Anomalously Slow Cyanide Binding to *Glycera dibranchiata* Monomer Methemoglobin Component II: Implication for the Equilibrium Constant†

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ABSTRACT: In comparison to sperm whale metmyoglobin, metleghemoglobin *a*, methemoglobins, and heme peroxidases, the purified *Glycera dibranchiata* monomer methemoglobin component II exhibits anomalously slow cyanide ligation kinetics. For the component II monomer methemoglobin this reaction has been studied under pseudo-first-order conditions at pH 6.0, 7.0, 8.0, and 9.0, employing 100–250-fold mole excesses of potassium cyanide at each pH. At 20 °C, with micromolar protein concentrations, k_{obsd} varies between 9.11×10^{-5} s^{-1} at pH 6.0, 100-fold KCN mole excess, and 1.12×10^{-2} s^{-1} at pH 9.0, 250-fold KCN mole excess. Our analysis shows that the concentration-independent bimolecular rate constant (k_1^{app}) is small in comparison to those of the other heme proteins. For example, at pH 7.0 it is 0.491 M^{-1} s^{-1}, compared to 1.1×10^5 M^{-1} s^{-1} for cytochrome *c* peroxidase; 111 M^{-1} s^{-1} for guinea pig methemoglobin; approximately 400 M^{-1} s^{-1} for sperm whale metmyoglobin; and 692 M^{-1} s^{-1} for soybean metleghemoglobin *a*, at the same pH and similar temperatures. Furthermore, our results show that the dissociation rate is extremely slow, with k_{-1}^{app} no larger than 10^{-6} s^{-1}. Separation of the bimolecular rate constant into contributions from k_{CN^-} (the rate constant for CN^- binding) and from k_{HCN} (the rate constant for HCN binding) shows that the former is approximately 90 times greater. These results indicate that cyanide ligation reactions are not instantaneous for this protein, which is important for those attempting to study the ligand-binding equilibria. From the results presented here we estimate that the actual equilibrium dissociation constant (K_D) for cyanide binding to this *G. dibranchiata* monomer methemoglobin has a numerical upper limit that is at least 2 orders of magnitude smaller than the value reported before the kinetic results were known.

Ligand-binding properties of the *Glycera dibranchiata* monomer hemoglobins (Seamonds, 1971; Seamonds et al., 1971, 1976; Parkhurst et al., 1980) have been of interest since the exceptional amino acid substitution at primary sequence position E-7 (E-7 His → Leu) was first identified by amino acid sequencing (Imamura et al., 1972) and X-ray crystallography (Padlan & Love, 1974). The possible implications of this substitution on ligand binding are obvious due to the close proximity of the E-7 residue to the heme ligand binding site and because the replacement creates a less polar heme ligand environment, one that contains a completely hydrocarbon amino acid side chain in place of a multifunctional amino acid side chain. Both equilibrium (Seamonds, 1971; Seamonds et al., 1971) and kinetics (Seamonds et al., 1976; Parkhurst et al., 1980) studies have been published, and these, indeed, revealed unusual equilibrium and kinetic binding constants for various ligands binding to the *G. dibranchiata* monomer hemoglobins.

In the case of cyanide binding, the only previous work on the *G. dibranchiata* monomer hemoglobins involved equilibrium ligation studies (Seamonds, 1971; Seamonds et al., 1971), and that work used the unseparated monomer hemoglobin fraction, which more recent work has shown to consist of three major, individual monomer hemoglobins (Kandler & Satterlee, 1983; Kandler et al., 1984; Cooke & Wright, 1985; Constantinidis & Satterlee, 1987). Establishment of a purity criterion for the component II monomer hemoglobin (Constantinidis & Satterlee, 1987) confirmed the globin homogeneity of our component II preparations and now allows us to report kinetics results for cyanide[1] (CN) binding to the purified component II methemoglobin. Component II was chosen because the suggestion has been made that this component of the monomer fraction most closely resembles that which was crystallized and for which the sequencing and crystal structure were performed (Cooke & Wright, 1985). Thus, we can be reasonably sure that this protein lacks the distal histidine at primary sequence position E-7.

Our results show that the rate of cyanide binding to monomer methemoglobin component II is anomalously slow in comparison to that of other heme proteins. This fact emerged from our earlier unsuccessful attempts to reproduce published equilibrium cyanide-binding results. Furthermore, this work shows that the protein preferably binds cyanide ion[1] (CN^-), that the dissociation of cyanide from the fully ligated protein is extremely slow, and that, under our experimental conditions (pH 6.0–9.0, μ = 1.0 M), there are no detectable protein ionizations that influence cyanide binding.

Materials and Methods

Protein Isolation. *G. dibranchiata* monomer hemoglobin component II was isolated as the ferric (met) protein as previously described (Kandler et al., 1984; Constantinidis & Satterlee, 1987). The apoprotein was subjected to high-voltage isoelectric focusing as a test of homogeneity, as previously

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[1] The term cyanide refers to all forms of KCN in aqueous solution at the appropriate pH. Cyanide ion will be specifically indicated by CN^-. Other abbreviations: Hb^+, methemoglobin; Hb-CN, cyanide-ligated methemoglobin; Mb^+, metmyoglobin; Mb-CN, cyanide-ligated metmyoglobin; Lb-CN, cyanide-ligated metleghemoglobin *a*; μ, ionic strength.

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described (Constantinidis & Satterlee, 1987). Samples of the protein preparation were also checked by proton NMR spectroscopy, which has been shown to be a valuable analytical tool for this hemoglobin (Constantinidis & Satterlee, 1987).

Determining k_{obsd}. The observed rate constants for cyanide binding were determined by using a Perkin-Elmer 559A UV–visible spectrometer equipped with a variable-temperature cell compartment. Variation in the absorption intensity of the Soret maximum (422 nm) as a function of time was followed after a buffered solution of the ferric unligated protein was mixed with a buffered solution (at identical pH) of KCN (Fisher, analytical grade). These experiments were all carried out under conditions in which the initial KCN concentration was in 100-, 150-, 200-, and 250-fold mole excess of the protein. In this way pseudo-first-order kinetics analyses could be applied. Our work at 20 °C and an ionic strength (μ) of 1.0 M necessitated calculating the pK_a of HCN(aq) by use of the van't Hoff equation and data reported in *Critical Stability Constants* (Smith & Martell, 1976) because the closest reported HCN pK_a (=8.95) is at 25 °C and μ = 1.0 M. For our conditions (20 °C, μ = 1.0 M) the pK_a is found to be 8.82. By use of this value the actual concentrations of HCN and CN^- present in each of our solutions can be calculated, as previously described (Kassner et al., 1985).

Stock solutions of hemoglobin and KCN were freshly made and stored in tightly stoppered flasks immediately prior to each experiment. The KCN solutions were prepared in flasks with no observable gas space in order to minimize loss of HCN gas from solution. Similarly, cuvettes were completely filled with liquid. The linearity of the data shown in Figure 3 indicates that the impact of KCN solution handling is within the experimental reproducibility. Each solution was heavily buffered at the pH of the experiment in order to eliminate pH fluctuation that might occur as a result of excess cyanide being present in less well buffered solutions. Such preparation also eliminated the need for titrating the KCN solution to the appropriate pH, as commonly done in other cyanide-binding studies. This necessitated the use of 0.3 M potassium phosphate (Fisher) buffer that has an ionic strength of 0.9 M at pH 9.0, the maximum pH employed. Since the ionic strength of our standard buffer solutions was less at the lower pH values, we chose to work consistently in μ = 1.0 M solutions. Adjustment of the ionic strength to 1.0 M was carried out by the addition of calculated amounts of KCl (Fisher) required for each solution at each pH. The stock hemoglobin solutions were 6.0–7.5 × 10^{-6} M, whereas the stock KCN solutions were 3.0–4.5 × 10^{-3} M. The precise hemoglobin concentration of each solution was determined from UV–visible extinction coefficients immediately before it was mixed with the appropriate amount of cyanide solution to start the kinetic run. Final hemoglobin concentrations were 5.0 (±0.05) × 10^{-6} M. Parallel mixtures of methemoglobin and KCN were monitored for pH variation at the beginning of each experiment by using a Beckman pH 60 meter and a Fisher combination electrode. The actual reaction mixture pH was also determined at the end of each experiment. The maximum detected pH variation in all experiments was 0.02 pH unit. Each experiment was repeated two or three times. The graphical error bars shown in the figures indicate the range of reproducibility in multiple experiments at a given pH.

Results and Discussion

Kinetic Constants. The chemistry of ligand binding by monomeric ferric heme proteins in aqueous solutions of potassium cyanide has been studied by many workers (Vega-Catalan et al., 1986; Kassner et al., 1985; Job et al., 1980; Erman, 1974; Seamonds, 1971; Seamonds et al., 1971; Ellis & Dunford, 1968; George & Tsou, 1952). Those studies have shown that, in general, one must consider the presence of both HCN and CN^- in aqueous solutions (Izatt et al., 1962). In principle a ferriheme protein may react with both species, so that the overall rate of formation of cyanide-ligated protein could depend upon the following two reactions:

$$\text{protein} + \text{HCN} \underset{}{\overset{k_{HCN}}{\rightleftharpoons}} \text{protein-CN} + H^+ \quad (1)$$

$$+H^+ \uparrow\downarrow -H^+$$

$$\text{protein} + CN^- \overset{k_{CN^-}}{\rightleftharpoons} \text{protein-CN} \quad (2)$$

It has been customary to treat KCN-binding data by considering the reaction to be dependent upon the initial KCN concentration, that is, to let CN (cyanide) represent the total amount of cyanide present (Kassner et al., 1985; Erman, 1974; Ellis & Dunford, 1968; George & Tsou, 1952). Our experiments and analyses follow those previous procedures. We have studied the cyanide-binding ability of the *G. dibranchiata* monomer methemoglobin component II under pseudo-first-order conditions based on the initial KCN concentration being much greater than the monomer methemoglobin component II concentration. The reaction was studied for four CN mole excesses: 100–, 150–, 200–, and 250-fold at each of the pH values 6.0, 7.0, 8.0, and 9.0.

The ligation reaction can be clearly followed because it causes large UV–visible and NMR spectral changes. The changes induced in the UV–visible spectrum for the reaction carried out at pH 6.0 and pH 9.0 are shown in Figure 1. An important point shown in Figure 1 is that the pH 9.0 (B) spectrum for the unligated methemoglobin is essentially identical with the pH 6.0 spectrum (A). In view of the large spectral changes demonstrated when hydroxo-ligated monomer Hb^+ forms (Seamonds et al., 1971), we may conclude that at the high pH extreme we are dealing with a protein that has not formed the hydroxy form. The spectra of the monomer methemoglobin presented in this figure possess the unusual properties (λ_{max} = 393; 637 nm) noted by Seamonds et al. (1971) for the unseparated monomer mixture. Thus, our unusual binding results cannot be construed as being atypical of the monomer methemoglobins. A shift in the Soret band maximum from 393 nm (unligated Hb^+) to 422 nm (Hb–CN) is characteristic of CN binding.

Therefore, the course of the cyanide-binding reaction for all KCN mole excesses, at each pH, was monitored by following increasing absorbance at 422 nm vs time. Such raw data were accurately fitted by a nonlinear least-squares method to a single exponential that yielded k_{obsd} (data not shown). All reactions were followed to completion. These data are consistent with an overall process that is characteristic of a bimolecular reaction, as written in eq 3, being carried out under pseudo-first-order conditions.

$$\text{Hb} + \text{CN} \underset{k_{-1}^{app}}{\overset{k_1^{app}}{\rightleftharpoons}} \text{HbCN} \quad (3)$$

In this case the observed rate constant (k_{obsd}) is dependent upon the cyanide concentration according to

$$k_{obsd} = k_1^{app}[\text{CN}] + k_{-1}^{app} \quad (4)$$

Five important points emerge from treating the raw cyanide-binding data in this manner. (1) Logarithmic graphs of all reactions (cf. Figure 2A,B) were linear, with correlation coefficients of 0.991 or better, over at least 7 half-lives, indicating conformity to pseudo-first-order kinetics. (2) This corresponds to completion of 99.2% of the reaction and shows

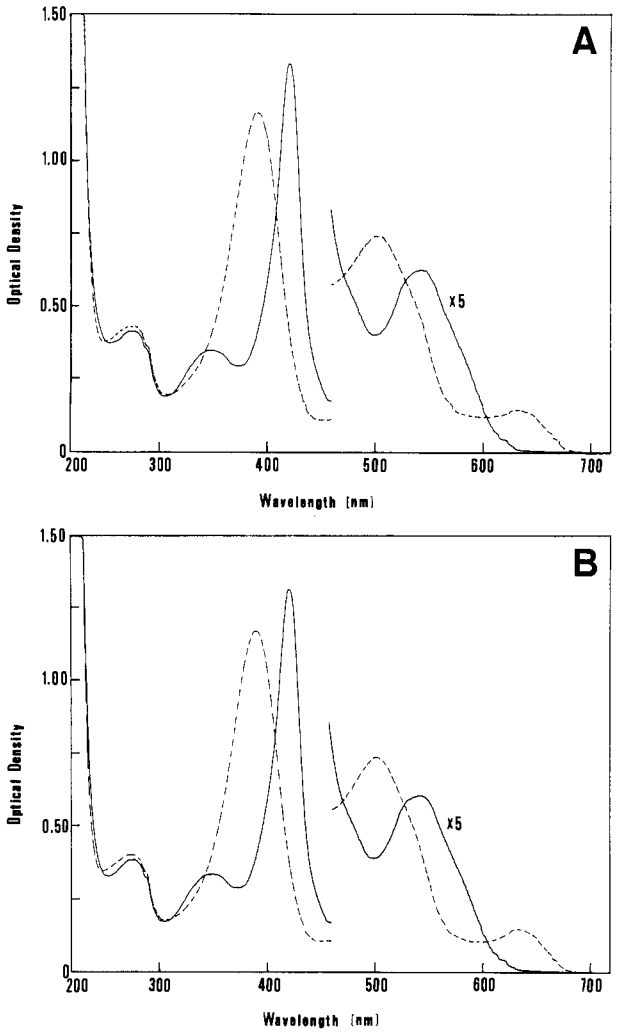

FIGURE 1: UV–visible spectra of the *G. dibranchiata* monomer methemoglobin component II (---) and the cyanide-ligated form (—) at pH 6.0 (A) and pH 9.0 (B), 20 °C, μ = 1.0 M.

Table I: Observed Pseudo-First-Order Rate Constants at 20 °C for Cyanide Binding to *G. dibranchiata* Monomer Methemoglobin Component II as a Function of pH and Mole Ratio of KCN to Hemoglobin

pH	$[KCN]_{initial}/[Hb]$	k_{obsd}[a] (s^{-1})
6.0	100	9.11 (±0.38) × 10^{-5}
	150	1.52 (±0.05) × 10^{-4}
	200	2.05 (±0.05) × 10^{-4}
	250	2.39 (±0.10) × 10^{-4}
7.0	100	2.58 (±0.06) × 10^{-4}
	150	4.26 (±0.20) × 10^{-4}
	200	5.32 (±0.31) × 10^{-4}
	250	6.61 (±0.34) × 10^{-4}
8.0	100	1.25 (±0.05) × 10^{-3}
	150	1.93 (±0.14) × 10^{-3}
	200	2.55 (±0.05) × 10^{-3}
	250	3.46 (±0.05) × 10^{-3}
9.0	100	4.33 (±0.05) × 10^{-3}
	150	6.67 (±0.29) × 10^{-3}
	200	9.22 (±0.33) × 10^{-3}
	250	1.12 (±0.05) × 10^{-2}

[a] μ = 1.0 M; errors in parentheses are variations in reproducibility; [Hb] = 5.0 μM.

that reactions which are more complicated than those shown in eq 1 and 2 do not occur. The data shown in Figure 2 are representative of all of our results (most not shown), which were obtained at each pH, at each cyanide mole excess. (3) At a fixed mole excess of KCN, k_{obsd} increases with increasing pH (Table I, Figures 3 and 4). (4) For a given pH, k_{obsd}

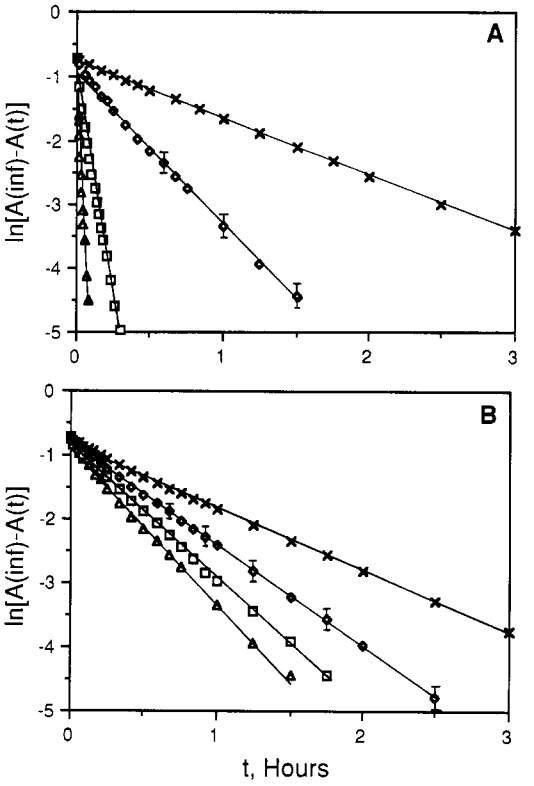

FIGURE 2: Logarithmic plots of raw cyanide-binding data for the *G. dibranchiata* monomer component II methemoglobin used for evaluating pseudo-first-order rate constants. (A) Data taken under the constraint of fixed mole ratio [KCN]/[Hb] = 250 and varying pH: pH 6.0 (×), pH 7.0 (◇), pH 8.0 (□), pH 9.0 (△); 20 °C, μ = 1.0 M. Data at pH 6.0 and 7.0 not shown entirely to completion. (B) Data taken at constant pH 7.0 but as a function of varying [KCN]/[Hb]: [KCN]/[Hb] 100 (×), 150 (◇), 200 (□), 250 (△); 20 °C, μ = 1.0 M.

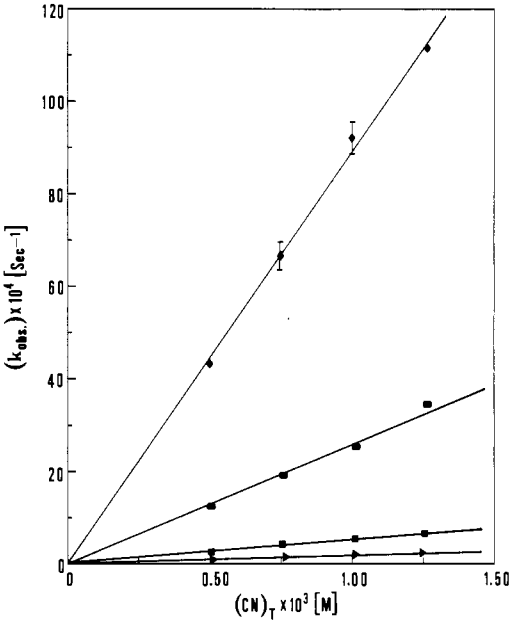

FIGURE 3: Plots of k_{obsd} for cyanide binding to *G. dibranchiata* monomer component II methemoglobin as a function of the initial KCN concentration at pH 6.0 (▲), 7.0 (■), 8.0 (●), 9.0 (◆), 20 °C; μ = 1.0 M, [Hb] = 5.0 × 10^{-6} M.

increases linearly with increasing KCN concentration (Figures 2B and 3) as predicted by eq 4. For example, in comparison to the pH 6.0, 250-fold KCN mole excess cited above, the ligation reaction at pH 6.0, 100-fold KCN excess requires 17 h for completion. (5) The k_{obsd} at a given pH, KCN mole

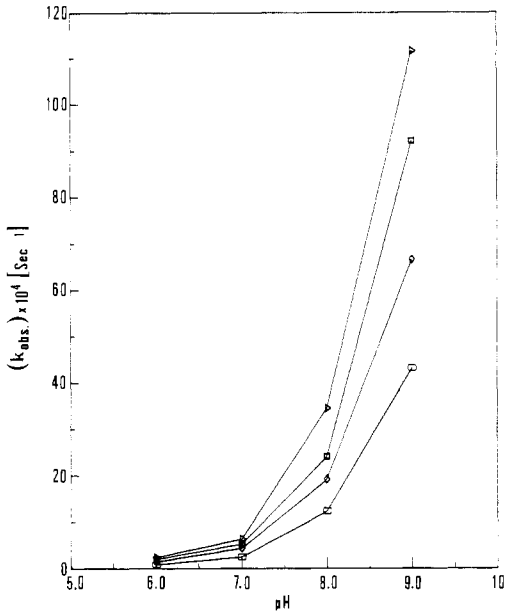

FIGURE 4: Variation of k_{obsd} with pH at ratios [KCN]/[Hb] = 100 (○), 150 (◇), 200 (□), 250 (△); 20 °C, μ = 1.0 M.

Table II: Apparent Bimolecular Rate Constant (k_1^{app}) for Cyanide Binding to the *G. dibranchiata* Monomer Component II Methemoglobin as a Function of pH at 20 °C Derived from the Slopes of the Graphs in Figure 5

pH	k_1^{app} [a] (M^{-1} s^{-1})	corr coeff[b]
6.0	2.03 (±0.10) × 10^{-1}	0.998
7.0	4.91 (±0.24) × 10^{-1}	0.994
8.0	2.81 (±0.14)	0.995
9.0	9.05 (±0.45)	0.998

[a] μ = 1.0 M; reported errors are in reproducibility propagated from data in Table I. [b] Correlation coefficient for data plotted in Figure 3.

excess and hemoglobin concentration is independent of ionic strength between μ = 0.3 M and μ 1.0 M (data not shown).

According to eq 4 the slopes of the (least-squares fit) lines shown in Figure 3 yield the apparent bimolecular rate constant, k_1^{app}, at each pH. In each case the extrapolated intercepts defined the apparent dissociation rate constant, k_{-1}^{app}. Values for k_1^{app} determined in this manner are presented in Table II. For all cyanide mole excesses studied k_1^{app} followed the same trend found for k_{obsd} (Figure 4, Table I) in that k_1^{app} increased with pH in the following order: pH 6.0 < pH 7.0 < pH 8.0 < pH 9.0. As for determination of the dissociation rate constant, k_{-1}^{app}, the intercepts of all four lines in Figure 3 are close to zero. We interpret this to indicate only that k_{-1}^{app} is exceedingly small, which, in turn, indicates that the cyanide dissociation rate from the ligated protein is very slow. Similar slow cyanide dissociation rates have been reported for sperm whale Mb–CN and soybean Lb–CN (Job et al., 1980). Faster dissociation rates are observed for the cyanide-ligated peroxidases (Erman, 1974; Ellis & Dunford, 1968).

Literature values of k_1^{app} for cyanide binding by several ferriheme proteins are collected in Table III. Comparing those values with the values of k_1^{app} determined here, under similar conditions, reveals that the *G. dibranchiata* monomer methemoglobin component II binds cyanide much more slowly than these other heme proteins. The k_1^{app} values for the *G. dibranchiata* component II monomer methemoglobin are from approximately 230 to 2200 times smaller than for the other hemoglobins and myoglobin and over 5 orders of magnitude smaller than for the heme peroxidases.

The results presented so far indicate that k_{obsd} (Figure 4) and k_1^{app} (Figure 5) both increase with increasing pH. This

Table III: Comparison of Apparent Bimolecular Rate Constants, k_1^{app}, for Cyanide Binding to Various Ferric Heme Proteins

protein[a]	temp (°C)	pH	μ (M)	k_1^{app} (M^{-1} s^{-1})	ref
HRP	25	7.05	0.11	9.8×10^4	Ellis and Dunford (1968)
CCP	25	7.0	0.15	1.1×10^5	Erman (1974)
guinea pig Hb^+	20	6.9	0.05	111	Vega-Catalan et al. (1986)
human HbA^+	20	7.0	0.05	200[b]	Vega-Catalan et al. (1986)
SW Mb^+	25	6.0	0.15	140[b]	Awad and Badro (1967)
SW Mb^+	25	7.0	0.15	400[b]	Awad and Badro (1967)
Lba^+	25	6.0	0.11	447[b]	Job et al. (1980)
Lba^+	25	7.0	0.11	692[b]	Job et al. (1980)
GD II Hb^+	20	6.0	1.00	0.203	this work
GD II Hb^+	20	7.0	1.00	0.491	this work

[a] Abbreviations: HRP, horseradish peroxidase; CCP, cytochrome *c* peroxidase; Hb^+, methemoglobin; SW, sperm whale; Mb^+, metmyoglobin; Lba^+, soybean metleghemoglobin *a*; GD, *G. dibranchiata*. [b] These values estimated from graphical data.

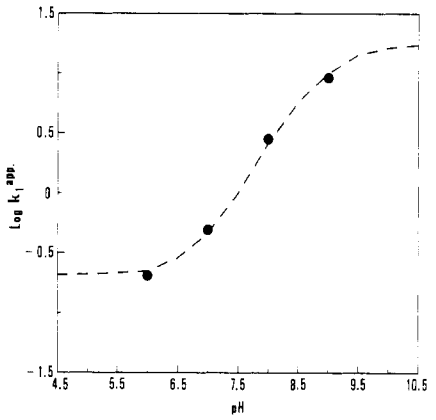

FIGURE 5: Graph of log k_1^{app} for cyanide binding to the monomer component II methemoglobin, determined at 20 °C, μ = 1.0 M, versus pH. The errors of reproducibility (<±10%) lie within the filled data points. The line through the data points represents the fit to eq 5 using k_{CN^-} = 17.4 and k_{HCN} = 0.22, as discussed in the text.

trend is also observed for cyanide binding to other metheme proteins (Vega-Catalan et al., 1986; Erman, 1974). These rate constants also increase with increasing cyanide concentration at a given pH (Figure 3). In view of the discussion presented earlier with regard to eq 1 and 2, this is not surprising. Taking into consideration the hydrolysis of KCN in aqueous solution (which forms HCN and CN^- in equilibrium amounts) and the HCN pK_a at our ionic strength (see Materials and Methods), we may calculate the ratio $[CN^-]/[HCN]$ at the four pH values of our experiments (Izatt et al., 1962). This ratio varies as follows: pH 6.0 = 1.51×10^{-3}; pH 7.0 = 1.51×10^{-2}; pH 8.0 = 1.51×10^{-1}; pH 9.0 = 1.51. Therefore, increasing pH and increasing the cyanide mole excess (by increasing the initial KCN concentration) both effectively increase the cyanide ion (CN^-) concentration in the reaction mixture. Consequently, the results described in Figures 3 and 4 are interpreted as the protein exhibiting a marked preference for binding CN^- over HCN.

Is HCN binding (eq 1) an important contribution to k_1^{app} (or k_{obsd})? To shed light on this question, we call attention to the data of Figure 4, which indicate that at low pH k_{obsd} tends to a minimum value. The shape of the curve suggests that this minimum value may be pH independent at low pH's, as previously suggested to be the case for ferricytochrome *c*

(George & Tsou, 1952). This pH dependence of k_1^{app} can be attributed to the ionization of HCN and different rates of binding for HCN and CN^-. Figure 4 indicates that the rate constant for eq 1, k_{HCN}, may be small, but nonzero, and may be important at high cyanide concentrations at low pH. In order to test this, we applied two graphical procedures suggested by others (George & Tsou, 1952; Goldsack et al., 1966; Ellis & Dunford, 1968). From eq 1 and 2 one can derive the following expression for k_1^{app} (George & Tsou, 1952):

$$k_1^{app} = \frac{k_{CN^-}K_a}{[H^+] + K_a} + \frac{k_{HCN}[H^+]}{[H^+] + K_a} \quad (5)$$

This equation predicts that data conforming to the mechanism shown in eq 1 and 2 should yield a straight line graph for a plot of k_1^{app} ($[H^+] + K_a$) vs $[H^+]$, with a slope equal to k_{HCN} and an intercept proportional to k_{CN^-}. A more accurate estimate of k_{CN^-} can be obtained from a slightly rearranged form of eq 5, namely, a graph of $k_1^{app}([H^+] + K_a)/[H^+]$ plotted against $1/[H^+]$. It is obvious that this latter plot yields a *slope* from which k_{CN^-} can be calculated. As suggested by Goldsack et al. (1966) and George and Tsou (1952), both types of graph have been made for our data (not shown). In the graph using $[H^+]$ the pH 6.0 data are overemphasized and dominate determination of the slope, whereas in the graph using $[H^+]^{-1}$ the pH 9.0 data are overemphasized. To obviate this impact we have plotted the data using all pH values and separately using only the three values that are not overemphasized. All four graphs were linear (with correlation coefficients of at least 0.991). The averages of the rate constants for both three- and four-point graphs are reported here and used to calculate the fit to the experimental data shown in Figure 5.

The important results from these data manipulations are the following: (i) $k_{CN^-} = 17.4$ (± 3.0) M^{-1} s^{-1}, $k_{HCN} = 0.22$ (± 0.06) M^{-1} s^{-1}, and the ratio $k_{CN^-}/k_{HCN} = 90 \pm 39$, which shows that the cyanide ion binding rate is, relatively, the most important process for the *G. dibranchiata* monomer methemoglobin. (ii) The linearity of each graph (not shown) conforms to a criterion that was shown to confirm the correctness of the mechanism shown in eq 1 and 2 as the simplest one that adequately describes our system (Ellis & Dunford, 1968; Goldsack et al., 1966). It has also been taken to indicate that no protein ionizations mediate the ligand binding over the pH range studied (Goldsack et al., 1966). (iii) Additional support for these conclusion comes from the graph of log k_1^{app} vs pH shown in Figure 5. The dashed line was calculated from eq 5 by using the values for k_{CN^-} and k_{HCN} that were determined above and show an excellent fit to the experimental data.

Impact of Kinetics Results on Equilibrium Constant Determination. These kinetics results directly impact the measurement of equilibrium constants for cyanide binding in two ways. First, the slow on-rate means that the standard mix-and-measure optical ligand titration method cannot be used at pH's <8.0. For example, at pH 7.0 each addition of cyanide to a monomer methemoglobin solution requires as much as 200 h to reach equilibrium at 20 °C (Mintorovitch and Satterlee, unpublished results). In our experience the protein's integrity is lost far sooner than one can accumulate sufficient titration points to accurately define an equilibrium constant. Before this slow ligation phenomenon was realized, the cyanide equilibrium dissociation constant (K_D) was reported to be 1.2×10^{-3} M at pH 7.0 for the unseparated monomer methemoglobin fraction, using the ligand titration method (Seamonds, 1971; Seamonds et al., 1971). Second, this reported K_D was calculated by using the Hill formalism, whose equations are valid only when the system under study is at true equilibrium, which it apparently was not in the previous work. It can be seen from the following discussion that these two complicating factors led Seamonds (1971) to report a dissociation equilibrium constant that is approximately 2 orders of magnitude larger than the maximum possible value that we estimate (see next paragraph) from the kinetics results presented here.

In order to estimate K_D for cyanide binding to the component II monomer methemoglobin, we employ the relationship (eq 3) $K_D = k_{-1}^{app}/k_1^{app}$. The problem we encounter is identifying a realistic value for k_{-1}^{app}. Estimates of k_{-1}^{app} can be obtained from the y intercepts of the graphs in Figure 3, and although a significant error may be associated with this extrapolation, the largest value (6.8×10^{-6} s^{-1} at pH 7.0) provides an illustrative example for estimating K_D from our data. Employing this number for k_{-1}^{app}, we calculate the value for K_D at pH 7.0 as 1.3×10^{-5} M. This value is approximately 100 times smaller than the previously reported value (Seamonds et al., 1971) and is probably only an upper limit. The actual K_D is probably much smaller as the following reveals.

In a separate experiment a monomer methemoglobin component II solution (5.26×10^{-6} M) was treated with a 1.19 mole excess of KCN (6.26×10^{-6} M), and ligation was followed at pH 8.0 and 20 °C for 96 h. At that point 88% of the methemoglobin was ligated, and the reaction was terminated. By that time subsequent spectral changes were very small. For example, the next detectable spectral change was an increase in the Soret absorption of 0.001 (OD units) after 120 h of reaction. On the cosmic time scale this reaction had probably not achieved true equilibrium; however, using this information, one can estimate that *if the system had achieved equilibrium at the time of termination* K_D would be at least as small as 10^{-7} M. For this sample the actual calculated value for K_D estimated in this way is 2×10^{-7} M. The clear implication from these results is that the equilibrium affinity of the monomer methemoglobin component II for cyanide is much higher than previously suspected.

The correlation between the unusually slow cyanide-binding rates presented here for the *G. dibranchiata* component II monomer methemoglobin and the unusually fast rate of CO and O_2 binding reported for monomer (reduced) hemoglobin components by Parkhurst et al. (1980) is not immediately clear. These phenomena are probably the result of a less polar heme environment, which is a consequence of the E-7 substitution. Certainly, substitution of the distal histidine by leucine results in loss of the hydrogen bonding that is expected to occur between the distal histidine and the heme ligands O_2 and CN^-. Any further conclusion must await completion of the *G. dibranchiata* monomer globin gene sequencing and site-directed mutagenesis experiments currently in progess in this lab and the rationalization of the two monomer hemoglobin isolations (Parkhurst et al., 1980; Kandler & Satterlee, 1983; Kandler et al., 1984; Constantinidis & Satterlee, 1987).

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Interactive Binding between the Substrate and Allosteric Sites of Carbamoyl-Phosphate Synthetase†

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ABSTRACT: The interaction between *Escherichia coli* carbamoyl-phosphate synthetase (CPS) and a fluorescent analogue of an allosteric effector molecule, 1,N^6-ethenoadenosine 5′-monophosphate (ε-AMP), has been detected by using fluorescence techniques and kinetic measurements. From fluorescence anisotropy titrations, it was found that ε-AMP binds to a single site on CPS with K_d = 0.033 mM. The nucleotide had a small activating effect on the rate of synthesis of carbamoyl phosphate but had no effect on the K_m for ATP. To test whether ε-AMP binds to an allosteric site, allosteric effectors (UMP, IMP, and CMP), known to bind at the UMP/IMP site, were added to solutions containing the ε-AMP–CPS complex. With addition of these effector molecules, a progressive decrease of the fluorescence anisotropy was observed, indicating that bound ε-AMP was displaced by the allosteric effectors examined. From these titrations, the dissociation constants for UMP, IMP, CMP, ribose 5-phosphate, 2-deoxyribose 5-phosphate, and orthophosphate were determined. When MgATP, a substrate, was employed as a titrant, the observed decrease in anisotropy was consistent with the formation of a ternary complex (ε-AMP–CPS–MgATP). The effect of ATP binding, monitored at the allosteric site, was magnesium dependent, and free magnesium in solution was required to obtain a hyperbolic binding isotherm. Solvent accessibility of ε-AMP in binary (ε-AMP–CPS) and ternary (ε-AMP–CPS–MgATP) complexes was determined from acrylamide quenching, showing that the base of ε-AMP is well shielded from the solvent even in the presence of MgATP. Using the theory of ligand binding [Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83], we computed the free energy of coupling between MgATP and ε-AMP to be +0.48 kcal/mol. Thus, the experiments described in this paper provide evidence for antagonism (anticooperative interaction) between the substrate and allosteric sites and suggest that this interaction provides a method of regulating the physiological activity of CPS.

The first step of the biosynthesis of pyrimidine nucleotides in *Escherichia coli* is the formation of carbamoyl phosphate from glutamine, bicarbonate, and two molecules of ATP. Since carbamoyl phosphate is also utilized by the arginine pathway, the enzyme that performs its synthesis, carbamoyl-phosphate synthetase (CPS),[1] is regulated by a number of metabolites; these include ammonia, potassium ions, ornithine, and IMP which activate CPS and UMP which is an allosteric inhibitor of the enzyme.

The enzyme is composed of two nonidentical subunits (Matthews & Anderson, 1972). The small subunit (M_r 48 000) has the glutamine binding site whereas the large subunit (M_r 130 000) possesses binding sites for ATP, bicarbonate, and allosteric effectors (Trotta et al., 1971; Matthews & Anderson, 1972).

NMR, EPR, and fluorescence energy-transfer experiments in this laboratory have established a partial topographical map of the enzyme based on point-to-point vectors between various binding loci (Raushel et al., 1979, 1983; Kasprzyk et al., 1983).

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[1] Abbreviations: ε-AMP, 1,N^6-ethenoadenosine 5′-monophosphate; CPS, carbamoyl-phosphate synthetase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate.

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