Functional Differences in the Multiple Hemocyanins of the Horseshoe Crab, *Limulus polyphemus L.*

(chloride effects/copper proteins/oxygen binding/hemocyanin components/Bohr effects)

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ABSTRACT Hemocyanin in the hemolymph of the horseshoe crab, *Limulus polyphemus* L., is a high-molecular-weight copper protein which binds oxygen cooperatively and shows a higher oxygen affinity at pH 7 than at pH 8.9. Treatment with EDTA (ethylenediaminetetraacetate) disaggregates the hemocyanin molecules and abolishes both the reverse Bohr effect and cooperative oxygen binding. Chloride ions interact with the EDTA-treated material and, in the presence of saturating amounts of NaCl, a reverse Bohr effect is restored, but cooperativity is not. The EDTA-treated hemocyanin contains at least five electrophoretically distinct hemocyanins. These hemocyanins have similar molecular weights (about 66,000) but are functionally dissimilar. They have different oxygen affinities and different responses to chloride ions. The effect of chloride ions on unfraccionated hemocyanin is due to pH-dependent chloride interactions with only two of the five hemocyanin components. The functional differences between the hemocyanin components may provide *Limulus* with a valuable respiratory flexibility in its interaction with the environment. The kinetics of oxygen combination and dissociation for the various hemocyanin preparations show that variations in the rate of oxygen dissociation are primarily responsible for the observed differences in oxygen affinity. The rate of oxygen dissociation varies 20-fold under conditions where the apparent rate of oxygen combination shows less than a 2-fold variation. Cooperative interactions in the untreated hemocyanin are most obvious in the "off" reaction, which increases in rate as successive oxygen molecules are released.

Hemolymph of the horseshoe crab, *Limulus polyphemus*, contains hemocyanin, a copper-containing respiratory protein of high molecular weight. Unlike most hemocyanins and hemoglobins, oxygen binding by *Limulus* hemocyanin shows a reverse Bohr effect. Hemocyanins have been found dissolved in the hemolymph of gastropods and cephalopods of the phylum Mollusca and in xiphosurans, arachnids, and crustaceans of the phylum Arthropoda. The spectral properties in the visible range, the amino-acid compositions, and the 2:1 stoichiometry of copper to molecular oxygen are remarkably similar for molluscan and arthropod hemocyanins (1, 2). On the other hand, there are significant differences between hemocyanins from the two phyla, including differences in molecular architecture as revealed by electron microscopy (3), circular dichroism (4), and subunit dissociation properties. The minimum functional unit of arthropod hemocyanin is about 74,000 daltons, contains 2 copper atoms and corresponds nicely to the minimum molecular weight (5, 6). We report a somewhat lower molecular weight, 66,000, for subunits of *Limulus* hemocyanin. The minimum functional unit of molluscan hemocyanins is about 50,000 daltons, contains 2 copper atoms, but does not appear to correspond to the minimum molecular weight (7–11). Although low molecular weights (<100,000) have been reported for some molluscan hemocyanins (12–16), the data indicating that these represent the minimal functional subunit are not particularly convincing. Thus, the question as to whether molluscan and arthropod hemocyanins are similar by convergence or are homologous and have descended from a common, ancestral gene remains unanswered.

Workers have had great difficulty isolating electrophoretically pure hemocyanin components in a functional state. Because native hemocyanin molecules have very high molecular weights (1 to 10 × 10⁶) and because their state of aggregation depends upon small changes in pH, salts, and the degree of ligation, conventional purification methods such as electrophoresis and chromatography have not produced electrophoretically pure fractions. The extreme genetic polymorphism which exists at the hemocyanin loci of many arthropods also hampers purification (unpublished results). The heterogeneity of hemocyanin preparations is reflected in complex oxygen-binding curves whose shapes show a dependence upon pH and cation concentration (17–21). Complex kinetics are also associated with functional heterogeneity in hemocyanin preparations. In spite of this complication, Millikan showed that the equilibrium constant of dialyzed *Limulus* serum as calculated from the overall rates of oxygen dissociation and combination agrees with that obtained by direct measurement (22).

We have found that hemocyanin from the horseshoe crab, *Limulus polyphemus*, can be fractionated after treatment with EDTA (ethylenediaminetetraacetate). No hemocyanin polymorphism has been observed in this species. We report here the kinetics and equilibria of oxygen-binding by unfractionated *Limulus* hemocyanin and by the isolated hemocyanin components. In addition, we report on the allosteric effects of chloride ions on two of the hemocyanin components.

**MATERIALS AND METHODS**

Specimens of *Limulus polyphemus* L. were obtained from the waters in the vicinity of Pipers Island, North Carolina. The animals were kept outdoors in large tanks of circulating seawater. Hemolymph was obtained by cardiac puncture. Unfractionated hemocyanin was obtained by allowing the hemolymph to clot and expressing the hemocyanin solution from the clot through 4–6 layers of cheese cloth. Hemocyanin prepared by extensive dialysis against 0.052 M Tris-glycine, 0.01 M EDTA at pH 8.9 is referred to as stripped hemocyanin. Estimates of the molecular weights of the native hemocyanin preparations were made by chromatography on Sephadex G-200 (3 columns, 1.8 × 118 cm connected in tandem) equilibrated either with 0.052 M Tris-glycine, pH 8.9 or 0.052 M Tris-glycine, 0.01 M EDTA, pH 8.9. Estimates of the denatured
RESULTS

Unfractionated Preparations. The pH dependence of oxygen binding by untreated Limulus hemocyanin, stripped Limulus hemocyanin, and stripped Limulus hemocyanin + NaCl is shown in Fig. 1. Untreated Limulus hemocyanin exhibits its lowest affinity at about pH 8.5. We confirm a previous report that the log PaO₂ values at a given pH depend on the extent of dilution (28). The dilution effect appears to be due to dilution of inorganic ions, since, as shown in Fig. 1, a hemocyanin sample diluted 1:30 with seawater exhibits the same oxygen affinity as an undiluted sample (filled and open circles, respectively). A 15-fold dilution of the hemocyanin sample with 0.05 M Tris·HCl buffer at pH 8.0 has a significantly higher oxygen affinity (log PaO₂ = 0.76), thus demonstrating the high sensitivity of the untreated hemocyanin to ionic conditions. As shown in Fig. 1, stripped Limulus hemocyanin is nearly devoid of a Bohr effect. The change in log PaO₂ between pH 7.0 and pH 9.0 is only 0.1 log units. In contrast to the untreated hemocyanin, stripped Limulus hemocyanin is homogeneous with respect to molecular weight. Sephadex G-200 chromatography (at pH 9.0 and pH 7.0) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis both give molecular weights of about 66,000 for stripped Limulus hemocyanin. Gel filtration on Sephadex G-100 shows that in the presence of NaCl the monomers present in stripped hemocyanin become somewhat aggregated. Fig. 1 shows that addition of NaCl to stripped Limulus hemocyanin partially restores the reverse Bohr effect.

Fig. 2 shows Hill plots for untreated and stripped Limulus hemocyanin. Throughout the pH range examined, untreated Limulus hemocyanin shows cooperative oxygen binding. Values of n₅₀ increase from 1.2 at pH 6.0 to 1.7-1.9 at pH 9.0. The Hill plots are heterogenous with higher n values above 50% saturation. Stripped Limulus hemocyanin shows non-cooperative oxygen binding. Values of n₅₀ vary from 0.9 to 1.1 and are independent of pH. The addition of NaCl to stripped hemocyanin does not restore cooperative oxygen binding.

Kinetic studies were undertaken to understand better the basis for the changes in affinity observed in equilibrium experiments. The time course of oxygen dissociation was measured by rapid-mixing of air-equilibrated hemocyanin preparations with degassed buffers containing sodium dithionate. The “off” rates were independent of dithionite concentration in the range examined. The kinetic difference spectrum (deoxy minus oxyhemocyanin) showed wavelength dependence like that observed in static difference spectra. Table 1 shows that the kinetics of deoxygenation are correlated directly with equilibrium data for the various hemocyanin preparations. Preparations with high oxygen affinity have low rates of oxygen dissociation and vice versa. As shown in Table 1, the untreated preparations of Limulus hemocyanin which show cooperative oxygen binding exhibit distinctly autocatalytic oxygen dissociation kinetics. This is the primary kinetic expression of subunit interaction.

The rate of oxygen combination was measured by mixing gasometrically deoxygenated hemocyanin with buffers containing varied amounts of oxygen. These measurements were complicated because much of the reaction was lost in the 2.4 msec dead-time of the stopped-flow apparatus. The observed rate was, however, linearly dependent on oxygen concentra-
tion and as the oxygen concentration was decreased, more of the reaction could be followed. The kinetic difference spectrum shows a peak at 340 nm in agreement with the static difference spectrum. The rate of oxygen binding decreases slightly during oxygen binding to untreated or stripped Limulus hemocyanin. This is probably a reflection of the functional heterogeneity of unfractionated material. Under pseudo first order conditions about 80% of the "on" reaction can be described by a single exponential rate. The second order combination velocity constant, \( k' \), was calculated from the oxygen-concentration dependence of the rate observed under pseudo first order conditions (with ratios of \( O_2/Cu > 10 \)). The value of \( k' \) does not vary by more than a factor of two, regardless of the pretreatment of the hemocyanin, i.e., EDTA, NaCl, or pH. This is to be contrasted with the marked sensitivity of the "off" constant to these variables. As previously reported (22) the ratios of the kinetic constants, \( k'/k \), predict values for the oxygen equilibrium constant which are in reasonable agreement with the measured values.

Equimolar concentrations of KCl and NaCl produce equivalent changes in the oxygen affinity of stripped Limulus hemocyanin. Titrations with both salts showed the effect on \( P_{O_2} \) to be half saturated at about 0.6 M. In order to determine the specificity of the ionic response, the following kinetic experiments were done. The rate of oxygen dissociation from stripped Limulus hemocyanin in Tris-acetate buffer, pH 7.4, is about 7 sec\(^{-1}\) with about 25% of the reaction occurring at a faster rate. This Tris-acetate buffer is 0.2 M with respect to Tris and 0.17 M with respect to acetate. When hemocyanin in this buffer is made 1 M with respect to sodium acetate (at the same pH) the dominant rate of oxygen dissociation is 8 sec\(^{-1}\), and is relatively unchanged by the large increase in both sodium and acetate ion concentrations. If the solution is made 1 M with respect to NaCl instead of sodium acetate, the dominant rate of oxygen dissociation (representing 65% of the reaction) increases to about 13 sec\(^{-1}\). This increase and the absence of an increase in rate after sodium acetate addition strongly suggest that it is the chloride ion of NaCl which has a specific effect on Limulus hemocyanin. Larger anions such as adenosine triphosphate, inositol hexaphosphate, inorganic phosphate, inorganic sulfate, and acetate under similar experimental conditions have little, if any, effect on the kinetics of oxygen dissociation from stripped Limulus hemocyanin.

Properties of Isolated Hemocyanin Components. Limulus hemolymph is a complex mixture of at least five hemocyanin components. These components contribute unequally to the overall oxygen affinity, the pH dependence, and the chloride ion dependence of the unfractionated material. The five major zones isolated from stripped Limulus hemocyanin by DEAE-Sephadex chromatography are shown in Fig. 3 and are designated Hcy I through Hcy V. The proportions of these zones in the whole hemocyanin preparation are given in Table 2. As is also shown in Table 2, these components show marked differences in their oxygen affinities. The stripped components show only slight pH dependence in oxygen binding. Table 2 shows that the sum of the weighted contributions of the oxygen affinities of the five components roughly equals the measured oxygen affinity of the mixture prior to chromatography. This implies that there is no oxygen-linked interaction between the components in the stripped material.

Fig. 4 shows the large difference in the kinetics of oxygen dissociation from Hcy IV and Hcy V at pH 7.5. As in mea-

![Fig. 3. Elution profile of 8 ml of whole hemocyanin applied to a 1.5 X 28-cm column of DEAE-Sephadex, A-50, and developed as described in the text.](image-url)
measurements with unfractionated hemocyanin, the rates of oxygen dissociation from the stripped, isolated components are correlated with their oxygen affinities. Hcy V has the highest rate of oxygen dissociation and the lowest oxygen affinity, and Hcy IV has the lowest rate of oxygen dissociation and the highest oxygen affinity. The time courses of deoxygenation of Hcy I, Hcy II, and Hcy IV may be represented as single exponential processes. Hcy III and Hcy V show biphasic kinetics of deoxygenation; Hcy III has a small (about 20%) fast phase and Hcy V has a small (about 15%) slow phase. Unlike Hcy I, II, and IV, both Hcy III and V show minor bands upon disc-gel electrophoresis. The presence of functionally distinct hemocyanin components is probably the major cause for heterogeneous kinetics of deoxygenation observed most clearly with unfractionated, stripped material.

Fig. 5 shows the effect of 3 M NaCl on the oxygen affinities of hemocyanins I through V at pH 8.0. It is apparent that only components II and III are significantly influenced by this concentration of NaCl. In the presence of 2–4 M NaCl, both Hcy II and Hcy III show reverse Bohr effects. The other components do not respond to NaCl and thus do not contribute to the reverse Bohr effect observed in the stripped, unfractionated hemocyanin in the presence of NaCl (Fig. 1). We have not yet examined the aggregation properties of isolated hemocyanins I through V in the presence of NaCl. Like the unfractionated stripped hemocyanin, the isolated components show non-cooperative oxygen binding both in the presence or absence of NaCl.

DISCUSSION

The oxygen-binding properties of many hemocyanins have been studied (17–21, 29–31). It has not been possible previously, however, to make preparations which are electrophoretically homogeneous, nonaggregated and functional throughout the physiological pH range. Functional heterogeneity of components in unfractionated hemocyanin preparations and variations in extent of aggregation must in part explain the heterogeneous binding curves observed for many hemocyanins (17–21). These complicating conditions seem to be absent in preparations of hemocyanin from Limulus which have been treated with EDTA and purified by ion-exchange chromatography (except that components III and V are not electrophoretically homogeneous).

**Table 2. Proportions, affinities, and rates of oxygen dissociation for stripped hemocyanin components of Limulus hemolymph**

<table>
<thead>
<tr>
<th>Component</th>
<th>% in Hemolymph</th>
<th>$P_{50}$ (mm Hg) at pH 7.5</th>
<th>$P_{10}$ (mm Hg) at pH 7.5</th>
<th>$k$ (sec$^{-1}$) at pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>13</td>
<td>7.5</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>II</td>
<td>25</td>
<td>7.14</td>
<td>1.4</td>
<td>1.62</td>
</tr>
<tr>
<td>III</td>
<td>37</td>
<td>4.0*</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>IV</td>
<td>16</td>
<td>2.4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>33.3*</td>
<td>5.9</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Predicted affinity of whole stripped hemocyanin from weighted average of components' affinities: 1.8 1.7

Observed oxygen affinity of whole stripped hemocyanin: 1.7 2.0

* Biphasic kinetics.

Oxygen binding by unstripped, unfractionated Limulus hemocyanin shows a reverse Bohr effect and cooperative interactions in accord with earlier studies (28, 32). The unstripped preparations contain aggregated material (about 60 S) which will dissociate (60 S → 42, 25, 16, and 5 S) under conditions of high or low pH (33). These sedimenting species may represent 64, 32, 16, 8, and 1 subunits (34). Our observations suggest that stripped (EDTA-treated) Limulus hemocyanin does not aggregate in the physiological pH range in the absence of divalent cations or chloride ions. The oxygen affinity of stripped hemocyanin is higher than unstripped hemocyanin; the negative Bohr effect and cooperativity are abolished. Addition of chloride ions reduces the oxygen affinity, restores the negative Bohr effect, causes partial aggregation but does not restore cooperativity. The failure of chloride to restore the original binding properties is probably related in part to the binding of EDTA to the copper (35). This binding may hinder aggregation of the subunits or exert a direct effect on oxygen affinity and cooperativity.

Isolation of the hemocyanin components from Limulus blood has made it possible to establish that only two of the five components contribute to the reverse Bohr effect that characterizes the oxygen affinity of the stripped material in the presence of NaCl. The stripped hemocyanin components which respond to pH and NaCl thus behave as allosteric molecules, whose oxygen affinities may be regulated by protons or chloride ions.
From our data we conclude that the chloride ion, not the sodium ion, of NaCl causes the affinity transition in stripped Limulus hemocyanin. The decreased sensitivity to chloride at low pH was unexpected since positively-charged binding sites would be generally more abundant at low pH. This reemphasizes the point, however, that the action of chloride as an allosteric effector must be exerted either through differential binding to the oxy and deoxy forms or by causing a change in ligand-linked aggregation, or both. The decreased effect of chloride at low pH may be due either to a much decreased chloride affinity for the deoxy form, or to an increasing chloride affinity for the oxy form at the lower pH. Differential binding would be decreased by either of these possibilities and the chloride effect on the oxygen affinity would consequently be reduced. Alternatively, at low pH, chloride may be ineffective in causing aggregation. The possibility has not been ruled out that the chloride ions may be exerting a fairly direct effect by competing for the oxygen binding site.

Most hemocyanins have a positive Bohr effect, but reverse Bohr effects have been reported for hemocyanins from Limulus and, interestingly, from several gastropod molluscs (17–21, 28–31). The presence of a reverse Bohr effect has been proposed to have physiological significance. Limulus hemolymph often has low O$_2$ and high CO$_2$ concentrations as, for example, when the animal burrows in mud or is exposed to air during egg laying. Measurements of external and internal pO$_2$'s and pH under conditions of environmental stress suggest that the biological significance of a reverse Bohr effect is to insure a constant supply of O$_2$ to the tissues (36). When O$_2$ is less available, Limulus blood pH normally drops due to an increase in CO$_2$ concentration. It has been proposed that the reverse Bohr effect compensates for the lowered pO$_2$'s (36).

Isolation of the hemocyanin components of Limulus has brought to light a feature which may have expression in many biological systems, i.e., the existence in the hemolymph of multiple structurally similar proteins which exhibit different functional properties. We have shown that isolated hemocyanin components not only show differences in their responses to chloride, but also show marked differences in electrophoretic behavior and in oxygen binding. The functional differences between the hemocyanin components of Limulus hemolymph may provide the organism with a valuable respiratory flexibility in meeting extreme environmental changes (i.e., salinity, oxygen pressure, pH, and temperature). We may point to some fish hemoglobin systems as a possible parallel case. In several species of fish two major hemoglobin isoforms are found whose differences in pH and temperature dependence allow them to play diverse physiological roles (37–40). Perhaps the multiple components of hemocyanin systems also serve different physiological needs.

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