Influence of Coordinating Ligands on Structure and Spectra of Hemerythrin*

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ABSTRACT: Changes in electrophoretic mobility of hemerythrin in different ionic solutions show that anions are bound to this protein. Large shifts in spectra on binding of ions point to the iron in the protein as the locus of attachment. All of the ions showing these effects, HS^-, N_3^-, SCN^-, CNO^-, Cl^-, F^-, and OH^-, are known to form coordination complexes with Fe(III). The main features of the spectra can be interpreted as due to charge transfer processes from the ligands to Fe(III), and point strongly to the presence of Fe—O—Fe bridges. Probable magnetic properties of the complexes are correlated with their spectra and behavior. Association constants have been evaluated for several of the complexes by spectrophotometric titration of aquomethemerythrin. The iron/ligand ratio was also determined in the case of the azide complex and found to be 2:1, just as in oxyhemerythrin. Complexes have also been obtained with the subunit form of the protein, merohemerythrin. The reaction of hemerythrin with mercaptan reagents, such as *N*-ethylmaleimide, has been found to be very slow in the absence of iron-coordinating ligands, but rapid in the presence of ions such as N_3^- or SCN^-. This cooperative interaction can be accounted for readily if it is assumed that undissociated hemerythrin is always in equilibrium with a very small amount of subunits. Ligand ions would shift this equilibrium toward the dissociated monomer. Such an equilibrium would also readily account for the all-or-none effect previously found in the dissociation of intact hemerythrin by mercurials, and hence may be the basis of similar behavior of other proteins. It is also possible, however, that all of these effects merely reflect changes in conformation of undissociated protein. Further experiments are needed to distinguish between these two mechanisms for cooperative interactions.

In earlier work we have established that hemerythrin, the iron-containing, oxygen-carrying (nonheme) protein of sipunculids, has a molecular weight of 107,000, and contains sixteen Fe atoms per molecule (Klotz and Keresztes-Nagy, 1963). In the deoxygenated state, the protein is colorless and the iron is in the Fe(II) state (Klotz and Klotz, 1955; Klotz *et al.*, 1957). The oxygenated violet-pink protein is obtained when one molecule of hemerythrin combines with eight molecules of O_2. The spectrum of oxyhemerythrin shows bands in the visible and near-ultraviolet regions which are absent in deoxyhemerythrin. On the basis of similarities in the spectra of oxy- and of methemerythrin (in which the iron is Fe[III]), as well as from specific color tests for iron, it has been argued (Klotz and Klotz, 1955; Klotz *et al.*, 1957) that the iron in oxyhemerythrin should also be classified as Fe(III). There have been, however, vigorous objections (Williams, 1955; Boeri and Ghiretti-Magaldi, 1957; Manwell, 1960) to this conclusion.

New studies of the electrophoretic behavior of hemerythrin have revealed that this protein combines with anions, and spectroscopic studies have shown that coordination occurs with the Fe(III) of methemerythrin. A detailed examination of the spectra and other properties of these complexes of Fe(III) methemerythrin with ions such as F^-, CN^-, CNO^-, SCN^-, N_3^-, and HS^- has therefore been undertaken. The results have provided some further insights into the state of iron in hemerythrin. They have also revealed the important role which complexing anions may play in determining the structure and behavior of this protein, particularly in controlling access to SH groups.

Experimental

Materials. Crystalline oxyhemerythrin and methemerythrin were prepared, by procedures described previously (Klotz *et al.*, 1957; Klotz and Keresztes-Nagy, 1963), from the coelomic fluid of the marine worm *Golfingia gouldii* (also known as *Phascolosoma gouldii*).

All salts used were of reagent grade. Other substances were the best grade available from general commercial sources.

Electrophoresis. On the basis of preliminary experiments with a variety of buffer salts, it became apparent that the highest isoelectric point would be obtained with Tris-cacodylate buffer. The isoelectric point of methemerythrin was lower in all other buffers, a behavior which indicates that anions are bound by the protein. Since the buffering range of cacodylic acid

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(*pK ca.* 6.2) is from about *p*H 5 to 7.5, and that of Tris (*pK ca.* 8.8 at 0°) from about *p*H 7.5 to 10, the combined buffer covers continuously almost the whole *p*H range of stability of hemerythrin. Therefore buffers of 0.05 ionic strength were prepared from these components. Protein was dissolved in buffer[1] to a concentration of 0.3%, and was then dialyzed overnight at 4° against the same buffer. The *p*H of the dialyzed protein solution was measured with a Beckman Zeromatic *p*H meter at 4°. Electrophoresis was then carried out in a Spinco Model H electrophoresis and diffusion apparatus at 1°. Schlieren patterns of both the ascending and descending limbs were photographed.

Preparation of Coordination Complexes. The aquo complex of methemerythrin, $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{H}_2\text{O})]_8$, was prepared as follows. Crystals of oxyhemerythrin, $[\overline{\text{Hr}}\text{Fe}_2(\text{O}_2)]_8$, were dissolved in 0.1 M NaClO_4, and the oxygen was removed by treatment with ferricyanide until the protein solution changed in color from violet-pink to yellow. This solution was then dialyzed exhaustively against 0.1 M NaClO_4 to remove all anions other than perchlorate. Since ClO_4^- ions show little if any tendency to coordinate with metal ions, the open coordination sites of iron in methemerythrin should now be filled by H_2O or OH^- groups, and the resultant complex will be designated as $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{H}_2\text{O})]_8$. The spectrum of the aquo protein complex so prepared was not affected by added carbonate, acetate, sulfate, or cacodylate ions, or by Tris. Thus buffers containing these constituents were also used to prepare the aquo complex at a definite *p*H. A particularly rapid procedure for preparing the aquo complex is to remove the oxygen from oxyhemerythrin by addition of ferricyanide in the presence of Cl^- ions. Less ferricyanide is required under these conditions because the Cl^- complex of methemerythrin, $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{Cl}^-)]_8$, is formed quickly. This complex can be converted easily into the aquo form by dialysis against 0.1 M NaClO_4 or any of the other noncomplexing constituents listed.

The aquo complex of methemerythrin is unstable above *p*H 10 and tends to become insoluble below *p*H 7.5. Nevertheless it will not precipitate or crystallize from solutions of 0.1–1% concentration if the *p*H is lowered slowly by dialysis. Thus the spectrum of the fully aquo form $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{H}_2\text{O})]_8$ was obtained readily even at *p*H 6. On the other hand, the absorption spectrum of pure $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{OH}^-)]_8$ (see Figure 1) could only be approximated, because at *p*H values near 11 the protein cannot be maintained unchanged.

The F^-, Cl^-, CN^-, CNO^-, SCN^-, and N_3^- complexes of methemerythrin, $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{L}^-)]_8$, are formed almost instantaneously, as judged by the conversion of the aquo spectrum to a new one, when either potassium or sodium salts of these ions are added to a solution of the aquo complex. These anion complexes can also be prepared directly from oxyhemerythrin by addition of ferricyanide in the presence of the particular anion whose complex is desired. In fact ferricyanide is not actually necessary if high concentrations of anion are used. For example, the violet-pink color of oxyhemerythrin is changed to pale yellow within minutes if 1 M F^- is added to the solution. With the other anions, except Cl^-, overnight dialysis of oxyhemerythrin versus 0.1 M solutions[2] of the particular ion is sufficient to bring about complete conversion to $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{L}^-)]_8$. Chloride ion is least effective in this respect, for oxyhemerythrin in 0.1 M Cl^- maintains its color for days at room temperature, and for weeks at 4°. For the other anions, however, the overnight dialysis procedure was the technique used to prepare the solutions required for spectroscopic studies. With F^-, N_3^-, and SCN^-, unbuffered solutions were used for dialysis, and with CN^- and CNO^- the solution was adjusted to *p*H 9 with Tris and perchloric acid. The Cl^- complex was prepared with the assistance of added ferricyanide as described.

The interrelationships between these complexes and various other states of hemerythrin may be visualized readily by examination of the chart in Figure 1.

Spectrophotometric Methods. The general shapes of the spectra of various complexes were obtained with the Beckman Model DK-1 recording spectrophotometer. For the calculation of molecular extinction coefficients, precise optical densities were read in a Zeiss PMQ II spectrophotometer. Extinction coefficients were calculated both in terms of protein concentration (in g/liter), and of total iron concentration (in moles/liter).

Results

Electrophoretic Mobilities. The variation of mobility with *p*H is summarized in Figure 2. The original objective of these experiments was to test for the presence of a Bohr effect by looking for a shift in isoelectric point upon oxygenation. As the data in Figure 2 show, there is no such shift, and hence the absence of a Bohr effect in hemerythrin of *Golfingia* (Manwell, 1958) is verified. Furthermore the mobilities of deoxy- and oxyhemerythrin are indistinguishable at small distances away from the isoelectric *p*H.

In contrast the slope of the mobility line for aquomethemerythrin is lower than that for oxyhemerythrin. Despite this difference there is no significant change in isoelectric point when oxy- is converted to methemerythrin.

Of most interest in the context of this paper is the large shift, toward lower *p*H, in isoelectric point of

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[1] For experiments with deoxyhemerythrin, 0.01 M sodium hydrosulfite was added to the buffer to reduce oxyhemerythrin and to protect the reduced protein against oxygenation by air while the cells were filled. At the end of the electrophoresis experiment the reduced protein was removed from the cell and reoxygenated, and the electrophoretic mobility measured again. In this way mobilities of oxy- and deoxyhemerythrin were compared with exactly the same samples of protein.

[2] With CN^- only 0.05 M solutions were used because higher concentrations extract Fe^{III} from the protein and form ferricyanide.

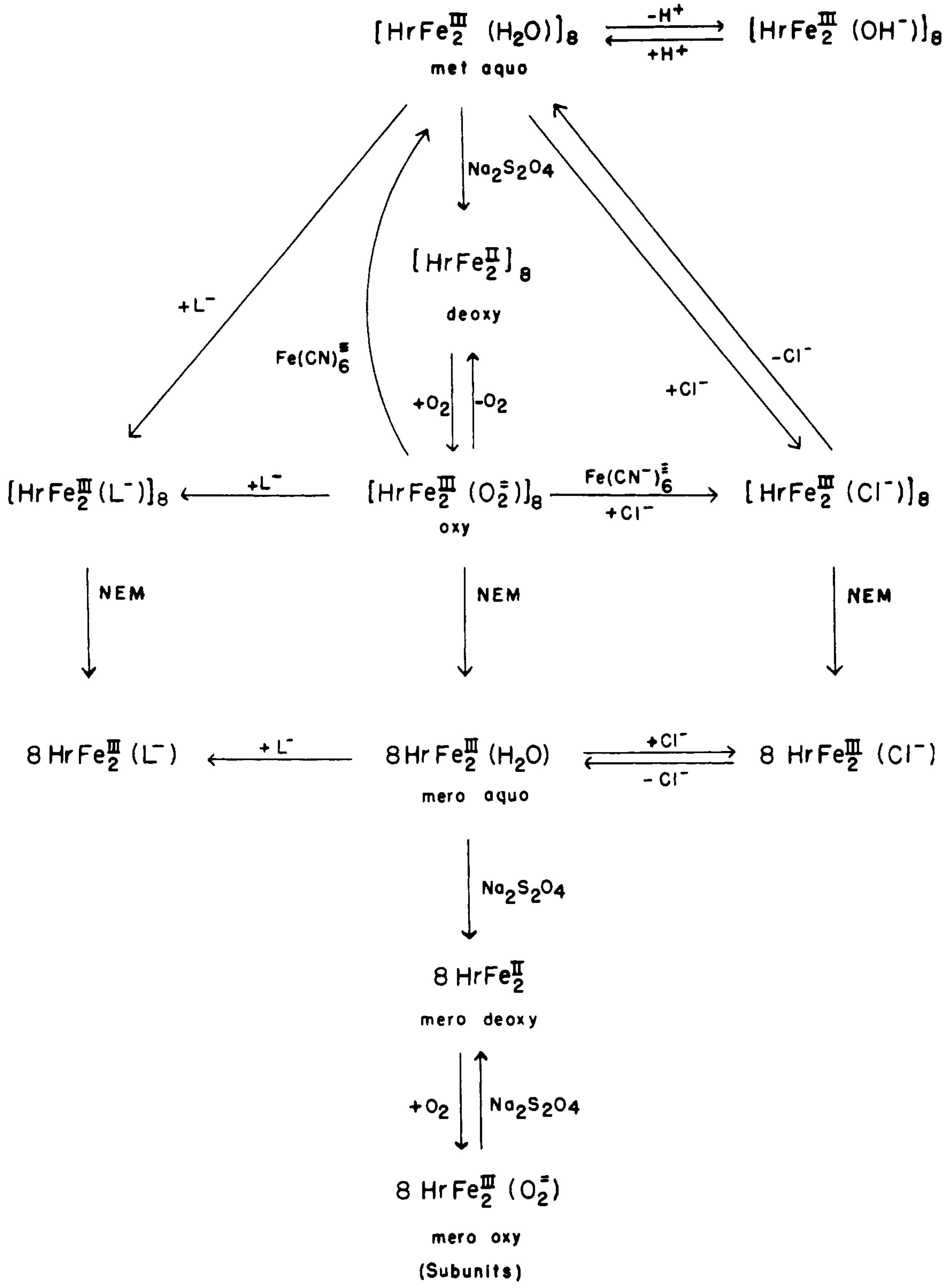

FIGURE 1: Relationships between different forms of hemerythrin. L represents the anionic ligands such as N_3^-, SCN^-, CNO^-, F^-. NEM is *N*-ethylmaleimide, which blocks sulfhydryl groups. Merohemerythrin designates the subunit containing one oxygen-binding site.

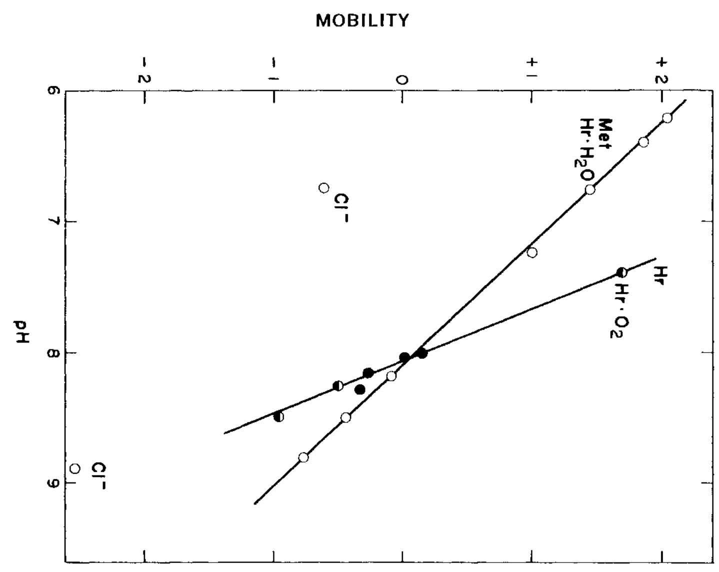

FIGURE 2: Electrophoretic mobilities at different pH values in buffer of Tris and cacodylic acid. ●, deoxyhemerythrin; ◐, oxyhemerythrin; ○, methemerythrin; Cl⁻○, methemerythrin, with 0.05 M Cl⁻ in buffer in place of cacodylate.

methemerythrin in the presence of chloride ions.[3] Such behavior is a clear indication of the binding of anions by this protein. This binding could be, in principle, either specifically to the iron atoms or nonspecifically to various side chains of the protein. The marked changes in absorption spectra, to be described herein, show however that binding occurs at the Fe sites.

Spectra of Undissociated Hemerythrin. Reproductions of the absorption spectra of a series of coordination complexes of methemerythrin are shown in Figures 3 and 4. The main features of the spectra of the complexes with anions are essentially independent of pH, in the range of stability of the protein. The aquo complex, however, changes with increasing pH as it is converted to the OH^- complex (Figure 3). A similar although small change also appears in the spectrum of the oxygenated protein (Figure 5) at the extremes of its pH stability range. The shoulder near 360 mμ in the neutral pH region is reduced to a slight inflection at pH 5 but develops into an almost discrete absorption peak above pH 10.

The extinction coefficients for these complexes are assembled in Table I. In general there are four regions in which peaks appear: 330, 360, 500, and 650

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[3] It is of interest to note that the isoelectric pH of 5.8 reported in the literature (Roche, 1933) was measured in solutions containing 1% NaCl.

TABLE I: Absorption Peaks and Extinction Coefficients of Hemerythrin Complexes.

Ligand	λ_{max} (mμ)	ε, cm liter/g	ε, cm liter/mole	λ_{max} (mμ)	ε, cm liter/g	ε, cm liter/mole	λ_{max} (mμ)	ε, cm liter/g	ε, cm liter/mole	λ_{max} (mμ)	ε, cm liter/g	ε, cm liter/mole
(O_2, O_2^-, O_2^{2-})	(~750)	~0.014	~100	500	0.160	1100	(~360)	0.395	2725	330	0.495	3400
N_3^-	680	~0.014	~100	445	0.270	1850	(~380)	(~0.32)	(~2100)	328	0.50	3450
SCN^-	675	~0.014	~100	452	0.370	2550	(~368)	(~0.350)	(~2400)	330	0.500	3450
Cl^-	650	~0.014	~100	(~485)	(~0.050)	(~360)	379	0.440	3040	330	0.500	3450
CNO^-	655	~0.014	~100	(~490)	(~0.060)	(~400)	377	0.51	3520	334	0.51	3520
CN^-	690	~0.014	~100	495	0.058	400	375	0.412	2840	332	0.495	3400
F^-	(~590)	~0.014	~100	(~480)	(~0.028)	(~200)	362	0.365	2516	317	0.405	2790
OH^-	(~570)	~0.014	~100	(~480)	(~0.044)	(~300)	362	0.46	3170	~317	~0.55	~3780
H_2O	(~580)	~0.014	~100	(~490)	0.040	(~275)	355	0.565	3220			

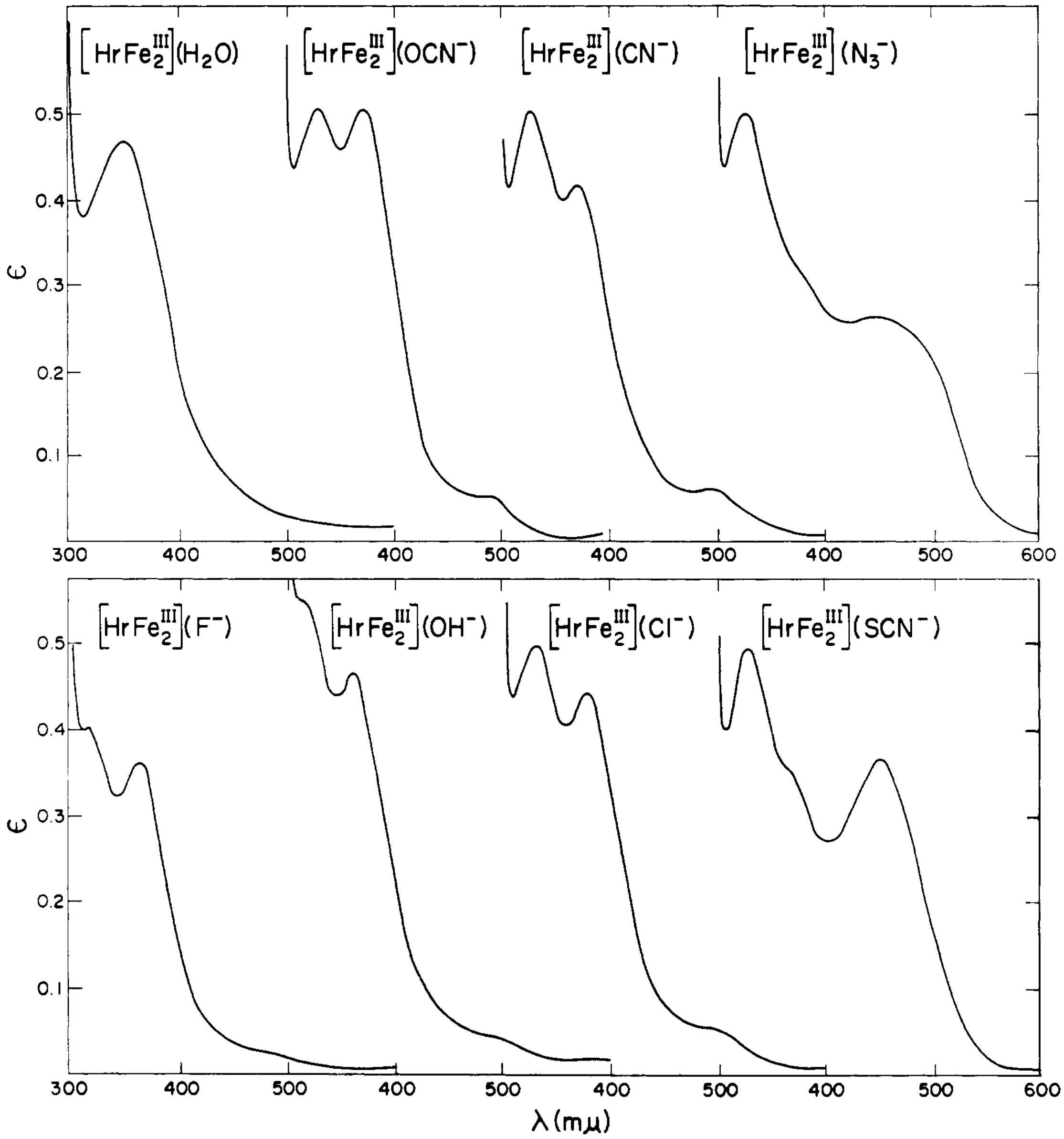


FIGURE 3: Absorption spectra of coordination complexes of methemerythrin. The extinction coefficients given are in terms of protein concentration, in grams/liter.

mμ. For purposes of comparison it should be mentioned that deoxyhemerythrin is colorless, and shows no absorption between 300 and 1000 mμ.

Spectra were also examined for coordination complexes of Fe(III) of methemerythrin with inorganic sulfides such as Na_2S or H_2S, and with organic mercaptans such as cysteine or cysteine ethyl ester. The two types of sulfur behave differently, however, when added to aquomethemerythrin. The organic mercaptans reduce the iron in the protein and turn it colorless. Evidently Fe(II) deoxyhemerythrin is formed, for if the solution is shaken with air it becomes pink temporarily, and then fades. Repeated admission of air again produces a pink color which in turn slowly fades. It would seem, therefore, that RS^- is being oxidized to RSSR by the Fe in hemerythrin, as it can be by inorganic iron (Lamfrom and Nielsen, 1957).

Very different behavior is observed with the inorganic sulfides. If H_2S is bubbled through an anaerobic solution of aquomethemerythrin in a buffer (Tris-perchlorate) at *p*H 8, a pink color is obtained directly, without any preliminary fading of the yellow of methemerythrin. The intensity of this color is comparable to that of oxyhemerythrin. A spectrum of the (bisulfide or) sulfide complex of methemerythrin is shown in Figure 6. The position of the peak at 510 mμ is close to that (500 mμ) for oxyhemerythrin.

To verify that the sulfur complex was with Fe(III) and not Fe(II), aquomethemerythrin was first reduced with sodium hydrosulfite and then exposed to H_2S

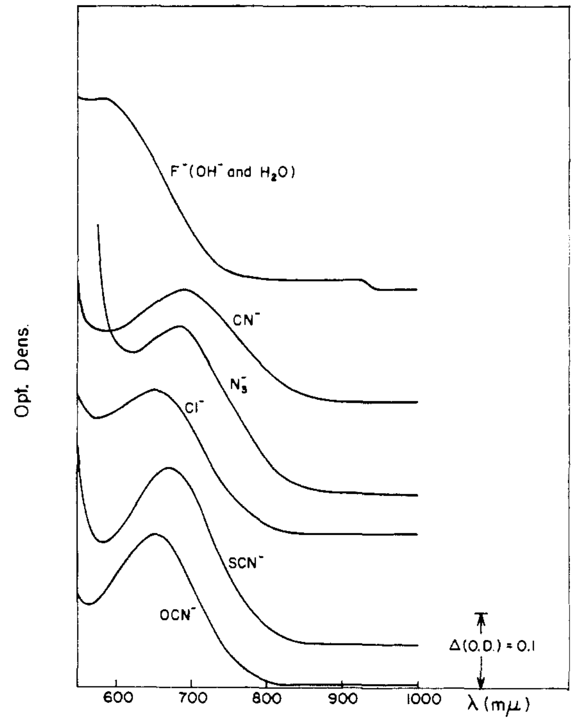


FIGURE 4: Absorption spectra of coordination complexes of methemerythrin in the region of d-d transitions. Ordinate axis gives optical densities. Curves have been displaced vertically so that they could all be presented together. Scale of Δ (optical density) shown by arrow in lower right corner.

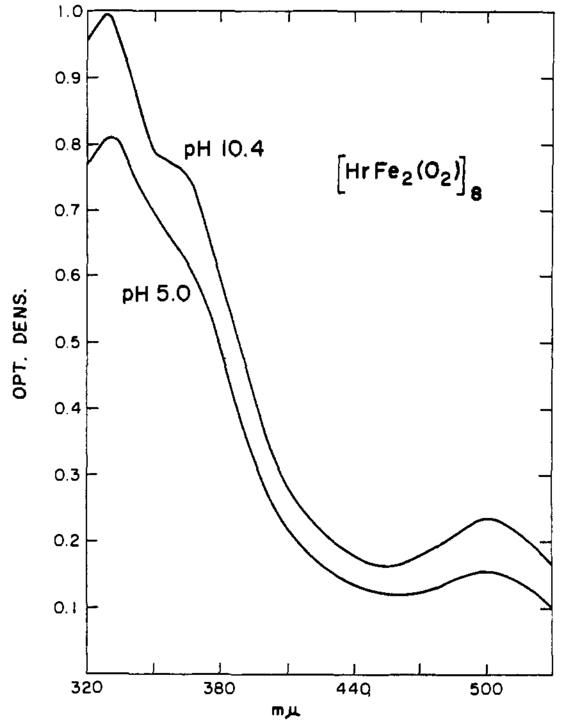


FIGURE 5: Absorption spectra of oxyhemerythrin at *p*H 5.0 and 10.4.

gas under anaerobic conditions. No color developed. In another experiment, oxygen from oxyhemerythrin was removed, by a stream of nitrogen, until 90% of the protein was in the deoxy form. Admission of H_2S at this point produced no increase in absorption in the visible region. That the protein was nevertheless still fully active was shown by the restoration of absorption at 500 mμ as soon as O_2 was admitted.

Efforts to prepare a peroxide complex with Fe(III) in methemerythrin have uniformly failed. It may be pertinent in this regard that pK_1 for H_2O_2 is about 12 (Pearson and Edgington, 1962), whereas that for H_2S is near 7. Thus HS^- ion is readily available for complexation, whereas very little HO_2^-, and very much less O_2^{2-}, can be generated at *p*H values at which hemerythrin is stable.

Spectra of Subunits. With the exception of oxyhemerythrin, dissociation of hemerythrin does not change the spectra of the complexes (Keresztes-Nagy and Klotz, 1963). The exceptional behavior of oxyhemerythrin is not surprising, for in this case (see chart in Figure 1) dissociation (by addition of *N*-ethylmaleimide) is accompanied by a marked change in the nature of the ligands coordinated with the Fe; in buffer containing Tris and perchlorate, the aquo complex $\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{H}_2\text{O})$ is formed; in the presence of Cl^- ions, $\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{Cl}^-)$ is formed. (It is also possible to transform the latter into the aquo complex by dialyzing out the Cl^- in the presence of perchlorate [see Figure 1].)

Aquomerohemerythrin, $\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{H}_2\text{O})$, is very unstable compared to undissociated hemerythrin, and it loses its iron easily. On standing, the protein precipitates even in the cold, and even at *p*H values (8–9) normally very favorable to stability of hemerythrin. Below *p*H 7, the precipitation is so fast that the aquo form cannot be obtained. The iron is removed much more readily from the subunits than from undissociated protein by Tiron (disodium salt of 4,5-dihydroxy-*m*-benzenedisulfonic acid), *o*-phenanthroline, or sodium dodecylsulfate. Merohemerythrin subunits are greatly stabilized, however, upon coordinating with anionic ligands, particularly azide. An exception in this regard is CN^- which removes Fe at a lower concentration than is required to abstract this metal from the undissociated protein.

It has now also been found possible to prepare the oxy form of merohemerythrin subunits and to obtain its spectrum. Oxyhemerythrin in 0.1 M $(NH_4)_2CO_3$ solution was dissociated by the addition of *N*-ethylmaleimide approximately to the point where it was stoichiometric with the sulfhydryl groups of the protein. The aquo subunit was then dialyzed against 10^{-4} M sodium hydrosulfite in 0.1 M $NaClO_4$ until the characteristic yellow color of $\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{H}_2\text{O})$ had disappeared. Thereafter, oxygenation of the subunit to produce oxymerohemerythrin, $\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(\text{O}_2^{2-})$, was effected by dialyzing the reduced protein, $\overline{\text{Hr}}\text{Fe}_2^{\text{II}}$-

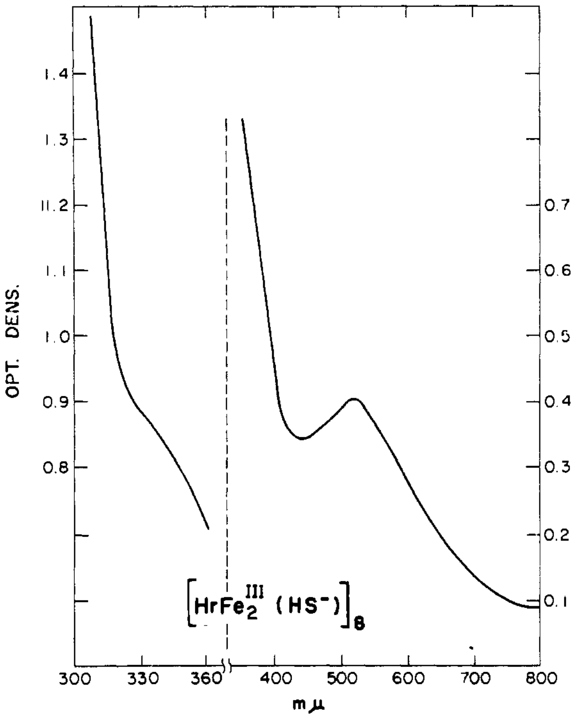


FIGURE 6: Absorption spectrum of complex of methemerythrin with sulfide ion, HS^- (or S^{2-}).

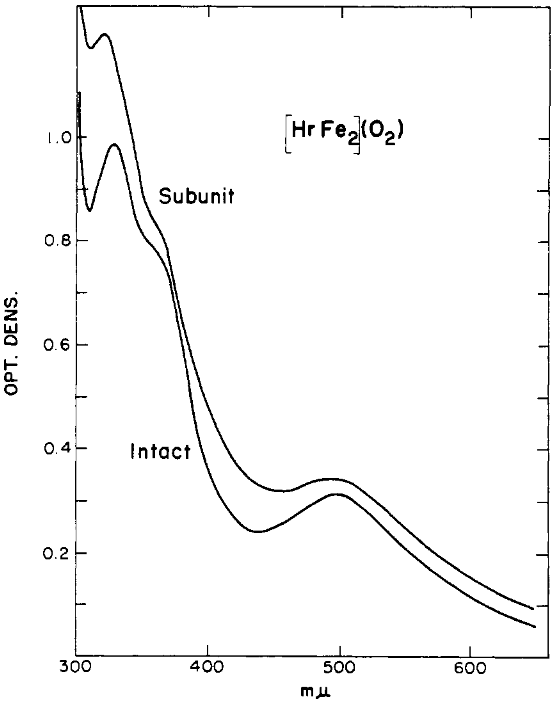


FIGURE 7: Absorption spectrum of oxygenated subunits of hemerythrin compared with that of intact undissociated oxygenated protein.

(H_2O), against a buffer (Tris, at pH 8) which had been saturated with air. When the dissociation and reduction was carried out gently, the color produced on oxygenating the subunit persisted for more than an hour, during which time an absorption spectrum was recorded and the state of disaggregation was verified by a sedimentation experiment. The spectrum of oxygenated subunits is shown in Figure 7.

Oxymerohemerythrin was not stable enough to be returned to the deoxy form when O_2 was removed by bubbling N_2 through the solution. However the back conversion could be effected by dialyzing oxygenated subunits against hydrosulfite. That reduction, and not merely denaturation, had been accomplished is shown by the observation that subsequent dialysis against air-saturated buffer once again produced oxygenated subunits.

Stability and Interaction Constants. From spectrophotometric titrations of the aquo complex with various ligands one can obtain stability constants for the formation of the complex, as well as n, the interaction parameter of the Hill equation originally derived to correlate oxygenation equilibria. For this purpose we write the following (oversimplified) equation for the equilibria:

$$[\overline{Hr}Fe_2^{III}(H_2O)_m]_8 + n\ L^- \rightleftharpoons [\overline{Hr}Fe_2^{III}(L^-)_m]_8 + 8m\ H_2O \quad (1)$$

Following a procedure analogous to that used for the Hill equation, we can express the formation constant, K, as

$$K = \frac{\{[\overline{Hr}Fe_2^{III}(L^-)_m]_8\}}{\{[\overline{Hr}Fe_2^{III}(H_2O)_m]_8\}} \frac{1}{(L^-)^n} \quad (2)$$

Rearrangement of equation (2) yields

$$\log \frac{\{[\overline{Hr}Fe_2^{III}(H_2O)_m]_8\}}{\{[\overline{Hr}Fe_2^{III}(L^-)_m]_8\}} = -\log K - n \log (L^-) \quad (3)$$

The left-hand side of equation (3), that is, the composition of an equilibrium mixture, can be obtained from the optical density of the mixture and the molecular extinction coefficients of the individual complexes, $[\overline{Hr}Fe_2^{III}(H_2O)_m]_8$ and $[\overline{Hr}Fe_2^{III}(L^-)_m]_8$. A graph of the left-hand side of equation (3) versus the logarithm of the free ligand concentration, (L^-), when it gives a straight line, can be used to evaluate the slope and intercept, n and pK, respectively.

In titrations with Cl^- and SCN^-, the amount of anion removed from the solution by hemerythrin is small, and hence the total concentration of added anion is essentially equal to the free concentration (L^-) required in equation (3). This is not the case under ordinary conditions for N_3^-, which is very strongly bound. To circumvent this difficulty, the azide equilibrium was studied, not by direct additions of salt to hemerythrin, but by dialysis of hemerythrin against large volumes of

TABLE II: Equilibrium Constants and Interaction Parameters.[a]

Reaction	n	K
$[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{II}}]_8 + n\,\mathrm{O}_2 = [\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{O}_2^{2-})_m]_8$	1.16	1×10^5
$[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{H}_2\mathrm{O})_m]_8 + n\,\mathrm{N}_3^- = [\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{N}_3^-)_m]_8 + n\,\mathrm{H}_2\mathrm{O}$	1.16	2.5×10^4
$[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{H}_2\mathrm{O})_m]_8 + n\,\mathrm{SCN}^- = [\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{SCN}^-)_m]_8 + n\,\mathrm{H}_2\mathrm{O}$	1.06	4×10^3
$[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{H}_2\mathrm{O})_m]_8 + n\,\mathrm{Cl}^- = [\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{Cl}^-)_m]_8 + n\,\mathrm{H}_2\mathrm{O}$	1.	4×10^1
$\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{H}_2\mathrm{O})_n + n\,\mathrm{N}_3^- = \overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{N}_3^-)_n + n\,\mathrm{H}_2\mathrm{O}$	1.	2×10^5
$[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{H}_2\mathrm{O})_m]_8 = [\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{OH}^-)_m]_8 + n\,\mathrm{H}^+$	0.9	1×10^{-9}

[a] All experiments with ions were carried out in Tris-cacodylate buffer, pH 6.5–7.5, ionic strength 0.1, 25°.

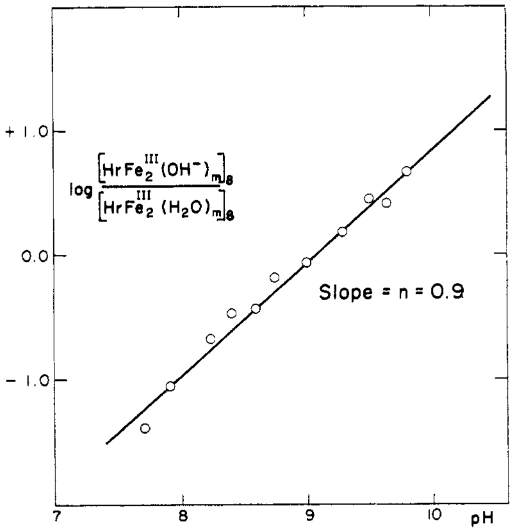


FIGURE 8: Optical titration of formation of OH^- complex of methemerythrin.

dilute solutions of azide. The volumes of outside solution were made so large that azide uptake did not significantly change the original concentration of this anion. The spectrum of the total azide complex was obtained after addition of solid NaN_3 to the hemerythrin.

In practice, linear relations of the form of equation (3) were found to fit the data (see, e.g., Figure 8) for all the anionic ligands examined. Values of K and n are assembled in Table II. Included in the table are parameters for the oxygenation reaction, calculated from measurements of Peticolas (1954), and also for the dissociation of an H^+ ion by the aquo complex:

$$[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{H}_2\mathrm{O})_m]_8 \rightleftharpoons [\overline{\mathrm{Hr}}\mathrm{Fe}_2^{\mathrm{III}}(\mathrm{OH}^-)_m]_8 + n\,\mathrm{H}^+ \quad (4)$$

Some difficulties were encountered in this dissociation titration, which required high pH values. To avoid any artifacts due to simultaneous ionization of tyrosine groups of the protein, the spectrophotometric titration was carried out at 380 mμ, a region far removed from the tyrosine spectrum. To circumvent the effects of instability of the OH^- form of the protein, the high pH spectrum (pH ~ 11) was measured near 0°. The titration was carried out in 0.1 M NaClO_4, and the pH was adjusted by addition of small quantities of solid cacodylic acid, sodium carbonate, or by Tris, so that protein concentration and ionic strength should be changed minimally during the titration.

Also included in Table II is one spectrophotometric titration (fifth row) with subunits, merohemerythrin. Here too difficulties were encountered owing to the lability of the protein. Subunits were prepared by the addition of *N*-ethylmaleimide to oxyhemerythrin in 0.1 M $(\mathrm{NH}_4)_2\mathrm{CO}_3$. The titration with azide was then carried out by the same dialysis procedure used with the undissociated protein. The data obtained were much more scattered for the subunit owing to its instability. In regard to n, one can merely say the results are compatible with a value of unity; there is no doubt, however, that the formation constant, K, is greater for the subunit. In other words, the subunit forms a much stronger complex with N_3^- than does the original undissociated protein.

As the results in Table II show, the value of n lies near unity in all cases, and we have in fact assumed it to be exactly 1.0 for the calculation of K (from the midpoint of the titration curve). We may conclude, therefore, that the eight binding sites in undissociated hemerythrin act essentially independently, each picking up one ligand.

To find out the actual ratio of Fe/L^- in a single active site, one must carry out stoichiometric experiments.

Stoichiometry of Iron to Ligand. The stoichiometric experiments are most easily carried out with N_3^- because it is so strongly bound. The procedure used was essentially a double spectrophotometric titration. First a dialysis experiment was run with large quantities of solution containing azide so that the free (N_3^-) was known to be equal to the total. In this way the spectrophotometric readings were related to free (N_3^-). Then a second set of experiments was run in which a known small amount of azide was added directly to a measured quantity of fresh solution of aquo methemerythrin of known (iron) concentration. From the spec-

TABLE III: Fe/N_3^- Ratios for Undissociated Methemerythrin.

Total Fe (μmoles)	Total N_3^- (μmoles)	Conversion (%)	Fe Converted (μmoles)	N_3^- Free (μmole)	$\frac{\text{Converted Fe}}{N_3^- \text{ Bound}}$
2.06	0.1500	13.25	0.273	0.0163	2.04
2.06	0.2500	22.5	0.4635	0.0283	2.09
2.06	0.3500	31.12	0.641	0.0410	2.07
2.06	0.4500	39.6	0.8158	0.0584	2.08
2.06	0.6000	52.03	1.072	0.0907	2.10
2.06	0.7000	59.06	1.217	0.1155	2.08
2.06	0.8000	61.56	1.268	0.1266	1.88
2.06	0.9000	70.88	1.460	0.1827	2.04
2.06	1.0500	78.94	1.626	0.2709	2.09
2.06	1.1500	84.44	1.739	0.3740	2.24
2.06	1.3500	89.25	1.838	0.5380	2.26

TABLE IV: Fe/N_3^- Ratios for Hemerythrin Subunits.

Total Fe (μmoles)	Total N_3^- (μmole)	Conversion (%)	Fe Converted (μmoles)	N_3^- Free (μmole)	$\frac{\text{Converted Fe}}{N_3^- \text{ Bound}}$
0.93	0.050	9.3	0.0865	0.002	1.80
1.65	0.100	10.95	0.1807	0.005	1.90
0.93	0.065	12.2	0.1135	0.0025	1.82
0.93	0.085	16.5	0.1535	0.003	1.87
1.65	0.200	22.7	0.0374	0.006	1.93
1.65	0.300	34.6	0.571	0.010	1.97
1.65	0.400	46.6	0.769	0.014	1.99
1.65	0.450	51.1	0.843	0.015	1.94
1.40	0.400	55.8	0.780	0.020	1.94
1.65	0.500	56.6	0.934	0.018	1.94
1.40	0.485	63.3	0.886	0.0267	1.90
1.65	0.600	66.0	1.089	0.028	1.90
1.65	0.700	73.6	1.214	0.035	1.82
1.65	0.800	77.90	1.285	0.040	1.70
0.93	0.450	85.2	0.792	0.038	1.92

trum, the fraction of hemerythrin complexed with N_3^- could be determined, as well as the free (N_3^-) that must be in equilibrium with the protein complex. From these observations it is possible to compute the moles of bound azide and the molecules of protein iron present in the solution, and hence the Fe/N_3^- stoichiometry at the active site. The results for undissociated hemerythrin and for subunits are assembled in Tables III and IV, respectively.

It is obvious that 1 N_3^- is associated with 2 Fe in both forms of hemerythrin. This is the same as the ratio of Fe/O_2 in oxyhemerythrin. We have assumed, therefore, that Fe/L^- is 2 for all ligands.

Effect of Ions on Sulfhydryl Interactions. In the course of the spectrophotometric investigations several attempts were made to prepare aquo subunits directly from aquomethemerythrin by reaction of protein SH groups with *N*-ethylmaleimide. These were uniformly unsuccessful. It seemed, therefore, that in the absence of ligand ions, the SH groups are masked and so do not react with the sulfhydryl reagent.

That such is indeed the situation was strikingly demonstrated by the experiment charted in Figure 9. To a 1% solution of aquomethemerythrin dissolved in 0.1 M $(NH_4)_2CO_3$ was added a 20-fold excess (over moles of SH groups present) of solid *N*-ethylmaleimide. After the reagent had dissolved, the solution was divided into two parts. To one of these NaN_3 was added to form $[\overline{\text{Hr}}\text{Fe}_2^{\text{III}}(N_3^-)]_8$. Then both solutions were allowed to stand overnight at 4°. Ultracentrifuge experi-

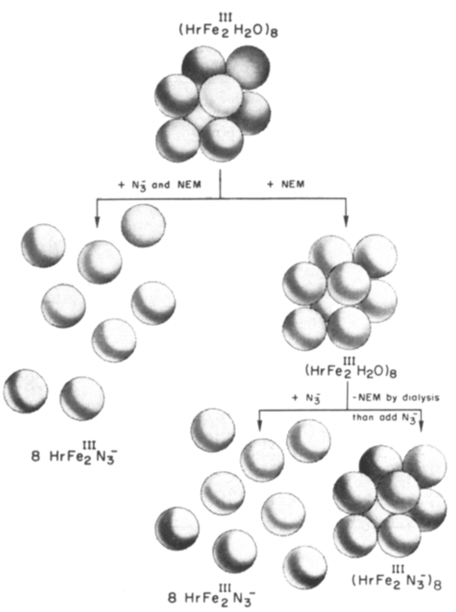


FIGURE 9: Chart outlining effect of azide ion on structure of hemerythrin and its reactivity toward *N*-ethylmaleimide (NEM).

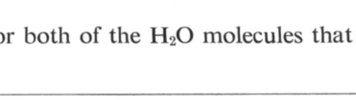
ments thereafter showed that hemerythrin in the azide-containing solution contained only particles with a sedimentation coefficient of 2 S, in other words, only subunits (Klotz and Keresztes-Nagy, 1963). The other solution, without azide, showed only 6 S particles, undissociated hemerythrin. Clearly the *N*-ethylmaleimide was not effective in dissociating hemerythrin in the absence of ligand anion. Its ineffectiveness might be due to either of two causes, however: (1) it did not combine with protein SH groups; (2) it combined with SH groups but dissociation would not proceed until L^- was bound. To settle this point the following experiment was carried out. Part of the solution containing aquomethemerythrin and *N*-ethylmaleimide was dialyzed to remove unreacted maleimide. If any reagent had reacted with protein mercaptan, it would not dialyze out, since a covalent —S—C— bond is formed. After dialysis, azide was added to the solution. If maleimide had reacted with protein but needed the azide to dissociate hemerythrin, 2 S particles should have appeared in the ultracentrifuge. If maleimide had not reacted in the absence of azide it should have been removed during the dialysis, and the subsequent addition of azide should have had no effect on particle size since addition of N_3^- to undissociated aquomethemerythrin $[\overline{HrFe_2^{III}(H_2O)}]_8$ merely leads to $[\overline{HrFe_2^{III}(N_3^-)}]_8$. The actual sedimentation experiment after dialysis and subsequent addition of azide showed only 6 S particles. Clearly *N*-ethylmaleimide had not reacted with aquo protein in the original solution in the absence of azide;[4] in other words the SH groups of aquomethemerythrin are masked.

Another reagent that we have used to dissociate hemerythrin is succinic anhydride (Klotz and Keresztes-Nagy, 1962; Hass, 1964; Okazaki *et al.*, 1964). We have found now that this reagent, too, is ineffective in producing 2 S particles from aquomethemerythrin in the absence of an anionic ligand. It seems, therefore, that when succinic anhydride dissociates hemerythrin it does so because of reaction with SH groups rather than because of the large electrostatic repulsion produced; for when the mercaptans are masked, succinic anhydride is ineffective, even though it has presumably acylated lysyl side chains. To substantiate this point, the SH groups of hemerythrin were blocked with salyrganic acid (Keresztes-Nagy and Klotz, 1963) and subunits produced. These subunits were then heavily succinylated. The succinylated subunits were then treated with cysteine ethyl ester to remove the mercurial from the mercaptan groups; 6 S particles were reconstituted despite succinylation of the lysyl side chains.

Discussion

Environment of Iron without Added Ligands. The most important conclusion from the electrophoretic observations for our present concern is that anions are bound by hemerythrin. In addition, the mobility experiments offer some hints in regard to the environment of the Fe ion in hemerythrin in the absence of added ligands.

Comparing methemerythrin with deoxyhemerythrin we recognize that the former, containing (sixteen) Fe^{III} ions in place of Fe^{II} ions of the latter, must contain sixteen more positive charges. Nevertheless, the isoelectric point of methemerythrin is not distinguishable from that of deoxy. Consequently the conversion of Fe^{II} to Fe^{III} must be accompanied by the introduction of compensating negative charges. Since it is well known that ferric iron has a much greater affinity for OH^- ions than does ferrous (Basolo and Pearson, 1958; Milburn, 1957), i.e., $Fe^{III}(H_2O)$ is much more acidic than $Fe^{II}(H_2O)$, it seems very likely that upon conversion of deoxyhemerythrin to methemerythrin each pair of irons in a site picks up two OH^- ions. In this way the extra positive charge on each Fe^{III}, as compared to Fe^{II}, is counterbalanced by an OH^- negative charge. Furthermore, since it is very unlikely that Cl^- could displace an OH^- from Fe^{III} at neutral or basic *p*H values, and yet we know that Cl^- can become attached to the iron of methemerythrin, the metal ion is presumably coordinated with an H_2O also. Thus we may represent the active site in aquomethemerythrin by structure (I):



(I)

It is one or both of the H_2O molecules that is presum-

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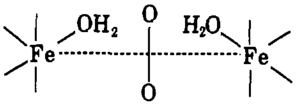
[4] The second part of the original solution, which was *not* dialyzed, showed 2 S particles as soon as NaN_3 was added to it (see chart in Figure 9).

ably displaced by Cl^- and other anions with a concurrent shift in isoelectric point and of absorption spectrum.

Likewise it is probably this H_2O that ionizes when aquomethemerythrin changes spectrum in the region above *p*H 8. In $Fe^{III}(H_2O)_n$ the first dissociation constant (Basolo and Pearson, 1958; Milburn, 1957) has a pK_a of about 2; in methemerythrin this probably corresponds to the uptake of the first OH^- anion by each Fe^{III}. On the other hand, the observed spectroscopic pK_a for the basic titration of aquomethemerythrin, *ca.* 9, would be reasonable[5] for the dissociation of a proton from the H_2O ligand shown in structure (I).

Similarly, if the structure of oxyhemerythrin is represented in a corresponding way[6] by (II), the variation of its spectrum with *p*H in the region around 8 (Figure 5) could be attributed to dissociation of H^+ from the coordinated H_2O molecule(s).



(II)

Spectra and Nature of Ion Complexes. The effect of ions on the electrophoretic mobility of methemerythrin shows that ions are bound to this protein, and the large changes in spectra on binding point to the Fe(III) as the locus of attachment. Furthermore the simple stoichiometry of 2 for Fe/L^- reinforces this conclusion. All of the ions examined, HS^-, N_3^-, SCN^-, CNO^-, Cl^-, CN^-, F^-, and OH^-, are known to form coordination complexes with iron.

In general the absorption bands of transition metal complexes fall into two classes: (1) allowed transitions with extinction coefficients greater than 2000; (2) transitions forbidden by the selection rules with extinction coefficients below 200.

These two classes of transition can be recognized in the complexes of hemerythrin (Figures 3 and 4, Table I). These complexes show a low-intensity absorption band in the range of 640–700 mμ, most probably owing to forbidden d → d transitions between the split $3d_e$ and $3d_{t_2}$ orbitals.

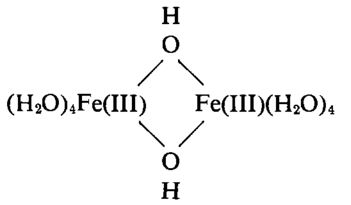
With regard to high-intensity absorption bands, methemerythrin complexes may be placed into three groups. Group I, including the ligands N_3^-, SCN^-, Cl^-, CN^-, CNO^-, is distinguished by the presence of a strong band at 330 mμ, with ε near 3500, and another in the visible or near-visible range. It seems reasonable to ascribe the latter band to a charge transfer from ligand to Fe(III). The shift of this band position from 450 mμ in the N_3^- complex to 375 mμ in the CN^- adduct is in accord with such an assignment since electron transfer would take place more readily from N_3^- or SCN^- than from Cl^- or CN^-. These complexes exchange their ligands slowly and hence are probably low-spin complexes.

The aquo complex comprises another class. It does not have a 330 mμ peak. It can exchange its ligand very rapidly, as is characteristic of high-spin or ionic complexes.

Group III includes the OH^- and F^- complexes of methemerythrin, and shows spectroscopic features intermediate between structures I and II. A peak appears at 317 mμ, which probably corresponds to the 330 mμ peak of Group I, and a second peak occurs at 362 mμ. One might expect the OH^- and F^- complexes of methemerythrin to display intermediate magnetic properties, as they are a mixture of low-spin and high-spin complexes.

In connection with the presumed magnetic states of hemerythrin it is relevant to mention that the Fe(III) in the aquo complex is easily reduced to Fe(II) with hydrosulfite but is almost impossible to reduce in the azide complex. The Fe(II) in deoxyhemerythrin is stated to be in a high-spin state (Kubo, 1953). Thus the reduction of the azide complex would require a change from a paired- to an unpaired-spin state and hence should be slower than reduction of the aquo form in which the spin state is unchanged.

In regard to the complexes showing an absorption band at 330 mμ, it is pertinent to recall that Fe(III) in perchlorate solutions forms a dimer (Mulay and Selwood, 1954, 1955) that displays an absorption peak at 335 mμ. Its structure may be represented by (III):



(III)

Magnetic measurements with this dimer show no unpaired electrons. Thus the fifth 3d electrons of the two Fe(III) atoms must be coupled. Complexes of hemerythrin with anionic ligands showing a 330 mμ peak may also be binuclear and contain an Fe—O—Fe bridge.

Finally, it is of interest to compare the spectra of the anionic complexes of methemerythrin (Figures 3 and 6) with that of oxyhemerythrin (Figure 5). It is clear that the more polarizable the ligand L^- in $[\overline{\mathrm{Hr}}\mathrm{Fe}^{III}(L^-)]_8$ the more nearly the absorption band in the visible region approaches that of oxyhemerythrin. Particularly striking are the positions of the absorption bands of HS^-, N_3^-, and SCN^- complexes. There is also a remarkable coincidence of peaks at 330 mμ. It seems reasonable therefore to describe oxyhemerythrin as $[\overline{\mathrm{Hr}}\mathrm{Fe}_2^{III}(O_2^{2-})]_8$, and to attribute the visible absorption band to a charge transfer to the Fe(III), analogous to corresponding methemerythrin-L^- complexes. In this way one can fit the position of the charge-transfer bands

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[5] Thus hydrated ferrous ion $Fe^{II}(H_2O)^{2+}$, which carries the same net charge as does $Fe^{III}(OH^-)^{2+}$, has a pK_a of 8.3 (Basolo and Pearson, 1958).

[6] The O_2 in structure II is represented as lying perpendicular to the Fe···Fe axis since this is the position it occupies in inorganic complexes of O_2 whose structures have been worked out recently by X-ray diffraction (Vannerberg and Brosset, 1963; Ibers and La Placa, 1964).

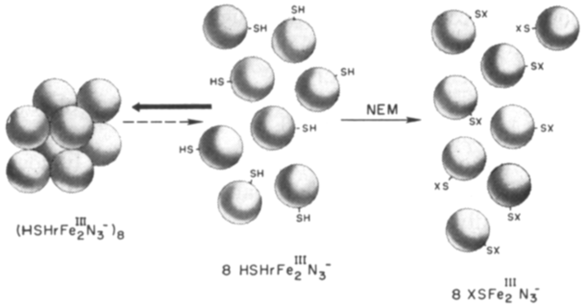

FIGURE 10: Schematic representation of equilibrium between intact hemerythrin and its subunits, as basis of cooperative interaction between N_3^- at iron locus and *N*-ethylmaleimide (NEM) at sulfhydryl site.

into a series which correlates with the reducing character of the ligand: HS^-, O_2^{2-}, N_3^-, SCN^-, CNO^-, CN^-.

Effect of Ions on Structure of Molecule. As far as one can tell by ordinary sedimentation analysis, all the anionic complexes of hemerythrin continue to exist in the octameric state with sedimentation coefficient of 6 S. On the other hand it is evident, particularly from the effect of N_3^- on the reactivity of the protein with *N*-ethylmaleimide (Figure 9), that complex formation at the iron locus has an effect on the reactivity of the sulfhydryl group. Since blocking of the sulfhydryl group does not change the spectrum of hemerythrin significantly, it seems evident that the mercaptan is not directly bonded to the iron, and hence it is at a different locus on the protein. Thus an interaction at one locus on the protein affects the reactivity at a second locus, and we have a clear example of a *cooperative interaction* or *allosteric effect* (Monod *et al.*, 1963).

In the present case, a reasonable molecular interpretation of this cooperative interaction is provided by the assumption that octameric hemerythrin is always in equilibrium with a *very small* amount of monomeric merohemerythrin (Figure 10). We know from experiments described here as well as from earlier work (Klotz *et al.*, 1957) that the SH groups in intact hemerythrin are masked. It seems reasonable to assume that they are more exposed in the subunits. Furthermore it is clear from the equilibrium constants in Table II that N_3^- has a greater stabilizing effect on subunits than on intact hemerythrin. It follows, therefore, that N_3^- ions would shift the octamer $\rightleftharpoons$ monomer equilibrium (Figure 10) to the right, increasing the concentration of exposed SH groups, and hence accelerating the rate of reaction with *N*-ethylmaleimide.

Such a picture explains not only why *N*-ethylmaleimide reacts more readily with hemerythrin in the presence of N_3^- than in its absence, but also another important feature of sulfhydryl reactions, the all-or-none effect.[7] It has been shown previously (Keresztes-Nagy and Klotz, 1963) that there is a linear one-to-one relationship between the percentage of SH groups titrated by a mercurial and the percentage dissociation of the original octamer. Such titration behavior is an immediate consequence of the picture shown in Figure 10, for if the added mercurial reacts only with subunits it would tie them up rapidly and completely until all the mercurial had been depleted from solution; then the octamer would dissociate to a very slight extent to regenerate a very small amount of subunit.

Despite the attractiveness of these interpretations in terms of an equilibrium with subunits, it must be recognized that all of the effects described could also be accounted for by the alternative assumption that hemerythrin undergoes a change in conformation without dissociation when it binds small molecules. Such a conformational rearrangement could provide greater accessibility for additional small molecules.

There are differences in prediction which each of these mechanisms makes in regard to some aspects of the behavior of hemerythrin, particularly in regard to hybridization phenomena. We hope, therefore, that new experiments may provide a choice between the two pictures of the molecular basis for cooperative interactions in this protein.

Acknowledgment

The authors are indebted to Dr. C. K. Jørgensen for helpful comments.

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[7] A similar conclusion has been reached independently by Dr. H. K. Schachman (private communication).

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Dialyzable Thyroid Hormone–binding Material in Human Serum*

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ABSTRACT: Human serum was dialyzed through cellophane against four times its volume of water, and the freeze-dried dialysate was extracted with 0.3% methanolic ammonia. The extract contained a material which interacted with added L-thyroxine on paper chromatography. In aqueous solution, in the presence of the major electrolytes of serum, it decreased the rate coefficient of escape of L-thyroxine and 3′,3,5-triiodo-L-thyronine through cellophane. Thyroxine and triiodothyronine were quantitated with the ceric sulfate–arsenious acid fading test for iodine.

The method, adapted for the present work, had 95% precision limits of ±0.055 ng iodine in the range of 0.1–2 ng iodine. The small-molecular-weight material interacting with thyroxine and triiodothyronine might conceivably be of importance as a regulator for the concentrations of thyroid hormones not bound to protein.

Models used for describing the behavior of thyroxine in blood include as an essential feature the equilibrium for thyroxine between free and bound states. The association constants for the thyroxine complexes with thyroxine-binding globulin, thyroxine-binding prealbumin and albumin, accepted for the physiological situations, are those obtained from dialysis equilibria and electrophoretic data. No allowance is made for the possible presence of a dialyzable substance that interacts with thyroxine. By reducing the chemical activity, such a substance would increase the concentration of thyroxine not bound to the three proteins. Furthermore, the possibility exists that this substance, by combining with thyroxine, could change the biological reactivity of the latter. The present work is concerned with evidence for a dialyzable material in human serum which binds, or interacts with, thyroxine and with triiodothyronine.

Materials and Methods

Sera from healthy men, 20–50 years old, were used fresh or after storage in the deep-freeze. L-Thyroxine and 3′,3,5-triiodo-L-thyronine were supplied by Sigma Chemical Co., St. Louis, Mo., or donated by Warner-Lambert (Morris Plains, N.J.). Analytical Reagent grade anhydrous methanol was supplied by Mallinckrodt. NH_4OH (Fisher) was purified by distillation with 3 volumes CH_3OH. From this distillate, CH_3OH-NH_4OH solutions I and II were prepared by diluting with 7 or with 24 volumes CH_3OH. The chemicals used in the determination of iodine were the same as described previously (Hoch *et al.*, 1964) except for $KClO_3$, which was recystallized four instead of three times from hot water. Reagent solutions: (1) 73% $HClO_4$: approximately constant-boiling, sold as "double vacuum distilled from Vycor, 70% $HClO_4$ and shipped in Vycor ampuls" by G. Frederick Smith Chemical Co., Columbus, Ohio. (2) $HClO_3$ reagent: 22.5 ml 73% $HClO_4$ was added slowly to a hot solution of 29 g $KClO_4$ in 58 ml H_2O, and after cooling the supernatant was separated and stored at −14° before use. (3) CrO_3 solution: the aqueous solution, approximately 0.6%, was adjusted with water so that when 0.050 ml was added to 1.35 ml H_2O the absorbancy was 0.30 at 430 mμ. (4) Sodium arsenite reagent: stock solution, 19 g As_2O_3, 7.8 g NaOH, and 2.0 g NaCl diluted with H_2O to 500 ml; for use, diluted further 1:10 with H_2O and stored at room temperature. (5) Ceric sulfate reagent: 3.32 g $Ce(SO_4)_2$, anhydrous, purified (Fisher), was suspended in 4.8 ml 10 N H_2SO_4, diluted to 100 ml with H_2O, and stored several days to let the undissolved and the freshly forming precipitate settle. It was accurately standardized so

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