Heme-Linked Effect in the Reaction of Sperm Whale Ferrimyoglobin with Cyanide*

Elias S. Awad and Riad G. Badro†

ABSTRACT: The detailed pH profile in the range of pH 5–7 was determined for the reaction of sperm whale ferrimyoglobin with cyanide at 25.0 and 15.6° in Tris-maleate–potassium chloride buffer of constant ionic strength 0.15 M.

The reaction shows a heme-linked effect dependent on five simultaneous prototropic ionizations on the globin moiety, suggesting a concomitant conformational change in the molecule. The intrinsic pK_a of these prototropic sites was found to be 5.66 at 25°, $\Delta H^\circ = +0.69$ kcal/mole, and $\Delta S^\circ = -24$ eu. In addition, there is an over-all heme-linked charge effect, which manifests itself as a dependence of the observed second-order rate constant on the one-half power of hydrogen ion concentration. This effect is interpreted as a reflection of pH-dependent changes in the intramolecular dielectric constant of the myoglobin molecule.

The kinetics of ligand-bonding reactions of heme proteins provide a means for the investigation of interactions between the heme group and the globin moiety. The heme proteins are particularly suited to this indirect approach, since the attachment of a ligand to the heme group occurs at a specific point in the molecule, the 6th coordination position of the heme iron atom, and gives rise to a distinct alteration in the spectrum of the heme protein. The kinetics of the reaction between the heme protein and a ligand can be measured by following absorbancy changes in the reaction mixture at a suitable wavelength, such as in the sensitive and specific Soret region of the spectrum.

In this manner, George and Tsou (1951) have studied the reaction between cytochrome *c* and cyanide and George and Hanania (1954a–c, 1955a,b) have investigated the effect of the variation of pH, temperature, and ionic strength on the reaction of horse ferrimyoglobin with cyanide. George and Hanania (1955b) interpreted the pH dependence of the reaction in terms of one heme-linked ionizing group. Blanck *et al.* (1961) have measured the rate of reaction of horse ferrimyoglobin with cyanide in a comparative study of various ligands. Diven *et al.* (1965) and Goldsack *et al.* (1965, 1966) investigated the kinetics of the reaction of sperm whale ferrimyoglobin with imidazole, azide, cyanate, benzimidazole, and hydrosulfide as a function of pH. They conclude that the best fit to their data is given by a reaction scheme involving two heme-linked ionizing groups with $pK_1 = 4.7$ and $pK_2 = 5.7$; pK_1 was attributed to a propionic acid side chain on the heme and pK_2 to the E7 (distal) histidine residue. Duffey *et al.* (1966), in their study of the sperm whale ferrimyoglobin–azide system, have argued that it is unnecessary to include heme-linked ionizations in the interpretation of the pH dependence of the ligand-bonding kinetics. The heme-linked effect has been recently discussed by Rossi-Fanelli *et al.* (1964), Wyman (1964), and Riggs (1965). The aim of the present work was to measure in detail the pH dependence of the cyanide reaction with sperm whale ferrimyoglobin in the range of pH 5–7 and to try to elucidate the nature of the heme-linked effect.

Experimental Section

Materials. Sperm whale skeletal muscle ferrimyoglobin was obtained from Seravac Laboratories (Maidenhead, Berks.). The iron content was 0.307% on a dry weight basis. Chromatography on Sephadex G-75 (method of Awad *et al.*, 1963) showed that the sample contained at least 95% myoglobin. Myoglobin solutions were prepared immediately before use.

Potassium cyanide (British Drug Houses, Laboratory Reagent) assayed 96% by silver nitrate titration. Allowance was made for this in calculating concentrations and only freshly prepared solutions were used.

Tris (British Drug Houses, Laboratory Reagent) was recrystallized from ethanol. All other chemicals were of reagent grade and solutions were made up with distilled deionized water.

Buffer Solutions. TMPC[1] buffers of ionic strength 0.15 M (0.05 M Tris, 0.05 M in maleate) and of pH values in the range of 5–7 were carefully prepared as

* From the Department of Chemistry, American University of Beirut, Beirut, Lebanon. *Received December 13, 1966.* This work was supported by U. S. Public Health Service Grant HE-04994. A preliminary report was presented at the 2nd International Biophysics Congress, Vienna, Sept 1966.

† Present address: Department of Physics, American University of Beirut, Beirut, Lebanon.

[1] Abbreviations used: TMPC, Tris-maleate–potassium chloride buffer; Mb, myoglobin; $Mb \cdot FeOH_2^+$, ferrimyoglobin hydrate (acidic ferrimyoglobin); Mb·FeOH, ferrimyoglobin hydroxide (basic ferrimyoglobin); Mb·FeCN, ferrimyoglobin cyanide.

follows. A stock solution 1.00 M in Tris and 1.00 M in maleic acid was prepared. Of this solution 5 ml was transferred to a 100-ml volumetric flask. A calculated volume of standard 0.2 N sodium hydroxide was added to give the desired final pH and the volume was brought to 100 ml with water. The pH of this solution was measured and a calculated amount of solid potassium chloride was added to bring the ionic strength to 0.15 M. The volume change due to the addition of solid potassium chloride was negligible. The pH of the final buffer solution containing potassium chloride was identical with the first pH measurement. The conductivities of all the buffer solutions prepared in this manner in the range of pH 5–7 were the same (Conductivity Bridge Model RC, 16B2, Industrial Instruments, Inc.). The effect of temperature on the pH of the TMPC buffers was negligible between 10 and 30°.

The amount of potassium chloride added to give an ionic strength of 0.15 M was calculated as follows. Let T and TH^+ represent Tris and its conjugate acid and let MH_2, MH^-, and M^{2-} represent maleic acid, acid maleate, and maleate. The equilibria involved are

$$MH_2 \overset{K_1}{\rightleftharpoons} MH^- + H^+$$

$$MH^- \overset{K_2}{\rightleftharpoons} M^{2-} + H^+$$

$$TH^+ \overset{K_3}{\rightleftharpoons} T + H^+$$

where at 25° $K_1 = 1.42 \times 10^{-2}$, $K_2 = 8.57 \times 10^{-7}$ (Handbook of Chemistry and Physics, 1960), and $K_3 = 8.40 \times 10^{-9}$ (Edsall and Wyman, 1958). Above pH 5, the concentration of MH_2 is negligible. It may be readily shown that the ionic strength, I, of a solution prepared as described above, before addition of potassium chloride, is given by the expression $I = 0.05 + 0.10K_2/(K_2 + H)$, where H is the concentration of hydrogen ion. Hence, to obtain a total ionic strength of 0.15 M, potassium chloride must be added to a concentration given by $[KCl] = 0.10\ H/(K_2 + H)$.

Measurement of pH was carried out with an accuracy of ±0.002 pH unit (Radiometer pH-meter 4, type RH.M4C). The instrument was standardized with phosphate and phthalate buffers prepared as recommended by the U. S. National Bureau of Standards (Bates, 1964).

Spectra of Acidic Ferrimyoglobin and Ferrimyoglobin Cyanide. The molar absorbancies of acidic ferrimyoglobin and ferrimyoglobin cyanide in TMPC buffers of ionic strength 0.15 M were determined in the spectral regions 404–426 and 535–565 mμ. No effect on the absorbancy of ferrimyoglobin cyanide due to pH was detected in the range of pH 5–7 and none due to temperature in the range of 10–30°. The same was true for the absorbancy of ferrimyoglobin hydrate (acidic ferrimyoglobin), although at pH above 7 the conversion to ferrimyoglobin hydroxide (basic ferrimyoglobin) becomes perceptible. The value of the maximum difference in the molar absorbancies of ferrimyoglobin hydrate and ferrimyoglobin cyanide was $\Delta\epsilon = 103{,}400\ M^{-1}\ cm^{-1}$ at 408 mμ and $\Delta\epsilon = 55{,}600\ M^{-1}\ cm^{-1}$ at 555 mμ. The former value was used in most kinetic experiments, the latter when the myoglobin solutions were more concentrated. The concentrations of myoglobin solutions were determined by complete conversion of the myoglobin to ferrimyoglobin cyanide and measurement of the absorbancy at 423 mμ, $\epsilon = 109{,}500\ M^{-1}\ cm^{-1}$.

Measurement of the Forward Rate of Reaction. The kinetic experiments were performed in a Zeiss PM QII spectrophotometer cuvet which was thermostated by circulating water from a constant temperature bath controlled to ±0.1°, through a specially built jacket around the cuvet. In a typical run, 3.00 ml of 5.0×10^{-6} M solution of ferrimyoglobin in TMPC buffer was placed in the spectrophotometer cuvet and 10.0 μl of 0.096 M potassium cyanide in the same buffer was added manually by means of a plunger. The addition and mixing took less than 5 sec. At the end of the run the temperature was checked with a thermistor probe (Tele-Thermometer, Yellow Springs Instrument Co.), which was previously standardized against a certified mercury thermometer from the National Physical Laboratory, England. The progress curve of the reaction was obtained on a Sargent Model M.R. recorder.

Kinetic Results

The reaction of cyanide with sperm whale ferrimyoglobin obeyed good first-order kinetics up to about 50% reactions, this being the extent to which the reaction was followed. The first-order rate constant, k_{obsd}, was not affected by variation of the initial myoglobin concentration over a 30-fold range (Table I). The dependence of k_{obsd} on the stoichiometric concentration

TABLE I: Effect of Myoglobin Concentration on k_{obsd}.[a]

$[Mb]_0$[b]	Wavelength[c] (mμ)	$10^3\ k_{obsd}$ (sec^{-1})
3.7	408	68.1 ± 0.8
12.7	408	70.7 ± 1.7
17.4	408	69.0 ± 0.1
23.4	408	71.3 ± 1.4
23.7	555	68.2 ± 0.6
58.9	555	69.5 ± 1.3
127.3	555	69.9 ± 1.1

[a] The reaction conditions were pH 5.06, 25.0°, stoichiometric cyanide concentration $C_0 = 9.57 \times 10^{-4}$ M, and TMPC buffer of ionic strength 0.15. The average of the values listed gives: $k_{obsd} = 69.5 \times 10^{-3}\ sec^{-1}$ (± 0.1×10^{-3}) and $k_F = 72.7\ M^{-1}\ sec^{-1}$ (±1.3). [b] Initial myoglobin concentration. [c] Wavelength at which the reaction was followed.

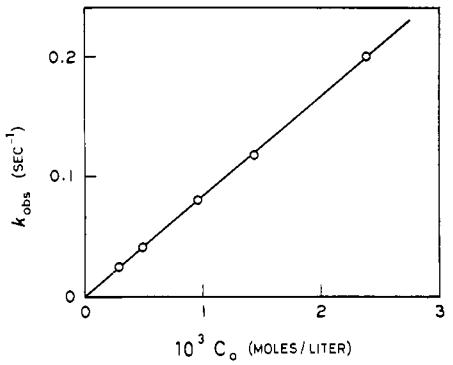

FIGURE 1: Effect of initial cyanide concentration, C_0, on the first-order rate constant, k_{obsd}. The reaction conditions were pH 5.28, 25.0°, TMPC buffer of ionic strength 0.15 M.

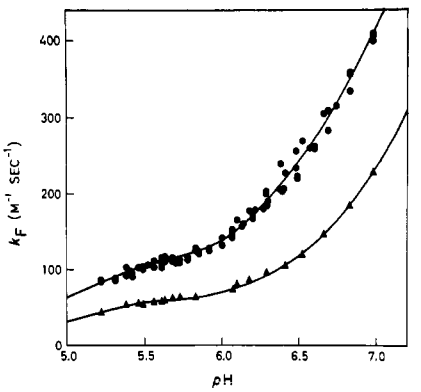

FIGURE 2: pH profile of the second-order rate constant k_F. Circles represent data at 25.0°, triangles at 15.6° The runs were made in TMPC buffers of ionic strength 0.15 M.

of cyanide was linear over a tenfold range of cyanide concentration (Figure 1). The second-order rate constant for the reaction, k_F, was, therefore, calculated from the simple relation $k_F = k_{obsd}/C_0$, where C_0 is the initial stoichiometric concentration of cyanide.

The pH profiles of k_F in the range of pH 5–7 at 25.0 and 15.6° are shown in Figure 2. Each point represents the average of triplicate runs. The existence of a shoulder in the pH profiles indicates a heme-linked effect.

Analysis of Kinetic Data

It is convenient to start with certain assumptions and to set up a reaction scheme upon which the following analysis will rest. The assumptions will be commented on in the Discussion section.

It is postulated that in ferrimyoglobin hydrate, $Mb \cdot FeOH_2^+$, there are a number, n, of heme-linked prototropic groups on the globin moiety which ionize in the pH region where the shoulder appears in the pH profile (Figure 2). To a first approximation, we shall assume that these groups are identical and electrostatically independent. We shall assume that cyanide ion is the reacting partner and that hydrocyanic acid is not a reacting species. The reaction of ferrimyoglobin hydrate with cyanide may then be described by the reaction scheme of eq 1–4, where GH represents one of the heme-linked acidic groups on the globin moiety and G^- its conjugate base.

$$(GH)_n \cdot Mb \cdot FeOH_2^+ + CN^- \xrightarrow{k_1} (GH)_n \cdot Mb \cdot FeCN + H_2O \quad (1)$$

$$(G^-)_n \cdot Mb \cdot FeOH_2^+ + CN^- \xrightarrow{k_2} (G^-)_n \cdot Mb \cdot FeCN + H_2O \quad (2)$$

$$(GH)_n \cdot Mb \cdot FeOH_2^+ \overset{K_nG}{\rightleftharpoons} (G^-)_n \cdot Mb \cdot FeOH_2^+ + nH^+ \quad (3)$$

$$GH \overset{K_G}{\rightleftharpoons} G^- + H^+ \quad (4)$$

The observed rise in the value of k_F with increasing pH must be attributed, at least in part, to a rise in the concentration of cyanide ion, which is due to the acid dissociation equilibrium of hydrocyanic acid

$$HCN \overset{K_{CN}}{\rightleftharpoons} H^+ + CN^-$$

governed by $K_{CN} = 6.16 \times 10^{-10}$ at 25°, $\Delta H° = 10.4$ kcal/mole, $\Delta S° = -3.9$ cal/deg mole (Izatt *et al.*, 1962). From these data $K_{CN} = 3.39 \times 10^{-10}$ at 15.6° was calculated. The concentration of cyanide ion at any particular pH is given by $[CN^-] = C_0K_{CN}/(K_{CN} + H)$, where C_0 is the stoichiometric concentration of cyanide and H is the concentration of hydrogen ion. Since at pH below 7, $K_{CN} \ll H$, we have $[CN^-] = C_0K_{CN}/H$. It follows that, for the reaction scheme of eq 1–4

$$k_F = (1 - \alpha)k_1\left(\frac{K_{CN}}{H}\right)f_1(H) + \alpha k_2\left(\frac{K_{CN}}{H}\right)f_2(H) \quad (5)$$

where

$$\alpha = \frac{[G^-]}{[G^-] + [GH]} = \frac{[(G^-)_n \cdot Mb \cdot FeOH_2^+]}{[(G^-)_n \cdot Mb \cdot FeOH_2^+] + [(GH)_n \cdot Mb \cdot FeOH_2^+]} \quad (6)$$

and $f_1(H)$ and $f_2(H)$ are some functions of the hydrogen ion concentration.

The experimental data were plotted in the form of log k_F against pH (Figure 3). At both temperatures, the plot is linear in the region of pH 6.0–7.0 and has a slope of 0.5. In the region of pH 5.2–5.5 the data are insufficient to be interpreted conclusively, but the plot is consistent with a linear relation having a slope

of 0.5. The variation of the rate constant k_F may therefore be described empirically by the equation

$$k_F = \text{constant} \cdot H^{-1/2} \quad (7)$$

Comparing eq 5 and 7, we obtain

$$k_F = (1 - \alpha)k_1\left(\frac{K_{CN}}{H}\right)q_1H^{1/2} + \alpha k_2\left(\frac{K_{CN}}{H}\right)q_2H^{1/2} \quad (8)$$

where q_1 and q_2 are constants with respect to pH. The physical significance of $q_1H^{1/2}$ and $q_2H^{1/2}$ will be discussed later. Equation 8 may be written more compactly as

$$k_F = (1 - \alpha)k_{F_1} + \alpha k_{F_2} \quad (9)$$

where k_{F_1} and k_{F_2} are defined by reference to eq 8. The functions of k_{F_1} and k_{F_2} correspond to the linear portions of the plot of log k_F against pH (Figure 3). It is clear that the rate constant k_{F_1} corresponds to the pH region where the ferrimyoglobin is essentially completely in the protonated form, $(GH)_n \cdot Mb \cdot FeOH_2^+$, and the formation of ferrimyoglobin cyanide is proceeding according to eq 1. Similarly, k_{F_2} corresponds to the pH region where the conjugate base form, $(G^-)_n \cdot Mb \cdot FeOH_2^+$, predominates and the reaction is proceeding according to eq 2. In the intermediate pH region, k_F receives significant contributions from both k_{F_1} and k_{F_2}, as expressed by eq 9, signifying that the reactions of eq 1 and 2 are proceeding in parallel.

From the definition of k_{F_1} by eq 8 and 9, it follows that $\log k_1q_1 = \log k_{F_1} + pK_{CN} - 0.5pH$, from which the value of k_1q_1 can be readily calculated. Since the values of k_1 and q_1 are experimentally not separable, the activation functions $\Delta F_1^{\pm}$, $\Delta H_1^{\pm}$, and $\Delta S_1^{\pm}$ pertaining to k_1 can not be determined. However, the following composite functions pertaining to the product term k_1q_1 can be obtained

$$(\Delta F_1^{\pm} + \phi_1) = -4.576\,T \log k_1q_1$$

$$(\Delta H_1^{\pm} + \eta_1) = -4.576\,\frac{\mathrm{d} \log k_1q_1}{\mathrm{d}(1/T)}$$

$$(\Delta S_1^{\pm} + \sigma_1) = \frac{(\Delta H_1^{\pm} + \eta_1) - (\Delta F_1^{\pm} + \phi_1)}{T}.$$

Here ϕ_1, η_1, and σ_1 are the thermodynamic contributions pertaining to q_1. Similarly, the corresponding functions for k_2q_2 were calculated. The numerical values obtained are listed in Table II.

We shall now proceed to determine the number, n, of the heme-linked prototropic sites and the thermodynamic parameters pertaining to the equilibrium of eq 4. For the equilibrium of eq 3

$$K_{nG} = \frac{[(G^-)_n \cdot Mb \cdot FeOH_2^+]}{[(GH)_n \cdot Mb \cdot FeOH_2^+]}H^n = \frac{\alpha}{1 - \alpha}H^n = RH^n$$

$$pK_{nG} = pR + n\text{pH} \quad (10)$$

where the degree of dissociation, α, is given by eq 6 and by definition, $R = \alpha/(1 - \alpha)$. Hence

$$R = \frac{k_{F_1} - k_F}{k_F - k_{F_2}} \quad (11)$$

Differentiating eq 10 with respect to pH, we have

$$-\frac{\mathrm{d}pR}{\mathrm{dpH}} = n \quad (12)$$

For the acid dissociation equilibrium of the prototropic site GH (see eq 4)

$$K_G = \frac{[G^-]}{[GH]}H = \frac{\alpha}{1 - \alpha}H = RH$$

$$pK_G = pR + \text{pH} \quad (13)$$

From eq 10 and 13, it follows that when $pR = 0$

$$\text{pH} = pK_G = \frac{1}{n}pK_{nG} \quad (14)$$

By eq 12, the value of n can be obtained from the slope of a plot of pR against pH, and, by eq 14, the intercept of this plot on the abscissa gives the value of pK_G. A plot of pR against pH, calculated from the kinetic data by means of eq 11 is given in Figure 4. The experimental points for 25.0 and 15.6° can be fitted well by straight lines of slope −5. Hence $n = 5$. The pK values and the corresponding thermodynamic functions calculated from the relations $\Delta F° = 4.576T\text{-}pK_G$, $\Delta H° = -4.576T^2(\mathrm{d}pK_G/\mathrm{d}T)$, and $\Delta S° = (\Delta H° - \Delta F°)/T$ are listed in Table II. The evaluation of n and pK_G according to the above considerations

TABLE II: Kinetic and Thermodynamic Data at 25.0°.[a]

	10^{-8} kq	$(\Delta F^{\pm} + \phi)$, kcal/mole	$(\Delta H^{\pm} + \eta)$, kcal/mole	$(\Delta S^{\pm} + \sigma)$, eu
k_1q_1	3.16	−11.6	+0.4	+40
k_2q_2	2.19	−11.4	+1.2	+42

	pK_G	$\Delta F°$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (eu)
GH	5.664	+7.72	+0.69	−24
CH_3COOH	4.874	+6.647	−0.163	−22.8

[a] The values for acetic acid are taken from Edsall and Wyman (1958).

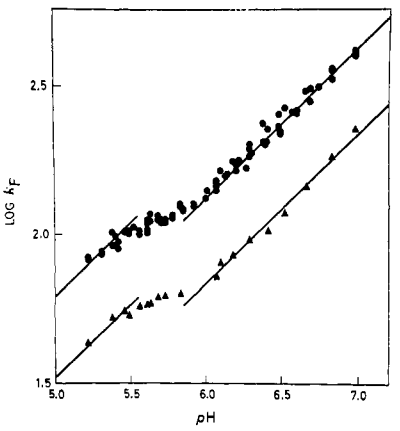

FIGURE 3: pH profile of log k_F. Circles represent data at 25.0°, triangles at 15.6°.

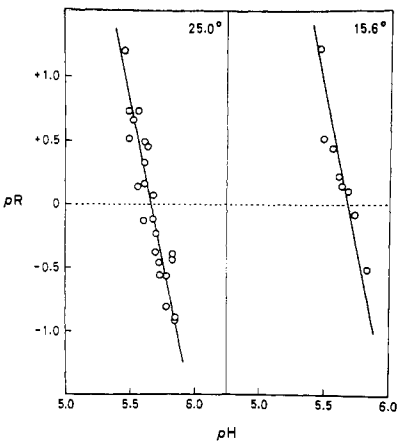

FIGURE 4: Plot used for the determination of the number, n, of heme-linked prototropic sites and their intrinsic pK.

is valid whatever kinetic interpretation is assigned to the functions k_{F_1} and k_{F_2}.

Using the numerical values given in Table II and making use of eq 8 and 13, the theoretical variation of k_F with pH was calculated and is shown in Figure 2 by the solid lines.

Discussion

Myoglobin in the ferric oxidation state can exist in aqueous solution in one of two forms, ferrimyoglobin hydrate (Mb·$FeOH_2^+$) and ferrimyoglobin hydroxide (Mb·FeOH), depending on the pH of the solvent. The equilibrium

$$Mb \cdot FeOH_2^+ \overset{K_a}{\rightleftharpoons} Mb \cdot FeOH + H^+$$

involves a prototropic change in the water molecule bound to the heme iron atom. Since the pK_a of this equilibrium for sperm whale ferrimyoglobin is 8.96 (Hanania *et al.*, 1966), the myoglobin dealt with in this investigation can be considered to be entirely in the ferrimyoglobin hydrate form.

When ferrimyoglobin hydrate reacts with cyanide ion, ferrimyoglobin cyanide is formed by displacement of the water molecule according to the stoichiometric equation

$$Mb \cdot FeOH_2^+ + CN^- \longrightarrow Mb \cdot FeCN + H_2O$$

The assumption that hydrocyanic acid enters into reaction as a chemical species with the heme group was made by George and Tsou (1951) and by George and Hanania (1954c). In both cases, the values reported for the rate constant of the hydrocyanic acid reaction were very small compared with the corresponding rate constants for cyanide ion. We have neglected the reaction of hydrocyanic acid in our analysis, because had this reaction been significant, a deviation from linearity should have been seen in the logarithmic pH profile (Figure 3). Furthermore, it is known that the sole product of the reaction of ferrimyoglobin with cyanide (whether the reacting species be cyanide ion or hydrocyanic acid) is ferrimyoglobin cyanide (Mb·FeCN not Mb·$FeHCN^+$) (Clark, 1948; George and Hanania, 1954b). In fact, a mechanism of the SN2 type involving hydrocyanic acid in the transition state would seem improbable in view of the steric environment of the heme group.

The existence of a heme-linked effect in the reaction of sperm whale ferrimyoglobin is clearly shown in the present work by the pH-dependent transition of the second-order rate constant from k_{F_1} and k_{F_2}. The transition reflects an acid–base equilibrium, which is dependent on the fifth power of hydrogen ion concentration. That is to say, we have an all-or-none process involving five simultaneous proton transfers within an individual myoglobin molecule. The fifth power dependence leads to the view that there is a concomitant pH-induced conformational transition, possibly a helix–coil transition of the type that is well known in protein denaturation (Steinhardt, 1937; Kauzmann, 1954; Scheraga, 1961). Such a *conformational heme-linked effect* is an example of the *allosteric effect* described by Wyman (1963). The thermodynamic parameters (Table II) associated with the ionizing groups on the globin moiety involved in this heme-linked effect suggest that these groups may be hydrogen-bonded carboxyls, which interact electrostatically with the heme group.

It is not clear what physical meaning should be assigned to the functions $q_1H^{1/2}$ and $q_2H^{1/2}$ in eq 8. The ionization of hydrocyanic acid is already accounted for by the factor K_{CN}/H. The parameters q_1 and q_2 may be temperature dependent. The possibility of side reactions can be eliminated by the fact that the algebraic form of the expressions which may be derived for the second-order rate constant are in conflict with the experimental data. Yet the linearity of the logarith-

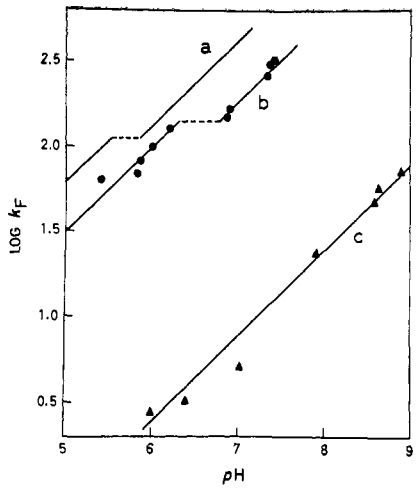

FIGURE 5: Comparison of heme-linked effects in sperm whale ferrimyoglobin, horse ferrimyoglobin, and cytochrome *c*. Curve a: sperm whale ferrimyoglobin + cyanide at 25.0°, simplified presentation of data taken from Figure 3. Curve b: horse ferrimyoglobin + cyanide at 20°, data of George and Hanania (1955b). Curve c: cytochrome *c* + cyanide at 24.6°, data of George and Tsou (1951).

mic pH profiles in the pH region of 6.0–7.0 (Figure 3) establishes the dependence of the second-order rate constant on the one-half power of hydrogen ion concentration. Furthermore, the data of George and Hanania (1955b) for the heme-linked effect in horse ferrimyoglobin and the data of George and Tsou (1951) for cytochrome *c* are strikingly consistent with a dependence on the one-half power of hydrogen ion concentration, as may be seen in Figure 5. In the absence of any other explanation, the following argument may be tentatively given.

The transmission of an electrostatic effect from a charged site of the globin moiety to the heme group is a function of spatial parameters and also of the dielectric constant of the medium. It may be argued that the charge effect is transmitted through the solvent or through the protein molecule. The two regions have different dielectric constants. The dielectric constant of the solvent will hardly be affected by a change in the hydrogen ion concentration in the pH range of 5–7. However, the intramolecular dielectric constant of the protein is a function of the polarity or hydrophobic character of the region within the protein molecule. Since the ionic state of the globin is a function of the pH of the solvent, it follows that the pH of the buffer will influence the charge transmission. It may be suggested, therefore, that the term $qH^{1/2}$ accounts for pH-dependent changes in the internal dielectric constant of the globin. Such an effect may be termed a *dielectric heme-linked effect*. It is distinct from the effect of changes in the dielectric constant of the medium, such as when organic solvents are added to the medium.

With the uncertainty in the meaning and magnitude of the parameters q_1 and q_2, it would be premature to try to comment in detail on the numerical values of k_1q_1 and k_2q_2 and their thermodynamic parameters, which are shown in Table II. However, it may be noted that the entropy changes are large, indicating that some kind of order–disorder phenomenon plays an important role in the pH effect on k_{F_1} and k_{F_2}. Possibly, the structuring of water molecules of hydration, which are associated with the myoglobin molecule, is involved in the dielectric effect. The enthalpy terms are small, while the values of k_1q_1 and k_2q_2 are nearing values of diffusion-controlled rate constants.

The fact that k_{F_1} is greater than k_{F_2} is to be expected, since ferrimyoglobin in the state of protonation corresponding to k_{F_1} has a more positive over-all charge than in the state of protonation corresponding to k_{F_2}. In the latter state, less attraction is exerted on the reacting cyanide ion in the transition state.

The rate of reaction of sperm whale ferrimyoglobin with cyanide is greater than the rate of reaction of horse ferrimyoglobin. This is consistent with the fact that the sperm whale ferrimyoglobin molecule is significantly more positively charged than that of horse, as evidenced by the chromatographic behavior of these myoglobins on CM-Sephadex C-50 cation exchanger (Awad and Kotite, 1966) and as may be expected from their amino acid compositions (sperm whale in Edmundson, 1965; horse in Akeson and Theorell, 1960).

References

Akeson, A., and Theorell, H. (1960), *Arch. Biochem. Biophys. 91*, 319.

Awad, E. S., Cameron, B. F., and Kotite, L. (1963), *Nature 198*, 1201.

Awad, E. S., and Kotite, L. (1966), *Biochem. J. 98*, 909.

Bates, R. G. (1964), Determination of pH, New York, N. Y., Wiley.

Blanck, J., Graf, W., and Scheler, W. (1961), *Acta Biol. Med. Germ. 7*, 323.

Clark, W. M. (1948), *Federation Proc. 7*, 499.

Diven, W., Goldsack, D. E., and Alberty, R. A. (1965), *J. Biol. Chem. 240*, 2437.

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

Edmundson, A. B. (1965), *Nature 205*, 883.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. I, New York, N. Y., Academic.

George, P., and Hanania, G. I. H. (1954a), *Nature 174*, 33.

George, P., and Hanania, G. I. H. (1954b), *Biochem. J. 56*, xxxvii.

George, P., and Hanania, G. I. H. (1954c), *Biochem. J. 56*, xxxviii.

George, P., and Hanania, G. I. H. (1955a), *Nature 175*, 1034.

George, P., and Hanania, G. I. H. (1955b), *Discussions Faraday Soc. 20*, 216.

George, P., and Tsou, C. L. (1951), *Biochem. J. 50*, 440.
Goldsack, D. E., Eberlein, W. S., and Alberty, R. A. (1965), *J. Biol. Chem. 240*, 4312.
Goldsack, D. E., Eberlein, W. S., and Alberty, R. A. (1966), *J. Biol. Chem. 241*, 2653.
Hanania, G. I. H., Yeghiayan, A., and Cameron, B. F. (1966), *Biochem. J. 98*, 189.
Handbook of Chemistry and Physics (1960), Hodgeman, C. D., Ed., 42nd ed, Cleveland, Ohio, Chemical Rubber, p 1755.
Izatt, R. M., Christensen, J. J., Pack, R. T., and Bench, R. (1962), *Inorg. Chem. 1*, 828.
Kauzmann, W. (1954), *Symp. Mech. Enzyme Action, Baltimore, Md., Johns Hopkins Univ.*, 70.
Riggs, A. (1965), *Physiol. Rev. 4*, 619.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964), *Advan. Protein Chem. 19*, 73.
Scheraga, H. A. (1961), Protein Structure, New York, N. Y., Academic.
Steinhardt, J. (1937), *Kgl. Danske Videnskab. Selskab Mat. Fys. Medd. 14*, 11.
Wyman, J. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 483.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.

Fluorometric and Spectrophotometric Study of Heme Binding on the Apoprotein from a Cytochrome b_2 Derivative*

Françoise Labeyrie, Ann di Franco, Motohiro Iwatsubo, and Alain Baudras

ABSTRACT: A derivative of cytochrome b_2 (L-lactate: cytochrome *c* oxidoreductase, EC 1.1.2.3) has previously been obtained by trypsic digestion of the enzyme; a similar product is found in the supernatant upon recrystallization of the same enzyme. Both are called noyau cytochromique b_2. It has been possible to separate the heme constituent from this protein and to describe some of the properties of the apoprotein. When added, heme is bound quantitatively to the apoprotein at neutrality; the reconstituted hemoprotein seems quite similar to the original one; tyrosine and tryptophan fluorescence are then 95% quenched. The ultraviolet spectrum allows the determination of tyrosine and tryptophan content. Quantum yields for tyrosine and tryptophan, calculated from the fluorescence emission spectrum and aromatic acid content, gave values of 0.030 and 0.025, respectively, for the apoprotein, the latter increasing to 0.21 when in the presence of 7 M urea.

These data, along with previous results, permit the discussion of the relative position of the heme and flavin prosthetic groups.

While the first spectroscopically observed reconstitutions of hemoglobin from hematin and globin date from the end of the last century, only recently similar data have been obtained for *b*-type cytochromes. These experiments were performed by Strittmatter (1960) on cytochrome b_5 from liver microsomes (mol wt 12,700), then by Shichi and Hackett (1962) for cytochrome b_{555} and b_{561} from mung bean seedlings (mol wt 12,000).

It is known that cytochrome b_2 from bakers' yeast (L-lactate:cytochrome *c*-oxidoreductase EC 1.1.2.3.) is a molecule having a molecular weight of order of 200,000 and containing one heme and one FMN[1] prosthetic group per 77,000*g* protein (Appleby and Morton, 1954; Appleby *et al.*, 1960). One of these prosthetic groups, the flavin, is easily removable as was shown independently by Morton (1961) and Baudras (1962); the resulting apoenzyme can be reactivated (up to 80%) by saturation with FMN and the reconstituted flavoenzyme was even crystallized (Baudras, 1965). As far as the heme prosthetic group of cytochrome b_2 is concerned, it has been impossible in spite of many efforts in Morton's (1961) laboratory and ours (M. Rippa, A. Baudras, and M. Iwatsubo, unpublished results) to obtain a separation without extensive denaturation of the protein moiety.

Recently, a hemoprotein derivative of low molecular weight, called noyau cytochromique b_2 "t," was prepared from cytochrome b_2 by trypsic hydrolysis. Oxidation–reduction and spectral investigations have shown that the heme–protein linkage is essentially the same in cytochrome b_2 and in this derivative obtained by elimination of 85% of the protein moiety (Labeyrie *et al.*, 1966). A hemoprotein very similar if not identical which is called noyau cytochromique b_2 "s," was found in the supernatant upon recrystallization of cytochrome b_2 (M. Iwatsubo and A. di Franco, unpublished results).

The experiments presented in this paper show that it is possible to remove the heme from both the noyau

* From the Service de Biophysique, Institut de Biologie Physicochimique, Paris, France. *Received March 27, 1967.*

[1] Abbreviation used: FMN, flavin mononucleotide.