Assembly and Calcium-Induced Cooperativity of *Limulus* IV Hemocyanin: A Model System for Analysis of Structure–Function Relationships in the Absence of Subunit Heterogeneity†

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ABSTRACT: Hemocyanins, the high molecular weight copper proteins which serve as oxygen carriers in many arthropods and molluscs, are representative of multisubunit complexes which are capable of reversible dissociation and assembly. Although reversible, in many hemocyanins these processes are not in true thermodynamic equilibria, and it has been suggested that there is "microheterogeneity" among the molecules in solution. An alternative explanation is that their complex behavior is due to the existence of quaternary interactions between structurally distinct types of subunits within the native molecule which have varying pH and ionic strength sensitivity. *Limulus* IV hemocyanin was used as a model system to examine structure–function relationships in the absence of subunit heterogeneity. Purified subunit IV of *Limulus polyphemus* hemocyanin is homogeneous by a number of electrophoretic and immunological criteria and is capable of undergoing pH-dependent self-assembly into hexamers. The monomer–hexamer transition was found to be an equilibrium whose rate is dependent on the presence or absence of calcium ions. The observation that the assembly of this homopolymer behaves as a true equilibrium suggests that the nonequilibrium dissociation profiles observed for native *Limulus* hemocyanin are related to the extensive subunit heterogeneity of the native protein. In calcium-containing buffers, the monomer–hexamer transitions of *Limulus* IV hemocyanin can be described by a cooperative mechanism with approximately six protons per hexamer lost on assembly from acid pH and three protons gained on assembly from alkaline pH. Increased ionic strength or increased temperature favors dissociation. Like the native molecule, *Limulus* IV hemocyanin behaves as an allosteric protein. In the absence of calcium ions, hexamers of *Limulus* IV are of higher oxygen affinity than monomers. Addition of calcium to the hexamers of *Limulus* IV hemocyanin results in cooperative oxygen binding. The cooperative oxygen binding of subunit IV hexamers is a clear demonstration that subunit heterogeneity is not a requirement for cooperativity in hemocyanins. The effects of monovalent and divalent cations on the function and self-assembly of *Limulus* IV hemocyanin are consistent with the proposal that this subunit is directly involved in the calcium-dependent stabilization of the 48-subunit ensemble, which is a unique feature of horseshoe crab hemocyanin.

Limulus hemocyanin is a 48-subunit heteropolymer with a molecular weight of 3.3 million (Johnson & Yphantis, 1976). The constituent subunits are heterogeneous with regard to electrophoretic and immunochemical properties and have been purified to homogeneity by ion-exchange chromatography (Sullivan et al., 1974, 1976; Brenowitz et al., 1981). The isolated subunits differ in both their oxygen binding characteristics and their roles in reassembly of the native oligomer (Bijlholt et al., 1979; Brenowitz, 1982). It is clear that the oxygen binding, dissociation, and reassembly characteristics of the native oligomer reflect a complex set of interactions between the subunits within the different levels of aggregation of the native molecule.

The dissociation and assembly of both arthropod and mollusc hemocyanins in general do not behave as true equilibria. The dissociation profiles reflect a nonequilibrium mixture of dissociation products, the proportions of which are not dependent on protein concentration. The concept of "microheterogeneity", which postulates that the hemocyanin molecules in solution are not homogeneous but differ slightly from one another in the pH of dissociation, has been developed as one explanation of this phenomenon (Siezen & Van Driel, 1973; Engleborghs & Lontie, 1973; Kegeles, 1977). Subunit heterogeneity has been found in all arthropod hemocyanins studied to date (for examples, see Markl et al. (1979a,b)]. Microheterogeneity could arise by way of assembly of the subunits into native molecules of identical molecular weight but with different subunit compositions. Alternatively, populations of identical molecules whose integrity is due to nonuniform intersubunit interactions could also exhibit the nonequilibrating dissociation reactions which have been observed.

Purified *Limulus* IV hemocyanin migrates as a single band upon native and sodium dodecyl sulfate (SDS) electrophoresis and isoelectric focusing and appears as a single symmetrical peak upon crossed immunoelectrophoresis (Brenowitz et al., 1981). The subunit is also capable of self-assembly into hexamers (Bijlholt et al., 1979). We have undertaken a systematic analysis of this subunit as a model system. Its properties enable us to study the properties of a hemocyanin oligomer in the absence of subunit heterogeneity. As will be shown, the self-assembly of *Limulus* IV hemocyanin is a true thermodynamic equilibrium by all criteria tested. Its oxygen binding properties are of interest in that subunit heterogeneity is shown not to be a requirement for cooperativity and allosteric control of oxygen binding by oligomeric hemocyanins. The properties of *Limulus* IV hemocyanin thus help to differentiate those properties of hemocyanins that are the result of polymerization of like subunits from those that are the consequences of subunit heterogeneity within a polymeric system. A preliminary report of this work has been presented by Brenowitz et al. (1982).

Materials and Methods

Hemocyanin Preparations. Limulus IV hemocyanin was prepared as described by Brenowitz et al. (1981). The protein

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was stored in liquid nitrogen until use. Similar structural properties and functional behavior were found for samples that had never been frozen. The unfrozen samples, however, did show a small but significant trend toward more favorable subunit interactions, as monitored by hexamerization and the degree of cooperativity in oxygen binding. All experiments were performed in $I = 0.1$ buffers (Long, 1961). Acetate was used from pH 5 to 6, cacodylic acid from pH 6 to 6.8, tris(hydroxymethyl)aminomethane (Tris) from pH 7 to 9, and glycine above pH 9. When required, either $CaCl_2$ or ethylenediaminetetraacetic acid (EDTA) was added to the buffers from pH-neutralized stock solutions. All solutions were dialyzed at room temperature for 24 h. Protein concentrations were estimated from the extinction coefficient at 340 nm determined for native *Limulus* hemocyanin by Nickerson & Van Holde (1971).

Ultracentrifugation. Sedimentation velocity experiments were carried out at 20 °C in a Beckman Model E ultracentrifuge, equipped with electronic speed control and an RTIC unit. Schlieren optics were used for all experiments and the plates measured with a Nikon comparator. Sedimentation coefficients were measured from the rate of movement of the peak maxima. All sedimentation coefficients were corrected to standard conditions in the usual way with the assumption that $\bar{v} = 0.725$ as for other arthropod hemocyanins. The protein concentration was 5 mg/mL unless otherwise noted. The relative areas of the peaks were determined by projecting the plates onto graph paper and counting the squares under each peak. Radial dilution corrections were applied in each case.

Oxygen Equilibria. Oxygen binding was measured by the method of Riggs & Wolbach (1956) with the fractional saturation determined from the absorption at 340 nm. The same buffer solutions and protein concentration were used as for the sedimentation velocity experiments. All samples were dialyzed at room temperature for 24 h against the appropriate buffer prior to the experiments.

Results

Self-Assembly. $s^0_{20,w}$ values of 16.0 and 5.4 S, respectively, were extrapolated for *Limulus* IV hemocyanin in pH 7, $I = 0.1$ Tris buffer containing 10 mM $CaCl_2$ and pH 9, $I = 0.1$ Tris buffer containing 10 mM EDTA. A linear dependence of the sedimentation coefficient on protein concentration was observed in these buffers, behavior characteristic of homogeneous, noninteracting protein systems (Gilbert & Gilbert, 1973). That the 16S component corresponds to a hexameric aggregate of monomers of $M_r = 70\,000$, $s_{20,w} = 5.4$ S can be inferred from the following information.

An average molecular weight of 70 000 was determined by sedimentation equilibrium for dissociated but unfractionated *Limulus* hemocyanin (Johnson & Yphantis, 1976). Molecular weight values of 69 000, 73 000, and 65 000 have been reported (using slightly different protocols) from SDS electrophoresis of *Limulus* IV hemocyanin (Sullivan et al., 1976; Markl et al., 1979a; Brenowitz et al., 1981). In addition, electron micrographs of *Limulus* IV hemocyanin under varied conditions show molecules identical in appearance with the monomers and hexamers observed for the hemocyanins of other species (Bijlholt et al., 1979). We calculated a molecular weight of 458 000 for the 16S component, by using the diffusion coefficient determined for the 16S hemocyanin of *Bathynomus giganteus* with their assumption that $\bar{v} = 0.725$ (Van Holde & Brenowitz, 1981). Hexamers of subunits with molecular weights in the range determined by SDS electrophoresis would have molecular weights of 390 000–438 000.

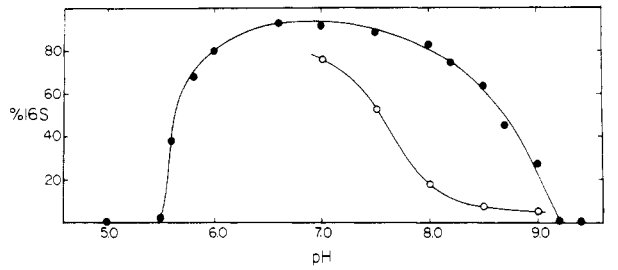

FIGURE 1: pH dependence of self-assembly of *Limulus* IV hemocyanin. Closed circles represent experiments in ionic strength 0.1 buffers containing 10 mM $CaCl_2$. Open circles are for experiments in ionic strength 0.1 buffers containing 10 mM $CaCl_2$ and 1 M NaCl. The buffers used at each pH are described under Materials and Methods.

These features support the conclusion that *Limulus* IV hemocyanin can exist as monomers or hexamers. Its two aggregation states will be referred to by their approximate sedimentation coefficients, "5S" and "16S", as in previous hemocyanin studies.

The pH dependence of the self-assembly reaction was first studied in $I = 0.1$ buffer containing 10 mM $CaCl_2$. *Limulus* IV hemocyanin is a monomer at both acid and alkaline pH, with the amount of hexamer increasing as neutral pH is approached (Figure 1). The proportion of 16S material reaches a maximum of 93% at pH 7. Temperature effects studied at pH 8.6 revealed a tendency toward greater dissociation at higher temperatures. Approximately 20% more hexamers are found at 10 °C than at 20 °C. Symmetrical, well-resolved boundaries with constant sedimentation values were observed for both the 5S and 16S components at each pH (despite differences in the proportion of the components), suggesting that the system is not in rapid equilibrium. If we postulate that the system is in a slow equilibrium (relative to the transport time of a centrifuge run), it is necessary to show that the data points of Figure 1 represent true equilibrium mixtures. The dissociation mixtures were tested to see if this is the case.

(*a*) *Reversibility.* When *Limulus* IV hemocyanin was dialyzed vs. pH 7, $I = 0.1$ Tris–10 mM $CaCl_2$ buffer, 93% of the total protein was competent to assembly into hexamers (Figure 1). The proportion of the material which remained monomeric at neutral pH did not vary with protein concentration, showing that it was not in equilibrium with the hexamers (data not shown). The possibility of contamination by subunits which do not hexamerize is ruled out by the homogeneity of subunit IV preparations as assayed by crossed immunoelectrophoresis (Brenowitz et al., 1981). The residual monomer may be due to contamination by partially denatured protein resulting from the chromatographic purification of the subunit.

As a quantitative test of reversibility, a solution of *Limulus* IV hemocyanin at pH 8.5 (73% 16 S) was dialyzed vs. pH 9.0 buffer. The percentage of 16S material decreased to 35% (the value expected from Figure 1). Upon redialysis against the pH 8.5 buffer, the protein reequilibrated to 72% 16S hemocyanin.

(*b*) *Monomer–Hexamer Separation.* Concentrated (20 mg/mL) solutions of *Limulus* IV hemocyanin at pH 8.8 and 5.7 (each sample with approximately 65% hexamer) were centrifuged in a moving partition separation cell until the 16S boundary cleared the partition's rest position. The upper portion of the sample was removed from the cell and reequilibrated by dialysis vs. the pH 8.8 or 5.7 buffer for 24 h. Following dialysis, these samples had protein concentrations of 8.6 mg/mL at pH 8.8 and 7.5 mg/mL at pH 5.7. As controls, samples which had not been centrifuged were also

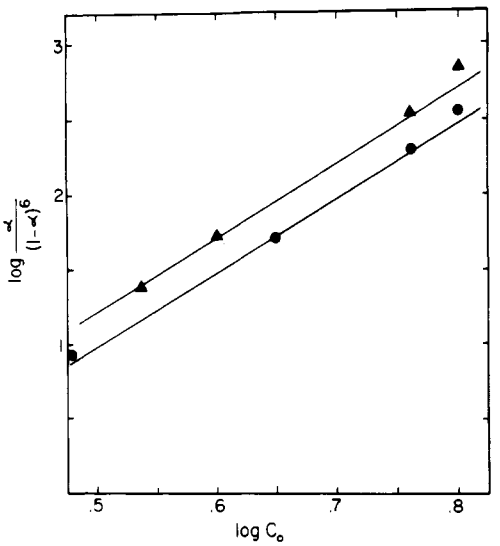

FIGURE 2: Test of the mass-action law for monomer–hexamer equilibrium. *Limulus* IV hemocyanin in $I = 0.1$ Tris buffer, pH 8.5, and 10 mM $CaCl_2$ (▲); *Limulus* IV hemocyanin in $I = 0.1$ acetate buffer, pH 5.8, and 10 mM $CaCl_2$ (●). Protein concentration, denoted by C_0, is in milligrams per milliliter. α is the fraction of hexamer. The slopes calculated by a linear, least-squares fit are 5.3, $r = 0.99$, at pH 8.5 and 5.1, $r = 0.99$, at pH 5.8 where r is the correlation coefficient. See text for further details.

dialyzed at concentrations identical with those of the reequilibrating samples. Upon centrifugation of the reequilibrated samples and their controls, identical monomer to hexamer ratios were observed (49% and 51% hexamer at pH 8.8 and 57% and 55% hexamer at pH 5.7, respectively). Reequilibration of the isolated monomers clearly occurs at both acid and alkaline pH. The fact that separation could occur at all reaffirms that the equilibration rate is slow.

(*c*) *Test of Mass-Action Law.* Varying concentrations of *Limulus* IV hemocyanin were dialyzed vs. pH 8.5, $I = 0.1$ Tris–10 mM $CaCl_2$ and pH 5.8, $I = 0.1$ cacodylate–10 mM $CaCl_2$ buffers for 24 h. The solutions were analyzed by ultracentrifugation and the relative peak areas determined and corrected for radial dilution. To test if the dependence on protein concentration is that expected for a monomer–hexamer equilibrium, the analysis of Miller & Van Holde (1981) as described for a monomer–hexamer system by Van Holde & Brenowitz (1981) was applied. For a monomer–hexamer equilibrium

$$6\mathrm{M} \rightleftharpoons \mathrm{H} \tag{1}$$

the equilibrium constant may be written as

$$K = [\mathrm{H}]/[\mathrm{M}]^6 \tag{2}$$

where [H] and [M] represent the concentrations of hexamer and monomer, respectively. With C_0 as the total weight concentration, α as the weight fraction of hexamer, and M_1 as the molecular weight of the monomer, this becomes

$$K = \frac{C_0\alpha/(6M_1)}{(1-\alpha)^6 C_0^6/M_1^6} = \frac{1}{C_0^5}\frac{\alpha}{(1-\alpha)^6}\frac{M_1^5}{6} \tag{3}$$

or

$$\log \frac{\alpha}{(1-\alpha)^6} = 5 \log C_0 + \log K - \log \frac{M_1^5}{6} \tag{4}$$

Figure 2 shows the graph of $\log [\alpha/(1-\alpha)^6]$ vs. $\log C_0$ for *Limulus* IV hemocyanin at pH 8.5 and at pH 5.8. The two sets of data points fall on lines with the expected slope of 5,

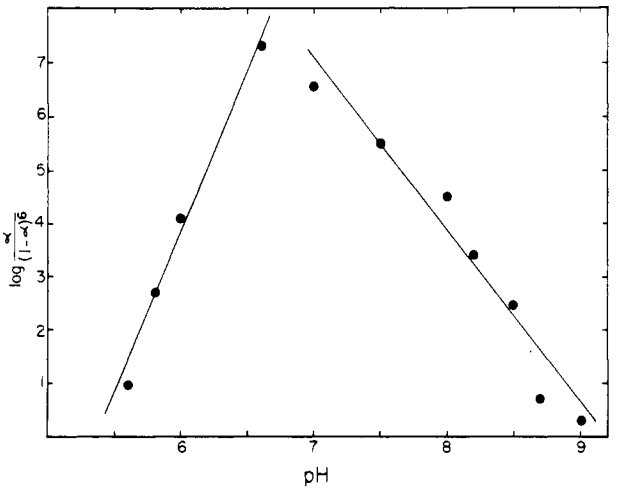

FIGURE 3: Test of the cooperative model of proton-mediated association. *Limulus* IV hemocyanin was in $I = 0.1$ buffers (described under Materials and Methods) containing 10 mM $CaCl_2$. α is the fraction of hexamer. See text for details.

clearly the behavior expected for a monomer–hexamer equilibrium. This is a particularly sensitive test as all errors in α are greatly magnified in the expression $(1-\alpha)^6$. Thus, we can conclude that the mass-action law is obeyed by the assembly reactions of *Limulus* IV hemocyanin.

It is clear from the above results that this system can be regarded as a slowly equilibrating monomer–hexamer system. Therefore, the model for a cooperative, proton-mediated association used by Van Holde & Brenowitz (1981) for the monomer–hexamer interaction of *Bathynomus* hemocyanin was applied. A model invoking appreciable concentrations of reaction intermediate did not adequately explain the data (calculations not shown). A cooperative mechanism in which the concentration of reaction intermediates is negligible can be expressed as

$$6\mathrm{M} + n\mathrm{H}^+ \rightleftharpoons \mathrm{H} \qquad K = \frac{[\mathrm{H}]}{[\mathrm{M}]^6[\mathrm{H}^+]^n} \tag{5}$$

where n, the number of protons, can have a positive or negative sign, depending on whether assembly to the hexameric state is approached from acid or alkaline pH. This scheme assumes that in the assembly from alkaline pH there is no appreciable buildup of monomers which are protonated at the site(s) involved in the association reactions. Likewise, in the assembly from acid pH, there is presumed to be no appreciable buildup of unprotonated monomers. This is equivalent to assuming that virtually all "active" monomers are immediately incorporated into hexamers.

The expression of eq 5 can be rewritten in terms of weight concentration and α, the weight fraction of hexamer. Rearranging and taking the log of the expression yield the final form

$$\log \frac{\alpha}{(1-\alpha)^6} = A - n(\mathrm{pH}) \tag{6}$$

where

$$A = \log K + \log \frac{6C_0^5}{M_1^5} \tag{7}$$

M_1 being the molecular weight of the monomer.

This theory predicts that a graph of $\log [\alpha/(1-\alpha)^6]$ vs. pH should yield a straight line with a slope equal to $-n$. Such graphs for the data at both acidic and alkaline transitions are shown in Figure 3. The slopes of the lines are −3.18 ($r = 0.98$)

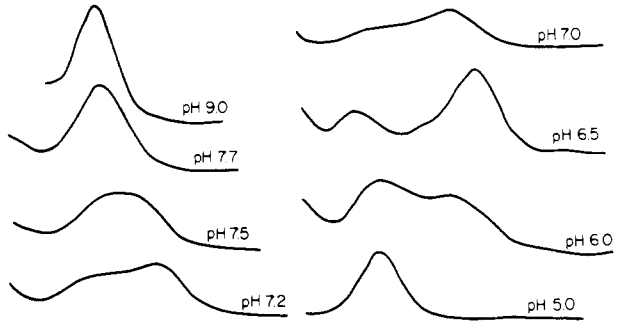

FIGURE 4: Tracings of the schlieren patterns of *Limulus* IV hemocyanin at various pH values in $I = 0.1$ buffers (described under Materials and Methods) containing 10 mM EDTA. Protein concentrations were 5 mg/mL. Rotor speed was 60 000 rpm. The photographs were all taken approximately 40 min after reaching the rotor speed.

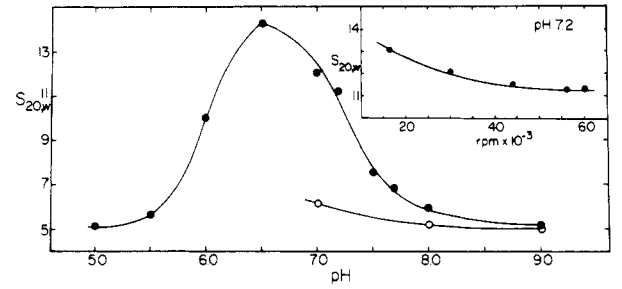

FIGURE 5: pH dependence of the sedimentation coefficient of *Limulus* IV hemocyanin in 0.1 ionic strength buffers (described under Materials and Methods) containing 10 mM EDTA (●). (○) Experiments in $I = 0.1$ Tris buffer containing 10 mM EDTA and 1 M NaCl. The sedimentation values were calculated from the movement of the maximum of the leading peak of the boundary (see Figure 4). Insert: Dependence of s on the rotor velocity at pH 7.2.

for the alkaline pH to neutral pH association and 6.26 ($r = 0.99$) for the acidic pH to neutral pH association where r is the correlation coefficient calculated from the linear least-squares fit of the data. As the slopes of the lines yield $-n$, approximately six protons per hexamer are lost on assembly from acid pH and approximately three protons per hexamer are gained on assembly from alkaline pH. Clearly, there are different groups involved for the transitions at high and low pH.

The behavior of *Limulus* IV hemocyanin in $I = 0.1$ buffers containing 10 mM EDTA differs from that observed in $CaCl_2$-containing buffers. Symmetric boundaries are observed only at pHs 5 and 9, conditions under which the subunit is monomeric. At intermediate pH values, broad asymmetric peaks, with velocities between the monomer and hexamer boundaries, are observed. These results are shown in Figure 4. The sedimentation coefficient of the leading peak increases as neutral pH is approached as shown in Figure 5. As neutral pH is approached, it appears that the association constant increases, favoring hexamer formation. As will be shown below, the behavior of *Limulus* IV hemocyanin in EDTA-containing buffers is consistent with a rapid equilibration between monomers and hexamers.

Based on Gilbert's theory (1959), Cox (1969) performed computer simulations of the sedimentation profiles expected for a monomer–hexamer system taking diffusion into account. The size of the monomer component, 6 S, and the rotor speed, 59 780 rpm, chosen for the simulations are close to those used for this study, facilitating a direct comparison with data on *Limulus* IV hemocyanin. The simulation predicts that as the association constant increases, the sedimentation coefficient of the trailing peak will remain close to that of the monomer

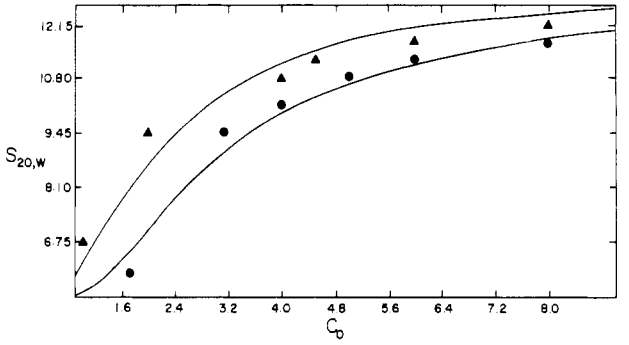

FIGURE 6: Dependence of the sedimentation coefficient on the concentration of *Limulus* IV hemocyanin in 0.1 ionic strength buffers containing 10 mM EDTA. Sedimentation coefficients were measured from the peak maxima of the boundaries shown in Figure 7. (●) pH 6.3 cacodylic acid; (▲) pH 7.2 Tris. The solid lines show the curves calculated for the weight-average sedimentation coefficient by using the values of K (9.7×10^{-9} μM^{-1} at pH 6.3 and 7.8×10^{-8} μM^{-1} at pH 7.2) determined by nonlinear least-squares analysis. See text for further details. Concentration is in milligrams per milliliter.

while the sedimentation coefficient of the leading peak increases. It also predicts minima in the schlieren boundaries, which remain constant as the proportions of high and low molecular weight peaks change with increasing association constant. The schlieren profiles of *Limulus* IV hemocyanin show the characteristics of the simulation. At the pH extremes, a symmetrical 5S peak is observed with the velocity of the slower peak unchanged from pH 7.2 to pH 9.4 (calculation not shown). As neutral pH is approached, the velocity of the fast component increases with its increasing concentration (Figures 4 and 5). Below pH 7.2, a single peak of increasing velocity is observed, rather than the appearance of a shoulder on the monomer peak predicted by the simulation. Clearly defined minima, predicted by the simulation, are not present in the *Limulus* IV hemocyanin experiments (Figure 4). A possible explanation for this is the effect of rotor speed (hydrostatic pressure) on *Limulus* IV hemocyanin (Figure 5, insert), which elevates base lines between the two peaks, possibly obscuring minima (Harrington & Kegeles, 1973).

The model also predicts that as the protein concentration increases, the low molecular weight peak will become skewed and develop a leading shoulder. Once the leading peak has appeared, the sedimentation coefficient of the trailing peak will remain constant while the leading peak will increase in both size and sedimentation value (Cox, 1969). Figure 6 shows that for *Limulus* IV hemocyanin the sedimentation coefficient of the leading peak increases with increasing protein concentration as predicted. The expected dependence of the sedimentation coefficient on total protein concentration was calculated from

$$s = (1 - gC_0)[16.0\alpha + 5.4(1 - \alpha)] \quad (8)$$

where α was determined from eq 4 and $g = 0.007$ L/g. The curves shown in Figure 6 were generated from the values of K determined by nonlinear least-squares analysis of the data to eq 4 and 8. The tracings of the boundaries shown in Figure 7 also show the predicted characteristics.

To confirm the reversibility of the interaction, *Limulus* IV hemocyanin in pH 7.5, $I = 0.1$ Tris–10 mM EDTA buffer ($s_{20,w} = 7.8$ S) was dialyzed vs. pH 7 buffer. A sedimentation coefficient of 12.4 S was measured. Upon redialysis vs. pH 7.5 buffer, a sedimentation coefficient of 7.8 S was observed, with a boundary identical with that of the original. Based on the above tests, we conclude that the monomer–hexamer interaction of *Limulus* IV hemocyanin in EDTA-containing buffer is a rapid equilibrium.

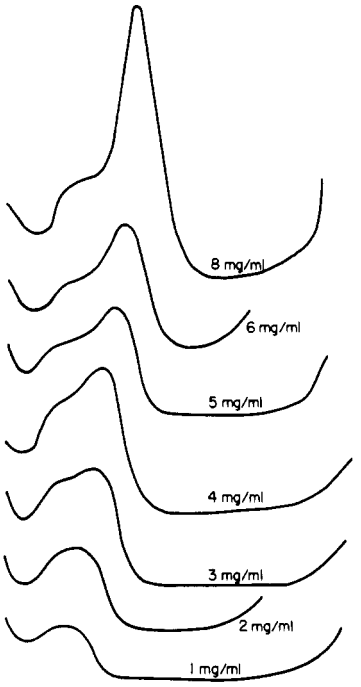

FIGURE 7: Schlieren boundaries of *Limulus* IV hemocyanin in pH 7.2, $I = 0.1$ Tris buffer containing 10 mM EDTA at decreasing protein concentration. Rotor velocity was 60 000 rpm. The photographs were taken about 35 min after reaching speed.

Inhibition of Self-Assembly. A series of experiments were performed with 1 M NaCl present in both the $CaCl_2$- and EDTA-containing buffers between pH 7 and 9. The results are shown by the open symbols in Figures 1 and 5. In both cases, NaCl inhibits the self-assembly of *Limulus* IV hemocyanin. Identical results were obtained when NaCl was added before or after transition toward neutral pH (which favors hexamerization). The symmetrical, well-resolved boundaries with constant sedimentation coefficients which were observed in the low ionic strength $CaCl_2$-containing buffers were also observed in high NaCl concentrations. Temperature effects studied with the protein in EDTA-containing buffers at pH 7.6 showed a tendency toward greater dissociation at higher temperature, as was found in the presence of calcium.

Oxygen Binding. The oxygen binding characteristics of *Limulus* IV hemocyanin were measured between pH 5 and 9 in $I = 0.1$ buffers containing 10 mM $CaCl_2$ or EDTA. In $CaCl_2$-containing buffers, the oxygen affinity was constant with changing pH as shown in Figure 8A. In contrast, in EDTA-containing buffers, an increase in affinity is observed at neutral pH (Figure 8A). In Figure 8B, the Hill coefficient is plotted vs. pH. An unexpected result is the requirement of $CaCl_2$ for cooperative oxygen binding, with n_{50} reaching about 1.4 at pH 7. Neither $MgCl_2$ nor NaCl were competent to induce cooperativity. In $CaCl_2$-containing buffers, the increase in n_{50} roughly parallels the increase in the concentration of the 16S component (compare Figures 1 and 8B). When samples which were never frozen were contrasted with those stored in liquid nitrogen (as used throughout this study), the unfrozen materials were found to be slightly (<10%) more associated at a given pH, with n_{50} values correspondingly increased (maximal n_{50} of 1.7).

The heterogeneity of oxygen binding seen in EDTA-containing buffer (Figure 8B) is clearly shown in the Hill plots presented in Figure 9. The protein at pH 9 is pure monomer while the curve at pH 7.0 shows a high proportion of hexamer (see Figure 4). The curve at pH 7.7 (where appreciable quantities of both monomer and hexamer are present) clearly

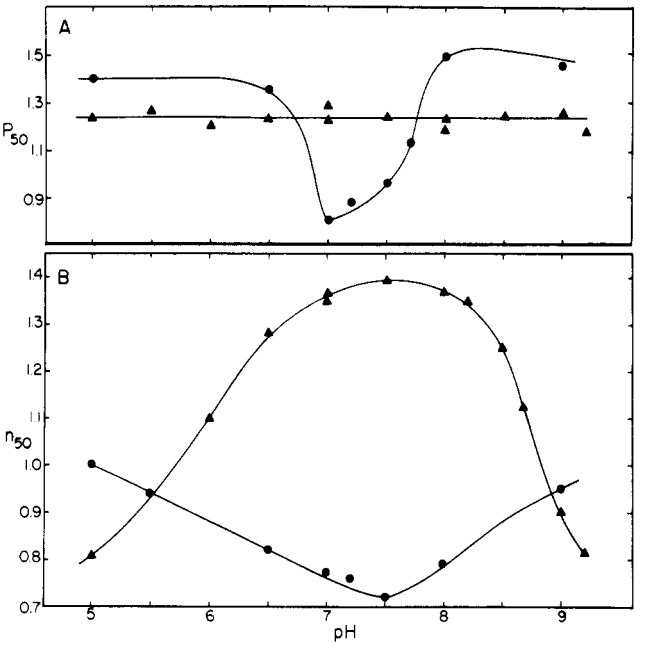

FIGURE 8: (A) Effect of pH on the oxygen affinity of *Limulus* IV hemocyanin in ionic strength 0.1 buffers (described under Materials and Methods) containing 10 mM $CaCl_2$ (▲) or 10 mM EDTA (●). P_{50} is in millimeters of mercury. (B) Effect of pH on the Hill coefficient of *Limulus* IV hemocyanin at 50% saturation in ionic strength 0.1 buffers (described under Materials and Methods) containing 10 mM $CaCl_2$ (▲) or 10 mM EDTA (●).

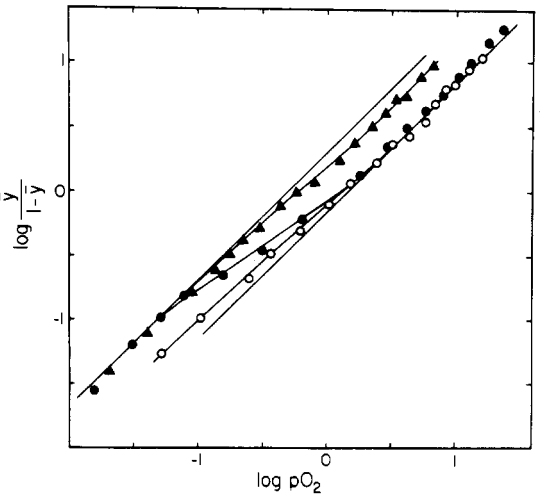

FIGURE 9: Hill plots of oxygen binding to *Limulus* IV hemocyanin in 0.1 Tris buffer containing 10 mM EDTA. Curves represent experiments at pH 7.0 (▲), 7.7 (●), and 9.0 (○).

is a transition from the "hexamer" curve to the "monomer" curve (Figure 9). It is clear that under these conditions a hexamer has a higher oxygen affinity than a monomer.

Discussion

The self-assembly reaction of *Limulus* IV hemocyanin is best described as a proton-mediated monomer–hexamer equilibrium. In the presence of $CaCl_2$, the association reactions shown by this homogeneous model system are very similar to those of the native hemocyanin isolated from the isopod *Bathynomus* (Van Holde & Brenowitz, 1981). For both proteins, the hexamerization of monomers can be described by a cooperative model in which protons are gained on assembly from alkaline pH. Calcium ions extend the alkaline pH stability of both. These facts suggest that they may share a common association mechanism. The apparent similarity

of the assembly mechanisms of *Limulus* IV hemocyanin and *Bathynomous* hemocyanin suggests that their interhexamer contacts may be similar. Conservation of the general features of intersubunit contacts is also suggested by the ability to form hybrid hemocyanins by using subunits isolated from three species of chelicerata (van Bruggen et al., 1980). The surface contacts within hexamers are in different locations than those which link hexamers and higher order structures. It is probably for this reason that the unique structural features which enable *Limulus* IV hemocyanin to stabilize the interface between two 24-mers in the native molecule have not fundamentally altered its interhexamer contacts, preserving the ability of the subunit to self-assemble. However, only in the assembly of *Limulus* IV hemocyanin hexamers does $CaCl_2$ affect the rate of monomer–hexamer equilibration. This observation is particularly significant in light of the requirement for subunit IV and calcium ions in reassembly of 48-subunit *Limulus* hemocyanin molecules from mixtures of its subunits (Bijlholt et al., 1979; Brenowitz, 1982). The observation by Bijlholt et al. (1979) of the lack of an effect of $CaCl_2$ on the self-assembly of *Limulus* IV hemocyanin (studied only at pH 7) seemed, at first, to indicate that two distinct effects were involved. However, the data presented here indicate that calcium ions do in fact affect *Limulus* IV hemocyanin by (a) changing the rate of equilibration of the monomer–hexamer interaction and (b) inducing cooperative interactions between the oxygen binding sites. Additional evidence that *Limulus* IV hemocyanin may be directly involved in the 24-subunit to 48-subunit aggregation of the native structure is the inhibitory effect of high NaCl concentrations both on the hexamerization of *Limulus* IV hemocyanin and on the assembly of the 48-mer. In contrast, high concentrations of NaCl generally promote self-assembly of the other *Limulus* subunits (Brenowitz, 1982).

The self-assembly of purified *Limulus* IV hemocyanin provides us with a model for hemocyanin polymerization in the absence of subunit heterogeneity. It appears that electrophoretic heterogeneity between subunits does not necessarily introduce complexity into assembly and dissociation processes. We note that simple monomer–hexamer equilibria occur with *Bathynomus* hemocyanin, a native hemocyanin whose subunits are electrophoretically but not immunochemically heterogeneous (unpublished results). Immunochemical identity between some of the electrophoretically distinct *Limulus* subunits (Brenowitz et al., 1981) and between some of the electrophoretically distinct subunits of crustacean hemocyanins (Markl & Kempter, 1981) indicates that this is a general phenomenon. In the case of *Limulus* hemocyanin, immunochemically identical subunits show identical functional and assembly properties (Brenowitz, 1982). Although more assembly profiles must be analyzed, it is possible that complex assembly reactions occur only when immunochemically distinct subunits are present.

The functional properties of the model homohexamer formed by *Limulus* IV hemocyanin are somewhat unusual compared to those of native hexameric hemocyanins from the crustaceans *Cherax*, *Penaeus*, and *Panulirus*, and the isopod *Bathynomous*, all of which contain electrophoretically distinct kinds of subunits (Jeffrey & Treacy, 1980; Brouwer et al., 1978; Kuiper et al., 1975; Van Holde & Brenowitz, 1981). Each of these hexameric hemocyanins shows substantial pH sensitivity in oxygen binding. In contrast, *Limulus* IV hemocyanin shows no pH dependence in the presence of calcium and only slight aggregation-dependent pH sensitivity in EDTA-containing buffers. Calcium ions have not been reported to be required for cooperative oxygen binding in crustacean hemocyanin systems, although with *Cherax* and *Panulirus* hemocyanins a reduction in cooperativity was noted in the absence of calcium ions [also see Klarman & Daniel (1977)].

All the native hexameric hemocyanins bind oxygen cooperatively. A Hill coefficient greater than 1 was noted for the hexamer formed by the isolated M_1 monomer of *Cherax* hemocyanin, although subsequent electrophoretic studies have shown this monomer fraction to be composed of two components, $M1_1$ and $M1_2$ (Jeffrey & Treacy, 1980; Marlborough et al., 1981). Thus, while cooperative oxygen binding of hemocyanin hexamers is a general phenomenon, only with subunit IV hexamers is it clear that heterogeneous subunits are not required for the interactions necessary for this process.

The strong linkage between calcium ions and the expression of cooperative interactions between the copper sites in *Limulus* IV hemocyanin were unexpected, as was the fact that neither $MgCl_2$ nor NaCl could substitute for $CaCl_2$ in this regard. Recent studies of this laboratory have shown, however, that the calcium-linked expression of cooperativity shown by *Limulus* IV hexamers is paralleled by calcium ion effects on native *Limulus* hemocyanin. In this system, calcium ions act as allosteric effectors, increasing the oxygen pressure necessary for half-saturation and also increasing the extent of cooperative interactions between subunits (Brouwer et al., 1983). It is tempting to speculate that these effects are attributable to the calcium ion sensitivity of *Limulus* IV hemocyanin molecules within the 48-subunit ensemble.

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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†

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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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