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Metal Ion Interactions with *Limulus polyphemus* and *Callinectes sapidus* Hemocyanins: Stoichiometry and Structural and Functional Consequences of Calcium(II), Cadmium(II), Zinc(II), and Mercury(II) Binding†

Marius Brouwer,* Celia Bonaventura, and Joseph Bonaventura

ABSTRACT: Hemocyanins are oligomeric metalloproteins containing binuclear copper centers that reversibly combine with oxygen molecules. The structural stability and functional properties of these proteins are modified by divalent cations. Equilibrium dialysis was used to study the reversible interaction of *Callinectes sapidus* and *Limulus polyphemus* hemocyanins with the divalent cations calcium, cadmium, zinc, copper, and mercury. The number of binding sites and association constants for each cation were obtained from an analysis of the binding data by a nonlinear least-squares minimization procedure. Spectral analysis showed *Limulus* hemocyanin to possess two mercury-reactive sulfhydryl groups per subunit ($K_{assoc} = 2.02 \times 10^{45}$ M^{-1}). *Callinectes* hemocyanin contains only one such group ($K_{assoc} = 2.29 \times 10^{34}$ M^{-1}). Cadmium and zinc are shown to substitute for calcium ions. Oxygen binding studies with *Limulus* hemocyanin showed that all five divalent metal ions increase its oxygen affinity. Calcium ions increase cooperativity of oxygen binding, while heavy-metal ions have an opposite effect. Binding of two mercuric ions per *Limulus* hemocyanin subunit irreversibly fixes the 48 subunit aggregate in a high-affinity noncooperative conformational state. These results offer a striking contrast to the functional consequences of heavy-metal ion interactions with *Callinectes* hemocyanin [Brouwer, M., Bonaventura, C., & Bonaventura, J. (1982) *Biochemistry 21*, 2529–2538]. The functional alterations associated with metal ion interactions are discussed within the context of an extension of the two-state model for allosteric transitions of Monod et al. [Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118]. Incubation of *Limulus* oxy- or deoxyhemocyanin with mercuric chloride results in the conversion of 60% of the binuclear copper sites to stable half-apo sites. The remaining active sites are stable with respect to mercury-induced copper displacement when oxygen is bridging both coppers. In the absence of oxygen these sites will eventually lose both copper atoms. Under the same conditions 50% of the binuclear copper sites of *Callinectes* deoxyhemocyanin are converted to half-apo sites. In this case oxygen completely protects against copper displacement [Brouwer, M., Bonaventura, C., & Bonaventura, J. (1982) *Biochemistry 21*, 2529–2538]. The binuclear copper center of *Busycon carica* is not affected at all, demonstrating profound differences between the active sites of hemocyanins of a chelicerate arthropod (*Limulus*), a crustacean arthropod (*Callinectes*), and a gastropod mollusc (*Busycon*).

A very large number of proteins require a metal ion for their structural stability or their biological function. In addition, enzymes and other proteins bind metal ions at other than structural or functional sites. In fact, the elucidation of three-dimensional structures by X-ray diffraction is dependent upon these other metal sites (Lipscomb, 1980). Hemocyanins, e.g., the multisubunit proteins that function as reversible oxygen carriers in arthropods and molluscs (Van Holde & van Bruggen, 1971; Bonaventura et al., 1977; Hendrickson, 1977; Lamy & Lamy, 1981; Van Holde & Miller, 1982), interact in vivo, as well in vitro, with a number of metal ions. Their oxygen binding site consists of a binuclear copper center containing both an endogenous (protein) and exogenous (dioxygen) ligand bridge (Himmelwright et al., 1980). EXAFS (extended X-ray absorption fine structure) (Brown et al., 1980; Co et al., 1981) and resonance Raman data (Larrabee &

† From the Marine Biomedical Center, Duke University Marine Laboratory, Beaufort, North Carolina 28516. *Received March 15, 1983.* This paper is dedicated to Professor Eraldo Antonini of the University of Rome, Italy, a dear friend and colleague whose recent death was a scientific and personal loss. This work was supported by grants from the National Oceanic and Atmospheric Administration (NOAA NA80RAD00063) and the National Science Foundation (PCM 79-06462).

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Spiro, 1980) strongly suggest imidazole nitrogens as the primary copper ligands.

In addition to copper, hemocyanins contain variable amounts of tightly bound zinc ions (Martin et al., 1977; Brouwer & Engel, 1982). In vitro experiments have demonstrated that these ions affect the oxygen binding characteristics of blue crab, *Callinectes sapidus*, hemocyanin (Brouwer et al., 1982a; Brouwer & Engel, 1982). A third divalent cation that plays an important role in controlling hemocyanin structure and function is calcium. It has been extensively demonstrated that calcium (or magnesium) is required for maintaining the structural stability of many arthropodan and molluscan hemocyanins. Removal of calcium from these proteins at alkaline pH results in their dissociation into subunits. In addition to its structural role, calcium ions also act as modulators of hemocyanin function. It has been suggested that cooperative oxygen binding in molluscan hemocyanins is conditional upon the presence of calcium (Klarman & Daniel, 1977). However, at high ionic strength, oxygen binding by *Helix pomatia* has been reported to be cooperative, even in the absence of calcium (van Driel & van Bruggen, 1974). In view of the important role that calcium ions play in controlling hemocyanin structure and function, it is surprising that only a few direct studies have been reported concerning calcium binding by hemocyanins. Such studies have been used to determine an overall binding constant (Kuiper et al., 1979) or the number of calcium-binding sites (Arisaka & Van Holde, 1979). It is only very recently that a quantitative analysis has been published concerning calcium binding by *Panulirus interruptus* hemocyanin (Andersson et al., 1982).

In a previous paper we have demonstrated that other divalent metal ions such as Cd(II), Zn(II), Cu(II), and Hg(II) have profound effects on structure and function of *Callinectes sapidus* hemocyanin. The purpose of this study was to examine in more detail the stoichiometry and functional consequences of divalent metal ion binding to *Callinectes sapidus* and *Limulus polyphemus* hemocyanin. It is demonstrated that Cd(II) and Zn(II) substitute for Ca(II). This observation opens the possibility to probe the calcium-binding sites of hemocyanins by means of ^{113}Cd NMR spectroscopy. Finally, we demonstrate that the binuclear copper centers of hemocyanins of the merostomata (*Limulus polyphemus*), crustacea (*Callinectes sapidus*), and gastropoda (*Busycon carica*) can be distinguished by their response to Hg(II) ions. A preliminary report of our findings has appeared elsewhere (Brouwer et al., 1982b).

Materials and Methods

Specimens of the horseshoe crab, *Limulus polyphemus*, the blue crab, *Callinectes sapidus*, and the knobbed whelk, *Busycon carica*, were obtained from waters in the vicinity of Beaufort, NC. *Limulus and Callinectes* hemocyanin were purified as described earlier (Brenowitz et al., 1981; Brouwer et al., 1982a). Hemolymph obtained from the pedal hemocoel of *Busycon* was allowed to drip into a beaker containing a few milligrams of the protease inhibitor phenylmethanesulfonyl fluoride. The hemolymph was centrifuged for 20 min at 12000*g* to remove particulate matter and then concentrated on a Diaflow YM10 ultrafiltration membrane. A volume of 5 mL, at a protein concentration of 70 mg/mL, was applied to a 2.4 × 50 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).

Hg(II), Cd(II), Cu(II), and Zn(II) binding by 60S *Limulus* and 25S *Callinectes* hemocyanin were studied at 20 °C in 50 mM Tris and 10 mM $CaCl_2$, ionic strength 0.13, pH 7, by using a Spectrum Medical Industries equilibrium dialyzer equipped with 1-mL Teflon cells and Spectra/Por 2 membranes. Cells were rotated at 20 rpm in a temperature-controlled water bath at 20 °C. Protein concentration was 3 mg/mL. The 60S *Limulus* hemocyanin and 25S *Callinectes* hemocyanin used for Ca^{2+} binding studies were extensively dialyzed against 50 mM Tris, pH 7, ionic strength 0.1, to which 25 μM $CaCl_2$ was added; 1 mL of these solutions at protein concentrations of 3–15 mg/mL was added to the protein compartment of the dialysis cell and dialyzed against 1 mL of 50 mM Tris, pH 7, ionic strength 0.1, containing 0–6 mM $CaCl_2$. Although initial experiments demonstrated that equilibrium was attained within 60 min, dialysis was usually carried out for at least 180 min. The concentration of 25S *Callinectes* hemocyanin was calculated from the extinction coefficient as given by Herskovits et al. (1981): $E^{1\%}_{1cm} = 14.7$ at 280 nm. The extinction coefficients for *Limulus* hemocyanin were determined from the weight of lyophilized hemocyanin samples, corrected for the weight of buffer salts, of known optical density and volume. For native undissociated, 60S hemocyanin at 280 and 340 nm we found $E^{1\%}_{1cm} = 13.04$ and 3.31, respectively, while for the dissociated 5S subunits we found $E^{1\%}_{1cm} = 11.71$ and 2.64, respectively. Molar concentrations were calculated by using a value of 75 000 for the molecular weight of *Callinectes* hemocyanin subunits (Hamlin & Fish, 1977) and 70 000 for the *Limulus* hemocyanin subunits (Johnson & Yphantis, 1978). Calcium, zinc, cadmium, and copper concentrations were determined by flame absorption spectroscopy with a Varian AA6 spectrometer. Mercury was measured with a Spectro Products, Inc., HG-3 mercury monitor with continuous background correction (Whaling et al., 1977). The concentration of free mercuric, cadmium, and zinc ions in 50 mM Tris and 10 mM $CaCl_2$, ionic strength 0.13, pH 7, was calculated as described previously (Brouwer et al., 1982a) from the total metal ion concentration and the stability constants for the various metal ion–chloride, –hydroxide, and –Tris complexes as given by Smith & Martell (1976) and Hanlon et al. (1966): $[Hg_{total}] = [Hg^{2+}_{free}](7.06 \times 10^{11})$; $[Cd_{total}] = [Cd^{2+}_{free}](12.86)$; $[Zn_{total}] = [Zn^{2+}_{free}](14.65)$. The activity coefficients of Ca^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+} were calculated from the Debye–Hückel limiting law as extended by Davies (Stumm & Morgan, 1981):

$$\log \gamma_+ = -AZ^2\left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I\right) \qquad (1)$$

with $A = (1.82 \times 10^6)(\epsilon T)^{-3/2}$ and $\epsilon = 80.36$ at 20 °C. The effect of the excluded volume of the hydrated protein on the results of the equilibrium dialysis experiments was calculated to be negligible at the protein concentrations used. The binding data were presented as Scatchard plots (Scatchard, 1949; Rodbard & Feldman, 1975). The experimental data were fitted by a nonlinear least-squares minimization procedure (Johnson et al., 1976; Turner et al., 1981) to the following function:

$$r = \sum_{i=i}^{3} \frac{n_i K_i(X)}{1 + K_i(X)} \qquad (2)$$

where r = average number of bound metal ions per hemo-

Table I: Metal Binding by 60S *Limulus* Hemocyanin[a]

	calcium	mercury	zinc	cadmium
N_1[b]	0.63	2.83	0.77	2.20
	(0.45-0.82)[d]	(2.62-3.08)	(0.68-0.89)	(1.78-2.82)
K_1[c]	2.47×10^5	2.02×10^{45}	1.03×10^8	1.79×10^6
	$[(0.86\text{-}6.47) \times 10^5]$	$[(1.06 \times 10^{18}\text{-}7.81 \times 10^{45})]$	$[(0.74\text{-}1.45) \times 10^8]$	$[(1.43\text{-}2.11) \times 10^6]$
N_2	2.69	5.68	8.00	6.92
	(1.87-3.61)	(4.78-6.49)	(5.88-10.63)	(5.16-9.12)
K_2	1.74×10^3	1.32×10^{16}	8.47×10^5	1.16×10^5
	$[(0.78\text{-}3.60) \times 10^3]$	$[(1.01\text{-}1.70) \times 10^{16}]$	$[(5.08\text{-}12.74) \times 10^5]$	$[(0.53\text{-}1.96) \times 10^5]$

[a] Metal binding by 60S *Limulus* hemocyanin in 50 mM Tris, pH 7, $I = 0.1$ (Hg, Zn, and Cd samples contain 10 mM $CaCl_2$). [b] N1, number of binding sites in class 1. [c] K1, association constant of N1 (M^{-1}) etc. [d] Numbers in brackets are confidence limits corresponding to one standard deviation.

Table II: Metal Binding by 25S *Callinectes* Hemocyanin[a]

	calcium	mercury	zinc	cadmium
N_1[b]	0.45	0.84	2.04	1.00
	(0.37-0.48)[d]	(0.71-0.98)	(1.88-2.24)	(0.66-1.35)
K_1[c]	6.32×10^4	2.29×10^{34}	5.34×10^7	5.45×10^6
	$[(4.29\text{-}9.05) \times 10^4]$	$[(2.04 \times 10^{18}\text{-}7.95 \times 10^{45})]$	$[(4.26\text{-}6.74) \times 10^7]$	$[(3.13\text{-}9.37) \times 10^6]$
N_2	4.20	2.32	22.4	12.8
	(2.28-6.96)	(1.93-2.72)	(15.7-30.6)	(11.4-14.1)
K_2	5.18×10^2	6.89×10^{16}	1.92×10^5	1.12×10^5
	$[(1.82\text{-}10.50) \times 10^2]$	$[(4.71\text{-}9.79) \times 10^{16}]$	$[(1.14\text{-}3.08) \times 10^5]$	$[(0.84\text{-}1.57) \times 10^5]$
N_3		(12.7-17.5)		
K_3		2.61×10^{15}		
		$[(2.09\text{-}3.16) \times 10^{15}]$		

[a] Metal binding by 25S *Callinectes* hemocyanin in 50 mM Tris, pH 7, $I = 0.1$ (Hg, Zn, and Cd samples contain 10 mM $CaCl_2$). [b] N1, number of binding sties in class 1. [c] K1, association constant of N1 (M^{-1}) etc. [d] Numbers in brackets are confidence limits corresponding to one standard deviation.

cyanin subunit, n_i = number of binding sites with association constant K_i, per hemocyanin subunit, and X = metal ion activity.

Calcium Replacement by Heavy-Metal Ions. In order to determine whether heavy-metal ions are capable of substituting for Ca^{2+}, 60S *Limulus* hemocyanin (10 mg/mL) and 25S *Callinectes* hemocyanin (7.5 mg/mL) in 50 mM Tris, pH 7, $I = 0.1$, buffer containing 4 mM $CaCl_2$ were incubated for 2 h at room temperature with 1.6 mM $HgCl_2$, $CdCl_2$, $ZnCl_2$, or $CuSO_4$. Subsequently the hemocyanin samples were centrifuged at 130000*g* for 2.5 h for *Limulus* and 8 h for *Callinectes* hemocyanin to pellet the protein. The clear supernatants were then analyzed for calcium by atomic absorption spectroscopy. All incubations and centrifugations were carried out in duplicate. A sample not subjected to centrifugation was used to determine the total (free and bound) calcium and hemocyanin concentration. The difference between the calcium concentration in the hemocyanin solution and in the supernatant was assumed to represent the calcium bound to the hemocyanin.

Effect of $HgCl_2$ on the Binuclear Copper Sites of 60S Limulus Hemocyanin. Three-milliliter samples of native 60S oxy- or deoxyhemocyanin (3 mg/mL) in 50 mM Tris and 10 mM $CaCl_2$, pH 7, $I = 0.13$, were incubated at room temperature in the presence of 1 mM $HgCl_2$. Under these conditions the protein precipitates due to indefinite self-association. The mercury treatment was stopped at various time intervals by the addition of 1 mL of 50 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.9, which also dissociates the protein to the level of 5S monomers. Samples were then extensively dialyzed against the EDTA-containing buffer. The fraction of intact active sites was determined from the remaining 340-nm copper–oxygen charge-transfer band. The fraction of active sites containing a single copper (half-apo) was calculated from the total copper concentration of the samples and the concentration of intact active site copper. Fraction of copperless (apo) active sites was calculated from the total protein concentration and the concentration of intact and half-apo active sites.

Determination of the Number of Mercury-Reactive Sulfhydryl Groups in 5S and 60S Limulus Hemocyanin. A 1-mL sample of 60S *Limulus* hemocyanin (0.44 mg/mL) in 50 mM Tris, pH 7, and 10 mM $CaCl_2$, $I = 0.13$, was incubated with 0.5 mM $HgCl_2$ for 30 min. The reaction was then stopped by addition of 200 μL of 50 mM Tris and 10 mM EDTA, pH 8.9. The samples were subsequently extensively dialyzed vs. the EDTA-containing buffer. Under these conditions the 60S structure dissociates into 5S subunits. The numer of mercury–mercaptide chromphores per hemocyanin subunit was calculated from the difference between the molar absorptivities at 250 nm of mercury-treated and control samples by using a value of 2908 ± 85 M^{-1} cm^{-1} for the molar absorptivity of the mercury–mercaptide chromophore at 250 nm (Brouwer et al., 1982a). To determine the number of sulfhydryl groups in 60S *Limulus* hemocyanin that are reactive to mercury, the hemocyanin was incubated with $HgCl_2$ as described above and then dialyzed against 50 mM Tris, pH 7, and 10 mM $CaCl_2$, $I = 0.13$, to which 2 mM EDTA was added (the affinity of EDTA for Hg^{2+} is approximately $10^{11.5}$ times larger than for Ca^{2+}). Under these conditions the 60S structure is preserved. The number of mercury–mercaptide chromphores was calculated from the molar absorptivity at 250 nm, as described above.

Results

Stoichiometry of Calcium Binding. Scatchard plots of calcium binding by 25S *Callinectes* and 60S *Limulus* hemocyanin are shown in Figure 1. The number of binding sites and their binding constants are given in Tables I and II. Both hemocyanins contain between three and four high-affinity ($K \approx 10^5$ M^{-1}) calcium binding sites per hexamer (less than one per subunit). Additionally, low-affinity sites also appear to

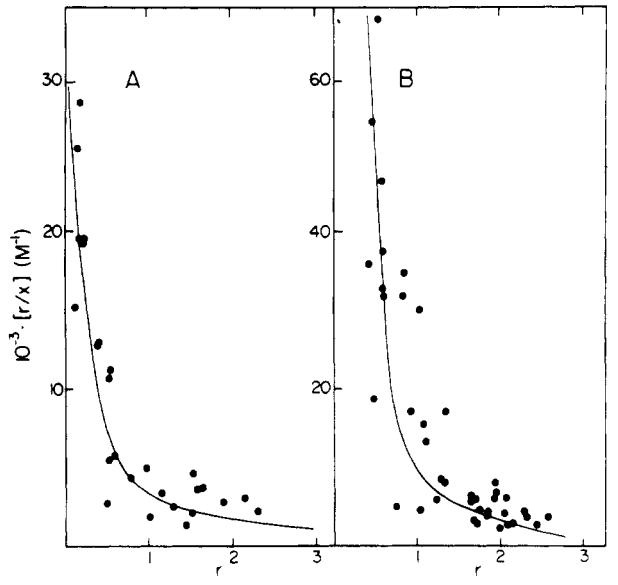

FIGURE 1: Scatchard plot of $r/(X)$ vs. r for binding of Ca^{2+} to *Callinectes* (A) and *Limulus* (B) hemocyanin. r is the binding ratio Ca^{2+}/hemocyanin subunit, and X is the free Ca^{2+} activity. The line is the theoretical curve obtained from eq 2. The number of Ca^{2+} binding sites and corresponding binding constants are given in Tables I and II.

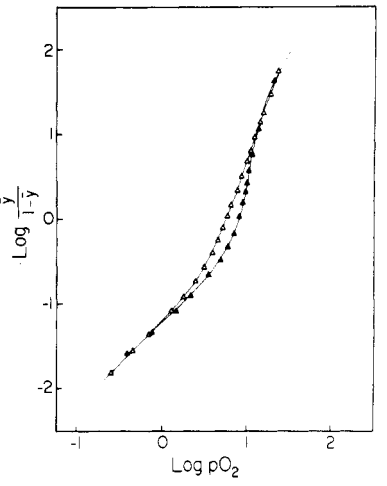

FIGURE 2: Hill plots of oxygen binding by 60S *Limulus* hemocyanin (4 mg/mL) at two different $CaCl_2$ concentrations. (△) 50 mM Tris and 4 mM $CaCl_2$, pH 7.8, ionic strength 0.112; (▲) 50 mM Tris and 20 mM $CaCl_2$, pH 7.8, ionic strength 0.130. Symbols are experimental data, and lines are calculated according to the two-state model for allosteric transitions, with the model parameters given in Table III.

be present (Tables I and II). The high-affinity sites are involved in mtaintaining the molecules in their normal aggregation states. The low-affinity sites modulate the oxygen-binding characteristics of both hemocyanins (see below).

Functional Consequences of Hemocyanin Interactions with Calcium. Hill plots of oxygen binding at pH 7.8 by 60S *Limulus* hemocyanin at two calcium concentrations are shown in Figure 2. Figure 3 summarizes the effect of calcium ions on the functional properties of both *Limulus* and *Callinectes* hemocyanins at pH 7 and at pH 7.8. At pH 7 calcium does not have a noticeable effect on oxygen binding by *Limulus* hemocyanin but increases the oxygen affinity and cooperativity of *Callinectes* hemocyanin. At pH 7.8 the cooperativity of oxygen binding by both hemocyanins was increased upon addition of calcium ions. Calcium has opposite effects on the oxygen affinity of the two hemocyanins. The oxygen affinity of *Limulus* hemocyanin decreases, but the affinity of *Callinectes* hemocyanin increases as a function of calcium ion concentration.

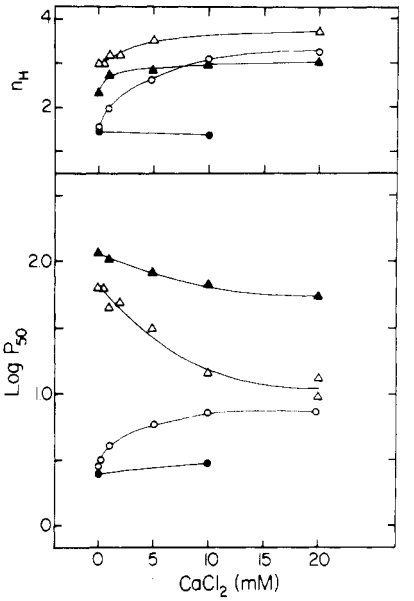

FIGURE 3: Effect of $CaCl_2$ on oxygen binding characteristics of 25S *Callinectes* (triangles) and 60S *Limulus* (circles) hemocyanin. Protein concentration 4 mg/mL. (●, ▲) 50 mM Tris, pH 7, ionic strength 0.13; (○, △) 50 mM Tris, pH 7.8, ionic strength 0.13. P_{50}, partial pressure of oxygen needed for half-saturation of hemocyanin (expressed in mmHg) n_H, Hill's coefficient, i.e., slope of the Hill plots (e.g., see Figure 2).

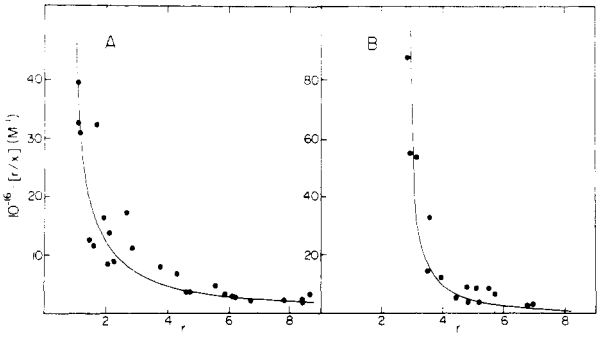

FIGURE 4: Scatchard plot of $r/(X)$ vs. r for binding of Hg^{2+} to *Callinectes* (A) and *Limulus* (B) hemocyanin. r is the binding ratio Hg^{2+}/hemocyanin subunit, and X is the free Hg^{2+} activity. The line is the theoretical curve obtained from eq 2. The number of Hg^{2+} binding sites and corresponding binding constants are given in Tables I and II.

Stoiochiometry of Heavy-Metal Binding. The Scatchard plots for Hg^{2+}, Zn^{2+}, and Cd^{2+} binding are shown in Figures 4–6. Mercuric ion binding by *Callinectes* hemocyanin could best be described with three classes of binding sites (Figure 4A and Table I). This hemocyanin possesses 18 Hg^{2+} binding sites per subunit, one site being of very high affinity ($K_{assoc} = 10^{34}$ M^{-1}). Figure 4B implies that *Limulus* hemocyanin binds eight to nine mercuric ions per subunit; approximately three of these are bound with an extremely high affinity ($K_{assoc} = 10^{45}$ M^{-1}; Table II). Generally, *Callinectes* hemocyanin seems to have a higher metal ion binding capacity than *Limulus* hemocyanin. Analysis of the binding curves in Figures 5 and 6 demonstrates that *Callinectes* hemocyanin binds up to 24 Zn^{2+} and 14 Cd^{2+} per subunit, while the corresponding values for *Limulus* hemocyanin are 9 and 9, respectively (Tables I and II).

Functional Consequences of Hemocyanin Interactions with Heavy-Metal Ions. The effect of heavy-metal ions on oxygen binding by *Callinectes* hemocyanin has been reported previ-

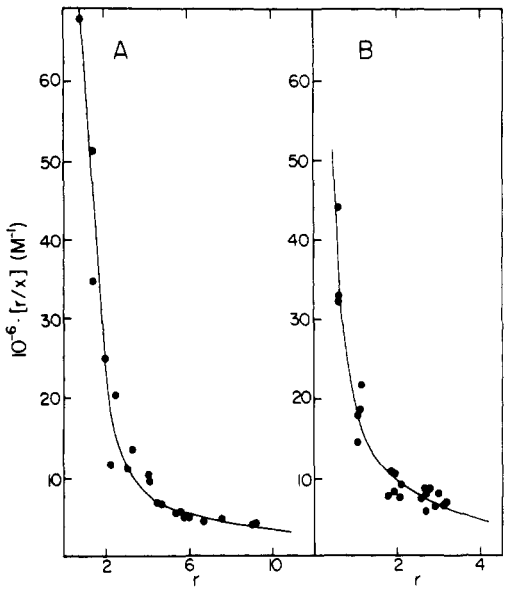

FIGURE 5: Scatchard plot of $r/(X)$ vs. r for binding of Zn^{2+} to *Callinectes* (A) and *Limulus* (B) hemocyanin. r is the binding ratio Zn^{2+}/hemocyanin subunit, and X is the free Zn^{2+} activity. The line is the theoretical curve obtained from eq 2. The number of Zn^{2+} binding sites and corresponding binding constants are given in Tables I and II.

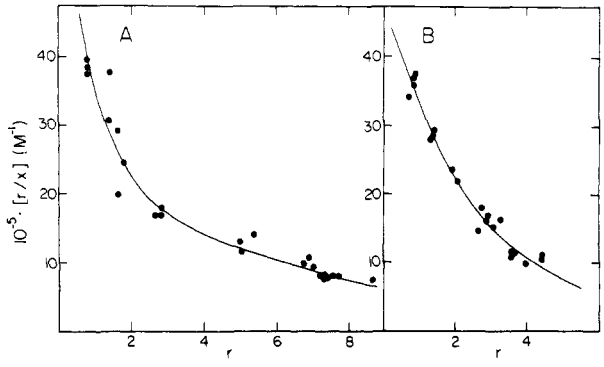

FIGURE 6: Scatchard plot of $r/(X)$ vs. r for binding of Cd^{2+} to *Callinectes* (A) and *Limulus* (B) hemocyanin. r is the binding ratio Cd^{2+}/hemocyanin subunit and X is the free Cd^{2+} activity. The line is the theoretical curve obtained from eq 2. The number of Cd^{2+} binding sites and corresponding constants are given in Tables I and II.

ously (Brouwer et al., 1982a). As will be discussed, there are striking differences in the functional alterations brought about by metal binding to *Callinectes* and *Limulus* hemocyanin. Copper, zinc, cadmium, and mercuric ions increase the oxygen affinity of 60S *Limulus* hemocyanin and decrease the cooperativity of oxygen binding as shown in Figure 7. Mercuric ions have a dramatic effect on *Limulus* hemocyanin function. These ions completely abolish the interactions between the oxygen binding sites and freeze the high molecular weight hemocyanin molecule irreversibly in a noncooperative conformational state as shown in Figure 8. Copper and zinc do not seem to interfere with the Bohr effect, and even in the presence of mercuric ions a small Bohr effect is observed (Figure 7). Only one data point at pH 8 in the presence of cadmium chloride is included because the deoxyhemocyanins at pH 7 and 7.5 self-associate into extended aggregates, thereby making tonometric oxygen binding experiments impossible. The different response of oxy- and deoxyhemocyanin to self-association induced by heavy metals demonstrates that removal of oxygen from the active site results in a structural alteration at the surface of the 60S molecule. We note that

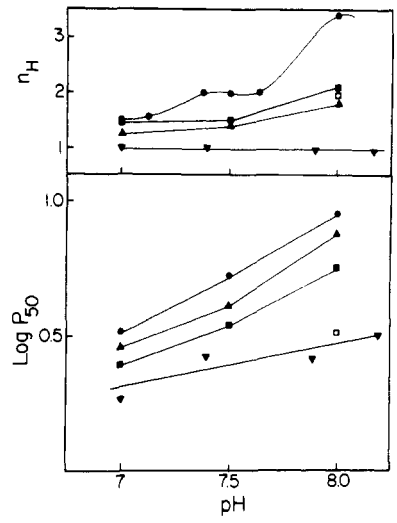

FIGURE 7: Effect of heavy metal ions on the oxygen binding characteristics of 60S *Limulus* hemocyanin. Protein concentration 4 mg/mL. (●) 50 mM Tris and 10 mM $CaCl_2$, ionic strength 0.13; (▲) same buffer containing 0.12 mM $CuSO_4$; (■) same buffer containing 0.06 mM $ZnCl_2$; (□) same buffer containing 0.25 mM $CdCl_2$; (▼) same buffer containing 0.3 mM $HgCl_2$. P_{50}, partial pressure of oxygen (expressed in mmHg) needed for half-saturation of hemocyanin; n_H, Hill's coefficient, i.e., slope of the Hill plots (see, e.g., Figure 8).

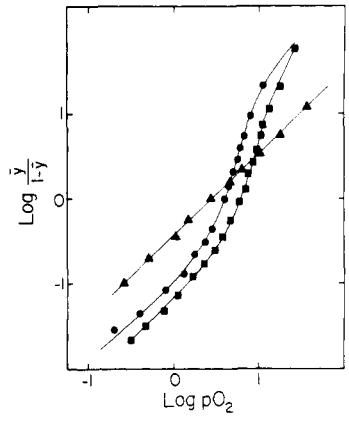

FIGURE 8: Hill plots of oxygen binding by 60S *Limulus* hemocyanin as a function of heavy-metal ions, in 50 mM Tris and 20 mM $CaCl_2$, pH 7.8, ionic strength 0.13. Protein concentration 4 mg/mL. (●) In the presence of 0.2 mM $CdCl_2$; (■) in the presence of 0.06 mM $ZnCl_2$; (▲) in the presence of 0.3 mM $HgCl_2$. Symbols are experimental data. Curves for Cd^{2+} and Zn^{2+} calculated according to the two-state model for allosteric transitions, with the model parameters in Table III.

removal of oxygen also results in structural changes in the intersubunit contact regions of 60S *Limulus* hemocyanin (Brouwer et al., 1981a,b). It should be emphasized that the oxygen-binding experiments described here were carried out under conditions where the aggregation state of the hemocyanins was unaltered.

Calcium Replacement Experiments. We wished to determine whether any of the heavy-metal ions used in this study are capable of substituting for Ca^{2+}. The simple, rapid, but semiquantitative, approach described under Materials and Methods was used to answer this question. With this approach the number of calcium-binding sites per *Limulus* and *Callinectes* hemocyanin subunits was found to be approximately five and seven, respectively, which is within, or very close to, the confidence limits as determined by careful analysis of equilibrium dialysis experiments (Tables I and II). Cadmium and zinc ions were found to be the most effect in substituting for calcium. Under the experimental conditions described,

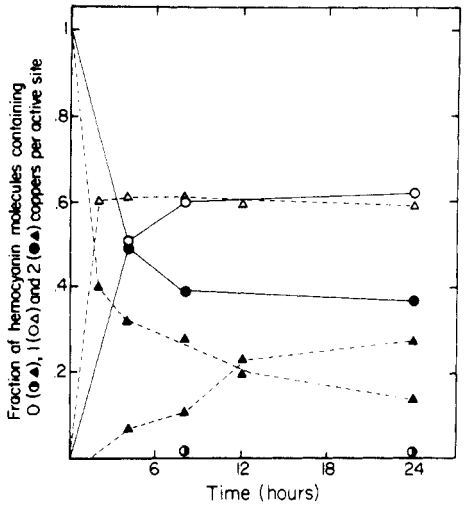

FIGURE 9: Formation of half-apo and apo active sites induced by incubation of oxygenated (circles) and deoxygenated (triangles) by 60S *Limulus* hemocyanin with 1 mM $HgCl_2$. Hemocyanin (30 μM) was in 50 mM Tris and 10 mM $CaCl_2$, pH 7, ionic strength 0.13. The 340-nm absorption band and the copper content of the samples were followed as a function of incubation time.

cadmium ions replaced 2.3 and 3.4 calcium ions per *Limulus* and *Callinectes* hemocyanin subunit, respectively. Zinc substituted for 3 and 2.6 calcium ions, respectively. Mercuric ions did not have an appreciable effect on the number of calcium ions bound by *Callinectes* or *Limulus* hemocyanin. Copper seemed to significantly increase the number of calcium ions bound by both hemocyanins, a puzzling observation for which we have no explanation at present.

Effect of $HgCl_2$ on the Binuclear Copper Sites in 60S Limulus Hemocyanin. The consequences of incubation of *Limulus* oxy- and deoxyhemocyanin with mercuric chloride as a function of time are shown in Figure 9. The incubation progressively decreases the number of normal, two-copper, oxygen-binding sites. This decrease is accompanied by a conversion of 60% of the active sites in both oxy- and deoxyhemocyanins into stable half-apo sites containing one copper atom. In deoxyhemocyanin a 2-h incubation period is required to attain this level of conversion. Incubation of deoxyhemocyanin up to 24 h results in a further decrease of the number of intact active sites, down to 14%, accompanied by an increase of copperless or apo sites. The fraction of half-apo (single copper) sites remains constant during the extended incubation. In oxyhemocyanin it takes about 8 h of incubation before the level of 60% stable half-apo sites is reached. The remaining 40% of intact active sites in oxyhemocyanin are stable. No appreciable formation of apohemocyanin is observed upon prolonged incubation of the oxygenated protein.

Determination of the Number of Mercury-Reactive Sulfhydryl Groups in 5S and 60S Limulus Hemocyanin. The number of sulfhydryl groups in *Limulus* hemocyanin that are reactive to mercury was determined spectroscopically. The molar extinction coefficient of mercury-treated 5S *Limulus* hemocyanin at 250 nm was found to have a value of 35 350 $\pm$ 579 M^{-1} cm^{-1}. The value for non-mercury-treated 5S hemocyanin was 28 711 $\pm$ 698 M^{-1} cm^{-1}. From the ΔOD_{250} and the molar absorptivity of the mercury–mercaptide chromophore at 250 nm (2908 $\pm$ 85 M^{-1} cm^{-1}; Brouwer et al., 1982a) the presence of 2.28 $\pm$ 0.32 mol of mercury–mercaptide chromophore/mol of hemocyanin subunit was calculated. Direct mercury measurements by atomic absorption spectroscopy showed 2.08 $\pm$ 0.21 non-EDTA removable mercuric ions per oxyhemocyanin subunit. Undissociated 60S, mercury-treated, EDTA-dialyzed, *Limulus* hemocyanin was prepared as described under Materials and Methods. Atomic absorption demonstrated the presence of 2.89 $\pm$ 0.24 non-EDTA removable mercuric ions per hemocyanin subunit in the 60S structure. Spectral analysis, as described above for the 5S particles, revealed the presence of 1.98 $\pm$ 0.41 mercury–mercaptide chromophores per hemocyanin subunit in the 60S structure.

Discussion

It is well-known that calcium ions play a crucial role in stabilizing the quaternary structure of hemocyanins and also in the modulation of their biological function (Van Holde & van Bruggen, 1971; Van Holde & Miller, 1982). In a recent paper we described how other divalent metal ions interact with *Callinectes sapidus* hemocyanin and how these interactions lead to altered structural and functional properties of the protein (Brouwer et al., 1982a). In the present paper we have extended these studies by determining the stoichiometry of calcium, mercury, zinc, and cadmium ion binding by 60S *Limulus* hemocyanin and 25S *Callinectes* hemocyanin. In addition we examined the structural and functional consequences of hemocyanin–metal interactions.

Scatchard plots for calcium binding by *Callinectes* and *Limulus* hemocyanin are shown in Figures 1. It has recently been shown by Klotz (1982) that extrapolation of Scatchard plots to the abscissa in order to obtain the total number of binding sites is an uncertain procedure, unless reliable data can be obtained at high ligand concentration. It is clear from Figure 1 that no such data exist for calcium binding by either of the hemocyanins under study. This limitation is a simple reflection of the fact that, at the high ligand concentrations needed to saturate low-affinity binding sites, the difference in concentration between free and total ligand in buffer and protein compartments becomes too small to be statistically significant, thereby making this part of the binding curve unobservable. In order to avoid graphical extrapolation, the experimentally determined values of free ligand concentration and bound ligand per mole of protein were fit to eq 2, assuming n_i classes of independent binding sites, by nonlinear least-squares analysis yielding the estimated parameters and confidence limits shown in Tables I and II. The values of the obtained parameters were then used to calculate the Scatchard plots. It should be emphasized here that the results are optimal in a statistical sense, irrespective of the choice of graphical coordinate system used for display of the data (Munson & Rodbard, 1983). The curve-fitting program minimizes the sum of the squares of the residuals (SSR: difference between actual dependent variable and the fitted function) divided by the standard deviation of the dependent variable, in order that data of different relative accuracy may be combined: $SSR = \sum[[Y - F(x)]/SD]^2$. Since the independent variable (the free ligand activity) is not a constant, but is subject to error in its determination, it is virtually impossible to determine the actual standard deviations of the dependent variables (amount of ligand bound). It was generally found, however, that the average error in the determination of r (binding ratio metal ion/hemocyanin subunit) was about $\pm$10%. Therefore, we decided to assign a standard deviation of $\pm$10% to each of the data points. The uniqueness of the fits was judged from the following criteria: (1) The variance of the fit and the values of the estimated parameters were independent of the initial guesses of the parameters; i.e., the minimum converged upon is a true minimum in residual space. (2) The distribution of the residuals is random. (3) The estimated parameters are independent from each other as judged by the values of their

cross-correlation coefficients [see Johnson et al. (1976) and Turner et al. (1981) for an extensive discussion].

The number of direct studies of calcium binding to hemocyanins is very limited. Klarman et al. (1972) reported the presence of approximately 20 Ca^{2+}-binding sites with $K_{assoc} = 10^2$ M^{-1} per M_r 50 000 unit in hemocyanin of the mollusc *Levantina*. Hemocyanin of the arthropod *Callianassa californiensis* possesses about eight calcium-binding sites per subunit, a few of which have very high affinity (Arisaka & Van Holde, 1979). Calculations based on the data as shown in Figure 10 of their paper imply that the high-affinity sites have binding constants in the range 10^4–10^5 M^{-1}. Hemocyanin of the arthropod *Panulirus interruptus* is reported to bind up to 10 calcium ions per subunit at pH 7.6, with an overall binding constant of approximately 2×10^4 M^{-1} (Kuiper et al., 1979). Recent studies by means of ^{43}Ca and ^{23}Na nuclear magnetic resonance spectroscopy revealed at least two classes of calcium-binding sites on this hemocyanin: 0.7 ± 0.3 strong binding sites [$K = (3 \pm 1) \times 10^4$ M^{-1}] and between 3 and 17 weak binding sites [$K = (1–5) \times 10^3$ M^{-1}] (Andersson et al., 1982). From the studies mentioned above and our own data it appears that arthropod hemocyanins typically possess a small number of high-affinity calcium-binding sites per subunit ($K = 10^4$–10^5 M^{-1}) and a large number of low-affinity sites ($K = 10^2$–10^3 M^{-1}). The high-affinity site will be saturated at the physiological concentration of calcium in the hemolymph (≈10 mM; Robertson, 1970). We have demonstrated that a concentration of only 100 μM $CaCl_2$ is sufficient to prevent dissociation of both *Callinectes* and *Limulus* hemocyanins at protein concentrations of 60 μM in the pH range from pH 7 to pH 8 (M. Brouwer, unpublished data). We conclude therefore that the high-affinity calcium-binding sites will be involved in stabilizing the quaternary structure of both proteins. The low-affinity sites are the allosteric modulator sites. It is generally observed that extracellular enzymes which are activated or stabilized by Ca^{2+} have low calcium affinities ($K = 10^3$–10^4 M^{-1}), consistent with the fact that the concentration of Ca^{2+} in the extracellular environment is about 1 mM. In contrast, intracellular enzymes or enzyme activators which are calcium sensitive usually have calcium affinities between 10^5 and 10^8 M^{-1} in accordance with the cytosol concentrations of free Ca^{2+} (10^{-6}–10^{-8} M) (Kretsinger, 1976). It seems therefore that hemocyanins, enzymes, and other calcium-modulated proteins have evolved calcium-binding sites with affinities adapted to the free Ca^{2+} concentration in their environment. The underlying mechanism remains unclear. The coordination geometries of calcium-binding sites of known structure are approximately octahedral, and the coordination number is usually six. There is no correlation between the number of carboxyl ligands and the calcium affinity. Nonetheless, most of the proteins, including hemocyanins, that bind Ca^{2+} strongly and specifically are acidic (Kretsinger, 1976; Lipscomb, 1980).

Our studies of heavy-metal binding demonstrate that both *Callinectes* and *Limulus* hemocyanin possess extremely high-affinity binding sites for mercuric ions (Figure 4). These high-affinity sites cause the Scatchard plots for mercury binding to curve upward very steeply at low mercury/hemocyanin ratios, which makes the binding data difficult to analyze. This is reflected in the rather wide confidence limits of the stability constants associated with these high-affinity sites: between 1.06×10^{18} M^{-1} and 7.81×10^{45} M^{-1} for *Limulus* hemocyanin and between 2.04×10^{18} M^{-1} and 7.95×10^{45} M^{-1} for *Callinectes* hemocyanin. The single high-affinity site on *Callinectes* hemocyanin has been demonstrated to correspond to a sulfhydryl group (Brouwer et al., 1982a). The number of sulfhydryl groups in *Limulus* hemocyanin that are reactive to mercury was determined spectroscopically. These studies demonstrated the presence of 2.28 ± 0.32 mol of mercury–mercaptide chromophore/mol of hemocyanin subunit. This value agrees well with direct mercury measurements by atomic absorption spectroscopy that show 2.08 ± 0.21 non-EDTA removable mercuric ions per oxyhemocyanin subunit. However, our equilibrium dialysis experiments revealed an average of 2.83 extremely high-affinity sites ($K_{assoc} = 2.02 \times 10^{45}$ M^{-1} cm^{-1}) per hemocyanin subunit in the 60S molecule. Are there then an average of three mercury–mercaptide bonds per hemocyanin subunit in the 60S structure, one of which is lost upon dissociation into 5S subunits? To explore this possibility, mercury-treated, 60S EDTA-dialyzed, *Limulus* hemocyanin was prepared (see Materials and Methods). Atomic absorption demonstrated the presence of 2.89 ± 0.24 non-EDTA removable mercuric ions per hemocyanin subunit in the 60S structure, a value in excellent agreement with our binding studies, which showed 2.83 high-affinity sites per subunit. Spectral analysis, as described above for the 5S particles, revealed the presence of 1.98 ± 0.41 mercury–mercaptide chromophores per hemocyanin subunit in the 60S structure, the same number as found for the subunits free in solution. UV-difference spectroscopy of *Limulus* hemocyanin vs. *Limulus* hemocyanin titrated with mercuric chloride does not show the spectral characteristics expected for mercury–tryptophan interactions (Brouwer et al., 1982a; Brouwer & Engel, 1982). We conclude that 60S *Limulus* hemocyanin contains three very high-affinity sites for Hg^{2+} and that two of these sites are sulfhydryl groups. The nature of the third high-affinity site, which is lost upon dissociation, is unknown. This situation contrasts to that for *Callinectes* hemocyanin where there is a single mercury-reactive sulfhydryl group per subunit and a second high-affinity site which was identified as the indole side chain of a tryptophan residue (Brouwer et al., 1982a).

Studies of heavy-metal binding were made difficult by the fact that *Callinectes* and *Limulus* hemocyanin self-associate at high heavy metal to hemocyanin ratios, to the extent that the formation of high molecular weight hemocyanin polymers results in turbid and opaque solutions, which are unfit for analysis (Brouwer et al., 1982a). Therefore, severe limitations were put on the highest concentration of heavy-metal ions that could be used during the binding studies, resulting in rather wide confidence limits for the numbers of low-affinity metal-binding sites (Tables I and II).

Studies of copper binding by both hemocyanins were very difficult, or impossible, to analyze, due to the large amount of scatter in the data points. Nevertheless it was clear that both *Limulus* and *Callinectes* hemocyanins possess a fairly large number of high-affinity copper-binding sites. The binding data for *Limulus* at pH 7, 10 mM $CaCl_2$ indicated approximately six copper-binding sites with an average binding constant of 8×10^8 M^{-1}.

Effect of $HgCl_2$ on Binuclear Copper Sites of Hemocyanins. In a previous publication we demonstrated that incubation of *Callinectes sapidus* deoxyhemocyanin with 1 mM $HgCl_2$ results in the conversion of 50% of the active sites to stable, one-copper, half-apo active sites, whereas the remaining 50% of the sites were not affected. Oxyhemocyanin was not susceptible to mercury-induced copper displacement (Brouwer et al., 1982a). These observations prompted us to examine the effect of $HgCl_2$ on *Limulus* hemocyanin. The results reveal heterogeneity within the active sites of undissociated 60S *Limulus* hemocyanin. We find that 60% of the active sites in both oxy- and deoxyhemocyanin are converted to stable

half-apo forms. The remaining active sites in oxyhemocyanin are stable with respect to mercury-induced copper displacement, whereas these sites in deoxyhemocyanin lose their coppers in pairs upon prolonged exposure to $HgCl_2$. As opposed to *Callinectes*, removal of oxygen from the active site of *Limulus* hemocyanin does not result in the exposure of an additional high-affinity binding site for reaction with mercury. Under both oxy and deoxy conditions two sulfhydryl-bound mercuric ions were found to be present. Differences and similarities between the active sites of *Callinectes* and *Limulus* hemocyanin are clearly revealed by these studies. In summary, for both *Callinectes* and *Limulus* deoxyhemocyanins 50–60% of the active sites may be readily converted to the half-apo form. The remaining sites in *Callinectes* deoxyhemocyanin are stable, and those in *Limulus* deoxyhemocyanin are not and lose both copper atoms. Oxygen bound to *Callinectes* hemocyanin protects completely against mercury-induced copper displacement. Oxygen bound to *Limulus* hemocyanin protects only 40% of the active sites, whereas the remainder is converted to half-apo sites. It is clear that $HgCl_2$ can be used as a tool to probe the active sites within undissociated arthropodan hemocyanins. Subunit heterogeneity in dissociated arthropodan hemocyanins is fairly well established (Van Holde & Miller, 1982). It is not known, however, if this subunit diversity is also reflected in the active sites of the associated intact molecules. Despite the fact that oxygen binding by *Limulus* and *Callinectes* hemocyanin subunits show functional heterogeneity, as judged from the value of the slope of Hill plots, oxygen-binding studies of the undissociated molecules strongly suggest that the functional properties of the binding sites in the aggregates are identical (Brouwer et al., 1977, 1982a,c). The data presented here demonstrate, however, that the structural properties of the active sites in the associated *Callinectes* and *Limulus* hemocyanins are not identical. This structural heterogeneity of the binding sites is apparently not associated with detectable differences in their oxygen affinities.

To further explore the active sites of hemocyanins by means of mercury perturbation, we incubated the hemocyanin of the mollusc *Busycon carica* with $HgCl_2$ under the same conditions as for *Limulus* and *Callinectes* hemocyanin. No decrease in the absorbance of the copper–oxygen band was observed. $HgCl_2$ binding studies are therefore also capable of distinguishing between the active sites of hemocyanins obtained from different species. The susceptibility to mercury-induced copper displacement decreases from the merostomata (*Limulus*), through crustacea (*Callinectes*) to the gastropoda (*Busycon*). Differences between the active sites of the hemocyanins obtained from organisms corresponding to these three classes have also been demonstrated by chemical and spectroscopic techniques [Himmelwright et al., 1980; Solomon et al., 1982; for review, see Van Holde & Miller (1982)]. The spectroscopic studies of the aggregated hemocyanins obtained from a single species have been interpreted by assuming a single active-site model. It has been reported, however, that preparation of either *Callinectes* and *Limulus* methemocyanin by two-electron oxidation of the protein leads to a disruption of approximately 35% of the active sites (Solomon et al., 1982), again a clear indication of the nonequivalence of the binuclear copper sites in these proteins.

Functional Consequences of Hemocyanin Interactions with Divalent Metal Ions. The effect of calcium ions on oxygen affinity and cooperativity of oxygen binding by *Limulus* and *Callinectes* hemocyanin is shown in Figure 3. The function of both hemocyanins is modulated by calcium ions in a concentration range from 1 to 20 mM, clearly demonstrating that the low-affinity calcium-binding sites (Tables I and II) are responsible for the observed modulation. It is notable that interaction of both hemocyanins with calcium ions results in opposite effects: a decrease in oxygen affinity for *Limulus* hemocyanin and an increase for *Callinectes* hemocyanin. This behavior is not completely unexpected, however, since we previously observed that other allosteric effectors such as chloride and H^+ also have opposite effects on the two hemocyanin systems. Thus, increases in pH or in chloride ion concentration increase the oxygen affinity of *Callinectes* hemocyanin but decrease the affinity of *Limulus* hemocyanin (Brouwer et al., 1977; Brouwer et al., 1982a). It seems rather perplexing that the interaction of the same small anions or cations with two supposedly similar hemocyanin systems can lead to such opposite effects.

Divalent cations of copper, zinc, cadmium, and mercury were found to decrease the degree of cooperative oxygen binding and to increase the oxygen affinity of *Limulus* hemocyanin (Figure 7). The effect of mercuric ions on oxygen binding by 60S *Limulus* hemocyanin is most drastic. For structural reasons yet to be clarified low levels of mercury completely freeze the 60S structure in a noncooperative conformational state. As mentioned before, *Limulus* hemocyanin possesses three extremely high-affinity sites for Hg^{2+} per subunit. Two of these sites correspond to sulfhydryl groups. How many of these sites, if any, have to be occupied in order to abolish cooperative interactions? To answer this question, 60S *Limulus* hemocyanin molecules in 50 mM Tris, pH 7, and 10 mM $CaCl_2$, ionic strength 0.13, containing 0.5, 1, 1.5, 2, and 2.5 mercuric ions per subunit were prepared by incubating 40 μM hemocyanin with 20, 40, 60, 80, and 100 μM $HgCl_2$. Equilibrium dialysis of these samples showed all of the Hg^{2+} to be protein bound. Oxygen binding experiments with these samples showed decreases in the Hill coefficients of oxygen binding from 1.8 to 0.98, while the P_{50} values decreased from 3.3 to 2 mmHg. The lowest values of the Hill coefficient and P_{50} were attained with two Hg^{2+} per hemocyanin subunit and did not decrease further when the number of Hg^{2+} per subunit increased. Therefore, we may conclude that the binding of two Hg^{2+} ions per *Limulus* hemocyanin subunit is sufficient to irreversibly abolish all cooperative interactions. These results show us that the mercuric cyanide isomorphous replacement methods used to solve the crystallographic phase problem in X-ray diffraction analysis of a *Limulus* hemocyanin subunit (Magnus & Love, 1981) should be considered with great care. On the other hand, the preparation of hemocyanin subunits, possessing one, two, and three Hg^{2+} per subunit, and knowledge of the position of the high-affinity mercuric ions in this subunit might provide us with information about the conformational changes involved in cooperative oxygen binding.

In order to understand the effect of Cd^{2+} and Zn^{2+} on hemocyanin function, we examined the possibility that these ions could substitute for calcium, the physiological effector. Our semiquantitative experiments described under Results unequivocably demonstrate that this is indeed the case. This observation also provides us with the exciting possibility of probing the allosteric sites on *Limulus* and *Callinectes* hemocyanin by means of ^{113}Cd NMR studies. This technique has been effectively used to probe the calcium binding sites of carp parvalbumin and concanavalin A (Lee & Sykes, 1980).

Oxygen binding by arthropodan hemocyanins generally 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, one with a low affinity

Table III: Effect of Calcium, Cadmium, and Zinc Ions on the Allosteric Parameters Describing the Oxygen by *Limulus polyphemus* Hemocyanin in 50 mM Tris, pH 7.8, $I = 0.1$

	K_R[a]	K_T[b]	L[c]	N[d]	SSR[e]	SSR ($N = 6$)
20 mM $CaCl_2$	0.31	16.2	6.95×10^{12}	9.90	28.70	673
	(0.27-0.35)[f]	(15.8-16.6)	[$(1.38\text{-}36.3) \times 10^{12}$]	(9.61-10.18)		
4 mM $CaCl_2$	0.11	15.7	2.44×10^{8}	5.03	5.72	13.79
	(0.09-0.13)	(15.2-16.2)	[$(0.92\text{-}6.48) \times 10^{8}$]	(4.95-5.12)		
20 mM $CaCl_2$ + 60 μM $ZnCl_2$	0.092	14.6	1.16×10^{10}	5.72	23.50	24.14
	(0.06-0.14)	(14.4-14.9)	[$(0.10\text{-}15.3) \times 10^{10}$]	(5.64-5.81)		
20 mM $CaCl_2$ + 200 μM $CdCl_2$	0.33	9.3	7.30×10^{6}	6.80	14.24	22.78
	(0.29-0.37)	(9.0-9.5)	[$(4.23\text{-}12.6) \times 10^{6}$]	(6.57-7.03)		

[a] Oxygen dissociation constant of the R state, expressed in mmHg. [b] Oxygen dissociation constant of the T state. [c] Allosteric equilibrium constant. [d] Number of interacting oxygen-binding sites. [e] Sum of the squares of the residuals [i.e., differences between the actual dependent variable, Y, and the fitted function (eq 3), divided by the standard deviation]. [f] Numbers in brackets represent confidence limits corresponding to one standard deviation.

for oxygen (T state) and one with a high affinity for oxygen (R state). The equilibrium between these two states is shifted by the binding of oxygen and by the binding of allosteric effectors. The saturation function describing oxygen binding within the two state model has the form

$$\bar{Y} = \frac{\alpha(1 + \alpha)^{n-1} + L'c\alpha(1 + c\alpha)^{n-1}}{(1 + \alpha)^n + L'(1 + c\alpha)^n} \quad (3)$$

in which $\alpha = pO_2/P_{50,R}$, $c = P_{50,R}/P_{50,T}$, and L' is the apparent allosteric equilibrium constant. The P_{50} values of the hypothetical R and T states are $P_{50,R}$ and $P_{50,T}$, and n is the number of interacting sites. It is generally agreed that arthropodan hemocyanins exist as oligomers assembled on a theme of hexameric units, each unit containing six oxygen-binding sites (Lamy et al., 1980, 1981). Most of the studies describing oxygen binding by arthropodan hemocyanins have therefore assumed the number of interacting oxygen binding sites to be six. Keeping the number of sites fixed at six, theoretical curves were then calculated and compared with the experimental ones. In this way it was found that oxygen binding by *Limulus* hemocyanin could be described, as a first approximation, by the two-state model of allosteric transitions (Brouwer et al., 1977). Later studies by the same authors revealed that the number of interacting sites seemed to be pH dependent, increasing to 12 at pH 8.9 (Brouwer et al., 1981a, 1982c). Oxygen binding by two crustacean hemocyanins was found to be best described by a modification of the original two-state model by allowing for a symmetrical R_3T_3 state ($n = 6$) (Miller & Van Holde, 1974; Brouwer et al., 1978; Arisaka & Van Holde, 1979). The four parameters of eq 3 can be obtained by the least-squares minimization procedure. If the two-state model applies, this procedure should, ideally, converge upon $n = 6$, or multiples thereof. Therefore we collected an extensive set of oxygen-binding data for *Limulus* hemocyanin as a function of calcium, cadmium, and zinc ion concentration. Three identical hemocyanin solutions in three tonometers of the same volume were subjected to the same changes in partial pressure of oxygen to generate one set of binding data, for which the standard deviations of Y, the fractional saturation of hemocyanin with oxygen, was known. These values of $Y \pm SD$ were then fitted to eq 3 with L, $P_{50,R}$, $P_{50,T}$, and n as variable parameters. The estimated parameters, and the Hill plots of oxygen binding calculated with these parameters, are shown in Table III and Figures 2 and 8, respectively. From the parameters in Table III it can be seen that the number of interacting sites in the presence of 4 mM $CaCl_2$, or in 20 mM $CaCl_2$ with added Cd^{2+} or Zn^{2+}, results in binding curves that can be fitted with a number of interacting sites ranging from five to seven (Table III). When the number of interacting sites for binding curves obtained under these conditions is fixed at six, the accuracy of the fit decreases only slightly, as judged from the small increase of the sum of squared residuals. Since the difference between the statistically best fit to eq 3 and the physically most plausible fit is small, this analysis shows that, under certain conditions, the two-state model is adequate to describe oxygen binding by the 48-subunit *Limulus* hemocyanin, with the hexamer as the allosteric unit. However, the number of interacting sites, *as interpreted within the context of the two-state model*, seems to have increased from 6 to 10 in the presence of 20 mM $CaCl_2$. Keeping n fixed at 6 dramatically increases the sum of residuals squared from 28.7 to 673, demonstrating the poor fit obtained when the number of interacting sites is assumed to be 6 (Table III). The same phenomenon has been observed for *Limulus* hemocyanin where the number of interacting sites gradually increases from 6 to 12 with increasing pH values, and for *Helix pomatia* β-hemocyanin where the number of interacting sites doubles with decreasing pH values (Brouwer et al., 1981a, 1982c; Zolla et al., 1978). These casual observations warrant a more extensive examination and thorough analysis of the effect of calcium and protons on both hemocyanins. Direct physical evidence that the number of interacting oxygen binding sites in certain arthropodan hemocyanins is not limited to six has been reported for spider hemocyanins (Loewe et al., 1977) and a scorpion hemocyanin (Lamy et al., 1980). Oxygen binding by the hemocyanins of both species is characterized by Hill coefficients, which represent the minimal number of interacting binding sites, slightly larger than nine. It should be noted from Table III that K_R and K_T may change as a function of the concentration of an allosteric effector, a phenomenon which is common to other hemocyanins as well (Van Holde & Miller, 1982).

The studies reported in this paper have demonstrated that both *Limulus* and *Callinectes* hemocyanin possess a large number of binding sites for divalent metal ions. Cadmium ions may, in future studies, be used to probe the binding site of calcium, the physiologically important effector. Mercuric ions can be used to probe the oxygen-binding site. Apart from being useful tools in probing hemocyanin structure and function, do heavy-metal ions also interact in vivo with hemocyanins, and if so, is there any physiological significance to be attached to this interaction? To explore this possibility, we determined the amount of Cd^{2+} and Zn^{2+} bound to hemocyanins of three species of marine invertebrates collected from the unpolluted waters around Beaufort, NC. Cadmium was not detectable. Zinc, however, was found to be bound to hemocyanins purified by gel-permeation chromatography or preparative ultracentrifugation from *Callinectes sapidus*, *Limulus polyphemus*, and *Busycon carica*. The Zn/O_2 binding site ratio was approximately 0.2, 0.18 and 0.3, re-

spectively. In view of the high hemocyanin concentration in the hemolymph (0.5–1 mM of O_2-binding sites) these numbers represent a considerable concentration of zinc. The presence of hemocyanin-bound zinc has also been demonstrated in *Carcinas maenas* (Martin et al., 1977) and *Homarus vulgaris* (Bryan, 1964, 1971, 1976). The latter author has suggested that zinc uptake may be mediated by hemocyanins. A similar situation seems to apply to the uptake of cadmium from seawater by *Carcinas maenas*. During cadmium uptake by *Carcinas*, this ion appears in the hemolymph, bound to protein, which may act as the transport medium to the hepatopancreas (Wright 1977a,b; Wright & Brewer, 1979). In vivo studies of cadmium accumulation by the blue crab, *Callinectes sapidus*, as a function of metal-ion exposure time showed part of the cadmium to be associated with hemocyanin (M. Howard and M. Brouwer, unpublished results). These data suggest that hemocyanins may indeed play a role in trace-metal accumulation and transport in marine invertebrates. This hypothesis is presently under investigation.

Acknowledgments

We thank Thea Brouwer-Hoexum and John Dickson for their excellent technical assistance.

Registry No. Ca, 7440-70-2; Cd, 7440-43-9; Zn, 7440-66-6; Hg, 7439-97-6; O_2, 7782-44-7.

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Interaction of R17 Coat Protein with Synthetic Variants of Its Ribonucleic Acid Binding Site†

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ABSTRACT: The specificity of the interaction between R17 coat protein and its site of translational repression on R17 RNA was studied by enzymatically synthesizing 23 sequence variants of the RNA binding site and measuring their affinity to the coat protein by a nitrocellulose filter binding assay. Experiments using oligomers truncated on the 3′ and 5′ termini allowed precise determination of the edges of the binding domain. Several oligomers which disrupted one or more of the base pairs in the binding site failed to bind coat protein, establishing the importance of RNA secondary structure for the interaction. Substitution at two single-stranded positions with each of the common bases affected K_a very differently. In one case, K_a was reduced substantially no matter which base was substituted for an adenine. At the other position, when a uracil was substituted with a purine, K_a decreased 10–100-fold, whereas when it was substituted by a cytosine, K_a increased about 5-fold. These studies indicate that the protein and the RNA hairpin loop interact over an extensive area and that several different types of contacts form to stabilize the complex.

The translational repression of the bacteriophage R17 replicase gene by the phage coat protein is a convenient system in which to study the molecular basis of a specific protein–RNA interaction. About 10 min after phage infection, phage coat protein accumulates to a concentration high enough to saturate a specific binding site on the phage RNA (Lodish & Zinder, 1966; Bernardi & Spahr, 1972). Since this site contains the initiator region for translation of the phage replicase subunit, repression of synthesis of replicase protein occurs. The binding site for coat protein consists of a single small RNA hairpin (Steitz, 1974; Jansone et al., 1979), the total synthesis of which has been recently achieved (Krug et al., 1982). The synthetic fragment binds coat protein with unit stoichiometry and an equilibrium constant identical with that of intact R17 RNA (Carey et al., 1983). Since other RNAs compete poorly with the fragment for coat protein binding, the interaction appears to be highly specific.

In this paper, we report the synthesis and coat protein binding properties of 23 sequence variants of the RNA binding site. The approach of using synthetic variants to define a protein binding site has been used successfully to identify the type and number of protein–nucleic acid contacts in the *lac* repressor–operator interaction (Caruthers, 1980). Since several of the physical properties of the R17 system differ substantially from those of *lac* (Carey & Uhlenbeck, 1983), it was of interest to compare the binding properties of variants. The results show that, like the *lac* system, there are several points on the RNA which contact the protein and other sites that are not essential for binding. In addition, the RNA secondary structure is found to be an important element in the specific interaction with the protein.

Materials and Methods

Synthesis of Variant Binding Fragments. RNA binding fragments were synthesized enzymatically according to the general protocol described by Krug et al. (1982) and summarized in Figure 1. The 21-nucleotide fragment is made by the ligation of a dodecanucleotide acceptor with a nonanucleotide donor. These two "half"-molecules were each prepared by the addition of two "quarter"-molecules. The synthesis of the 23 variants is summarized in Table I. In most cases, a variant was prepared by introducing one or sometimes two unique quarter-molecules in the synthetic scheme. These are identified by underlining in Table I.

Variant quarter-molecules were prepared in polynucleotide phosphorylase reactions using buffer A [0.4 M NaCl, 10 mM $MgCl_2$, and 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.2] or in RNA ligase reactions with buffer B [20 mM $MgCl_2$, 3 mM dithiothreitol, and 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 8.0]. Reactions were terminated by heating to 65 °C for 5 min. In many cases, 100 μg/mL bacterial alkaline

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0006-2960/83/0422-4723$01.50/0