Kinetic Study of the Slow Cyanide Binding to *Glycera dibranchiata* Monomer Hemoglobin Components III and IV†

Jan Mintorovitch, David van Pelt, and James D. Satterlee*,‡

Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87107

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ABSTRACT: Compared to other monomeric heme proteins and the heme peroxidases, the *Glycera dibranchiata* monomer hemoglobin components III and IV exhibit very slow cyanide binding kinetics. This is in agreement with the previously reported behavior of component II. Similar to component II, components III and IV have been studied under pseudo-first-order conditions at pH 6.0, 7.0, 8.0, and 9.0 by using a 100–250-fold excess of potassium cyanide at each pH. At 20 °C with micromolar protein concentrations, k_{obs} for component III varies between 7.08×10^{-5} s^{-1} at pH 6.0 and 100-fold cyanide excess and 1.06×10^{-2} s^{-1} at pH 9.0 and 250-fold cyanide excess. For component IV, the values are 2.03×10^{-4} s^{-1} for 100-fold cyanide excess at pH 6.0 and 4.13×10^{-2} s^{-1} for 250-fold cyanide excess at pH 9.0. In comparison to other heme proteins, our analysis shows that the bimolecular rate constant (k_1^{app}) is small. For example, at pH 7.0, it is 3.02×10^{-1} M^{-1} s^{-1} for component III and 1.82 M^{-1} s^{-1} for component IV, compared to 400 M^{-1} s^{-1} for sperm whale metmyoglobin, 692 M^{-1} s^{-1} for soybean metleghemoglobin a, 111 M^{-1} s^{-1} for guinea pig methemoglobin, and 1.1×10^5 M^{-1} s^{-1} for cytochrome *c* peroxidase. Our results also show that the dissociation rates (k_{-1}^{app}) are extremely slow and no larger than 10^{-6} s^{-1}. Furthermore, separation of the bimolecular rate constant (k_1^{app}) into contributions from the binding of CN^- (k_{CN^-}) and from the binding of HCN (k_{HCN}) allows the comparison k_{CN^-}/k_{HCN} to be made. For component III, this ratio is 106. For component IV, it is 220. In agreement with the results from the component II study, these results indicate that cyanide ligation reactions of these hemoglobins are not instantaneous. These kinetic results mean that accurate equilibrium constants are impractical when the standard optical measurement technique is used because the time needed to reach equilibrium is so long. From the results presented here, the actual equilibrium dissociation constant (K_D) can only be estimated. However, these estimates yield K_D values for components III and IV that have a numerical upper limit at least 2 orders of magnitude smaller than the value previously reported.

Since the exceptional amino acid substitution at primary sequence E-7 (E-7 His → Leu) was first identified for one of the *Glycera dibranchiata* monomer hemoglobins (Imamura et al., 1972; Padlan & Love, 1974), the ligand binding properties of these proteins have attracted much interest (Seamonds, 1971; Seamonds et al., 1971, 1976; Parkhurst et al., 1980). It is expected that the substitution at position E-7, which is in close proximity to the heme ligand binding site, would have detectable effects on ligand binding dynamics. The reason for this expectation is that, in place of a multifunctional histidine molecule that can promote hydrogen bonding with a ligand, one finds a leucine with a totally aliphatic side chain that cannot have the same function. This substitution also creates a less polar distal heme environment.

Prior to publication of the component II cyanide binding data (Mintorovitch & Satterlee, 1988), both equilibrium (Seamonds, 1971; Seamonds et al., 1971) and kinetics (Seamonds et al., 1976; Parkhurst et al., 1980) studies for various ligands binding to various preparations of the *G. dibranchiata* monomer hemoglobins were published. They all revealed unusual equilibrium and kinetic binding constants. However, in these studies, including the equilibrium studies for cyanide binding, all investigators used either the unseparated monomer fraction or monomeric components of undocumented purity. Recent work has demonstrated the reproducibility with which three major monomer hemoglobins can be isolated (Kandler & Satterlee, 1983; Kandler et al., 1984; Cooke & Wright, 1985) and established a high-resolution purity criterion for the individual monomer hemoglobins isolated in this laboratory (Constantinidis & Satterlee, 1987). The homogeneity and uniqueness of the three major monomer components isolated in this laboratory have recently been confirmed by partial amino acid sequencing of the individual globins and by successful cloning and sequencing of cDNA for one of the monomer globins (Simons and Satterlee, unpublished data). Consequently, we can be confident that studies of ligand binding kinetics are carried out on purified and well-characterized individual *G. dibranchiata* monomer hemoglobins. Our first study was done on component II because previous work suggested that hemoglobin component II was the one that was crystallized and sequenced and, therefore, shown to lack the distal histidine at primary sequence position E-7 (Parkhurst et al., 1980).

The results for monomer hemoglobin components III and IV reported herein are similar to those for component II with the principal result being that the rate of cyanide[1] (CN)

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* Author to whom correspondence should be addressed.

‡ Address after July 1, 1989: Department of Chemistry, Washington State University, Pullman, WA 99164.

[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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binding to these monomer hemoglobins is anomalously slow in comparison to other heme proteins. Furthermore, this work also shows that these proteins also preferably bind cyanide ion (CN^-), that the dissociation of cyanide from the fully ligated proteins is extremely slow, and that, under our experimental conditions (pH 6.0–9.0 and μ = 1.0), there are no detectable protein ionizations that influence the ligand binding.

Materials and Methods

Protein Isolation and Purification. *G. dibranchiata* monomer hemoglobin components III and IV were isolated as the ferric (met) proteins as previously described (Kandler et al., 1984; Constantinidis & Satterlee, 1987). The apoproteins were subjected to high-voltage isoelectric focusing as a test of homogeneity, as previously described (Constantinidis & Satterlee, 1987), and all preparations thus assayed focused as single lines. Samples of the proteins from each isolation and purification were also checked by proton NMR spectroscopy, which has been shown to be a valuable tool for the characterization of the *G. dibranchiata* monomer hemoglobins (Constantinidis et al., 1987).

Determination of k_{obs}. The Soret band maxima for the unligated and the cyanide-ligated met forms of the two proteins are 393 and 422 nm, respectively. An isosbestic point occurs at 412 nm for both. Furthermore, due to the slow ligand binding kinetics for these proteins, the ligation can easily be monitored by UV–visible spectroscopy (Perkin-Elmer 559A; Mintorovitch & Satterlee, 1988).

Stock solutions of hemoglobin and KCN were made and stored in tightly stoppered flasks immediately prior to each experiment. The KCN solutions were prepared in flasks with no observable gas space to minimize loss of HCN gas from solution. Similarly, cuvettes were completely filled with liquid. The linearity of the data shown in Figures 2 and 3 indicates that the impact of KCN solution handling is within experimental reproducibility. Each solution was heavily buffered so that pH fluctuations due to the additions of KCN were minimized. This required the use of 0.3 M potassium phosphate (Fisher, analytical grade) buffer that has an ionic strength of 0.9 M at pH 9.0, the maximum pH employed. All other procedures were precisely those previously described (Mintorovitch & Satterlee, 1988).

Final hemoglobin concentrations were 5.0 ($\pm$0.05) $\times$ 10^{-6} M. The actual reaction mixture pH was monitored in parallel solutions at the beginning of each experiment by using a Beckmann pH 60 meter equipped with a Fisher combination electrode. The final pH was also determined at the end of each experiment. The maximum detected pH variation during the course of each experiment was 0.02 pH unit. Each experiment was repeated at least once so that each point in the figures represents the average of at least duplicate runs, and the error bars shown in the figures indicate the range of reproducibility for the multiple experiments.

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, 1986; George & Tsou, 1952). Those studies have shown that, in the pH range 6.0–9.0, one has to consider at least two reactions giving rise to the overall rate of formation of the cyanide-ligated protein, the first with HCN and the second with CN^-. Consequently, the simplest possible mechanism for the ligation is

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

pH	$[KCN]_{initial}$/[Hb]	component III k_{obs}[a] (s^{-1})	component IV k_{obs}[a] (s^{-1})
6.0	100	7.08 ($\pm$0.07) $\times$ 10^{-5}	2.03 ($\pm$0.05) $\times$ 10^{-4}
	150	1.09 ($\pm$0.05) $\times$ 10^{-4}	3.40 ($\pm$0.07) $\times$ 10^{-4}
	200	1.47 ($\pm$0.05) $\times$ 10^{-4}	4.58 ($\pm$0.07) $\times$ 10^{-4}
	250	1.80 ($\pm$0.08) $\times$ 10^{-4}	5.42 ($\pm$0.10) $\times$ 10^{-4}
7.0	100	1.89 ($\pm$0.05) $\times$ 10^{-4}	9.22 ($\pm$0.10) $\times$ 10^{-4}
	150	2.56 ($\pm$0.05) $\times$ 10^{-4}	1.32 ($\pm$0.03) $\times$ 10^{-3}
	200	3.36 ($\pm$0.07) $\times$ 10^{-4}	1.73 ($\pm$0.04) $\times$ 10^{-3}
	250	4.11 ($\pm$0.05) $\times$ 10^{-4}	2.28 ($\pm$0.03) $\times$ 10^{-3}
8.0	100	1.02 ($\pm$0.03) $\times$ 10^{-3}	4.94 ($\pm$0.06) $\times$ 10^{-3}
	150	1.47 ($\pm$0.03) $\times$ 10^{-3}	7.33 ($\pm$0.04) $\times$ 10^{-3}
	200	1.88 ($\pm$0.07) $\times$ 10^{-3}	9.85 ($\pm$0.15) $\times$ 10^{-3}
	250	2.30 ($\pm$0.05) $\times$ 10^{-3}	1.23 ($\pm$0.03) $\times$ 10^{-2}
9.0	100	4.24 ($\pm$0.03) $\times$ 10^{-3}	1.65 ($\pm$0.03) $\times$ 10^{-2}
	150	5.83 ($\pm$0.06) $\times$ 10^{-3}	2.42 ($\pm$0.03) $\times$ 10^{-2}
	200	7.87 ($\pm$0.11) $\times$ 10^{-3}	3.23 ($\pm$0.03) $\times$ 10^{-2}
	250	1.06 ($\pm$0.03) $\times$ 10^{-2}	(*)4.13 $\times$ 10^{-2}

[a] μ = 1.0 M; errors in parentheses are variations in reproducibility; [Hb] = 5.0 μM; (*) = extrapolated value (see text).

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

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

(k_a connects the two reactions)

It has been customary to treat KCN binding data by considering the reaction to be dependent upon initial KCN concentration and to let "CN" (cyanide) represent the total amount of cyanide present (Kassner et al., 1985; Erman, 1978; Ellis & Dunford, 1968; George & Tsou, 1952). Our experiments and analyses follow these previous procedures and are identical with those used in studying cyanide binding to component II (Mintorovitch & Satterlee, 1988).

We have studied the cyanide ligation of *G. dibranchiata* monomer hemoglobin components III and IV at pH 6.0, 7.0, 8.0, and 9.0. To ensure pseudo-first-order conditions, we employed a 100-, 150-, 200-, and 250-fold mole excess of cyanide at each pH.

Similar to component II, changes in the UV–visible spectra, for the unligated and the cyanide-ligated forms of the component III and IV hemoglobins, are large (data not shown) and provide the spectroscopic parameter needed to study the ligand binding reaction. Like component II, the spectra for each unligated protein at pH 6.0 and at pH 9.0 are essentially identical (not shown), allowing us to conclude that, at the highest pH employed, the hydroxy form of the protein is not appreciably formed. Also similar to component II, a 30-nm red shift, without detectable intermediates (clean isosbestic points, not shown), from 393 to 422 nm, occurs upon ligation with cyanide for these two proteins at all pH values employed. The rate of ligation was, therefore, monitored by the time-dependent increase in the intensity at 422 nm (not shown). All reactions were followed up to at least 7 half-lives (99.2% completion), and the data obtained were accurately fitted by a linear least-squares fit to a semilog plot (Mintorovitch & Satterlee, 1988). As shown previously (Mintorovitch & Satterlee, 1988), the slope of the best-fit line from these graphs yields k_{obs}. Figure 1 shows the fits for binding data obtained for (A) component III and (B) component IV.

All of the calculated values for k_{obs} derived from this work are collected in Table I. The value for k_{obs} at pH 9.0 and

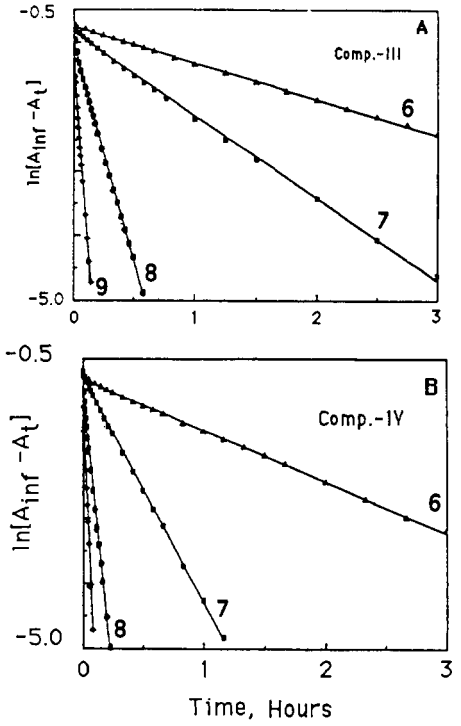

FIGURE 1: Logarithmic plots of cyanide binding data for *G. dibranchiata* monomer methemoglobin components. (A) Component III with [KCN]/[Hb] = 200 at pH 6.0 (▲), pH 7.0 (■), pH 8.0 (●), and pH 9.0 (◆); 20 °C, μ = 1.0 M. Data at pH 6.0 (11 h) and pH 7.0 (6 h) not shown to completion. (B) Component IV with [KCN]/[Hb] = 100 at pH 6.0 (▲), pH 7.0 (■), pH 8.0 (●), and pH 9.0 (◆); 20 °C, μ = 1.0 M. Data at pH 6.0 (8 h) not shown to completion.

250-fold excess KCN for component IV could not be accurately measured by this method because the ligation proceeded too rapidly. Therefore, this value (marked with an asterisk in Table I) is an estimated one predicted by extrapolation of the linear least-squares fit of the three lower cyanide ratios (100-, 150-, and 200-fold excesses; Figure 3; R = 0.998). This k_{obs} is not included in the calculation of k_1^{app} (Table III; Figures 3 and 4) since it is only an estimate. The correlation coefficient for each of the semilog graphs was 0.991 or better, showing that intermediate steps, or more complicated reactions than those shown in eq 1 and 2, are not detectable within the pH range studied.

All of the results described above are consistent with formulation of the overall ligand binding process given by

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

Under pseudo-first-order conditions, the observed rate constant (k_{obs}) is dependent upon the total cyanide concentration and is given by

$$k_{obs} = k_1^{app}[\mathrm{CN}] + k_{-1}^{app} \qquad (4)$$

If this equation holds, k_{obs} should increase linearly with increasing cyanide concentration, and Figures 2 and 3 show that plots for all k_{obs} values obtained for KCN excesses, for components III and IV, conform to this expectation. The least-squares fits for these data are excellent, with correlation coefficients of 0.994 or better.

According to eq 4, the slopes of the lines plotted in Figures 2 and 3 should give the value for the apparent bimolecular rate constant (k_1^{app}), at each pH, and the extrapolated intercepts define the apparent dissociation rate constant (k_{-1}^{app}). Values for k_1^{app}, determined from slopes in this manner, are presented in Table II. It can be seen from Figures 2 and 3 and Table II that k_1^{app} follows the same trend as k_{obs}: an increase with increasing pH. However, accurate determination of the dissociation rate constant, k_{-1}^{app}, is complicated by the fact that the y intercepts of all four lines in Figures 2 and 3 are zero

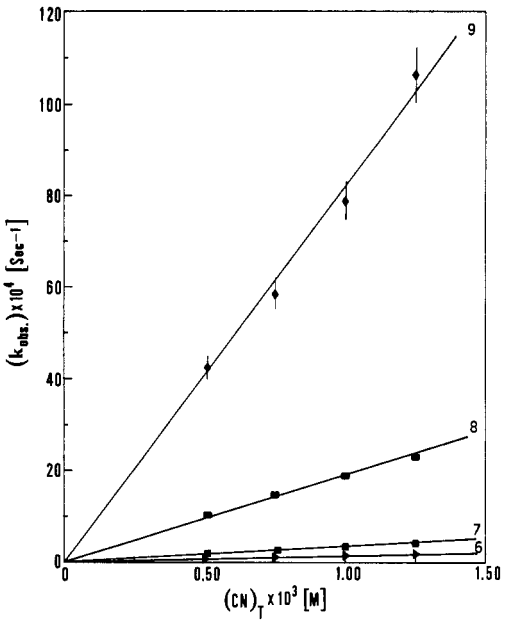

FIGURE 2: Plots of k_{obs} for cyanide binding to monomer methemoglobin component III as a function of the initial KCN concentration at pH 6.0 (▲), pH 7.0 (■), pH 8.0 (●), and pH 9.0 (◆); 20 °C, μ = 1.0 M, [Hb] = 5.0 × 10^{-6} M.

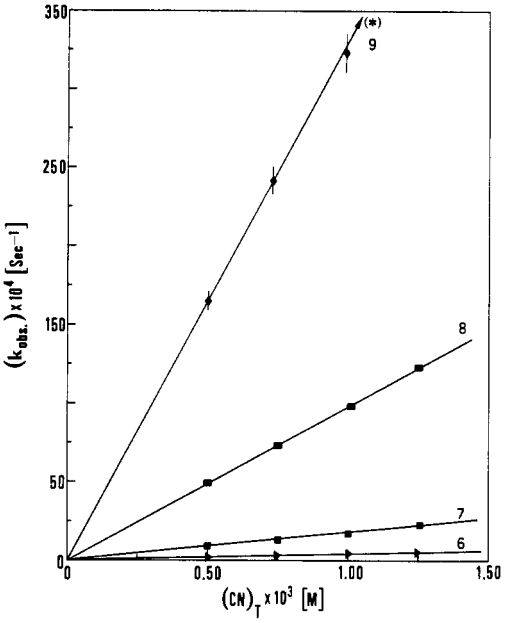

FIGURE 3: Plots of k_{obs} for cyanide binding to monomer methemoglobin component IV as a function of the initial KCN concentration. Other details are identical with those of Figure 2.

Table II: Apparent Bimolecular Rate Constant (k_1^{app}) for Cyanide Binding to the *G. dibranchiata* Monomer Hemoglobin Components III and IV as a Function of pH at 20 °C[a]

	component III		component IV	
pH	k_1^{app}[b] (M^{-1} s^{-1})	R[c]	k_1^{app} (M^{-1} s^{-1})	R[c]
6.0	1.47 (±0.10) × 10^{-1}	0.9978	4.53 (±0.14) × 10^{-1}	0.9946
7.0	3.02 (±0.06) × 10^{-1}	0.9986	1.82 (±0.03)	0.9968
8.0	1.71 (±0.06)	0.9992	9.90 (±0.10)	0.9998
9.0	8.60 (±0.05)	0.9936	3.23 (±0.03) × 10^1	0.9998

[a] The values are derived from the slopes in Figures 3 and 4. [b] μ = 1.0 M; reported errors are in reproducibility propagated from data in Table I. [c] Correlation coefficients for data plotted in Figures 2 and 3.

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)
g. 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	7.0	0.15	400[b]	Awad and Badro (1967)
Lba^+	25	7.0	0.11	692[b]	Job et al. (1980)
GD II Hb^+	20	7.0	1.0	0.491	Mintorovitch and Satterlee (1988)
GD III Hb^+	20	7.0	1.0	0.302	this work
GD IV Hb^+	20	7.0	1.0	1.82	this work

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

within experimental error. We interpret this to indicate merely that k_{-1}^{app} is very small, which, in turn, indicates that the cyanide dissociation rate from each of the ligated proteins 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 (Ermann, 1974; Ellis & Dunford, 1968). Published values for k_1^{app} for cyanide binding to several ferriheme proteins are collected in Table III. Comparison of these values with those for k_1^{app} determined here, under similar experimental conditions, reveals that the *G. dibranchiata* components III and IV bind cyanide at approximately the same rate as component II. However, the rates determined for all of the *G. dibranchiata* monomers are 2–3 orders of magnitude less than those of the other hemoglobins and myoglobin and over 5 orders of magnitude less than for the heme peroxidases.

The trend of increasing k_{obs} and k_1^{app} with increasing pH has also been observed for cyanide binding to other heme proteins (Vega-Catalan et al., 1986; Erman, 1974). 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 and the pK_a for HCN under our experimental conditions, 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}, and pH 9.0 = 1.51. Therefore, increasing the pH of the protein solution and increasing the cyanide mole excess, by increasing the initial KCN concentration, are both ways to effectively increase the cyanide ion concentration in the reaction mixture. Consequently, the results described in Figures 2 and 3 are interpreted as the proteins' exhibiting a marked preference for binding CN^- over HCN.

In view of this interpretation, an interesting issue to address is whether the binding of HCN contributes significantly to k_{obs} (or k_1^{app}). It is known from earlier studies of cyanide ligation that, for other heme proteins, the sole product of the reaction is protein–CN (and not protein–HCN) regardless of whether the reacting species was CN^- or HCN (George & Hanania, 1955). To answer this question for the *G. dibranchiata* monomers, one can first consider the values for k_{obs} in Figures 2 and 3 and Table I. These data suggest that k_{obs} tends to a minimum at low pH and that this minimum value may even

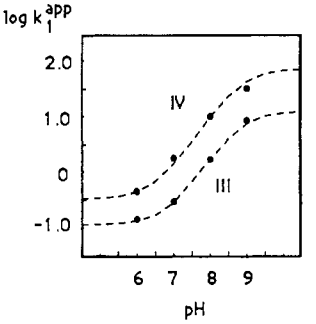

FIGURE 4: Graph of log k_1^{app} vs pH for cyanide binding for the *G. dibranchiata* monomer methemoglobin component III (lower) and component IV (upper) (20 °C, μ = 1.0 M). The errors of reproducibility (≤5%) lie within the solid data points. The dashed lines through the data points represent the fits to eq 5 as discussed in the text.

be pH independent at lower pH values than we have used. This leads to the suggestion that k_{HCN}, the rate constant for eq 1, although small compared to k_{CN^-}, may be nonzero. Moreover, due to the ionization of HCN (pK_a = 8.82 under our conditions), HCN binding may be significant at low pH values and high cyanide concentrations. To test the significance of the two second-order rate constants, k_{HCN} and k_{CN^-}, we have applied two graphical procedures suggested by others (George & Tsou, 1952; Goldsack et al., 1966; Ellis & Dunford, 1968).

Equations 1 and 2 show that the bimolecular rate constant is given by

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

This expression can be transformed into the two linear functions

$$k_1^{app}([H^+] + K_a) = k_{HCN}[H^+] + K_a k_{CN^-} \quad (6)$$

$$k_1^{app}\frac{([H^+] + K_a)}{[H^+]} = (K_a k_{CN^-})\frac{1}{[H^+]} + k_{HCN} \quad (7)$$

Therefore, if eq 5 and, consequently, the mechanism predicted by eq 1 and 2 hold, plots of (1) k_1^{app} $([H^+] + K_a)$ versus $[H^+]$; and (2) $k_1^{app}([H^+] + K_a)/[H^+]$ versus $1/[H^+]$ should be linear with slopes of k_{HCN} and $K_a k_{CN^-}$, respectively. Since K_a is known (pK_a = 8.82), k_{CN^-} can be determined from the latter slope. These plots are linear for both components III and IV (not shown), and values for the two rate constants were determined as before (Mintorovitch & Satterlee, 1988). An indication of the accuracy of these rate constants should be the fit obtained when values k_{HCN} and k_{CN^-} are inserted into eq 5 and used to calculate the fit to the experimental data. The dashed curves in Figure 4 show very good fits.

The important results from these data manipulations are the calculated rate constants. For component III, k_{CN^-} = 12.33 (±2.1) M^{-1} s^{-1}, k_{HCN} = 0.115 (±0.02) M^{-1} s^{-1}, and the ratio k_{CN^-}/k_{HCN} = 106 ± 2. For component IV, k_{CN^-} = 72.50 (±8.0) M^{-1} s^{-1}, k_{HCN} = 0.329 (±0.04) M^{-1} s^{-1}, and the ratio k_{CN^-}/k_{HCN} = 220 ± 6. These results show that the CN^- binding rate is, relatively, the most important process for the *G. dibranchiata* monomer methemoglobins component III and IV at neutral pH and above.

This behavior at neutral pH is in contrast to that exhibited by metmyoglobin, which exhibits a preference for binding the conjugate acids of azide and fluoride ions (Duffey et al., 1966; Goldsack et al., 1966; George & Hanania, 1955). Cyanide binding to ferrimyoglobin is more ambiguous, however, with

cyanide ion and HCN exhibiting comparable reactivity at pH 7 (Chance, 1952; Ver Ploeg et al., 1971). It is likely that the distal histidine (E-7) plays a role as a hydrogen ion acceptor in these processes. In the *G. dibranchiata* monomer methemoglobin components, Leu E-7 is incapable of fulfilling such a role and CN^- is the most reactive species.

In comparing these results to metmyoglobin, one must note the much smaller k_1^{app} for the monomer methemoglobins (Table III). The E-7 His → Leu substitution must play a paramount role in mediating cyanide binding, as this work shows. However, other, more subtle effects may also be important in determining reactivity. Recently we have cloned and sequenced the cDNA for monomer component IV (Simons and Satterlee, unpublished data). Comparing this inferred complete primary sequence with that of sperm whale myoglobin reveals that the polarities of the heme pockets are different. This difference could well be the cause of different preferences for ligand species.

The results presented above show that differences exist in the rate constants for cyanide binding among the monomer methemoglobins of *G. dibranchiata* as a group (Table III). Such differences are probably the result of slight differences in the structures of the individual proteins. Partial amino acid sequencing and complete cDNA sequences have revealed differences in primary sequences among the three monomer globins (Simons and Satterlee, unpublished data) that could account for these results. More important, however, is the fact that the differences among the three monomer methemoglobins are much less than the orders of magnitude differences demonstrated between myoglobin and the monomers. These large differences between different species are most likely attributable to the fact that all of the *G. dibranchiata* monomer hemoglobins lack a distal histidine and display a less polar distal heme environment.

Impact of the Kinetics Results on Equilibrium Constants Determination. These kinetics results directly impact the measurement of the equilibrium constants for *G. dibranchiata* monomer methemoglobin components III and IV in two ways.

First, the slow on-rates mean that the standard mix-and-scan optical ligand titration method cannot be used at pH ≤9.0. Additional experiments using this method in attempts to measure the equilibrium binding constant for cyanide have shown that, particularly at low pH and a low excess of KCN, it can take up to 200 h for the system to reach equilibrium after one cyanide addition (Mintorovitch and Satterlee, unpublished results). Our experience shows that a given protein's integrity may be lost far sooner than one can collect sufficient data to accurately define an equilibrium constant. Before this slow ligation phenomenon was known, the equilibrium dissociation constant (K_D) was reported to be 1.2×10^{-3} at pH 7.0 for the unseparated monomer methemoglobin fraction, using the ligand titration mix-and-scan method (Seamonds, 1971; Seamonds et al., 1971).

Second, the previously reported K_D value was determined by using the Hill formalism, whose equations are only valid when the system under study is at true equilibrium. It apparently was not in the earlier studies. It is obvious from the following discussion that these two complicating factors led Seamonds (1971) to report a value for K_D for the unseparated monomer fraction that is approximately 2 orders of magnitude larger than the one we estimate (see next paragraph) from the kinetics results presented here.

To estimate K_D for cyanide binding to *G. dibranchiata* methemoglobin components III and IV, we employ the relationship $K_D = k_{-1}^{app}/k_1^{app}$ (from eq 3). However, the problem we encounter is identifying an accurate value for k_{-1}^{app}. As mentioned before, estimates of k_{-1}^{app} can be obtained from the y intercepts in Figures 3 and 4, although a significant error may be associated with this extrapolation, especially when all the intercepts are this close to zero. However, the largest values obtained for components III and IV are 2.9×10^{-6} and 2.2×10^{-5} s^{-1}, respectively (both at pH 7.0). These values can be used to calculate an upper limit for K_D allowed by our data. The results are an estimated upper limit for K_D (for component III at pH 7.0) of 1.0×10^{-5} M and an upper limit for K_D (for component IV at pH 7.0) of 1.2×10^{-5} M. Both of these values agree in magnitude with previous findings for component II. Since components II–IV make up approximately 90% of the unseparated monomer fraction, it does not seem plausible that the unseparated monomer fraction has a value for K_D approximately 2 orders of magnitude larger than that of any of the major components.

The correlation between the unusually slow cyanide binding rates for the *G. dibranchiata* monomer hemoglobin components III and IV presented here and the unusually fast rates for CO and O_2 binding reported for several monomer (reduced) hemoglobin components by Parkhurst et al. (1980) is not immediately clear. These phenomena are probably the result of the less polar heme environment that is a consequence of the E-7 substitution. Any further conclusion must await the completion of the *G. dibranchiata* monomer globin gene sequencing and site-directed mutagenesis experiments currently under way in our laboratory and the rationalization of the two monomer hemoglobin isolations (Parkhurst et al., 1980; Kandler & Satterlee, 1983; Kandler et al., 1984; Constantinidis & Satterlee, 1987).

Registry No. CN, 57-12-5; HCN, 74-90-8; methemoglobin III, 121124-58-1; methemoglobin IV, 121124-59-2.

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A Histidine-Rich Protein from the Vitellaria of the Liver Fluke *Fasciola hepatica*†

J. Herbert Waite*,‡ and Allison C. Rice-Ficht§

Marine Biology/Biochemistry Program, College of Marine Studies, University of Delaware, Lewes, Delaware 19958, and Department of Medical Biochemistry and Genetics, Texas A&M University, College Station, Texas 77843

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ABSTRACT: The vitellaria are an extensive network of glandular cells and ducts distributed throughout the peripheral tissues of the liver fluke *Fasciola hepatica*. Eggshell precursor proteins are produced and stockpiled in the vitelline cells of mature flukes. Vitelline protein C has an extraordinary composition: the amino acid 3,4-dihydroxyphenyl-L-alanine (DOPA) and histidine each comprise about 20% of the residues, while glycine represents 41-42% in all variants of what appears to be a microheterogeneous protein family. Protein C has an apparent molecular weight of 16 000-17 000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although the protein appears homogeneous following polyacrylamide gel electrophoresis in Tris-glycine with SDS and in acetic acid-urea, electrophoresis in borate, however, suggests that the vitelline protein consists of four or more closely related proteins weighing from 16 000 to 18 500. Isoelectric focusing of the protein family in the presence of 8 M urea resolves only two species having p*I* values of 6.89 and 6.99. A single N-terminus having the sequence H-H-W-D-G-DOPA-G-DOPA-G was detected. The primary structure of vitelline protein C is characterized by a repeated motif consisting of $(G\text{-}X)_n$, where X is Ser, DOPA, or His. Most of the His occurs as G-H repeats in a pepsin-resistant fragment of the protein. Previously, a 31-kDa protein, representing up to 6% of the total protein in the fluke, was reported [Waite, J. H., & Rice-Ficht, A. (1987) *Biochemistry 26*, 7819-7825] to contain significant levels of DOPA. In other respects, however, it is distinct from vitelline protein C. Present studies suggest the existence of at least one other distinct DOPA-containing protein in the vitellaria.

The formation of eggshells in helminths has long captivated the attention of parasitologists [see reviews by Smyth and Clegg (1959) and Cordingley (1987)]. This is due in part (i) to the large proportion (20-30%) of the total energy budget of helminths devoted to eggshell production (Wharton, 1983), (ii) to pathological complications caused by the entrapment of helminth eggs in host tissues (Malek, 1980), and (iii) to the extraordinary chemical and physical stability of the eggshells, which are not unlike well-tanned leather bags (Stephenson, 1947). Despite all the attention, however, it is ironic that very little is actually known about the biochemistry of helminth eggshells. The protein precursors for trematode eggshells are generally thought to be synthesized in the vitelline cells that, when mature, migrate from the vitellaria through specialized conducting tubules to Mehli's gland and the proximal uterus. There, following some as yet unidentified biochemical cue, the cells release globules of eggshell precursors that coalesce to form the nascent eggshell around the fertilized ovum (Irwin & Threadgold, 1970). In recent years, researchers have speculated that the expression of gender-specific genes in blood flukes (*Schistosoma mansoni*) and gonad-specific genes in the liver fluke (*Fasciola hepatica*) is linked to putative eggshell precursor proteins (Johnson et al., 1987; Köster et al., 1988; Kunz et al., 1987; Bobek et al., 1986, 1988; Zurita et al., 1987). The gene sequences code for proteins especially rich in glycine, tyrosine, aspartic acid, histidine, and lysine. Recently, a protein representing perhaps 6% of the total protein in mature *F. hepatica* was purified from the vitellaria. This protein (M_r 31 000) was distinctive in containing up to 10 residues/100 of 3,4-dihydroxyphenyl-L-alanine (DOPA)[1] and having a composition generally reminiscent of that of *F. hepatica* eggshells, i.e., high levels of Asp/Asn, Gly, and Lys (Waite & Rice-Ficht, 1987). In the present paper, we describe

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‡ University of Delaware.
§ Texas A&M University.

[1] Abbreviations: Bis, *N*,*N*'-methylenebis(acrylamide); DOPA, 3,4-dihydroxyphenyl-L-alanine; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; vpC, vitelline protein C.

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