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Allosteric Modulation of *Callinectes sapidus* Hemocyanin by Binding of L-Lactate†

Bruce A. Johnson,* Celia Bonaventura, and Joseph Bonaventura

ABSTRACT: Hemocyanin of the blue crab *Callinectes sapidus* has the typical structure of crustacean hemocyanins in that its smallest in vivo structure is a hexamer of subunits each having a molecular mass of approximately 75 000. As found in the blood, *Callinectes* hemocyanin consists of a mixture of hexamers and dodecamers (typically 1:4). As in other crustacean hemocyanins, the affinity with which oxygen binds to the binuclear copper site has been reported to be very sensitive to pH and to a variety of inorganic allosteric effectors. We report here the interaction of L-lactate, a natural metabolite, with the native hemocyanin and with chromatographically purified hexamers and dodecamers. Under ionic conditions that approximate those found physiologically, the addition of 10 mM L-lactate to native *Callinectes* hemocyanin substantially increases its oxygen affinity ($\Delta \log P_{50} = -0.28$). The data from lactate titrations were fit to a theoretical equation, and the best fit was obtained with a lactate dissociation constant of 1.8 mM for the oxy state and 2.2 lactate binding sites for every 6 oxygen binding sites. Independent measurements by ultrafiltration techniques indicated a dissociation constant of 3.2 mM with 2.8 lactate binding sites per 6 oxygen binding sites. The two sets of data clearly indicate that there is less than one lactate binding site per oxygen binding site. The fit to the titration was not improved with the assumption of more than one class of lactate binding site. The hexamers and dodecamers of native *Callinectes* hemocyanin are not in equilibrium and are stable after separation by gel-filtration chromatography. Polyacrylamide gel electrophoresis of the subunits of the dissociated dodecamers shows five major bands. Two of these bands, which constitute one-sixth of the total dodecameric hemocyanin, do not appear upon gel electrophoresis of dissociated hexamers. The oxygen affinities of the hexameric and dodecameric hemocyanin forms are similar to one another but show differences in their sensitivity to L-lactate. The oxygen affinity of native *Callinectes* hemocyanin was increased appreciably more by L-lactate than by glycolate, D-lactate, and pyruvate (listed in decreasing order of effectiveness). Propionate, acetate, succinate, D-alanine, and L-alanine were without effect, thus illustrating the selectivity of the L-lactate effect on the hemocyanin. We found the magnitude of the Bohr effect to be unchanged by the addition of L-lactate over the pH range 7.5-8.0. Moreover, there is no significant effect of L-lactate on the aggregation state of the hemocyanin or on its 340-nm copper–oxygen absorption band. The foregoing results are consistent with the role of L-lactate as a specific allosteric effector of *Callinectes* hemocyanin that acts by preferential binding to a stereospecific site of the oxyhemocyanin.

Hemocyanins are the copper-containing, oxygen-transport proteins that occur in some arthropods and molluscs. In these proteins, oxygen is bound between two copper atoms as a peroxide bridge (Solomon, 1981). The oxygen affinity of the binuclear copper site can be altered by a variety of allosteric ligands in a manner analogous to the effect of protons and organic phosphates on the oxygen affinity of vertebrate hemoglobins (Van Holde & Miller, 1982). In recent years, the allosteric interactions of protons, chloride, calcium, and magnesium with various hemocyanins have been extensively studied and modeled by using various extensions of the two-state model of Monod, Wyman, and Changeux (Brouwer et al., 1977, 1982b). We still know little, however, about the exact mechanisms of action of allosteric effectors of hemocyanin. Direct measurements of the number of binding sites and their affinities for allosteric effectors have been made in only a few cases (Arisaka & Van Holde, 1979; Kuiper et al., 1979; Chiancone et al., 1981; Andersson et al., 1982). Additionally, almost no information concerning the location or identity of amino acid residues involved in these interactions has been obtained.

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The situation of the hemocyanins is in direct contrast to that of tetrameric hemoglobins where detailed mechanistic models of allostery have been proposed (Perutz, 1979). Of singular importance in the understanding of the function of vertebrate hemoglobins is their interaction with organic phosphates (Chanutin & Curnish, 1967; Benesch & Benesch, 1974). An awareness of the regulatory effect of 2,3-diphosphoglycerate (DPG) on hemoglobin function was necessary for understanding red cell oxygen affinity and its modulation in conditions of health, disease, and environmental stress. Furthermore, the study of these interactions provided a great deal of insight into the molecular mechanism of oxygenation and conformational transitions in this protein.

Only recently has a similar interaction between hemocyanin and an organic molecule that affects hemocyanin function been described. Truchot (1980) reported that the oxygen affinity of hemocyanins from the crabs *Carcinus maenas* and *Cancer pagurus* was increased by L-lactate. We became interested in the use of this interaction as a probe of allosteric mechanisms in the crustacean hemocyanins. Accordingly, we demonstrated the presence of this effect in hemocyanin from the blue crab, *Callinectes sapidus* (Johnson & Becker, 1981; Johnson et al., 1982). In this paper we report on further characterization of the interaction of L-lactate with *Callinectes* hemocyanin.

Rigorous demonstration that lactate is an allosteric effector of a hemocyanin should include more than a demonstration of an altered function of the oxygen binding site; a physical binding of the effector to a site other than the oxygen binding site in a manner consistent with the observed effect should also be demonstrated. Therefore, we not only have investigated the effect of lactate on the equilibrium properties of oxygen binding by *Callinectes* hemocyanin but also have measured the number of lactate binding sites and their affinities by an ultrafiltration technique. As a completely independent measure of the lactate–hemocyanin equilibrium, we have determined the number and affinities of oxygen-linked lactate binding sites by analysis of oxygen equilibria in the presence of various lactate concentrations.

Reference to a "lactate effect" presumes that the effect is in fact specific to lactate and not, for example, a general feature of small organic anions or ionic strength. We also investigated, therefore, the specificity of this interaction. Finally, the physiological pH change that accompanies the metabolic addition of lactate to the blood during exercise and hypoxia, along with the known strong Bohr effect, led us to investigate the allosteric interaction between lactate and protons in altering the oxygen affinity of this hemocyanin.

Like other arthropod hemocyanins (Bonaventura & Bonaventura, 1980; Hamlin & Fish, 1977), blue crab hemocyanin is a polymer of subunits having molecular weights of about 75 000. The arthropod hemocyanin subunits, each containing a single oxygen binding site, nearly always assemble in multiples of six. The native structure of hemocyanin found in blue crab hemolymph consists of a mixture of dodecamers whose molecular weight is approximately 900 000 and hexamers whose molecular weight is approximately 450 000 (Herskovits et al., 1977). These components are stable after separation and thus are not in rapid equilibrium with each other (Herskovits et al., 1981). The dodecamer is the major component, typically 80% of the total hemocyanin in our experiments, although its proportion may vary seasonally (Herskovits et al., 1981). As is the case for other arthropod hemocyanins (Markl et al., 1979), *Callinectes* hemocyanin is electrophoretically heterogeneous at the subunit level (Brouwer et al., 1982a). Again, as with many arthropod hemocyanins, allosteric interactions are clearly present in *Callinectes* hemocyanin. The oxygen affinity of undissociated hemocyanin exhibits a strong Bohr effect, a high degree of cooperativity, and a sensitivity to inorganic ions (Bonaventura et al., 1974; Brouwer et al., 1982a).

Materials and Methods

Male *Callinectes sapidus* were obtained from a commercial fisherman operating near Beaufort, NC. Hemolymph was obtained from a severed walking leg. The hemolymph was allowed to clot and then homogenized with a glass–Teflon tissue homogenizer. The broken clot was then removed by centrifugation at 27000*g* for 20 min. The hemocyanin was sedimented from the resulting supernatant by preparative ultracentrifugation at 140000*g* for 7 h. The pellet was resuspended in artificial sea water solution that approximates the ionic composition of crab hemolymph (423 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 26 mM $MgSO_4$, 9.3 mM $CaCl_2$, pH 7.5). The solutions were buffered with 50 mM Tris [tris(hydroxymethyl)aminomethane]; the pH was then adjusted as desired with HCl. Removal of any endogenous lactate was ensured by dialysis against this same buffer. Unless otherwise specified, all experiments were performed without further purification of the hemocyanin.

The hexameric and dodecameric components of *Callinectes* hemocyanin were separated by chromatography through Fractogel 55-S (EM Science). A 2.5 by 47 cm column was eluted at a flow rate of approximately 80 mL/h by using 50 mM Tris-HCl buffer, pH 7.5, ionic strength 0.13, and 10 mM $CaCl_2$. The sample load was 7 mL of protein at a concentration of approximately 15 mg/mL. To maximize recovery of the hexameric fraction (approximately 20% of the total), fractions enhanced in hexamers were rechromatographed. Pooled fractions containing hexamers were concentrated by ultrafiltration and then subjected to a rechromatography as described above. The hexamer to dodecamer ratio was determined by analytical ultracentrifugation with a Beckman Model E centrifuge equipped with scanner optics.

Subunit heterogeneity was studied by gel electrophoresis in 7.5% polyacrylamide gels. A discontinuous buffer system with a pH 8.9 resolving gel was used. Gels were stained with 0.1% Coomassie Brilliant Blue G-250 in 25% trichloroacetic acid. The gels were scanned at 570 nm for protein density with a Gilford Model 2520 gel scanner. The area under each peak was measured with a digitizing tablet connected to an Apple II Plus microcomputer.

Oxygen equilibrium experiments were performed at 20 °C according to the tonometric method of Riggs & Wolbach (1956) with either a Cary 14 or a Cary 219 spectrophotometer. The latter instrument was connected with a digital interface to an Apple II Plus microcomputer for automated instrument control and data collection. This automated instrument was also used for spectral comparisons of the hemocyanin in the presence and absence of L-lactate.

Measurement of lactate binding to hemocyanin was carried out by ultrafiltration of the protein–ligand solution (Sophianopoulos et al., 1978) with an Amicon MPS micropartition system. Air-equilibrated hemocyanin solutions (0.7 mL, approximately 1.68 mM binuclear copper sites), with various concentrations of ^{14}C-labeled L-lactate, were ultrafiltered through a YMT membrane by centrifugation. This provided approximately 100 μL of ultrafiltrate for analysis.

The volume occupied by hydrated hemocyanin (V_h) was determined by control experiments where various amounts of ^{14}C-labeled glucose were dissolved in 2.1 mM hemocyanin

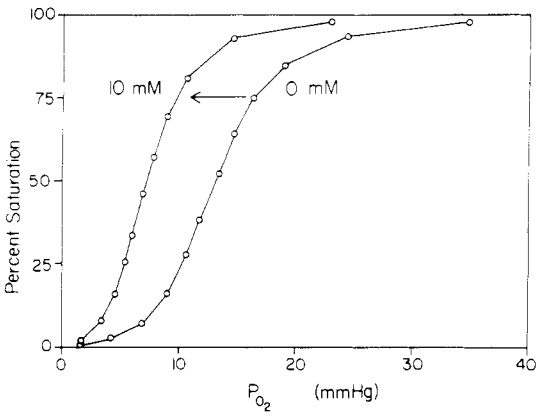

FIGURE 1: Oxygen equilibrium curve of *Callinectes* hemocyanin in the presence (10 mM) and absence of L-lactate. Conditions were Tris-buffered (50 mM) artificial seawater (see Materials and Methods), pH 7.5, 20 °C.

solutions and then subjected to ultrafiltration as above (Achilles et al., 1981). V_h was calculated from the slope of a plot of the concentration of ultrafiltered glucose, $[G_{uf}]$, vs. the concentration of glucose per solution volume, $[G_t]$, where

$$[G_{uf}] = [G_t]/(1 - V_h) + [G_b] \quad (1)$$

and $[G_b]$ is glucose bound to the hemocyanin. Lactate or glucose concentrations in the ultrafiltrates were determined by liquid scintillation counting. The Donnan effect was assumed to be insignificant in the presence of the high salt concentrations used, and hence, no correction was made for this effect.

The molar absorptivity used for the calculation of hemocyanin concentrations was determined by a measurement of copper content with atomic absorption spectroscopy. The value determined by this method is $E^{1\%}_{278,1cm} = 13.9$. A value of $E^{1\%}_{278,1cm} = 13.1$ was determined by weighing hemocyanin solutions dryed to constant weight at 105 °C. The weight of buffer salts was subtracted from the hemocyanin weight after correcting for the volume of solution occupied by hemocyanin.

The fitting of experimental data to theoretical equations was done with a nonlinear, least-squares, regression program (Johnson et al., 1981). Goodness of fit was judged by the magnitude of the variance and the appearance of plots of the residuals vs. the independent and dependent variables. The best fit values are reported along with their 65% confidence intervals (corresponding to approximately 1 standard deviation).

Results

Lactate increases the oxygen affinity (decreases P_{50}, the P_{O_2} in millimeters of Hg at which the hemocyanin is half-saturated with oxygen) of blue crab hemocyanin as shown in Figure 1. At pH 7.5, the log P_{50} is decreased 0.28 unit by the addition of L-lactate to a final concentration of 10 mM. The isolated hexameric and dodecameric forms have the same oxygen affinity in the absence of L-lactate. The affinity of purified hexamers is increased to a somewhat greater extent ($\Delta \log P_{50} = -0.39$) than that of purified dodecamers ($\Delta \log P_{50} = -0.31$) as a result of lactate addition.

Gel electrophoresis of the dissociated *Callinectes* shows the presence of five major bands. Scans of the dissociated hexameric and dissociated dodecameric fractions are shown in Figure 2. The dodecameric fraction contains two bands that are essentially absent in the hexameric fraction. Table I shows the relative proportion of the different types of electrophoretic bands in the hexamer and dodecamers, as well as their presumed compositions. It should be noted that the bands have not yet been unequivically established to contain single types of polypeptide chains.

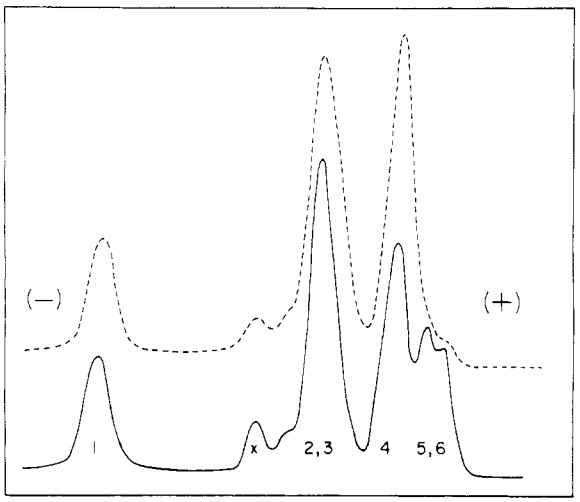

FIGURE 2: Densitometric scans of polyacrylamide gel of dissociated hexameric and dodecameric hemocyanin. In these gels, peak 3 occurs only as a slight shoulder on peak 2. Under certain environmental conditions, it becomes more abundant in *Callinectes* hemocyanin (Mason et al., 1983). Only the scan of the portion of gel containing presumed hemocyanin subunits is shown. Peaks at the position labeled X on gels of *Callinectes* occur in variable amounts and were not included in calculation of peak areas.

Table I: Relative Area of Electrophoretic Zones and the Presumed Subunit Composition of Dissociated Hexamers and Dodecamers[a]

	hexamers		dodecamers	
zone	area	composition	area	composition
1	0.8	1	0.9	2
2 and 3	2.8	3	2.4	5
4	2.3	2	1.7	3
5 and 6	0.1	0	1.0	2

[a] Relative area is calculated as area under each peak in Figure 2 divided by one-sixth of the total area.

The effect of various lactate concentrations on the oxygen affinity was analyzed according to

$$P_{50,L} = P_{50,0}([1 + (L/{}^0k)]/[1 + (L/{}^6k)])^{n/m} \quad (2)$$

where $P_{50,L}$ and $P_{50,0}$ are the P_{O_2}s corresponding to half-saturation of the hemocyanin in the presence and absence of lactate. ${}^0k$ and ${}^6k$ are the microscopic dissociation constants of lactate from deoxy- and oxyhemocyanin. The number of binding sites for lactate is n [see Cantor & Schimmel (1980) for a derivation of eq 2). Equation 2 is presented on the assumption that ${}^6k$ and ${}^0k$ are independent of the number of lactates bound. A similar approach has been used by Benesch et al. (1971) in the analysis of the DPG–Hb interaction. As derived by Szabo & Karplus (1976), and used here, m refers to the number of oxygen binding sites (six), not the value of the Hill coefficient as used by Benesch et al. (1971). Ackers (1979) has derived this equation in an independent manner and clarified the significance of the meaning ascribed to m. Equation 2 is valid so long as the concentration of the ligand in question (lactate) does not change significantly in response to oxygenation. This condition is satisfied if the ligand concentration is at least 5 times higher than the protein concentration (Ackers, 1979) as was the case in our experiments. To be strictly correct, eq 2 should be expressed in terms of the median ligand pressure (P_m) rather than the P_{50}. For highly

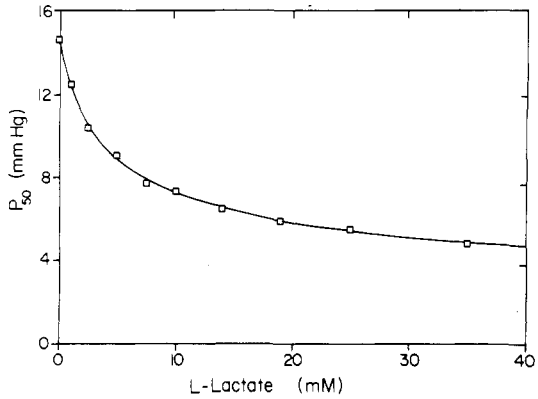

FIGURE 3: Effect of L-lactate on oxygen affinity of *Callinectes* hemocyanin. Conditions were Tris-buffered (50 mM) artificial seawater, pH 7.5, 20 °C. The solid line corresponds to best fit of eq 2 to the experimental data. P_{50} is the partial pressure of oxygen (mmHg) needed to half-saturate the hemocyanin.

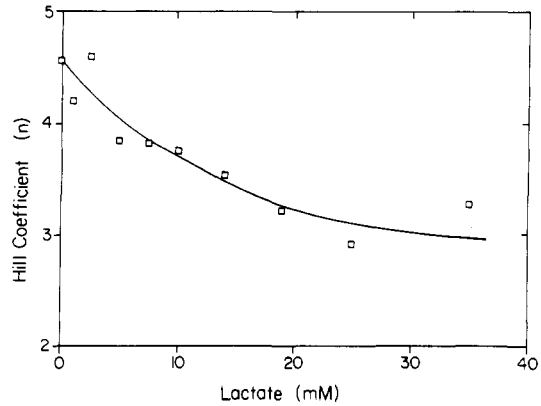

FIGURE 4: Effect of L-lactate on the cooperativity of oxygen binding by *Callinectes* hemocyanin. n_h is the slope of a Hill plot over the range 30–70% saturation. Conditions were Tris-buffered (50 mM) artificial seawater, pH 7.5, 20 °C. The solid line is fit by eye to the data.

cooperative systems, the parameters have approximately equal values (Szabo & Karplus, 1976). Using an Imai-type apparatus (Imai, 1981), we have confirmed that this is a good approximation for *Callinectes* hemocyanin (unpublished results).

The best fit of the data to eq 2 is obtained with a lactate dissociation constant for the oxy state of ${}^6k$ = 1.8 mM (65% confidence interval 1.6–2.0) and with n = 2.2 (65% confidence interval 2.1–2.3). The data and theoretical curve are shown in Figure 3. The lactate dissociation constant for the deoxy state is ${}^0k$ = 190 mM. However, no decrease in variance occurs by assuming that lactate does not bind to the deoxy state. Although the data on isolated hexamers and dodecamers suggest a difference in lactate binding between these two forms, no improvement in fit is obtained by allowing for heterogeneity in the affinity of the binding sites.

Cooperative interactions between the oxygen binding sites as measured by the Hill coefficient n are reduced by the addition of L-lactate. These results are shown in Figure 4.

Ultrafiltration experiments were used to analyze the number and affinity of lactate binding sites. In these experiments, protein and total ligand concentrations were adjusted for the excluded volume of the hemocyanin by dividing their solution concentrations by the fraction of solution that is solvent, F, where

$$F = 1 - V_h C \quad (3)$$

and C is the protein concentration (mol of hexamer/L). The value of V_h, obtained from the glucose ultrafiltration experiment for the volume of hydrated hemocyanin solution that is not accessible as solvent, is 400 L/mol of hexamer (0.89 cm^3/g).

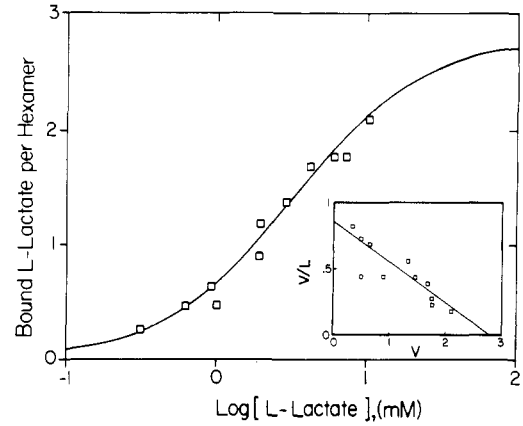

FIGURE 5: Binding of L-lactate to *Callinectes* hemocyanin as measured by ultrafiltration analysis. Conditions were Tris-buffered (50 mM) artificial seawater, pH 7.5, 20 °C. The solid line was calculated from parameters of the best fit of eq 5 to the experimental data. The inset shows a Scatchard plot of the data and best fit line.

For a protein at concentration C, with n identical binding sites in equilibrium with ligand at concentration L_f, and dissociation constant k, the concentration of bound ligand is

$$L_b = nCL_f/(k + L_f) \quad (4)$$

In an ultrafiltration experiment of the type we performed, the free ligand concentration is the dependent (measured) variable and total ligand concentration is the independent variable. Therefore, eq 2 should be rearranged to express free-ligand concentration as a function of total ligand resulting in

$$L_f = [-(nC - L_t + k) + [(nC - L_t + k)^2 + 4kL_t]^{1/2}]/2 \quad (5)$$

The experimental data were fit to this equation. The best fit is obtained with a dissociation constant of k = 3.2 mM (65% confidence interval 2.4–4.4) and the number of binding sites per hexamer being n = 2.8 (65% confidence interval 2.2–3.3). The experimental data and the fitted curve are shown in Figure 5. Neither a Scatchard plot (Figure 5, inset) nor a Hill plot with n = 2.8 (not shown) of the lactate binding data shows any indication of heterogeneity or interaction of the binding sites.

Spectral analysis of the 335-nm Cu–O_2 absorption band does not show any difference resulting from addition of L-lactate to hemocyanin. Nor is any significant change observed in the absorption spectra in the region associated with the aromatic amino acids (260–300 nm). This pattern is consistent with lactate binding at sites other than the oxygen binding site.

The effect of L-lactate on the proportion of hemocyanin in the dodecameric, hexameric, and monomeric forms was studied by analytical ultracentrifugation. The preparation studied for this purpose contained 77.8% dodecamers. The fraction of dodecameric hemocyanin was found to be the same for oxy- and deoxyhemocyanin in the presence and absence of 10 mM L-lactate. No monomers were detected.

Specificity of the lactate effect was determined by measuring the oxygen affinity in the presence and absence of a 10 mM concentration of nine compounds. Of those tested, the compounds L-lactate, glycolate, D-lactate, and pyruvate, in decreasing order of effectiveness, brought about increases in the oxygen affinity. These results are shown in Table II. The increase in the oxygen affinity in the presence of D-lactate is significantly less than that in the presence of L-lactate, indi-

Table II: Specificity of Response of Oxygen Affinity of *Callinectes* Hemocyanin to Various Small Organic Compounds[a]

	n	$\Delta \log P_{50}$[b]	P[c]
D-alanine	2	0.004	NSS[d]
L-alanine	2	0.007	NSS
propionate	1	0.006	
acetate	3	−0.003	NSS
succinate	2	−0.012	NSS
pyruvate	4	−0.038	>99
D-lactate	4	−0.084	>99.9
glycolate	3	−0.115	>99.9
L-lactate	7	−0.277	>99.9

[a] Tris-buffered (50 mM) artificial seawater, pH 7.5, 20 °C, was used. [b] $\log P_{50}$ in the presence of 10 mM of the indicated compound minus $\log P_{50}$ in its absence. [c] Probability level for Student's t test comparison of the P_{50} in the presence and absence of effector. [d] Not statistically significant.

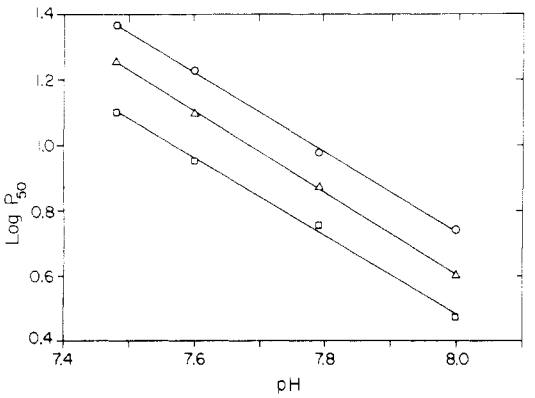

FIGURE 6: Effect of pH and L-lactate on the oxygen affinity of *Callinectes* hemocyanin. Conditions were Tris-buffered (50 mM) artificial seawater, 20 °C, and 0 (circles), 2.86 (triangles), and 9.80 (squares) mM L-lactate.

cating the stereoselective nature of the lactate binding site.

The number of oxygen-linked protons can be calculated from

$$\Delta \log P_{50}/\Delta \mathrm{pH} = -\Delta \mathrm{H}^{+} \qquad (6)$$

where ΔH^+ is the difference in the number of bound protons per oxygen binding site between the deoxy and oxy forms. As pointed out by Bucci & Fronticelli (1981), the linkage equations are valid provided that the solution activities of the other linked ligands are not significantly changed by binding of oxygen to the protein. Since all other known or likely effectors of *Callinectes* hemocyanin (calcium, chloride, lactate) are present in at least 10-fold abundance over the protein concentration, this condition is satisfied. The derivative in eq 6 corresponds to the slope of the lines plotted in Figure 6. Approximately 1.2 protons per oxygen binding site are released when *Callinectes* hemocyanin is oxygenated in this pH range. Analysis of covariance (Sokal & Rohlf, 1981) indicates that the slopes of the Bohr effects in the presence and absence of L-lactate are not significantly different. Thus, over at least the pH range 7.5–8.0 (the physiological range), the magnitude of the Bohr effect is unchanged by the addition of L-lactate.

Discussion

The preceding results comprise, to our knowledge, the first documentation of the binding of a physiological, organic molecule to a hemocyanin in a manner consistent with its action as an allosteric effector. Binding parameters for the interaction of lactate with *Callinectes* hemocyanin have been obtained, although measurements were hampered by the low lactate affinities of the binding sites. Measurements of protein–ligand equilibria are best performed at protein concentrations near the dissociation constant of the complex to ensure a measurable difference between bound and free ligand concentrations (Weber, 1975). With equilibria having low association energies (low affinity), this criterion is difficult to meet due to the high protein concentrations necessary. At high protein concentrations, a correction must be made for the volume of solution occupied by the hydrated protein and, hence, inaccessible to the ligand (Achilles et al., 1981).

We have measured the excluded volume of a concentrated hemocyanin solution. This value was used to correct ligand and protein concentrations used in the ultrafiltration analysis of the lactate–hemocyanin equilibrium. Our measured volume excluded by hemocyanin (0.89 mL/g) is somewhat larger than the partial specific volume (0.724 mL/g) calculated from the amino acid composition of *Callinectes sapidus* hemocyanin (Hamlin & Fish, 1977). The difference is probably due to the presence of a bound water layer that is inaccessible as solvent. The hydrated volume we measured is less than that calculated by assuming the binding of 0.3 mL of water/g, a value that is typical of many proteins (Cantor & Schimmel, 1980) and used by Arisaka & Van Holde (1979) to correct their measurements of ligand binding to *Callianassa* hemocyanin. This difference is similar to that reported by Achilles et al. (1981) for human hemoglobin. The difference between our copper-based extinction coefficient and that calculated from dry weights of *Callinectes* hemocyanin is consistent with the presence of tightly bound water that is not released even upon drying to constant weight. In our calculations, we have used the value obtained from glucose ultrafiltration experiments as that value most nearly corresponds to the conditions of the lactate ultrafiltration experiments.

At high protein concentrations, the binding parameters are very sensitive to changes in the value of the hydrated volume. Additionally, at high concentration the thermodynamic activity of the protein is not necessarily in direct proportion to its concentration (Weber, 1975). For these reasons the values of the binding parameters reported should be regarded as first approximations of the true values. Confirmation by an independent method that does not require high protein concentrations would be desirable.

An alternative method for the calculation of binding parameters involves fitting a theoretical equation (eq 5) to the oxygen-affinity measurements at a series of different ligand concentrations. Data analyzed by this method show substantial agreement with the binding parameters obtained by the ultrafiltration analysis. The two methods agree within the confidence intervals for the number of binding sites and are nearly in agreement for the dissociation constants. The ultrafiltration method measures all lactate binding sites, whereas the thermodynamic-linkage relations are sensitive only to the oxygen-linked binding sites. The agreement between the two methods suggests that all the lactate binding sites are oxygen linked.

It is clear that there is not a 1:1 stoichiometry between the number of lactate binding sites and the number of subunits. By analogy to the DPG–Hb interaction, it is possible that the binding sites are located between subunits. The "dimer of trimers" substructure of hexameric arthropod hemocyanins would allow for a number of symmetrical arrangements with either two or three sites (Van Schaick et al., 1982). The marked electrophoretic heterogeneity of the subunits, however, suggests a second possibility: that lactate does not bind between subunits but rather only to certain subunit types. The now considerable evidence for the functional role of subunit heterogeneity in arthropod hemocyanins is consistent with this possibility (Brenowitz, 1982; also, see below). Our own work

with purified subunits from the spiny lobster *Panulirus interruptus* indicates that homohexamers prepared from different subunit types have different responses to lactate (Johnson et al., 1983). These two possibilities are not, of course, mutually exclusive.

Additional support for the existence of a direct binding of lactate to the *C. sapidus* hemocyanin comes from a comparison of the effect of D-lactate with that of L-lactate. Aside from their symmetry differences, these two compounds are chemically and physically identical. Only through a direct interaction with the protein could a distinction be made. The observed stereoselectivity indicates that the L-lactate molecule must have multiple points of interaction with the protein. The total interaction energy is so low, however, that loss of a single functional group can be critical. Comparison with propionate, for example, indicates the essential role played by the hydroxyl group. In glycolate, the hydroxyl group is free to rotate to the appropriate position. Accordingly, glycolate has a relatively strong effect. Rotation of the hydroxyl carbon of D-lactate to a position favorable for an interaction with the protein is presumably hindered by the interaction of the methyl group. It has been suggested (Graham et al., 1983) that the effect of pyruvate on the oxygen affinity of *Cancer magister* hemocyanin is due to a slight (0.5%) contamination of the pyruvate with L-lactate. Although the magnitude of the effect of pyruvate on *Callinectes* hemocyanin is similar to that reported for *Cancer*, we cannot ascribe this effect solely to L-lactate contamination. From eq 2, one can calculate that 0.5 mM L-lactate would have to be present to reach the $\Delta \log P_{50}$ (with a control P_{50} of 14.2) reported in Table II. This is 10 times the suspected level of contamination.

While both lactate and proton binding are clearly linked to oxygen binding, there is no interaction between the two. Apparently, the pK_a's of the Bohr groups are unaffected by lactate binding (and probably, therefore, not located at the lactate binding site). The pK_a of lactate is low enough so that binding does not significantly alter the degree of lactate ionization, and thus its binding does not contribute to the Bohr effect.

The lack of any effect of L-lactate on the 335-nm $Cu–O_2$ absorption band makes it unlikely that lactate interacts directly with the oxygen binding site. While subtle effects of L-lactate on the association equilibria remain a possibility, our ultracentrifugation experiments did not indicate any major changes in hexamer to dodecamer ratio as a result of either lactate or oxygen binding. We did not detect monomeric hemocyanin under the conditions used for oxygen binding experiments. This lack of monomers is consistent with the report of Herskovits et al. (1981), who found monomers only under conditions of high pH or in the presence of denaturants. These findings strongly support the concept that L-lactate acts as a classical allosteric effector, that is, by binding preferentially to oxyhemocyanin at a site other than the oxygen binding site.

Considerable evidence now indicates that specific hemocyanin subunits are involved in the formation of aggregation states of crustacean hemocyanins above that of the hexamer (Markl, 1979; Terwilliger, 1982). The electrophoretic data on dissociated *Callinectes* hemocyanin are consistent with a role of electrophoretic bands 5 and 6 in the linkage of two hexamers to form a dodecamer. The concentration of these bands suggests that they constitute one out of every six subunits in dodecameric hemocyanin and are not present in the hexameric hemocyanin.

From Figure 3 it is clear that even at relatively low lactate concentrations the oxygen-dissociation curve can be shifted significantly toward a higher affinity. Reported lactate concentrations in the blood of other crabs range up to 10 mM (Bridges & Brand, 1980; Truchot, 1980; Graham et al., 1983). Thus, accurate estimation of in vivo oxygen binding curves in species with lactate-sensitive hemocyanin will require measurement of in vivo lactate concentrations and a determination of the magnitude of the effect. During periods of lactate production, exercise or hypoxia for example, blood lactate will rise, resulting in a metabolic acidosis. The Bohr effect and the lactate effect, being opposite in direction, will tend to counterbalance each other, resulting in a smaller change in affinity than expected to result from either effect alone. Mangum (1982) has suggested that the Bohr effect alone would result in a maladaptive drop in oxygen affinity, resulting in insufficient oxygenation at the gills.

Whether a lactate effect is a general feature of crustacean hemocyanins remains to be seen. Besides Truchot (1980), Graham et al. (1983) report that lactate increases the oxygen affinity of another crab hemocyanin (*C. magister*). Our own work with the spiny lobster indicates that the effect is not limited to the crabs [see also Mangum (1983)]. We have, however, observed the absence of the effect in certain crab species under the same experimental conditions used here (unpublished results). The varied distribution of the DPG–hemoglobin effect among mammalian species has led to very interesting results concerning the mechanism, evolution, and adaptation of mammalian hemoglobins (Bunn, 1980). We expect similarly interesting results to be forthcoming from the further study of the lactate–hemocyanin interaction.

Finally, it is interesting to compare the lactate–hemocyanin effect with the DPG–hemoglobin effect (Benesch & Benesch, 1974). The most striking difference, of course, is the fact that the effects are opposite in direction. DPG decreases oxygen affinity by binding preferentially to the deoxy conformation of hemoglobin. Lactate increases oxygen affinity by preferentially binding to the oxy conformation. Both effects, however, can be modeled by the use of thermodynamic relations developed for the DPG–hemoglobin interaction. Binding of DPG alters the pK_a of groups involved in the Bohr effect and of its own ionizable groups. These combine to produce a Bohr effect that is dependent on the degree of DPG binding. Such a dependence is absent with the lactate–hemocyanin interaction. Owing to the symmetry of the binding site in hemoglobin, there is no difference in the decrease in oxygen affinity produced by the binding of L-DPG as compared to D-DPG (the natural form). On the contrary, the lactate binding site must be asymmetrical as L-lactate binds preferentially as compared to D-lactate. The affinity of hemocyanin for lactate is substantially lower than that of human hemoglobin for DPG. For both interactions, the affinities have probably been optimized with respect to the physiological effector concentrations.

Registry No. Pyruvic acid, 127-17-3; D-lactic acid, 10326-41-7; glycolic acid, 79-14-1; L-lactic acid, 79-33-4; oxygen, 7782-44-7.

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