Slow Interconversion of Acid and Base Forms of Methemerythrin and Some Implications for Methemerythrin Reactivity†

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ABSTRACT: The acid and base forms of methemerythrin from *Phascolopsis gouldii* interconvert slowly in solution. The kinetics were studied by a pH-perturbation method, monitoring the relaxation both directly and indirectly (with indicators). The return to equilibrium following the perturbation was always first order with rate constant k_{obsd} (s^{-1}) = $3.3 \times 10^{-3} + (2.5 \times 10^{3})[H^+] + (5.0 \times 10^{-12})(1.6 \times 10^{-10} + [H^+])^{-1}$. A number of reaction schemes were considered. From pH 7.0 to 10.5, the scheme PH $\rightleftarrows$ P + H^+ ($K_a = 1.4 \times 10^{-10}$ M); P $\rightleftarrows$ P* ($k_f = 3.2 \times 10^{-2}$ s^{-1}; $k_r = 3.6 \times 10^{-3}$ s^{-1}) fitted the data. Below pH 7.0, the conformational change was acid catalyzed. A number of large anions besides ClO_4^- (already reported), BF_4^-, PF_6^-, and Ph_4B^- slowly converted the base to the acid form, with similar kinetics to those of pH perturbation. The slow base $\rightleftarrows$ acid change plays a role in anation of methemerythrin by SCN^- (but not N_3^-) and probably in reduction by dithionite.

Hemerythrin is an iron-containing respiratory protein in certain marine organisms whose structure and properties have been well characterized (Hendrickson, 1978; Kurtz et al., 1977; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979; Wilkins & Harrington, 1983). Each subunit of hemerythrin contains two non-heme irons. The deoxy form contains both irons in oxidation state +2 and interacts reversibly with oxygen to give the oxy form. Both deoxy and oxy forms are easily oxidized to a met species containing irons in the oxidation state +3. The met form is no longer O_2 sensitive but reacts with a number of anions to form adducts with distinctive spectra (Keresztes-Nagy & Klotz, 1965; Meloon & Wilkins, 1976; Olivas et al., 1979). Keresztes-Nagy & Klotz (1965) first described neutral- and high-pH forms of methemerythrin and considered that these were respectively "metaquo" and "methydroxo" hemerythrin. The spectrum of the latter species is similar to those of other anionic adducts and may contain an Fe^{3+}–OH^- linkage. However, crystal structure of metaquo shows no iron-coordinated water molecule (R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, private communication), so that the nature of the acid–base change is still conjectural. We shall refer to the two forms as $(Hr)_a$ (acid) and $(Hr)_b$ (base). Certain anions, particularly ClO_4^- and to a lesser extent NO_3^-, have the ability to raise the pK_a for the acid–base transformation [for example, from 7.8 (no ClO_4^-) to 8.7 (1 mM ClO_4^-)]. Thus, one is able to convert the base to the acid form by the addition of perchlorate (Darnall et al., 1968; Garbett et al., 1971a,b). This effect is apparently associated with ClO_4^- binding away from the iron site (Darnall et al., 1968) near Cys-9 and Cys-50. The ClO_4^- is probably held by peptide amides and lysine side chains, at least in *Themiste dyscritum* hemerythrin with which crystallographic studies have been carred out (Stenkamp et al., 1978).

We now report that the acid–base equilibration and the ClO_4^--induced base → acid change occur slowly, taking minutes at room temperature, and have studied the detailed kinetics of these changes and their effect on the anation and reduction reactions of methemerythrin. The octamer from the coelomic fluid of *Phascolopsis gouldii* has been used throughout.

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Materials and Methods

The marine worms *P. gouldii* were obtained from Marine Biological Laboratory, Woods Hole, MA. Oxyhemerythrin was obtained from the coelomic fluid of the worms (Klotz et al., 1957). Methemerythrin was prepared by dialyzing oxyhemerythrin against $Fe(CN)_6^{3-}$ and then several times against the appropriate buffer system. For the reactions with dithionite, the preparation and manipulation of solutions were with scrupulous exclusion of oxygen. A Beckman 24 recording spectrophotometer and a Dionex stopped-flow apparatus interfaced with an OLIS data collecting system were used. Chemicals were used as purchased except $NaBF_4$ (PCR Research Chemicals Inc.) and NaN_3, which were recrystallized from water, and $NaPF_6$ (Alfa), which was recrystallized from ethanol/ether.

In the majority of studies, 0.05–0.15 mM methemerythrin was used (based on a monomer molecular weight of 13 500). The following buffer systems were employed: pH ~4.4–6.5, 5–30 mM 4-morpholineethanesulfonic acid (Mes) adjusted with NaOH; pH ~6.0–7.7, sodium phosphate buffer adjusted to I = 0.15 M with a Boyd (1965) nomogram; pH ~7.5–9.5, 50–100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) adjusted with H_2SO_4; pH ~9.0–10.5, 50 mM (cyclohexylamino)propanesulfonic acid (CAPS) adjusted with NaOH. For the determination of the pK_a, separate pH-preadjusted solutions were mixed with concentrated protein (~2 mM) and the spectrum and pH measured at equilibrium (1 h allowed). For the pH jumps, methemerythrin at pH ~9 (1 mM Tris) was dropped in pH, and methemerythrin at pH 5–6 (50 μM sodium phosphate or 5 mM Mes buffers) was raised in pH, by appropriate mixing. In the temperature jump at pH 7.7, 2 mM methemerythrin at 0 °C was plunged into 10 times its volume of 0.15 M phosphate buffer at pH 7.7 and 25 °C. The reactions of met with SCN^- were studied directly at 452 nm and those with $S_2O_4^{2-}$ at 425 nm. In experiments where small pH changes were monitored, these were kept to ~0.1-unit change by light buffering of the solutions. Indicators used (5–20 μM) were bromothymol blue (pH ~7, λ = 620 nm), cresol red (pH ~8, λ = 575 nm), thymol blue (pH ~9, λ = 600 nm), and phenolphthalein (pH ~9, λ = 552 nm). When appropriate, solutions of protein were incubated for 1 h with or without ClO_4^- before measurements. Most of the reactions were monitored at 320 nm, some at 425 nm with similar results. Most pH-jump experiments were carried out

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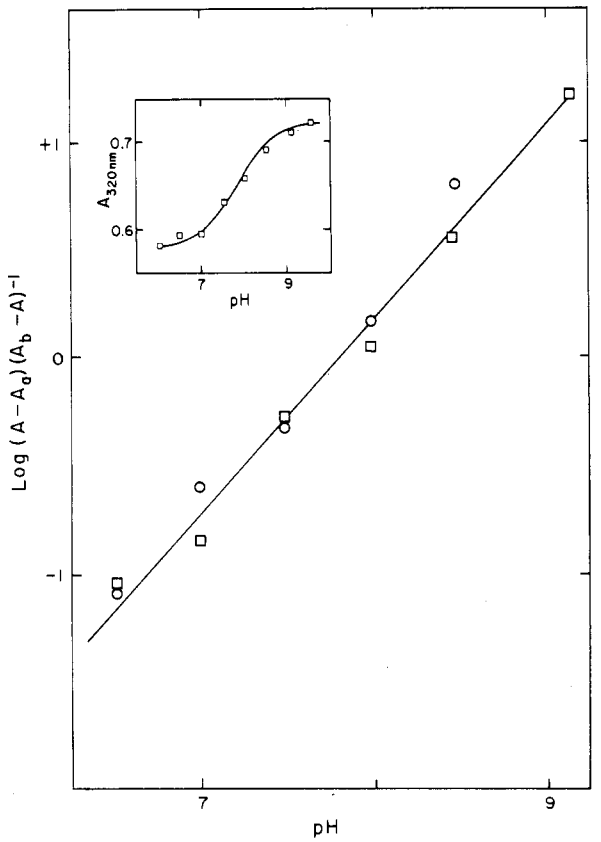

FIGURE 1: Plot of log $[(A - A_a)(A_b - A)^{-1}]$ vs. pH. (Inset) A_{320nm} vs. pH. Full line is theoretical equation for $A_a = 0.575$, $A_b = 0.72$, and $pK_a = 7.8$. Data at 320 nm (□) and 450 nm (○). 0.1 mM met, 25 °C, $I = 0.15$ M, Tris and phosphate buffers.

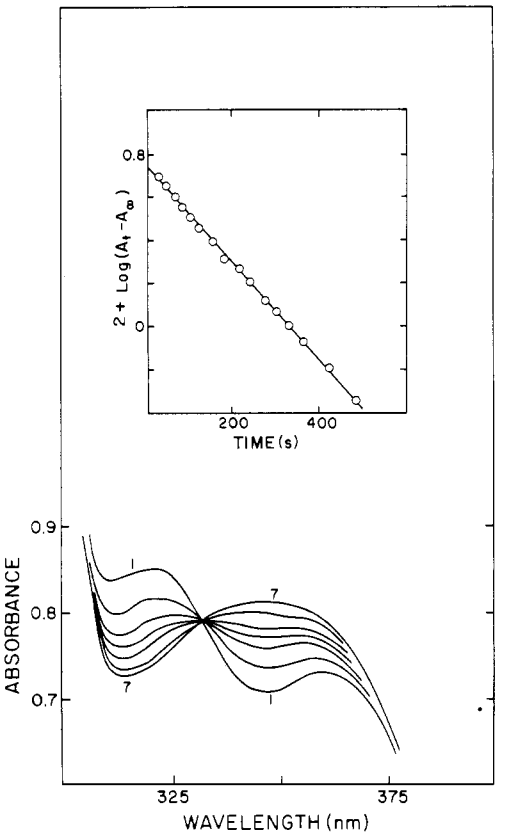

FIGURE 2: Spectra of methemerythrin, with time, after plunge from pH 9.0 to 6.0: t = 0 s (1), 60 s (2), 140 s (3), 220 s (4), 320 s (5), 580 s (6), and 1 h (7). (Inset) First-order plot of absorbance with time. 0.14 mM met, 25 °C $I = 0.15$ M, phosphate buffer, wavelength of observation 320 nm.

at 25 °C, $I = 0.15$ M. In the SCN^- reactions, the ionic strength was usually 0.2 M.

Results

Equilibrium between Acid $(Hr)_a$ and Base $(Hr)_b$ Forms. The spectra of methemerythrin at a number of pH's between 6.0 and 9.5 showed a clean isosbestic point at 332 nm. The spectra at pH 6.0 and 9.5 were considered to represent (>98%) those of the acid and base forms, $(Hr)_a$ and $(Hr)_b$, respectively. No denaturing was observed over this pH range during the time of the experiments. For equilibrium 1, it is easily shown

$$(Hr)_a \rightleftarrows (Hr)_b + nH^+ \quad (1)$$

$$K_a = [(Hr)_b][H^+]^n/[(Hr)_a] \quad (2)$$

that, at a single wavelength, the absorbance at any pH is related to that at pH 6 (A_a) and pH 9.5 (A_b) by eq 3. The

$$\log [(A - A_a)/(A_b - A)] = n\text{pH} - pK_a \quad (3)$$

appropriate plot is shown in Figure 1. Consistent results are obtained at two observation wavelengths, and $n = 0.95$ and $pK_a = 7.8$. When methemerythrin at pH 9 is plunged into a buffer at pH 6, $(Hr)_b$ is converted into $(Hr)_a$. The time dependence of this change is shown spectrally in Figure 2. An isosbestic point at 332 nm was observed. The change is first order, as shown by continual observation at 320 nm (Figure 2). The dynamics of the $(Hr)_a \rightleftarrows (Hr)_b$ equilibrium were therfore studied by pH jump (both up and down). The change following the pH jump was monitored at a number of wavelengths, particularly 320 and 425 nm, as well as indirectly with an indicator by using the small attendant pH change. A very small absorbance change accompanied the temperature jump from 0 to 25 °C of methemerythrin at pH 7.7, and this was monitored. This small change is consistent with near zero values for ΔH for the $(Hr)_a \rightarrow (Hr)_b$ change (Gorman & Darnall, 1981). Within experimental error, the rate constant measured at any pH was independent of the starting pH and independent of a pH jump up or down. Small changes in the perturbing pH obviously effected small absorbance changes, and the errors in the measured rate constants were large. The spectral or pH change associated with the $(Hr)_a \rightleftarrows (Hr)_b$ equilibration had to be utilized for determination of the rate constants at pH <6 or >9. No spectral changes, direct or with indicator, were observed when pH changes were initiated from or to pHs $\ll pK_a$ or pHs $\gg pK_a$. All the changes observed were single first-order reactions, and the results are shown in Figure 3. From measurements of the first-order rate constants from 13.5 to 35 °C, after a pH jump from 6.0 to 9.4 was intitiated, values of $\Delta H^\ddagger = 14.4$ kcal mol^{-1} and $\Delta S^\ddagger = -19$ eu were determined. The complete reversibility of the $(Hr)_a \rightleftarrows (Hr)_b$ change was demonstrated by initiating a pH change from 9 to 6.6 and, after equilibrium was reached, by reconverting the solution to pH = 9.5. Similar rate constants were obtained in this cycle, as was observed with the separate pH jumps (down and up).

Anion-Initiated $(Hr)_b \rightarrow (Hr)_a$ Conversion. The addition of ClO_4^- ion (>9 mM) to a solution of methemerythrin at pH 9.0 promotes conversion of $(Hr)_b$ into $(Hr)_a$. The slow change is accompanied by an isosbestic point at 333 nm and is nicely first order. There is a slight effect of perchlorate concentration on the first-order rate constant (Figure 4). The change can be monitored directly (320 nm and other wavelengths) and indirectly by following, with an indicator, the small pH change (up) that accompanies the $(Hr)_b \rightarrow (Hr)_a$ transformation. The reversibility of the change at pH 8.5 was demonstrated as

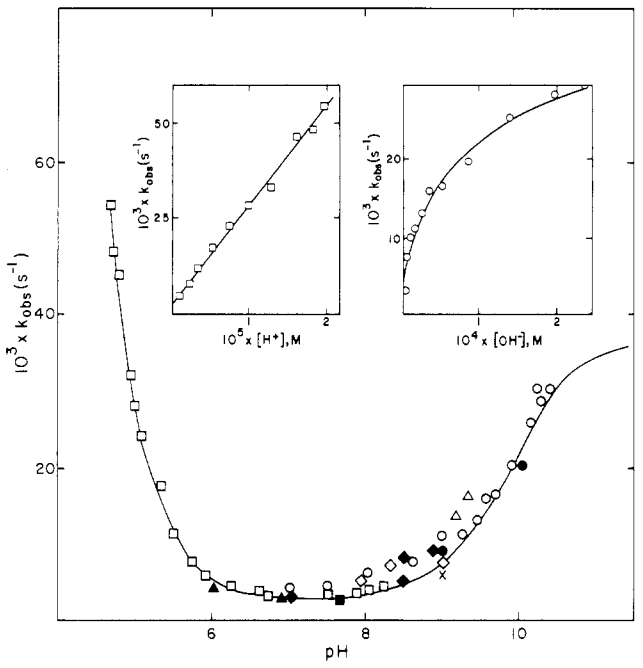

FIGURE 3: Plot of $10^3 k_{obsd}$ vs. pH for acid–base equilibration of methemerythrin: pH jump from 6.0 into 0.05 M CAPS (○); pH jump from 5.0 by adding solid Tris (●); pH jump from 6.0 using indicator method (Δ); pH jump from 9.0 into Tris, Mes, or phosphate (□); pH jump from 9.0 or 8.5 using indicator method (▲); ClO_4^--induced base → acid form (♦); BF_4^-- (50–150 mM) or PF_6^-- (15–100 mM) induced base → acid form (×); slow phase of SCN^- reaction (◇); temperature jump 0 → 25 °C (■). All data at 25 °C and I = 0.15 M. (Inset) $10^3 k_{obsd}$ vs. $10^5[H^+]$ and vs. $10^4[OH^-]$. Solid lines conform to eq 5; see text.

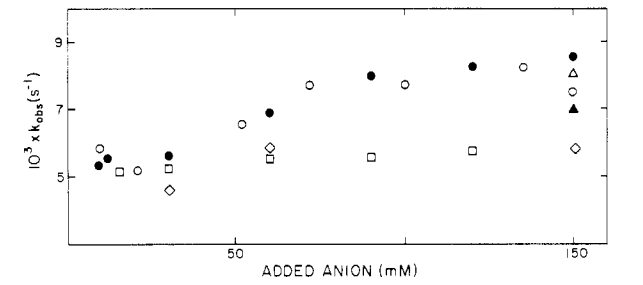

FIGURE 4: Effect of anions on first-order rate constant for base → acid methemerythrin conversion: ClO_4^- (○), phenolphthalein (Δ), thymol blue (▲), PF_6^- (□), and BF_4^- (◇), all at pH 9.0; ClO_4^- (●) at pH 8.5. All data at 25 °C and I = 0.15 M.

follows (results are the mean of five separate experiments): a concentrated solution of methemerythrin (1 mM) and perchlorate (1–5 mM) maintained for 1 h at 25 °C was then diluted 10-fold. The relaxation of the acid to the acid–base mixture had a rate constant of 4.7×10^{-3} s^{-1}. If concentrated perchlorate solution was then added to the diluted solution (final $[ClO_4^-]$ = 13–30 mM), reconversion to the acid form was first order ($k = 5.5 \times 10^{-3}$ s^{-1}). The perchlorate perturbation at pH 8.5 had attendant activation parameters of $\Delta H^{\ddagger}$ = 13.7 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −22 eu. It was also shown that other large anions (BF_4^-, PF_6^-, and Ph_4B^-) could induce the $(Hr)_b \rightarrow (Hr)_a$ change. The results for BF_4^- and PF_6^- are shown in Figure 4 and indicate, as with ClO_4^-, a very small dependence of the rate on the anion concentration. Data for all these anions are also included in Figure 3. The behavior of Ph_4B^- differed from that of the other anions. The conversion rate was linearly dependent on $[Ph_4B^-]$; rate (M s^{-1}) = $0.55[Hr^+][Ph_4B^-]$. The $(Hr)_a$ spectrum was somewhat modified by Ph_4B^- ions but was almost unaffected by the other anions.

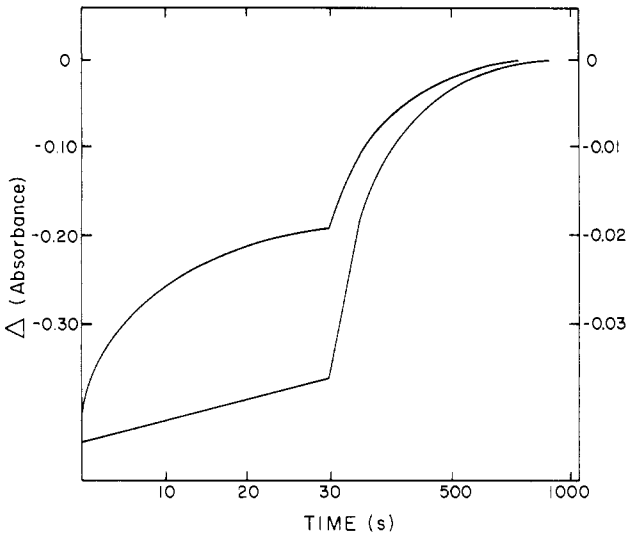

FIGURE 5: Absorbance changes with time following addition of SCN^- ion (5.0 mM) to methemerythrin (69 μM) at 25 °C, pH 7.9, and I = 0.20 M. The upper curve and left ordinate indicate direct spectral change at 452 nm. The lower curve and right ordinate refer to indicator (cresol red, 10 μM) change at 575 nm. The solutions are lightly buffered (0.2 mM Tris), and there is a small (~0.05 pH unit) change accompanying complete anation. Note the scale change on the time ordinate. k_{obsd}(fast) = 9.1×10^{-2} s^{-1}; k_{obsd}(slow) = 4.8×10^{-3} s^{-1}.

Reaction of Methemerythrin with SCN^- Ion. The reaction of methemerythrin with SCN^- ion was studied from pH 6.0 to 9.5 in the absence and in the presence of ClO_4^- ion (50 and 100 mM). The ClO_4^- had been incubated with the protein for at least 1 h at 25 °C. Nearly all the reactions studied were biphasic, but the amount of the contribution of the slower rate, which was always well separated from the faster (Figure 5), was dependent on the pH and the concentration of ClO_4^-. The contributions of the two phases could be assessed from the relative absorbance changes at 452 nm, since the same product (methemerythrin–SCN^- adduct) results for each phase [over *complete* reaction, isosbestic point at 385 nm (pH 9.0) and 388 nm (pH 7.6) and retention of peak at ~450 nm]. In addition, the SCN– adduct has a much higher absorbance coefficient at 452 nm (ϵ = 5100 M^{-1} cm^{-1}) than $(Hr)_a$ (ϵ ~ 800 M^{-1} cm^{-1}) and $(Hr)_b$ (ϵ ~650 M^{-1} cm^{-1}). Each phase was first order, and the two first-order rate constants were designated k_{obsd}(fast) and k_{obsd}(slow). The value of k_{obsd}(fast) was dependent on SCN^- (and ClO_4^-) concentration as shown in Figure 6. The slopes of the linear plots gave the second-order formation rate constants (k_f) and the intercepts gave the dissociation rate constant (k_d) for the anation reaction (eq 4) (Meloon & Wilkins, 1976):

$$\text{met} + SCN^- \rightleftarrows \text{met–}SCN^- \qquad k_f, k_d \qquad (4)$$

The value of k_{obsd}(slow) was almost independent of the concentrations of SCN^- and ClO_4^- ions (Figure 6). The rate constants and relative contributions of the fast and slow step at a number of pHs are collected in Table I. In experiments at pH 7.9, it was shown that a very small pH change attended the fast phase (four half-lives in 30 s) but that the slow reaction had a much larger pH change (Figure 5). The majority of the small pH change could be shown to arise from a contribution from the slower phase during the first 30 s. No such biphasic changes, however, accompanied the reactions of methemerythrin with N_3^- ion at pH 8.5, either in the absence or presence of ClO_4^- ion. The rate constant (0.078 s^{-1}, $[N_3^-]$ = 150 mM) was reduced in the presence of ClO_4^- (0.039 s^{-1}, $[N_3^-]$ = 150 mM, $[ClO_4^-]$ = 150 mM). A few experiments

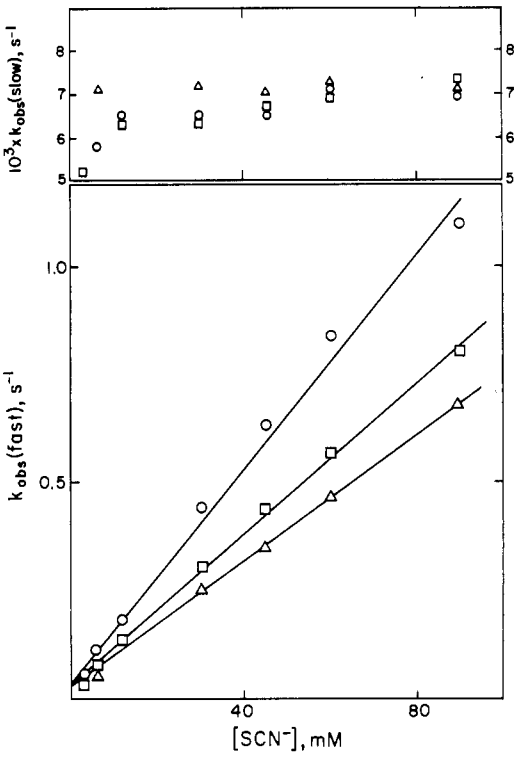

FIGURE 6: Plot of k_{obsd}(fast) and k_{obsd}(slow) vs. [SCN$^-$] for biphasic reaction at pH 8.3: no perchlorate added (○); 50 mM ClO_4^- (□); 100 mM ClO_4^- (△).

Table I: Rate Constants for Fast and Slow Phases of Reaction of Methemerythrin with SCN$^-$ and $S_2O_4^{2-}$ Ions

pH	[$NaClO_4$] (mM)	k_f (M^{-1} s^{-1})	k_d (s^{-1})	k_{obsd}^- (slow) (M^{-1} s^{-1})	% fast reaction exptl	% fast reaction calcd[a]
			SCN$^-$			
6.0	0	22.3	0.11	~5	~97	98
6.0	50	13.7	0.06		>97	100
7.9	0			5.0	47[b]	44
8.3	0	12.4	0.05	7.0[c]	29[b]	24
8.3	50	8.8	0.03	6.3[c]	92	~100
8.3	100	7.3	0.03	6.8[c]	95	~100
9.0	0			5.0[d,e]	<5	6
9.0	100	4.7	0.03		90	85
9.5	0			5.7[d]		
			$S_2O_4^{2-}$			
6.3	0	0.68[f]		1.4	82[g]	97
6.3	50	0.22[f]		1.6	80[g]	~100
9.0	0	0.17[f]		3.0	20[g,h]	6
9.0	135	0.07[f]		2.7	68[g,h]	~85

[a] % $(Hr)_a$ in methemerythrin estimated spectrally. [b] Allowance is made in assessing this value for a small contribution to the absorbance change for the fast phase from the slow phase. [c] At 10–90 mM SCN$^-$, see Figure 6. [d] At 10 mM SCN$^-$, slightly higher value in 100 mM SCN$^-$. [e] Same value using pH change at 10 mM SCN$^-$. [f] Fast phase (s^{-1}). [g] % total absorbance change associated with fast phase. [h] ~7–10% absorbance change for third step.

were carried out in which ClO_4^- and SCN$^-$ ions or ClO_4^- and N_3^- ions were mixed with methemerythrin. In all cases, the results obtained were similar to those in which ClO_4^- had been incubated with the protein. This meant that the ClO_4^- bound more rapidly to the protein than did SCN$^-$ ion ($k_{obsd} > 1$, 10 mM ClO_4^-, pH 6.0) or N_3^- ion ($k_{obsd} > 0.04$ s^{-1}, 150 mM ClO_4^-, pH 8.5).

Reduction of Methemerythrin by Dithionite Ion. The reactions were studied by using 10 mM $S_2O_4^{2-}$ at pH 9.0 and 6.3 in the absence and presence of ClO_4^- ion. The results are included in Table I. The bulk (≥90% of the total absorbance change) could be dissected into two first-order changes, well separated in rate. The assignment of these absorbance changes to the extent of reduction was however not simple, since the extent of reduction of $(semi\text{-}met)_R$ hemerythrin, the product of the fast step, was uncertain during the slow step.

Discussion

Figure 3 contains most of the kinetic data obtained in the present study. The majority of the kinetic results were obtained by a pH perturbation of the $(Hr)_a \rightleftarrows (Hr)_b$ equilibrium and subsequent monitoring of the relaxation. A perturbation could also be imposed by the addition of ClO_4^-, BF_4^-, PF_6^-, and SCN$^-$ ions, and similar (slow) relaxation rates were observed as the base form converted into the acid form. Only with SCN$^-$ ion did binding occur at the iron center. Thus, several perturbation techniques were available, and their interrelationship could be established as discussed below.

$(Hr)_a \rightleftarrows (Hr)_b$ Equilibrium. Spectral titration of methemerythrin from pH 6.0 to 9.5 indicates that there is predominantly one acid and one base form, differing by the equivalent of one proton, with our conditions, $pK_a = 7.8$ (Figure 1). Previously, pK_a values of 7.83 (Darnall et al., 1968) and 7.44 at $I = 0.2$ M (Gorman & Darnall, 1981) associated with a single ionization have been reported (all for *P. gouldii*). The proposal that two protons are involved (Garbett et al., 1971b) is not considered likely (Gorman & Darnall, 1981).

The attainment of equilibrium is surprisingly slow (Figure 2), a feature not previously commented upon. The process is first order, there is no sign of an absorbing intermediate (Figure 2), and at pH 6–8 and 25 °C, 20 min is required for equilibrium to be reached. Extensive study of the dynamics by using a variety of perturbations gave the k_{obsd}/pH profile shown in Figure 3.

The first-order equilibration rate constant/pH profile (Figure 3) is described by

$$k_{obsd} = a + b[H^+] + c[d + [H^+]]^{-1} \quad (5)$$

with $a = 3.3 \times 10^{-3}$ s^{-1}, $b = 2.5 \times 10^3$ M^{-1} s^{-1}, $c = 5.0 \times 10^{-12}$ M s^{-1}, and $d = 1.6 \times 10^{-10}$ M. The agreement of experimental and calculated values on the basis of eq 5 is indicated in Figure 3 for the acid and base regions in the insets and over the full pH range by the line drawn. It is difficult to attach a completely satisfactory reaction scheme to this rate equation. The slow alkaline conversion of horseradish peroxidase has been considered to involve a conformational change of the protein (Epstein & Schejter, 1972; Iizuka et al., 1976; Kihara et al., 1978) although this has been disputed (Araiso & Yamazaki, 1978). The expression of the first-order relaxation rate constant k_{obsd} for acid–alkaline conversion of peroxidase [see also Job et al. (1977)]

$$k_{obsd} = a + b[H^+] + c[OH^-] \quad (6)$$

is obviously incompatible with eq 5 in the alkaline region. An alternative treatment of the peroxidase data is in terms of the reaction scheme shown in eq 7–9 (Kihara et al., 1978). There

$$PH_2 \rightleftarrows PH + H^+ \qquad K_7 \quad (7)$$

$$PH \rightleftarrows {}^*PH \qquad k_8, k_{-8} \quad (8)$$

$$^*PH \rightleftarrows P + H^+ \qquad K_9 \quad (9)$$

would be two very rapid relaxations associated with the proton reactions 7 and 9 while the equilibrium between the two conformers PH and *PH would be established slowly, with a k_{obsd} given by eq 10 (Kihara et al., 1978). Our data (Figure

$$k_{obsd} = k_8(1 + [H^+]/K_7)^{-1} + k_{-8}(1 + [H^+]/K_9)^{-1}([H^+]/K_9) \quad (10)$$

3) give a reasonably good fit to this equation (except for the pH 7–9 region where $k_{obsd} > k_{calcd}$) with $K_7 = 3.4 \times 10^{-10}$ M, $K_9 = 5.9 \times 10^{-5}$ M, $k_8 = 0.03$ s^{-1}, and $k_{-8} = 0.21$ s^{-1}. However, this scheme requires that conversion of PH_2 into P occurs in the pH 7–9 region, and our [and others, Darnall et al. (1968) and Gorman & Darnall (1981)] titration data are inconsistent with a two-proton change. The alkaline isomerization of oxidized cytochrome *c* has a rate/pH profile similar to that for our system from pH 7.0 to 10.5 (Figure 3) but much higher rates (Davis et al., 1974). This pH dependence can be described by a scheme (eq 11 and 12) in which the deprotonated form P undergoes a conformational change to the stable form P*:

$$PH \rightleftarrows P + H^+ \qquad K_{11} \quad (11)$$

$$P \rightleftarrows P^* \qquad k_{12}, k_{-12} \quad (12)$$

with

$$k_{obsd} = k_{-12} + k_{12}K_{11}/(K_{11} + [H^+]) \quad (13)$$

Our data are reproduced well by expression 13 with $k_{12} = 3.2 \times 10^{-2}$ s^{-1}, $k_{-12} = 3.6 \times 10^{-3}$ s^{-1}, and $K_{11} = 1.4 \times 10^{-10}$ M. The value of K_a for ionization 14, determined spectrally, will

$$PH \rightleftarrows P^* + H^+ \quad (14)$$

thus be given by $(K_{11}k_{12}/k_{-12})$. This calculated value, 1.24×10^{-9} M, is in poor agreement with the experimental value of K_a (1.6×10^{-8} M). If the value of $K_{11}k_{12}/k_{-12}$ is constrained to be 1.6×10^{-8} M, then the best fit of the experimental data of Figure 3 to eq 5 is given by $b = 2.3 \times 10^3$ M^{-1} s^{-1}, $a = 3.2 \times 10^{-4}$ s^{-1}, $c = 9.9 \times 10^{-12}$ M s^{-1}, and $c = 3.2 \times 10^{-10}$ M, which implies that $k_{12} = 3.1 \times 10^{-2}$ s^{-1}, $k_{-12} = 3.2 \times 10^{-4}$ s^{-1}, and $K_{11} = 3.2 \times 10^{-10}$ M. It should be emphasized that this is a much poorer fit than that shown in Figure 3, particularly in the pH 6–8.5 region. If scheme 11 and 12 is the correct interpretation of the data, then the enhanced values in the acid region might arise from acid catalysis of the P ⇄ P* change and increased k_{12} and k_{-12} values. The enhanced rate in the acid and base regions can only be observed if changes are initiated from pH > pK_a (7.8) or pH < pK_a, respectively; i.e., the spectral change associated with the apparent pK_a = 7.8 must be utilized.

Anion-Initiated $(Hr)_b \rightarrow (Hr)_a$ Conversion. The quantitative aspects of the binding of ClO_4^- to methemerythrin (*P. gouldii*) have been previously studied at pH 7.2 by utilizing the ClO_4^- effect on the binding characteristics of SCN^- at the iron site. Two (and possibly three) successive formation constants for ClO_4^- binding must be assumed to explain the data (Garbett et al., 1971a). The $(Hr)_b \rightarrow (Hr)_a$ transformation, induced by ClO_4^- ion, is again slow with no apparent intermediate showing up during the spectral scan. The binding of ClO_4^- to the protein was shown to be more rapid than that of SCN^-, and if the ClO_4^- binding is second order, the rate constant exceeds 10^2 M^{-1} s^{-1}. The rapidity of binding of ClO_4^- is consistent with the very small, if any, effect of ClO_4^- concentration on the first-order equilibrium constant. Other anions such as BF_4^- and PF_6^- also induce the $(Hr)_b \rightarrow (Hr)_a$ transformation, and the associated rate constants are similar for all three anions and for the $(Hr)_b \rightarrow (Hr)_a$ pH-induced changes (Figure 3). Slight differences may arise from the anions' binding affecting the rate of the slow conformational change by modifying the active site structure [observed in crystallographic studies on the methemerythrin (*T. dyscritum*)–ClO_4^- system (Stenkamp et al., 1978)]. That similar changes are associated with nonanionic and anionic perturbations is also strongly supported by similar activation parameters for each process. The ClO_4^- binding then causes a conformational change that is reflected in the structure at the active site (Stenkamp et al., 1978).

Reaction of Methemerythrin with Anions. All the data for the SCN^- reaction with methemerythrin (Table I) can be accommodated by the scheme:

$$(Hr)_a \rightleftarrows (Hr)_b \qquad k_{15}, k_{-15}, K_{15} \quad (15)$$

$$(Hr)_a + SCN^- \rightleftarrows \text{adduct} \qquad k_{16}, k_{-16} \quad (16)$$

Since equilibrium 15 is established much more slowly than that of (16) with the SCN^- concentrations used, then the equilibrium amount of $(Hr)_a$ in (15) reacts rapidly with SCN^- in a second-order reaction (Figure 6). The further conversion of $(Hr)_b$ to $(Hr)_a$ in (15) limits the rate of further formation of adduct and gives rise to the slow first-order step, k_{-15}. A very slight effect of SCN^- on the rate of the slow step may arise from a small contribution from a direct reaction of $(Hr)_b$ with SCN^- or may result from a slight modification of k_{-15} caused by SCN^- binding to the secondary site. Since the addition of ClO_4^- increases the amount of $(Hr)_a$ in equilibrium 15, the contribution from the fast reaction increases in the presence of perchlorate. The second-order rate constant k_{16} decreases with increasing perchlorate (Table I and Figure 6), an effect already observed (Garbett et al., 1971b). The slow reaction rate remains almost unchanged. The relative contributions from the fast and slow stages can be calculated from the known amounts of $(Hr)_a$ and $(Hr)_b$, respectively, and the values are in excellent agreement with those found experimentally (Table I). The fast reaction associated with (15) is unaccompanied by a pH change, but the slow step determined by k_{16} will have a pH increase as protons are taken up in the $(Hr)_b \rightarrow (Hr)_a$ change. This effect is shown in Figure 5.

The absence of such biphasic character in the reaction of methemerythrin with N_3^- ion at pHs ~8 probably reflects the stronger binding tendencies of this anion toward hemerythrin (Keresztes-Nagy & Klotz, 1965; Meloon & Wilkins, 1976) although the striking differences in behavior are puzzling. As with SCN^-, ClO_4^- ions reduce the rate constant for N_3^- binding. The data obtained for reduction of methemerythrin by dithionite (Table I) may also be interpretable in a similar fashion to that for thiocyanate binding. Unfortunately, it is difficult to interpret the absorbance changes and their associated rates in terms of specific changes. However, the data at pH 9.0 were consistent with only $(Hr)_a$ being easily reduced by $S_2O_4^{2-}$ and the conversion of $(Hr)_b \rightarrow (Hr)_a$ limiting the further reduction of methemerythrin. Monitoring of $(semimet)_R$ hemerythrin by electron paramagnetic resonance (EPR) (Muhoberac et al., 1980) during the reduction would greatly aid the interpretation of the results.

Conclusions

The important result and crux of this study are that the interconversion of the acid and base forms of methemerythrin from *P. gouldii* is remarkably slow. The first-order rate constant for the $(Hr)_a \rightleftarrows (Hr)b$ equilibration is almost constant from pH 6.0 to 8.5 but accelerated at pHs outside this range according to eq 5. Difficulties are encountered in assigning a satisfactory mechanisms to all the kinetic data.

Large anions, ClO_4^-, BF_4^-, and PF_6^-, promote conversion of $(Hr)_b$ into $(Hr)_a$, and the similar slow rate suggests the same underlying cause, i.e., a conformational change in the protein. This slow acid–base reaction also controls the rate of reaction

of $(Hr)_b$ with SCN^-, but not N_3^- ions, and probably the reduction of $(Hr)_b$ by $S_2O_4^{2-}$ ion. The reactivity of methemerythrin will thus depend on factors such as pH or the concentration of added large anions that control the amount of the base form. Finally, it should be emphasized that in any investigation of the reactions of these species, particularly at lowered temperatures, sufficient time should be allowed for equilibration of the acid and base forms to be achieved. This might require several hours at 4 °C.

Registry No. BF_4^-, 14874-70-5; PF_6^-, 16919-18-9; Ph_4B^-, 4358-26-3; SCN, 302-04-5; perchlorate, 14797-73-0; dithionite, 14844-07-6.

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Kinetic and Equilibrium Studies on the Role of the β-147 Histidine in the Root Effect and Cooperativity in Carp Hemoglobin†

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ABSTRACT: In fish possessing a swim bladder, the alkaline Bohr effect is much enhanced. At pH ≤6, oxygen binding becomes noncooperative and the oxygen affinity very low. This phenomenon is known as the Root effect. In mammalian hemoglobin a major fraction of the alkaline Bohr effect is contributed by His-HC3(146)β. Perutz proposed that the Root effect is a consequence mainly of the replacement of Cys-F9(93)β in mammalian hemoglobin by Ser in fish hemoglobin. Model building showed that this Ser would stabilize the salt bridges between His-HC3 and Asp- or Glu-FG1 in the T structure by the formation of two additional hydrogen bonds. If this theory is correct, then enzymatic cleavage of His-HC3β should inhibit the Root effect. We have prepared des-His-HC3(147)β carp hemoglobin and compared its ligand-binding equilibria and kinetics with those of native carp hemoglobin and have interpreted the results in terms of the allosteric model. Our results show that removal of His-HC3(147)β halves the alkaline Bohr effect as determined from $\partial \log P_{50}/\partial$pH, accelerates the binding of CO at acid pH, and diminishes the pH dependence of the CO on rate. In native carp hemoglobin at pH 7, recombination of CO after photolytic dissociation of only part of the CO is faster than after full dissociation. In des-His carp hemoglobin at pH 7, this acceleration is not observed. These results show that removal of His-HC3 inhibits all the phenomena associated with the Root effect. Our measurements indicate that this is due to a destabilization of the T structure by the equivalent of 3.1 kcal/mol of tetramer.

The effects of pH on the oxygen affinities of the bloods and hemoglobins of a number of fish have been examined (Green & Root, 1933; Root & Irving, 1941; Scholander & Van Dam, 1954; Rossi-Fanelli & Antonini, 1960). For the bony fishes possessing a swim bladder, the pH changes altered the O_2 pressures for half-saturation (Bohr effect) and the shapes of the O_2 equilibrium curves. At low pH, the binding curves often became heterogeneous and leveled off before complete saturation could be reached. Scholander & Van Dam (1954) reported that at low pH certain fish hemoglobins remained only partially saturated when the O_2 pressure was as high as 100 atm. Tan et al. (1973) have shown how the effects for carp hemoglobin can be interpreted within the framework of the allosteric model. At low pH (5.6 or 6 in the presence of 1 mM

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