Kinetics of Reaction of Anions with Methemerythrin Derivatives†

D. R. Meloon‡ and R. G. Wilkins*

ABSTRACT: The kinetics of anation of methemerythrin over a wide range of pH and concentration of anions have been studied at 25 °C. The azide and thiocyanate ions have been most intensively investigated but experiments with fluoride and chloride are also reported. The replacement of anion in methemerythrin-anionic adducts by other anions has also been studied. Except for replacement of met-fluoride by azide, all replacements can be explained by a dissociative mechanism via the aquated species. Anations are second-order and an associative mechanism is preferred. The second-order rate constant decreases with increasing anion concentrations (from 20 μM to 20 mM). This is attributed to the effect of a secondary anion binding site. The behavior of octameric and monomeric forms of the protein toward thiocyanate is identical. A comparison of results with simple Fe(III) complexes and certain metalloproteins is made.

Hemerythrin is the oxygen-transporting protein found in certain species of marine invertebrates (Klotz, 1971; Llinas, 1973; Okamura and Klotz, 1973). It has a molecular weight of 108 000 and consists of eight units, each containing two nonheme iron(II) centers. It forms an interesting contrast to myoglobin and hemoglobin, which have a similar function in mammals and which have been investigated extensively (Antonini and Brunori, 1971). Colorless deoxyhemerythrin reacts rapidly with oxygen (Bates et al., 1968) to form dark-pink oxyhemerythrin, in which the stoichiometry is one oxygen molecule for every two iron centers. Deoxyhemerythrin and oxyhemerythrin can be oxidized (e.g., by $Fe(CN)_6^{3-}$ ion) to the met form, which is no longer oxygen sensitive. Methemerythrin exists in acid and basic forms in which the iron is intimately involved. The interrelating pK is 7.8 in the absence of certain anions such as perchlorate (Garbett et al., 1971a,b). Methemerythrin combines with a variety of anions and, where the stoichiometry has been firmly established (for N_3^- and SCN^-), the iron(III):anion ratio in the product is 2:1 (Keresztes-Nagy and Klotz, 1965, 1967; Klapper and Klotz, 1968). Marked and intense spectral changes accompany anation (Keresztes-Nagy and Klotz, 1965; Garbett et al., 1969) and these have been used to characterize these species and to determine their formation constants (Garbett et al., 1971a). Chemical, magnetic, Mossbauer, and other spectral studies have been made on hemerythrin and derivatives in attempts to define the nature of the iron sites. An oxy moiety (O^{2-}) is believed to bridge the two iron(III) ions in the oxy- and methemerythrin derivatives, and this is reinforced by a peroxide (O_2^{2-}) or ligand bridge, respectively. (Garbett et al., 1969; Dawson et al., 1972). A nonbridged formulation for the azide compiex has, however, been proposed on the basis of infrared studies (York and Bearden, 1970). Recent resonance Raman spectral studies strongly support a Fe(III)-O_2^{2-}-Fe(III) formulation for oxyhemerythrin (Dunn et al., 1973).

The octamer is readily and reversibly dissociated by urea, by mercuration, or in the case of the met derivatives by simply diluting greater than 20 μM solutions to less than 5 μM concentrations of protein (Klotz, 1971; Okamura and Klotz, 1973). Only the monomer is produced, there being no oligomeric intermediate (e.g., dimeric) species (Langerman and Klotz, 1969). Some preliminary anation experiments indicate that these are easily measurable (Garbett et al., 1971a) but no kinetic studies have been reported. We have examined in detail the rates of reaction of the met form of hemerythrin from *Golfingia gouldii* with azide and thiocyanate ions over a wide range of anion concentrations and pH, mainly with the octamer, but also with the monomer (merohemerythrin) form. We have also (much less extensively) examined reactions with fluoride and chloride ions. Finally, we have examined the replacement of anion in the methemerythrin-anionic adduct by other anions. We are thus enabled to compare the behavior of binuclear iron(III) in the marine worm protein with other systems, both biochemical and simple Fe(III) complexes.

Methods

Golfingia gouldii obtained live from Marine Biological Laboratories, Woods Hole, Massachusetts, was used as the source of hemerythrin. The coelomic fluid of some 75 worms was treated essentially as described in the literature (Klotz et al., 1957) and yielded about 2 g of oxyhemerythrin crystals, containing some met form. These were stored at 4 °C, immersed in a 20% ethanol-0.4% NaCl aqueous solution. Solutions containing methemerythrin at the desired pH were obtained by $Fe(CN)_6^{3-}$ oxidation (Keresztes-Nagy and Klotz, 1965; Klapper and Klotz, 1968; Garbett et al., 1971a,b). Chemically pure commercial samples of the potassium salts of azide, thiocyanate, fluoride, and chloride anions were used, and solutions of these were prepared by weight. The buffers used were 2-(N-morpholino)ethanesulfonic acid (Mes[1]), N-2-hydroxyethylpiperaz-

† From the Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003. *Received June 18, 1975.* Support by National Science Foundation is gratefully acknowledged.

‡ This work is taken in part from the dissertation submitted by D. R. Meloon in partial fulfillment of the requirements for the Ph.D. degree from the State University of New York at Buffalo.

* To whom correspondence should be addressed at Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003.

[1] Abbreviations used: Mes, 2-(N-morpholino)ethanesulfonic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

ine-*N'*-2-ethanesulfonic acid (Hepes) (Good et al., 1966), and tris(hydroxymethyl)aminomethane (Tris). The concentration of methemerythrin solutions used in the studies is given in terms of the monomer concentration and was usually around 20 μM (0.27 mg of protein/cc). The concentration of metaquahemerythrin solutions was determined from absorbance at 355 nm at pH 6.3, ϵ 6.4 $\times$ 10^3 M^{-1} cm^{-1} (Garbett et al., 1969), and from the concentration of total iron determined spectrophotometrically as the phenanthroline complex (ϵ_{510} 1.10 $\times$ 10^4 M^{-1} cm^{-1}) after protein decomposition at pH 2-3 (Darnall, 1974). In experiments using low anion concentrations, 6-12 μM protein was used to ensure pseudo-first-order kinetics. In experiments with merohemerythrin, 2.8 μM protein was employed. The diluted solutions were left for at least an hour to ensure dissociation of the octamer (Langerman and Sturtevant, 1971, in calorimetry studies, report dissociation complete in less than 45 min). The aquomerohemerythrin gave excellent kinetic behavior in the reaction with thiocyanate ion. No precipitation of protein was observed during the study. The monomer has been reported to be unstable at pH 7-9, although it is stabilized by coordination to anions (Keresztes-Nagy and Klotz, 1965). For experiments at pH 6.3, and above, the pH of a concentrated solution of the protein was adjusted to the desired pH by dialysis against the appropriate buffer. A portion of this protein solution was then diluted to the desired concentration with buffer solution. Below pH 6.3, concentrated protein solutions had to be added to large amounts of buffered solutions since, in the time of dialysis, substantial denaturation occurs in acid medium.

All studies were at 25 °C and an ionic strength of 0.1 M. For runs at pH 4.0-5.6, a mixture of Mes and K_2SO_4, adjusted with NaOH, was used. Since Mes is essentially nonionized at these pH, K_2SO_4 was included for ionic strength adjustment. At pH 6.3, 7.5, and 9.0, mixtures of Mes and NaOH, Hepes and NaOH, and Tris and Mes, respectively, were employed. For the anation runs at pH 7.0, where the relative reactivities of monomer and octamer toward SCN^- were examined, a buffer of 0.1 M Tris and cacodylic acid was used, in order to simulate the conditions of the molecular weight study (Langerman and Klotz, 1969). Chloride ion in the cacodylic acid was replaced by sulfate ion using an anion exchanger. Perchlorate and nitrate ions were avoided in all studies since they bind to the protein and modify the anion interactions (Garbett et al., 1971a,b).

The equilibrium constants for anation were determined spectrally using the same conditions as in the kinetic experiments. The spectrum of a solution of the met-aqua or methydroxy form was obtained, a known concentration of anion was added and, after equilibrium had been attained, the spectrum was redetermined. This was repeated for other concentrations of anion. The spectrum of the met anionic form was determined by using a large concentration of anion. Thus the dependence of the extent of coordination upon free anion concentration could be measured and the formation constant determined (Garbett et al., 1971a). The kinetics of anation were measured with a Cary 14, but in a few experiments where high concentrations of SCN^- or N_3^- ions were studied, a Gibson-Durrum stopped-flow spectrophotometer was used. The anation progress was monitored near the spectral maximum of the products, 345 (F^-), 390 (Cl^-), and 450-470 nm (SCN^-, N_3^-). In anion interchange experiments, which were all conducted at pH 6.3, the met-L species (see eq 13) was prepared by treating met-H_2O with L^-, and allowing equilibration. X^- ion was added and the formation of met-X was monitored. The formation of some met-X directly from X^- and met-H_2O (still present in the equilibrated solutions) was rapid and did not interfere with the interchange observations.

Table I: Values of k_f and k_r for Interaction of Methemerythrin with Anions at 25 °C (I = 0.1 M).

Anion	pH	k_f (M^{-1} s^{-1})	$10^3 \times k_r$ (s^{-1})
N_3^-	4.0	35 (35)[a]	3.7 (3.7)[b]
	4.5	36 (28)	0.96 (1.1)[b]
	5.0	20 (19)	0.53 (0.37)[b]
	5.6	9.2 (11.1)	0.21 (0.10)[b]
	6.3	7.6 (8.0)	<0.05
	7.5	4.4 (4.7)	<0.05
	9.0	0.13 (0.40)	<0.05
SCN^-	4.0	160	22
	5.0	89	9.0
	6.3	82 (88)[c]	6.9 (6.8)[c]
	7.0	50 (40)[c]	8.0 (8.0)[c]
	7.5	11	6.2
F^-	6.3	0.035	0.39
Cl^-	6.3	0.006	0.075

[a] Values in parentheses are calculated on the basis of eq 8, see text. [b] Values in parentheses are calculated on the basis of eq 10, see text. [c] Values in parentheses for monomeric form.

Results

The acid form of methemerythrin, metaquahemerythrin, which is abbreviated met-H_2O, is in acid-base equilibrium with a basic form, methydroxyhemerythrin, met-OH[2]

$$\text{met-}H_2O \rightleftarrows \text{met-OH} + H^+ \qquad K_1 \qquad (1)$$

When the entering anion exists both in a protonated LH and nonprotonated form L^-

$$LH \rightleftarrows L^- + H^+ \qquad K_2 \qquad (2)$$

the predominant forms of both protein and ligand will depend on pH. The anation can then be represented as

$$(\text{protein})_T + (L)_T \rightleftarrows (\text{protein} \cdot L)_T \qquad (3)$$

where the bracketted species refer to all the rapidly equilibrating protononic forms of protein and ligand. The concentration of anion reacting with protein was always in large excess. The rate of approach to equilibrium was first order. The associated rate constant, k_{obsd}, is related to the total anion concentration $[L]_T$ by expression 4

$$\text{rate}/[\text{protein}]_T = k_{obsd} = k_f[L]_T + k_r \qquad (4)$$

k_f and k_r are forward and reverse rate constants for reaction 3 and in general are composite and pH dependent (see eq 8 and 9). Plots of k_{obsd} vs. $[L]_T$ are shown in Figures 1 and 2 for reaction of methemerythrin with azide and thiocyanate at a variety of pH's. The reactions of fluoride and

[2] Only one pK associated with the iron centers has been observed. The acid-base equilibrium may be related to one between a diaqua species (in which there is one water molecule attached to each iron) and a hydroxy-aqua species; or it may involve a hydroxy-aqua species and a dihydroxy complex (in which a hydroxide ion is attached to each iron). In the former case a second pK > 9.5 might exist; in the latter, a second pK < 4.0 would be expected. Such pK's would be difficult to detect in the protein and, therefore, to use for diagnostic purposes. Existing evidence supports the second formulation (Garbett et al., 1971b).

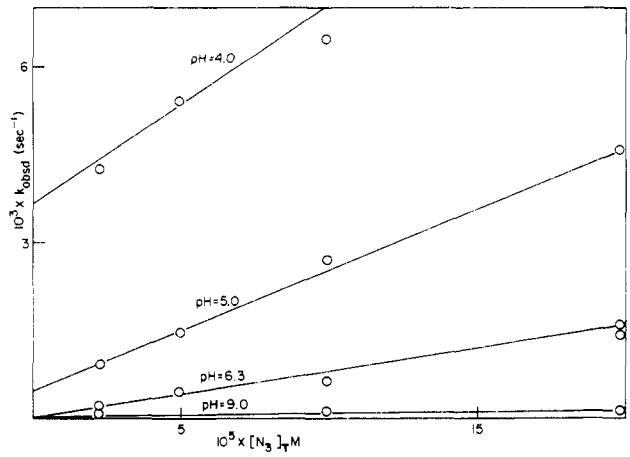

FIGURE 1: Anation of hemerythrin by azide ion at 25 °C and various pH, I = 0.1 M. Plot of k_{obsd} vs. total azide concentration, $[N_3]_T$.

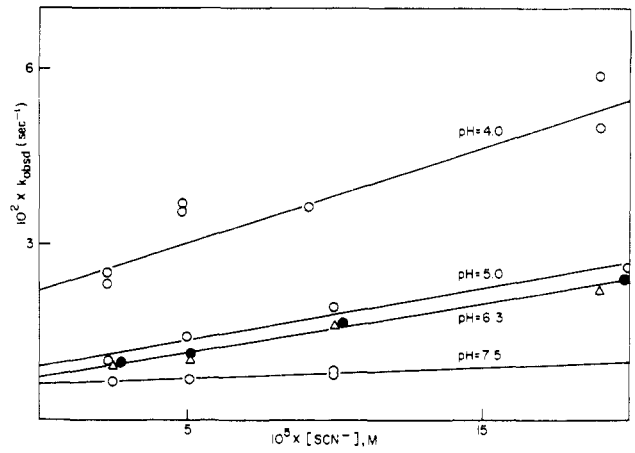

FIGURE 2: Anation of hemerythrin by thiocyanate ion at 25 °C and various pH, I = 0.1 M. Plot of k_{obsd} vs. $[SCN^-]$. Values for monomer (●).

chloride were studied at pH 6.3 only. Except for azide ion, all anions studied are aprotic over the pH range of examination and for these, $[L]_T = [L^-]$. The slopes and intercepts of such plots yield values for k_f and k_r, respectively, and these are recorded in Table I.

Reaction with Azide Ion. It was first determined that reaction of methemerythrin with azide ion at pH 6.3 produced no sizable concentrations of intermediates. Isosbestic points at 343 and 387 nm were rigorously maintained (Figure 3). Further, semilog plots of absorbance vs. time remained linear for some 4–5 $t_{1/2}$'s. The principal species present at pH 4 are HN_3 and met-H_2O, at pH 6.3, N_3^- and met-H_2O and at pH 9.0, N_3^- and met-OH. Since it transpires that the reactivity of met-OH is small (see Figures 1 and 2), the system 3 is modified to (1) and (2) plus (5)–(7)

$$\text{met-}H_2O + L^- \rightleftarrows \text{met-L} + H_2O \qquad k_1, k_{-1}, K_L \quad (5)$$

$$\text{met-}H_2O + HL \rightleftarrows \text{met-LH} + H_2O \qquad k_2, k_{-2} \quad (6)$$

$$\text{met-LH} \rightleftarrows \text{met-L} + H^+ \qquad K_3 \quad (7)$$

Since the pK_a of HN_3 at 25 °C is 4.75 (Cotton and Wilkinson, 1973), the rate constants k_f at pH 4.0 and pH 6.3 (Table I) represent approximately the reactions of HN_3 (k_2) and N_3^- (k_1), respectively. Since k_f at pH 6.3 is about 20% of k_2, this rate cannot arise simply from a contribution from the small amount of HN_3 present at pH 6.3 (~3% of total azide). The protein itself might be expected to have different reactivities at pH 4.0 and 6.3 but this is a relatively unimportant effect, judging from the results at pH 4.0, 5.0, and 6.3 (Table I) with SCN^-, where acid and base forms of the anion do not arise.

It can be shown that

$$k_f = k_2\left(\frac{[H^+]}{[H^+]+K_1}\right)\left(\frac{[H^+]}{[H^+]+K_2}\right) + k_1\left(\frac{[H^+]}{[H^+]+K_1}\right)\left(\frac{K_2}{[H^+]+K_2}\right) \quad (8)$$

and a fair fit with experimental data is obtained when values are assigned for k_1 = 7.0 M^{-1} s^{-1}, and k_2 = 40 M^{-1} s^{-1}. Values for $K_1 = 1.6 \times 10^{-8}$ M (Garbett et al., 1971a,b) and $K_2 = 1.8 \times 10^{-5}$ M (see Table I) are taken from the literature and rate constants for reaction of met-OH are assumed negligibly small.

The variation of k_r with pH is given by

$$k_r = \frac{k_{-1} + k_{-2}[H^+]K_3^{-1}}{1 + [H^+]K_3^{-1}} \quad (9)$$

When K_3, the ionization constant of met-LH, $\gg [H^+]$, i.e., the proton dissociates completely from the coordinated HN_3 entity even at pH as low as 4.0, expression 9 reduces to

$$k_r = k_{-1} + \frac{k_{-2}[H^+]}{K_3} \quad (10)$$

A linear plot for k_r vs. $[H^+]$ is quite well obeyed. Calculated values for k_r on the basis of a negligibly small k_{-1} and k_{-2}/K_3 = 37, are shown in parentheses alongside the experimental value in Table I. The data for azide anation so far discussed (Figure 1) relate to the range 20–200 μM N_3^-. Studies at higher anion concentrations were carried out at pH 6.3. As the concentration of azide ion was increased (finally up to 80 mM), a curious behavior was observed. The slope of the k_{obsd} vs. $[N_3^-]$ plot continuously decreased, as did therefore the associated values of k_f, while those of k_r increased (see Figure 4). A limiting *positive* slope was observed corresponding to values of k_f and k_r of 2.3 and 0.009 M^{-1} s^{-1}, respectively. Similar behavior was observed in a much less detailed study at pH 7.5. Garbett et al. (1971a) have reported that perchlorate ion, even in millimolar concentrations, retards the rate of formation of the met-azide complex. We have confirmed this finding. In 8.0 mM N_3^-, ionic strength = 0.1 M, pH 6.3, and k_{obsd} equals 0.029 s^{-1}. This is reduced to 0.012 s^{-1} when 0.01 M sodium perchlorate is included in the 0.1 M ionic medium.

It would be extremely difficult to measure the value of the equilibrium constant K_L directly at pH 6 for the formation of the very stable azide complex. Very low concentrations of protein and azide ion are needed to prevent complete formation of the azide adduct, and then spectral changes would be small and difficult to measure. At pH 9.0 however, OH^- ion competes effectively with N_3^- for the iron center. The equilibrium concentrations of reactants in the reaction

$$\text{met-OH} + N_3^- \rightleftarrows \text{met-}N_3 + OH^- \qquad K_4 \quad (11)$$

can be accurately assessed and K_4 determined. It is easily shown that

$$K_L = K_4K_1/K_W \quad (12)$$

Values of K_L are shown in Table II. It was not possible to determine accurate values of $k_r(k_{-1})$ at pH 6.3 (Figure 1) and so the value of K_L could not be compared with a kinetic

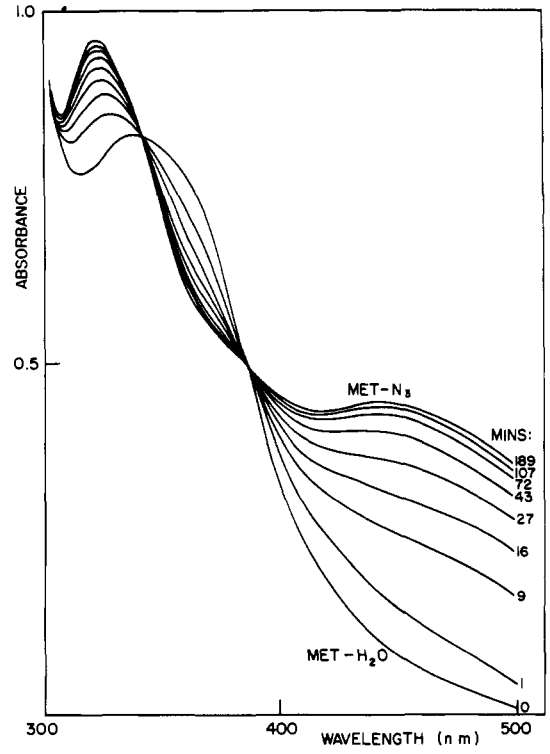

FIGURE 3: Observation of isosbestic points in reaction of methemerythrin with azide ion, at 25 °C, pH 6.3, and I = 0.1 M. Protein was 28 μM and $[N_3^-]$ = 100 μM, 5-cm cell. Scan time: 400 s.

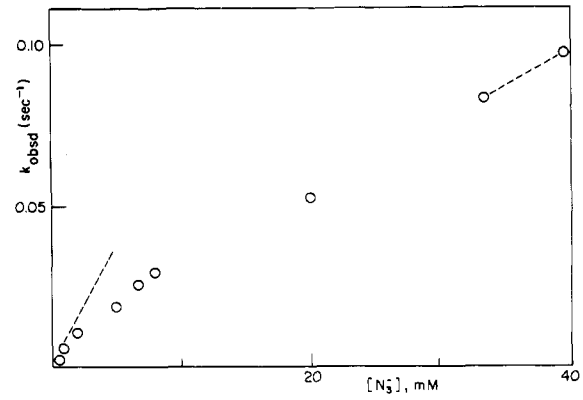

FIGURE 4: Anation by wide range of $[N_3^-]$ at 25 °C, pH 6.3.

Table II: Kinetics and Thermodynamics of Anation of Metaquohemerythrin at 25 °C and pH 6.3 (Reaction 5).

L^-	k_1 (M^{-1} s^{-1})	$10^4 k_{-1}$ (s^{-1})	K_L (Kinetic) (M^{-1})	K_L (Spectral) (M^{-1})
F^-	3.5×10^{-2}	3.9	90	95
N_3^-	7.0	≤0.5	≥1.4×10^5	1.0×10^{6a} (10^{6})[c]
SCN^-	82 (88)[b]	69 (68)[b]	1.2×10^4 (1.3×10^4)[b]	0.9×10^4 (3.8×10^4)[c]

[a] From data at pH 9. [b] Monomeric form of protein. [c] Garbett et al., 1971a,b.

one. This is in contrast to a successful comparison of K_L values from spectral and kinetic data for the other anations (vide infra).

Reaction with Thiocyanate Ion. Anation of methemerythrin by thiocyanate was studied over a wide range of anion concentration (2×10^{-5} to 4×10^{-2} M), and from pH 4.0 to 7.5. Value of k_f and k_r for reaction 5, L = SCN, are shown in Table I. The slight increase in k_f and k_r from pH 6.3 to 4.0, about 2-3-fold for a 200-fold increase in $[H^+]$, contrasts with the marked change for the corresponding constants for anation by azide over the same pH range. This undoubtedly arises from a much weaker basicity for free (Morgan et al., 1965) and coordinated thiocyanate group and the nonparticipation of reactive protonated species in the reaction. The small increase in k_f might arise from protonation of an amino acid residue lying near the iron centers (Garbett et al., 1971b). The increased positive charge would be expected to increase the rate of anation. The combination of k_f and k_r at pH 6.3 gives a value of K_L in excellent agreement with that determined spectrally (Table II). At higher concentration of thiocyanate, the slope of the $k_{obsd}/[SCN^-]$ plot decreased as the $[SCN^-]$ increased, although it never reached a limiting zero value. The second-order rate constants (M^{-1} s^{-1}) for the formation of methemerythrin–thiocyanate adduct at pH 6.3 are 82 (0.025–0.19 mM SCN^-), 45 (0.19–0.80 mM SCN^-), and 26 (0.60–40 mM SCN^-).

The reaction with thiocyanate ion was used to study the relative reactivity of the octameric and monomeric forms of hemerythrin. The dependence of the weight-average molecular weight of aquamethemerythrin upon protein concentration is known (Langerman and Klotz, 1969). Solutions of the protein could therefore be prepared which contained (predominantly) the octameric form (6–20 μM) or the monomeric form (2.8 μM) and the rates of reaction of these species with SCN^- ion were measured. Anation rate constants at pH 6.3 were virtually identical (Figure 2) as well as at pH 7.0 (Tables I and II) where the molecular weight determinations were carried out (Langerman and Klotz, 1969).

Reaction with Other Ions. The reaction with fluoride ion was studied only at pH 6.3 and over a limited range of anion concentration from 0.017 to 0.16 M. This use of relatively high concentrations was mainly necessitated by the lower formation constant for the fluoride complex. The formation rates were relatively slow and a series of spectra was obtained. An isosbestic point at 324 nm was maintained, strongly suggesting that no intermediate in substantial concentration was formed. The values of k_f and k_r lead to a value for K_L which is in excellent agreement with that obtained by spectral examination of equilibrated solutions and based on a 2Fe:1F^- stoichiometry (Table II). A series of kinetic runs only at pH 6.3 was made involving reaction with chloride and one run only for bromide ions. These were used for comparison purposes, to give an idea of the relative reactivity of these anions, compared with the anions which had been more extensively studied.

Interchange of Anions. The reaction

$$\text{met-L} + X^- \rightleftarrows \text{met-X} + L^- \qquad (13)$$

was studied at pH 6.3 and I = 0.1 M for a number of combinations of L and X including L = SCN, X^- = N_3^- and OH^-; L = F, X^- = N_3^- and SCN^-; and L = Cl, X = N_3^-. In all cases $[X^-]$ and $[L^-]$ were kept in excess of [met-L], and excellent first-order loss of met-L (k_{obsd}) was observed. In the interchange 13, L = Cl, X^- = N_3^-, the reaction was followed for $6t_{1/2}$'s and linearity was maintained for the semilog plot. Except for the combination (F, N_3^-) all results (Table III) could be interpreted in terms of the scheme

Table III: Rate Constant for Anion Interchange in Reaction 13.[a]

[L⁻] (mM)	[X⁻] (mM)	$10^3 k_{obsd}$ (s^{-1})
SCN^-	N_3^-	
5.1	8.3	1.0 (1.2)[b]
0.34	33.3	7.4 (7.5)[c]
0.34	50.0	9.0
0.34	66.7	8.7
0.34	83.3	9.3
SCN^-	OH^-	
0.1	0.0083	6.9
0.1	0.013	7.6
0.1	0.020	7.7
F^-	SCN^-	
67.0	10.0	1.2
67.0	20.0	1.1
67.0	41.0	1.1
F^-	N_3^-	
67.0	6.7	1.9
67.0	13.0	2.8
67.0	33.0	5.4
67.0	67.0	9.5
Cl^-	N_3^-	
50.0	25.0	0.066
25.0	50.0	0.062

[a] In all experiments pH 6.3 (except $X^- = OH^-$), I = 0.1 M, 25 °C, and [protein] ~ 0.03 mM except 0.20 mM for met-F experiments. [b] Calculated value in parentheses on basis of (16) using values of k_1 = 26 M^{-1} s^{-1}, $k_{-1} = 9 \times 10^{-3}$ s^{-1}, k_3 = 2.3 M^{-1} s^{-1}, and $k_{-3} = 7 \times 10^{-6}$ s^{-1}. [c] Calculated value in parentheses on basis of (16) using k_1 = 45 M^{-1} s^{-1}, other values as in *b*.

$$\text{met-L} + H_2O \rightleftarrows \text{met-}H_2O + L^- \qquad k_{-1}, k_1 \qquad (14)$$

$$\text{met-}H_2O + X^- \rightleftarrows \text{met-X} + H_2O \qquad k_3, k_{-3} \qquad (15)$$

If a steady-state approximation for [met-H_2O] is assumed

$$k_{obsd} = \frac{k_{-1}k_3[X^-] + k_1k_{-3}[L^-]}{k_3[X^-] + k_1[L^-]} \qquad (16)$$

The agreement of k_{obsd} with k_{calcd} on the basis of expression 16 is shown in Table III. Usually the replacement was complete and thus k_{-3} could be ignored. Except for the combination (SCN, N_3^-), in the conditions which were used, $k_3[X^-] \gg k_1[L^-]$ and thus a limiting value for k_{obsd} ($=k_{-1}$) only was observed. This represented the dissociation of the met-L species. These values (Table III) were similar to those obtained directly from anation studies (Table II). The differences probably reflect different anionic composition of the medium for the two studies. These affect the composition of the secondary site and the dissociation rate behavior (vide infra). Comparison of the rate of replacement of fluoride in met-fluoride by thiocyanate and azide ions proved interesting. The k_{obsd} value was constant for a range of [SCN^-] from 10 to 40 mM and represented the first-order dissociation rate constant of the met-fluoride species (Figure 5). The value of k_{obsd} was however dependent on [N_3^-] which was varied from 6.7 to 67 mM. Extrapolation of the data for k_{obsd} to zero concentrations of N_3^- ion gave the same value as obtained in the thiocyanate replacement (Figure 5).

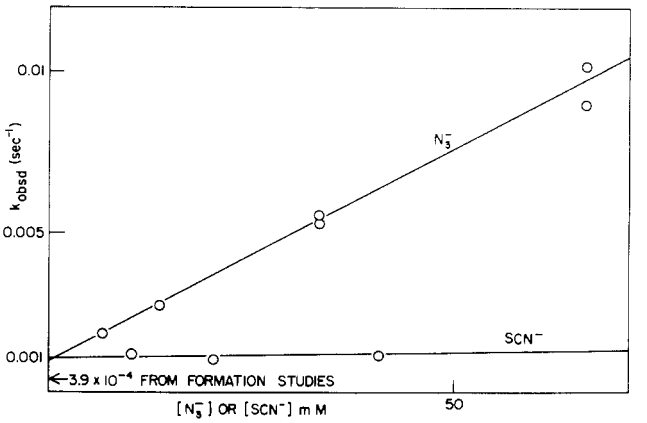

FIGURE 5: Dependence of k_{obsd} upon [N_3^-] and [SCN^-] for the replacement of F^- in met-fluoride at 25 °C, pH 6.3, and I = 0.2 M. [F^-] = 67 mM.

Discussion

Isosbestic points were observed for the series of spectra which accompanied anation of metaquahemerythrin by N_3^- and F^- (Figure 3) ions at pH 6.3. Additionally, no initial rapid reaction accompanied these anations, spectra at the beginning of the reaction conforming to the aqua species. If the azide product is bridged therefore (Klotz, 1971), the intermediate in which azide ion is associated with only one of the irons does not build up during the reaction and must rapidly react with the other iron center to ring close. It has been suggested that, in the fluoride product, the ratio of iron(III) to fluoride is 2:2 and not 2:1 as with the other anions (Garbett et al., 1969). If this is true, the presence of isosbestic points would indicate that the 2:1 species is unstable. Our (admittedly limited) spectral work on equilibrated solutions is, however, more consistent with a 2:1 Fe:F ratio in the product, although we worked at much lower fluoride concentrations than those previously reported (Garbett et al., 1969; Okamura and Klotz, 1973).

The anation studies were limited to the approximate pH range of 4 to 9. Denaturing of hemerythrin solutions occurs at pH 4 and a cloudiness develops within an hour. Its extent is, however, negligible in the short times necessary to study the anation ($t_{1/2} \leq 3$ min) and the final spectra, at higher anion concentrations, correspond to complete conversion to the anion complex. The protein denatured very quickly at pH 3.5. Anation proceeds very slowly and to a limited extent at pH ≥9, the methydroxy species being unreactive and stable. The protein is unstable at pH above 10 (Keresztes-Nagy and Klotz, 1965). The effect of pH was studied in most detail for the reaction of methemerythrin with azide ion. The kinetic data conformed very well with the scheme indicated in (5)-(7). The results showed that coordinated HN_3 had a pK < 4 compared with that of free HN_3 (pK = 4.8). This behavior resembles that of simple metal complexes as well as cobalamins (Randall and Alberty, 1967). The basicity of ligands is invariably markedly reduced when metal coordinated (Wilkins, 1974).

The unreactivity of the hydroxy form of methemerythrin toward N_3^- and SCN^- ions observed in our study finds parallelisms in the similar behavior of *aplysia* metmyoglobin toward N_3^- (Giacometti et al., 1975), and of hydroxycobalamin toward N_3^- and SCN^- ions (Randall and Alberty, 1966, 1967). Our kinetic data therefore suggest that the basic form of methemerythrin has no coordinated water (which would be easily replaced by entering anions) and this supports the dihydroxy formulation.[2]

Kinetic and equilibria data for reaction of azide, thiocyanate, and fluoride ions are collected in Table II. In gener-

al, there is good agreement between the values for equilibrium constants obtained from kinetic studies and from spectral examination of equilibrated solutions. Both agree reasonably well with the literature values available (Table II). The formation rate constants are of the same order of magnitude as those for the corresponding reactions of $Fe(H_2O)_6^{3+}$ ion. For example, the latter reacts with HN_3 and SCN^- with rate constants of 4.0 and 127 M^{-1} s^{-1} (Seewald and Sutin, 1963). Hence it appears that anions can as readily approach the iron pocket in hemerythrin as they can the hydrated iron(III) ion, and a "non-polar binding site" in the protein (Garbett et al., 1971a) is not supported by (at least) this kinetic result. A comparison of the reactivities of the various anions at pH 6.3 is interesting. Using in all cases 0.067 M anion, the pseudo-first-order rate constants (s^{-1}) are 1.7, 0.16, 0.003, 0.003, and 0.0004 for reactions of SCN^-, N_3^-, F^-, Br^-, and Cl^- ions. This wide range of rate constants is supportive of an associative mechanism rather than a dissociative one which would be expected to yield similar rate constants (Wilkins, 1974). Both mechanisms lead to a second-order rate law under certain circumstances. A relatively high stability, particularly for the azide and thiocyanate complexes compared with Fe(III) aquated complexes must reside then in the *remarkably* small values for their dissociation rate constants. The half-lives for dissociation of the met-azide, fluoride, and thiocyanate complexes are >230, 30, and 2 min, respectively, at pH 6.3, while those of the Fe(III) complexes are of the order of seconds or less. This behavior could be construed as evidence for a bridged formulation for the anionic species, if the behavior of simpler complexes and chelates can be extrapolated to these systems (Wilkins, 1974).

So far the discussion has been confined to behavior at low anion concentrations. The protein system could be studied over a large range (almost four orders of magnitude) of anion concentration. It was found that there was a reduction in the formation rate constant, and an increase in the dissociative rate constant as the total anion concentration increased (Figure 4). This was shown as a decrease in the k_{obsd} vs. [anion] slope. However, a limiting *positive* slope was obtained at the highest anion concentration. A limiting *zero* slope, corresponding to a limiting value for k_{obsd}, was not obtained. This would have characterized a dissociative mechanism (Wilkins, 1974) and the behavior lends further support to an associative SN2 type of process for anation. The explanation for the changing formation rate constant with increasing entering anion concentration may reside in the existence of a secondary binding site (or sites) near to the iron locus (Garbett et al., 1971a,b). Binding of perchlorate (Garbett et al., 1971a,b) and chloride (Rao and Keresztes-Nagy, 1973) ions even at low concentrations to those secondary sites has been documented. Our work shows that such secondary binding is rapid, and that it retards the coordination of azide to the iron center. If an anion such as N_3^- can bind to the secondary site, then this binding would also be expected to impede, via electrostatic effects, the coordination of N_3^- to the primary (iron) site. At low $[N_3^-]$, the secondary site is not occupied (perchlorate and other binding anions being excluded in all the experiments). At high $[N_3^-]$, the secondary site is saturated with azide. Each condition will give an associated rate constant, with an intermediate value at concentrations of azide between the two extremes. A similar behavior and interpretation would cover the results with thiocyanate interaction. The decrease in the ratio k_f/k_r as the anion concentration increases, agrees with the observation of a reduced value for K_L as anions bind at the secondary site (Garbett et al., 1971a,b). The presence of a secondary site in the protein may also be the underlying cause for differences in dissociation rate constant values for the met-thiocyanate and met-fluoride complexes obtained from anation and interchange studies (Tables II and III). In the former, extrapolation of k_{obsd} to zero anion concentration (except for buffer contribution) affords the value for k_{-1}, whereas in the interchange studies the value refers to a medium containing free anion (thiocyanate or fluoride) and therefore anion at the secondary site. Loss of anion (dissociation) from such an environment would be anticipated to be more rapid, as is in fact observed.

Examination of the kinetic data for the reaction of azide with whale ferrimyoglobin indicates that the second-order anation rate constant also decreases with increasing azide concentration, at constant ionic strength, although the trend is not commented upon by the authors (Duffey et al., 1966). Secondary site binding may also account for discrepancies in the values of anation formation constants derived from static and kinetic measurements with myoglobins (Antonini and Brunori, 1971). The anation rate process being influenced by secondary binding processes is unique to proteins and has hardly any counterpart in complex ion chemistry. Obviously it needs always to be considered in any study of anion interaction with the metal center of proteins. Compounding this problem is that of strong catalysis by buffers, which has been documented for addition of cyanide to myoglobins (Ver Ploeg et al., 1971).

The data in Table II and Figure 2 indicate that monomeric and octameric forms of methemerythrin exhibit the same (within experimental error) formation and dissociation rate constants in their reaction with SCN^- ion.[3] This necessarily means that the affinities of the two states toward thiocyanate (k_1/k_{-1}) are the same. The noncooperativity shown in the oxygen uptake by *Sipunculus nudus* deoxyhemerythrin (Bates et al., 1968) and *Golfingia gouldii* (DePhillips, 1971) is therefore also displayed in anation of the met derivative. Earlier work (Klapper and Klotz, 1968) indicated that thiocyanate bound more strongly to the monomer than to the octamer. However in these experiments, perchlorate ion was present and in higher concentration in the octamer solutions. This would lead to an apparent lower equilibrium constant for the octamer-SCN^- interaction via an electrostatic repulsion from the ClO_4^- ion bound at the secondary site.

The usual path for anion interchange in octahedral complexes is via a sequence of aquation and anation, comparable to (14) and (15) (Wilkins, 1974). This mechanism for anion interchange is also observed in metmyoglobin for a number of anion combinations (Sweigart and Bern, 1974), and now in the present work (with one exception) for methemerythrin. However for the replacement of fluoride in met-fluoride by azide (but not thiocyanate) ion, a direct path operates in addition to the aquation process, i.e.

$$\text{met-F} + N_3^- \rightleftarrows \text{met-}N_3 + F^- \qquad (17)$$

$$\text{rate} = 1.1 \times 10^{-3} + 0.13[N_3^-] \qquad (18)$$

[3] If the equilibrium between monomer and octamer is either more slowly or more rapidly established than anation, it is easily shown that identical reaction rate constants and *complete* anation of solutions containing (predominantly) monomer and octamer can only result if both species are equally reactive.

This means that a species in which both N_3^- and F^- are associated with the iron is important, and this is reminiscent of the behavior of four-(planar) and five-coordinated species, rather than associated with six coordination (Wilkins, 1974). A conclusion that the iron centers are therefore four- or five-coordinated would, however, be tenuous since direct replacement is not observed with any other anion interchange studied. Other anion replacements are being examined in an attempt to find the reason for the behavior of the azide ion, which incidentally is the most strongly coordinating anion. It is interesting that hydroxide ion, at 10^{-5} M concentration, does not accelerate removal of thiocyanate ion (Table III). Base-catalyzed hydrolysis is common with metal complexes and is ascribed to proton removal from ammonia and amine ligands associated with the metal in the so-called conjugate-base mechanism (Wilkins, 1974). Ligands believed associated with the iron, namely tyrosine and histidine, do not contain such acidic hydrogens.

Acknowledgment

We are very grateful to Drs. Dennis Darnall and Ron Conrad for many helpful discussions. Dr. Darnall introduced us to the techniques for the isolation of hemerythrin. This work was supported by a National Science Foundation grant (GP-36783).

References

Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobin in Their Reactions with Ligands, Amsterdam, North-Holland.

Bates, G., Brunori, M., Amiconi, G., Antonini, E., and Wyman, J. (1968), *Biochemistry 7,* 3016.

Cotton, F. A., and Wilkinson, G. (1973), Advanced Inorganic Chemistry, New York, N.Y., Wiley, p 353.

Darnall, D. W. (1974), unpublished experiments.

Dawson, J. W., Gray, H. B., Hornig, H. E., Rossman, G. R., Schredder, J. M., and Wang, R. H. (1972), *Biochemistry 11,* 461.

DePhillips, Jr., H. A. (1971), *Arch. Biochem. Biophys. 144,* 122.

Duffey, D., Chance, B., and Czerlinski, G. (1966), *Biochemistry 5,* 3514.

Dunn, J. B. R., Shriver, D. F., and Klotz, I. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2582.

Garbett, K., Darnall, D. W., and Klotz, I. M. (1971a), *Arch. Biochem. Biophys. 142,* 455.

Garbett, K., Darnall, D. W., and Klotz, I. M. (1971b), *Arch. Biochem. Biophys. 142,* 471.

Garbett, K., Darnall, D. W., Klotz, I. M., and Williams, R. J. P. (1969), *Arch. Biochem. Biophys. 135,* 419.

Giacometti, G. M., DaRos, A., Antonini, E., and Brunori, M. (1975), *Biochemistry 14,* 1584.

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5,* 467.

Keresztes-Nagy, S., and Klotz, I. M. (1963), *Biochemistry 2,* 923.

Keresztes-Nagy, S., and Klotz, I. M. (1965), *Biochemistry 4,* 919.

Keresztes-Nagy, S., and Klotz, I. M. (1967), *J. Polym. Sci., Part C 16,* 561.

Klapper, M. H., and Klotz, I. M. (1968), *Biochemistry 7,* 223.

Klotz, I. M. (1971), in Subunits in Biological Systems, Part A, Timasheff, S. N., and Fasman, G. D., Ed., New York, N.Y., Marcel Dekker, Chapter 2.

Klotz, I. M., Klotz, T. A., and Friess, H. A. (1957), *Arch. Biochem. Biophys. 68,* 284.

Langerman, N. R., and Klotz, I. M. (1969), *Biochemistry 8,* 4746.

Langerman, N., and Sturtevant, J. M. (1971), *Biochemistry 10,* 2809.

Llinas, M. (1973), *Struct. Bonding (Berlin) 17,* 135.

Morgan, T. D. B., Stedman, G., and Whineup, P. A. E. (1965), *J. Chem. Soc.,* 4813.

Okamura, M. Y., and Klotz, I. M. (1973), in Inorganic Biochemistry, Eichorn, G. L., Ed., Amsterdam, Elsevier, p 336.

Rao, A. L., and Keresztes-Nagy, S. (1973), *Biochim. Biophys. Acta 313,* 249.

Randall, W. C., and Alberty, R. A. (1966), *Biochemistry 5,* 3189.

Randall, W. C., and Alberty, R. A. (1967), *Biochemistry 6,* 1520.

Seewald, D., and Sutin, N. (1963), *Inorg. Chem. 2,* 643.

Sweigart, D. A., and Bern, C. (1974), *Inorg. Chem. 13,* 3033.

Ver Ploeg, D. A., Cordes, E. H., and Gurd, F. R. N. (1971), *J. Biol. Chem. 246,* 2725.

Wilkins, R. G. (1974), The Study of Kinetics and Mechanism of Reactions of Transition Metal Complexes, Boston, Mass., Allyn and Bacon.

York, J. L., and Bearden, A. J. (1970), *Biochemistry 9,* 4549.