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Calorimetric Studies of Quaternary Structure and Ligand-Binding Hemerythrin*

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ABSTRACT: The association behavior of the oxygen-binding protein, hemerythrin, isolated from the marine worm *Golfingia gouldii* has been studied by microcalorimetric techniques. A small positive enthalpy of association was observed at 25° and pH 7.0. The pH dependence of this enthalpy suggests that a group with a pK near 8 is involved in the stabilization of the quaternary structure. One possible candidate for this group is the side chain of cysteine. A small positive heat capacity change was also observed for the association reaction. The enthalpy of binding of oxygen to reduced deoxyhemerythrin was shown to be -9.2 ± 0.4 kcal mole^{-1} at 25° and pH 7.0. This value is compared to similar values for other oxygen-binding proteins. Binding heats are also reported for the formation of metazide- and metthiocyanatehemerythrin from metaquohemerythrin. The enthalpy of oxidation of reduced hemerythrin was determined by the application of Hess' law to a suitable series of reactions and found to be equal to 24.5 ± 2.3 kcal (mole of monomer)$^{-1}$. These various data are examined in the context of the known chemistry of this protein.

The nonheme, iron-containing, oxygen-transport protein, hemerythrin, isolated from the sipunculid worm, *Golfingia gouldii*, has been shown to consist of an octamer in chemical equilibrium with its monomers, the reaction being adequately described by

$$8\mathrm{Hr} \rightleftarrows \mathrm{Hr}_8 \quad K = (\mathrm{Hr}_8)/(\mathrm{Hr})^8 = 3.4 \times 10^{36}\ \mathrm{M}^{-7} \qquad (1)$$

at 25° and pH 7 (Langerman and Klotz, 1969). Application of the van't Hoff equation indicated that the enthalpy for this association is near zero. In order to obtain a better estimate of the heat for this reaction and to demonstrate the application of microcalorimetry to the study of quaternary structure we have carried out direct calorimetric measurements of reaction 1. We have also examined the temperature variation of the enthalpy to obtain an estimate of the magnitude of the heat capacity change upon association.

The native protein reversibly binds one molecule of oxygen per two (presumably reduced) iron atoms (Keresztes-Nagy and Klotz, 1965), as well as a wide variety of other ligands, with the same stoichiometry, when the irons are oxidized to the Fe(III) state. In order to obtain some insight into the thermodynamic characteristics of these binding reactions, we have also determined the enthalpy of binding of oxygen, azide, and thiocyanate, as well as the heat of oxidation of the irons. The values for the heat of oxygenation are especially interesting since comparison to similar data for the hemerythrin from *Sipunculus nudus* is possible (Bates *et al.*, 1968). This value can also be compared to values for human hemoglobin (Hill and Wolvekamp, 1936), sheep hemoglobin (Roughton *et al.*, 1955), and ox hemoglobin (Roughton *et al.*, 1936).

Materials and Methods

Oxyhemerythrin was prepared from the coelomic fluid of the marine worm *Golfingia gouldii* obtained from the Marine Biological Institute (Woods Hole, Mass.) by procedures previously described (Klotz *et al.*, 1957). The oxyprotein was oxidized to the Fe(III) form, methemerythrin, by the ferricyanide method described by Keresztes-Nagy and Klotz (1965) except that exhaustive dialysis was against Tris-cacodylate buffer (pH 7.0). Dialysis was continued until the spectrum of the sample was that of metaquohemerythrin. The octameric nature of the protein and the absence of any "crippled" monomer were routinely confirmed by sedimentation velocity experiments in the Spinco Model E ultracentrifuge prior to calorimetric experiments (Langerman and Klotz, 1969).

All buffers used in this work were prepared by adjusting an

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TABLE I: Heat of Dissociation of Metazidehemerythrin Measured in Various Buffers at 25 and 5°.

Buffer and pH[a]	Temp (°C)	No. of Expt	C_t (mg/ml)[d]	% [b]	Measured Heat (mcal)	$-\Delta H$ (kcal/mole)
Uncorrected for Effect of Buffer Heat of Ionization						
TS 7	25	5	3.85	65	0.73 ± 0.12	8.7[c]
TS 7	25	5	4.65	63	1.0 ± 0.10	9.8
TS 7	25	4	2.04	82	0.34 ± 0.21	7.3
TS 7	25	3	3.12	73	0.53 ± 0.15	8.8
P 7	25	3	2.04	82	0 ± 0.1	0
Corrected for Effect of Buffer Heat of Ionization						
TC 5.6	25	3	2.72	74	0.10 ± 0.08	1
TC 7.6	25	3	3.12	73	0.13 ± 0.05	0.7
TC 8.5	25	3	2.86	77	0 ± 0.09	0
TC 9.2	25	2	1.90	92	0 ± 0.14	0
B 9.2	25	2	1.90	92	0 ± 0.12	0
TC 7	5	3	1.36	98	0 ± 0.02	0
TS 7	5	3	4.3	65	0 ± 0.01	0

[a] Buffers and pH: TS 7 = 0.1 M Tris-sulfate (pH 7.0); P 7 = 0.1 M phosphate (pH 7.0); TC 5.6, TC 7, TC 7.6, TC 8.5, TC 9.2 = 0.1 M Tris adjusted to pH 5.6, 7.0, 7.6, 8.5, and 9.2, respectively, with solid cacodylic acid; B 9.2 = 0.1 M borate (pH 9.2). All buffers contained 10^{-3} M mercaptoethanol. [b] Per cent dissociated at final concentration. [c] The corrected value of the enthalpy based on these data is +0.7 kcal mole^{-1} when $n_H = 0.6$ is used as shown in Figure 3. [d] C_t is the initial concentration of protein.

0.1 M solution of the appropriate salt to the desired pH with solid cacodylic acid (Tris-cacodylate buffers) or concentrated sulfuric acid or sodium hydroxide, as required. In order to prevent oxidation of the single sulfhydryl group of the monomer, all buffers (except those used during oxidation experiments) contained about 10^{-3} M mercaptoethanol. Sodium azide was purified as previously described (Langerman and Klotz, 1969). Metazidehemerythrin was prepared by dialysis of metaquohemerythrin against the appropriate buffer which contained 0.05 M NaN_3.

Calorimetric measurements were performed on either the Model 190B microcalorimeter (Beckman Instruments, Inc., Palo Alto, Calif.) for "batch" experiments or the flow modification (Lyons and Sturtevant, 1969) of the 190B for "flow" experiments. All "batch" experiments, unless otherwise indicated, were performed using dimple cells with a dimple capacity of approximately 200 μl. Typically, 150 μl of sample or reference solution were placed in each of the two dimples of the cell. The reference solutions were chosen so as to compensate for heats of mixing or heats of dilution. Separate heats of dilution determinations on the species $[HrFe^{III}_2(N_3)\text{-}SSCy]_1$ showed the heat of dilution of the monomer to be negligible. Each cell had an outer volume of 15 ml.

In order to determine the heat of oxygenation of deoxyhemerythrin, techniques were developed to perform anerobic calorimetry. A Plexiglass glove box was built to enclose the flow system. The dimensions (length, width, and height) of the enclosure were 56 × 33 × 53 cm and it was designed in two sections such that the upper 46 cm could be removed without disturbing the flow system. The sealed enclosure was maintained with a positive pressure of nitrogen from which the last traces of oxygen were removed using a four station scrubbing system (Meites and Meites, 1948). The enclosure was purged for at least 2 hr prior to use. All manipulations were performed through two glove ports. A solution of oxygen-saturated buffer was maintained outside the enclosure by bubbling O_2. It was drawn directly into the gas-tight syringe of the flow system through a Hamilton two-way valve (Hamilton Co., Whittier, Calif.) inserted into a 22 needle which was sealed into the top of the enclosure. The syringe was emptied and refilled prior to every experiment.

The molar concentration of free dissolved oxygen was determined in a separate parallel experiment using a Clark-type polarographic electrode. The electrode was calibrated using the data of Linke and Seidell (1958).

The molar concentration of hemerythrin was determined using $\epsilon_{445} = 1850$ cm l. mole^{-1} (Keresztes-Nagy and Klotz, 1965) for metazidehemerythrin and the orthophenanthroline method of Massey (1957) for all other species. The molecular weight of the monomer used in all calculations was 1.36×10^4.

Reduced deoxyhemerythrin, $[HrFe_2(II)]_8$, was prepared in a nitrogen atmosphere using formamidinesulfinic acid (H. DePhillips, in preparation). Because reduction with this reagent would not proceed in Tris-cacodylate buffer (pH 7.0) (probably due to interference from the arsenic of cacodylic acid), the reduction was performed in Tris-sulfate (pH 7.0) and the buffer then changed to the former one by dialysis.

Results

Enthalpy of Association. The primary objective of this study was to determine the enthalpy for reaction 1 by measuring the heat associated with the reaction

$$8[HrFe^{III}_2(N_3)] \rightleftharpoons [HrFe^{III}_2(N_3)]_8 \qquad (2)$$

which has been shown (Langerman and Klotz, 1969) to be essentially identical with reaction 1. The reaction was carried out by dilution of a small volume of metazidehemerythrin into a large volume of dialysate.[1] The results are shown in Table I. The data in the upper portion of the table indicate that the observed heat of dissociation is dependent on the buffer used. This suggests that there is a change in protonation upon dissociation. From the relation

$$\Delta H_{app} = \Delta H_{dissocn} - n_H \Delta H_{ionizn} \qquad (3)$$

where ΔH_{app} is the measured enthalpy, $\Delta H_{dissocn}$ is the heat for reaction 2, n_H is the number of protons involved, and ΔH_{ionizn} is the heat of ionization[2] of the buffer, the number of protons liberated (or bound) may be estimated. Since ΔH_{ionizn} for Tris is +11.34 kcal mole^{-1} (Öjelund and Wadsö, 1968) and for phosphate is +1.13 kcal mole^{-1} (G. D. Watt, 1968, unpublished observations), it is apparent that somewhat less than one proton is involved. Thus, reaction 1 should properly be written as

[1] Complete dissociation was confirmed by sedimentation velocity experiments following the calorimetric experiment. The time required for complete dissociation (<45 min) was confirmed by observing the calorimetric response for a total of 3 hr.

[2] Other relevant heats of ionizations are: boric acid, +3.4 kcal mole^{-1} (Sober, 1968); cacodylic acid, −1.1 kcal mole^{-1}; Tris-cacodylate (pH 7), +5.36 kcal mole^{-1}; and Tris-cacodylate (pH 9.2), +10.3 kcal mole^{-1} (N. R. Langerman, 1969, unpublished data).

TABLE II: Heat of Association for Metaquo- and Methydroxyhemerythrin at 25°.[a]

	C_t (mg/ml)	No. of Expt	Measured Heat (mcal)	ΔH_{assocn} (kcal/mole)
$[HrFe^{(III)}_2(H_2O)]$, TS 7[b]	3.03	3	0.54 ± 0.15	1.0
$[HrFe^{(III)}_2(OH)]$, TC 9.2[b]	2.95	3	0 ± 0.2	0.0

[a] Data are corrected for heat of ionization of the buffer as described in the text. [b] See list of buffers in Table I.

$$8Hr + 8nH^+ \rightleftharpoons [Hr(H^+)_n]_8 \quad 0.3 < n < 0.9 \qquad (4)$$

at pH 7.0. When these data are used in eq 3, an average value of $n_H \simeq 0.6 \pm 0.3$ and an enthalpy of association of about 1 ± 0.5 kcal (mole of monomer)$^{-1}$ are obtained.

The data in the second portion of Table I demonstrate the effect of varying pH on the dissociation enthalpy of metazidehemerythrin. As can be seen, the enthalpy, corrected by the application of eq 3, drops to about zero at high pH. Also shown in Table I are data obtained at 5° and pH 7.0, which indicate a small positive heat capacity change upon association.

Previous work (Keresztes-Nagy and Klotz, 1965) has shown that in the absence of any other ligand, the iron atoms of hemerythrin will bind water (pH 7) or hydroxyl ions (pH 9). Langerman and Klotz (1969) have shown that the free energy of association for metaquo- and metazidehemerythrin are not significantly different, while the free energy for methydroxyhemerythrin is slightly more negative. In order to examine the thermodynamic parameters of these systems we have performed dissociation experiments on metaquo- and methydroxyhemerythrin. These results, reported in Table II, are consistent with the data observed for metazidehemerythrin.

Binding of Azide and Thiocyanate. Metaquohemerythrin is capable of binding a wide variety of ligands, L, according to general reaction 5 (Keresztes-Nagy and Klotz, 1965). Of

$$[HrFe^{III}_2(H_2O)_m]_8 + 8L \rightleftharpoons [HrFe^{III}_2(L)]_8 + 8mH_2O \qquad (5)$$

this group of ligands, the affinity of the protein for azide and thiocyanate is the largest. The enthalpy of binding for each of these ligands according to eq 5 is reported in Table III. The mean value of each of a series of measurements is, for L = N_3^-, $\Delta H = -15.2 \pm 1.5$ kcal (mole of ligand)$^{-1}$, and for L = SCN^-, $\Delta H = -14.4$ kcal ± 0.45 (mole of ligand)$^{-1}$. Unfortunately, no other estimates of these values (from van't Hoff measurements, for example) have been reported. Keresztes-Nagy and Klotz (1965) have reported values for the equilibrium constant for each of these cases under approximately the same conditions to be, for L = N_3^-, $K = 2.5 \times 10^4$ M^{-1}, and for L = SCN^-, $K = 4 \times 10^3$ M. That no proton was involved in reaction 5 at pH 7 was shown in a separate pH-Stat experiment.

Oxygenation. The apparent biological function of hemerythrin is oxygen binding. Using the anerobic enclosure described above, we were able to measure the heat of oxygenation at pH 7.0 and 9.2 and 25°, with fair precision. The oxygen binding reaction is

$$[HrFe_2^{II}]_8 + O_2 \rightleftharpoons [HrFe_2O_2]_8 \qquad (6)$$

TABLE III: Enthalpy of Binding of Azide and Thiocyanate to Metaquohemerythrin in Tris-cacodylate Buffer, pH 7.0 and 25°.

Hemerythrin Concn (mg/ml)	Ligand Concn [L], mM	Ratio[b] of Ligand Concn to Protein Concn	Measured Heat (mcal)	$-\Delta H$ (kcal/mole of Monomer)
		Binding of Azide		
2.99	0.61	2.8	47.3	14.4
1.44	1.25	11.8	17.9	10.4
2.99	3.03	13.8	55.9	16.9
2.99	6.06	27.5	47.5	14.5
2.45	6.06	33.7	52.7	19.8
			av	15.2 ± 1.5[a]
		Binding of Thiocyanate		
3.54	7.0	36.8	55.0	14.3
3.54	14.0	53.8	54.4	14.2
2.58	20.0	105.6	24.5	12.8
3.54	28.0	107.8	59.6	15.5
2.58	39.9	209.0	28.8	15.1
			av	14.4 ± 0.45[a]

[a] Standard error of the mean. [b] Ratio of initial ligand concentration to initial protein concentration.

Figure 1 summarizes the results of such oxygenation experiments for which complete data are shown in Table IV. While the involvement of protons was not established directly, the fair agreement of the data at the two pH values suggests that none are involved.[3] (The difference in the enthalpy of oxygenation between pH 7.0 and 9.2 is about 1 kcal, while the difference in the heat of ionization of the mixed buffer, Tris-cacodylate, at the same two pH values is about 10 kcal.) As shown in Figure 1, the data can be fit reasonably well by an enthalpy and a single equilibrium constant which suggests that there is no cooperativity between oxygen binding sites. This is in agreement with the observations of Bates *et al.* (1968) on *Sipunculid nudus* hemerythrin and Ferrelli and Kitto (1970) on *Dendrostomum pyroides* hemerythrin.

Darnall *et al.* (1968) have shown that a strong binding site for ClO_4^- and a few other anions exists and while the anion does not coordinate to the irons, its presence does affect the iron locus. In an attempt to determine if the presence of an anion at this site will affect oxygen binding, oxygenation experiments were performed in Tris-cacodylate buffer (pH 7.0) 25°, in the presence of 0.03 M $NaClO_4$. These data are shown in Table V and summarized graphically in Figure 1. The results indicate that the oxygen affinity of hemerythrin is decreased as is the enthalpy of binding.

Because of the approximately 3-kcal difference between the enthalpy of oxygenation in the absence and in the presence of perchlorate, experiments were attempted to determine if the source of this difference was a preferential binding of perchlorate to oxy- or deoxyhemerythrin. These data are shown in Figure 2 and summarized in Table VI. It appears that

[3] M. Okamura and I. M. Klotz (1970, unpublished data) have reached a similar conclusion based on attempts to measure the hydrogen ion release upon oxygenation directly.

TABLE IV: Oxygenation of Hemerythrin at pH 7.0 and 9.2 in 0.1 M Tris-cacodylate Buffer, 25°.

$[O_2]_{free}$ (mM)	[Hr] Monomer M	$-\Delta H$ (kcal/mole)	$[O_2]_{free}$ (mM)	[Hr] Monomer M	$-\Delta H$ (kcal/mole)
	pH 7.0			pH 9.2	
0.013	0.91×10^{-3}	0.31	0.026	0.28×10^{-3}	2.35
0.013	0.28	1.89	0.026	0.34	2.06
0.016	0.25	1.66	0.026	0.40	1.42
0.016	0.25	4.58	0.061	0.31	4.94
0.026	0.21	8.42	0.061	0.25	4.31
0.026	0.28	7.50	0.061	0.25	5.22
0.230	0.51	8.35	0.14	0.21	9.18
0.230	0.51	10.9	0.14	0.29	8.10
0.230	0.51	8.52	0 14	0.31	9.32
0.230	0.51	8.80	0.29	0.23	10.1
0.56	0.34	9.07	0.29	0.29	10.8
0.80	0.20	10.1	0.29	0.16	9.3
0.80	0.20	8.6	0.46	0.17	10.5
	av[a] 9.2 ± 0.4[b]			av[a] 10.2 ± 0.3[b]	

[a] Only data obtained at free oxygen concentrations greater than 0.2 mM were used. [b] Standard error of the mean.

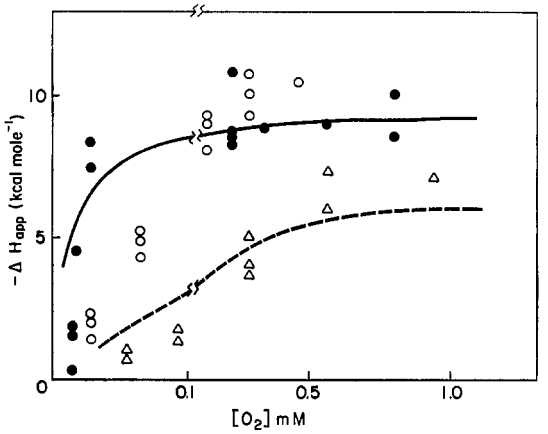


FIGURE 1: Enthalpy of binding of oxygen to hemerythrin at 25° in Tris-cacodylate buffer, pH 7.0 (●) and pH 9.2 (○). Similar data (△) at pH 7.0 in the presence of 0.03 M $NaClO_4$. The solid line was calculated using $\Delta H = -9.2$ kcal mole^{-1} and $K = 10^5$ M^{-1} and the dashed line using $\Delta H = -6.9$ kcal mole^{-1} and $K = 10^4$ M^{-1}. The scatter in the data in the presence of perchlorate precludes any interpretation of the significance of these values (other than the overall binding enthalpy) as well as any suggestion as to the need for more than one equilibrium constant to fit the data in the presence of perchlorate.

preferential binding does not completely explain the difference. It is apparent that the enthalpy of binding of ClO_4^- to deoxyhemerythrin is about 1 kcal more favorable than to oxyhemerythrin.

Oxidation of Hemerythrin. The conversion of hemerythrin from its Fe(II) into its Fe(III) form is easily and quantitatively carried out by ferricyanide. $Fe(CN)_6^{-3}$ is a useful oxidant to employ for the determination of the heat of oxidation since it allows the calculated enthalpy to be referred to the enthalpy of the standard hydrogen electrode, for which the

$$H^- + e^- \rightleftharpoons 0.5\ H_2$$

standard enthalpy is set equal by definition to zero at all values of temperature and pH (Watt and Sturtevant, 1969).

TABLE V: Oxygenation of Hemerythrin in 0.1 M Tris-cacodylate Buffer (pH 7), 25° in the Presence of 0.30 M $NaClO_4$.

$[O_2]$ (mM)	[Hr] Monomer M	$-\Delta H$ (kcal/mole)
0.052	1.49×10^{-3}	1.03
0.052	1.49	0.80
0.084	1.25	1.52
0.084	1.25	1.80
0.29	0.95	4.16
0.29	0.95	5.19
0.29	0.95	3.70
0.56	0.62	7.46
0.56	0.62	6.09
0.94	0.21	7.22
		av[a] 6.9 ± 0.4

[a] Only data obtained at free oxygen concentrations greater 0.3 mM were used in calculating the average value.

A determination of ΔH_{oxidn} for hemerythrin may be conveniently carried out by mixing a sample of oxyhemerythrin with potassium ferricyanide in the batch calorimeter using equipartition cells filled with 7 ml of each solution. The amount[4] of methemerythrin in the oxy sample was determined spectrally and subtracted from the total amount of protein present to determine the number of moles of monomer oxidized. A summary of the thermochemical calculations is given below.

The (reverse of the) measured reaction of the octamer expressed per monomeric unit is

$$[HrFe^{III}_2(H_2O)_m] + 2Fe(II) + O_2 \rightleftharpoons [HrFe_2O_2] + 2Fe(III) + mH_2O;\ \Delta H_7 \quad (7)$$

[4] After oxygenation and deoxygenation, 20–25% methemerythrin was usually found.

TABLE VI: Heat of Binding of ClO_4^- to Oxy- and Deoxyhemerythrin in 0.1 M Tris-cacodylate Buffer (pH 7.0), 25°.

$[ClO_4^-]$ (mM)	[Hr] Monomer M	$-\Delta H$ (kcal/mole)	$[ClO_4]$ (mM)	[Hr] Monomer M	$-\Delta H$ (kcal/mole)
	Deoxyhemerythrin			Oxyhemerythrin	
0.18	4.43×10^{-4}	1.57	0.33	5.91×10^{-4}	0.80
0.33	13.4	1.37	0.33	8.41	1.07
0.33	4.19	2.02	0.60	5.32	1.41
1.00	3.14	3.92	0.60	7.57	1.85
1.50	7.55	4.41	0.67	8.41	2.61
1.50	7.55	4.35	1.00	6.31	3.02
1.50	2.35	4.84	1.20	7.57	3.95
2.00	5.03	4.76	1.50	4.73	3.35
2.36	3.45	4.80	2.00	3.15	3.26
2.40	3.02	4.50	2.40	1.89	4.00
			5.30	1.05	3.18
	av[a] 4.5 ± 0.1			av[a] 3.5 ± 0.2	

[a] Average of $-\Delta H$ values for $[ClO_4^-]$ greater than or equal to 1.0×10^{-3} M.

where Fe(II) and Fe(III) refer to ferro- and ferricyanide, respectively. The ferro–ferricyanide reaction with respect to the hydrogen electrode is

$$Fe(III) + 0.5\ H_2 \rightleftharpoons Fe(II) + H^+;\ \Delta H_8 \qquad (8)$$

for which, at all pH values above 6, $\Delta H_8 = 26.7 \pm 0.03$ kcal/mole (Hanania *et al.*, 1967). The enthalpy of oxygenation under these conditions is given by eq 6 or, per monomer unit, as

$$[HrFe_2O_2] \rightleftharpoons [HrFe^{II}_2] + O_2;\ \Delta H_9 = -\Delta H_{oxy} = +9.2 \text{ kcal/mole} \qquad (9)$$

When eq 8 is multiplied by 2 (the number of irons oxidized per monomeric unit) and eq 7, 8, and 9 are added and referred to the standard hydrogen electrode, we obtain the reaction of interest

$$2e^- + [HrFe^{III}_2(H_2O)_m] \rightleftharpoons [HrFe^{II}_2] + mH_2O \qquad (10)$$

Thus, $\Delta H_{10} = -\Delta H_7 + 2\Delta H_8 + \Delta H_9$. Using the data given in Tables IV and VII, we find ΔH for reaction 10 is -24.5 ± 2.3 kcal/mole of monomer or $-12.2 + 1.7$ kcal/iron oxidized. This value may be compared to that obtained (Watt and Sturtevant, 1969) for the reduction of ferricytochrome *c* of -14.1 ± 0.2 kcal mole^{-1}.

TABLE VII: Heat of Reaction of $Fe(CN)_6^{-3}$ with Oxyhemerythrin in 0.1 M Tris-cacodylate Buffer (pH 7.0), 25°.

$[HrFe_2(O_2)]$[a] (M)	$[Fe(CN)_6^{-3}]$ (M)	Obsd Heat (mcal)	$-\Delta H_7$ (kcal/mole)
4.32×10^{-4}	5.31×10^{-3}	48.4	16.0
4.32	5.31	59.6	19.7
3.78	16.0	55.6	21.0
3.78	5.30	58.48	22.1
		av = 19.7×1.3[b]	

[a] Protein concentration is corrected for the amount of met present and expressed in units of moles of monomer per liter. [b] Standard error of the mean.

Discussion

While the number of proteins known to be composed of subunits is large (Klotz, 1967; Klotz and Darnall, 1970), those for which a model for the association and the related thermodynamic parameters have been deduced is quite limited (Klotz *et al.*, 1970). Hemerythrin is one such system. It also provides a good example of the utility of sedimentation equilibrium (Langerman and Klotz, 1969) and calorimetry in deducing information about the quaternary structure of proteins.

If the data in Table I are plotted as a function of pH, as in Figure 3, we are able to rationalize the pH dependence of the association indicated by reaction 4. From the association data of Langerman and Klotz (1969), we can deduce that the difference ΔG°_{assocn} (pH 7) $-\ \Delta G^\circ_{assocn}$ (pH 8.5) is somewhat less than 1 kcal. The calorimetric data shown in Figure 3 then indicate that there is little or no change in ΔS° between pH 7 and 9.

Another way to examine this relation between ΔG° and ΔH for association is to apply the relation[5]

$$\Delta G\ (\text{pH } 7) - \Delta G\ (\text{pH } 9) = 2.3RT \int_{\text{pH } 7}^{\text{pH } 9} n_H d(\text{pH}) \qquad (11)$$

(Lebowitz and Laskowski, 1962). Treatment of the data in Table I according to eq 3 gives values of n_H shown in Figure 3. Using these data as indicated by eq 11, we again find a difference in free energy of about 1 kcal.

The solid curve in Figure 3 suggests that a group with a pK near 8 is involved in the association reaction. One very likely candidate is the sulfhydryl group of cysteine (residue 50) (Klippenstein *et al.*, 1968). Physical and chemical evidence shows that if this group is modified, by blockage with a mer-

[5] While ΔG° and ΔS° are standard state quantities, a calorimetrically determined enthalpy becomes equal to the standard state enthalpy only in the limit of infinite dilution. The equality of these two enthalpies at the concentration used in this work is a good approximation.

TABLE VIII: Summary of Thermodynamic Parameters for Various Reactions for *G. gouldii* Hemerythrin.[a]

Reaction and Conditions	*K* Molar Scale	ΔG° (kcal/mole)	ΔH (kcal/mole)	ΔS° (eu)
$8Hr \rightleftharpoons Hr_8$; pH 7.0	3.4×10^{36} [b]	−5.8[b]	1 ± 0.5	23
$8Hr \rightleftharpoons Hr_8$; pH 8.5	$>3.4 \times 10^{36}$ [b]	<-5.8[b]	0	
$[HrFe^{(III)}{}_2(H_2O)_m + L \rightleftharpoons [HrFe^{(III)}{}_2(L)] + mH_2O$				
$L = N_3^-$	2.5×10^4 [c]	−5 98	−15.2 ± 1.5	−31.0
$L = SCN^-$	4.0×10^3 [c]	−4.76	−14.4 ± 0.5	−32.5
$[HrFe^{(II)}{}_2] + O_2 \rightleftharpoons [HrFe_2O_2]$				
pH 7.0			−9.2 ± 0.4	
pH 9.2			−10.2 ± 0.3	
pH 7 plus 0.03 M $NaClO_2$			−6.9 ± 0.4	
$[HrFe^{(II)}{}_2] + ClO_4^- \rightleftharpoons [HrFe^{(II)}{}_2(ClO_4^-)]$			−4.5 ± 0.1	
$[HrFe_2O_2] + ClO_4^- \rightleftharpoons [HrFe_2O_2(ClO_4^-)]$			−3.5 ± 0.2	
$[HrFe^{(III)}{}_2(H_2O)_m] + 2H^+ \rightleftharpoons [HrFe^{(II)}{}_2] + H_2 + mH_2O$			−24.5	

[a] All values are for the indicated reaction in Tris-cacodylate buffers at the indicated pH and 25°. [b] Langerman and Klotz (1969). [c] Keresztes-Nagy and Klotz (1965).

curial, for example, the octamer dissociates to monomers (Klotz and Keresztes-Nagy, 1963).

While the value for n_H has not been measured directly, we may infer an average value of about 0.6 ± 0.3 mole of proton/mole of monomer from three lines of evidence: (a) the observed behavior in buffers of different heats of ionization (Table I and Figure 3); (b) the agreement between the ultracentrifugal data of Langerman and Klotz (1969) and the result of the application of eq 11; and (c) the agreement of the calculated curve (Figure 3) with the experimental points when the assumed reaction is as written in eq 4.

As shown in Table VIII, the free energy and enthalpy data combine to give, at 25° a value for ΔS_{assoc} of about +23 eu/mole of monomer. This is in marked contrast to the roughly −8 eu expected (Kauzmann, 1959) from purely statistical arguments. The data in Table I indicate a heat capacity change of about +50 cal deg^{-1} (mole of monomer)$^{-1}$.

It is apparent that the thermodynamic driving force is primarily entropic. The source of this increased entropy, however, is not at all clear. Three important thermodynamic observations have been established concerning the association. (1) The enthalpy of association is small and positive. (2) The partial specific volume of the monomer and the octamer are essentially identical (Langerman and Klotz, 1969). (3) The heat capacity change upon association is small and positive.

The first observation suggests that some sort of hydrophobic bonds are involved in the association (Kauzmann, 1959); however, if this were all that was occurring, we would expect a volume change of about 20 ml/mole of aliphatic residue transferred from a nonaqueous to an aqueous environment and a negative ΔC_P. Thus, we must conclude that a simple molecular interpretation of the association is not feasible based on our present knowledge.

Oxygenation. Our results indicate that the binding of oxygen occurs with a release of about 9 kcal (mole of $HrFe_2O_2$)$^{-1}$ formed. This value agrees well with the value reported by Bates *et al.* (1968) of about −10 kcal/mole for the hemerythrin from *S. nudus*. The value reported for hemoglobin is about −7.5 kcal/mole (Hill and Wolvekamp, 1936), for sheep hemoglobin −10.9 kcal/mole (Roughton *et al.*, 1955), and for ox hemoglobin −9.45 kcal/mole (Roughton *et al.*, 1936). A lack of pH dependence of the $p_{1/2}$ data for the hemerythrins of both *G. gouldii* (H. DePhillips, in preparation) and *S. nudus* (Bates *et al.*, 1968) has been demonstrated. This is in agree-

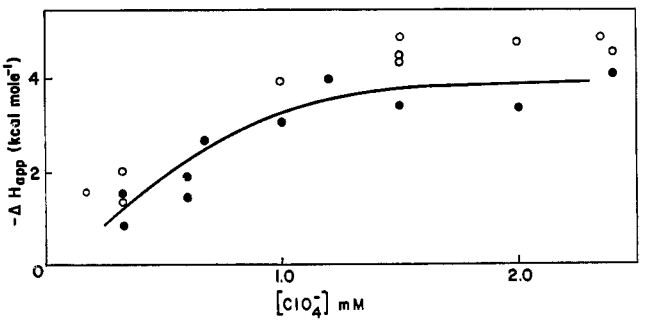


FIGURE 2: Enthalpy of binding of perchlorate to (●) oxy- and (○) deoxyhemerythrin. The data were obtained at pH 7.0 and 25° in Tris-cacodylate buffer. The solid line is the approximate line through the oxyhemerythrin data.

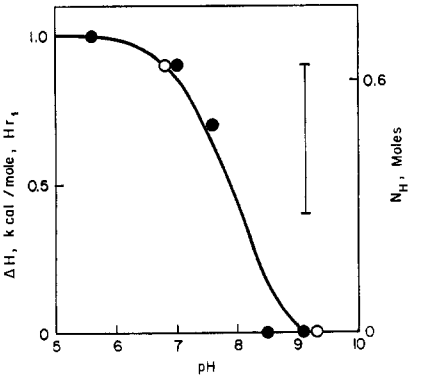
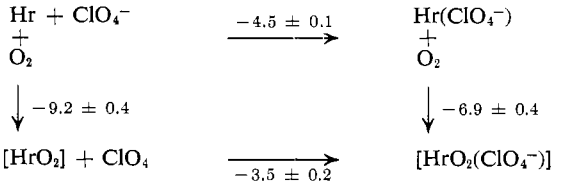


FIGURE 3: Enthalpy of association for hemerythrin at 25° as a function of pH. Data for variation of ΔH (left axis) and proton binding (right axis) are concurrent. The error estimate is the standard error of the mean for the pH 7.0 data. The solid line is calculated using $\Delta H = 1.0$ and $pK = 7.8$. (●) $[HrFeIII_2(N_3)]$; (○) $[HrFeIII_2(H_2O)]$ at pH 7.0 or $[HrFeIII_2(OH)]$ at pH 9.2.

ment with our observations of only a small pH dependence of the enthalpy data.

The source of the observed decrease in ΔH_{oxy} in the presence of ClO_4^- remains obscure. However, the work of Okamura *et al.* (1969) suggests a possible, though not conclusive, explanation. They have suggested that oxygen may bind as the peroxide, Fe(III)–O_2^{-2}–Fe(III). (This was also suggested by Klotz and Klotz, 1955.) Darnall *et al.* (1968) have suggested that the ClO_4^- binding site is close to the oxygen. If oxygen binds as the peroxide, then it is reasonable to expect a decreased oxygen affinity (manifested by a decreased enthalpy in the presence of the anion, perchlorate), as well as a reciprocal effect for the binding of perchlorate to oxyhemerythrin.

These relations are more easily visualized from the thermodynamic cycle



where the numbers quoted are the enthalpies for the indicated reactions. The path, Hr → $Hr(ClO_4)$ → $[HrO_2(ClO_4)]$, exhibits a total enthalpy change of -11.4 ± 0.5 kcal mole^{-1} while the alternate path, Hr → $[HrO_2]$ → $[HrO_2(ClO_4)]$, exhibits a total enthalpy change of -12.7 ± 0.6 kcal mole^{-1}. While the ranges of the values do not overlap, the average values are close enough to imply that the final state is independent of the path. The data certainly suggest that no gross irreversible changes occur along the two paths: however, the present data certainly do not rule out steric factors or changes in the iron–iron distance arising from the bound perchlorate.

Summary

Table VIII presents a summary of the thermodynamic parameters for the various reactions of octameric hemerythrin deduced from this and from previous reports.

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