Preparation of Two-Dimensional Arrays from Purified Beef Heart Cytochrome *c* Oxidase†

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ABSTRACT: A method for preparing two-dimensional crystals from highly purified beef heart cytochrome *c* oxidase is described. This involves mixing the enzyme with phosphatidylcholine and then extracting excess lipid with deoxycholate. The reconstituted crystals show *P*1 symmetry. Alternating rows of monomers are more closely packed (along the *b* axis) than in the previously described $P12_1$ crystals. However, the monomer structure in projection is the same in the two crystal forms. The $P12_1$ crystal form has been reacted with trypsin. This treatment did not alter the crystals but removed most of the impurities present in these cytochrome *c* oxidase preparations of low purity.

Our studies of the structure and function of cytochrome *c* oxidase have progressed along two so far independent lines. First, we have prepared two different, two-dimensional crystal forms of the enzyme and studied each of these by electron microscopy and image reconstruction procedures (Henderson et al., 1977; Fuller et al., 1979). This work has provided information about the size, shape, and other gross features of the structure of the protein. Second, we have been conducting cross-linking and labeling experiments directed toward detailing the arrangement of polypeptides and prosthetic groups within the cytochrome *c* oxidase complex [e.g., Downer et al. (1976), Briggs & Capaldi (1977, 1978), Ludwig et al. (1979), Prochaska et al. (1980), and Bisson et al. (1980)].

A major limitation to combining the two approaches and using the same preparations for all experiments is that, to date, crystallization of the enzyme has only been obtained from impure samples of cytochrome *c* oxidase. The $P2_12_12$ crystal form, prepared by treating mitochondria with Triton X-100, has a heme *a* to protein ratio of no more than 8 nmol/mg, which is only 60–70% of that of the most pure preparations of beef heart cytochrome *c* oxidase. Crystals of the $P12_1$ type, made with deoxycholate, have a maximal heme *a* content of 5 nmol/mg of protein. Both preparations thus contain many contaminating polypeptides, which would interfere with the execution and interpretation of cross-linking, labeling, or other protein modification studies. We have, therefore, sought methods of obtaining two-dimensional crystals of purified beef heart cytochrome *c* oxidase that are amenable to both structural analyses as well as chemical probing of the arrangement of subunits. Here we describe two approaches that provide highly ordered two-dimensional crystals of the enzyme, one beginning with purified enzyme and the other using crystals of crudely prepared cytochrome *c* oxidase and removing impurities from this preparation.

Materials and Methods

Enzyme Preparations. Purified beef heart cytochrome *c* oxidase was prepared by the method of Steffens & Buse (1976). Triton X-100 derived $P22_12_1$ crystals were prepared by a modification of the method of Sun et al. (1968) as described by Vanderkooi et al. (1972). Deoxycholate-derived $P12_1$ crystals were prepared as described in Fuller et al. (1979).

Gel Techniques. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the procedure of Swank & Munkres (1971) with 12.5% acrylamide and *N,N'*-methylenebis(acylamide) (1:10). Gels were stained and destained as described by Downer et al. (1976).

Preparation of Reconstituted Crystals. L-α-Phosphatidylcholine (0.5 mL, 100 mg/mL) in chloroform–methanol (2:1) was mixed with 5 μL of 10 mM butylated hydroxytoluene (used to prevent lipid peroxidation) in ethanol and then dried with nitrogen. Ethanol (1 mL) was added and the mixture dried first with nitrogen and then under vacuum for 1 h. Ten milliliters of 50 mM Tris–acetate,[1] 0.5 mM EDTA, and 5 μM butylated hydroxytoluene (pH 7.0) was added to the dried lipid and the mixture sonicated with a Branson bath sonicator. The sonication step was continued until the phospholipid solution was optically clear (usually 1 h). Purified oxidase (200 μL, 30 mg/mL) prepared by the method of Steffens & Buse (1976) was added to 2.4 mL of L-α-phosphatidylcholine vesicles (5 mg/mL). This mixture was stirred on ice for 12 h and then dialyzed against 0.66 M sucrose, 50 mM Tris-HCl, and 5 μM butyrated hydroxytoluene for 7 h to remove residual cholate. Samples at this stage were examined by electron microscopy. The reconstituted material was treated with 1 mg of deoxycholate/mg of protein, solid KCl was added to a concentration of 1 M, and the solution was stirred on ice. After 48 h, the material was placed on top of a sucrose gradient (1.5–2.0 M/5 mL of total volume) in 10 mM Tris-HCl and 2 mM sodium deoxycholate, pH 8.0, and spun in an SW 50.1 rotor for 20 h at 40000*g*. The crystals were found in a tight band near 1.8 M sucrose.

Trypsin Treatment. Preparations containing deoxycholate-derived $P12_1$ crystals were incubated with trypsin TPCK (Worthington) in 0.66 M sucrose and 50 mM Tris-HCl, pH 8.0, for 1 h at room temperature. The reaction was stopped by addition of 5 mg of soybean trypsin inhibitor/mg of trypsin. The mixture was diluted 5-fold with 0.66 M sucrose and 50 mM Tris-HCl, pH 8.0, and the crystals were separated from trypsin, inhibitor, and cleaved fragments by centrifugation.

Other Methods. Microscopy and image reconstruction were performed as described in Fuller et al. (1979) except that

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DOC, deoxycholate. We use here the nomenclature for two-sided plane groups proposed by Holser (1958). An outline of this scheme is given in Fuller et al. (1979).

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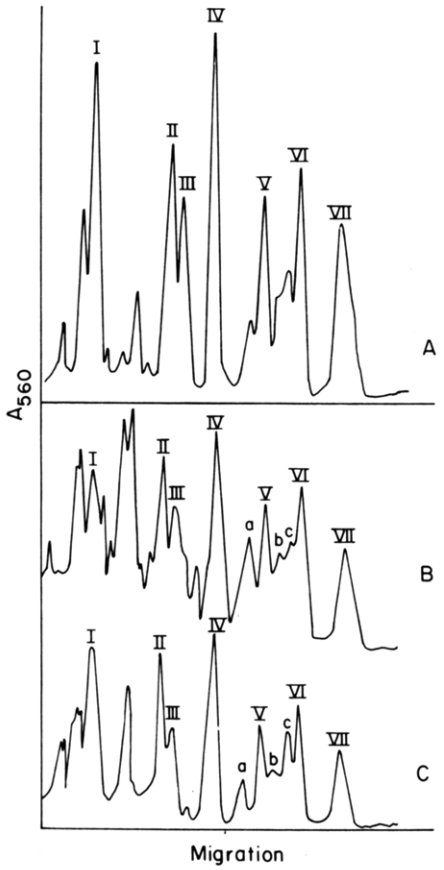

FIGURE 1: Densitometric traces of $NaDodSO_4$–polyacrylamide gels of different crystalline preparations of beef heart cytochrome *c* oxidase. (A) Reconstituted crystals prepared with enzyme isolated according to Steffens & Buse (1976). (B) Preparation containing deoxycholate-derived crystals. (C) Preparation containing Triton X-100 derived crystals.

symmetry averaging was not applied to the *P*1 crystals. All images processed were taken under minimum dose conditions.

Protein concentration was determined by the method of Lowry et al. (1951). Heme *a* was estimated as described by Williams (1974). Cytochrome *c* oxidase activity was measured polarographically according to Vik & Capaldi (1977). Lipid composition was analyzed as described in Robinson & Capaldi (1977).

Cholic acid was purchased from Aldrich and purified by recrystallization from ethanol. Deoxycholic acid (Gold label) was purchased from Aldrich; L-α-phosphatidylcholine and butylated hydroxytoluene were obtained from Sigma. TPCK trypsin and soybean trypsin inhibitor were obtained from Worthington.

Results

Preparation of Two-Dimensional Crystals from Purified Cytochrome c Oxidase. Lipid-depleted cytochrome *c* oxidase, isolated according to Steffens & Buse (1976), was used as the starting material for the preparation of two-dimensional arrays. Typically, this enzyme preparation contained 10–11 nmol of heme *a*/mg of protein and 50 μg of phospholipid/mg of protein, most of which was cardiolipin. The polypeptide composition as resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis is shown in Figure 1A. Seven major bands (I–VII) that are characteristic of beef heart cytochrome *c* oxidase were clearly separated. Bands I, II, III, IV, V, and VI are polypeptides with approximate molecular weights of 50 000, 28 000, 33 000, 17 000, 12 500, and 8000, respectively [for reviews, see Capaldi (1979) and Buse et al. (1981)]. Band VII has been

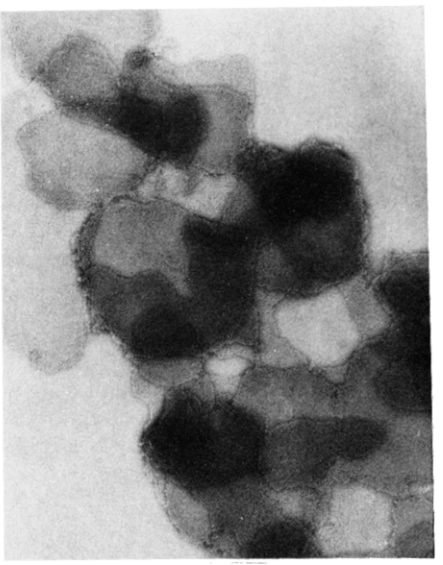

FIGURE 2: Electron micrograph of a negatively stained preparation of reconstituted crystals. Protein appears white and stain black in these positive images.

shown to contain a mixture of three different polypeptides with Ile, Phe, and Ser, respectively, as N-terminal amino acids (Buse et al., 1981). One or more of these small polypeptides may be subunits of the enzyme. Substoichiometric amounts of polypeptides labeled a–c in Figure 1 were also present. Figure 1 also shows the gel profile of samples of the previously described two-dimensional crystals made with Triton X-100 (gel B) and deoxycholate (gel C), respectively. The much greater purity of the preparation used in the reconstitution experiments is clearly evident from Figure 1.

Two-dimensional arrays of cytochrome *c* oxidase were formed by mixing cholate-solubilized enzyme and vesicles of phosphatidylcholine (protein:lipid ratio of 1:2 w/w) with stirring overnight and then by dialyzing the solution to remove detergent. This procedure generated a heterogeneous population of vesicles, seen in the electron microscope to range in size from less than 500 Å up to several microns in diameter. The smaller vesicles appeared devoid of protein. The vesicular preparation was incubated in the presence of deoxycholate (1 mg/mg of protein) at high-salt concentration (1 M KCl) and then centrifuged through a sucrose gradient to remove excess detergent and any phospholipid not associated with protein. The cytochrome *c* oxidase containing fractions obtained showed large two-dimensional crystals as evident in the electron micrograph of the negatively stained material shown in Figure 2.

When less deoxycholate was used (0.5 mg/mg), a mixture of vesicles and small crystals resulted. Ordered arrays were never obtained in the absence of salt even when higher concentrations of deoxycholate (i.e., greater than 1 mg/mg of protein) were used. The two-dimensional arrays obtained with purified cytochrome *c* oxidase will be called reconstituted crystals to distinguish them from previously described arrays for which the nomenclature Triton X-100 derived ($P22_12_1$ form) and deoxycholate-derived ($P12_1$ form) crystals will be used.

Electron Microscopy and Optical Diffraction of the Reconstituted Crystals. The reconstituted crystals showed many features in common with the deoxycholate-derived crystals. Both have the same ragged sheet morphology. However, the reconstituted crystals were better ordered with fewer dislocations and irregularities. The *a* axis of the unit cell in both crystal forms was close to 68 Å. In the reconstituted crystal,

the length of the *b* axis was found to vary from 144 to 154 Å within a single preparation. The a^*/b^* ratio (2.12–2.26) was still considerably closer to the a^*/b^* ratio of the deoxycholate-derived crystals (2.5) than that of the Triton X-100 derived crystals (1.25). Optical diffraction showed that the reconstituted crystals exhibit lower projection symmetry than either the deoxycholate-derived crystals (*Pg*) or Triton X-100 derived crystals (*Pgg*). Examination of the phases of computational transforms indicates that the projection displays a symmetry no higher than *P*1. Average departure from *Pg* symmetry varied between 53° and 67° for all reflections to a resolution of $^1/_{20}$ Å in the phases of five images tested. This lack of symmetry does not appear to result from lattice disorders because the reflections do not display phase gradients or split intensity profiles characteristic of such disorders (Fuller et al., 1979). Uneven staining of the two sides of the crystal could be responsible, but the reconstituted crystals show only *P*1 symmetry under several staining conditions including those which give even staining of the deoxycholate-derived crystals. For these reasons we believe that the intrinsic symmetry of the reconstituted crystals is *P*1 and have generated filtered projections without phase and amplitude averaging.

A filtered projection of the reconstituted crystal is shown in Figure 3. The lack of symmetry averaging in the processing leads to differences in the projection image of the two monomers in the unit cell. Both monomers show a large and a small domain, similar in dimensions and separated by the same distance as the CM_1 and M_2 domains of the deoxycholate-derived crystals. The differences between images are easily accounted for by a small variation in staining or a slight tilt between the two monomers. Importantly, the disconnected feature in the reconstitution of the deoxycholate-derived crystals, labeled X in our previous work (Fuller et al., 1979), is missing from the filtered projections of the reconstituted crystals. This confirms our original conclusion that this feature represents a disorder artifact in the crystal rather than a discrete piece of protein.

Trypsin Digestion of Preformed Crystals of Cytochrome c Oxidase. The second approach used for preparing two-dimensional crystals of pure enzyme was to begin with crystals of an impure preparation and remove contaminants from this. The starting point for these experiments was the well-characterized deoxycholate-derived crystals. Recent studies have shown that incubation of isolated and detergent-dispersed cytochrome *c* oxidase with low levels of trypsin removes most of the impurities with only a small effect on the electron-transfer and proton-pumping function of the enzyme (Ludwig et al., 1979; F. Malatesta and R. A. Capaldi, unpublished results). Figure 4 shows the effect of trypsin treatment on the polypeptide composition of a sample of this crystalline preparation. As discussed already, the DOC-derived crystals are relatively impure (heme *a* content of 4 nmol/mg of protein) and contain a large number of polypeptides not seen in purer oxidase preparations. Incubation of the crystalline sheets with trypsin (1 mg/25 mg of protein) increased the purity as judged by heme *a* content (from 4.0 to 10.5 nmol of heme *a*/mg of protein) and removed many bands from the gel profile. In particular it is important to note that polypeptides b and c were both cleaved by the protease treatment, an effect seen with the isolated and detergent-solubilized enzyme [see Ludwig et al. (1979)]. Figure 3C shows the filtered projection of the negatively stained trypsin-treated crystals. A comparison with Figure 3B shows that the protein digestion did not alter the filtered projection and thus has not altered significantly the structure of the repeating protein unit.

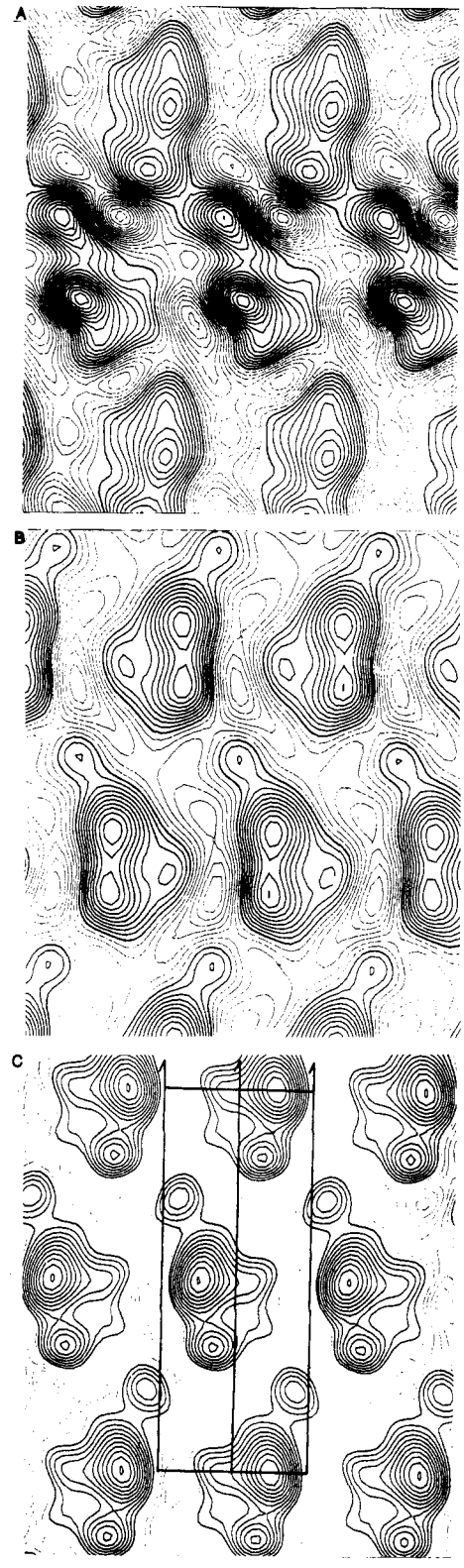

FIGURE 3: Projections of maps of different crystalline preparations. All projections are down the *c* axis of the three-dimensional structure, i.e., perpendicular to the sheet. (A) Reconstituted crystals. (B) Deoxycholate-derived crystals that have been treated with low levels of trypsin. (C) Deoxycholate-derived crystals.

The gel profile in Figure 4C is of DOC-derived crystals incubated with higher concentrations of trypsin (1 mg/10 mg of protein). This treatment not only led to removal of impurities but resulted in the cleavage of subunit IV (17 500 daltons) to a smaller fragment (15 500 daltons). There was no obvious change in the other subunits of the oxidase. This more complete digestion effected more than 50% loss of

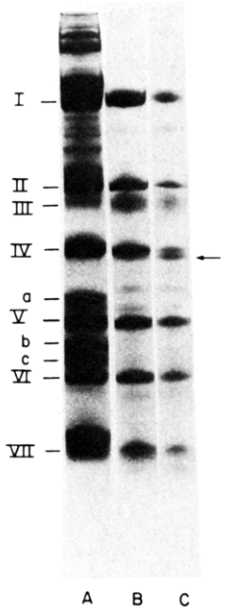

FIGURE 4: $NaDodSO_4$–polyacrylamide gels of deoxycholate-derived cytochrome *c* oxidase crystals treated with trypsin. (A) Untreated $P12_1$ crystalline preparation. (B) The same preparation as in (A) after incubation with trypsin (1 mg/25 mg) for 1 h at room temperature, followed by centrifugation to remove cleaved fragments. (C) Incubation of the crystalline enzyme with trypsin (1 mg/10 mg) for 1 h at room temperature.

electron transfer activity and caused disruption of the two-dimensional array.

Discussion

Two different two-dimensional arrays of cytochrome *c* oxidase have been studied to provide a low-resolution, three-dimensional structure of this protein (Henderson et al., 1977; Fuller et al., 1979). The protein is seen as Y shaped and thus as a three domain structure. The arms of the Y, called the M domains, each span the membrane. One of these (M_1) is about twice the mass of protein of the second (M_2). The stalk of the Y extends from the surface of the membrane for 55 Å on what would be the outer (cytoplasmic) side of the mitochondrial inner membrane.

Beef heart cytochrome *c* oxidase contains at least seven different subunits as well as two hemes *a* and two copper atoms as prosthetic groups (Downer et al., 1976). The arrangement of these components with respect to the C, M_1, and M_2 domains seen on the low-resolution structure of the enzymic complex can in some cases be inferred. Thus the C domain must carry the cytochrome *c* binding site by virtue of being on the outside of the inner mitochondrial membrane. It must contain subunits II and III as both can be covalently cross-linked to cytochrome *c* bound into its high-affinity site (Bisson et al., 1978, 1980; Birchmeier et al., 1976; Fuller et al., 1981). In order to obtain direct evidence for the location of subunits it is now important to combine the biochemical and structural approaches on the same crystalline preparation. It should be possible to tag specific subunits of cytochrome *c* oxidase with heavy atom chemical reagents and thereby locate a particular subunit within the three-dimensional structure. For example, there is a highly reactive sulfhydryl on subunit III that can be modified with electron-dense derivatives (Darley-Usmar et al., 1981). Subunits could also be localized by antibody binding and the cytochrome *c* binding site identified by covalently binding cytochrome *c* to crystals and then by locating this small molecule with the larger Fab fragment made against cytochrome *c*.

Experiments such as those described above require crystals of purified enzyme so that the modification of cytochrome *c* oxidase by heavy atom derivatives or binding of cytochrome *c* can be analyzed and quantitated. Here we describe two approaches to making crystals of cytochrome *c* oxidase suitable for the above kinds of experiments.

Crystals of cytochrome *c* oxidase have been made from an enzyme preparation isolated according to Steffens & Buse (1976). This preparation is of high heme content and contains the seven to nine subunits of cytochrome *c* oxidase with few impurities and only low concentrations of endogenous phospholipid. The purified enzyme was mixed with phosphatidylcholine in the presence of cholate and treated with deoxycholate and salt, and the excess lipid and detergent was then removed by sucrose density gradient centrifugation. The final preparation contained sheetlike crystals similar to the deoxycholate-derived $P12_1$ crystals in their morphology and in the ratio of unit-cell axes.

The two-sided plane group of the reconstituted crystals is *P*1. Filtered images of the reconstituted crystals showed that the unit cell contained two monomers. The density profile of the monomer in projection was superimposable on the density profile of the monomer in the $P12_1$ crystals. The differences between these crystals result from differences in packing. The absence of "X", a disconnected piece of density seen in the $P12_1$ reconstruction, and the shortening of the *b* axis both result from the tighter packing of monomers in the *P*1 cell. Comparisons between simulations of the *P*1 projection using the $P12_1$ monomer and the observed projection suggest that the monomers along *b* alternate in their orientation with respect to the plane of the sheet as in the $P12_1$ form. However, the M_2 domains are juxtaposed along *b*, allowing tighter packing of molecules than in the $P12_1$ form.

Protease digestion of the deoxycholate-derived crystals was used as a second approach to obtaining crystals of pure cytochrome *c* oxidase. Cleavage by low levels of trypsin was found to remove many of the larger molecular weight impurities in the preparation without disrupting the crystals or altering the projection. This treatment also cleaved both polypeptides b and c. Studies on detergent-solubilized cytochrome *c* oxidase have shown that component c is completely removed by this treatment (Ludwig et al., 1979). Component b is removed from the polypeptide profile but may be digested to a smaller fragment that is retained by the complex.

The trypsin cleavage experiments also provide information about the state of the enzyme in the $P12_1$ crystals. These crystals of cytochrome *c* oxidase display the same pattern of resistance to trypsin cleavage as the purified enzyme in either the membranous or detergent-solubilized form. This resistance is a characteristic of the native conformation of the enzyme since treatment with $NaDodSO_4$ renders the enzyme very trypsin sensitive. Trypsin treatment also increases the heme to protein ratio of the preparation in proportion to the purification achieved (assessed from gels), indicating that heme has not been lost during crystallization. These results taken together with the published activity measurements confirm that the conformation of cytochrome *c* oxidase in $P12_1$ crystals is similar to that of the native enzyme.

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Heavy Metal Ion Interactions with *Callinectes sapidus* Hemocyanin: Structural and Functional Changes Induced by a Variety of Heavy Metal Ions†

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ABSTRACT: Hemocyanins are oligomeric proteins that reversibly bind oxygen. The oxygen binding site is a binuclear copper center bound to the protein by amino acid side chains. The hemocyanin of the blue crab, *Callinectes sapidus*, occurs in vivo as a mixture of 25S dodecamers and 16S hexamers, whose oxygen binding properties are identical. Four heavy metals have been used as probes of structure and function in this hemocyanin system. Divalent cations of cadmium, copper, mercury, and zinc induced an indefinite self-association of the hemocyanin molecule. These higher ordered association states can be dissociated by ethylenediaminetetraacetic acid. *Callinectes* oxyhemocyanin possesses at least three mercury binding sites: (1) a sulfhydryl group which forms a mercaptide bond with a single mercuric ion, (2) a tryptophanyl side chain which forms a noncovalent 1:1 complex with mercuric ions with an association constant of 5.7×10^{15} M^{-1}, and (3) lower affinity site(s) involved in the self-association process also observed with cadmium, copper, and zinc. Sites 1 and 2 are most likely also involved in the binding of cadmium. Upon removal of oxygen from the active site of hemocyanin, an additional binding site becomes available for the reaction with mercury. Binding of mercury to this site leads to loss of one of the coppers from the binuclear oxygen binding site. Both the binuclear copper center and allosteric sites on the hemocyanin are affected by heavy metal binding. Cadmium and zinc ions increase the oxygen affinity; mercury and copper ions have the opposite effect. All four heavy metal ions decrease the degree of cooperative oxygen binding. The mercury-induced changes in oxygen binding by 25S *Callinectes* hemocyanin appear to be the result of that metal's interaction with the high-affinity tryptophan binding site. Mercury binding to the available sulfhydryl group in oxyhemocyanin occurs without functional consequences. Heavy metal, hydrogen, and chloride ions affect the affinity of the first or last oxygen molecules bound to the hemocyanin, which results in the appearance of multiple T (low oxygen affinity) and R (high oxygen affinity) states. Additionally, these ions shift the equilibrium between the low and high oxygen affinity states. The appearance of additional R states at high pH is accompanied by the cleavage of a tyrosine hydrogen bond.

Hemocyanins are blue copper proteins that exist extracellularly in the hemolymph of arthropods and molluscs. In both groups of organisms, they function as reversible oxygen carriers. Fundamental differences in the molecular architecture of molluscan and arthropodan hemocyanins exist. In the molluscs, the hemocyanins are 300 by 350 Å cylinders made of about 20 very large subunits. The hemocyanins of the arthropods exist as oligomers assembled on a theme of hexameric units of molecular weight 450 000 (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977; Hendrickson, 1977; Klarman et al., 1979; Lamy et al., 1980, 1981). Aside from their intrinsic interest, their high in vivo concentration and the relative ease of isolation make hemocyanins good

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