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Mercury Binding to Hemerythrin. Coordination of Mercury and Its Effects on Subunit Interactions†

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ABSTRACT: The reaction of hemerythrin, an oxygen-carrying protein in marine invertebrates, with K_2HgI_4 was investigated to determine the mode of mercurial binding to the protein in the heavy atom derivative used for solving the crystal structure of *Themiste dyscritum* hemerythrin. Spectrophotometric titrations established that the *T. dyscritum* protein reacts with approximately 1.5 molecules of K_2HgI_4 per protein subunit and that the reaction involves mercury–sulfur bond formation. Crystallographic studies using difference density maps showed that one mercury is coordinated to the sulfur of cysteine-50 as HgI and that a second mercury is shared between two cysteine-9 sulfurs of adjacent subunits, resulting in a linear S–Hg–S coordination. Gel filtration chromatography of K_2HgI_4-treated hemerythrin demonstrated that the protein retains its octameric structure in solution after reacting with K_2HgI_4, as had been observed in the crystalline state. The roles of the cysteine-9 and -50 residues in the subunit interactions of *T. dyscritum* hemerythrin were determined from the effects of the bulkier sulfhydryl reagents, *p*-hydroxymercuribenzoate and *N*-ethylmaleimide. Binding of either reagent to cysteine-50 resulted in the disruption of all subunit interactions and the conversion of the native octamers to monomers. Binding of either reagent to cysteine-9 caused the isolated monomers to reassociate as dimers. The critical effect of cysteine-50 modification on subunit interactions was found to be due to its proximity to the major points of subunit contact, as seen in the crystal structure of the protein.

The nature of the interaction of mercury compounds with proteins is of interest from a number of points of view. Mercury binding to proteins and consequent poisoning of active sites or disruption of protein structure play an important role in the toxic effects of mercurials (Bremner, 1974). As protein sulfhydryl groups are the primary targets of mercury binding, reactions with mercurials are useful for elucidating the involvement of cysteine sulfhydryl groups in enzymatic catalysis and in the maintenance of tertiary and quaternary structure (Webb, 1966). On the other hand, protein crystallography depends on mercurials which do not disrupt protein structure in order to obtain heavy atom derivatives by the isomorphous replacement technique (Blundell & Johnson, 1976). Thus, the effects of mercury compounds vary considerably depending on the nature of the mercurial and the protein to which it binds.

One protein which has been shown to undergo dissociation upon treatment with sulfhydryl reagents is hemerythrin, a respiratory protein found in marine invertebrates. Hemerythrin from the sipunculid, *Phascolopsis gouldii*, has been studied in considerable detail (Klotz, 1971). The native, octameric protein can be dissociated by reaction with sulfhydryl reagents such as *p*-hydroxymercuribenzoate (PHMB),[1] *N*-ethylmaleimide (NEM), or salyrganic acid (Kereztes-Nagy & Klotz, 1963). As the protein contains only a single cysteine at residue 50, this must be the residue whose modification results in subunit dissociation.

Hemerythrin from *Themiste dyscritum* appears to be very similar to *P. gouldii* hemerythrin in its molecular weight and subunit composition (Loehr et al., 1975), amino acid sequence (Loehr et al., 1978), spectroscopic properties (Dunn et al., 1977), and protein conformation as judged by X-ray crystallography at approximately 5-Å resolution (Stenkamp et al., 1976; Ward et al., 1975). However, in addition to the cysteine at position 50, there is a second cysteine at position 9 in the polypeptide chain (Loehr et al., 1978). The crystal structure of K_2HgI_4-treated hemerythrin at 2.8-Å resolution indicates that the isomorphous derivative has one mercury binding site close to cysteine-50 on each subunit and another mercury site close to cysteine-9 residues on adjacent subunits related by noncrystallographic twofold axes (Stenkamp et al., 1978b).

The above information leads to the conclusion that, if K_2HgI_4 has reacted with cysteine sulfurs in *T. dyscritum* hemerythrin, it has not caused any disturbance of the protein structure, in contrast to the results observed with mercurials like PHMB (Kereztes-Nagy & Klotz, 1963). However, mercurials used in protein crystallography are quite often bound to groups other than cysteine (Blundell & Johnson, 1976). The present study was undertaken to determine

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[1] Abbreviations used: PHMB, *p*-hydroxymercuribenzoate; NEM, *N*-ethylmaleimide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

0006-2960/79/0418-0684$01.00/0

whether the mercury in the heavy atom derivative of *T. dyscritum* hemerythrin is, in fact, covalently bound to cysteine sulfur atoms and, if so, to elucidate the structure of the mercury–sulfur complexes in the protein and the forces involved in subunit interactions.

Experimental Procedures

Preparation of Hemerythrin. The marine worms *Phascolopsis gouldii* and *Themiste dyscritum* were obtained, respectively, from Marine Biological Laboratory, Woods Hole, MA, and Oregon Institute of Marine Biology, Charleston, OR. Hemerythrin from the coelomic fluid of both marine worms was purified by the method of Klotz et al. (1957) and crystallized as described previously (Dunn et al., 1977). The only modification was that 0.01 M sodium azide was added to the laked blood and was present at this concentration in all subsequent steps. The sodium azide treatment served to inhibit bacterial growth and to promote crystallization of *T. dyscritum* hemerythrin which occurs more readily after oxidation to metazidohemerythrin. The purity of crystallized *T. dyscritum* hemerythrin was checked by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Weber & Osborn, 1969) and found to be greater than 95% pure. The protein subunit concentration was determined spectrophotometrically for the metazide complexes using $\epsilon_{327} = 7200\ M^{-1}\ cm^{-1}$ for *P. gouldii* hemerythrin and $\epsilon_{325} = 7750\ M^{-1}\ cm^{-1}$ for *T. dyscritum* hemerythrin (Dunn et al., 1977).

Spectrophotometric Titrations. Because azide absorbs strongly at 250 nm, the wavelength used to detect mercury–sulfur bond formation (Boyer, 1954), the metazidohemerythrin samples were dialyzed vs. 0.5 M KCl, 0.05 M Tris-Cl (pH 7) to remove excess azide. The hemerythrin, however, remained in the metazide form, as judged by the visible absorption spectrum (Garbett et al., 1969). The protein subunit concentration ranged from 3 to 7×10^{-5} M. Solutions of K_2HgI_4 in the above buffer were made by dissolving HgI_2 in a tenfold excess of KI, while dissolution of *p*-hydroxymercuribenzoate required the addition of 0.025 N KOH. Fresh mercurial titrant was prepared for each experiment at such concentrations that a maximum of 0.4 mL of titrant was added per 25 mL in the reaction vessel.

The titrations were performed at 7 ± 3 °C using a temperature-controlled titration apparatus designed for the Cary 14 spectrophotometer. The cell holder was an aluminum block, bored to permit circulation from a temperature-controlled water bath. The block was fitted with two round quartz windows which passed the light beam through a rectangular, 30-mL quartz cuvette with a 2-cm pathlength. The other side of the cuvette contained a Teflon stirrer and the glass tip of the Gilmont micrometer buret which was used to add titrant. The sample chamber was purged with nitrogen to prevent condensation of water vapor on optical surfaces. The reference cell (1-cm path length) was at room temperature and contained a hemerythrin solution with an absorbance equal to that of the protein in the sample cell.

Upon addition of an aliquot of mercurial, there was a rapid rise in absorbance at 250 nm due to the mercurial itself. This was followed by a slow change (increase or decrease) in absorbance indicative of a reaction of the respective mercurial with the protein. Titration with PHMB led to an increase in absorbance, as expected for Hg–S bond formation in which $\Delta\epsilon_{250nm} = 0.76 \times 10^4\ M^{-1}\ cm^{-1}$ (Boyer, 1954). Titration with K_2HgI_4 led to a decrease in absorbance upon reaction of the mercurial with the protein due to the higher absorptivity of K_2HgI_4 ($\epsilon_{250nm} = 1.72 \times 10^4\ M^{-1}\ cm^{-1}$) than of the subsequent Hg–S complex. Each reaction was allowed to go to completion before the next aliquot of titrant was added. Reaction times varied from about 30 min for 0.25 mol of mercurial per mol of subunit to about 2 h for 1 mol of mercurial per mol of subunit. Although the entire titration required as long as 12 h in certain cases, the spectrophotometer was found to be stable over this time period and no protein denaturation was observed in either the reference or the sample cell. Each titration experiment was performed at least two times. Titration end points were determined with an accuracy of ±5%.

Crystallographic Studies. Difference electron density maps were calculated using X-ray diffraction data from isomorphous crystals of metaquohemerythrin and K_2HgI_4-treated metaquohemerythrin from *T. dyscritum*. The metaquohemerythrin data were those used in the refinement of the protein model at 2.5-Å resolution (Stenkamp et al., 1978a). The data for K_2HgI_4-treated hemerythrin (Stenkamp et al., 1976) were reprocessed to include the 2θ dependence of the radiation decay and yielded 10526 reflections with $I > 2\sigma(I)$ out to 2.8-Å resolution. After scaling the derivative map structure factors to those of metaquohemerythrin in shells of $\sin\theta/\lambda$, the quantity $\sum||F_{derivative}| - |F_{aquo}||/\sum|F_{aquo}|$ was 0.286. The differences between the structure factors for the two forms were used as coefficients for the electron density maps (Blundell & Johnson, 1976) with phases from the 2.5-Å resolution refined protein model. The electron density at the six binding sites in the asymmetric unit was then contoured and superimposed on a skeleton model of the protein at 2.5-Å resolution (Stenkamp et al., 1978a).

Gel Filtration Chromatography. All chromatography was performed at 4 °C on a 1.5 × 30 cm column of Sephadex G-100 (40–120 μm) equilibrated with 0.5 M sodium azide, 0.05 M Tris-Cl (pH 8.0–8.5). Sample volumes were less than 0.5 mL. Column eluant was collected in 0.5–1-mL fractions and assayed for protein content by its absorbance at 326 nm. The column was calibrated with proteins of known molecular weight in order to establish the relationship between elution volume and molecular weight. The proteins used for calibration were ribonuclease (mol wt = 13 700), myoglobin (17 200), trypsin inhibitor (21 500), ovalbumin (45 000), bovine serum albumin (66 000), and conalbumin (85 000). The calibration was verified with *P. gouldii* hemerythrin in the native, octameric form (107 000) and in the PHMB-dissociated, monomeric form (13,500), which had been previously characterized by Klotz & Kereztes-Nagy (1963).

Hemerythrin solutions at 10^{-3} M in subunit concentration were mixed with small volumes of sulfhydryl reagent and incubated at 4 °C for various lengths of time. All of the hemerythrin samples were dissolved in 0.5 M KCl, 0.05 M Tris-Cl, 0.01 M sodium azide (pH 8) with the exception of hemerythrin which was reacted with *N*-ethylmaleimide (NEM). Since NEM is more rapidly hydrolyzed in the presence of azide at high pH, it was reacted with hemerythrin in 0.5 M KCl, 0.05 M Tris-Cl (pH 7.0).

Results and Discussion

K_2HgI_4 Binding to Hemerythrin. Spectroscopic evidence for mercury–sulfur coordination in a protein can be obtained from the increase in absorbance at 250 nm upon addition of mercurial (Boyer, 1954). Spectrophotometric titrations of hemerythrin (Figure 1) exhibit an additional increase in absorbance at the end point of the titration due to free K_2HgI_4 having a greater absorptivity than cysteine-bound mercury. The data in Figure 1 show that *P. gouldii* hemerythrin reacts with 0.9 mol of K_2HgI_4 per mol of subunit, while *T. dyscritum* hemerythrin reacts with 1.4 mol of K_2HgI_4 per mol of subunit. These findings are consistent with cysteine-50 being the sole

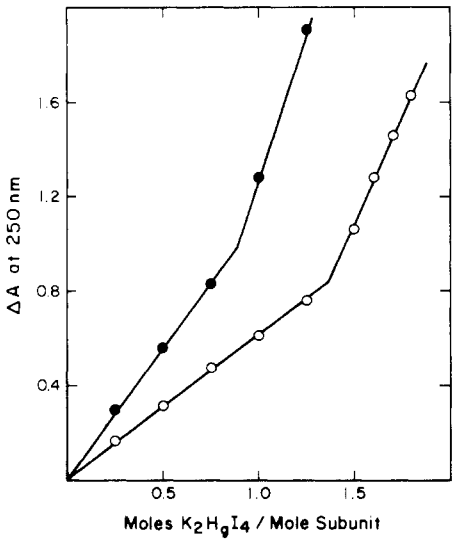

FIGURE 1: Spectrophotometric titration of metazidohemerythrin with K_2HgI_4. (○) *T. dyscritum* hemerythrin, 3.6×10^{-5} M in subunit concentration. (●) *P. gouldii* hemerythrin, 6.4×10^{-5} M in subunit concentration. Reference cell (1-cm path length) and sample cell (2-cm path length) contained hemerythrin solutions of equal absorbance.

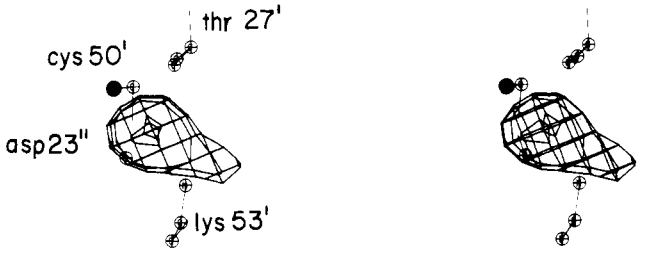

FIGURE 2: Stereoview of the 2.8-Å resolution K_2HgI_4 difference density near cysteine-50 in a representative subunit in the asymmetric unit of *T. dyscritum* hemerythrin. Since cysteine-50 lies at the interface of two subunits, superscripts indicate the subunit to which each amino acid belongs. Positive density is indicated by solid contour lines. The amino acid structures in the vicinity of the difference density are from the 2.5-Å resolution refined protein model (Stenkamp et al., 1978a) with filled-in circles representing the cysteine sulfur atoms. There are four such sites in the asymmetric unit; the difference density at the other three independent sites is nearly identical in shape and orientation to the one shown here.

site of mercury binding in *P. gouldii* hemerythrin and two cysteine-9 residues on adjacent subunits providing an additional site for mercury bridging in *T. dyscritum* hemerythrin.

Crystallographic studies based on difference Fourier maps of K_2HgI_4-treated *T. dyscritum* hemerythrin yield further evidence for mercury–sulfur coordination. Figure 2 contains a stereoview of the difference electron density at 2.8-Å resolution near cysteine-50, superimposed on the protein model refined at 2.5-Å resolution (Stenkamp et al., 1978a). The elongated shape of the difference density indicates that the bound mercurial is Hg–I. The larger density toward one end identifies the Hg atom and shows it to be in close proximity to the sulfur of cysteine-50. The ϵ-amino group of lysine-53 from the same subunit as cysteine-50 and the β-carboxyl group of aspartate-23 from an adjacent subunit are located at positions in the underivatized protein which would be within bonding distance of the mercury in the K_2HgI_4-treated protein. If these two residues retain their orientation during the binding of Hg–I to cysteine-50, this would result in tetrahedral mercury coordination. Tetrahedral structures are quite common for divalent mercury complexes with sulfur-containing ligands and are also seen in mixed-ligand complexes (Grdenić, 1965). The nearby threonine-27 (Figure 2) is not a ligand because its

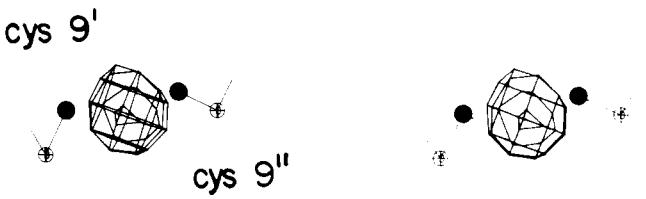

FIGURE 3: Stereoview of the 2.8-Å resolution K_2HgI_4 difference density near cysteine-9 residues for one pair of subunits related by a noncrystallographic twofold axis in the asymmetric unit of *T. dyscritum* hemerythrin. The other independent pair of subunits in the asymmetric unit has essentially the same difference density as the pair shown here. Additional details as in Figure 2.

terminal atoms are 3 Å or more away from the Hg atom.

The mercury atom which is shared by two subunits lies on a noncrystallographic twofold axis (Stenkamp et al., 1976). The difference density contour for this mercury atom is spherical in shape (Figure 3), indicating that no iodide remains attached to the mercury. The cysteine-9 side chains are disordered in the metaquohemerythrin crystals used to obtain the refined protein model and two alternative positions are possible for each cysteine-9 side chain, either with the sulfur atoms pointed toward one another (Figure 3) or with the sulfur atoms pointed away from one another. In the presence of mercury it is likely that all of the cysteine-9 side chains achieve the orientation shown in Figure 3 and that the difference density expected from such a reorientation of cysteine residues is too small to be observed at the contoured density levels used in this map. The mercury appears to be bridging the sulfur atoms of the two cysteine-9 residues in Figure 3 in a linear arrangement. This type of mercury coordination has been observed in $Hg(cysteine)_2$ complexes (Taylor & Carty, 1977) and in proteins treated with inorganic mercury after disulfide bond reduction (Sperling et al., 1969; Arnon & Shapira, 1969).

Although the cysteine-9 residues on adjacent subunits are close enough to be cross-linked by mercury, there is no evidence for a disulfide bond in the native protein. The electron density map of the native protein (Figure 3) shows that the sulfur atoms of the two cysteine-9 residues are at least 4 Å apart and, thus, not within bonding distance. It is possible that the cysteine-9 sulfurs are prevented from closer approach by neighboring tyrosine-8 residues whose phenolic groups are buried inside the protein. Furthermore, cysteine-9 in *T. dyscritum* hemerythrin can be modified directly by treatment with K_2HgI_4, PHMB, or NEM (see below). Sulfhydryl reagents, in general, and organic mercurials, in particular, do not react with disulfides in the absence of reducing agents (Cecil & McPhee, 1959; Jocelyn, 1972). Since no reducing agents were added in our experiments, cysteine-9 must be in the free sulfhydryl form.

Effect of K_2HgI_4 on Quaternary Structure. Having shown that K_2HgI_4 reacts with hemerythrin by forming covalent Hg–S bonds, it was of interest to compare the effects of K_2HgI_4 with those of sulfhydryl reagents known to promote hemerythrin dissociation (Kereztes-Nagy & Klotz, 1963). Hemerythrins from *P. gouldii* and *T. dyscritum* were reacted with sufficient reagent to modify all of the sulfhydryl groups and were incubated for long times at 4 °C to allow the reaction to approach completion. The reaction products were separated by gel filtration on Sephadex G-100 and the subunit composition of each product was identified by its elution volume, V_e, relative to the void volume, V_0. Native octameric hemerythrin (mol wt = 107 000) eluted at $V_e/V_0 = 1.3$. Dissociation products were identified as dimers ($V_e/V_0 = 1.7$) and monomers ($V_e/V_0 = 2.0$) from their apparent molecular weights of 27 000 and 13 000, respectively.

Table I: Hemerythrin Species Resolved by Gel Filtration Chromatography Following Treatment with Sulfhydryl Reagents

reagent	source of hemerythrin	mol of reagent/mol of subunit	incubation time (h)[a]	chromatographic products (%) octamer	dimer	monomer
K_2HgI_4	*P. gouldii*	1.0	24	80		20
	T. dyscritum	1.5	20	100		
PHMB	*P. gouldii*	2.0	4			100
	T. dyscritum	2.0	0	5	10	85
	T. dyscritum	2.0	6		70	30
NEM	*P. gouldii*	10.0	53			100
	T. dyscritum	10.0	52	60	40	
NEM followed by PHMB	*T. dyscritum*	10.0	4			
		2.0	2		90	10

[a] Time of exposure to sulfhydryl reagent before applying protein sample to column.

The results presented in Table I show that K_2HgI_4 treatment causes no dissociation of *T. dyscritum* hemerythrin in solution. This agrees with the crystallographic studies which depended on K_2HgI_4-treated crystals being isomorphous with untreated crystals (Stenkamp et al., 1976). In contrast to the data with *T. dyscritum* hemerythrin, the protein from *P. gouldii* shows 20% dissociation upon treatment with K_2HgI_4. The greater stability of the *T. dyscritum* octamer may be due to the formation of cross-links between subunits by mercury coordination to cysteine-9 residues. These cysteine residues are not present in *P. gouldii* hemerythrin and the binding of mercury to cysteine-50 alone apparently causes some of the octamers to dissociate. However, the extent of dissociation of *P. gouldii* hemerythrin resulting from the reaction of K_2HgI_4 with cysteine-50 is considerably less than that caused by other sulfhydryl reagents (Table I). The less disruptive effect of K_2HgI_4 could be due to the relatively small size of the bound –HgI or to the availability of vacant coordination sites for aspartate-23 and lysine-53 from adjacent subunits, thereby providing additional cross-linking.

Effect of PHMB on Quaternary Structure. As has been reported previously (Kereztes-Nagy & Klotz, 1963; Rao & Kereztes-Nagy, 1973), treatment of *P. gouldii* hemerythrin with PHMB results in complete conversion of the native octamers to monomers (Table I). Hemerythrin from *T. dyscritum* is also completely dissociated by PHMB (Table I). It is likely that the disruption of hemerythrin subunit interactions by this sulfhydryl reagent is due to the introduction of a bulky substituent at cysteine-50.

The dissociation behavior of *T. dyscritum* hemerythrin is complicated by the tendency of the dissociated species to reassociate into dimers (Table I). Since modification of cysteine-50 in *P. gouldii* hemerythrin does not lead to the formation of dimers, it is likely that modification of the additional cysteine at position 9 in *T. dyscritum* hemerythrin is responsible for its dimerization. It is possible to distinguish the different roles of cysteine-50 and cysteine-9 in subunit interactions because the two cysteines differ in their reactivity toward PHMB and NEM.

Under the mild conditions (low concentrations and incubation times less than 1 h) used in spectrophotometric titrations, *T. dyscritum* hemerythrin reacts with PHMB at a similar rate and to a similar extent (1 mol of PHMB bound per subunit) as *P. gouldii* hemerythrin (Figure 4). When PHMB-treated *T. dyscritum* hemerythrin is subjected to gel filtration after similarly mild reaction conditions (higher concentrations but no incubation time), the primary dissociation product is the monomeric species (Table I). Longer incubation times (Table I) result in the progressive appearance of dimers. Thus, PHMB modification of a rapidly reacting sulfhydryl group results in the production of monomers, and subsequent PHMB modification of a slowly reacting sulfhydryl group leads to the production of dimers. By analogy to the effect of PHMB on *P. gouldii* hemerythrin, the rapidly reacting sulfhydryl in *T. dyscritum* hemerythrin is most likely cysteine-50 and the slowly reacting sulfhydryl is cysteine-9. The dimerization process does require the reaction of cysteine-9 with PHMB, rather than simple disulfide bond formation, because isolated PHMB-treated monomers are converted to dimers only in the presence of PHMB.

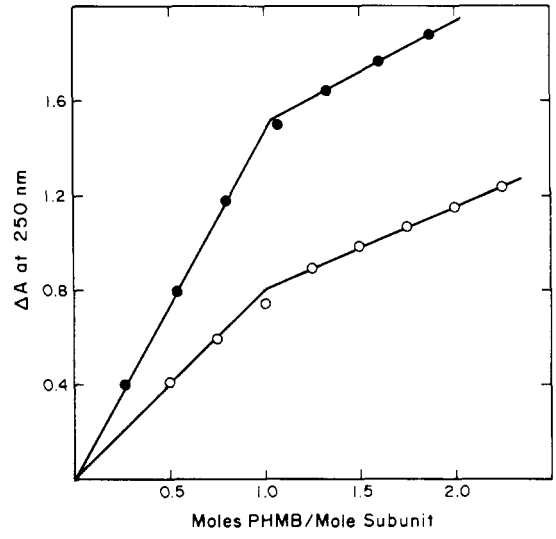

FIGURE 4: Spectrophotometric titration of metazidohemerythrin with PHMB. (○) *T. dyscritum* hemerythrin, 3.2×10^{-5} M in subunit concentration. (●) *P. gouldii* hemerythrin, 6.1×10^{-5} M in subunit concentration. Conditions as in Figure 1.

Due to the difficulty in controlling reaction times in a chromatographic process, a more quantitative measure of the conversion of monomers to dimers was obtained by limiting the amount of PHMB instead of the reaction time. As can be seen in Figure 5, at PHMB/subunit ratios below 0.5, the primary dissociation product is a monomer. As the PHMB/subunit ratio increases above 0.5, dimers become the major dissociation product. Although the end point cannot be determined with certainty, the dimerization reaction appears to level off after the addition of 1.5 mol of PHMB per mol of subunit.

Effect of NEM on Quaternary Structure. A further difference between the two hemerythrins is seen in their sensitivity to dissociation by NEM. As observed previously (Kereztes-Nagy & Klotz, 1963), *P. gouldii* hemerythrin is rapidly dissociated to monomers by treatment with NEM (Table I). Hemerythrin from *T. dyscritum* is more slowly dissociated by

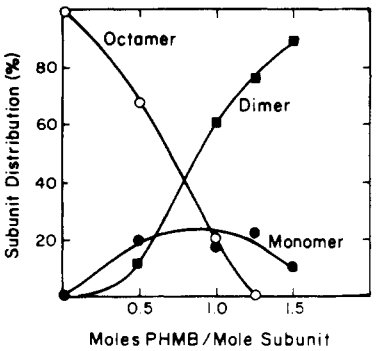

FIGURE 5: Effect of PHMB on the distribution of octamers (○), dimers (■), and monomers (●) in *T. dyscritum* hemerythrin. Protein samples were incubated with PHMB for at least 20 h at 4 °C prior to separation by gel filtration.

NEM and yields only dimers as dissociation products. The slowness of the dissociation reaction could be ascribed to cysteine-50 being less accessible to NEM, while the speed of the dimerization reaction could be ascribed to cysteine-9 being very accessible to NEM. By analogy to PHMB-related dimerization, hemerythrin octamers which have reacted with two molecules of NEM per subunit probably dissociate to monomers which then rapidly reassociate to dimers. In both cases, the dimers are stable species and they maintain their identity upon rechromatography.

Support for the above interpretation comes from the last experiment listed in Table I. Hemerythrin from *T. dyscritum* was treated with NEM under conditions where essentially no dissociation occurs. After removal of unreacted NEM by dialysis, the protein was exposed to PHMB under conditions where monomers are the major dissociation product. As can be seen in Table I, this experiment resulted in an unexpectedly large production of dimers after the brief exposure to PHMB. This indicates that the cysteine modification responsible for dimer formation had already occurred during the initial treatment with NEM. Thus, it appears that cysteine-9 reacts rapidly with NEM and that the addition of a bulky group at this position does not interfere enough with subunit interactions to cause dissociation.

In summary, dimer formation appears to require the cysteine-50-dependent dissociation of the *T. dyscritum* protein into monomeric subunits and the reaction of cysteine-9 with PHMB or NEM. The former occurs more rapidly with PHMB, while the latter occurs more rapidly with NEM. Since both PHMB and NEM are monovalent sulfhydryl reagents (Cecil & McPhee, 1959), the dimerization processes must involve noncovalent interactions between subunits rather than chemical cross-linking. It is possible that the introduction at cysteine-9 of a negatively charged group from the reaction with PHMB leads to new electrostatic interactions resulting in dimerization. A similar possibility exists for the reaction of NEM with cysteine-9, since histidine residues can cause hydrolysis of the bound NEM to yield the *N*-ethylsuccinimate derivative (Benesch & Benesch, 1961). In any case, the dimers resulting from reaction of cysteine-9 with PHMB or NEM are probably different than any dimeric structures present in the native octamer, since the concurrent modification of cysteine-50 disrupts normal subunit interactions (see below).

Effect of Sulfhydryl Reagents on Subunit Contact Regions. The above data show that *T. dyscritum* hemerythrin is more likely to dissociate upon modification of cysteine-50 than upon modification of cysteine-9. The reason for the tendency of cysteine-50 substituents to cause dissociation is that cysteine-50 is located in close proximity to the subunit–subunit contact

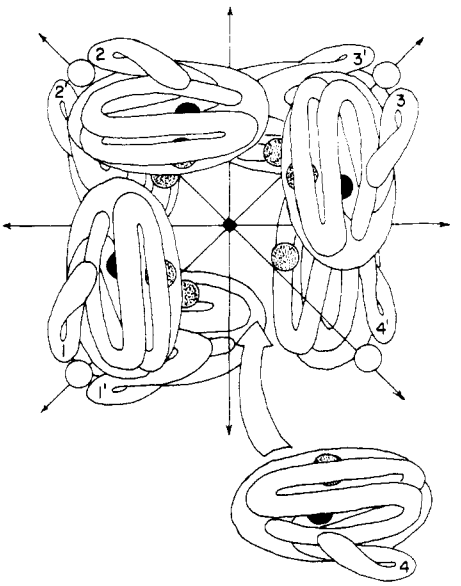

FIGURE 6: Schematic drawing of *T. dyscritum* hemerythrin showing the positions of subunits relative to the twofold and fourfold axes of the molecule. Each subunit has been surrounded by an oval ring to help distinguish between subunits in the upper and lower layers. Filled-in circles represent the position of the iron dimer at the oxygen-binding site of each subunit. Open circles indicate the locations of the four mercury atoms in the K_2HgI_4 derivative which bridge cysteine-9 residues and lie on the noncrystallographic twofold axes through the corners of the molecule. Stippled circles indicate the eight mercury atoms in the K_2HgI_4 derivative which are bound to cysteine-50 residues and are located at the interface between subunits in different layers. One subunit has been removed from the upper layer of the octamer to expose the mercury sites in the lower subunit. The mercury atoms attached to cysteine-50 residues lie near the two subunit contact regions responsible for the octameric structure. The major contact region is between twofold related subunits (e.g., 1 and 1′) near the twofold axis running through the corners of the molecule. The other contact region occurs between fourfold related subunits (e.g., 1 and 2) in each layer of the molecule.

Table II: Amino Acid Interactions at Subunit Contact Regions in *T. dyscritum* Hemerythrin[a]

contact region	subunit 1′ (A helix)	subunit 1 (B helix and BC turn)	subunit 2 (B helix)
twofold corner	Arg-15 ····· Asp-42		
	Thr-19 ····· Arg-49		
	Asp-23 ····· Lys-53		
fourfold layer		Ser-65 ····· Lys-53 (NH)	
		Gln-66 (CO) ····· Arg-48	

[a] From averaged electron density map at 2.8-Å resolution (Stenkamp et al., 1968b). Approximate distribution of residues in helical regions: A helix (15–35), B helix (42–60), C helix (70–85), D helix (95–113). Unless otherwise noted, interactions are between amino acid side chains; atoms in parentheses refer to peptide bond contributions. Subunit designations as in Figure 6.

regions responsible for maintenance of the octameric structure (Figure 6). The major contact region occurs between subunits (1 and 1′) related by a twofold axis through the corner of the molecule and is stabilized by the electrostatic and hydrogen-bonding interactions listed in Table II. Due to the twofold symmetry in this region, each pair of subunits is stabilized by two such sets of interactions. A second contact region occurs between subunits (1 and 2) related by the fourfold axis in a single layer of the molecule (Figure 6 and Table II). This is probably a weaker contact because it contains fewer interacting amino acids and involves only one set of interactions per subunit pair. From Table II it can be seen that modification of cysteine-50 with bulky reagents could disrupt the elec-

trostatic and hydrogen-bonding interactions of neighboring B helix amino acids such as Arg-48, Arg-49, and Lys-53 and, thereby, cause disruption of both subunit contact regions. Furthermore, the monovalent character of organic mercurials such as PHMB precludes the possibility of the cysteine-bound mercury forming additional ligations to the protein, as may be the case with inorganic mercurials such as K_2HgI_4.

Conclusions

In octameric hemerythrins, cysteine-50 is located in a region of the protein which is critical for subunit interactions. Treatment with K_2HgI_4 results in the coordination of –HgI to the sulfur of cysteine-50 but causes little or no dissociation of the protein. Binding of bulkier substituents such as PHMB and NEM to the sulfur of cysteine-50 disrupts subunit interactions and leads to complete dissociation. The ability of K_2HgI_4 to bind to proteins like hemerythrin without causing dissociation suggests that this mercury complex may be more useful for the isomorphous replacement technique in the X-ray crystallographic determination of protein structure than has been previously recognized.

At the K_2HgI_4 concentrations used in these experiments, it can be calculated from known stability constants (Eliezer & Marcus, 1963; Hansen et al., 1963) that the major mercury species present are HgI_3^- and HgI_4^{2-}. However, in apolar solvents HgI_2 becomes the predominant species (Grdenić, 1965). Since protein sulfhydryl groups tend to be located in more hydrophobic environments, it is likely that HgI_2 would be the reactive species. The smaller size and electroneutrality of HgI_2 help to explain the ease with which it reacts with hemerythrin sulfhydryl groups and its minimal disruptive effects.

In the case of *T. dyscritum* hemerythrin, K_2HgI_4 appears to react rapidly with both cysteine-50 and cysteine-9. The other sulfhydryl reagents studied, PHMB and NEM, showed considerably greater selectivity in their rates of reactions with the different cysteines. PHMB reacted more rapidly with the relatively buried cysteine-50, and NEM reacted more rapidly with the relatively exposed cysteine-9. The difference in reactivity at cysteine-50 could be due to the greater affinity of PHMB for buried sulfhydryl groups (Means & Feeney, 1971) or to electrostatic effects related to the fact that PHMB is charged whereas NEM is neutral. The slow reaction of PHMB with cysteine-9 could be due to electrostatic repulsion by the side chains of aspartate-6 and aspartate-12 which are in the vicinity of cysteine-9.

This study also has implications regarding the toxicity of mercury-containing compounds. It is well known that inorganic mercury is less toxic than organic mercury (Bremner, 1974; MacGregor & Clarkson, 1974). Much of this difference lies in the greater lipid solubility and consequent membrane permeability of organic mercury compounds. Furthermore, the multivalent character of inorganic mercury increases its ability to be detoxified by binding to the kidney protein, metallothionein (Magos, 1973). The present work indicates that inorganic mercury may also have a less deleterious effect on the tertiary and quaternary structures of proteins, thereby providing an additional factor to account for the lower toxicity of inorganic mercury compounds.

Acknowledgments

Themiste dyscritum was kindly supplied by Robert C. Terwilliger of the Oregon Institute of Marine Biology. The temperature-controlled cell compartment for the Cary 14 spectrophotometer was designed and generously loaned by Dennis W. Barnum of Portland State University. We thank Therese Westrup for her technical help in performing this work, Gordon L. Kilgour, Mary L. Taylor, and Horace F. White for their assistance in the design of experiments, and Richard Doty, Peter Lammers, Malkanthi Paulis, Thomas M. Loehr, and Kenneth A. Walsh for their interest and valuable suggestions. We also thank Lyle H. Jensen for his continued enthusiasm and support.

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