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Anomalous Azide Binding to Metmanganomyoglobin†

Brian M. Hoffman* and Quentin H. Gibson*

ABSTRACT: The reaction of metmanganomyoglobin ($Mn^{III}Mb$) with azide presents a novel pattern with direct evidence for kinetic complexity. Although the kinetic analysis may not be unique, it appears that the azide complex cannot be fully formed, as judged by spectrophotometric changes, even with an infinitely great $[N_3^-]$. This is interpreted as resulting from an equilibrium between the final spectroscopically observable azide complex and an intermediate species whose spectrum is not substantially different from that of $Mn^{III}Mb$ itself. The two forms of the azide complex appear to exhibit roughly equal proportions at 3 °C. We propose that this intermediate is a weak Mn^{3+}–azide complex in which the metal ion remains out-of-plane toward the imidazole of the proximal histidine, but that the metal lies toward the anion in the "final" complex.

Although it is usually possible to represent heme-protein, ligand reactions by one or more simple second-order forward (on) reactions and first-order reverse (off) reactions, this does not mean that the reactions themselves are simple, but more probably, that single steps are rate determining. There are, indeed, indications that this simple approach may not always be sufficient. For example, Gibson and Roughton (1955) found a discrepancy between the rate of oxygen dissociation from oxymyoglobin as measured by a CO replacement procedure, and as measured by mixing with dithionite. More recently Gibson and Kamen (1966) found multiple deviations from simple expectations in examining the reaction of CO with cytochrome *c*'s from *Rhodospirillum* and *Chromatium,* and their observations were confirmed and extended by Cusanovich and Gibson (1973). Their results could be explained by supposing that the cytochrome *c*'s are able to bind CO in two ways, only one of which is associated with the characteristic spectrophotometric change to the CO cytochrome. We report here an analogous situation in the binding of azide by the Mn^{3+} form of the manganese-substituted globins, metmanganoglobin ($Mn^{III}Hb$) and metmanganomyoglobin ($Mn^{III}Mb$).[1]

The parallels between the functional (Thiele et al., 1964; Yonetani and Asakura, 1969; Bull et al., 1974; Gibson et al., 1974; Hoffman et al., 1975) and structural (Moffat et al., 1974) properties of hemoglobin and myoglobin and the manganese-substituted proteins are particularly close. However, the ligation properties of Mn^{3+} and Fe^{3+} porphyrins are drastically affected by the difference of a single electron in the d-orbital populations. Thus, for example, cyanide, nitrite, and azide bind to met-Hb and met-Mb producing the low-spin, six-coordinate hemochrome (see Antonini and Brunori, 1971), whereas only azide binds to $Mn^{III}Mb$ and $Mn^{III}Hb$ (Thiele et al., 1964) and the resultant six-coordinate metal complex remains high spin ($S = 2$) (Boucher, 1972). Furthermore, water as the sixth ligand to met-Hb undergoes an ionization to OH^- with $pK \sim 8$, whereas $Mn^{III}Hb$ shows no such ionization below

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[1] Abbreviations used: $Mn^{III}Hb$; metmanganoglobin, the Mn^{3+} substituted hemoglobin; $Mn^{III}Mb$; metmanganomyoglobin, the Mn^{3+} substituted myoglobin; Tris, tris(hydroxymethyl)aminomethane; TPP, tetraphenylporphinato; IHP, inositol hexaphosphate.

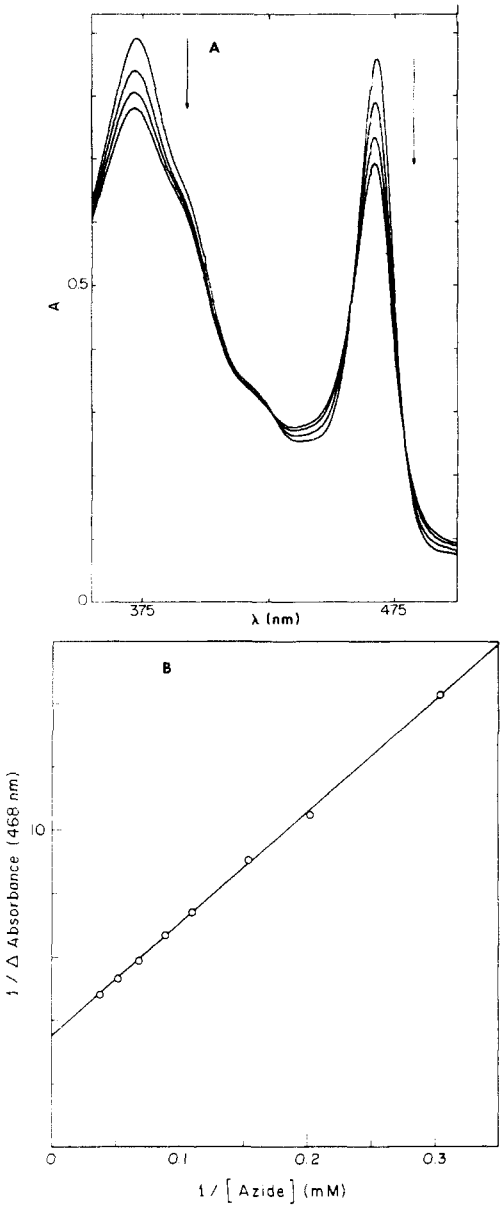

FIGURE 1: Titration of $Mn^{III}Mb$ with azide. (A) Typical spectral changes; $[N_3]$ increases as indicated by arrows. (B) Relation between absorbance excursion and azide concentration. Ordinate, reciprocal of absorbance excursion; abscissa, reciprocal of $[N_3^-]$ in mM^{-1}. $[Mn^{III}Mb]$ = 12.5 mM; phosphate buffer, 0.05 M, pH 7; 22 °C; 1-cm path, 468 nm.

pH ~10 (Hoffman et al., 1975).

We have examined the binding of azide to both sperm whale met-Mb and $Mn^{III}Mb$. Although on the stopped-flow time scale the binding by the Fe^{3+} protein *appears* to be kinetically simple, in azide binding to the Mn^{3+} protein we see two kinetically separable steps.[2] This complexity does not appear to relate to a possible requirement that *any* ligand (e.g., N_3^-) be in an uncharged form (e.g., HN_3) in order to approach the metal ion. We tentatively attribute the behavior difference between metals to the different spin states and attendant stereochemistries of the resultant azide complexes: that of the Mn^{3+} protein is considered to exhibit an equilibrium in which the Mn^{3+} ion moves through the porphyrin central hole (inverts) at a rate which is observable on the time scale of a stopped-flow experiment. In addition, azide binding to $Mn^{III}Hb$ shows the chain heterogeneity observed for binding to met-Hb (Gibson et al., 1969).

Experimental Procedure

Materials. Metmanganoglobin and manganomyoglobin were prepared from sperm whale Mb described by Hoffman et al. (1975). The stock solutions were diluted with buffers as required. Solutions of sodium azide were prepared in the appropriate buffers and their pH checked.

Methods. Stopped-flow kinetic measurements were made with the Durrum Instrument Corp. version of the apparatus of Gibson and Milnes (1964) equipped with the data acquisition devices of De Sa and Gibson (1969). The concentration of manganoglobins was determined spectrophotometrically at 468 nm using ϵ_{mM} 70 cm^{-1} mM^{-1}.

Results

Reaction of $Mn^{III}Mb$ with Azide. The "split Soret" spectrum of $Mn^{III}Mb$ (and $Mn^{III}Hb$) is independent of pH below pH ~9.5. It is not qualitatively changed by the reversible binding of azide, but does undergo a reduction in absorbance, particularly at the longer wavelength maximum (Figure 1A). Apparent equilibrium azide binding curves for $Mn^{III}Mb$ were obtained from the observed spectral changes and are hyperbolic, as expected (Figure 1B). The half-saturation azide concentration ($C_{0.5}$) obtained at pH 7, 5 °C, from these studies (8 mM) decreases by only ~20% on going to pH 6 (6 mM). The observed $C_{0.5}$ increases by slightly less than threefold on warming from 3 °C to room temperature (23 mM, pH 7; 17 mM, pH 6), corresponding to an apparent enthalpy of binding of −9.5 kcal/mol.

The kinetics of azide binding to $Mn^{III}Mb$ was examined at pH values of 6, 7, 7.6, and 9, at 3 °C and 20 °C; several independent protein preparations were employed. The results cannot be reconciled with a simple kinetic scheme involving a bimolecular reaction and a single rate-limiting step. For example, at pH 6 and 7 (2 °C), the observed binding rates are closely proportional to $[N_3^-]$, whereas at pH 9 (2 °C) the observed rate is approximately independent of $[N_3^-]$. In presenting the results we begin by discussing one set of experiments at pH 7.6 and 3 °C. This is done because we find under these conditions that two rate-determining steps contribute observably to the binding process.

A solution of $Mn^{III}Mb$ was mixed with six different solutions of azide covering a concentration range from 0.4 to 25 mM, and the reaction was followed to 90% completion in each case. Plotting the pseudo-first-order rate constants against azide concentration (Figure 2A) gives a reasonably straight line with an intercept at 10^{-1} s and slope of 0.6 mM^{-1} s^{-1}. This result is consistent with a simple reversible reaction with a binding rate of 0.6 mM^{-1} s^{-1} and dissociation constant 17 mM. The measured rates are low enough to permit an accurate estimate of the total absorbance excursion corresponding to each point in Figure 2A, and the reciprocals of these excursions are plotted against the reciprocal of azide concentration in Figure 2B (the lowest point is omitted because of scale considerations—it lies well on the line). A straight line is obtained, again consistent with a simple reversible reaction. The dissociation constant derived from Figure 2B is 11 mM, in satisfactory agreement with the equilibrium $C_{0.5}$ given above; however, this value is significantly lower than that derived from the rate constants.

[2] In a paper published after completion of this work (Giacometti et al., 1975), it was reported that Aplysia Mb reacting with N_3^- exhibits non-simple kinetics. The mechanism they propose links ligand binding with the acid methydroxymet-Hb transition. Since $Mn^{III}Hb$ and $Mn^{III}Mb$ show no such transition between pH 6 and 9.5 (Hoffman et al., 1975), this mechanism is not applicable here.

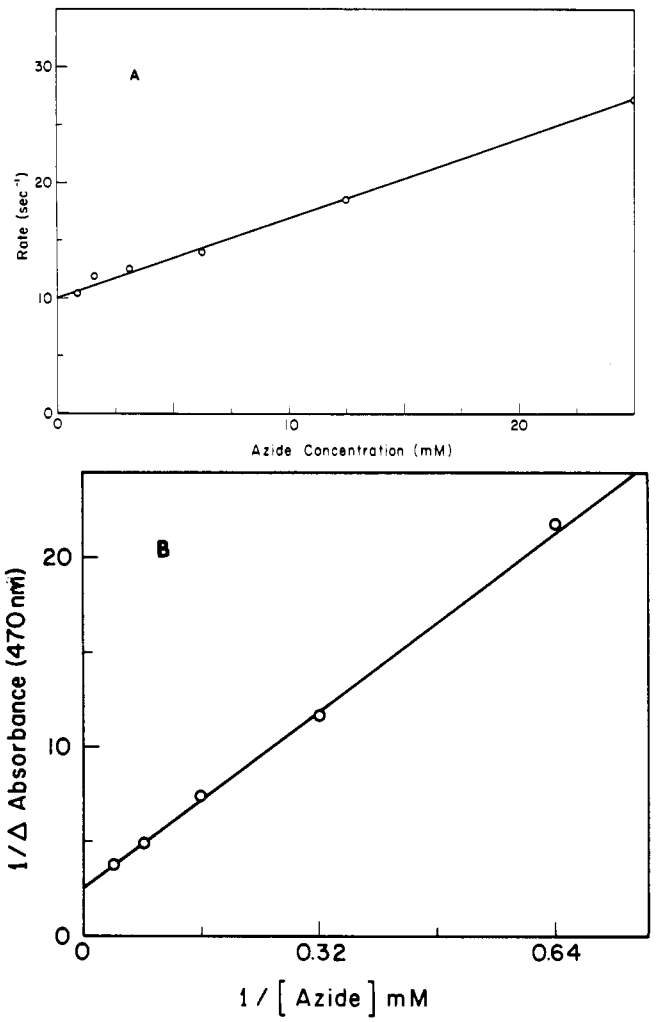

FIGURE 2: Reaction of 9.1 μM $Mn^{III}Mb$ with azide. Phosphate buffer, 0.1 M, pH 7.6; 3 °C; 470 nm. (A) Relation between observed pseudo-first-order rate constant in s^{-1} (ordinate) and azide concentration in mM (abscissa). (B) Relation between total absorbance excursion and azide concentration. Ordinate, reciprocal of absorbance excursion; abscissa, reciprocal of azide concentration in mM^{-1}.

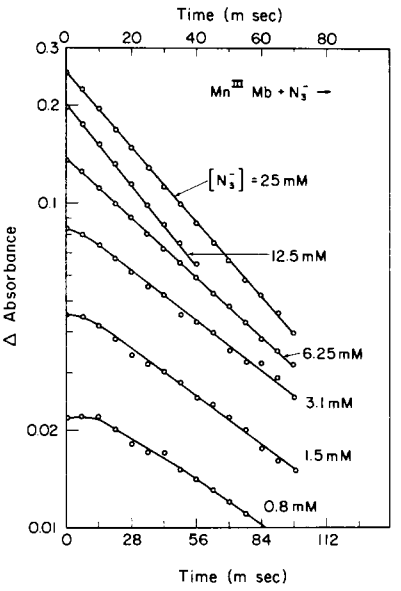

FIGURE 3: Progress curves for the reaction of 9.1 μM $Mn^{III}Mb$ with azide. Phosphate buffer, 0.1 M, pH 7.6; 3 °C; 470 nm, 2-cm path. Upper time scale for 25 mM azide; lower scale for all other curves; azide concentrations adjacent to curves.

Furthermore, a more detailed examination of the time course of azide binding shows that the reactions are only approximately first order and, at the lower azide concentrations, there is regularly observed an appreciable lag before the rate for the main part of the reaction is established (Figure 3).

These findings require a more complicated reaction scheme. In selecting one, the nature of the absorbance changes upon azide binding was taken into account. The solution used had an absorbance of 1.08 at 470 nm in 1 cm before mixing. The total change at 470 nm from Figure 2B was estimated to be about 0.4 at infinite azide concentration so that there was still strong absorbance (0.7) at that wavelength and, as the family of titration curves of Figure 1A shows, the "split Soret" spectrum of $Mn^{III}Mb$ is weakened on addition of azide, but by no means abolished.

A simple mechanism which will accommodate the results is schematically

$$Mn^{3+} + N_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (Mn^{3+}(N_3^-))^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Mn^{3+}\,(N_3^-) \quad (1)$$

where the starred form is not substantially spectrophotometrically different from Mn^{3+}, while the transition to the final (unstarred) azide complex produces a majority of the overall observed change in spectrum. There are many possible alternative schemes, some of which would involve binding of two azide molecules, but that given is simple and sufficient to account for the experiments so far available and is consistent with structural evidence discussed below. A lag in the spectrophotometric progress curves of Figure 3 is introduced by the finite values of k_1 and k_{-1}.

Writing $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$, the equilibrium equation describing the formation of the final, spectroscopically observable form is

$$Y = K_1K_2[N_3^-]/(1 + K_1[N_3^-] + K_1K_2[N_3^-])$$
$$= K_1K_2[N_3^-]/(1 + K_1(1 + K_2)[N_3^-]) \quad (2)$$

where $[N_3^-]$ is the azide concentration and Y the fraction of Mn^{3+} (N_3^-) formed. The equilibrium curve is thus hyperbolic as required by the data of Figure 2B. However, this equation sets a limit to the fraction of metmanganomyoglobin which can be converted to the final azide complex, for, at high $[N_3^-]$, Y tends to $K_2/(1 + K_2) < 1$. Thus, the scheme involves an intrinsic equilibrium between $(Mn^{3+}\,(N_3^-))^*$ and $Mn^{3+}\,(N_3^-)$. Because of this equilibrium the value of $C_{0.5}$ observed in equilibrium measurements is not that azide concentration at which $Y([N_3^-]) = 0.5$, but that at which $Y([N_3^-])/Y(\infty) = Y([N_3^-])(1 + K_2)/K_2 = 0.5$.

In applying this simple scheme to the kinetic data in Figure 3 (pH 7.6, 3 °C), we required that both kinetic and equilibrium results be reproduced simultaneously, by requiring that the observed $C_{0.5} = 11$ mM be reproduced. Use of the *limiting assumption* that Mn^{3+} and $(Mn^{3+}(N_3^-))^*$ have identical spectra leaves undetermined the fractional change in extinction at 468 nm upon $(Mn^{3+}\,(N_3^-))$ formation, $\delta = [\epsilon(Mn^{3+}) - \epsilon(Mn^{3+}(N_3^-))]/\epsilon(Mn^{3+})$. A fixed value of δ was also employed in the fitting procedure, which was then repeated for a range of values of δ.

For $\delta = 0.74$, the observed absorbance changes correspond to a limiting ratio of $Mn^{3+}(N_3^-)$ to $(Mn^{3+}(N_3^-))^*$ of 0.75. Using this value, the time course of azide binding at all six concentrations is reproduced with a mean residual of 0.0024 in absorbance or 0.5% of the total absorbance change. The

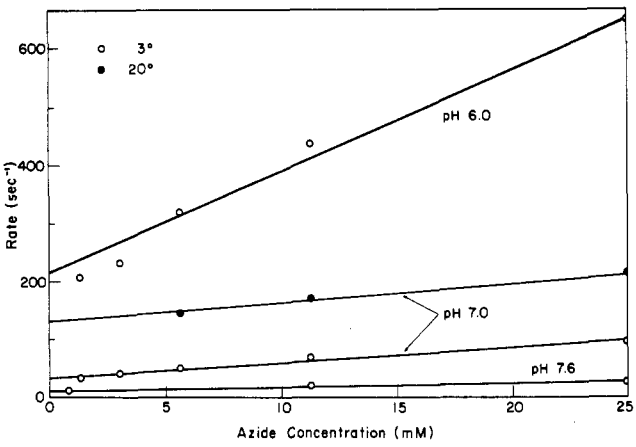

FIGURE 4: Reaction of $Mn^{III}Mb$ with azide at 3 °C (○) and 20 °C (●) (470 nm). Ordinate: pseudo-first-order rate constant (s^{-1}) of observed reaction. Abscissa: azide concentration in mM. Buffers: 0.1 M phosphate at indicated pH.

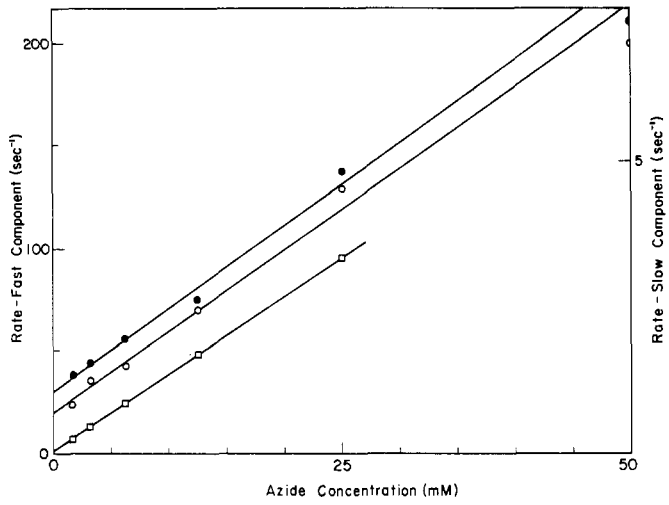

FIGURE 5: Relation between pseudo-first-order rate constants in s^{-1} and azide concentration in mM (abscissa) for the reaction of 13.5 μM $Mn^{III}Hb$ with azide (3 °C, 465 nm): fast component, left ordinate (○); slow component, right ordinate (●). Also shown, reaction of met-myoglobin with azide (22 °C, 434 nm), left ordinate (□). Phosphate buffer: 0.05 M, pH 7.0.

residuals are satisfactorily distributed both between and within the six runs. The numerical values obtained in the fitting procedure are: $k_1 = 1.1\ mM^{-1}\ s^{-1}$; $k_{-1} = 21\ s^{-1}$; $k_2 = 80\ s^{-1}$; $k_{-2} = 108\ s^{-1}$.

It is of particular importance to note that employing the above rate constants in the scheme of eq 1 reproduces quite well the lag in absorbance change observed at low $[N_3^-]$ (Figure 3). In contrast, although using values of δ between 0.45 and 1.0 did not appreciably increase the mean residuals, the lag was not adequately reproduced if δ was not in the vicinity of 0.7 to 0.8.

The above rate constants thus reproduce both kinetic and equilibrium results and are quite well defined, both as to the fitting procedure and to variations in δ. Nevertheless, there can be no assurance that they form an unique set. The curve fitting shows primarily that the simple scheme proposed can accommodate the observations.

The observed pseudo-first-order reaction rates are strongly influenced by pH (Figure 4). At pH 7, k_1 and k_{-1} are increased by factors of 8 and 4, respectively, whereas k_2 and k_{-2} change negligibly (0 and 30% increase, respectively.) An experiment at 20 °C and pH 7 is also included in Figure 4. The intercept (rate at zero azide concentration) is much more affected than the slope, and the affinity is less at 20 °C, just as observed in the equilibrium binding studies. At pH 6, 2 °C, the rates approach the limits of the stopped flow and the reaction is seen to be approximately second order, indicating that the first, bimolecular step is rate limiting. On the other hand, at pH 9, 2 °C, the velocity is approximately $[N_3^-]$ independent for $2 \lesssim [N_3^-] \lesssim 50$ mM, with a phenomenological rate constant of $k \sim 2\ s^{-1}$. Thus, at pH 9 the second, intramolecular step in eq 1 appears to be rate limiting.

Reaction of $Mn^{III}Hb$ with Azide. The rate of azide binding to $Mn^{III}Hb$ was measured at pH 6, 7, and 8 at 20 °C and at pH 7 at 3 °C, using a series of azide concentrations under each set of conditions. The reaction was notably biphasic under all conditions studied, and the results could be fitted satisfactorily to the sum of two exponentials (see ref 8, Figure 3). The biphasic nature of the reaction may reasonably be attributed to chain differences, though no wavelength dependence could be demonstrated. This identification is supported by experiments with iron–manganese hybrids which point to the β chains as the rapidly reacting components (Hoffman et al., 1975; unpublished observations, M. J. McDonald, Q. H. Gibson, and B. M. Hoffman). Each individual component resembles $Mn^{III}Mb$ qualitatively in its behavior, but the components differ from one another by about 20-fold in apparent combination and dissociation velocities (Figure 5). Their affinities are similar, with a dissociation constant of about 5 mM for each. The amplitude of the absorbance change is similar for each component at each azide concentration, with a tendency for the slow component to dominate at high azide concentrations and the fast component at low concentrations. The effect of pH was similar to that on $Mn^{III}Mb$, and similar for each component.

No attempt was made to apply an analogue of eq 1 with the α and β chains independently exhibiting such a binding scheme because the data must define eight rate constants when such a mechanism is assumed. However, the simplest possible scheme was examined, of the form

$$\alpha^{Mn^{III}} + N_3^- \rightleftharpoons \alpha^{Mn^{III}}(N_3^-) \quad (3)$$

$$\beta^{Mn^{III}} + N_3^- \rightleftharpoons \beta^{Mn^{III}}(N_3^-) \quad (4)$$

and assuming that the fast and slow components are independent and may be represented by a single, spectrophotometrically observable, reversible binding reaction. This did not lead to a satisfactory fit or to a random distribution of residuals, the mean residual being about 5% of the total absorbance excursion. The implication is that the reactions of each type of chain in metmanganoglobin resemble the metmanganomyoglobin reaction.

The marked heterogeneity in kinetic measurements has no obvious manifestations in the equilibrium binding of azide to $Mn^{III}Hb$, as expected from the similar affinity of the two components as observed kinetically. Hill plots give straight lines of slopes (n) not significantly different from unity. At 20 °C, total azide concentrations for half-saturation are ≈22 mM at pH 7 and ≈42 mM at pH 8.9 with no significant effect produced by the presence of excess IHP (0.05 M Tris). The affinity at pH 7 is the same as that for $Mn^{III}Mb$, but again differs from that derived from the plot of Figure 5, which implicitly assumes the validity of eq 3 and 4.

Reaction of Myoglobin with Azide. As an overall control on procedures, similar experiments were performed with sperm

whale metmyoglobin. The results (Figure 5) show an accurate proportionality between rate and azide concentration in the range plotted. In the experiment, further dilutions were made to 25 μM. These indicated a dissociation velocity constant of 0.1 s^{-1}. There was no sign of a lag at the beginning of any of the reaction records; the first-order rates decreased by less than 10% within each trace, presumably because of heterogeneity of the protein.

The apparent second-order rate constant for the association of azide with metmyoglobin under the conditions of Figure 5 is $\approx$3.8 mM^{-1} s^{-1}, considerably less than that for the reaction of $Mn^{III}Mb$ with azide under the same conditions. The initial lag in the progress curves for azide binding by $Mn^{III}Mb$ (Figure 2) cannot therefore be attributed to contamination with ferric myoglobin.

Discussion

The results reported here indicate that the electronic structure differences between Mn^{3+} and Fe^{3+} porphyrins result in the observation of different kinetic behavior in azide binding. Met-Mb exhibits an ordinary bimolecular reaction and normal pseudo-first-order kinetics in the presence of excess azide. The reaction of $Mn^{III}Mb$ presents a novel pattern with direct evidence for kinetic complexity. Although the kinetic analysis may not be unique, it appears that the azide complex cannot be fully formed, as judged by spectrophotometric changes, even with an indefinitely great $[N_3^-]$. This is interpreted as resulting from an equilibrium between the spectroscopically observable azide complex and another, "starred", species whose spectrum is not observably different from that of $Mn^{III}Mb$ itself. As both the formation and breakdown of the observable complex are slow (i.e., within the stopped-flow time scale), it may be separated by a significant energy barrier from the starred complex. At pH 7.6, 3 °C, as $[N_3^-] \rightarrow \infty$, the observable and "starred" complexes are in roughly comparable concentration.

The suggestion that a change in sixth ligand can occur with a minimal change from the "Mn^{3+}" spectrum seems unusual, but is readily documented in this instance. It is well known that the character of the split Soret spectra of Mn porphyrins is primarily determined upon coordination of a single neutral nitrogenous base, such as the proximal histidine, and the spectrum differs substantially from that observed with a coordinated anion (Boucher, 1972). More directly, the Soret spectra of $Mn^{III}Hb$ and $Mn^{III}Mb$ are quite similar, yet an x-ray structure study of $Mn^{III}Hb$ (Moffat et al., 1974, 1976) shows that its spectrum may be that of a mixture of five-coordinate (β chain) and six-coordinate (α chain) porphyrins. Furthermore, in mixed-metal hybrid hemoglobins with Mn(III) porphyrin in one type of chain and Fe^{3+} porphyrin in the other, the components of the Soret spectra arising from the Mn^{3+} porphyrin appear to be largely independent of which chain contains the Mn^{3+} porphyrin (Yonetani and Asakura, 1969), despite the difference in Mn^{3+} coordination to be expected.

Noting that H_2O bound to Mn^{3+} is less acid than that bound to Fe^{3+} porphyrins, one might propose that the starred species represents a weak complex with HN_3, and that this final equilibrium involves proton loss. Moreover, it is extremely noteworthy that not only are all hemoglobin ligands neutral molecules (e.g., O_2, CO, NO, isocyanides), but also the ligands of met-Hb are either neutral molecules (e.g., NH_3, imidazole, NO) or else anions which are themselves salts of weak acids (e.g., CN^-, N_3^-, NO_2^-, SCN^-, F^-, acetate$^-$, formate$^-$) and therefore in equilibrium with the uncharged acid. This raises the possibility that only a neutral species can reach the central metal atom of the heme, and might explain why fluoride, the salt of a weak acid (pK_A = 3.2), can bind to met-Hb, but that chloride, the salt of a strong acid, cannot.

Although this correlation between the ability of a ligand to bind to the met-Hb heme iron and its ability to assume an uncharged form is extremely attractive, the proposal cannot explain the complex kinetics shown by the manganese proteins or their differences from the corresponding iron proteins. Thus, the observed values of k_2 and k_{-2} are implausibly small for a proton transfer process and this picture requires $k_{-2}\alpha[H^+]$ to decrease by about fourfold from pH 7.0 to 7.6, whereas the observed change is much smaller. Furthermore, the pK of the metal-coordinated H_2O molecule in met-Mb is $\approx$9, substantially reduced from that of free water, whereas such an interpretation of the starred form has the unreasonable consequence that HN_3 bound to $Mn^{III}Hb$ exhibits a p$K \approx 7$, increased from that of HN_3 in free solution (pK = 4.8).

We tentatively favor an identification of the starred species based on crystal structure studies of the six-coordinate complexes, azido-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinato(methanol) manganese(III) ($N_3(CH_3OH)MnTPP$) (Day et al., 1974) and Cl(pyridine)MnTPP (Kirner and Scheidt, 1975). In both cases, *including* the complex with pyridine as sixth ligand, the metal ion is substantially displaced from the porphyrin plane toward the anion and the sixth ligand is held by a weak, elongated bond. On the other hand, it is clear that the metal ion in $Mn^{III}Hb$ and $Mn^{III}Mb$ is out of plane toward and tightly bonded to the imidazole of the proximal histidine (Moffat et al., 1974, 1976).

We propose that the starred, spectroscopically unobservable, product of the reaction of azide with $Mn^{III}Mb$ is a weak Mn^{3+}-azide complex in which the metal ion remains out-of-plane toward the imidazole of the proximal histidine. The principle spectroscopic changes observed are the result of a subsequent activated "inversion" in which the metal ion pushes through the porphyrin central hole toward the anion, resulting in a tight azide complex with elongated bond to the imidazole nitrogen, analogous to the reported model complexes. The two forms of the azide complex are in equilibrium, with the rates of interconversion (k_2, k_{-2}) being relatively slow because of the energy required to achieve a transition in which the high-spin Mn^{3+} ion lies in the porphyrin plane.

The different kinetic behavior exhibited by the Mn^{3+} and Fe^{3+} proteins thus may be attributed to differences in the behavior of the metal ions upon adding the sixth ligand. The Mn^{3+} ion remains high spin, and the equilibrium between two "inversion"-related, out-of-plane metal-ion positions is observable. In contrast the Fe^{3+} ion of azidomet-Hb becomes low spin. Now, the x-ray study of the model compound N_3(pyridine)FeTPP (Adams et al., 1975) indicates that the Fe^{3+} will lie out of plane toward azide, but to a much lesser degree than does high-spin Mn^{3+}. The simple kinetics observed for azide binding to Fe^{3+} might be interpreted to suggest that the resulting low-spin Fe^{3+} actually lies in a well-defined energy minimum, exhibiting only one significant structural form. It is more likely that they reflect a complex scheme such as discussed here, but with all first-order processes, "inversion," for example, associated with such low energy barriers that the overall rates (e.g., k_2, k_{-2}) do not contribute significantly to determining the rates of combination under the conditions employed here.

The above interpretation leads to several further considerations. First, the $Mn^{III}Hb(N_3^-)$ optical spectra are somewhat temperature dependent, and thus the system would be open to

study using the temperature-jump techniques. The number of relaxation rates to be observed is predictable from the present scheme, and the greater time resolution should permit a wider variation in pH and temperature.

Second, the inversion equilibrium proposed is analogous to that observed by NMR for a high-spin Fe^{3+} porphyrin exchanging halide ligands in free solution (La Mar, 1973). If an (N_3)Mn porphyrin in solutions containing excess azide, or else a base such as pyridine or an imidazole, also shows an equilibrium, then it might be detectable in extensions of the recent careful NMR studies of La Mar and Walker (1975). In addition, resonance Raman spectroscopy in progress on solution manganese(III) porphyrins may help to confirm the present interpretation (D. Scholler, D. F. Shriver, and B. M. Hoffman, unpublished), and an x-ray study of an N_3 (imidazole) (Mn^{III}porphyrin) would also be an extremely valuable check.

Third, fluoromet-Hb is high spin and formally analogous to azido-Mn^{III}Hb and thus might appear to be a candidate in a search for kinetic complexity in Fe^{3+} proteins. However, fluoride is so weak a ligand that the form in which Fe^{3+} is out-of-plane toward the imidazole (the starred form) should be overwhelmingly favored and the second step of the above scheme undetectable.

Finally, the assumption of an elongated, weak imidazole-Mn^{3+} bond in the "inverted" form of the azide complex suggests that this species is analogous in structure to the HBNO complex since the x-ray structure of ON (*N*-methylimidazole) FeTPP (Piciulo et al., 1974) shows that the heme iron will also tend to be out-of-plane away from, and with an elongated bond to the proximal imidazole. This suggests that $Mn^{III}Hb(N_3^-)$ may also be forced toward the T state upon addition of IHP, as appears to be true for HbNO (Salhany et al., 1974), and experiments designed to test this suggestion are under way.

Viewed differently, the comparison with HbNO suggests that, during the binding of NO by Hb (or Mn^{II}Hb (Hoffman et al., 1975)), there might conceivably occur an intermediate in which the Fe (or Mn) remains out-of-plane toward imidazole. Such an intermediate might be kinetically observable, although the reaction is so rapid as to make this unlikely.

It is unusual to find direct evidence of kinetic complexity in ligand binding by hemoproteins under ordinary (room temperature, aqueous solution) conditions, but there are already several sets of observations which may be interpreted as arising from this cause. In the displacement of oxygen from myoglobin by carbon monoxide (Gibson and Roughton, 1955) it seems that the ratio of the rates of binding of oxygen and carbon monoxide is a function of the ligand concentration. In relative terms oxygen appears to bind faster at high concentrations. This may reflect a difference in weighting of individual steps in the reaction for the two ligands as the absolute rate changes. The effect is, however, quite small, perhaps 10% or so.

More strikingly, Cassoly and Gibson (1972) have found that the rate of the NO + hemoglobin reaction is insensitive to the occurrence of the R $\rightarrow$ T transition, whereas CO is strongly sensitive to it. A model porphyrin x-ray structure (Collman et al., 1976) shows that the Fe of HbCO will tend to lie in-plane and have a short, strong bond to imidazole, in contrast to the situation in HbNO. Thus, the kinetic differences between such ligands of similar size may reflect a differential weighting of individual reaction steps whose stereochemical basis involves the features discussed in this paper.

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