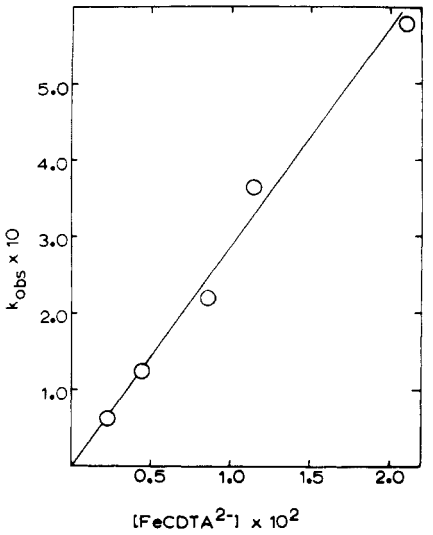

FIGURE 3: k_{obsd} (sec^{-1}) as a function of [FeCDTA^{2-}] (M) for the reaction of Mb$^+$ with FeCDTA^{2-} at 25° in 0.1 M phosphate buffer (pH 6.8). The protein concentration was 5×10^{-6} M.

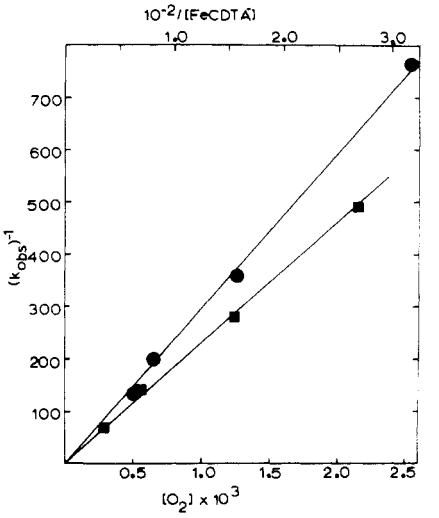

FIGURE 4: $(k_{obsd})^{-1}$ as a function of [FeCDTA$^-$]$^{-1}$ at a constant [O_2] of 5.1×10^{-4} M (■) and as a function of [O_2] $\times 10^3$ at a constant [FeCDTA$^-$] of 1.95×10^{-2} M (●) for the reaction of MbO_2 with FeCDTA$^-$ at 25° in 0.1 M phosphate (pH 6.8). The protein concentration was 7.5×10^{-5} M.

ges et al. (1974), we have also determined the activation parameters for the reduction of Mb$^+$ by FeEDTA^{2-}. For reduction by FeCDTA^{2-}, the enthalpy of activation, $\Delta H^{\ddagger}$, is equal to 13 ± 1.0 kcal/mol and the entropy of activation, $\Delta S^{\ddagger}$, is −11 ± 5 eu. For reduction by FeEDTA^{2-}, the results are nearly identical with a rate constant at 25° of 31 M^{-1} sec^{-1}, a $\Delta H^{\ddagger}$ of 12 ± 1 kcal/mol, and $\Delta S^{\ddagger}$ of −13 ± 5 eu.

Because of the difficulty of producing Mb^0, the kinetics of oxidation of Mb^0 were determined by making use of a previous observation that oxidation of MbO_2 by $Fe(CN)_6^{3-}$ occurs via the deoxy form (Antonini et al., 1965). The mechanism expected here is

$$MbO_2 \underset{k_a^{MbO2}}{\overset{k_d^{MbO2}}{\rightleftharpoons}} Mb^0 + O_2$$

$$Mb^0 + FeCDTA^- \xrightarrow{k_{ox}^{Mb0}} Mb^+ + FeCDTA^{2-}$$

Using the steady-state approximation, it can be shown that

$$\frac{d[Mb^+]}{dt} = \frac{k_d^{MbO_2} k_{ox}^{Mb^0} [MbO_2][FeCDTA^-]}{k_a^{MbO_2}[O_2] + k_{ox}^{Mb^0}[FeCDTA^-]} \quad (1)$$

If [O_2] and [FeCDTA$^-$] $\gg$ [MbO_2]

$$\frac{1}{k_{obsd}} = \frac{k_a^{MbO_2}[O_2]}{k_d^{MbO_2} k_{ox}^{Mb^0}[FeCDTA^-]} + \frac{1}{k_d^{MbO_2}} \quad (2)$$

The rates were determined spectrophotometrically by measuring the spectrum as a function of time in the visible region on the spectrophotometer. An excellent isosbestic point was obtained at 525 nm. The final spectrum clearly showed that the product was Mb$^+$. Plots of $(k_{obsd})^{-1}$ as a function of [O_2] at constant [FeCDTA$^-$] and as a function of [FeCDTA$^-$]$^{-1}$ at constant [O_2] are shown in Figure 4. In both plots the value of $1/k_d^{MbO_2}$ is sufficiently small that the intercept is near zero. The value of $k_a^{MbO_2}/k_d^{MbO_2} k_{ox}^{Mb^0}$ determined from the slope of the first plot is equal to 3.5×10^3, in excellent agreement with that of 3.8×10^3 determined from the slope of the second. Using a value of $k_a^{MbO_2}/k_d^{MbO_2}$ of 5.3×10^5 M^{-1} (calculated from the data on p 221 of Antonini and Brunori (1971), $k_{ox}^{Mb^0}$ is calculated to be 1.48×10^2 M^{-1} sec^{-1}. This value is in excellent agreement with that of 1.33×10^2 M^{-1} sec^{-1} calculated from the equilibrium constant of 0.21 and rate constant measured for the reduction of 28 M^{-1} sec^{-1}.

Effect of pH. The effect of pH on the rate of reduction of Mb$^+$ by FeCDTA^{2-} was measured from pH 8.8 to 9.6 to determine the effect of the ionization of the water molecule coordinated to the heme iron. In borate buffer two reactions were observed. Therefore, no buffer was used. Rather, excess CDTA was added to maintain the pH. KCl was added to maintain the ionic strength at 0.2 M. Under these conditions only one reaction was seen. In spite of the limited buffering capacity of CDTA at these pH's, the pH change was negligible during the course of the reaction due to the low protein concentrations used. At higher pH's the rate is slowed considerably. Thus, the ionized form, Mb$^+$OH$^-$, is being oxidized much more slowly than the protonated form as in the mechanism:

$$Mb^+ \overset{K_a^{Mb+}}{\rightleftharpoons} Mb^+OH^- + H^+$$

$$Mb^+ + FeCDTA^{2-} \xrightarrow{k_{red}^{Mb+}} Mb^0 + FeCDTA^-$$

$$Mb^+OH^- + FeCDTA^{2-} \xrightarrow{k_{red}^{Mb+OH-}} Mb^0 + FeCDTA^-$$

The rate law consistent with this mechanism is

$$\frac{-d[Mb^+]}{dt} = \frac{(k_{red}^{Mb^+}[H^+] + k_{red}^{Mb^+OH^-} K_a^{Mb^+})[Mb^+]_T[FeCDTA^{2-}]}{K_a^{Mb^+} + [H^+]} \quad (3)$$

where [Mb$^+$]$_T$ is the sum of the unreacted [Mb$^+$] and [Mb$^+$OH$^-$]. When FeCDTA^{2-} is present in large excess and the reaction sufficiently buffered:

$$\frac{k_{obsd}(K_a^{Mb^+} + [H^+])}{K_a^{Mb^+}[FeCDTA^{2-}]} = \frac{k_{red}^{Mb^+}[H^+]}{K_a^{Mb^+}} + k_{red}^{Mb^+OH^-} \quad (4)$$

The reactions were followed at 425 nm on the stopped-flow.

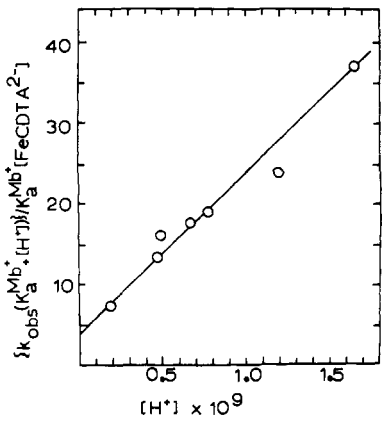

FIGURE 5: $\{k_{obsd}(K_a^{Mb^+} + [H^+])\}/K_a^{Mb^+}[FeCDTA^{2-}]$ as a function of $[H^+]$ for the reduction of Mb^+ by $FeCDTA^{2-}$ at 0.2 M ionic strength and 25°. The pK_a was 8.86 (George and Hanania, 1952). $[FeCDTA^{2-}]$ was varied from 4×10^{-3} to 1×10^{-2} M and the protein concentration was 5×10^{-6} M. KCl was added to bring the ionic strength to 0.2 M.

Using a value of 8.86 for $pK_a^{Mb^+}$ [calculated from George and Hanania (1952)] the right-hand side of eq 4 was plotted as a function of $[H^+]$ as shown in Figure 5. From the slope, the rate constant for the reduction of Mb^+, $k_{red}^{Mb^+}$, is equal to 31 M^{-1} sec^{-1}, in excellent agreement with the value of 28 M^{-1} sec^{-1} determined at low pH. From the intercept, the rate constant for the reduction[3] of Mb^+OH^- is equal to 4.0 M^{-1} sec^{-1}.

Effect of CN$^-$. Addition of KCN was observed to have a large inhibitory effect on both the equilibrium and rate of the reaction of Mb^+ with $FeCDTA^{2-}$. The effect on the equilibrium was determined by reacting Mb^+CN^- with $FeCDTA^{2-}$ and measuring the fraction of Mb^+CN^- reacted at 435 nm, as a function of added [KCN]. Good isosbestic points were obtained at 427 and 452 nm. The final spectrum shows that under these conditions the product is clearly Mb^0. The overall equilibrium is represented by

$$Mb^+CN^- + H^+ + FeCDTA^{2-} \overset{K_{red}^{Mb^+CN^-}}{\rightleftharpoons} Mb^0 + HCN + FeCDTA^-$$

with

$$K_{red}^{Mb^+CN^-} = \frac{[Mb^0][HCN][FeCDTA^-]}{[Mb^+CN^-][H^+][FeCDTA^{2-}]} \quad (5)$$

and

$$\log ([Mb^0][FeCDTA^-]/[Mb^+CN^-][FeCDTA^{2-}]) = \log K_{red}^{Mb^+CN^-} - pH - \log [HCN] \quad (6)$$

A plot of log $([Mb^0][FeCDTA^-]/[Mb^+CN^-][FeCDTA^{2-}])$ as a function of log [HCN] is shown in Figure 6A. From the intercept, log $K_{red}^{Mb^+CN^-}$ is equal to −0.25. The slope as expected is equal to −1. The above equilibrium can be broken down into the following steps:

[3] It was impossible to attain the higher pH's necessary to determine the intercept value more accurately because of precipitation and dimerization of $FeCDTA^-$. In the case of the reduction of methemoglobin, which exhibits a lower pK_a for the ionization of the coordinated water, we were able to show a definitive non-zero rate constant (J. C. Cassatt and C. P. Marini, unpublished).

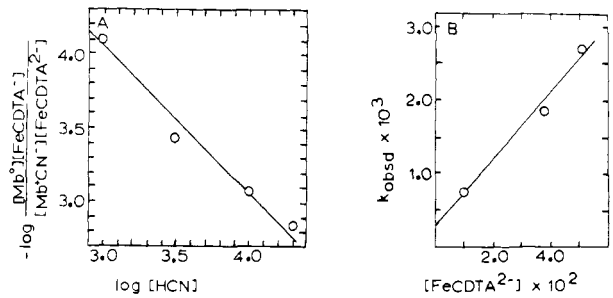

FIGURE 6: (A) $-\log ([Mb^0][FeCDTA^-]/[Mb^+CN^-][FeCDTA^{2-}])$ as a function of −log [HCN] at a constant $[FeCDTA^{2-}]$ of 2×10^{-2} M at 25° in 0.1 M phosphate buffer (pH 6.8). The protein concentration was 4.4×10^{-6} M. (B) k_{obsd} as a function of $[FeCDTA^{2-}]$ for the reduction of Mb^+CN^- by $FeCDTA^{2-}$ at a constant [HCN] of 5×10^{-5} M at 25° in 0.1 M phosphate buffer and pH 6.8.

$$Mb^+CN^- \overset{K_d^{Mb+CN-}}{\rightleftharpoons} Mb^+ + CN^-$$

$$HCN \overset{K_a^{HCN}}{\rightleftharpoons} H^+ + CN^-$$

$$Mb^+ + FeCDTA^{2-} \overset{K_{red}^{Mb^+}}{\rightleftharpoons} Mb^0 + FeCDTA^-$$

Thus $K_{red}^{Mb^+CN^-}$ is equal to $(K_d^{Mb^+CN^-}K_{red}^{Mb^+})/K_a^{HCN}$. Since $K_{red}^{Mb^+}$ and K_a^{HCN} are known, $K_d^{Mb^+CN^-}$ can be calculated. Our value of −8.8 for log $K_d^{Mb^+CN^-}$ is in reasonable areement with that of −8.4, calculated from the free energy (George, 1956). The slope of the plot in Figure 6A is an indirect measure of the "Hill" constant for the reaction of CN^- with Mb^+. Our expected value of 1 contrasts with abnormally high values reported earlier (Scheler et al., 1958).

The effect of KCN on the rate of reduction of Mb^+CN^- by $FeCDTA^{2-}$ was measured as a function of both added KCN and $FeCDTA^{2-}$. A good isosbestic point was observed at 427 nm and the product of the reaction was clearly Mb^0. Unfortunately, the range of concentration over which measurements could be made was limited by the large inhibitory effect of CN^- on the equilibrium such that at high $[CN^-]$ the reaction does not go to completion. Over a fourfold change in $[CN^-]$ from 1×10^{-4} to 4×10^{-4} M and a constant $[FeCDTA^{2-}]$ of 4×10^{-2} M, the observed rate constant remained virtually unchanged with the following values being observed: 1.6×10^{-5} sec^{-1} at 1×10^{-4} M; 1.8×10^{-3} sec^{-1} at 2×10^{-4} M; and 1.8×10^{-3} sec^{-1} at 4×10^{-4} M. With increasing $[FeCDTA^{2-}]$, however, the rate increased markedly. The results are shown in Figure 6B where k_{obsd} is plotted as a function of $[FeCDTA^{2-}]$ The observed behavior is consistent with the mechanism:

$$Mb^+CN^- \underset{k_a^{Mb+CN-}}{\overset{k_d^{Mb+CN-}}{\rightleftharpoons}} Mb^+ + CN^-$$

$$HCN \overset{K_a^{HCN}}{\rightleftharpoons} H^+ + CN^-$$

$$Mb^+CN^- + FeCDTA^{2-} \overset{k_{red}^{Mb+CN-}}{\longrightarrow} Mb^0 + FeCDTA^{2-} + CN^-$$

$$Mb^+ + FeCDTA^{2-} \overset{k_{red}^{Mb+}}{\longrightarrow} Mb^0 + FeCDTA^-$$

Using steady-state approximation it can be shown that

$$d[Mb^0]/dt = \{k_{red}^{Mb^+} k_d^{Mb^+CN^-} / ((k_a^{Mb^+CN^-} K_a^{HCN}[HCN]/[H^+]) + k_{red}^{Mb^+}[FeCDTA^{2-}]) + k_{red}^{Mb^+CN^-}\}[FeCDTA^{2-}][Mb^+CN^-] \quad (7)$$

Under the conditions of these experiments, $k_{red}^{Mb^+}[FeCDTA] \gg (k_a^{Mb^+CN^-} K_a^{HCN}[HCN])/[H^+]$; so that under pseudo-first-order conditions

$$k_{obsd} = k_d^{Mb^+CN^-} + k_{red}^{Mb^+CN^-}[FeCDTA^{2-}] \quad (8)$$

Thus a plot of k_{obsd} as a function of $FeCDTA^{2-}$ will appear[4] to have an intercept of $k_d^{Mb^+CN^-}$ with a slope of $k_{red}^{Mb^+CN^-}$. Furthermore, k_{obsd} will be independent of $[CN^-]$. From Figure 6B, $k_{red}^{Mb^+CN^-}$, the rate constant for the reduction of Mb^+CN^-, is equal to 4.02×10^{-2} M^{-1} sec^{-1}, and $k_d^{Mb^+CN^-}$, the rate constant for the dissociation of Mb^+CN^-, is equal to 3.5×10^{-4} sec^{-1}. The latter value is in poor agreement with that of 3×10^{-3} sec^{-1} calculated by Blanck et al. (1961) from the measurement of the association rate constant and equilibrium constant. We, therefore, determined $k_d^{Mb^+CN^-}$ directly under the conditions of our experiments by diluting a solution containing Mb^+ (1.0×10^{-4} M) and KCN (2×10^{-4} M) 100-fold and measuring the rate of dissociation induced by dilution spectrophotometrically in the Soret region. The results showed that the Mb^+CN^- complex initially formed was completely dissociated by this procedure with a rate constant of 7.6×10^{-4} sec^{-1}. This value, while closer to our value determined from the reduction experiment than that of Blanck et al. (1961), is nevertheless still not in satisfactory agreement. Similar discrepancies between dissociation rate constants determined directly and indirectly as a result of oxidation have been observed in the case of oxidation of MbO_2 and MbCO by $Fe(CN)_6^{3-}$ (Antonini et al., 1965; J. C. Cassatt, unpublished).

Effect of NO. NO is a unique ligand in that it binds with high affinity to both Mb^0 and Mb^+ (Antonini and Brunori, 1971, p 49). We thus decided to study the effect of NO on the rate of reduction of Mb^+ by $FeCDTA^{2-}$. Spectral studies showed complete formation of Mb^+NO at all concentrations used. Over an eightfold range of $[FeCDTA^{2-}]$ from 4×10^{-2} to 5×10^{-3} M, the pseudo-first-order rate constant is proportional to $[FeCDTA^{2-}]$ and over a 20-fold range of [NO], the highest of which was a saturated solution, independent of [NO]. These results indicate that $FeCDTA^{2-}$ reduces Mb^+NO directly without prior dissociation to Mb^+ and NO. The rate constant for the reduction was found to be equal to 10 M^{-1} sec^{-1}.

Discussion

The reduction of Mb^+ by $FeCDTA^{2-}$ follows simple second-order kinetics with no intermediates such as an aquo complex of Mb^0 being observed. The rate constant of 28 M^{-1} sec^{-1} is about 100 times less than that observed for the reduction of cytochrome *c* by $FeEDTA^{2-}$ (Hodges et al., 1974). This difference can be accounted for by the large difference of 6 kcal/mol in the activation enthalpies. The entropy of activation of −13 eu is a little lower than the value of −18 eu observed for the reduction of cytochrome *c*. Interestingly, the rate constant for the oxidation of Mb^0 by $FeCDTA^-$ of 148 M^{-1} sec^{-1} is about the same as that of 110 M^{-1} sec^{-1} calculated for the oxidation of reduced cytochrome *c* by $FeEDTA^-$.[5]

The most interesting and fundamental question involved in all redox reactions of heme proteins is that of how electrons are transported to the heme. Previous studies on the mechanism of redox reactions of cytochrome *c* have implicated the possibility of a conformational change as being important in the overall mechanism of electron transport (Yandell et al., 1973; Cassatt and Marini, 1974). The mechanism of reduction of cytochrome *c* by $FeEDTA^{2-}$ however, was compatible with a simple outer-sphere reaction (Hodges et al., 1974) although a more complex mechanism could not be ruled out. Our kinetic results for the reduction of myoglobin by $FeCDTA^{2-}$ are also consistent with a simple outer-sphere mechanism. There is no deviation of the reaction from second-order kinetics, and the activation parameters are of the right order of magnitude.

These observations alone, however, do not completely rule out an inner-sphere mechanism where the $FeCDTA^{2-}$ is bound to the heme iron either through a $CDTA^{4-}$ bridge or through a ligand attached to the heme iron. The latter is inconsistent with the data observed for Mb^+OH^- and Mb^+CN^-. These complexes would be expected to be reduced more rapidly than Mb^+ since CN^- and OH^- are better bridging groups than H_2O. Bridging through the $CDTA^{4-}$ cannot be ruled out as easily since the rates of reduction are similar to rates of substitution in Mb^+. We feel, however, that bridging through the $CDTA^{4-}$ moiety is highly unlikely. The heme pocket is not large enough to easily accommodate the relatively bulky $FeCDTA^{2-}$ molecule. The affinity would be further decreased by the necessity of bringing a highly hydrophilic molecule into the hydrophobic heme pocket. In this respect, it is important to note that reduction of Mb^+ by $FeEDTA^{2-}$, a much smaller molecule than $FeCDTA^{2-}$, occurs with a similar rate constant and activation parameters. Furthermore, in the reduction of Mb^+NO, displacement of NO followed by bridging through the $CDTA^{4-}$ molecule is ruled out by the lack of dependency of the rate constant on [NO]. Similarly, the fact that Mb^+CN^- can be reduced directly shows that such a bridging mechanism in this case is impossible.

The effect of the ligand coordinated to the heme iron on the rate of reduction is interesting. Both CN^- and OH^- decrease the reduction potential of myoglobin because they bind tightly to Mb^+ and either weakly or not at all to Mb^0 and thus stabilize Mb^+ relative to Mb^0. Both ligands markedly decrease the rate of reduction. NO, on the other hand, binds more tightly to Mb^0 than to Mb^+. Mb^+NO is reduced with a rate constant similar to that of Mb^+ and much greater than Mb^+CN^- which, like Mb^+NO, is a low-spin d^5 complex. Similarly, the reduction potential of myoglobin is lower than that of cytochrome *c* and is reflected by a rate constant for reduction 100 times that of myoglobin. Thus, the same factors that influence the relative stabilities of complexes of Fe^{III} heme proteins compared to Fe^{II} heme proteins play an important role in the mechanism of reduction. One reason for this may be that the iron–ligand bond could be loosened in the transition state. Such a loosening would not occur in the case of Mb^+NO since the product remains bound to NO.

[4] At very low $[FeCDTA^{2-}]$, k_{obsd} approaches zero. However, the concentrations of $FeCDTA^{2-}$ needed to produce deviations from eq 8 are two orders of magnitude less than the concentrations employed here.

[5] This value was calculated using values of 0.12 V (Kolthoff and Auerbach, 1952) and 0.26 V (Margalit and Schejter, 1973) for the reduction potentials of $FeEDTA^-$ and cytochrome *c*.

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Mode of Inhibition of Herpes Simplex Virus DNA Polymerase by Phosphonoacetate†

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ABSTRACT: Phosphonoacetate is a highly specific inhibitor of Herpes simplex virus-induced DNA polymerase. Sensitivity of herpesvirus type 1 or type 2 induced DNA polymerase to the drug was similar. However, DNA polymerases from other sources such as the host cells (Wi-38), *Micrococcus luteus*, and hepatitis B virus were highly resistant. In addition, *Escherichia coli* RNA polymerase and reverse transcriptase of Rous sarcoma virus were also insensitive to the drug. Enzyme kinetic studies showed that inhibition was noncompetitive with respect to deoxyribonucleotide triphosphates. The K_i value was about 0.45 μM. The apparent K_m values for dTTP, dATP, dCTP, and dGTP were 0.71, 0.75, 0.42, and 0.39 μM, respectively. The base composition of template has no profound effect on the extent of inhibition. The drug caused uncompetitive inhibition with respect to template which indicated that phosphonoacetate did not bind directly to template DNA. Results are presented which suggest that phosphonoacetate did not affect the formation of the enzyme-DNA complex but probably inhibited the elongation step of DNA polymerase reaction.

Sodium phosphonoacetate has been shown to suppress Herpes simplex virus (HSV)[1] replication in tissue culture (Overby et al., 1974) and in model animal systems (Shipkowitz et al., 1973). The former studies also demonstrated that this compound selectively inhibited viral DNA synthesis without effect on host cell DNA synthesis. This result was confirmed by a recent study (Mao et al., 1975), in which HSV-induced DNA-dependent DNA polymerase and normal cellular DNA polymerases were isolated and it was found that phosphonoacetate specifically inhibited HSV-induced DNA polymerase. However, the mode of action of phosphonoacetate was not elucidated.

Inhibition of DNA synthesis in vitro may occur at several different sites—the DNA template, the substrate, or the polymerase. Numerous antibiotics and synthetic compounds such as actinomycin, olivomycin, chromomycin, daunomycin, acridine, and ethidium bromide bind to DNA (Müller et al., 1971), thereby impairing the template function of DNA. These usually demonstrate little selective tox-

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[1] Abbreviation used is: HSV, Herpes simplex virus.