Bisson, R., Jacobs, B., & Capaldi, R. A. (1980) *Biochemistry 19*, 4173.
Briggs, M., & Capaldi, R. A. (1977) *Biochemistry 16*, 73.
Briggs, M., & Capaldi, R. A. (1978) *Biochem. Biophys. Res. Commun. 80*, 553.
Buse, G., Steffens, G. J., Steffens, G. C. M., Sacher, R., & Erdweg, M. (1982) *Interactions between Iron and Proteins in Oxygen and Electron Transport* (Ho, C., Ed.) Elsevier/North-Holland (in press).
Capaldi, R. A. (1979) *Membrane Proteins in Energy Transduction* (Capaldi, R. A., Ed.) p 201, Marcel Dekker, New York.
Darley-Usmar, V. M., Capaldi, R. A., & Wilson, M. T. (1981) *Biochem. Biophys. Res. Commun. 103*, 1223.
Downer, N. W., Robinson, N. C., & Capaldi, R. A. (1976) *Biochemistry 15*, 2930.
Fuller, S. D., Capaldi, R. A., & Henderson, R. (1979) *J. Mol. Biol. 134*, 305.
Fuller, S. D., Darley-Usmar, V. M., & Capaldi, R. A. (1981) *Biochemistry 20*, 7046.
Henderson, R., Capaldi, R. A., & Leigh, J. S. (1977) *J. Mol. Biol. 112*, 631.
Holser, W. T. (1958) *Z. Kristallogr. 110*, 266.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Ludwig, B., Downer, N. W., & Capaldi, R. A. (1979) *Biochemistry 18*, 1401.
Prochaska, L., Bisson, R., & Capaldi, R. A. (1980) *Biochemistry 19*, 3174.
Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375.
Steffens, G., & Buse, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1125.
Sun, F. F., Prezbindowski, K. S., Crane, F. L., & Jacobs, E. E. (1968) *Biochim. Biophys. Acta 153*, 804.
Swank, R. T., & Munkres, K. (1971) *Anal. Biochem. 39*, 462.
Vanderkooi, G., Senior, A. E., Capaldi, R. A., & Hayashi, H. (1972) *Biochim. Biophys. Acta 274*, 38.
Vik, S. B., & Capaldi, R. A. (1977) *Biochemistry 16*, 5755.
Williams, J. R. (1974) *Arch. Biochem. Biophys. 107*, 537.

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

Marius Brouwer,* Celia Bonaventura, and Joseph Bonaventura

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

† From the Marine Biomedical Center, Duke University Marine Laboratory, Beaufort, North Carolina 28516. *Received October 8, 1981.* This work was supported by a grant from the National Oceanic and Atmospheric Administration (NOAA NA80RAD00063).

0006-2960/82/0421-2529$01.25/0

model blue copper proteins. They are known to resemble tyrosinases (Jolley et al., 1974; Eickman et al., 1978), and their carbon monoxide and peroxide adducts may be analogous to some of the intermediate states of the copper containing subunits of cytochrome *c* oxidase. The oxygen binding sites of both mollusc and arthropod hemocyanins are similar to the extent that a binuclear copper center is directly liganded to amino acid side chains of the protein (Brown et al., 1980; Co & Hodgson, 1981; Co et al., 1981; Eickman et al., 1979). The amino acids involved in the binding of the copper atoms to the protein are still incompletely known. It has been suggested that the copper atom distance shortens on oxygenation (Co et al., 1981).

Homotropic and heterotropic allosteric interactions are well developed in the hemocyanins. The allosteric interactions observed in arthropod hemocyanins can generally be adequately explained by the two-state model for allosteric transitions (Monod et al., 1965) extended to include multiple T and R states. Heterotropic allosteric effectors exert their influence by differential binding to one of the extreme states, thus altering the allosteric equilibrium constant, *L*. The molecular mechanism of allosteric effects in hemocyanins is yet to be elucidated. Chemical cross-linking experiments have shown that, at least in the instance of molluscan hemocyanin, it is possible to "freeze" the molecule either in the T or R conformation (van Driel & van Bruggen, 1975). Little work on chemical modification of residues involved in allosteric interactions of hemocyanins has been done.

The hemocyanin of the blue crab, *Callinectes sapidus*, has two major components with sedimentation coefficients of approximately 16 S and 25 S (Herskovits et al., 1981; this study). The molecular weights of these components have been reported to be 450 000 and 900 000–940 000, respectively (Hamlin & Fish, 1977). The subunit molecular weight is around 75 000. Hence, the two components of *Callinectes* hemocyanin share the hexameric and dodecameric organization found in many arthropod hemocyanins (Hamlin & Fish, 1977; Herskovits et al., 1981).

Respiratory protein–heavy metal interactions are studied in our laboratory to determine whether these proteins act as carriers or targets in trace metal toxicity in marine organisms. During the course of these studies, it was noticed that heavy metal ions may be used to probe the oxygen binding and allosteric sites in hemocyanins. In this paper, we report on the effect of four heavy metal ions on the structural and functional characteristics of *Callinectes* hemocyanin.

Materials and Methods

Hemolymph was collected from adult male blue crabs by cutting off the first walking leg. The clotted hemolymph was homogenized with a tissue homogenizer and subsequently centrifuged at 27000*g* at 4 °C for 10 min. The supernatant was concentrated on a Diaflow YM 10 ultrafiltration membrane. A maximum volume of 8 mL, at a protein concentration of 70 mg/mL, was applied to a 2.4 × 52 cm Sepharose CL-4B column in 50 mM tris(hydroxymethyl)aminomethane (Tris) and 10 mM $CaCl_2$, pH 7, ionic strength 0.13. The column was eluted at 30 mL/h with the same buffer, and the hemocyanin content was monitored at 340 nm.

Sedimentation analysis and tonometric oxygen equilibrium experiments were performed as described elsewhere (Riggs & Wolbach, 1956; Brouwer et al., 1977). Samples were in Tris-HCl, made up to the desired ionic strength with NaCl (Bates, 1973).

Fluorescence emission spectra of *Callinectes* 25S hemocyanin in 50 mM Tris and 10 mM $CaCl_2$, pH 8, ionic strength 0.13, at several $HgCl_2$ concentrations were measured by using a Turner Model 430 fluorometer. The excitation maximum occurred at 287 nm, and the fluorescence emission was monitored at 340 nm. The absorbance of the protein samples used for the fluorescence studies was 0.35 at 280 nm.

Mercury-induced difference spectra in the ultraviolet region were measured with a Cary 219 double-beam spectrophotometer. The base line was set at sensitivity 0.1 with 3 mL of hemocyanin in 50 mM Tris and 10 mM $CaCl_2$, pH 7.94, ionic strength 0.13, in the sample and reference beam. The optical density of the protein sample was 1.0 at 280 nm. $HgCl_2$ was then added to the sample cuvette and buffer to the reference cuvette. pH-induced difference spectroscopy was carried out by the addition of 1 N NaOH to a hemocyanin solution in the sample cuvette and an equivalent amount of buffer to the reference solution (see figure legends for details).

Protein concentrations were calculated from the extinction coefficients as given by Herskovits et al. (1981): $E_{cm}^{1\%} = 14.7$ at 280 nm for the 25S molecule and 12.4 for the 16S and 5S molecules. In addition, the Coomassie Brilliant Blue method was used (Bradford, 1976). In the latter case calibration was done with *Limulus polyphemus* hemocyanin by using the extinction coefficients as published by Nickerson & Van Holde (1971). Molar concentrations were calculated by using a value of 75 000 for the molecular weight of the *Callinectes* hemocyanin subunits (Hamlin & Fish, 1977).

Copper and cadmium were determined by flame absorption spectroscopy with a Varian AA 6 spectrometer. Cadmium ion activities were measured with an Orion Model 94-48 A cadmium ion specific electrode. Mercury was determined as described by Whaling et al. (1977) using a Spectro Products, Inc., HG-3 mercury monitor with continuous background correction.

Gel electrophoresis of 5S *Callinectes* hemocyanin subunits in 50 mM Tris and 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.9, was carried out on 7.5% acrylamide slab gels (15 × 20 cm) by using the discontinuous buffer system described by Laemmli (1970). Proteins were stained with 1% Coomassie G-250 in 25% trichloroacetic acid.

Results

Hemocyanin eluted from Sepharose CL-4B as a broad asymmetrical peak. The leading fractions sedimented at 25 S and the trailing fractions at 16 S. These values are characteristic of dodecameric and hexameric hemocyanin molecules (Van Holde & van Bruggen, 1971). These species usually occurred in a ratio of 3:1 and were stable over a pH range of 7–8.5, in 50 mM Tris and 10 mM $CaCl_2$, ionic strength 0.13. Fluorescence and difference spectroscopy studies were done with the 25S species. Oxygen binding experiments showed the binding characteristics of the hexamer and the dodecamer to be identical. Therefore most of the oxygen equilibrium experiments reported in this paper were carried out with the 25S and 16S mixture.

Structural Changes Induced by Heavy Metal–Hemocyanin Interaction. (a) Indefinite Self-Association. When 25S *Callinectes* hemocyanin was titrated with copper, zinc, cadmium, or mercury chloride, a process of indefinite self-association was observed (Figure 1; see figure legends for experimental details). Free metal ion concentrations were calculated by using the Tris–metal complex formation constants as determined by Hanlon (1966) and the chloride–hydroxide–metal stability constants as given by Smith & Martell (1976). These calculations, which did not take into account the hemocyanin–metal interaction, showed that the association process is induced at a free metal ion concentration of 2.77×10^{-15}

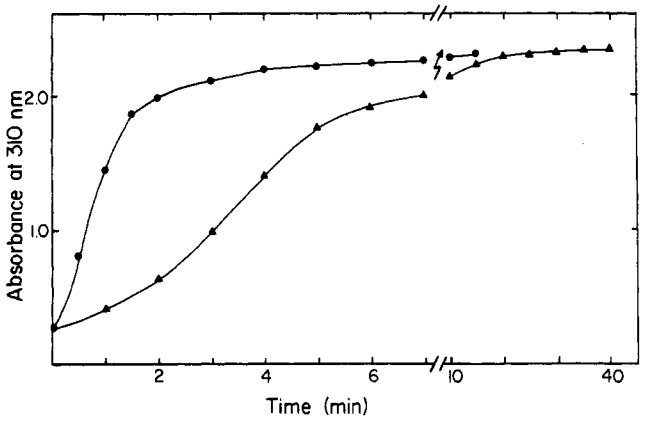

FIGURE 1: Zinc-induced self-association of *Callinectes* hemocyanin. 80 μL of 10 mM $ZnCl_2$ in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13, was added to 2.92 mL (1.3 mg/mL) of hemocyanin dissolved in the same buffer. The self-association was monitored by reading the optical density attributable to light scattering at 310 nm, which corresponds to a minimum in the absorption spectrum of *Callinectes* hemocyanin. (▲) Oxygenated hemocyanin; (●) deoxygenated hemocyanin.

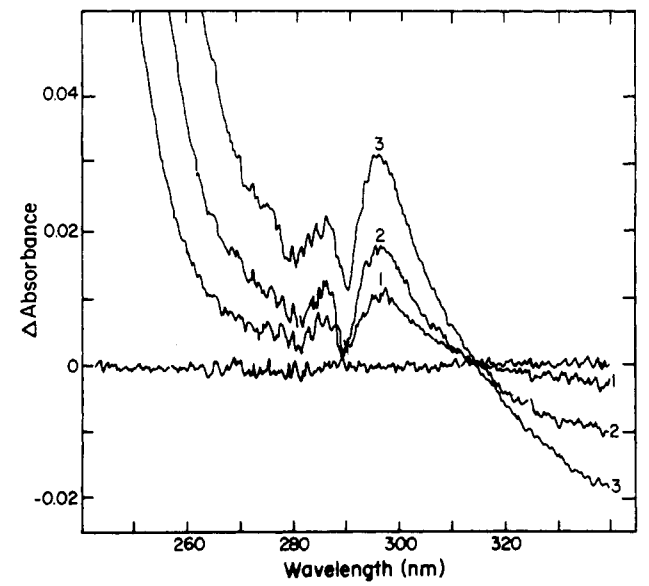

FIGURE 2: Mercury-induced spectral changes of *Callinectes* hemocyanin. 20, 40, and 80 μL of 10 mM $HgCl_2$ in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13, were added to 3 mL (0.7 mg/mL) of hemocyanin in 50 mM Tris and 10 mM $CaCl_2$, pH 7.94, ionic strength 0.13, in the sample beam of the spectrophotometer. Equivalent amounts of pH 7 buffer were added to 3 mL of hemocyanin, pH 7.94, in the reference beam. Difference spectra were recorded from 340 to 240 nm: (1) 0.066 mM $HgCl_2$ (free Hg^{2+} concentration: 9.3×10^{-17} M); (2) 0.132 mM $HgCl_2$ (free Hg^{2+} concentration: 1.87×10^{-16} M); (3) 0.26 mM $HgCl_2$ (free Hg^{2+} concentration: 3.68×10^{-16} M).

M (Hg^{2+}), 6.24×10^{-7} M (Cu^{2+}), 1.84×10^{-5} M (Zn^{2+}), and 1.36×10^{-4} M (Cd^{2+}). (Cadmium ion activity is 1.19×10^{-4} M as measured with the cadmium selective electrode.) Self-association of deoxyhemocyanin is much faster than oxyhemocyanin association (Figure 1). This observation shows that removal of oxygen from the active site results in a structural alteration at the surface of the 25S molecule.

(*b*) *Mercury-Induced Spectral Changes of Callinectes Hemocyanin.* Mercury chloride, at concentrations where no self-association occurs, induces profound changes in the ultraviolet spectrum of *Callinectes* hemocyanin (Figure 2). First of all there is a decrease in absorbance around 340 nm, where absorption is due to the copper–oxygen complex. Mercury chloride also decreases the oxygen affinity of *Callinectes* hemocyanin (Figure 5), e.g., from 8.5 to 40 mmHg under conditions corresponding to difference spectrum number 3. The negative absorption band is therefore most likely due to a slight dissociation of the copper–oxygen complex upon the addition of $HgCl_2$. A large positive peak occurs at 297 nm, due to a red shift of tryptophan absorption, indicative of the formation of a tryptophan–mercuric ion complex (see Discussion). The chief feature of the difference spectrum is the very strong positive absorption in the far-UV. This increase of the absorption is due to the formation of a Tris-HCl–Hg complex and the formation of a single sulfur mercaptide bond per hemocyanin subunit (see Discussion).

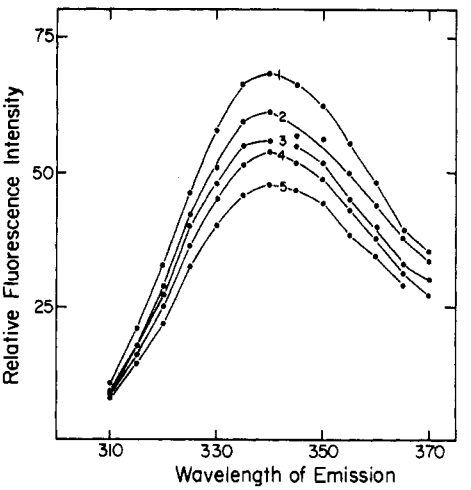

FIGURE 3: Fluorescence emission spectra of 25S *Callinectes* hemocyanin at 0.24 mg/mL, excited at 287 nm, and monitored at 340 nm. 50, 100, 150, and 250 μL of 10 mM $HgCl_2$ in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13, were added to 3 mL of hemocyanin in 50 mM Tris and 10 mM $CaCl_2$, pH 8, ionic strength 0.13. (1) 0 mM $HgCl_2$; (2) 0.16 mM $HgCl_2$; (3) 0.32 mM $HgCl_2$; (4) 0.48 mM $HgCl_2$; (5) 0.77 mM $HgCl_2$.

(*c*) *Fluorescence Quenching Due to Mercuric Ion–Hemocyanin Interaction.* Figure 3 shows the quenching of intrinsic hemocyanin fluorescence in the presence of $HgCl_2$. The observed emission intensities were corrected for trivial reabsorption due to the overlap of the emission and the 340-nm absorption band according to Er-El et al. (1972). Difference spectroscopy reveals a strong tryptophan–Hg^{2+} interaction. It is therefore likely that the mechanism involved in mercuric ion quenching of *Callinectes* hemocyanin fluorescence is a direct complex formation between mercuric ion and tryptophan.

(*d*) *Mercury-Induced Irreversible Decrease of the Copper–Oxygen Band of Callinectes Hemocyanin.* When oxyhemocyanin is incubated with mercury chloride, there is an instantaneous very small decrease of the copper–oxygen band (Figure 2). When deoxyhemocyanin is incubated with 1 mM $HgCl_2$, there is a very slow disappearance of the copper–oxygen band up to 50% of the original absorption at 335 nm (Figure 4). Mercury-treated oxy- and deoxyhemocyanin contain 1 and 1.5 sulfhydryl bound mercuric ions per subunit, after the noncysteine bound mercuric ions are removed by extensive dialysis against 50 mM Tris and 10 mM EDTA, pH 8.9. Removal of these ions does not restore the original absorption of the copper–oxygen charge transfer band. Mercury-treated oxyhemocyanin still contains two coppers per active site. Mercury-treated deoxyhemocyanin, however, contains 1.5 coppers per active site.

Functional Consequences of Heavy Metal Ion–Hemocyanin Interaction. Figure 5 shows that both cadmium and mercuric ions suppress cooperative interactions in *Callinectes* hemo-

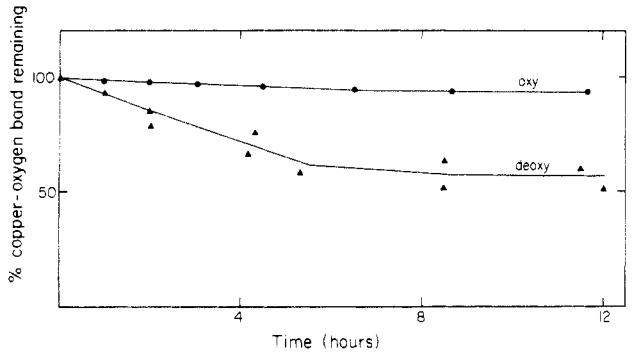

FIGURE 4: Decrease of the copper–oxygen band upon incubation of deoxygenated hemocyanin with 1 mM $HgCl_2$. Two *Callinectes* hemocyanin samples of 2.7 mL (4.8 mg/mL), in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13, were deoxygenated. One sample was flushed with water-saturated oxygen. 300 μL of 10 mM $HgCl_2$ in the same buffer was added to both samples, and the 335-nm absorption band was followed as a function of time. (●) Oxygenated hemocyanin; (▲) deoxygenated hemocyanin.

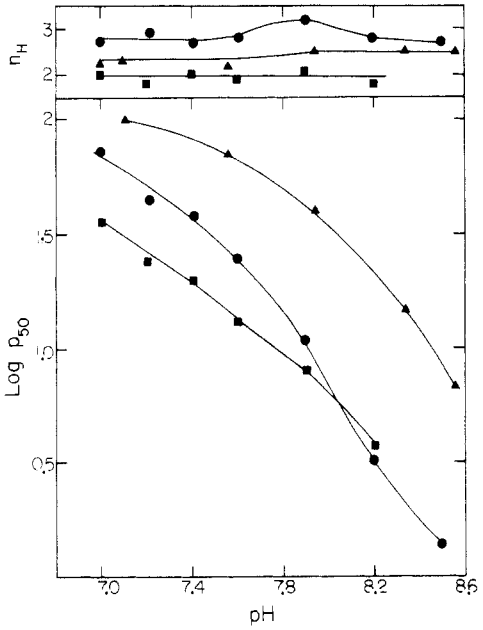

FIGURE 5: Effect of pH, cadmium chloride, and mercury chloride on oxygen binding characteristics of *Callinectes* hemocyanin. Protein concentration: 4 mg/mL. (●) 50 mM Tris, 10 mM $CaCl_2$, ionic strength 0.13; (▲) same buffer containing 0.24 mM $HgCl_2$; (■) same buffer containing 0.50 mM $CdCl_2$. P_{50}: Partial pressure of oxygen needed for half-saturation of *Callinectes* hemocyanin (expressed in mmHg). n_H: Hill coefficient, i.e., slope of the Hill plots (see Figure 6).

cyanin, as expressed in a decrease of the slope of the Hill plots. Mercuric ions decrease the oxygen affinity of *Callinectes* hemocyanin. Cadmium ions have an opposite effect. The effect of cadmium decreases with increasing pH, which is, at least partly, due to a decrease of the cadmium ion activity: 3.63×10^{-5} M at pH 7 and 8.77×10^{-6} M at pH 8.5.

Oxygen binding data, presented as Hill plots, are shown in Figure 6. $ZnCl_2$ and NaCl increase the oxygen affinity of *Callinectes* hemocyanin at pH 7 and shift the lower (deoxy) asymptote of the Hill plots (Figure 6A). The effect of NaCl on oxygen binding properties of *Callinectes* hemocyanin was investigated in order to obtain more accurate information about the upper part, or oxy asymptote, of the Hill plots. This information is necessary for a theoretical interpretation of the oxygen binding data, which will give us some insight into the molecular mechanism underlying the effect of heavy metal ions on the oxygen binding characteristics of *Callinectes* hemocyanin.

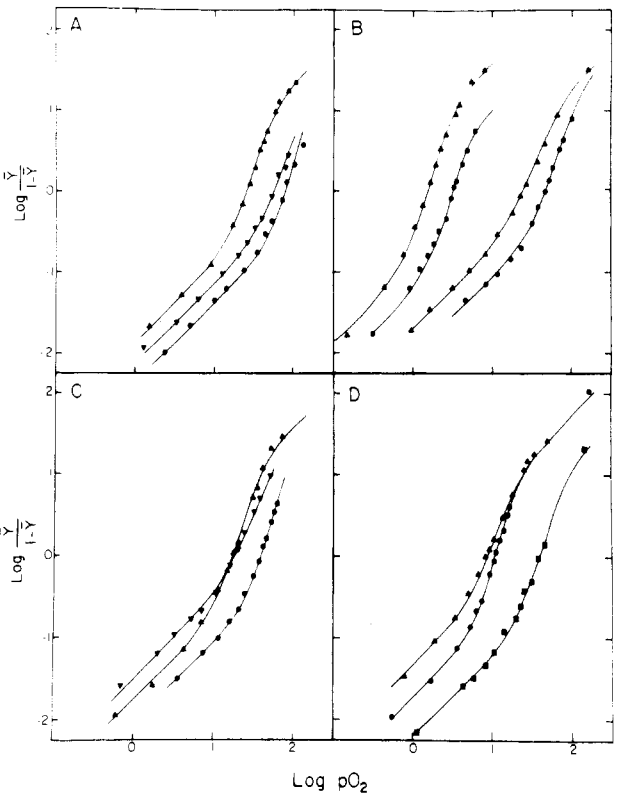

FIGURE 6: Effect of several allosteric effectors, pH and chloride, zinc, cadmium, and mercuric ions, on oxygen binding by *Callinectes* hemocyanin. Protein concentration: 4 mg/mL. (A) (●) 50 mM Tris, 10 mM $CaCl_2$, pH 7, ionic strength 0.13; (▼) same buffer containing 0.2 mM $ZnCl_2$; (▲) same buffer containing 1.48 M NaCl. (B) (▲) 50 mM Tris, 10 mM $CaCl_2$, pH 8.5, ionic strength 0.13 (left curve); (●) 50 mM Tris, 10 mM $CaCl_2$, pH 8.2, ionic strength 0.13 (second curve from left); (▲) 50 mM Tris, 10 mM $CaCl_2$, pH 7.2, ionic strength 0.13, containing 0.5 mM $CdCl_2$ (third curve from left); (●) 50 mM Tris, 10 mM $CaCl_2$, pH 7.2, ionic strength 0.13. (C) (●) 50 mM Tris, 10 mM $CaCl_2$, pH 7.4, ionic strength 0.13; (▲) same buffer containing 1.58 M NaCl; (▼) same buffer containing 0.5 mM $CdCl_2$. (D) (●) 50 mM Tris, 10 mM $CaCl_2$, pH 7.9, ionic strength 0.13; (■) same buffer containing 0.24 mM $HgCl_2$; (▲) same buffer containing 0.50 mM $CdCl_2$. Symbols are experimental data, and lines are calculated according to the two-state model for allosteric transitions, using the model parameters given in Table I.

cyanin. Figure 6B shows the tremendous effect of pH on the oxygen affinity of *Callinectes* hemocyanin (see also Figure 5). Increasing pH values not only increase the oxygen affinity but also shift the lower (deoxy) and upper (oxy) asymptotes of the Hill plots. Cadmium ions increase the oxygen affinity and shift the lower (deoxy) asymptotes to higher oxygen affinities (Figure 6B,C,D). The effect of mercuric ions is exactly opposite. They decrease the oxygen affinity and shift the lower (deoxy) asymptote to lower oxygen affinities. The oxygen binding data presented here will be analyzed under Discussion.

Discussion

The study of the interaction of mercuric ions and other heavy metal atoms with hemocyanins is of interest from the standpoint of preparation of isomorphic heavy metal derivatives used in X-ray crystallography. Additionally, as shown in this paper, heavy metals may be used as probes of structure and function in hemocyanin systems. Finally, such studies may lead to a better understanding of the mechanisms involved in trace metal transport and toxicity in marine organisms. It is well-known, for example, that heavy metals such as mercury, cadmium, zinc, copper, and lead are toxic to marine organisms (Bryan, 1976, 1979). It has been suggested that the hemocyanins of crustacea facilitate the uptake of zinc from the sea

water across the gill into the blood, by forming stable hemocyanin–zinc complexes (Bryan, 1964, 1971, 1976; Martin et al., 1977). Cadmium is taken up by the shore crab *Carcinas maenas* and appears in the hemolymph bound to hemolymph protein (Wright, 1977a,b). It is then passed on from the hemolymph to the hepatopancreas (Wright & Brewer, 1979).

Functional consequences of heavy metal binding to respiratory proteins have been reported for hemoglobin only.[1] Copper, for example, has been shown to catalyze the oxidation of Fe(II) hemoglobin, thereby interfering with the reversible oxygenation of hemoglobin resulting in a less effective oxygen transport (Rifkind, 1974, 1979; Rifkind et al., 1976). Zinc increases the oxygen affinity of sickle and normal red blood cells (Oehlschlegel et al., 1974; Rifkind & Heim, 1977; Gilman & Brewer, 1978), which results from an enhanced dissociation of hemoglobin tetramers to dimers (Gray, 1980). This catalogue of data shows that respiratory proteins may be involved in heavy metal transport, which in turn may lead to altered functional properties of these oxygen carriers.

In the present paper we focus our attention on the effect of heavy metal ions on the structural and functional characteristics of *Callinectes* hemocyanin, which occurs as a hexameric, 16 S, and dodecameric, 25 S, species in the hemolymph. The 16S and 25S components can be separated by gel permeation chromatography and ultracentrifugation and behave as distinct molecular species over a pH range from pH 7 to 8.5, in the presence of 10 mM $CaCl_2$, indicating the absence of equilibrium between the two species.

At heavy metal concentrations used in our oxygen binding experiments no change in sedimentation pattern of *Callinectes* hemocyanin was observed. At higher metal concentrations self-association occurred (Figure 1). Analytical ultracentrifugation did not reveal any distinct molecular weight species during the self-association process. Addition of EDTA resulted in an instantaneous dissociation of the high molecular weight aggregates. Similar behavior was observed with hemocyanins obtained from the horseshoe crab *Limulus polyphemus* and the shrimp *Penaeus setiferus*, indicating that hemocyanins in general have high affinity sites for a wide variety of heavy metal ions. The effect of binding of mercuric ions on spectral properties of *Callinectes* hemocyanin is shown in Figure 2. The decrease of the copper–oxygen absorption band around 340 nm is most likely due to a mercury-induced decrease in the oxygen affinity of *Callinectes* hemocyanin (Figures 5 and 6D), resulting in a dissociation of the copper–oxygen complex. This explanation is supported by the fact that the spectral change at 340 nm decreases with increasing pH values, which results in higher oxygen affinities (Figure 5). The absorption change at 340 nm can be reversed by the addition of EDTA, showing that the mercuric ion involved in changing the oxygen affinity of *Callinectes* hemocyanin is not bound to a sulfhydryl group. Further proof of this statement will be delivered when the effect of mercuric ions on oxygen binding will be discussed.

Interaction of mercuric acetate, mercuric nitrate, and mercuric chloride with tryptophan results in a broadened red-shifted UV absorption spectrum (Ramachandran & Witkop, 1964; Chen, 1971). This bathochromic shift is due to the formation of a 1:1 mercuric ion–tryptophan complex, with a molar extinction coefficient of 3240 M^{-1} cm^{-1} at 297 nm (Chen, 1971). The chief feature of a tryptophan vs. tryptophan–Hg^{2+} difference spectrum is a large positive peak at 297 nm and a minimum at 289 nm due to obliteration of the peak which occurs at this wavelength in the normal unperturbed tryptophan spectrum. When mercuric ions bind to *Callinectes* hemocyanin, exactly the same difference spectrum is observed (Figure 2). Therefore it seems reasonable to suppose that the positive peak at 297 nm in the difference spectrum of Figure 2 is due to a direct mercuric ion–tryptophan interaction. When the change in absorption at 297 nm is plotted vs. the $HgCl_2$ concentration, there is a steep linear increase of the optical density at low $HgCl_2$ concentrations, followed by a slower increase at higher $HgCl_2$ concentrations (the range of $HgCl_2$ concentrations used is limited due to the mercury-induced self-association of *Callinectes* hemocyanin). From difference spectra such as given in Figure 2 we determined the steep linear increase of the 297-nm absorption to have a value of 0.035 ± 0.003 per 0.7 mg/mL (9.2 μM) hemocyanin. This value corresponds to 1.17 ± 0.1 Hg^{2+}–tryptophan complex per hemocyanin subunit. In addition to this high affinity site, a lower affinity site seems to be present as judged from the slow increase of the OD_{297} at higher mercury concentrations. The noncovalent nature of these binding sites is indicated by the observation that the optical density change at 297 nm is EDTA reversible. The stability constant of the mercury–tryptophan complex can be calculated from the spectral data, taken into account the following considerations: The total mercury concentration in the buffer system used for the binding studies (see legends to Figure 2) is

$$[Hg]^{total} = [Hg^{2+}] + [HgCl^+] + [HgCl_2] + [HgCl_3^-] + [HgCl_4^{2-}]$$

At the relatively high chloride concentration used (0.12 M) the complexes of Hg^{2+} with hydroxyl ions [see Smith & Martell (1976) for stability constants] and Tris (Hanlon et al., 1966) can be ignored. Therefore

$$[Hg]^{total} = [Hg^{2+}](7.060 \times 10^{11})$$

In the presence of hemocyanin

$$[Hg]^{total} = [Hg^{2+}](7.060 \times 10^{11} + K[Hcy]_{free})$$

From this it follows that

$$[Hg^{2+}]_{free} = \frac{[Hg]^{total}}{7.06 \times 10^{11} + K([Hcy]_{total} - [Hcy{\sim}Hg])} \quad (1)$$

where

$$K = \frac{[Hcy{\sim}Hg]}{[Hg^{2+}]_{free}[Hcy]_{free}} = \frac{[Hcy{\sim}Hg]}{([Hcy]_{total} - [Hcy{\sim}Hg])[Hg]_{free}} \quad (2)$$

Here we neglect the binding of mercury to the sulfhydryl group of hemocyanin. The justification of this neglection is based on the observation that the mercury–tryptophan interaction is instantaneous, whereas the formation of the mercaptide bond is very slow, approximately 30 min at 0.25 mM $HgCl_2$ ($[Hg^{2+}]_{free} = 3.54 \times 10^{-16}$ M). Since the OD_{297} is measured 15 s after the addition of $HgCl_2$, we assume that virtually none of the mercaptide bond has been formed. Furthermore we disregard the contribution of the low affinity sites for Hg^{2+}. Equations 1 and 2 constitute two equations with two unknowns (K and $[Hg]_{free}$) and hence can be solved. [Hcy~Hg] is calculated by using a value of 3240 for the molar extinction coefficient of the Hg^{2+}–tryptophan complex (Chen, 1971).

[1] Recently, Kuiper et al. (1981) published a study concerning the effect of mercuric chloride on *Panulirus interruptus* hemocyanin.

The binding constant calculated this way is $K = 5.7 \times 10^{15}$ M^{-1}.

When 50 mM Tris-HCl is titrated with $HgCl_2$, at concentrations as used in the UV difference spectroscopy experiments, a very strong absorption from 280 to 240 nm is observed. The absorption is strongly dependent on the pH, more than doubling going from pH 8.5 to 7. This observation suggests the formation of a UV-absorbing Tris-HCl–Hg complex. The huge positive peak in the difference spectrum of Figure 2 is therefore in part due to the formation of this complex. When the mercury-treated samples are extensively dialyzed against 50 mM Tris and 10 mM EDTA, pH 8.9, there still is a positive absorption at 250 nm with respect to nontreated and dialyzed hemocyanin. This absorption is indicative of the formation of a mercury–mercaptide bond (Boyer, 1954; Vasak et al., 1981). The molar absorptivity of mercury-treated and dialyzed hemocyanin at 250 nm was found to be 34525 ± 368 M^{-1} cm^{-1}; the molar absorptivity of nontreated dialyzed hemocyanin was 31728 ± 461 M^{-1} cm^{-1}. The molar absorptivity of the mercury–mercaptide chromophore at 250 nm was determined by titrating 0.5 mM mercaptoethanol in distilled water, pH 6, with 10 mM $HgCl_2$ in water, pH 6. From the equivalence point in the titration curve a value of 2908 ± 85 M^{-1} cm^{-1} was obtained. This value agrees very well with that which can be calculated for the mercury–mercaptide chromophore in a protein. The molar absorptivity of mercury–metallothionein, possessing 7 g-atoms of mercury/mol of protein, is approximately 6.50×10^4 M^{-1} cm^{-1} at 250 nm (see Figure 1 in Vasak et al., 1981), or 9.28×10^3 M^{-1} cm^{-1}/Hg atom with an average of 3S ligands per Hg atom, or 3100 M^{-1} cm^{-1} for a single mercury–mercaptide chromophore. The validity of this calculation is based upon the absorption spectra of the cadmium–mercaptide chromophores in alcohol dehydrogenase and in metallothionein (Sytkowski & Vallee, 1979). From the spectral data presented above, the presence of 0.96 ± 0.28 g-atoms of mercury/mol of hemocyanin subunit was calculated. These results were supported by direct mercury determinations which show the presence of a single (1.038 ± 0.045) non-EDTA removable mercuric ion per oxyhemocyanin subunit (the standard deviations and means are obtained from six observations). Since the affinity of Hg^{2+} for SH groups in proteins is generally far greater than the affinity for any other functional groups (Vallee & Ulmer, 1972; Liu, 1977), it seems likely that the EDTA-resistant mercury binding site in *Callinectes* hemocyanin represents a sulfhydryl group. It might be argued, however, that this site does not represent a very high affinity site but that it is protected from the action of EDTA by its surroundings in the protein matrix. Evidence in favor of a high-affinity mercury binding site is obtained from equilibrium dialysis experiments. Analysis of the binding data by nonlinear regression shows the presence of 0.84 (0.71–0.98) mercury binding sites per hemocyanin subunit with an association constant of $10^{33.9}$ ($10^{17.9}$–$10^{45.5}$) (M. Brouwer, unpublished results; numbers in parentheses represent confidence limits corresponding to one standard deviation).

Summarizing we can say that *Callinectes* oxyhemocyanin contains at least three mercury binding sites per subunit, the highest affinity site being a sulfhydryl, which forms a mercaptide bond with a single mercuric ion. The second site is the indole side chain of a tryptophan residue which forms a 1:1 complex with a free Hg^{2+} ion, with a stability constant of 5.7×10^{15} M^{-1}. The third site, or more likely class of sites, is that involved in the mercury-induced self-association.

From Figure 3 it can be seen that up to 30% of the intrinsic protein fluorescence is quenched in the presence of 0.77 mM $HgCl_2$ ($[Hg^{2+}]_{free} = 1.09 \times 10^{-15}$ M). Hamlin & Fish (1977) reported that the amino acid composition of *Callinectes* hemocyanin was indistinguishable from that of *Cancer magister* hemocyanin (Carpenter & Van Holde, 1973), which contains 12 tryptophan molecules per subunit. Therefore we would expect that the formation of a single nonfluorescent Hg–tryptophan complex would result in an approximately 10% decrease of the fluorescence, which is considerably less than actually observed. As described earlier, mercuric ions may interact with a second tryptophan residue at high $HgCl_2$ concentration. Additionally part of the fluorescence quenching may be due to energy transfer from tryptophan to the tail end of the mercaptide absorption band, which extends above 300 nm (Chen, 1971).

Cadmium binding to *Callinectes* hemocyanin is accompanied by an increase of the absorption at 240 nm, indicative of the formation of a cadmium–mercaptide bond. This bond is EDTA sensitive. Cadmium ions quench the intrinsic protein fluorescence for about 10% and also give a red shift of the tryptophan absorption to 296 nm. These two facts suggest that cadmium is also capable of complexing with tryptophan in *Callinectes* hemocyanin.

When deoxyhemocyanin was incubated with 1 mM $HgCl_2$ at pH 7, a dramatic decrease of the absorption of the copper–oxygen band was observed (Figure 4). Oxygen protects against the change of spectral properties. Deoxyhemocyanin incubated with 0.24 mM $HgCl_2$ at pH 7 did not show a decrease of 335-nm absorption, showing that both the absence of oxygen from the active site and the additional binding of mercuric ions to low affinity sites are prerequisites for the irreversible change in spectral properties. Mercury analysis, after extensive dialysis against EDTA, showed the presence of 1.385 ± 0.049 ($N = 6$) mercuric ions per hemocyanin subunit in mercury-treated deoxyhemocyanin, as opposed to 1.038 ± 0.049 ($N = 6$) per subunit in mercury-treated oxyhemocyanin. The oxyhemocyanin samples still contained 1.948 ± 0.117 ($N = 6$) coppers per subunit, but the deoxyhemocyanin samples only 1.536 ± 0.006. The removal of oxygen from the active site of *Callinectes* hemocyanin apparently results in the exposure of a second high-affinity binding site for mercury, only in approximately 50% of the subunits. Once the mercury is bound, one copper is released from the active site, resulting in a 50% irreversible decrease of the copper–oxygen band. The Hg^{2+}-treated deoxyhemocyanin subunits with intact active sites were functionally equivalent to Hg^{2+}-treated oxyhemocyanin subunits, having a P_{50} value of 7.8 mmHg and a Hill coefficient of 0.91 in 50 mM Tris and 10 mM EDTA, pH 8.9. The fact that the decrease of the copper–oxygen band never exceeded the 50% level indicates that *Callinectes* hemocyanin contains at least two types of active sites. Subunit heterogeneity in arthropodan hemocyanins has been well documented (Sullivan et al., 1974; Bonaventura et al., 1975, 1977; Miller et al., 1977; Markl et al., 1980; Larson et al., 1981). Gel electrophoresis of *Callinectes* hemocyanin subunits shows the presence of at least eight distinct polypeptide chains.

Displacement of type I copper from the copper binding site of the blue plastocyanins by silver or mercuric ions, concomitant with the formation of a mercury–mercaptide bond, has been reported (Katoh & Takamiya, 1964; Bohner et al., 1981). X-ray crystallography showed the copper to be liganded by cysteine (Coleman et al., 1978), as in the blue copper (type I) protein azurin (Adman et al., 1978). X-ray absorption spectroscopy showed the type I copper of stellacyanin and the copper a_3 of cytochrome oxidase also to be liganded by cysteine

Table I: Effect of Hydrogen, Chloride, Cadmium, Mercuric, and Zinc Ions on the Allosteric and Thermodynamic Parameters Describing the Oxygen Binding by *Callinectes sapidus* Hemocyanin

pH	Cl^- (M)	Cd^{2+} (mM)	Hg^{2+} (mM)	Zn^{2+} (mM)	$L \times 10^{-4}$ [a]	K_T [b]	K_R [c]	$c \times 10^2$ [d]	$-\Delta G_{T\rightarrow R}$ [e]	$-\Delta F_I$ [f]
7.0	0.12				3426	237	3.68	1.55	42.3	10.15
7.0	1.48				4.42	73.8	3.68	5.25	26.1	7.15
7.0	0.12			0.2	519	133	3.68	2.77	37.6	8.74
7.2	0.12				152.7	112	3.75	3.35	34.7	8.28
7.2	0.12	0.5			3.12	51.3	3.75	7.31	25.2	6.36
7.4	0.12				773	115	2.37	2.06	38.6	9.45
7.4	1.58				8.22	57.5	2.37	4.12	27.6	7.78
7.4	0.12	0.5			7.10	31.6	2.37	7.50	27.2	6.32
7.9	0.12				4.59	54.0	1.78	3.31	26.1	8.28
7.9	0.12	0.5			0.727	21.6	1.78	8.23	21.7	6.07
7.9	0.12		0.25		2.81	160	7.00	4.37	24.9	7.61
8.2	0.12				0.301	17.8	0.89	5.00	19.5	7.28
8.5	0.12				5.23	7.5	0.24	3.20	26.4	9.08
8.5	0.12		0.25		0.297	30.0	1.78	5.93	19.4	6.86

[a] Allosteric equilibrium constant. [b] Oxygen dissociation constant of the T state as determined from the lower asymptotes of the Hill plots, expressed in mmHg. [c] Oxygen dissociation constant of the R state. [d] Nonexclusive binding coefficient, K_R/K_T. [e] Free energy for the transition from T to R state (kJ/mol). [f] Free energy of interaction (kJ/mol).

(Powers et al., 1981). The presence of sulfhydryl groups in the coordination sphere of the hemocyanin coppers (type III) has been suggested by Klotz & Klotz (1955) and Amundsen et al. (1977). In addition it has been shown that removal of oxygen from the hemocyanin oxygen binding site results in a considerable increase of the copper–copper distance concomitant with the breakage of a protein ligand bridge between the two coppers (Co & Hodgson, 1981; Brown et al., 1980). These data suggest that a cysteine sulfur forms a bridge between the two coppers in 50% of the oxygenated active sites of *Callinecetes* hemocyanin. Upon deoxygenation this cysteine residue becomes a ligand for Hg^{2+}, leading to the formation of a mercury–mercaptide bond and the loss of a single copper from the active site. The coppers in hemocyanins are known to be liganded by at least two histidines (Brown et al., 1980; Co & Hodgson, 1981; Eickman et al., 1978, 1979). In view of the strong tendency of the mercuric ion to form two coordinate complexes, the formation of a strong linear (His)N–Hg–N(His) complex, with the concomitant displacement of copper, in deoxyhemocyanin seems also possible. Further studies will be required to establish the validity of the proposed mechanisms. It seems very likely that the introduction of a highly scattering mercuric ion in the active site of *Callinectes* hemocyanin will contribute to a more detailed knowledge of the structure of the oxygen binding site.

Functional Consequences of Hemocyanin–Heavy Metal Interactions. Oxygen binding properties of hemocyanins are controlled by a number of physiologically important ions, such as hydrogen, sodium, calcium, and chloride. Detailed studies have shown that oxygen binding by a variety of hemocyanins can be described by the two-state model for allosteric transitions (Monod et al., 1965). According to this model the hemocyanin molecule occurs in two conformations, referred to as T and R. Each conformation can independently bind oxygen molecules. The T state has a lower affinity for oxygen than the R state. This difference in affinity is responsible for the cooperative oxygenation, as during the course of ligation the equilibrium between the T and R states is shifted toward the latter. The effect of allosteric effectors on the oxygen binding characteristics is due to preferential binding of the effector to either the T or R state. Oxygen binding by *Panulirus interruptus* and *Helix pomatia* α-hemocyanin can be described within the framework of this model (Colosimo et al., 1977; Kuiper et al., 1977, 1979). Oxygen binding by *Helix pomatia* β-hemocyanin and *Limulus polyphemus* hemocyanin can basically be described by the two-state model with the modification that K_T, the oxygen dissociation constant of the T state, is altered by allosteric effectors, such as pH and chloride ions (Zolla et al., 1978; Brouwer et al., 1977, 1981a,b, 1982). Oxygen binding studies with *Callianassa californiensis* and *Penaeus setiferus* hemocyanin demonstrated that the binding data could not be described by the original two-state model. However, the introduction of a single mixed hybrid state containing subunits in the R as well in the T conformation resulted in an excellent fit between theory and experiment (Miller & Van Holde, 1974; Brouwer et al., 1978; Arisaka & Van Holde, 1979).

The oxygen binding data presented as Hill plots in Figure 6 were analyzed as described earlier (Brouwer et al., 1977). It is obvious from this figure that the binding data cannot be described by the simple two-state model for allosteric transitions. Hydrogen ions, for example, significantly affect the binding constant of the first oxygen molecules to the deoxygenated hemocyanin, as indicated by the shift of the deoxy asymptotes to lower affinities. In addition, hydrogen ions affect the affinities of the oxygenated hemocyanins as well (Figure 6B). The shifts of the oxy and deoxy asymptotes of *Callinectes* hemocyanin imply the existence of multiple R and T states whose oxygen affinities depend on proton binding. At pH 7 the K_R/K_T ratio (Table I) indicates a 64-fold higher affinity for the last compared with the first oxygen molecules bound, a value which decreases to 20-fold at pH 8.2. This corresponds to a decrease of the free energy of interaction per site realized in saturating hemocyanin with oxygen of 2.9 kJ/mol. An increase from pH 7 to 8.2 facilitates the transition from the deoxy to the oxy state by as much as 22.8 kJ/mol (Table I).

The effect of NaCl, which is due to chloride ions (Brouwer et al., 1977, 1978, 1982), on oxygen binding by *Callinectes* hemocyanins is shown in Figure 6A,C. Chloride ions increase the binding constant of the first oxygen molecules to deoxygenated hemocyanin but have no effect on the oxygen affinity of the R state. Binding of chloride ions facilitates the T $\rightarrow$ R transition and reduces the free energy of interaction (Table I). The same behavior was exhibited by cadmium (Figure 6B–D) and zinc ions (Figure 6A). Mercuric ions, on the other hand, also decrease the association constant for the binding of the last oxygen molecules (Figure 6D and Table I). The effect of hydrogen, chloride, and heavy metal ions on the oxygen affinity of T-state hemocyanin can be explained by preferential binding of these ions to either oxygenated subunits

(Cl^-, Cd^{2+}, and Zn^{2+}) or deoxygenated subunits (H^+ and Hg^{2+}) in the T-state quaternary structure. In general, if a given ligand has preferential affinity for either oxy or deoxy subunits in the T state, then the apparent affinity of the T state will be a function of the binding constants and the concentration of that ligand (Brouwer et al., 1977). A similar line of argument applies to the R state as well. When the existence of multiple T and R state was taken into account, the allosteric parameters of oxygen binding were determined (Table I). When the values in Table I were used, Hill plots were calculated with the hexamer as the allosteric unit (Brouwer et al., 1977, 1978, 1981b; Kuiper et al., 1977, 1979; Arisaka & Van Holde 1979). The fit between the experimental results and the calculated Hill plots is good (Figure 6). From the foregoing considerations, we can conclude that heavy metal ions modulate the oxygen affinity of *Callinectes* hemocyanin, by shifting the equilibrium between two conformational states of this multisubunit protein, having different affinities for oxygen. In addition, heavy metal ions also modify the oxygen affinities of these conformational states.

It should be remembered that our mercury binding studies revealed the presence of at least two classes of active sites in *Callinectes* hemocyanin. Gel electrophoresis revealed considerable structural heterogeneity. Moreover, oxygen binding by *Callinectes* subunits in 50 mM Tris and 10 mM EDTA, pH 8.9, showed functional heterogeneity as well, as judged from the value of the slope of the Hill plot (0.95). The studies reported in this paper, however, strongly suggest that the binding sites in the undissociated molecule are identical. Apparently, the subunits only express their own identity when free in solution, as has been shown for *Limulus* (Brouwer et al., 1977, 1982) and *Helix pomatia* hemocyanin (van Driel, 1974). Conformational changes of *Callinectes* subunits upon assembly in the higher molecular weight aggregates are indicated by quenching of their intrinsic protein fluorescence (M. Brouwer, unpublished results).

As has been discussed before, *Callinectes* hemocyanin has two very high affinity mercury binding sites: a sulfhydryl and the indole moiety of a tryptophan residue. Which of these is the oxygen linked one? The P_{50} value and Hill coefficient of 25S *Callinectes* hemocyanin in 50 mM Tris and 10 mM $CaCl_2$, pH 7, are 72 mmHg and 2.8, respectively. In the presence of 0.24 mM $HgCl_2$ these values change to 102 mmHg and 2.1, respectively. After dialysis against 50 mM Tris, 10 mM $CaCl_2$, and 2 mM EDTA, pH 7, followed by dialysis against the same buffer without EDTA, the presence of a 25S *Callinectes* hemocyanin molecule containing a single cysteine-bound mercury per subunit was demonstrated. The functional properties of these labeled 25S molecules were identical with those of native 25S molecules. From this we infer that the high-affinity tryptophan binding site is the allosteric site involved in stabilizing the quaternary T-state structure. This also shows that hemocyanin containing the sulfhydryl-bound mercury is an excellent derivative for X-ray crystallography, since mercury binding leaves the 25S structure and its functional characteristics intact. This observation, unfortunately, does not seem to be valid for other hemocyanins as well. We have been able to prepare mercury-labeled 60S *Limulus polyphemus* hemocyanin. However, their functional characteristics were drastically and irreversibly altered (M. Brouwer, unpublished results).

The P_{50} of the R state changes from 3.68 mmHg at pH 7 to 0.24 mmHg at pH 8.5 (Table I). Not only the functional but also the structural characteristics of 25S R state molecules change with varying pH (Figure 7). The peaks in the difference spectrum at 279 and 286.5 nm are characteristic of the perturbation of phenolic chromophores (Herskovits, 1967). These two absorption maxima have been assigned to transitions from excited state vibrational modes of the tyrosine molecule to its electronic ground state (Horwitz et al., 1970). Interpretation of the difference spectrum in terms of the environment of the individual chromophores remains very difficult. A negative difference spectrum usually indicates that a larger number of chromophores are exposed to the solvent in the sample solution (Donovan, 1969, 1973). Accordingly Figure 7 suggests that tyrosine(s) become exposed to solvent when the pH of a solution of *Callinectes* hemocyanin is raised from pH 7 to 8.5. The small peak at 300 nm and the large peak at 240 nm are characteristic of tyrosine ionization. For comparison the pH difference of *N*-acetyltyrosine is shown in the same figure (Figure 7). Maxima are observed at 293 and 242 nm, showing that the spectrum of tyrosine ionization in the protein is red shifted over 7 nm. The characteristic ratio of 4.6 for $\Delta OD_{242}/\Delta OD_{293}$ is also observed in the protein difference spectrum. This ratio corresponds to tyrosine ionization only (Mihalyi, 1968). The very low p*K* value of this tyrosine suggests that it is hydrogen bonded. Whether the decrease in oxygen affinity of the R state at low pH values and the formation of the tyrosine hydrogen bond are two linked phenomena is still an open question.

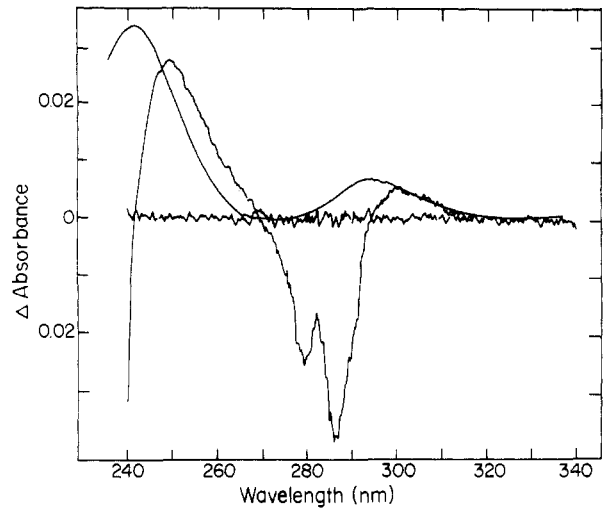

FIGURE 7: pH-induced spectral changes of *Callinectes* hemocyanin. (Irregular line) 100 μL of 1 N NaOH was added to 3 mL (0.8 mg/mL) of hemocyanin in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13, in the sample compartment of the spectrophotometer. pH after addition: pH 8.5. 100 μL of pH 7 buffer was added to 3 mL of pH 7 hemocyanin, in the reference cuvette. (Smooth line) 150 μL of 1 N NaOH was added to 3 mL of 3.3 μM *N*-acetyltyrosine in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13, in the sample cuvette. pH after addition: 10.6. 150 μL of pH 7 buffer was added to 3 mL of *N*-acetyltyrosine, pH 7, in the reference cuvette. The 249-nm peak in the hemocyanin difference spectrum corresponds to 2.4 μM ionized tyrosine ($\Delta\epsilon$ 11 100; Donovan, 1973). Hemocyanin concentration is 10 μM.

The effect of NaCl and pH on blue crab hemocyanin reported in this paper is of great physiological significance. *Callinectes sapidus* is exposed to different environments in its life. After development and metamorphosis to the adult form, the young crabs swim upstream, leaving behind the well oxygenated saline ocean waters for the hypoxic dilute waters of the estuaries. In the autumn of each year the impregnated females migrate to more saline waters. During migration from waters of 35‰ to 1‰ salinity the reduction in total blood salt is about 37%, which leads to a decrease in oxygen affinity of the hemocyanin. The respiratory stability in dilute waters is maintained by concomitant changes in blood pH, which oppose

the salt effect. The pH change results from the ammonia produced in deamination of the intracellular pool of free amino acids as the cells conform to osmotic changes in body fluids (Mangum & Towle, 1977). The allosteric effectors, H^+ and Cl^-, thus provide *Callinectes sapidus* with a respiratory stability in an environment which is unstable in both ions and oxygen. This fine tuning of hemocyanin function may well be disturbed by heavy metal ions.

Acknowledgments

We thank Thea Brouwer for her excellent technical assistance and Patrick Whaling for executing the heavy metal analyses. We are much indebted to Dr. David Engel for his generous supply of blue crab hemolymph.

References

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35–47.

Amundsen, A. R., Whelan, J., & Bosnich, B. (1977) *J. Am. Chem. Soc. 99*, 6730–6739.

Arisaka, F., & Van Holde, K. E. (1979) *J. Mol. Biol. 134*, 41–73.

Bates, R. G. (1973) *Determination of pH. Theory and Practice*, Wiley, New York.

Bohner, H., Sandmann, G., & Boger, P. (1981) *Biochim. Biophys. Acta 636*, 65–69.

Bonaventura, J., Bonaventura, C., & Sullivan, B. (1975) *J. Exp. Zool. 194*, 155–173.

Bonaventura, J., Bonaventura, C., & Sullivan, B. (1977) *Oxygen Physiol. Funct., Proc. Am. Physiol. Soc. Colloq. 1976*, 177–220.

Boyer, P. D. (1954) *J. Am. Chem. Soc. 76*, 4331–4337.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1977) *Biochemistry 16*, 3897–3902.

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1978) *Biochemistry 17*, 2148–2154.

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1981a) in *Invertebrate Oxygen-Binding Proteins: Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) pp 761–774, Marcel Dekker, New York.

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1981b) *Biochemistry 20*, 1842–1848.

Brouwer, M., Bonaventura, C., & Bonaventura, J. (1982) in *Physiology and Biochemistry of Horseshoe Crabs* (Bonaventura, J., & Bonaventura, C., Eds.) A. R. Liss Publishing Co., New York (in press).

Brown, J. M., Powers, L., Kincaid, B., Larrabee, J. A., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 4210–4216.

Bryan, G. W. (1964) *J. Mar. Biol. Assoc. U.K. 44*, 549–563.

Bryan, G. W. (1971) *Proc. R. Soc. London, Ser. B 177*, 389–410.

Bryan, G. W. (1976) in *Marine Pollution* (Johnston, R., Ed.) pp 185–302, Academic Press, London.

Bryan, G. W. (1979) *Phil. Trans. R. Soc. London, Ser. B 286*, 483–505.

Carpenter, D. E., & Van Holde, K. E. (1973) *Biochemistry 12*, 2231–2238.

Chen, R. F. (1971) *Arch. Biochem. Biophys. 142*, 552–564.

Co, M. S., & Hodgson, K. O. (1981) *J. Am. Chem. Soc. 103*, 319–330.

Co, M. S., Hodgson, K. O., Eccles, T. K., & Lontie, R. (1981) *J. Am. Chem. Soc. 103*, 984–986.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Vekatappa, M. P. (1978) *Nature (London) 272*, 319–324.

Colosimo, A., Brunori, M., & Wyman, J. (1979) in *Structure and Function of Haemocyanin* (Bannister, J. V. M., Ed.) pp 189–192, Springer-Verlag, Berlin.

Donovan, J. W. (1969) *J. Biol. Chem. 244*, 1961–1967.

Donovan, J. W. (1973) *Methods Enzymol. 27*, 497–548.

Eickman, N. C., Larrabee, J. A., Solomon, E. I., Lerch, C., & Spiro, T. G. (1978) *J. Am. Chem. Soc. 100*, 6529–6531.

Eickman, N. C., Himmelwright, R. S., & Solomon, E. I. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2094–2098.

Er-El, Z., Shaklai, N., & Daniel, E. (1972) *J. Mol. Biol. 64*, 341–352.

Gilman, J. G., & Brewer, J. (1978) *Biochem. J. 169*, 625–632.

Gray, R. D. (1980) *J. Biol. Chem. 255*, 1812–1818.

Hamlin, L. M., & Fish, W. W. (1977) *Biochim. Biophys. Acta 491*, 46–52.

Hanlon, D. P., Watt, D. S., & Westhead, E. W. (1966) *Anal. Biochem. 16*, 225–233.

Hendrickson, W. A. (1977) *Trends Biochem. Sci. (Pers. Ed.) 2*, 108–111.

Herskovits, T. T. (1967) *Methods Enzymol. 11*, 748–775.

Herskovits, T. T., Erhunmwunsee, L. J., San George, R. C., & Herp, A. (1981) *Biochim. Biophys. Acta 667*, 44–58.

Horwitz, J., Strickland, E. H., & Billups, C. (1970) *J. Am. Chem. Soc. 92*, 2119–2124.

Jolley, R. L., Evans, L. H., Makino, N., & Mason, H. S. (1974) *J. Biol. Chem. 249*, 335–345.

Katoh, S., & Takamiya, A. (1964) *J. Biochem. (Tokyo) 55*, 378–387.

Klarman, A., Gottlieb, J., & Daniel, E. (1979) *Biochemistry 18*, 2239–2244.

Klotz, I. M., & Klotz, T. A. (1955) *Science (Washington, D.C.) 121*, 477–480.

Kuiper, H. A., Brunori, M., & Antonini, E. (1977) *J. Mol. Biol. 116*, 569–576.

Kuiper, H. A., Forlani, L., Ghiancone, E., Brunori, M., & Wyman, J. (1979) *Biochemistry 18*, 5849–5854.

Kuiper, H. A., Zolla, L., Calabrese, L., Vecchini, P., Constantini, S., & Brunori, M. (1981) *Comp. Biochem. Physiol. 69C*, 253–257.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lamy, J., Lamy, J., Bonaventura, J., & Bonaventura, C. (1980) *Biochemistry 19*, 3033–3039.

Lamy, J., Bijlholt, M. M. C., Sizaret, P. Y., Lamy, J., & van Bruggen, E. F. J. (1981) *Biochemistry 20*, 1849–1856.

Larson, B. A., Terwilliger, N. B., & Terwilliger, R. C. (1981) *Biochim. Biophys. Acta 667*, 294–302.

Liu, T. Y. (1977) in *The Proteins* (Neurath, H., Hill, R. L., & Boeder, C. L., Eds.) pp 239–402, Academic Press, New York.

Mangum, C., & Towle, D. (1977) *Am. Sci. 5*, 65–75.

Markl, J., Savel, A., Decker, H., & Linzen, B. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 649–660.

Martin, L. J. M., Van Wormhoudt, A., & Ceccaldi, H. J. (1977) *Comp. Biochem. Physiol. A 58A*, 193–195.

Mihalyi, E. (1968) *Biochemistry 7*, 208–223.

Miller, K., & Van Holde, K. E. (1974) *Biochemistry 13*, 1668–1674.

Miller, K. I., Eldred, N. W., Arisaka, F., & Van Holde, K. E. (1977) *J. Comp. Physiol. 115*, 171–184.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.

Nickerson, K. W., & Van Holde, K. E. (1971) *Comp. Biochem. Physiol. B 39*, 855–872.
Oehlschlegel, F. J., Brewer, G. J., Knutse, H., Prasad, A. S., & Shoomaker, B. (1974) *Arch. Biochem. Biophys. 163*, 742–748.
Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465–498.
Ramachandran, L. K., & Witkop, B. (1964) *Biochemistry 3*, 1603–1611.
Rifkind, J. M. (1974) *Biochemistry 13*, 2475–2481.
Rifkind, J. M. (1979) *Biochemistry 18*, 3860–3865.
Rifkind, J. M., & Heim, J. M. (1977) *Biochemistry 16*, 4438–4443.
Rifkind, J. M., Lauer, L. D., Chiang, S. C., & Li, N. C. (1976) *Biochemistry 15*, 5337–5343.
Riggs, A. F., & Wolbach, R. A. (1956) *J. Gen. Physiol. 39*, 585–605.
Smith, R. M., & Martell, A. E. (1976) *Critical Stability Constants*, Vol. 4, Plenum Press, New York.
Sullivan, B., Bonaventura, J., & Bonaventura, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2558–2562.
Sytkowski, A. J., & Vallee, B. L. (1979) *Biochemistry 18*, 4095–4099.
Vallee, B. L., & Ulmer, D. D. (1972) *Annu. Rev. Biochem. 41*, 91–128.
van Driel, R., & van Bruggen, E. F. J. (1975) *Biochemistry 14*, 730–735.
van Driel, R., Brunori, M., & Antonini, E. (1974) *J. Mol. Biol. 89*, 103–112.
Van Holde, K. E., & van Bruggen, E. F. J. (1971) *Biol. Macromol. 5 (Part A)*, 1–55.
Vasak, M., Kagi, J. H. R., & Hill, H. A. O. (1981) *Biochemistry 20*, 2852–2856.
Whaling, P. J., Barber, R. T., & Paul, J. C. (1977) Report No. 123, Water Resources Research Institute of the University of North Carolina.
Wright, D. A. (1977a) *J. Exp. Biol. 67*, 137–146.
Wright, D. A. (1977b) *J. Exp. Biol. 67*, 147–161.
Wright, D. A., & Brewer, G. C. (1979) *Mar. Biol. (Berlin) 50*, 151–156.
Zolla, L., Kuiper, H. A., Vecchini, P., Antonini, E., Brunori, M., & Wyman, J. (1978) *Eur. J. Biochem. 87*, 467–473.

Calcium-Dependent α-Helical Structure in Osteocalcin†

Peter V. Hauschka* and Steven A. Carr

ABSTRACT: Osteocalcin is an abundant Ca^{2+}-binding protein of bone containing three residues of vitamin K dependent γ-carboxyglutamic acid (Gla) among its 49 (human, monkey, cow) or 50 (chicken) amino acids. Gla side chains participate directly in the binding of Ca^{2+} ions and the adsorption of osteocalcin to hydroxylapatite (HA) surfaces in vivo and in vitro. Osteocalcin exhibits a major conformational change when Ca^{2+} is bound. Metal-free chicken osteocalcin is a random coil with only 8% of its residues in the α helix as revealed by circular dichroism. In the presence of physiological levels of Ca^{2+}, 38% of the protein adopts the α-helical conformation with a transition midpoint at 0.75 mM Ca^{2+} in a rapid, reversible fashion which (1) requires an intact disulfide bridge, (2) is proportionally diminished when Gla residues are decarboxylated to Glu, (3) is insensitive to 1.5 M NaCl, and (4) can be mimicked by other cations. Tyr fluorescence, UV difference spectra, and Tyr reactivity to tetranitromethane corroborate the conformational change. Homologous monkey osteocalcin also exhibits Ca^{2+}-dependent structure. Integration of predictive calculations from osteocalcin sequence has yielded a structural model for the protein, the dominant features of which include two opposing α-helical domains of 9–12 residues each, connected by a β turn and stabilized by the Cys_{23}–Cys_{29} disulfide bond. Cation binding permits realization of the full α-helical potential by partial neutralization of high anionic charge in the helical domains. Periodic Gla occurrence at positions 17, 21, and 24 has been strongly conserved throughout evolution and places all Gla side chains on the same face of one α helix spaced at intervals of $\sim$5.4 Å, closely paralleling the interatomic separation of Ca^{2+} in the HA lattice. Helical osteocalcin has greatly increased affinity for HA; thus, the Ca^{2+}-induced structural transition may perform an informational role related to bone metabolism.

Specific interaction between ionic calcium and proteins serves numerous important biochemical and biological functions in areas as diverse as muscle contraction, cell motility, fertilization, neurotransmission, blood coagulation, and hormonal regulation of metabolic pathways [see Kretsinger (1976) and references cited therein]. Ca^{2+} has been called the "second messenger" (Rasmussen & Goodman, 1977), and indeed the targets for its action are generally known to be proteins. This interaction may be classified according to the nature and affinity of the Ca^{2+} binding site and by the free Ca^{2+} concentration in the natural environment of the protein (Kretsinger, 1977). Certain enzymes require Ca^{2+} as a cofactor, and this cation is tightly bound in or near the active site [phospholipase A_2 (Verheij et al., 1980) and staphylococcal nuclease (Tucker et al., 1979)]. Others are merely stabilized by Ca^{2+} bound at noncatalytic sites [thermolysin (Matthews & Weaver, 1974) and other proteases (Roche & Voordouw, 1977)]. Another class of proteins containing one or more "E-F hand" structural domains with closely related amino acid sequences exhibits high affinity for Ca^{2+} ($K_d \simeq 10^{-6}$–10^{-7} M) at binding sites formed by the junction of two α-helical segments and their connecting loop (Kretsinger, 1976, 1977).

† From the Department of Orthopaedic Surgery, Children's Hospital Medical Center, and the Department of Oral Biology, Harvard School of Dental Medicine, Boston, Massachusetts 02115 (P.V.H.), and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (S.A.C.). *Received October 30, 1981.* Supported by the March of Dimes Birth Defects Foundation and National Institutes of Health Grants AM 15671 and AM 16754 to the Children's Hospital Medical Center and GM 2625 to Harvard Medical School.

0006-2960/82/0421-2538$01.25/0