Determination of Protein–Ligand Equilibria by Difference Spectroscopy. Hemerythrin–Ligand Thermodynamic Studies†

Eileen G. Gorman‡ and Dennis W. Darnall*

ABSTRACT: A difference spectrophotometric method for the rapid determination of equilibrium constants for protein–ligand interactions has been developed. The method requires no knowledge of the extinction coefficient of either reactants or products. Furthermore the method allows rapid determination of the temperature dependence of a reaction and thus leads to rapid determination of thermodynamic parameters. The method has been tested by following the interactions of ligands with hemerythrin, the nonheme iron, oxygen storage protein isolated from *Phasocolopsis gouldii*. The reactions were studied at various temperatures and ionic strengths, and standard thermodynamic parameters were determined. The standard thermodynamic parameters for the conversion of metaquohemerythrin to methydroxyhemerythrin were found to be $\Delta H^\circ = 5.8 \pm 1.3$ kcal mol^{-1} and $\Delta S^\circ = -11.5 \pm 1.5$ cal mol^{-1} deg^{-1}. For the reaction of metaquohemerythrin with thiocyanate ion to produce metthiocyanatohemerythrin $\Delta H^\circ = -13.0 \pm 1.6$ kcal mol^{-1} and $\Delta S^\circ = -25.3 \pm 5.5$ cal mol^{-1} deg^{-1}. For the reaction of thiocyanate ion with methydroxyhemerythrin $\Delta H^\circ = -6.6 \pm 0.8$ kcal mol^{-1} and $\Delta S^\circ = -38.3 \pm 4.0$ cal mol^{-1} deg^{-1}. Perchlorate ion decreases the affinity of metaquohemerhythrin for thiocyanate ion. This is reflected in both the entropy and enthalpy being more unfavorable for the reaction in the presence of perchlorate ion.

The determination of binding constants for protein–ligand interactions is the subject of many investigations. By use of such techniques as equilibrium dialysis, the amount of ligand bound per mole of protein is determined as a function of ligand concentration, and the data can be analyzed as suggested by Klotz (1946) or by Scatchard et al. (1949). Less time consuming methods for determining equilibrium constants which are based upon electronic absorption changes have also been developed. Most of these methods are variations of the Benesi & Hildebrand (1949) method; however, problems that can arise by use of these methods have been discussed by Person (1965) and Deranleau (1969), and, more recently, by Bergeron & Roberts (1978).

In this paper we describe a difference spectral method which can be used to study protein–ligand interactions. The method requires no knowledge of the extinction coefficient of either reactants or products. Furthermore, the method allows rapid determination of the temperature dependence of a reaction and thus leads to rapid determination of thermodynamic parameters. The method has been tested by following the interaction of ligands with hemerythrin.

Hemerythrin is a nonheme, iron-containing, oxygen-carrying protein isolated from marine worms. The native protein, molecular weight to 107 000, is an octamer composed of eight identical subunits each of which contains two irons. Oxidation of iron(II) to iron(III) results in the formation of methemerythrin. Methemerythrin reacts with a variety of ligands such as halides or psuedohalides with only one such ligand being coordinated between the two irons in each subunit (Klotz, 1971; Loehr & Loehr, 1979; Stenkamp & Jensen, 1979). Ions such as perchlorate or nitrate bind at a site on the protein which is different from the iron locus, but such binding nevertheless affects the reactivity of the iron site for other ligands (Darnall et al., 1968; Garbett et al., 1971a,b).

Although the reactions between methemerythrin and a variety of ligands have been studied previously (Klotz, 1971), with the exception of a report by Langerman & Sturtevant (1971), few thermodynamic parameters have been determined for the system.

Theory

Consider the equilibrium

$$P + L \rightleftarrows PL$$

where P represents a protein, L a ligand, and PL the protein–ligand complex. If there is a change in the spectroscopic properties of P or L upon complexation, then the equilibrium constant for the above reaction can be determined without knowledge of the extinction coefficients by using a difference spectral approach. Assume for the moment that there is a spectral difference between P and PL and that L does not contribute to the absorbance at a wavelength where P and PL absorb. If an experiment is designed such that both the 1-cm sample and reference cells contain equal concentrations of P but the sample cell contains in addition a certain concentration of L, then the ΔA observed at a given wavelength is given by

$$\Delta A = \epsilon_P[P] + \epsilon_{PL}[PL] - \epsilon_P[P_T] \quad (1)$$

where ϵ_P and ϵ_{PL} are the extinction coefficients of the free protein and complexed protein, respectively, and $[P_T]$ represents the total protein concentration in the reference. Since the total protein concentrations in the sample and reference cells are identical, eq 1 can be rearranged to

$$\Delta A = \Delta\epsilon[PL] \quad (2)$$

where $\Delta\epsilon = \epsilon_{PL} - \epsilon_P$, i.e., the difference between the extinction coefficients of the complexed and uncomplexed protein. If another difference absorbance measurement is now made at the same wavelength but under a slightly different protein and/or ligand concentration (but maintaining identical protein concentrations in both the sample and reference cells) another equation results

$$\Delta A' = \Delta\epsilon[PL]' \quad (3)$$

† From the Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003. *Received July 3, 1980.* This study was supported in part by U.S. Public Health Service Grant GM-22241 and Alpha Lambda Delta. Portions of this work were performed when D.W.D. was the recipient of U.S. Public Health Service Research Career Development Award GM 32014.

‡ Present address: Section of Protein Chemistry, Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20205.

0006-2960/81/0420-0038$01.00/0

where the superscript indicates the second set of conditions. If eq 2 is divided by eq 3, then we obtain

$$\frac{\Delta A}{\Delta A'} = \frac{[PL]}{[PL]'} \tag{4}$$

In addition, we have the two equilibrium expressions under the two conditions:

$$K = \frac{[PL]}{[P][L]} = \frac{[PL]}{[P_T - PL][L_T - PL]} \tag{5}$$

$$K = \frac{[PL]'}{[P]'[L]'} = \frac{[PL]'}{[P_T - PL]'[L_T - PL]'} \tag{6}$$

Equations 4–6 thus represent three simultaneous equations with three unknowns, K, [PL], and [PL]′, which can be solved. In theory, then, only two difference absorption measurements at a single wavelength are needed to determine the equilibrium constant, although the precision is increased by making several such measurements.

Once the equilibrium constant has been evaluated at one temperature, it is a simple matter to determine the temperature dependence of the reaction. If the extinction coefficients do not change with temperature, then

$$\frac{\Delta A_{Temp1}}{\Delta A_{Temp2}} = \frac{[PL]_{Temp1}}{[PL]_{Temp2}} \tag{7}$$

Thus, using a single set of solutions, it is possible to determine the thermodynamic parameters of the reaction.

Acid–Base Equilibria. Many proteins are involved in acid–base reactions that can be followed spectrophotometrically. For example, hemerythrin undergoes reaction 8 with

$$[HRFe_2^{III}(H_2O)_n]_8 \rightleftarrows [HRFe_2^{III}(OH)_n^{n-}]_8 + 8nH^+ \tag{8}$$

concomitant spectral changes. It may be difficult to study such equilibria, however, if the protein is unstable at the pH extremes required to obtain the complete titration curve. Using a difference spectral approach, it is possible to determine the stoichiometry of a reaction such as eq 8 and to determine the equilibrium constant for the reaction by changing the pH only a few tenths of a unit. Even though hemerythrin exists as an octamer under normal conditions, each dimeric iron center acts independently, and thus only one association constant describes the system. However, it is not clear how many protons are lost from each iron center upon conversion of the aquoform to the hydroxy form, and hence the number n is unknown. This type of formulation is not necessary where the stoichiometry of the reaction is known. Consider then the general reaction:

$$P(H_2O)_n \overset{K_a}{\rightleftharpoons} P(OH)_n^{n-} + nH^+ \tag{9}$$

An experiment is designed so that the sample and reference cells contain identical concentrations of protein, but the sample cell contains an equilibrium mixture of $P(H_2O)_n$ and $P(OH)_n^{n-}$, whereas the reference cell contains only $P(H_2O)_n$.[1] If 1-cm cells are used, the resulting difference spectrum at a given wavelength is

$$\Delta A = \Delta\epsilon[P(OH)_n^{n-}] \tag{10}$$

where again $\Delta\epsilon$ represents the difference between the extinction coefficients of the protonated and deprotanated forms. Since $[P(OH)_n^{n-}]$ can be expressed in terms of $[P_T]$ (the total protein concentration) and K_a (the equilibrium expression for eq 9), eq 10 can be rewritten as

$$\Delta A = \frac{\Delta\epsilon K_a}{[H^+]^n + K_a}[P_T] \tag{11}$$

Since the pH can be buffered, a plot of ΔA as a function of total protein concentration, $[P_T]$, will be linear, and the slope of such a plot can be determined. Two independent experiments as slightly different pH values then allow for the determination of K_a if n is known:

$$K_a = \frac{\text{slope}_1[H^+]_1^n - \text{slope}_2[H^+]_2^n}{\text{slope}_1 - \text{slope}_2} \tag{12}$$

Subscripts 1 and 2 refer to data obtained at the two slightly different pH values. If n is not known, then three independent experiments at slightly different pH values allow n to be determined in addition to K_a.

Competing Equilibria: Reaction of Protein with Two Different Ligands. Proteins such as hemerythrin exhibit an aquo–hydroxy equilibrium (eq 8), but, in addition, both the aquo and hydroxy forms are capable of reacting with another ligand such as thiocyanate. Difference spectroscopy can also be used to obtain the equilibrium constants for such reactions. The relevant equilibria can be expressed by

$$P(H_2O) \overset{K_a}{\rightleftharpoons} P(OH^-) + H^+ \tag{13}$$

$$P(H_2O) + L \overset{K_1}{\rightleftharpoons} PL + H_2O \tag{14}$$

$$P(OH^-) + L \overset{K_2}{\rightleftharpoons} PL + OH^- \tag{15}$$

If the experiment is designed such that the sample and reference beams contain protein solutions of identical concentration, but the sample beam contains ligand L, whereas the reference beam contains only $P(H_2O)$ and $P(OH^-)$ in equilibrium, then the difference absorbance at a particular wavelength can be expressed as (assuming free ligand L does not contribute to the absorbance)

$$\Delta A = \left(\epsilon_{PL} - \frac{\epsilon_{H_2O}}{1 + K_a/[H^+]} - \frac{\epsilon_{OH}}{1 + [H^+]/K_a}\right)[PL] \tag{16}$$

where ϵ_{H_2O}, ϵ_{OH}, and ϵ_{PL} are the molar extinction coefficients of $P(H_2O)$, $P(OH)$, and PL, respectively. K_a is the equilibrium constant of eq 13 and can be determined as outlined by using eq 10–12. If another experiment is designed with slighlty different total protein or ligand concentrations, another ΔA will be obtained. The ratio of the two ΔA's will then be equal to the ratio of the concentrations of PL under the two conditions, resulting in an equation analogous to eq 4. The equilibrium constant for the reaction of L with $P(H_2O)$ (eq 14) can be expressed as

$$K_1 = \frac{[PL]}{\frac{[P_T] - [PL]}{1 + K_a/[H^+]}([L_T] - [PL])} \tag{17}$$

where $[L_T]$ and $[P_T]$ represent the total ligand concentration and total protein concentration and K_a is the equilibrium constant from eq 12. Thus, K_1 can be evaluated in a manner analogous to that shown by eq 4–6. Once K_a and K_1 have been evaluated, K_2 can be evaluated from

[1] In the case of hemerythrin, the metaquo-form, $P(H_2O)_n$, can be obtained at pH values near 7 by the inclusion of perchlorate ion in the reference cell (Garbett et al., 1971a).

$$K_2 = K_1K_w/K_a \quad (18)$$

where K_w is the ion product of water.

Materials and Methods

Live *Phascolopsis gouldii* were obtained from the Marine Biological Laboratory at Woods Hole, MA. Dowex 1-X8, chloride (20–50 mesh) was obtained from Dow Chemical. Baker analyzed potassium thiocyanate, potassium ferricyanide, sodium hydrogen phosphate, and sodium dihydrogen phosphate were used without further purification.

Hemerythrin was isolated from the marine worm *P. gouldii* by using the procedure of Klotz et al. (1957). All studies were performed with methemerythrin, which was prepared from oxyhemerythrin by the following procedure. Oxyhemerythrin was dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris) sulfate buffer at pH 8.5. The two irons per subunit were oxidized by adding potassium ferricyanide in a 10% excess over the iron present in the protein. The solution containing protein and ferricyanide was allowed to stand at room temperature for 1 h and then in the refrigerator overnight. Excess ferricyanide and ferrocyanide were removed from the solution by anion exchange with Dowex 1-X8 in the chloride form, 20–50 mesh. The protein solution was then dialyzed against the desired buffer.

The methemerythrin prepared by this method was found to be >99% homogeneous by analytical polyacrylamide gel electrophoresis. Ultracentrifugation and spectroscopic comparison of absorption spectra with published values (Kereszteş-Nagy & Klotz, 1965) also verified protein purity.

Metaquohemerythrin–Methydroxyhemerythrin Equilibria. The 1-cm sample cell in a Cary 14 spectrophotometer contained an equilibrium mixture of metaquo- and methydroxyhemerythrin. The 1-cm reference cell contained an identical concentration of 100% metaquohemerythrin, due to the presence of 0.1 M sodium perchlorate. Perchlorate ion shifts the pK of the metaquohemerythrin–methydroxyhemerythrin equilibrium to such an extent that under these experimental conditions no methydroxyhemerythrin exists (Garbett et al., 1971a). Difference spectra were recorded from 420 to 300 nm. The difference peaks occur in the 355-nm region, where metaquohemerythrin (reference cell) absorbs strongly, and near 320 nm, where methydroxyhemerythrin (sample cell) absorbs strongly. Three different pH values were used (pH 7.2, 7.5, and 7.7) to determine the equilibrium constant. The pH was maintained with a sodium hydrogen phosphate and sodium dihydrogen phosphate buffer prepared at a constant pH and ionic strength by using Boyd's nomogram (Boyd, 1965). Although Garbett et al. (1971a) showed that phosphate may bind hemerythrin at the perchlorate binding site, at the concentrations used here there is no detectable binding of phosphate. The temperature dependence of the aquo–hydroxy reaction was determined between 10 and 35 °C as outlined under Theory. Each series of solutions was allowed to equilibrate for 1 h at each desired temperature. Studies showed all temperature effects were completely reversible. That the extinction coefficients were insensitive to temperature was also verified.

Metaquohemerythrin–Metthiocyanatohemerythrin Equilibria. The reaction of thiocyanate ion with metaquohemerythrin was studied in Na_2HPO_4–NaH_2PO_4 buffer at pH 6.85. The sample cell contained an equilibrium mixture of metaquo- and methydroxyhemerythrin to which standardized potassium thiocyanate was added. The reference cell contained protein of concentration identical with that of the sample cell, but no thiocyanate ion was added. Difference spectra were recorded from 500 to 340 nm. Difference absorption peaks occur in the 452-nm region where metthiocyanatohemerythrin absorbs and in the 360-nm region where the metaquo-methydroxyhemerythrin absorb strongly.

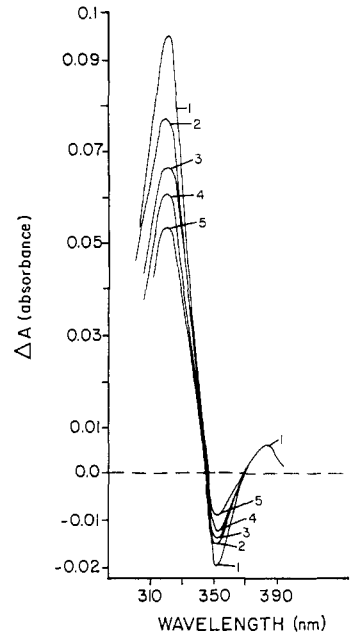

FIGURE 1: Differential absorption spectra between metaquo- and methydroxyhemerythrin and metaquohemerythrin at various hemerythrin concentrations. the 1.00-cm sample cell contained an equilibrium mixture of metaquo- and methydroxyhemerythrin, while the 1.00-cm reference cell contained metaquohemerythrin in 0.1 M sodium perchlorate. the spectra were obtained at pH 7.50, I = 0.20, in sodium hydrogen phosphate–sodium dihydrogen phosphate buffer. The total hemerythrin concentration was (1) 1.69×10^{-4} M, (2) 1.37×10^{-4} M, (3) 1.17×10^{-4} M (4) 1.05×10^{-4} M, and (5) 8.98×10^{-5} M.

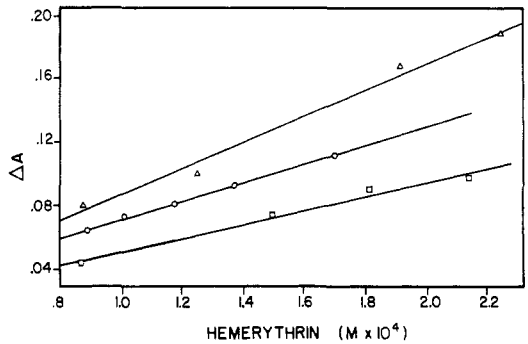

FIGURE 2: Plot of ΔA as a function of total hemerythrin concentration at an ionic strength of 0.20. solutions at pH 7.70 (Δ), 7.50 (○), and 7.20 (□) were prepared in sodium hydrogen phosphate–sodium dihydrogen phosphate buffers with an ionic strength of 0.20. For determination of ΔA, the 1.00-cm sample cell contained an equilibrium mixture of metaquo- and methydroxyhemerythrin. The 1.00-cm reference cell contained metaquohemerythrin in buffer with 0.1 M sodium perchlorate. The temperature was 25 °C.

Results

Metaquohemerythrin–Methydroxyhemerythrin Equilibria. Figure 1 shows the difference absorption spectra between an equilibrium mixture of metaquo- and methydroxyhemerythrin and metaquohemerythrin at pH 7.5 and an ionic strength of 0.20. Figure 2 shows plots (at different pH values) of ΔA (taken as the total difference in absorbance between 316 and 350 nm) as a function of protein concentration. From these data and by use of eq 11 and 12, the pK for eq 8 (calculated per monomer) is found to be 7.44 ± 0.15 at an ionic strength of 0.2. The value of n, the number of protons involved the equilibrium, was determined to be 1. Figure 3 shows the

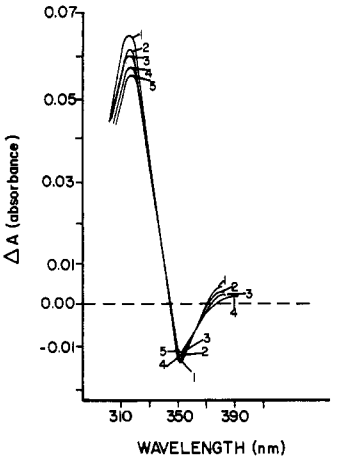

FIGURE 3: Temperature dependence of differential absorption spectra between metaquo- and metthiocyanatohemerythrin. The solutions at pH 7.50, ionic strength 0.20, and 1.05×10^{-4} M hemerythrin were equilibrated at five temperatures: (1) 29.5 °C; (2) 25.0 °C; (3) 22.0 °C; (4) 17.0 °C; (5) 12.7 °C. The 1.00-cm sample cell contained an equilibrium mixture of metaquo- and methydroxyhemerythrin while the 1.00-cm reference cell contained metaquohemerytrhin in the presence of 0.1 M sodium perchlorate. The buffer was sodium hydrogen phosphate–sodium dihydrogen phosphate.

Table I: Effect of Ionic Strength on Thermodynamic Parameters for Conversion of Metaquohemerythrin to Methydroxyhemerythrin[a]

I	pK	ΔG^b (kcal mol^{-1})	ΔH^c (kcal mol^{-1})	ΔS^d (cal mol^{-1} deg^{-1})
0.03	7.07 ± 0.08	9.6 ± 0.1	4.2 ± 0.2	−18.0 ± 0.7
0.05	7.15 ± 0.15	9.7 ± 0.2	3.0 ± 0.3	−22.5 ± 1.2
0.10	7.33 ± 0.15	10.0 ± 0.2	2.6 ± 0.4	−24.7 ± 3.0
0.20	7.44 ± 0.15	10.1 ± 0.2	1.0 ± 0.2	−30.5 ± 0.8

[a] Calculated per monomer. [b] Calculated from the standard deviation of mean of the equilibrium constant at 25 °C. [c] Calculated from the standard error of the slope of the van't Hoff plot. [d] Calculated from standard error of the intercept of van't Hoff plot.

temperature dependence of the reaction. Using eq 7, we calculated the equilibrium constant at various temperatures, constructed van't Hoff plots (which were linear), and determined the thermodynamic parameters for the reaction. In order to calculate standard thermodynamic terms, we studied the metaquohemerythrin–methydroxyhemerythrin equilibrium at various ionic strengths. The ionic strength was maintained with NaH_2PO_4–Na_2HPO_4 concentrations at a particular pH by using Boyd's nomogram (Boyd, 1964). This buffer system is especially well suited to temperature studies because of the absence of a temperature-dependent pH change. The results of the metaquohemerythrin–methydroxyhemerythrin equilibrium and thermodynamic studies are tabulated in Table I.

Metaquohemerythrin–Metthiocyanatohemerythrin and Methydroxyhemerythrin–Metthiocyanatohemerythrin Equilibria. The pertinent equations for the reaction of methemerythrin with thiocyanate ion are given by eq 19 and 20.

$$[HRFe_2^{III}H_2O]_8 + 8SCN^- \rightleftharpoons [HRFe_2^{III}SCN^-]_8 + 8H_2O \quad (19)$$

$$[HRFe_2^{III}OH^-]_8 + 8SCN^- \rightleftharpoons [HRFe_2^{III}SCN^-]_8 + 8OH^- \quad (20)$$

Figure 4 shows difference spectra that are obtained by the reaction of thiocyanate ion with methemerythrin. The data in Figure 4 along with the data in Table I were used to calculate the equilibrium constant for reaction 19 by using eq 16 and 17. Figure 5 shows the temperature dependence of the equilibrium. These data were then used to calculate the thermodynamic parameters. In this case, however, eq 7 could not be used directly to obtain the temperature dependence of the reaction, since ΔA is given by eq 16. Since K_a (the aquohemerythrin–hydroxyhemerythrin equilibrium constant) varies with temperature (Table I), the terms in brackets in eq 16 do not cancel at two different temperatures. Therefore, eq 16 was used directly to calculate [PL] at different temperatures by substituting with the known values of the extinction coefficients and K_a. Table II shows results of these studies and also shows the ionic strength dependence of these reactions.

Table II: Effect of Ionic Strength on Thermodynamic Parameters for Conversion of Metaquohemerythrin to Metthiocyanatohemerythrin[a]

I	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})
0.03	−5.9 ± 0.3	−15.7 ± 0.7	−34.5 ± 2.5
0.05	−5.9 ± 0.3	−17.1 ± 1.0	−38.0 ± 3.3
0.10	−5.9 ± 0.4	−16.6 ± 3.0	−35.2 ± 10
0.20	−6.1 ± 0.5	−18.8 ± 0.4	−43.2 ± 1.3

[a] Footnotes in Table I apply here as well.

Table III: Effect of Ionic Strength on Thermodynamic Parameters for Conversion to Methydroxyhemerythrin to Metthiocyanatohemerythrin

I	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})
0.03	4.3 ± 0.4	−6.3 ± 1.0	−35.5 ± 3.2
0.05	3.3 ± 0.5	−6.7 ± 1.3	−33.8 ± 4.2
0.10	2.7 ± 0.5	−5.6 ± 2.2	−27.9 ± 6.0
0.20	2.6 ± 0.6	−6.2 ± 0.6	−29.8 ± 2.1

Table IV: Effect of Perchlorate Ion on Thermodynamic Parameters for Conversion of Metaquohemerythrin to Metthiocyanatohemerythrin[a]

	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})
no perchlorate	−6.1 ± 0.5	−18.8 ± 0.4	−43.2 ± 1.3
0.1 M perchlorate	−4.1 ± 0.2	−9.4 ± 0.4	−17.5 ± 1.5

[a] Calculated per monomer at an ionic strength of 0.2. ΔG was calculated at 25 °C.

Once the metaquohemerythrin–methydroxyhemerythrin and the metaquohemerythrin–metthiocyanatohemerythrin equilibrium constants were determined, it was possible to determine the equilibrium constants for the methydroxy–metthiocyanate equilibrium using eq 18. The value of K_w at different ionic strengths was taken from Harned & Copson (1933). From the temperature dependence of the reaction, van't Hoff plots were constructed in order to obtain thermodynamic parameters. These are tabulated in Table III.

Effect of Perchlorate Ion on Metaquohemerythrin–Methiocyanatohemerythrin Equilibria. The effect of perchlorate ion on reactions of ligands at the iron center of hemerythrin has been studied previously (Darnall et al., 1968; Garbett et al., 1971a). Perchlorate ion decreases the affinity of metaquohemerythrin for thiocyanate by more than 1 order of magnitude. We have examined the effect of perchlorate ion on the thermodynamic parameters for the interaction of thiocyanate ion with metaquohemerythrin. Table IV shows results of these experiments and compares the results obtained for the interaction of thiocyanate with metaquohemerythrin

Table V: Standard Thermodynamic Parameters for Various Hemerythrin Reactions[a]

reaction	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal mol^{-1} deg^{-1})
$[HRFe_2^{III}(H_2O)]_8 \rightleftarrows [HRFe_2^{III}(OH)]_8 + 8H^+$	10.3	5.18 ± 1.3	−11.5 ± 1.5
$[HRFe_2^{III}(H_2O)]_8 + 8SCN^- \rightleftarrows [HRFe_2^{III}(SCN)]_8 + 8H_2O$	−5.5	−13.0 ± 1.6	−25.3 ± 5.5
$[HRFe_2^{III}(OH)]_8 + 8SCN \rightleftarrows [HRFe_2^{III}(SCN)]_8 + 8OH^-$	4.8	−6.6 ± 0.8	−38.3 ± 4.0

[a] Calculated per monomer and determined from extrapolation of plots of ΔH and ΔS as a function of the square root of ionic strength. ΔG° was calculated for 25 °C. Errors in ΔH° and ΔS° were calculated from the standard error of the plots.

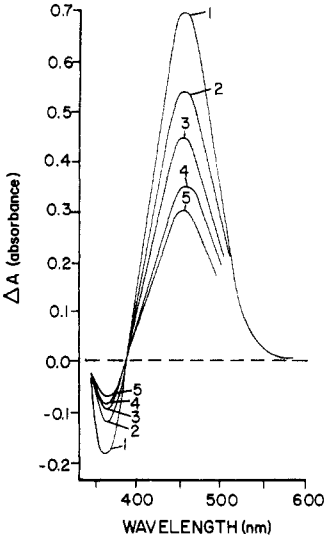

FIGURE 4: Differential absorption spectra between metaquo- and methydroxyhemerythrin and metthiocyanatohemerythrin at various thiocyanate and hemerythrin concentrations. the 1.00-cm sample cell contained an equilibrium mixture of metaquo- and methydroxyhemerythrin to which thiocyanate was added. The 1.00-cm reference cell contained an equilibrium mixture of metaquo- and methydroxyhemerythrin of total protein concentration equal to that in the sample cell. All solutions were prepared in sodium hydrogen phosphate–sodium dihydrogen phosphate buffer at pH 6.85, ionic strength 0.20, and temperature 25.0 °C. The total protein concentration in sample and reference and the total thiocyanate concentration in the sample cell were (1) 3.11×10^{-4} M; (2) 2.49×10^{-4} M; (3) 2.07×10^{-4} M; (4) 1.78×10^{-4} M; (5) 1.56×10^{-4} M.

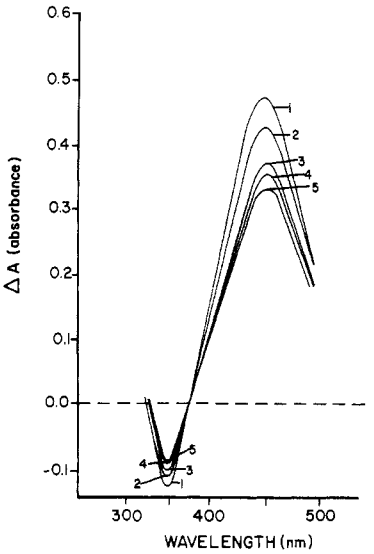

FIGURE 5: Temperature dependence of differential absorption spectra for the metaquohemerythrin–metthiocyanatohemerythrin equilibrium. The solutions at pH 6.85, an ionic strength of 0.20, and total hemerythrin concentration of 2.07×10^{-4} M were allowed to equilibrate at five temperatures: (1) 28.0 °C; (2) 25.0 °C; (3) 22.5 °C; (4) 16.5 °C; (5) 12.0 °C. The 1.00-cm sample cell contained an equilibrium mixture of metaquo- and metthiocyanatohemerythrin which was 1.99×10^{-4} M in thiocyanate. The 1.00-cm reference cell contained an equilibrim mixture of metaquo- and methydroxyhemerythrin. The buffer was sodium hydrogen phosphate–sodium dihydrogen phosphate.

in the absence of perchlorate ion.

Discussion

The reaction of methemerythrin with thiocyanate has been studied previously by using spectral methods (Garbett et al., 1971a,b), kinetic methods (Meloon & Wilkins, 1976), and calorimetric methods (Langerman & Sturtevant, 1971). However, many of these studies were carried out at pH values near 7.0 and thus represent the reaction between thiocyanate ion and an equilibrium mixture of metaquo- and methydroxyhemerythrin, since the metaquohemerythrin–methydroxyhemerythrin pK is 7.33 at an ionic strength of 0.1 (Table I). Meloon and Wilkins found an association constant of 1.2×10^4 (25 °C, ionic strength of 0.1) for this reaction at pH 6.3 where the reaction of thiocyanate is largely with metaquohemerythrin. This compares well with the value of 3.14×10^4 we found for the reaction of thiocyanate ion with pure metaquohemerythrin. Langerman & Sturtevant (1971), using calorimetric methods, determined the enthalpy of the reaction of thiocyanate with a mixture of metaquo- and methydroxyhemerythrin at pH 7 to be −14.4 kcal mol^{-1} subunit^{-1} which compares well with the value of −16.6 kcal mol^{-1} subunit^{-1} obtained for metaquohemerythrin at an ionic strength of 0.1. That we obtained a slightly more exothermic value for the enthalpy is easily explained by the presence of some methydroxyhemerythrin in the previous reaction measurements.

The difference spectral method outlined in eq 1–6 is especially well suited to reactions that have small equilibrium constants since it is often difficult to obtain the fully formed complex. The formation of the fully complexed species is necessary to obtain the extinction coefficients in order to determine the equilibrium constant. The method is also particularly well suited to reactions between proteins and ligands where it is desirable to maintain low concentration of ligand. This is the case for many metal–ligand interactions where higher complexes such as ML_2, ML_3, ..., ML_n can form as the ligand concentration increases. Also, in protein studies excess small ions or molecules may interact at secondary sites on the protein interfering with the reaction of importance. Thus, by working at lower ligand concentrations these problems can be alleviated.

Since the ratio of differences is required by the difference spectral technique, the method is sensitive to comparatively small experimental errors (on the order of 1–2%) in sample preparation. It is not always obvious that a single point is in error, and hence a plot of ΔA as a function of total protein concentration allows one to visually see if a particular point is in error. If the experimental data are of extremely good quality, the experimentally obtained ΔA's can be directly used to calculate a family of equilibrium constants. In some cases it may be desirable to fit the ΔA as a function of total protein concentration to a polynominal and use calculated rather than experimental values of ΔA to obtain a more internally con-

sistent set of equilibrium constants.

The presence of a sizable difference spectrum and accurately determined ΔA's is not the only limitation encountered in the application of the method. For determination of an equilibrium constant as developed in eq 1–6, the stoichiometry of the interaction must be known. This is in contrast to the method of Klotz (1946) or Scatchard (1949) which allows simultaneous determination of the number of ligands bound to a protein and an intrinsic equilibrium constant provided there is no interaction between subunits. However, in cases where 9–12 apply, as is the situation for the equilibrium between metaquo- and methydroxyhemerythrin, it is possible to determine the number of ligands simultaneously with equilibrium constant.

The method as described in eq 9–12 allows determination of acid–base equilibrium constants without pH titration. The use of pH titrations requires that the pH of a sample vary over several pH units in order to obtain end points. In dealing with proteins, this pH variation may denature the protein and may result in ionization of other amino acid side chains. The difference spectral method requires only a small change in pH (<1 pH unit) for the determination of an equilibrium constant. This small pH variation allows one to work in a pH region where the protein is stable and minimizes problems which may be encountered as a result of amino acid ionization.

The number of protons lost upon conversion of metaquohemerythrin to methydroxyhemerythrin was determined to be one. Garbett et al. (1971a) proposed that two protons are involved in this conversion even though only a single dissociation constant is available (Darnall et al., 1968; Garbett et al., 1971b). That two protons were released in the conversion of metaquohemerythrin to methydroxyhemerythrin was suggested from the slope of a Hill plot. The Hill plot reported by Garbett et al. (1971b) is nonlinear, indicating that K apparently changes with ligand concentration. Garbett et al. (1971b) state that the apparent equilibrium constant does not remain constant over the pH region studied but varies in a manner which reflects ionization of other groups close to the iron. The fact that the slope in the midregion of the graph is >1 (1.6) is more likely an indication of the effect of ionization of one group on another one in the protein (Cornish-Bowden & Koshland, 1975), rather than an indication that two protons are involved in the metaquo- to metthiocyanatohemerythrin conversion.

The data shown in Tables I–III were plotted as a function of the square root of ionic strength and were then extrapolated to zero ionic strength in order to obtain the standard thermodynamic parameters for the three hemerythrin reactions studied. These data are listed in Table V. It is interesting to note that in all three cases an unfavorable entropy term is observed. The effect of perchlorate ion (Table IV) is to change in opposing directions the enthalpy and entropy for the reaction of thiocyanate ion with metaquohemerythrin. X-ray studied have shown that there are two different binding sites for perchlorate in *Themiste dyscritum* hemerythrin (Stenkamp et al., 1978). Furthermore, binding of the perchlorate ions some 12 and 15 Å, respectively, from the iron centers produced structural changes at the iron center. This may then account for the remarkable changes in the thermodynamic parameters observed in the presence of perchlorate.

It is interesting to note that the free energy change for the conversion of metaquohemerythrin to either methydroxy- or metthiocyanatohemerythrin (Tables I and II) is relatively insensitive to changes in ionic strength. On the other hand, the enthalpy and entropy terms do change and compensate for one another. The enthalpy–entropy compensation phenomena which have been observed extensively in reactions of proteins and small molecules in water have been interpreted in terms of changes in hydration of those molecules (Lumry & Rajender, 1970; Imai, 1979). It may be that the changes in the enthalpy and entropy we observe are functions of ionic strength thus reflect changes in hydration of the protein.

References

Benesi, H. A., & Hildebrand, J. H. (1949) *J. Am. Chem. Soc. 71*, 2703.

Bergeron, K. J., & Roberts, W. R. (1978) *Anal. Biochem. 90*, 844.

Boyd, W. C. (1965) *J. Biol. Chem. 240*, 4097.

Cornish-Bowden, A., & Koshland, D. E., Jr. (1975) *J. Mol. Biol. 95*, 201.

Darnall, D. W., Garbett, K., & Klotz, I. M. (1968) *Biochem. Biophys. Res. Commun. 32*, 264.

Deranleau, D. A. (1969) *J. Am. Chem. Soc. 91*, 4044.

Garbett, K., Darnall, D. W., & Klotz, I. M. (1971a) *Arch. Biochem. Biophys. 142*, 455.

Garbett, K., Darnall, D. W., & Klotz, I. M. (1971b) *Arch. Biochem. Biophys. 142*, 471.

Harned, H. S., & Copson, H. R. (1933) *J. Am. Chem. Soc. 55*, 2206.

Imai, K. (1979) *J. Mol. Biol. 133*, 233.

Keresztes-Nagy, S., & Klotz, I. M. (1965) *Biochemistry 4*, 919.

Klotz, I. M. (1946) *Arch. Biochem. Biophys. 9*, 109.

Klotz, I. M. (1971) in *Subunits in Biological Systems* (Timasheff, S. N., & Fasman, G. D., Eds.) p 55, Marcel Dekker, New York.

Klotz, I. M., Klotz, T. A., & Fiess, H. A. (1957) *Arch. Biochem. Biophys. 68*, 284.

Langerman, N. R., & Sturtevant, J. M. (1971) *Biochemistry 10*, 2809.

Loehr, J. S., & Loehr, T. M. (1979) *Adv. Bioinorg. Chem. 1*, 235.

Lumry, R., & Rajender, S. (1970) *Biopolymers 9*, 1125.

Meloon, D. R., & Wilkins, R. G. (1976) *Biochemistry 15*, 1284.

Person, W. B. (1965) *J. Am. Chem. Soc. 87*, 167.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.

Stenkamp, R. E., & Jensen, L. H. (1979) *Adv. Bioinorg. Chem. 1*, 219.

Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 126*, 457.