Kinetics of Binding of Cyanide to Hemin Intercalated in Micellar Sodium Lauryl Sulfate†

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ABSTRACT: The kinetic and equilibrium properties of hemin, incorporated in micelles of sodium lauryl sulfate (SLS), with potassium cyanide, have been examined by stopped flow. A pH range from 9.0 to 13.0 with cyanide serving as both nucleophile and buffer over a portion of that range and temperatures varying from 15 to 35° were used to determine all accessible formation and decomposition rate constants, equilibrium constants, and associated activation parameters. It has been found that in the presence of 2% SLS, well above the critical micellar concentration, and 0.1 M tetramethylammonium bromide (TMAB), intercalated hemin monomers are observed to react rapidly with cyanide in a two-step process forming a dicyanohemin complex. The first cyanide goes on in the rate-determining step by displacing apically bound hydroxide or water with a rate constant of 3.35×10^3 M^{-1} sec^{-1}. The rate of formation has been found to be dependent upon the concentration of cyanide anion and upon the concentration of the counterion TMAB. The latter is believed to be a result of changing surface charge effects at the micelle–water interface due to strong micellar-TMAB electrostatic and hydrophobic interactions. Time scales for the observed reactions range from milliseconds to seconds. Two kinetically indistinguishable mechanisms are postulated that are consistent with kinetic and equilibrium studies. Decomposition rates of the dicyanohemin complex affected by strong base coupled with formation rate data have permitted a kinetic determination of the overall equilibirum which agrees favorably with that determined from spectrophotometric analysis. Application of concentration-jump-relaxation techniques further provides confirming evidence for the mechanisms of the formation and decomposition of the dicyanohemin complex. Kinetic and environmental comparisons of the hemin–micellar complex to metmyoglobin reaction with cyanide are discussed.

Both the hydrophobic and polar groups of hemoproteins can affect the kinetics of the active site, *i.e.*, the metalloporphyrin. To investigate one parameter to the exclusion of the other (since the protein environment imposes all to some degree) it is desirable to remove the metalloporphyrin from the native protein and study its reactivity in carefully selected modified environments, such as solvents with varying protic or polar character, thereby permitting parallels and contrasts to the more complex natural systems. It has been suggested that a major influence upon protein-free metalloporphyrin reactivity is either the protic character of the environment from studies in nonaqueous solutions (Alben *et al.*, 1968; Cohen and Caughey, 1968) or hydrophobic nature of the environments (Kao and Wang, 1965). The use of aqueous and nonaqueous solvents may be complicated by the fact that dimerization of metalloporphyrins may occur (Sadasivan *et al.*, 1969; Falk, 1964). In a companion paper (Simplicio, 1972) an equilibrium study has revealed that iron(III) porphyrins are monodispersed by SLS,[1] forming an intercalated hemin monomer in the micellar structure of the SLS similar to that pictured for porphyrins (Lowe and Phillips, 1961). Such micelles impose a highly nonpolar environment on the hemin, providing a model for the natural systems that does not share their complexities with regard to protein acid–base equilibria which are capable of inducing conformational changes affecting active-site reactivity or producing ambiguities of mechanism as a result of rapid acid–base equilibria.

SLS-induced, monodispersed systems of hemin (Simplicio, 1972) indicate that the cyanide nucleophile coordinates with the intercalated hemin moiety above pH $\simeq$ 9. Such a system provides a uniquely simple model for hemoprotein kinetic reactivity that could be compared to previous studies on the reactivity of metmyoglobin with cyanide (Awad and Badra, 1967; Ver Ploeg and Alberty, 1968; Ver Ploeg *et al.*, 1971; George and Hanania, 1955). Over a wide range of temperature (Shick, 1964; Reiss-Husson and Luzzati, 1964) and TMAB concentration (Stigter, 1964) neither the critical micelle concentration (cmc) nor the structure of the micelle is greatly affected, thus permitting a temperature study of the intercalated hemin reaction with cyanide.

In this report we describe a kinetic study of hemin, incorporated in micellar SLS, as a function of pH, concentration of counterion TMAB, and temperature.

Experimental Section

Materials and Methods. The preparation and standardization of all solutions were essentially as described in the preceding paper. The pH was checked before and after most runs and indicated no appreciable change. Cyanide acted as a buffer as well as nucleophile throughout most of the pH range studied. pH was controlled to ±0.03 and the temperature during pH adjustment and stopped-flow work was controlled to ±0.1°. The concentration of hemin in solution was obtained with a Cary 17 recording spectrophotometer.

Kinetic Measurements. Stopped-flow experiments were run pseudo-first order in cyanide and were driven to at least 95% completion to avoid reversible kinetics. Reversible kinetics were employed in the concentration-jump experiments. Flow

† From the Department of Chemistry, University of Miami, Coral Gables, Florida 33124. *Received November 12, 1971.* Supported in part by an Institutional National Science Foundation grant and by a grant from the National Institutes of Health (NIH 101RHE-13111-01A1).

[1] Abbreviations used are: SLS, sodium lauryl sulfate; TMAB, tetramethylammonium bromide; MOH·SLS and $M(CN)_2$·SLS refer to the monoaquomonohydroxyhemin and dicyanohemin complexes intercalated in the micellar SLS.

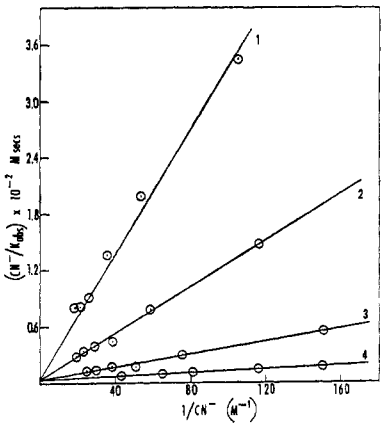

FIGURE 1: Plots of eq 4 for stopped-flow data at 25°. Curve 1 at pH 10.50, curve 2 at pH 10.00, curve 3 at pH 9.50, and curve 4 at pH 9.00. Reactions were all driven to completion and were carried out in 0.1 M TMAB and 2% SLS. The lines were drawn from a least-squares analysis of the data.

experiments were carried out by keeping the final concentration of hemin monomers between 0.4 and 0.91 μM measured by using an extinction coefficient for the monoaquomonohydroxyhemin MOH·SLS complex (λ_{max} 400 mμ) of 0.82×10^5 cm^{-1} M^{-1} (Simplicio, 1972). This insured that absorbance changes during the reaction did not exceed more than about 5% of the total absorption of the solutions and hence linearity between absorbance and extent of reaction was always maintained. Hemin solutions for flow work contained 0.1 M TMAB and 4% SLS while the potassium cyanide solutions only contained 0.1 M TMAB. It was found necessary to keep the cyanide separate from SLS prior to mixing in the flow since the SLS salted out in the presence of cyanide anion after about 5–20 min depending upon the pH, temperature, and potassium cyanide concentration. Precipitation upon mixing the hemin and cyanide solutions was not important in the flow work since the formation of the dicyanohemin complex was over before precipitation occurred. The final SLS concentration was always 2% and that of TMAB at 0.1 M, except where otherwise noted. Reactions were followed in the visible region either at 400 mμ, the peak of MOH SLS, or at 422 mμ the peak of $M(CN)_2$·SLS.

The stopped-flow apparatus used is a portion of a combination stopped-flow–temperature-jump apparatus previously described (Erman and Hammes, 1966; Faeder, 1971). Reactions were monitored with a 1P28 phototube powered by a high-voltage regulated D.C. power supply from Power Designs Co., Long Island, N.Y., and emitter follower and recorded on a Tektronix oscilloscope Type 549 (Storage) with a Type 1A7A high-gain differential amplifier. The traces were recorded permanently on Polaroid film and calculations of the half-lives were made by a secondary plot of these curves on semilog paper. Usually a minimum of three half-lives averaged from three separate traces were obtained for better accuracy. A computer program was then used to calculate free cyanide concentrations and observed rate constants. The program was also used to calculate rate constants by applying a least-squares analysis to all the data to obtain the best slopes and intercepts possible.

Results and Treatment of Data

Formation of the Dicyanohemin Complex. The kinetics of binding of cyanide over a concentration range from about 5×10^{-3} to 0.05 M with intercalated hemin essentially drives the reaction to completion. The data for the kinetic studies for the formation of the dicyanohemin complex at 15, 25, and 35° are summarized in Table I and plotted in Figure 1. The data presented here are consistent with the overall equilibrium expression given by eq 1 only valid in 2% SLS (Simplicio, 1972) (with charges omitted)

TABLE I: Summary of Formation Rate Data.

pH	Slope $\frac{k_{-1}(OH^-)}{k_1k_2}$ (M^2 sec)	$\frac{k_{-1}}{k_1k_2}$ (M sec)	T (°C)	$1/k_1$ (M sec)
10.50	3.12×10^{-4}	0.99	25	*a*
10.00	1.25×10^{-4}	1.25	25	1.74×10^{-4}
9.50	3.32×10^{-5}	1.05	25	4.52×10^{-4}
9.00	0.995×10^{-5}	0.995	25	2.69×10^{-4}
10.00	1.32×10^{-4}	1.32	15	2.50×10^{-3}
10.00	1.03×10^{-4}	1.03	35	*a*

$(k_{-1}/k_1k_2)_{25°}^{Avg} = 1.07$

[a] Intercepts were too sensitive to steep slopes and therefore unreliable.

$$K_{eq} = \frac{[M(CN)_2 \cdot SLS][OH^-]}{[MOH \cdot SLS][CN^-]^2} \quad (1)$$

Two different mechanisms consistent with the equilibrium expression in eq 1 and kinetically indistinguishable are expressed in the following two processes (neglecting charge)

mechanism 1

$$MOH \cdot SLS + CN^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MCN \cdot SLS + OH^- \quad (2a)$$

$$MCN \cdot SLS + CN^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M(CN)_2 \cdot SLS + H_2O \quad (2b)$$

or mechanism 2

$$MOH \cdot SLS + CN^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HOMCN \cdot SLS + H_2O \quad (3a)$$

$$HOMCN \cdot SLS + HCN \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M(CN)_2 \cdot SLS + H_2O \quad (3b)$$

$$K_3 = \frac{k_1k_2}{k_{-1}k_{-2}}$$

K_{eq} for mechanism 2a–2b is related to the overall equilibrium constant for 3a–3b as $K_{eq} = K_3(K_w/K_a)$ where K_a is the acid dissociation constant for HCN and K_w the dissociation constant for water. Since all reactions (except the concentration-jump-perturbation work) are driven to greater than 95% completion, the steps in the above two mechanisms that involve k_{-2} can be ignored. With MCN·SLS and HOMCN·SLS in steady state the following two respective rate equations are obtained. For mechanism 1 in which CN^- displaces OH^- first

$$\frac{[CN^-]}{k_{obsd}} = \frac{k_{-1}[OH^-]}{k_1k_2[CN^-]} + \frac{1}{k_1} \quad (4)$$

$$(k_{obsd} = 0.693/t_{1/2})$$

TABLE II: Decomposition of Dicyanohemin Complex with KOH.[a]

T (°C)	pH Final	$t_{1/2}$ (sec)
15		*b*
25	11.52	11.1
25	12.10	10.8
25	13.05	11.5
35	~13	3.9

[a] Followed at λ 422 mμ, *i.e.*, disappearance of $M(CN)_2$·SLS. [b] Salting out of SLS competed with decomposition of dicyanohemin complex. Conditions: 0.1 M TMAB, 2% SLS final, $Fe_T = 6.0\ \mu M$, $M(CN)_2$·SLS ≈ 0.4 μM.

For mechanism 2 in which CN^- first displaces H_2O

$$\frac{[CN^-]}{k_{obsd}} = \frac{k_{-1}[OH^-]}{k_1k_2[CN^-]}\left[\frac{K_a}{K_w}\right] + \frac{1}{k_1} \qquad (5)$$

It is noted that in each case a plot of $(CN^-)/k_{obsd}$ against $1/(CN^-)$ will give a straight line, at constant pH, with an intercept of $1/k_1$ but slopes, respectively, of k_{-1}/k_1k_2 and $(k_{-1}/k_1k_2) \times (K_a/K_w)$. The forms of both equations are identical and thus kinetically indistinguishable. The back-reaction, *i.e.*, the decomposition of $M(CN)_2$·SLS *via* the k_{-2}, has been evaluated by two independent techniques to be discussed shortly. This k_{-2} has been combined (see Table III) with the kinetically determined slopes (corrected for pH) for the above mechanisms and yields an overall equilibrium constant in agreement with that determined spectrally (see Tables II and III). The intercept at 35° is too sensitive to the slope of the plot. k_1 at this temperature was estimated by extrapolation from an Arrhenius plot. Activation energies and entropies of activation are listed in Table III. The hemin–cyanide formation reaction was studied by monitoring the disappearance of MOH·SLS at its wavelength of maximum absorbance, 400 mμ, or the appearance of $M(CN)_2$·SLS at its wavelength of maximum absorbance, 422 mμ. Both wavelengths yielded the same results as indicated in Figure 2. Previous spectral work (Simplicio, 1972) indicated the existence of an isosbestic point at 412 mμ. Observing the reactions at the isosbestic point on the flow apparatus showed no absorbance changes as would be expected on the basis of the mechanism postulated.

TABLE III: Summary of Rate and Equilibrium Constants.

Temp (°C)	k_1 (M^{-1} sec^{-1})	k_{-2} (sec^{-1})	K_3[a] (M^{-1})	Spectrally Determined[e]
35	2.8×10^4[b]	0.178	5.45	
25	$3.35 \times 10^3 \pm 1.85$	0.057	16.4	$14.4 \pm 4.0\ M^{-1}$
15	4.00×10^2	0.0155[c]	49.0	

Rate Constants	E_a[d]	ΔS
k_1	+38.2 kcal/mole ± 36.6	
k_2	+18.7 kcal/mole ± 3.8	3.6 eu ± 4.1
K_{eq}	ΔH° = −18.7 kcal/mole ± 4.1	

[a] $K_3 = (k_1k_2/k_{-1}k_{-2})$ (kinetically determined). [b] Extrapolated from 25° and 15°. [c] Extrapolated from 35° and 25°. The estimated error in k_1 at 15° and k_{-2} at 25° and 35° is ±7%. [d] The activation parameters are based upon data from only two different temperatures. [e] See Simplicio (1972).

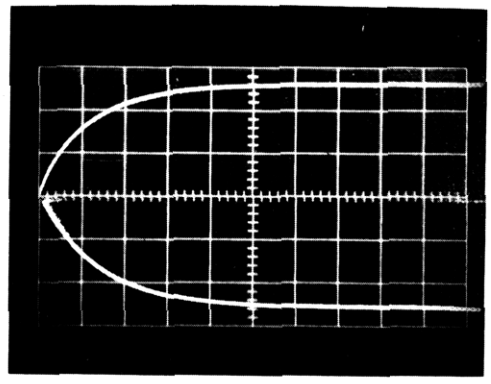

FIGURE 2: Traces for the formation of the dicyanohemin complex. Top trace is reaction monitored at 422 mμ. Bottom trace is reaction monitored at 400 mμ. Reaction conditions are T = 25°, MOH·SLS = 0.8 μM, 2% SLS, 0.1 M TMAB, pH 10.00, KCN = 4.0×10^{-2} M, time scale at 0.10 sec/div. The $t_{1/2}$ (top trace) = 0.077 sec and for the bottom trace = 0.077 sec. The abscissa has units of 100 msec/cm and the ordinate has units of 50 mV/cm.

Effect of TMAB. Reactions were usually carried out in the presence of 0.1 M TMAB. This served to minimize ionic strength changes during the course of the formation reaction of the dicyanohemin complex and between reaction conditions where the total KCN was changed. It was also needed to stabilize the SLS micellar solutions from salting out rapidly when mixed with cyanide solutions greater than about 0.01 M. It is observed (Figure 3) that the half-lives for the formation of the dicyanohemin complex, at constant pH and KCN, are sensitive to added TMAB, the half-life decreasing with increasing concentration of the counterion. In one experiment the concentration of TMAB was 0.15 M and KCl, 0.05 M. This mixture provides an ionic strength equivalent to 0.2 M TMAB but was found to give a half-life only slightly different from solutions that contain 0.15 M TMAB.

Decomposition of the Dicyanohemin Complex. The equilibria in mechanisms 1 and 2 indicate that the dicyanohemin complex equilibrium may be shifted away from the product side by the addition of hydroxide. Solutions containing 0.1 M TMAB and 4% SLS with $M(CN)_2$·SLS were rapidly mixed on the stopped flow with solutions containing varying concentrations of potas-

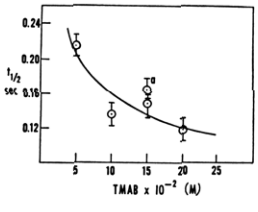

FIGURE 3: Graph of $t_{1/2}$ for the formation reaction as a function of TMAB. Reaction conditions: T = 25°, 2% SLS, KCN = 5.0×10^{-2} M, pH 10.50; note that point "a" contains 0.15 M TMAB and 0.05 M KCl.

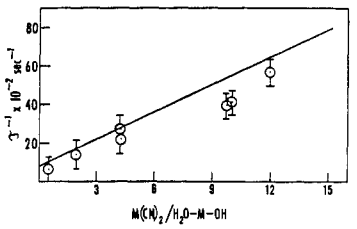

FIGURE 4: Plot of the reciprocal of the relaxation time against the ratio of $M(CN)_2 \cdot SLS/MOH \cdot SLS$. This is based on eq 7. Reaction conditions: $T = 25°$, 2% SLS, and 0.1 M TMAB. The concentrations used are the equilibrium values.

sium hydroxide and 0.1 M TMAB. The high base concentrations effectively cause the complete breakdown of the $M(CN)_2 \cdot SLS$. The half-lives of the decomposition (Table II) were monitored at 422 mμ by observing the disappearance of the dicyanohemin complex. It is noted that at constant temperature the half-lives are independent of the concentration of the base within experimental error. This suggests that the mechanism of decomposition is unimolecular and presumably the rate-determining step is the decomposition of the dicyanohemin complex, *i.e.*, k_{-2}. This k_{-2} has been combined with the slopes (k_{-1}/k_1k_2) of the formation plots, corrected for pH, and yields an overall equilibrium constant in excellent agreement with one obtained by an independent spectral determination (Table III). At temperatures lower than about 20° salting out of SLS becomes a competing process to the decomposition due to the high concentration of KOH needed for complete decomposition. The k_{-2} reported for 15° is based upon an extrapolation from 25 and 35° on an Arrhenius plot. The kinetic data at the two experimental temperatures may be used to determine the energy of activation and entropy of activation for the decomposition as well as being combined with the data of Table I to yield the overall equilibrium constant (eq 1) at three different temperatures. These data are presented in Table III. The spectrum of the final decomposed material at pH $\geq$ 12.5 differs slightly from that of $MOH \cdot SLS$ and is presumably due to some $M(OH)_2 \cdot SLS$ product. The fact that the product of this decomposition may be different from the initial reactant, $MOH \cdot SLS$, does not invalidate that what is being observed is k_{-2}, since this is observed to be the rate-determining step.

Concentration-Jump-Relaxation Investigations. A dilution perturbation or concentration jump, such as by the addition of hydroxide or cyanide to an equilibrium mixture, will cause a shift in the equilibrium indicated in eq 1. The observed relaxation spectrum, as equilibrium is reestablished, may be used as an independent check of the postulated mechanism as well as the evaluation of the accessible rate constants. If small perturbations are used (*i.e.*, on the order of a few per cent away from equilibrium) then the differential equations describing the system may be linearized (Eigen and DeMaeyer, 1964). Following the method of Castellan (1963) only one relaxation effect is predicted to be observed for the two-step mechanism. There are five concentration variables (neglecting H_2O—the solvent). Since there are two mass conservation equations, one steady-state assumption on $HOMCN \cdot SLS$ and one rapid acid–base equilibrium involving HCN and OH^-, there will only be one relaxation process given by eq 6.

$$\tau^{-1}_{exact} = \frac{k_1k_2(MOH \cdot SLS)}{k_1(MOH \cdot SLS)(CN^-) + k_{-2}(M(CN)_2 \cdot SLS)} \times \left[4(M(CN)_2 \cdot SLS) + \frac{(CN^-)(M(CN)_2 \cdot SLS)}{MOH \cdot SLS} + \frac{(CN^-)(M(CN)_2 \cdot SLS)}{(OH^-)} + (CN^-)\right] \quad (6)$$

τ^{-1} has units of sec^{-1}.

Under the experimental conditions $MOH \cdot SLS \approx M(CN)_2 \cdot SLS$, $Fe_{total} = MOH \cdot SLS + M(CN)_2 \cdot SLS \leqq 6.5$ mM, and $CN^- \approx 1 \times 10^{-2}$ M, and placing tentative values in the relaxation equation, $k_1 = 3.35 \times 10^3$ M^{-1} sec^{-1} and $k_{-2} = 5.7 \times 10^{-2}$ sec^{-1}, only a few of the terms in eq 6 will be important and may be simplified to

$$\tau^{-1} \cong k_{-2}\frac{(M(CN)_2 \cdot SLS)}{(MOH \cdot SLS)} + k_{-2} \text{ (reintroducing } k_{-2}) \quad (7)$$

This indicates that a plot of τ^{-1} against the ratio of the dicyanohemin complex to the monoaquomonohydroxyhemin complex should give a straight line with a slope and intercept both equal to k_{-2}. The data are presented in Table IV and the final results plotted in Figure 4. k_{-2} obtained from the slope of the line is 5.16×10^{-2} sec^{-1} and from the intercept 7.8×10^{-2} sec^{-1}. These values are in good agreement with each other and in excellent agreement with the k_{-2} value obtained from base decomposition studies. The range of pH variation 10.5–11.0 and the 20-fold ratio change of product to reactant indicates that the assumptions regarding eq 7 are valid.

TABLE IV: Concentration Perturbation Results.

τ_{obsd} (sec)	$MOH \cdot SLS^a$ (μM)	$M(CN)_2 \cdot SLS^a$ (μM)	$M(CN)_2 \cdot SLS/MOH \cdot SLS$
2.5[b]	0.495	4.82	9.75
4.5	0.825	3.46	4.20
2.4	0.390	3.88	9.95
18.5	2.92	1.26	0.432
1.75	0.500	6.00	12.0
3.7	1.07	4.46	4.17
7.5	1.49	2.80	1.88

[a] Refers to final equilibrium concentrations. [b] Error limits are ±10%; slope = 5.16×10^{-2} sec^{-1}; intercept = 7.8×10^{-2} sec^{-1}; $T = 25°$. pH varied from 10.50 to 11.0. Fe_T varied from ~4 to 6.5 μM.

Discussion

The kinetics of interaction between cyanide solutions and metmyoglobin have been extensively studied (George and Hanania, 1955; Awad and Badra, 1967; Ver Ploeg *et al.*, 1971). In these studies an ambiguity exists regarding the nature of the reactive cyanide specie. The data are consistent with either HCN or CN^- being the reactive nucleophile due to an acid–base equilibrium associated with the metmyoglobin itself. Thus a protonated form of metmyoglobin will react with CN^- to form a transition state identical with an unprotonated metmyoglobin with HCN. The present study has found that the attacking nucleophile on hemin is CN^- in the first step, in the pH range from 9.0 to 11.5. It is impossible kinetically to ascertain whether OH^- or H_2O is lost first when cyanide attacks in the first step (mechanisms 1 and 2, respectively). In

view of hydroxide being a stronger nucleophile than water, mechanism 2 is favored over mechanism 1. It is difficult to make a clear comparison between the observed rate constants for metmyoglobin and the present study on two accounts. First, the rate constants previously obtained (Ver Ploeg and Alberty, 1968) are quite sensitive to catalytic effects of the buffers used. The present study, carried out without the complications of added buffer agents, yields a k_1 of 3.35×10^3 M^{-1} sec^{-1} (0.10 M ionic strength) but is not too different from the $k_{apparent}$ varying from 0.1×10^3 M^{-1} sec^{-1} (0.0 M ionic strength) to 0.6×10^3 M^{-1} sec^{-1} (0.06 M ionic strength) (Ver Ploeg and Alberty, 1968) if ionic strength factors are considered. The reaction between metmyoglobin with azide at high pH gives a rate of 5×10^3 M^{-1} sec^{-1} (Duffy *et al.*, 1966), again quite close to the rate constant in the present study.

The relative insensitivity of the micelle to temperature suggests that the nature of the environment of the intercalated hemin remains the same and permits the cyanide–hemin interactions to be studied for evaluation of activation parameters. The energy of activation for k_1 is 38.2 kcal/mole ± 36.6. The error in the activation energy is due to the large error associated with the value of k_1 at 25° and largely precludes comparisons to $Fe(H_2O)_6^{3+}$ or $Fe(OH)^{2+}$ for which E_a is approximately 20 kcal/mole (Caldin, 1964). The $\Delta S^{\neq}$ for the k_{-2} reaction has a value quite close to zero. This would seem to suggest that the replacement of cyanide by water involves little difference in the steric accommodations in the transition state or in the structure of the micelle.

It has been suggested that the surface charge of metmyoglobin plays a role in its reactivity (Awad and Badra, 1967). In the present study the half-life for the k_1 reaction is sensitive to the concentration of the counterion TMAB. A 2% SLS solution is approximately an order of magnitude above the cmc (Shick, 1964); therefore, the effect of TMAB cannot be ascribed to influence on the cmc. The effect of added counterion is believed to lie in the charge neutralization of the sulfate anion heads of the SLS in the micelle. Charge neutralization would presumably facilitate the approach and ultimately penetration at the micellar–water interface by CN^-. Hemin intercalated in micelles of cetyltrimethylammonium bromide (J. Simplicio and K. S. Schwenzer, unpublished) reacts much faster with CN^- compared to the SLS. This is presumably due to the cationic micellar–water interface. Analogously the metal incorporation of Cu(II) into protoporphyrin IX is accelerated nearly 20,000-fold in the presence of SLS compared to cetyltrimethylammonium bromide (Lowe and Phillips, 1961). It has been suggested by the same authors that an important electrostatic effect is operative in the anionic sodium lauryl sulfate as contrasted to the cationic charge on the cetyltrimethylammonium bromide. The negative head would be expected to attract the Cu(II) cation and hence accelerate its incorporation into the intercalated porphyrin.

The rate constant for the loss of the first cyanide (*i.e.*, k_{-2}) is 5.7×10^{-2} at 25°. The apparent first-order dissociation rate constant for the metmyoglobin–cyanide reaction (Ver Ploeg *et al.*, 1971) in the pH region from 9.0 to 10.0 is 2×10^{-4} sec^{-1}. It is difficult to compare these two results in the absence of enthalpy and entropy data for the metmyoglobin system. The difference may have its origin in either or both of the activation parameters. The accelerated rate of the micellar hemin over the natural system may also be a reflection of the effect of two different trans groups in the two cases. In the micellar–hemin system the first CN^- leaving is trans to the second CN^- still present. However, in the metmyoglobin case, the imidazole portion of a histidine residue is presumably trans to the leaving CN^-. The strong π-bonding characteristics of the cyanide ligand (Parshall, 1966) as compared to imidazole nitrogen and the available t_{2g} orbitals on the metalloporphyrin (Falk, 1964) may be the reason for the increased lability for the first removed cyanide.

Confidence in the postulated mechanism is reinforced by a kinetically determined overall equilibrium constant for eq 1 that agrees quite well with that obtained by spectrophotometric techniques.

It would appear from the results that hemin incorporated in micellar material such as SLS can be used, with caution, as a model system for hemoproteins. The advantages offered by this type of system are a strong hydrophobic environment imposed about a monodisperse system, a system free from protein acid–base equilibria and one free from the added complexity of the role that axial coordination of amino acid residues to the metalloporphyrin play. Furthermore, changing surface charge on the macromolecular micelle, by the addition of a counterion, can give information on how the surface charge of a macromolecule affects the reactivity of the active site buried within such structures.

Acknowledgment

The author wishes to express his deep appreciation for funds given by Dr. Walter O. Walker for the partial construction of the stopped-flow–temperature-jump apparatus, also to the Tektronix Corporation for a partial donation of the Type 549 oscilloscope, to the Chemistry Department at the University of Miami for financial assistance for the partial construction of the experimental equipment, and finally to Mr. J. F. Hellenkamp of Hansa R & D for his invaluable work and imagination in constructing the rapid reaction apparatus.

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Proton Magnetic Resonance of Vitamin B_{12} Derivatives. Functioning of B_{12} Coenzymes†

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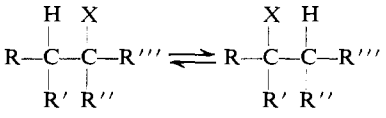
ABSTRACT: Proton magnetic resonance studies of 5,6-dimethylbenzimidazole B_{12} coenzyme (CoB_{12}) or 5′-deoxyadenosylcobalamin in water at different pH values and temperatures and in dimethyl sulfoxide at different temperatures are presented. The 5,6-dimethylbenzimidazole base is shown to be longer coordinated to cobalt at acid pH in water or in dimethyl sulfoxide at 88°. This result is confirmed by optical measurements. CoB_{12} is about 15–20% "base off" in water at 85° and neutral pH. The pK at 23° for the protonation of the base on CoB_{12} is 3.28 ± 0.04. The rate constant for base dissociation is greater than 550 sec^{-1}. The prochiral protons on the cobalt-bound carbon in CoB_{12} are found to be nonequivalent for both "base-on" and "base-off" configurations. This nonequivalence is attributed to incomplete averaging of proton environments through rotation about the carbon–cobalt bond. Upon loss of 5,6-dimethylbenzimidazole coordination, CoB_{12} is suggested to exhibit a significant change in the average orientation of its 5′-deoxyadenosyl moiety. The loss of base coordination appears to have little effect on the cobalt–carbon R′5 (on 5′-deoxyadenosyl) bond. A brief discussion of the implications of these studies for the functioning of CoB_{12} in enzymatic reactions is presented.

The enzymatically active derivatives of vitamin B_{12} known as B_{12} coenzymes were initially isolated by Barker *et al.* (1958), and have been demonstrated to catalyze a number of unusual enzymatic transformations. The geometrical structure and chemical properties of the coenzymes are of particular interest to biochemists because the coenzyme serves as the prosthetic group in several enzyme-dependent reactions of the following type



X-Ray diffraction studies (Lenhert, 1968) have demonstrated three-dimensional molecular structure of 5,6-dimethylbenzimidazole B_{12} coenzyme (CoB_{12}).[1]

Several previous investigators (Brodie, 1969; Babior, 1970) have emphasized the importance of geometrical accommodations in the coenzyme that must accompany B_{12}-catalyzed enzymic reactions and a recent report (Law *et al.*, 1971) has pointed out that even in aqueous solution, the average orientation of the deoxyadenosyl moiety with respect to the macrocycle must vary appreciably from the crystalline state. We now report proton magnetic studies on CoB_{12} under conditions of varying pH and temperature that provide insight into those structural and electronic properties of B_{12} coenzymes in solution that might be relevant to enzymatic catalysis. These and earlier studies (Brodie and Poe, 1971; Cockle *et al.*, 1970; Hill *et al.*, 1965, 1968, 1969; Law *et al.*, 1971; Doddrell and Allerhand, 1971) also permit a detailed assessment of the relationship of geometrical and magnetic states in corrinoids.

Materials and Methods

CoB_{12} (kindly donated by Dr. L. Mervin, Glaxo, Ltd., England) was purified by chromatography on CM-cellulose in the dark. Methylcobalamin was prepared and purified as described previously (Brodie and Poe, 1971). Proton magnetic resonance spectra were run on either Varian 220 MHz or Varian HA-100 spectrometer, and referenced internally to $(CH_3)_4Si$ or to the methyl resonance of the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid. Chemical shifts were measured in hertz or parts per million, with downfield shifts assigned positive values. The temperature of the sample zone of the 220-MHz spectrometer was determined from the resonance frequencies of the hydroxyl group of ethylene glycol to an estimated accuracy of ±0.5°. The HA-100 was "locked" on either external $(CH_3)_4Si$ or benzene (C_6H_6). The signal-to-noise characteristics of certain spectra were improved using a Varian C-1024 computer of average transients.

For proton magnetic resonance studies about 30 mg of CoB_{12} was dissolved in 1 ml of either deuterated dimethyl sulfoxide, $(CD_3)_2SO$, or D_2O. About 50 mg of methylcobalamin was dissolved in 2 ml of D_2O. All handling operations were carried out in the dark; proton magnetic resonance tubes containing B_{12} solutions were kept wrapped in aluminum foil. pH measurements were made at 23° in the nuclear mag-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CoB_{12}, 5,6-dimethylbenzimidazole B_{12} coenzyme; pmr, proton magnetic resonance; pH, uncorrected glass-electrode pH meter readings in D_2O.