hydrogen was effected by constant-current electrolysis of 0.1 N H_2SO_4 in the same electrolysis cell for a measured time interval and treatment of the resulting gas samples exactly as described above.

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Mercaptan Involvement in Dissociation and Reconstitution of Hemerythrin*

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The oxygen-carrying pigment hemerythrin can be dissociated into eight subunits (merohemerythrins) by the addition of a mercurial such as salyrganic acid. Other mercaptan-blocking reagents are also effective. The native macromolecule can be reconstituted from subunits if the mercurial is removed with cysteine ethyl ester and the iron is reduced to the ferrous state. Similarities and differences in macromolecular constitution between hemerythrin and hemoglobin are discussed.

The oxygen-carrying pigment hemerythrin contains eight oxygen-binding sites per particle of 107,000 mw (Klotz and Keresztes-Nagy, 1962, 1963). It has also been established that a hemerythrin macromolecule can be dissociated into eight subunits (merohemerythrins) by a variety of chemical treatments: exposure to low or high *p*H; treatment with a detergent, sodium dodecyl sulfate; reaction with succinic anhydride. These chemical effects show that in hemerythrin the subunits are held together by noncovalent bonds. It might be possible, therefore, to reverse the disaggregation process and convert merohemerythrin back to hemerythrin. The chemical treatments previously used, however, are relatively strong and generally irreversible, and hence an alternative method of dissociation is essential if reaggregation is to be achieved.

Earlier studies (Klotz *et al.*, 1957) have shown the presence of sulfhydryl groups in hemerythrin. Recalling the discovery of Madsen and Cori (1956) that phosphorylase can be dissociated by blocking of sulfhydryl groups we felt a similar approach might be effective with hemerythrin. Such indeed has proved to be true; the pigment can be dissociated with a mercurial (as well as other mercaptan reagents) and reconstituted upon removal of bound mercurial.

Experimental Procedures and Results

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*).

Salyrganic acid was purchased from Winthrop Laboratories and was warranted by the manufacturer to be 97–103% pure. *p*-Chloromercuribenzoic acid was purchased from Nutritional Biochemicals Corp., *p*-chloromercurisulfonic acid from Sigma Chemical Co., neohydrin from Lakeside Laboratories, *N*-ethylmaleimide from Mann Research Laboratories, and oxidized glutathione from Schwarz Bioresearch. The azomercurial 4-(*p*-dimethylaminobenzeneazo)phenylmercuric acetate was prepared by procedures described previously (Horowitz and Klotz, 1956). All other reagents were obtained from general commercial sources.

Sulfhydryl Content of Hemerythrin.—Previous analyses for mercaptan groups in oxyhemerythrin (Klotz *et al.*, 1957) using an amperometric silver titration (Benesch *et al.*, 1955) indicated an SH-Fe ratio of approximately 1. Since there are 2 Fe atoms per subunit (merohemerythrin) this result implies 2 SH groups in each subunit. Since then evidence has accumulated that silver titrations are not specific for mercaptan groups in hemerythrin. Using the colorimetric monofunctional mercurial titration developed recently (Klotz and Carver, 1961), we find that oxyhemerythrin in 8 M urea, after equilibration with a mercurial for 0.5–1.0 hour, shows an SH-Fe ratio of 0.42. This result indicates 0.84, or approximately 1, SH group per subunit of merohemerythrin (with 2 Fe). Amino acid analyses (by Mr. W. R. Groskopf) also give approximately one cysteic acid residue in merohemerythrin. It seems clear, therefore, that there is only 1 SH in each subunit, or 8 SH in the native hemerythrin macromolecule of 107,000 mw.

Dissociation of Hemerythrin.—Merohemerythrin subunits were obtained by the following procedure. Crystalline oxyhemerythrin was dissolved in 0.1 M sodium cacodylate buffer at *p*H 5.9, the buffer solution containing also 0.15 M sodium chloride. The protein concentration, measured and expressed in terms of iron content, was made 6.0×10^{-4} M. This corresponds to 3×10^{-4} M mercaptan. To a series of 2-ml portions of this solution were added increasing quantities of 3×10^{-3} M salyrganic acid, a mercurial, so as to pro-

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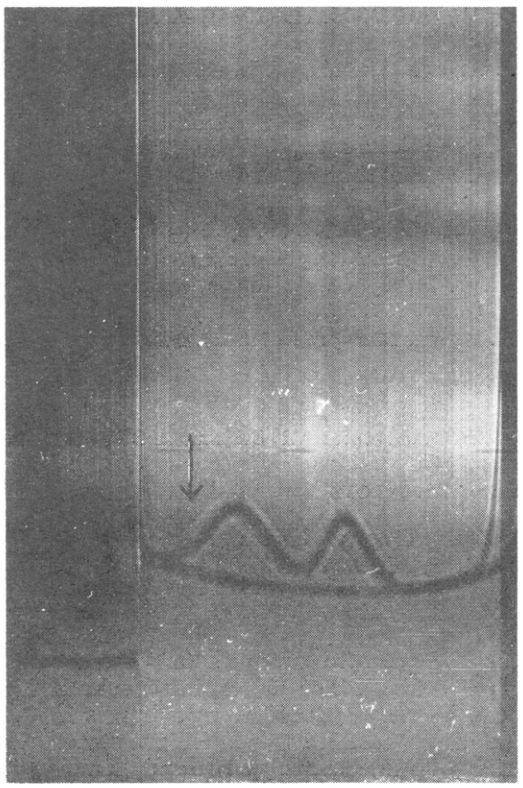

FIG. 1.—Schlieren photograph of hemerythrin in presence of salyrangic acid sedimented at 59,780 rpm. Arrow indicates position of synthetic boundary. Peaks become completely separated if sedimentation is continued.

duce the following ratios of Hg-SH: 0.25, 0.50, 0.75, and 1.00. The solutions were allowed to stand at room temperature for 15 minutes and then at 4° for 6 hours. Then each solution was dialyzed overnight in the cold room against 100 ml of isotonic NaCl. Thereafter the protein solution was placed in one compartment of a double-sector synthetic-boundary ultracentrifuge cell, the dialysate was placed in the other compartment, and the solutions were centrifuged at 59,780 rpm in the Spinco Model E ultracentrifuge. In this way the areas under the refractive index gradient (Figure 1) curve could be measured easily for both undissociated hemerythrin (with a sedimentation coefficient of about 6.5 S) and merohemerythrin (sedimentation coefficient of 2 S). Actual area measurements were made from a photographic enlargement with a planimeter. There is no evidence from sedimentation experiments of any intermediates between the 6.5 S and 2 S particles. If ultracentrifugation of a partially disaggregated system, such as that shown in Figure 1, was continued, complete separation of the peaks occurred.

TABLE I
CLASSIFICATION OF MERCAPTAN-BLOCKING REAGENTS IN THEIR EFFECT ON HEMERYTHRIN

Class I	Class II	Class III
Salyrganic acid, *o*-[(3-hydroxymercuri-2-methoxypropyl)-carbamyl]phenoxy-acetic acid	Neohydrin, 1-[3-(chloromercuri)-2-methoxy-propyl] urea	Glutathione
p-Chloromercuribenzoic acid		Ag^+
p-Chloromercuriphenylsulfonic acid	Iodoacetic acid	Hg^{++}
N-Ethylmaleimide	*p*-Dimethylamino-phenylazophenyl-mercuric acetate	
Iodoacetamide		
Cyanogen bromide		

The results of the addition of salyrganic acid to hemerythrin in cacodylate-chloride buffer are presented in Figure 2. As can be seen there is a linear one-to-one relationship between the percentage of SH groups titrated and the percentage dissociation of the macromolecule, and complete dissociation is achieved at the stoichiometric equivalence point. The linear relationship indicates an "all-or-none" reaction; that is, as soon as one mercurial molecule is bound to one site, binding to other sites on the same protein molecule, followed by dissociation, occurs in preference to binding on sites of a different protein molecule.

We have also examined several additional mercurials and other substances capable of reacting with mercaptan groups. In regard to their effect on the dissociation of hemerythrin these compounds fall into three classes (Table I). Those in class I produce yellow-colored merohemerythrins, native in structure as judged from sedimentation behavior. The reagents in class II had some effect in that at relatively high concentration they slowly generated a colorless subunit whose behavior indicated some denaturation. Substances in class III were completely ineffective in producing dissociation.

Salyrganic acid and *p*-chloromercuriphenylsulfonic

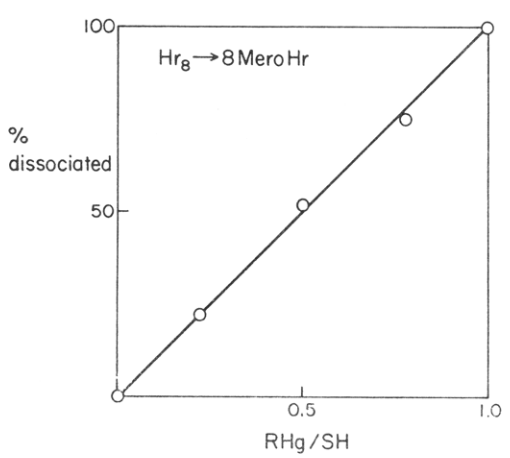

FIG. 2.—Percentage of hemerythrin dissociated into merohemerythrin subunits as a function of moles of mercurial salryganic acid added to block mercaptan groups. Cacodylate-chloride buffer, *p*H 6–7.

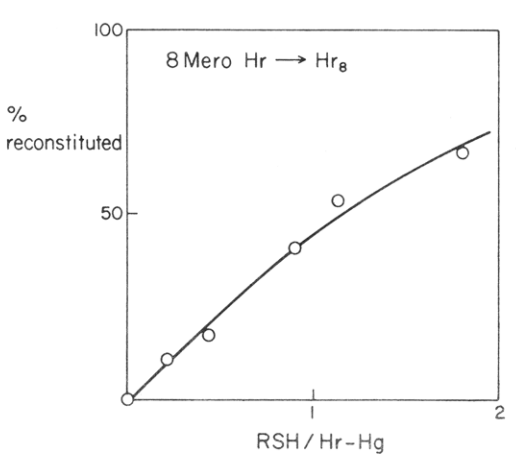

FIG. 3.—Percentage of reconstituted hemerythrin obtained from merohemerythrin subunits upon addition of cysteine ethyl ester in increasing stroichiometric ratios to the mercurial bound by merohemerythrin.

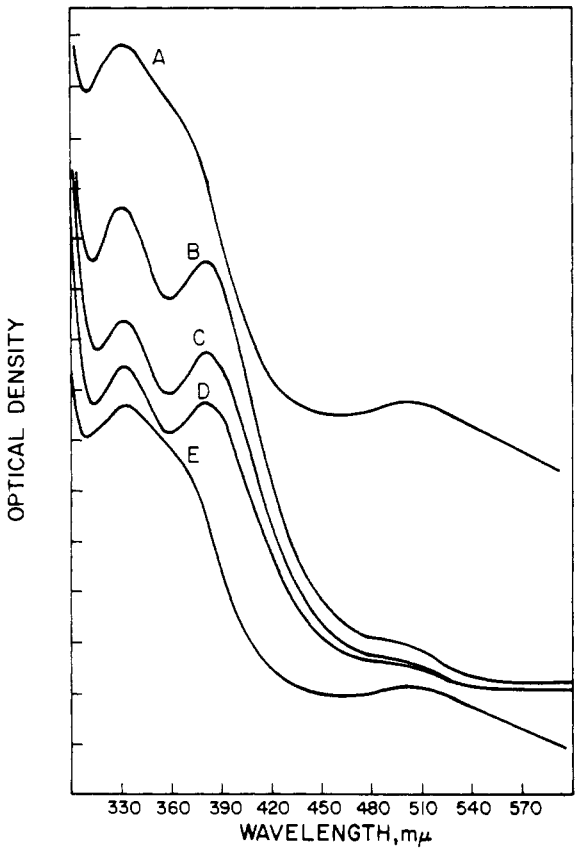

FIG. 4.—Spectra of hemerythrins at various stages in dissociation and reconstitution. A, original oxyhemerythrin; B, methemerythrin; C, merohemerythrin, subunits; D, reconstituted methemerythrin; E, reconstituted oxyhemerythrin. The curves have been shifted arbitrarily vertically, or the solutions were diluted, so that the spectra should not overlap. Divisions on the ordinate axis are 0.1 optical density units.

acid were most effective between *p*H's 6–7, whereas *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, and iodoacetamide worked best at *p*H's 8–9. A particularly interesting reagent is cyanogen bromide. When added to hemerythrin in slight stoichiometric excess (to the SH content) it produces rapid and complete dissociation throughout the *p*H range 6–9. This reagent converts SH groups to SCN (Swan, 1958):

$$P\text{—}SH + CNBr = P\text{—}SCN + HBr$$

It may also react with the thioether group of methionine (Swan, 1958) and then promote cleavage of the peptide chain (Gross and Witkop, 1962). However, it is unlikely that the methionine cleavage reaction occurs under our conditions since it is generally carried out under highly acidic conditions (0.1 M HCl).

Merohemerythrin produced by the reagents of class I is not stable at room temperature and tends to precipitate after about 30 minutes. On the other hand it can be kept at 4° for weeks without showing any signs of denaturation.

Reconstitution of Hemerythrin.—It seemed reasonable to expect that the addition of a small-molecule mercaptan would remove the mercurial from the conjugate with merohemerythrin and thus set the stage for reaggregation. Cysteine at low concentrations (*ca.* 10^{-3} M) did produce partial reaggregation to the 6 S particle, but larger quantities of cysteine caused some protein precipitation. On the other hand cysteine ethyl ester, with a lower pK_a for the SH group, was maximally effective in regenerating methemerythrin.

Variable quantities of this mercaptan as an 0.01 M solution in 0.15 M NaCl adjusted to *p*H 8 with dilute NH_4OH, were added to 1.0-ml aliquots of merohemerythrin dissolved in 0.15 M NaCl and at an iron concentration of 8.8×10^{-4} M. The following ratios of cysteine ethyl ester to mercurial in the protein were used: 0.23, 0.46, 0.92, 1.14, and 1.8. Within 15–30 minutes after mixing the solutions were sedimented in the Spinco Model E ultracentrifuge at 59,780 rpm. Again the schlieren photographs were magnified and the areas of merohemerythrin and hemerythrin were measured with a planimeter. The extent of re-formation of methemerythrin in this series of experiments is shown in Figure 3. In additional experiments it was found that nearly 100% reaggregation was obtained when the RSH-Hg ratio reached 5.

The reaggregated methemerythrin shows a spectrum (Figure 4) and sedimentation behavior (Figure 5) characteristic of the original methemerythrin (with Cl^- complexed to the Fe [III]). Absorption peaks at 330 and 380 mμ appear in both cases (Figure 4). The schlieren patterns (Figure 5) of the original and reaggregated methemerythrin are essentially indistinguishable. Sedimentation coefficients determined under identical conditions (0.5% protein in 0.01 μ cacodylate buffer containing 0.09 μ NaCl, *p*H 6.3) gave 6.79 S and 6.68 S, respectively, for s_{20}. Under the same conditions s_{20} was 1.95 S for merohemerythrin.

Reduction of methemerythrin (Fe [III]) to hemerythrin (Fe [II]) was achieved upon addition of $NaBH_4$ and Na_2SO_3, as well as by dialysis against 0.001 M $Na_2S_2O_4$. If oxygen was then admitted to the solution oxyhemerythrin was obtained, as was evident from the appearance of a pink color. The spectrum (Figure 5) of the reconstituted protein was essentially identical with that of the original native oxyhemerythrin.

Essentially complete reconstitution was obtainable if the complete cycle from oxyhemerythrin to oxyhemerythrin was carried out without delays. Thus in one experiment a sample of oxyhemerythrin in 0.15 M NaCl with an optical density (at 500 mμ) of 0.380 was disaggregated by addition of solid salyrganic acid. Completeness of dissociation was verified by ultracentrifugation of a portion of this solution. Less than 30 minutes later solid cysteine ethyl ester was added. Reaggregation was verified by ultracentrifugation of a portion of this solution. The remainder of the solution was dialyzed for about 24 hours against 0.001 M $Na_2S_2O_4$ in 0.15 M NaCl at *p*H 6. Oxygen was then admitted to the reduced protein solution; the optical density at 500 mμ was found to return to 0.340. There was probably some dilution of protein during dialysis. Even if such dilution is overlooked, approximately 90% reconstitution was achieved.

DISCUSSION

The relationships between the different forms of hemerythrin may be compactly represented by Figure 6. Starting with deoxyhemerythrin (upper left of Fig. 6), one has an octamer, each subunit of which contains a pair of Fe^{II} atoms. The protein can be reversibly oxygenated to oxyhemerythrin, again an octamer, but, at least in our opinion (Klotz *et al.*, 1957), containing iron as Fe^{III} and oxygen as O_2^{2-}. Oxidation of either deoxy- or oxyhemerythrin leads to methemerythrin, again an octamer but with iron definitely Fe^{III} and with O_2 (or O_2^-) replaced by Cl^- or by some other small anion (Keresztes-Nagy, 1963, unpublished observations). Methemerythrin may be dissociated into its merohemerythrin subunits upon addition of a mercurial, RHgX. It is also possible to go directly

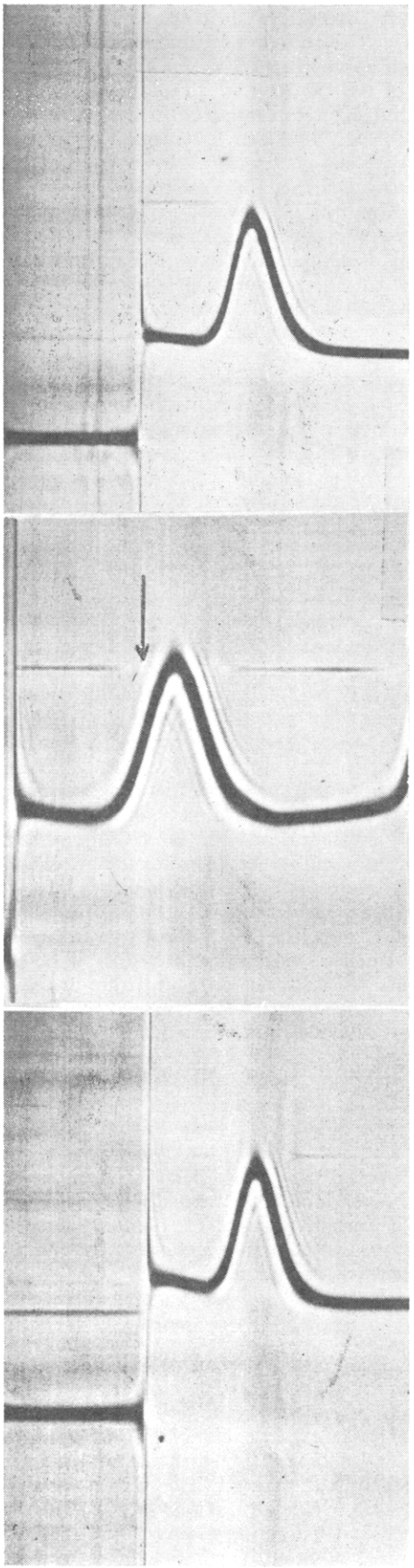

FIG. 5.—Sedimentation behavior of hemerythrins. Top, original methemerythrin; middle, merohemerythrin, subunits, arrow indicating position of synthetic boundary; bottom, reconstituted methemerythrin. All pictures taken at 20 minutes after attainment of speed of 59,780 rpm.

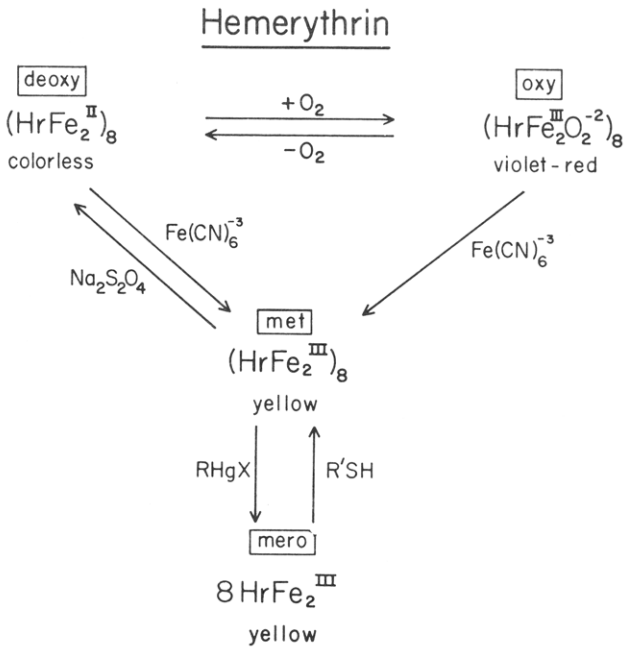

FIG. 6.—Interrelationships between various states of hemerythrin.

from oxyhemerythrin to merohemerythrin on addition of a mercurial, and the subunit obtained contains iron in the Fe^{III} state. The mero form is unable to bind oxygen either in the Fe^{III} state or after reduction to the (colorless) Fe^{II} state. If oxygen is admitted to the Fe^{II} mero-protein, one obtains only the yellow Fe^{III} compound.

One can return to the naturally occurring protein by the following steps. If one removes the mercurial from merohemerythrin by addition of a mercaptan, RSH, the subunits reaggregate into methemerythrin. The iron may then be reduced to Fe^{II} with $Na_2S_2O_4$, and deoxyhemerythrin is obtained. Admission of oxygen regenerates oxyhemerythrin and thus completes the cycle.

There are now a number of examples of a protein dissociating into subunits when its SH groups are blocked. In addition to phosphorylase (Madsen and Cori, 1956) and hemerythrin, potato virus X (Reichmann and Hatt, 1961), myosin (Kominz, 1961), turnip yellow mosaic virus (Kaper and Houwing, 1962) and formyltetrahydrofolate synthetase (Himes and Rabinowitz, 1962) have recently been found to do so. In almost all cases *p*-chloromercuribenzoate has been at least one of the reagents used to cause dissociation, and there has been some temptation to ascribe the effect of the mercurial to its bulk or charge. The same explanation could be applied to salyrganic acid, the mercurial used in most of our experiments with hemerythrin. However, at least with hemerythrin it is doubtful that bulk or charge is a crucial feature of the dissociating reagent since CNBr works well also and it introduces a small noncharged entity.

It is tempting to try to ascribe some specific role to the SH group in holding subunits together but as yet no convincing picture is available. With phosphorylase, kinetic experiments (Madsen and Cori, 1956) have established that the role of SH in maintaining activity of the enzyme cannot be directly related to its involvement in keeping the macromolecule in its associated form, for the rate of loss of activity in the presence of mercurial differs from the rate of dissociation. Similar observations, but still scanty, have been made with hemerythrin. In reactions with salyrganic

acid it is qualitatively obvious that the undissociated hemerythrin loses its violet-red color (and thus its oxygen-carrying capacity) rapidly. Likewise in experiments with the azomercurial dye, it was found that dye was bound to the protein but that the hemerythrin, standing at about 4°, did not dissociate until several days had passed. The molecular events following attachment of the mercurial to the SH are still obscure.

Since hemerythrin is an oxygen-carrying pigment it is of interest to compare its macromolecular constitution with that of hemoglobin. The native macromolecule in the latter case has four sites for oxygen, and four protein subunits, in contrast to eight for hemerythrin. In hemoglobin, the subunits generally fall into two categories (e.g., α and β); in hemerythrin the identity or nonidentity of merohemerythrins has yet to be established. In both proteins SH groups are present but not S–S. On the other hand, blocking of SH groups disaggregates hemerythrin but does not change the macromolecular size of hemoglobin, although it does affect interactions between oxygen-binding sites. In an evolutionary sense these two pigments seem very far apart, not only in the absence of a heme group in hemerythrin, but even in differences in protein component. The subunit merohemerythrin has a molecular weight near 13,500, whereas that of hemoglobin is near 17,000, although a *paramecium* hemoglobin with weight near 13,000 has been reported recently (Smith *et al.*, 1962). It seems more likely that hemerythrin may be related to other nonheme iron-containing proteins such as ferredoxin (Valentine, *et al.*, 1963) and homogentisate oxidase (Flamm and Crandall, 1963) than to the heme pigments, despite the closer similarity to the latter group in physiological function.

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Binding Sites, Reactivation Phenomena, and Possible —S—S— Groups of Rabbit Muscle Aldolase*

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Equilibrium dialysis and ultracentrifuge experiments with rabbit muscle aldolase in presence of added dihydroxyacetone phosphate or fructose 1,6-diphosphate establish the presence of one substrate binding site per aldolase molecule. The binding of fructose 1,6-diphosphate has a K_d of about 4×10^{-6}, and is considerably stronger than the binding of dihydroxyacetone phosphate. Removal of the terminal tyrosine residues lowers the affinity of the enzyme for fructose diphosphate. Aldolase exposed to *p*H 3 loses activity and shows structural disorganization and chain separation which is nearly completely reversible upon return to *p*H 8. This suggests a dominant role of primary structure in determination of configuration of this multichain enzyme. A rapid initial and much slower secondary rate of reactivation was observed, and addition of chelating agents prevented the second, slower reactivation. No metals which might be suspected of contributing to aldolase activity were found in the native enzyme, however. Upon reduction with mercaptoethanol in 8 M urea, aldolase shows appearance of about one —SH group per enzyme molecule. Thus native aldolase probably has one or less reducible —S—S— group per molecule.

The presence in aldolase of at least three peptide chains has been demonstrated by determinations of carboxy- and amino-terminal groups (Kowalsky and Boyer, 1960; Udenfriend and Velick, 1951). This raised the question of whether one or more substrate binding sites are present on the aldolase molecule, and one purpose of this paper is to report data on determination of the number of binding sites per molecule. Concomitant with these studies, other investigations were made on the regaining of activity of acid-inactivated aldolase. Results obtained led to interest in possible disulfide groups and undetected metals in muscle aldolase, and results of studies in this area are also reported.

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