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Oxidation–Reduction Reactions of Hemoglobin A, Hemoglobin M Iwate, and Hemoglobin M Hyde Park†

Tatsuo Yamada, Claudia P. Marini, and James C. Cassatt*

ABSTRACT: The kinetics and equilibrium of the redox reactions of hemoglobin A, hemoglobin M Iwate, and hemoglobin M Hyde Park using the iron(II) and iron(III) complexes of *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetate ($CDTA^{4-}$) as the reducing and oxidizing agents have been studied. With respect to the equilibrium it was found that hemoglobin M Iwate (where the β chains were reduced) was more readily reduced than hemoglobin M Hyde Park (where the α chains are reduced). This difference was shown to be a result of a difference in the rate constant for reduction but not oxidation. The observed rate constants for the reduction of all three hemoglobins were shown to decrease with increasing pH. This was attributed to a decrease in the [T]/[R] ratio. The observed rate constants for the oxidation reaction were shown to increase with increasing pH. Accompanying this increase was a change in the kinetic profile for hemoglobin A from pseudo first order to one in which the rate increased as the extent of reaction increased. Inositol hexaphosphate had no effect on the rate of oxidation of deoxyhemoglobin A. This was a result of binding of $FeCDTA^{2-}$ or $HCDTA^{3-}$ to the protein. However, in the presence of inositol hexaphosphate, the reduction of methemoglobin A exhibited biphasic kinetics. This result was interpreted in terms of the production of a small amount of a conformation which was more readily reduced.

The conformational changes involved in the oxidation of deoxyhemoglobin to methemoglobin are believed to be the same (T → R) as those involved in the oxygenation reaction. Nonetheless, in contrast to the oxygenation reaction, the "Hill" constant for the oxidation reaction varies with pH with increasing cooperativity being observed with increasing pH (Brunori et al., 1969; Antonini et al., 1964; Kilmartin, 1973). Two different mechanisms have been proposed for the variation of the "Hill" constant with pH. The first proposed by Perutz (1973) and Kilmartin (1973) is that the low value for the cooperativity is a result of the relatively high concentration of the T conformation present in methemoglobin at low pH, al-

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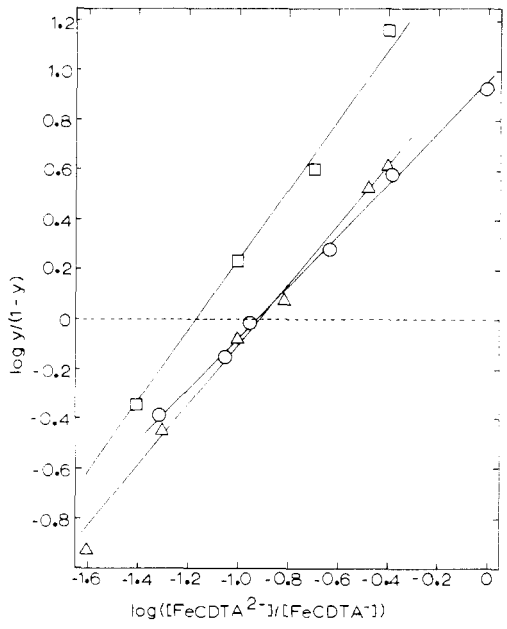

FIGURE 1: The $\log[y/(1-y)]$ (where y is the fraction reduced) determined at 555 nm as a function of $\log([FeCDTA^{2-}]/[FeCDTA^{-}])$ for Hb A (○); Hb M Iwate (□); and Hb M Hyde Park (△). The hemoglobin concentration was about 10^{-5} M, ionic strength was 0.2 M, pH 6.8, and temperature 25 °C.

though the predominant conformation is R. The basis for this mechanism is in part the observation that pronounced changes in the visible bands of the hemoglobin spectrum occur when IHP[1] is added to the solution (Perutz et al., 1974). These changes were interpreted to indicate that IHP causes an overall change from the R to the T conformation. Consistent with this observation was the result that addition of IHP resulted in a complete loss in the cooperativity of the redox reaction of hemoglobin (Kilmartin, 1973).

An alternative mechanism proposed by Brunori et al. (1968a) and supported by the studies of Edelstein and Gibson (1975) is that the low degree of cooperativity is a result of the heterogeneity in the α and β chains in the intact tetramer with respect to their redox potential. The effect of IHP on the cooperativity was explained by an increase in the degree of heterogeneity while the increase in cooperativity with pH was explained by an increase in the [T]/[R] ratio with increasing pH. Support for this hypothesis was obtained by the measurement of the relative amounts of oxidized α and β chains in mixtures of methemoglobin and deoxyhemoglobin by taking advantage of the fact that oxidized α and β chains react with azide at substantially different rates (MacQuarrie and Gibson, 1971; Edelstein and Gibson, 1975). These results showed that the β chain was preferentially reduced and that the difference amounted to about a factor of two in the equilibrium constant for reduction.

As a result of the importance of the redox process in hemoglobin, we decided to study both the oxidation and reduction reactions of hemoglobin using the $FeCDTA^{2-}/FeCDTA^{-}$ system. We had previously used this system for a study of the redox reaction of myoglobin over a wide range of pH (Cassatt et al., 1975). We thought this system would be ideal because the potential of 0.09 V is close to that of hemoglobin and the system is stable over the range of pHs indicated in this study.

[1] Abbreviations used are: IHP, inositol hexaphosphate; Hb, hemoglobin; $CDTA^{4-}$, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate; 2,3-DPG, 2,3-diphosphoglyceric acid; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

In addition, we decided to investigate the redox properties of the individual α and β chains by examining the redox properties of hemoglobin M's, hemoglobin M Iwate (α87 His → Tyr) and hemoglobin M Hyde Park (β92 His → Tyr) in which the α and β chains exist normally in the oxidized states as a result of substitution of a tyrosine for the proximal histidine.

Experimental Section

Materials. Hb A was obtained from lysates from out-dated blood from the Georgetown University Hospital Blood Bank. It was prepared according to the method of Drabkin (1949), recrystallized three times, and stored frozen. Hb M Iwate and Hb M Hyde Park were obtained from lysates obtained from patients heterozygous in these abnormal hemoglobins and kindly supplied by Dr. Paul Heller of the VA Hospital, Chicago, Illinois. The Hb M's were purified by ion-exchange chromatography (Nishikura et al., 1975; Ranney et al., 1968). The distinct spectra of the hemoglobin M samples were the same as those previously published (Nishikura et al., 1975; Hayashi et al., 1968).

In all cases, 2,3-DPG was stripped from hemoglobin samples by gel filtration with Sephadex G-25 after equilibration with 0.1 M NaCl. Where appropriate, methemoglobin was prepared by oxidation of oxyhemoglobin with 2 equiv of ferricyanide. Excess ferricyanide and ferrocyanide were removed by gel filtration on Sephadex G-25 in 0.1 M KCl. Traces of methemoglobin in oxyhemoglobin samples were removed by adding a small amount of dithionite in the cold and quickly pouring the solution on a column of mixed bed ion-exchange resin, AG-501 (from Bio-Rad), to remove the dithionite and its oxidation products (Brown and Mebine, 1969). Because of sensitivity to autoxidation, $FeCDTA^{2-}$ was prepared by addition of solutions of $HCDTA^{3-}$ (about a 10% excess to ensure complete complex formation) and buffer, which had been deaerated, to a deaerated solution of $FeCl_2$ via gas tight syringes (Cassatt et al., 1975). $FeCDTA^{-}$ was also prepared in situ by addition of $HCDTA^{3-}$ (a 10% excess) to $FeCl_3$ followed by addition of buffer. All other chemicals were reagent grade or better.

Methods. Kinetic experiments were carried out on an Aminco-Morrow stopped-flow apparatus with the temperature controlled to ±0.1 °C with a circulating water bath. Because of the sensitivity of most of the systems studied to atmospheric oxygen, solutions were generally deaerated prior to reaction with prepurified N_2 in flasks equipped with serum caps near the bottom. The stopped-flow apparatus was flushed several times with deaerated solutions before use. Spectra were determined on Beckman Acta CV spectrophotometer. In those cases where the solutions were oxygen sensitive, a cuvette equipped with a serum cap was deoxygenated with prepurified N_2 and the sample introduced by means of a gas-tight syringe.

Results

Equilibria. The equilibrium constants for the redox reactions at neutral pH (using 0.01 M Bistris buffer) and an ionic strength of 0.2 M (with added KCl) were measured by incubating methemoglobin (A and M) with varying ratios of $[FeCDTA^{2-}]/[FeCDTA^{-}]$ and determining the extent of reaction spectrophotometrically as had previously been done for myoglobin (Cassatt et al., 1975). In the case of Hb M's, it was clear from the spectra that only the normal chains were reduced. This result is expected because of the rather mild nature of the reducing agent. Values for $\log[y/(1-y)]$ were plotted as a function of $\log([FeCDTA^{2-}]/[FeCDTA^{-}])$ as shown in Figure 1. The equilibrium constant and "Hill" con-

TABLE I: Data for the Redox Reactions of Hemoglobin at Neutral pH Using $FeCDTA^{2-}$ and $FeCDTA^{-}$ as Electron-Transfer Agents.[a]

Species	K_{eq} (reduction)	"Hill" constant	e° [b]	K_{eq} (kinetic)[c]
Hb A	8.91	1.03	0.146	9.2
Hb M Iwate	45.7	1.43	0.185	7.8
Hb M Hyde Park	14.1	1.25	0.158	4.04

[a] The data were obtained at an ionic strength of 0.2 M and a temperature of 25 °C. [b] Obtained using an e° value of 0.90 V for $FeCDTA^{2-}/FeCDTA^{-}$ (Cassatt et al., 1975). [c] The ratio of the forward and reverse rate constants obtained from the limiting values of the rate constants at acidic pH (see Table II).

stant were then obtained from the equation:

$$\log[y/(1-y)] = n \log([FeCDTA^{2-}]/[FeCDTA^{-}]) + \log K \quad (1)$$

where n is the "Hill" constant and K the equilibrium constant for the reaction:

$$metHb + FeCDTA^{2-} \rightleftarrows deoxyHb + FeCDTA^{-}$$

Values for n, K, and the reduction potential obtained using a value of 0.09 V for the potential of $FeCDTA^{-}$ are shown in Table I. As is seen, the reaction is noncooperative with Hb A with a slight degree of cooperativity being observed for the Hb M's. It is also seen that the α chain is more difficult to reduce than the β chain and that the difference in the equilibrium constants is about a factor of two.

Kinetics. The kinetics of reduction of methemoglobin A by $FeCDTA^{2-}$ were determined under pseudo-first-order conditions at 25 °C and an ionic strength of 0.2 M (with added KCl). Bistris or Tris buffers (0.01 M) were generally used to maintain the pH. The reactions were generally first order for three half-lives except for a slight lag period near the beginning. This lag period was eliminated by maintaining more stringent anaerobic conditions or by using a wavelength isosbestic for oxy- and deoxyhemoglobin. The half-life, $t_{1/2}$, from which the observed rate constants were calculated was obtained from plots of $\log|A - A_{\infty}|$ as a function of time. The observed rate constant was found to be linear with $FeCDTA^{2-}$ concentration (1.0×10^{-3} to 1.0×10^{-2} M). The reaction was studied as a function of temperature. An Arrhenius plot yielded a straight line over a 25 °C range (10 to 35 °C) with an enthalpy of activation of 10.5 kcal/mol and entropy of activation of −18.5 eu. The rate constant was found to vary as a function of pH. The data were arbitrarily analyzed assuming a mechanism where there are two species connected by a protonation and

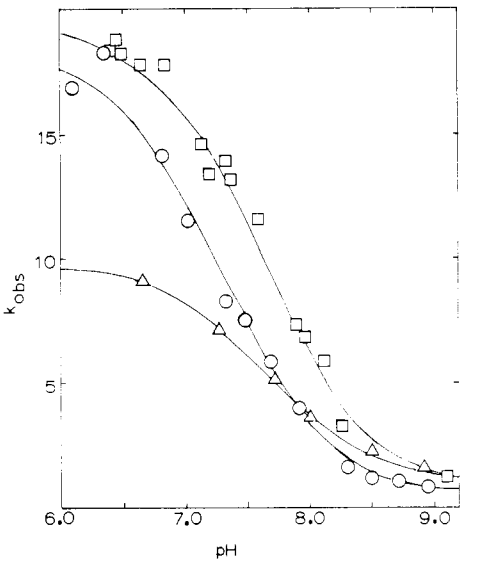

FIGURE 2: The observed second-order rate constant, k_{obsd}, as a function of pH for the reduction of Hb A (○), Hb M Iwate (□), and Hb M Hyde Park (△) by $FeCDTA^{2-}$. The ionic strength was 0.2 M and temperature was 25 °C. The hemoglobin concentration was about 10^{-5} M. The parameters used to calculate the curves shown are given in Table II.

that these two species are reduced with different rate constants as shown below:

$$metHbH^{+} \overset{K_a}{\rightleftarrows} metHb + H^{+}$$

$$metHbH^{+} + FeCDTA^{2-} \xrightarrow{k_1} deoxyHb + FeCDTA^{-} + H^{+}$$

$$metHb + FeCDTA^{2-} \xrightarrow{k_2} deoxyHb + FeCDTA^{-}$$

The rate law consistent with this mechanism is:

$$\text{rate} = \frac{k_1[H^{+}] + k_2K_a}{K_a + [H^{+}]}[metHb]_T[FeCDTA^{2-}] \quad (2)$$

or

$$k_{obsd} = \frac{k_1[H^{+}] + k_2K_a}{K_a + [H^{+}]} \quad (3)$$

where $[metHb]_T$ is the total unreacted hemoglobin and $k_{obsd} = t_{1/2}/0.693[FeCDTA^{2-}]$. Values for k_{obsd} as a function of pH are shown in Figure 2. The lines are calculated using the rate constants and equilibrium constant shown in Table II.

In a similar fashion, the kinetics for the reduction of the fully

TABLE II: Kinetic Parameters for the Redox Reactions of Hb A, Hb M Iwate, and Hb M Hyde Park Using $FeCDTA^{-}$ and $FeCDTA^{2-}$ as the Oxidizing and Reducing Agents.

Reactants	Oxidation[a]			Reduction[a]		
	k_1[b]	k_2[c]	pK_a[d]	k_1[b]	k_2[c]	pK_a[d]
Hb A (no IHP)	2.0	9.2	8.05	18.4	0.3	7.3
Hb A (10^{-3} M IHP)	2.5	6.3	8.10			
Hb M Iwate	2.5	4.66	8.30	19.4	0.55	7.6
Hb M Hyde Park	2.4	9.45	7.95	9.7	0.56	7.7
Myoglobin[e]				28.0	4.8	8.86

[a] Rate constants were obtained at an ionic strength of 0.2 M and a temperature of 25 °C. [b] Limiting rate constant in acid media. [c] Limiting rate constant in basic media. [d] Equilibrium constant for the acidic and basic forms obtained from the kinetic data. [e] From Cassatt et al. (1975).

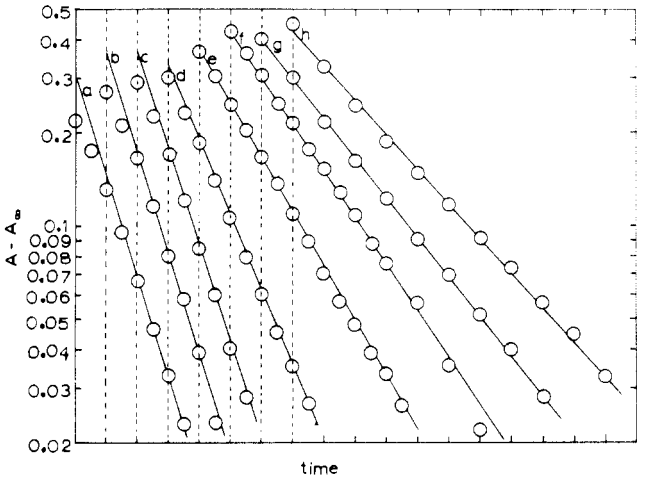

FIGURE 3: Semi-log plot of $|A - A_\infty|$ as a function of time for the oxidation of deoxyHbA by FeCDTA$^-$ at the following pHs: (a) 8.98; (b) 8.49; (c) 8.35; (d) 8.10; (e) 7.80; (f) 7.58; (g) 7.21; (h) 6.80. Each division on the abscissa is 10 s. The hemoglobin concentration was 5×10^{-5} M. The reaction was followed at 555 nm. The temperature was 25 °C and ionic strength was 0.2 M.

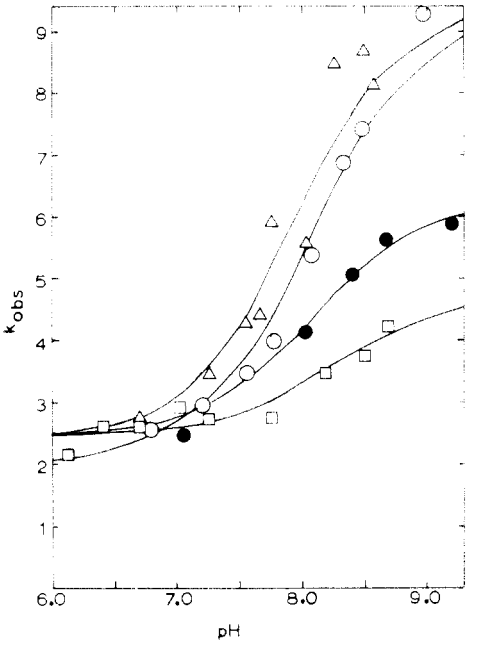

FIGURE 4: The observed second order rate constant, k_{obsd}, for the oxidation of: deoxyHb A (in the absence of IHP (○) or in the presence of 10^{-3} M IHP (●); half-reduced Hb M Iwate (□); and half-reduced Hb M Hyde Park (△) by FeCDTA$^-$ as a function of pH. The temperature was 25 °C and ionic strength was 0.2 M. The parameters used to calculate the curves shown are included in Table II.

oxidized forms of Hb M Iwate and Hb M Hyde Park were determined. A spectral analysis showed that the products were the half-reduced forms of the proteins. The rate constant was also found to vary with pH. The data were analyzed as described above for Hb A and the results are shown in Figure 2 with appropriate kinetic parameters shown in Table II.

The kinetics of oxidation of deoxyhemoglobin A by FeCDTA$^-$ were determined at 25 °C and an ionic strength of 0.2 M. Oxyhemoglobin was deoxygenated with prepurified N_2 or by addition of 0.04 to 0.08 mg/mL of dithionite under anaerobic conditions. This amount of dithionite was sufficient to remove O_2 but did not reduce the concentration of FeCDTA$^-$ significantly (present at 10^{-2} M). Both methods yielded identical rate constants. The observed rate constant was linear with [FeCDTA$^-$] from 10^{-3} to 10^{-2} M (dithionite was not added at the lower concentrations). Good first-order traces were observed at neutral pH. However, at pHs greater than eight, the rate was observed to increase as the extent of reaction increased as shown in Figure 3. This behavior had previously been observed by Antonini et al. (1965) and was seen at several different wavelengths (500, 555, and 630 nm) and was reproducible. To obtain the rate constants shown in Figure 4, the last portion of the curves was used. The rate constant was observed to increase with increasing pH and the data were arbitrarily analyzed according to eq 2 assuming a mechanism analogous to that assumed for the reduction of methemoglobin. The results are shown in Figure 4 and constants used to fit the data are shown in Table II.

In a similar fashion, the kinetics of oxidation of the normal chains of Hb M Iwate and Hb M Hyde Park by FeCDTA$^-$ were determined. Half-reduced Hb M was produced by making use of the difference in the rates of reduction of the chains with dithionite (Nishikura et al., 1975). Fully oxidized hemoglobin M was deoxygenated and a small amount (0.04 mg/mL)[2] of dithionite added. Within a few minutes (less than 5) the rate was measured on the stopped flow apparatus. A spectral analysis showed that this method did indeed produce half-reduced hemoglobin M. As above, the rate was observed to increase with increasing pH. The results were analyzed as above and are shown in Figure 4. The constants used to fit the data are shown in Table II.

Ionization Constants of Hb M's. Because of the previously observed role of the ionization of the coordinated water in the mechanism of reduction of metmyoglobin (Cassatt et al., 1975), we determined the pK_a of the ionization of the coordinated water in fully oxidized hemoglobin M Iwate and hemoglobin M Hyde Park by measuring the visible spectrum as a function of pH under the conditions of our other experiments, ionic strength of 0.2 M, temperature of 25 °C. Good isosbestic points were obtained. The data were analyzed according to the equation:

$$\mathrm{pH} = \mathrm{p}K_a + n \log \frac{[\mathrm{metHbOH}^-]}{[\mathrm{metHb}]}$$

where metHbOH$^-$ refers to methemoglobin with the heme coordinated water ionized. The results are consistent with a value for n of 1.02 in both cases and pK_as of 8.18 and 8.15 for Hb M Iwate and Hb M Hyde Park, respectively.

Influence of Phosphates. The effect of the addition of 10^{-3} M IHP on the oxidation of deoxyhemoglobin A by FeCDTA$^-$ is shown in Figure 4. As can be seen, there is virtually no effect at neutral pH but some inhibition at basic pH. In the case of the reduction of Hb$^+$ by FeCDTA^{2-}, addition of IHP results in a biphasic reaction as shown in Figure 5. These results are consistently and reproducibly obtained at all pHs (from 6.5 to 8.5) and several wavelengths (500, 555, and 630 nm). Rate constants obtained from the latter portions of the curve are similar to those obtained in the absence of IHP.

We attempted to measure binding of FeCDTA^{2-} to methemoglobin A by adding CaCDTA^{2-} (prepared in situ by addition of a 10% excess of HCDTA^{3-} to Ca^{2+}) to metHb$^+$ and measuring the difference spectrum thus produced, as had previously been done for binding of IHP to methemoglobin A (Perutz et al., 1974). Under the conditions of our kinetic experiments, no difference spectrum was observed. To measure the binding of FeCDTA^{2-} to deoxyhemoglobin A, we reduced

[2] This amount is considerably less than that used by Nishikura et al. (1975) to fully reduce the Hb M's.

methemoglobin A with $FeCDTA^{2-}$ in the presence of a fluorescent analogue of 2,3-DPG, 8-hydroxyl-1,3,6-pyrenetrisulfonate (MacQuarrie and Gibson, 1972). When compared with methemoglobin reduced in the presence of 10^{-3} M IHP, no quenching of fluorescence was observed. From these experiments, we conclude that some component, i.e., $FeCDTA^{2-}$, $FeCDTA^{-}$, H_2CDTA^{3-}, does bind to deoxyhemoglobin A and displaces the fluorescent analogue but that nothing in our solutions is binding at the phosphate binding site in methemoglobin A.

Discussion

Edelstein and Gibson (1975) proposed that the low level of cooperativity of the redox reactions of HbA was a result of heterogeneity in the α and β chains. They showed that the free energy for the reduction of the β chains was lower than for the α chain. Our data are consistent with this observation in that the equilibrium constant for reduction of Hb M Iwate, in which the β chains are reduced, is about twice that observed for Hb M Hyde Park, in which the α chain is reduced. This difference in equilibrium constant is manifested in a difference in the rate of reduction.

In the reduction of HbA by $FeCDTA^{-}$, the reaction is first order with no hint of a biphasic reaction. Thus, the observations that (1) the α chains are reduced more slowly than the β chains and (2) that the rate of reduction of the β chains is near that observed for normal hemoglobin suggests that in normal hemoglobin the sequence of reaction is first reduction of the β chains followed by reduction of the α chains. However, once the β chains are reduced, there are conformation changes sufficient to accelerate the reduction of the α chains so that in effect only one reaction is seen.

The shape of the pH profile for the reduction of hemoglobin is similar to that observed in the reduction reactions of myoglobin (Cassatt et al., 1975) and monomeric hemin dissolved in sodium dodecyl sulfate micelles (Cassatt et al., 1977). In these cases, the reduction in rate at high pH is due to a smaller rate constant being associated with the reduction of the hydroxide species than with the aquo. In the case of myoglobin, the pK_a calculated from the kinetic data is in excellent agreement with that observed for the ionization of the heme coordinated water molecule. This explanation is clearly not valid for the reduction of hemoglobin. The pK_a consistent with the kinetic data is 7.3 compared with 8.05 for the pK_a associated with the ionization of the heme coordinated water molecule (Brunori et al., 1968b). It is, however, near the midpoint for the change in the cooperativity in phosphate buffer. We, therefore, propose that the T form, a small amount of which is present in equilibrium with the R form, is preferentially reduced because of its resemblance to the product, deoxyhemoglobin. If the [T]/[R] ratio decreases with increasing pH as predicted by Edelstein and Gibson (1975), then, based on this proposed mechanism, the observed rate constant will decrease with increasing pH and follow the change in cooperativity. A similar mechanism involving electron transfer through a conformation which resembles the product has been proposed for the oxidation of reduced cytochrome c by ferricyanide (LeBon and Cassatt, 1977). Similar arguments could be made for the pH profiles of the Hb M's although the changes in the cooperativity would not be as dramatic nor need they be the same because of the presence of the abnormal chains.

At neutral pH the rates of oxidation of Hb A, Hb M Iwate, and Hb M Hyde Park are virtually the same. At high pH it is observed that Hb M Iwate is reduced more slowly than Hb A and Hb M Hyde Park, which are both reduced at about the same rate. In terms of the sequence of events, two are possible:

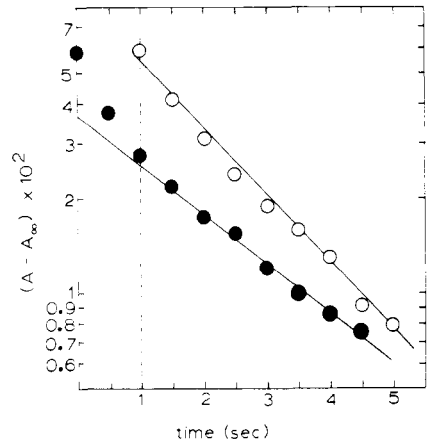

FIGURE 5: A semilog plot of $(A - A_\infty) \times 10^2$ as a function of time for the reduction of metHbA in the presence (●) and absence (○) of 10^{-3} M IHP. The reaction was followed at 630 nm. The hemoglobin concentration was 2×10^{-5} M, the pH 7.0, the temperature 25 °C, and ionic strength 0.2 M.

$$\alpha_2^0\beta_2^0 \rightarrow \alpha_2^0\beta_2^+ \rightarrow \alpha_2^+\beta_2^+$$

or

$$\alpha_2^0\beta_2^0 \rightarrow \alpha_2^+\beta_2^0 \rightarrow \alpha_2^+\beta_2^+$$

where the superscript 0 refers to the chain with iron(II) and the superscript + refers to the chain with iron(III). The intermediate states produced, $\alpha_2^0\beta_2^+$ and $\alpha_2^+\beta_2^0$, are similar, but not identical, to the starting materials for the oxidation of Hb M Hyde Park and Hb M Iwate, respectively. Based on the observation that the rates of oxidation of Hb M Iwate, Hb M Hyde Park, and Hb A are the same, and that the semilog plots yield good linear pseudo-first-order profiles we propose that at neutral pH the α and β chains are oxidized with the same intrinsic rate constant.

At basic pH, the situation is different. The semilog plots show a lag period and the rate of oxidation of Hb M Iwate is decidedly slower. From the latter part of the reaction of Hb A, a rate constant which is the same as that observed for the oxidation of Hb M Hyde Park is obtained. This suggests that the general sequence of events at basic pH is:

$$\alpha_2^0\beta_2^0 \rightarrow \alpha_2^0\beta_2^+ \rightarrow \alpha_2^+\beta_2^+$$

Thus, the rate of oxidation of the α chains is retarded more than the β chains so that the first step is oxidation of the β chains. However, once adjacent to oxidized β chains, the rate of oxidation of the α chains is increased—thus the observed profile. The ionizable group responsible for the rate change with pH is unknown.

The lack of an effect of IHP on the rate of oxidation of deoxyhemoglobin A by $FeCDTA^{2-}$ suggests that $FeCDTA^{2-}$ (or perhaps $HCDTA^{3-}$ which is also present) also binds to the IHP site and mimics any effect IHP might potentially have on the rate. The observation that $FeCDTA^{2-}$ effectively competes with a fluorescent analogue of IHP supports this hypothesis.

In the reduction of methemoglobin by $FeCDTA^{2-}$, addition of IHP results in a biphasic reaction being observed. These data are consistent with a mechanism where binding of IHP to methemoglobin does result in the production of a small amount of another conformation which is more easily reduced and where these two forms do not interconvert within the time

course of the experiment. Olsen (1976) has, in fact, shown that two forms (not R and T) are produced on addition of IHP to methemoglobin and that they are not rapidly interconvertible.

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Sedimentation Studies of the Reversible Dimer–Tetramer Transition Kinetics of Concanavalin A†

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ABSTRACT: The self-association of concanavalin A in solution at pH 7 was analyzed at different temperatures (4, 15, 23, 31 °C) using the analytical band-sedimentation technique linked with a computer simulation method. In the concentration range investigated, which is close to that used in studying the agglutinating and binding properties of concanavalin A, the polymerization of this lectin depends on temperature; up to 23 °C, concanavalin A is essentially subject to a reversible dimer–tetramer transition; above this temperature, the specific dimer–dimer interaction is disturbed by isodesmic associations, which are negligible at lower temperatures. The kinetic parameters of the dimer–tetramer transition were determined at 4, 15, and 23 °C: for 4 °C $\leq t \leq$ 23 °C the equilibrium constant K varied from 1.9×10^4 M^{-1} to 6.9×10^5 M^{-1}, and the relaxation time values were very large varying from 6500 to 440 s which indicated that important structural changes were taking place. The experimental results were used to calculate values for the following thermodynamic constants of the associative reaction: ΔG_0, ΔH_0, ΔS_0, and $\Delta G_{\pm}^{\ddagger}$, $\Delta H_{\pm}^{\ddagger}$, $\Delta S_{\pm}^{\ddagger}$. This reaction is essentially characterized by large and positive values of ΔH_0 (25–39 kcal) and ΔS_0 (118–160 cal/deg). These findings led us to conclude that the two dimers associate through a large hydrophobic binding region. This conclusion is supported by previous crystallographic results (Reeke, G. N., Jr., Becker, J. W., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1525, 1547) and allows us to propose a model for this interaction process.

The primary sequence and the three-dimensional structure of concanavalin A have been well established (Wang et al., 1975; Cunningham et al., 1975; Becker et al., 1975; Reeke et al., 1975). Moreover, the quaternary structure of concanavalin A in solution is pH and temperature dependent: at physiological pH (pH 7.2) and 0 °C the protein is a dimer, but at 37 °C it is a tetramer. At pH 5.6, in the same temperature range, it is always a dimer (McKenzie et al., 1972; Huet et al., 1974; Huet, 1975). Nevertheless, the thermodynamic and kinetic characteristics of the transition between these two forms of the molecule and between them and possibly more polymerized states have not been studied. Previous analytical centrifugation studies on the variation of the concanavalin A sedimentation

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