Oxygen Binding by *Limulus polyphemus* Hemocyanin: Allosteric Modulation by Chloride Ions†

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ABSTRACT: 60S *Limulus polyphemus* hemocyanin contains 48 oxygen-binding sites. The effect of chloride ions on the oxygen-binding properties of this huge molecule was examined by equilibrium and rapid-mixing techniques. Ultracentrifugation verified that the sedimentation coefficient of the hemocyanin was 60 S in all of the conditions examined. Chloride lowers the oxygen affinity of *Limulus* hemocyanin at all pH values, from pH 7.0 to 9.5, with maximum effectiveness at pH 8.5. Kinetically this decrease in affinity is reflected in a pH-dependent increase of the oxygen dissociation rate. Chloride also shifts the deoxy asymptotes of the Hill plots (the low affinity, $n = 1$, portion) and ultimately stabilizes the 60S structure in a noncooperative low-affinity state. Based on the shift of the deoxy asymptotes, we were able to calculate equilibrium association constants for the following reactions: Hcy + O_2 ↔ Hcy-O_2 (K_1), Hcy + Cl ↔ Hcy-Cl (K_2), Hcy-O_2 + Cl ↔ Hcy-Cl-O_2 (K_3), and Hcy-Cl + O_2 ↔ Hcy-Cl-O_2 (K_4), where Hcy represents hemocyanin in the initial portion of the binding curve where oxygen binding is noncooperative. The effect of chloride on oxygen binding by *Limulus* hemocyanin can be satisfactorily accounted for within a concerted two-state model (Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88–118) with the modification that this ligand not only changes the allosteric equilibrium constant, but also changes the nonexclusive binding coefficient by decreasing the oxygen affinity of the unliganded state ($K_1 > K_4$). This interpretation, which implies the presence of more than one apparent T state, is supported by the kinetic data. Curve fitting within the context of the modified MWC model suggests that the hexamer is the allosteric unit in the 60S molecule.

Hemocyanins are the large, copper-containing respiratory proteins of arthropods and molluscs. Arthropodan hemocyanins, with 70 000–75 000 dalton subunits, contain 6, 12, 24, or 48 oxygen-binding sites per undissociated molecule. Molluscan hemocyanins have a functional unit with a minimum molecular weight of 50 000 and contain as many as 180 binding sites per undissociated molecule (Van Holde and van Bruggen, 1971; Bonaventura et al., 1976).

In view of the large number of oxygen-binding sites per molecule in these respiratory proteins, it is of considerable interest to know whether the interactions responsible for cooperativity are confined to functionally independent allosteric units, each containing n sites, or whether these interactions radiate out to cover a large number of sites (Colosimo et al., 1974). Evidence for the latter hypothesis has been given by van Driel (1973) for *Helix pomatia* hemocyanin. In this large molecule, interactions between 1/10 molecules, each containing 18 binding sites, seem to be present. These interactions do not seem to be necessary for cooperativity in molluscan hemocyanins, for Klarman et al. (1975) showed that a 1/10 molecule of *Levantina hierosolima* hemocyanin can bind oxygen cooperatively. Cooperativity of the oxygen binding by these hemocyanins can be interpreted as an oxygen-linked transition from a state with a low oxygen affinity to a state with high oxygen affinity (Er-El et al., 1972; van Driel, 1973; van Driel and van Bruggen, 1975). A more detailed analysis of the oxygen-binding properties of the hemocyanin of the ghost shrimp, *Callianassa californiensis,* has been given by Miller and Van Holde (1974). The hexamer in this arthropodan hemocyanin is the allosteric unit, and the oxygen binding can be described by the theory of Monod et al. (1965), assuming the occurrence of hybrid R–T states (Johannes and Hess, 1973; Buc et al., 1973).

Undissociated *Limulus polyphemus* hemocyanin has a molecular weight of 3.3×10^6 and consists of 48 subunits with a molecular weight of 70 000 each (Johnson, 1973). These subunits can be fractionated into five major chromatographic zones, which differ in their oxygen-binding affinities, oxygen dissociation and combination rates, CO affinities, structure, and response to chloride ions (Sullivan et al., 1974; Bonaventura et al., 1974, 1975, 1977; Sullivan et al., 1976).

Allosteric modulations of the oxygen-binding properties of hemocyanins by anions are not found in the literature, whereas anions are well-known allosteric effectors in hemoglobins (Antonini and Brunori, 1971). In view of the response of some of the separated *Limulus* hemocyanin components to chloride ions (Sullivan et al., 1974), an analysis of the effect of this ligand on the equilibrium and kinetics of the oxygen binding by undissociated 60S *Limulus* hemocyanin was carried out.

It is shown that chloride lowers the oxygen affinity and increases the oxygen dissociation rate. The effect of chloride cannot be accounted for within the original MWC model (Monod et al., 1965). The observed binding curves can be described satisfactorily, with the hexamer as the allosteric unit, assuming that chloride not only changes the allosteric equilibrium constant, but also the nonexclusive binding coefficient.

Materials and Methods

Hemocyanin from the horseshoe crab, *Limulus polyphemus* L., was prepared as described previously (Sullivan et al., 1974). These preparations contain a small amount of a hemagglutinin (Fernandez-Moran et al., 1968; Marchalonis and Edelman, 1968). The 60S hemocyanin was separated from the 13.5S hemagglutinin by ultracentrifugation for 75 min at 45 000 rpm

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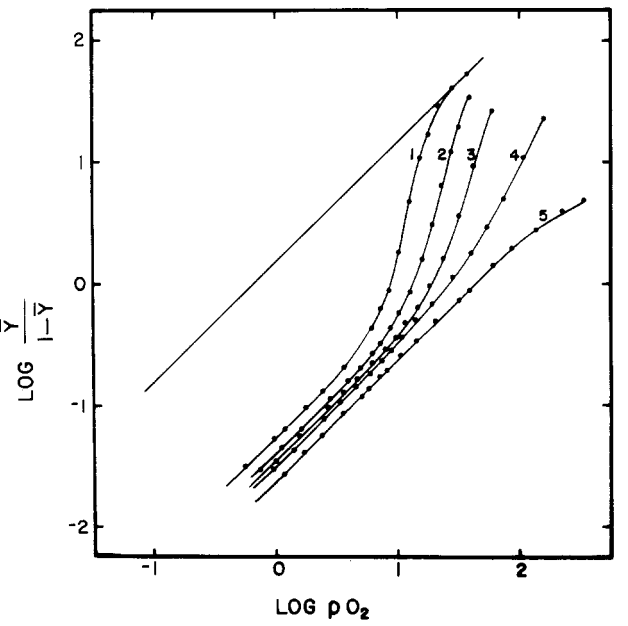

FIGURE 1: Hill plots for oxygen binding at 20 °C in a 50 mM Tris buffer, ionic strength 0.05, pH 8.0, to which 10 mM $CaCl_2$ and various amounts of sodium chloride were added. Protein concentration was about 4 mg/mL. (1) 0.07 M Cl; (2) 0.22 M Cl; (3) 0.52 M Cl; (4) 1.48 M Cl; (5) 3.77 M Cl. Curves at 0.03, 0.12, 1.02, 1.96, and 2.91 M Cl have been omitted for clarity. The straight line with slope unity represents the hypothetical binding curve of the R state.

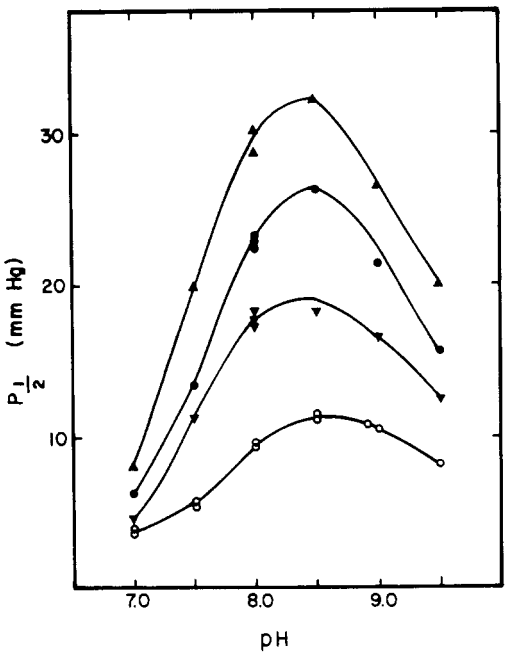

FIGURE 2: Effect of chloride on the oxygen affinity of *Limulus* hemocyanin at 20 °C in 50 mM Tris, ionic strength 0.05, buffers, pH 7.0–9.5, to which 10 mM $CaCl_2$ and various amounts of sodium chloride were added. Protein concentration was about 4 mg/mL. (○) 0.12 M Cl; (▼) 0.52 M Cl; (●) 1.02 M Cl; (▲) 1.96 M Cl.

in a Beckman Model L preparative ultracentrifuge. The hemocyanin pellets were dissolved in and dialyzed against the appropriate buffer. Tris-HCl[1] buffers, made up to the desired ionic strength with NaCl, were prepared according to Bates (1973).

Hemocyanin concentrations were determined using the extinction coefficients at 280 or 340 nm as given by Nickerson and Van Holde (1971).

Sedimentation velocity experiments were carried out at 20 °C using a Beckman Spinco Model E analytical ultracentrifuge with mechanical speed control and schlieren optics. Sedimentation coefficients were corrected to standard conditions of viscosity and density of water according to Svedberg and Pederson (1940).

Oxygen equilibrium experiments were performed using a spectrophotometric method (Riggs and Wolbach, 1956). Values for the percent saturation with oxygen were determined at 338 nm. When complete saturation of hemocyanin with oxygen could not be obtained, the absorbance value at 338 nm for the fully oxygenated protein was obtained from the experimentally determined relationship: $\Delta A_{338nm} = 0.281$ per mg of protein.

Rapid-mixing experiments were performed with a Gibson-Durrum stopped-flow apparatus. Data collection and analysis were accomplished via an analog to digital converter (Aminco DASAR) coupled to a PDP-11 computer (Digital Equipment Corp.).

Results

Sedimentation Analysis. Throughout all the different experimental conditions used in this study, oxy- as well as deoxyhemocyanin sedimented as a single symmetrical boundary with a sedimentation coefficient of 60 S.

Oxygen Equilibrium Experiments. Figure 1 shows a set of oxygen equilibrium curves, presented as Hill plots, at pH 8 as a function of the sodium chloride concentration. These curves show a number of important features.

The slope of all the curves at low oxygen concentration is always unity. This portion of the binding curves, the deoxy asymptote, shifts to a lower oxygen affinity with increasing NaCl concentration. There is a concomitant increase of the value of the fractional oxygen saturation, $\bar{Y}$, at which the slope of the Hill plot starts to be greater than unity. The $p_{1/2}$ values, the partial oxygen pressures at half saturation, increase with increasing NaCl concentrations. At high NaCl concentration the protein appears to be fixed in a noncooperative low-affinity state. Unexplained heterogeneity is observed in the upper part of curve 5.

The influence of NaCl on the oxygen affinity of *Limulus* hemocyanin at different pH values is shown in Figure 2. These results show that NaCl lowers the oxygen affinity of *Limulus* hemocyanin at all the pH values studied, with a maximum effect at about pH 8.5. At all NaCl concentrations, *Limulus* hemocyanin has its lowest oxygen affinity at about pH 8.5.

Rapid-Mixing Experiments. The kinetics of the oxygen dissociation from *Limulus* hemocyanin were followed by mixing the oxy protein with buffer containing sodium dithionite. The time course of oxygen dissociation at different NaCl concentrations is shown in Figure 3. At low NaCl concentrations the time course is clearly autocatalytic. The increase in the apparent first-order rate constants with the extent of the reaction is shown in Figure 4. It is clear that NaCl markedly increases the rate of oxygen dissociation from *Limulus* hemocyanin.

The effect of NaCl on the oxygen dissociation process at different pH values is shown in Figure 5. Since the apparent rate of oxygen dissociation increases with the extent of the reaction, we arbitrarily chose to plot k_{off} 50% (calculated from the slope of the first-order plots between 60 and 40% of the reaction) vs. pH. This does not imply, of course, that the oxygen dissociation rate observed at 50% of the reaction in the rapid-

[1] Abbreviation used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

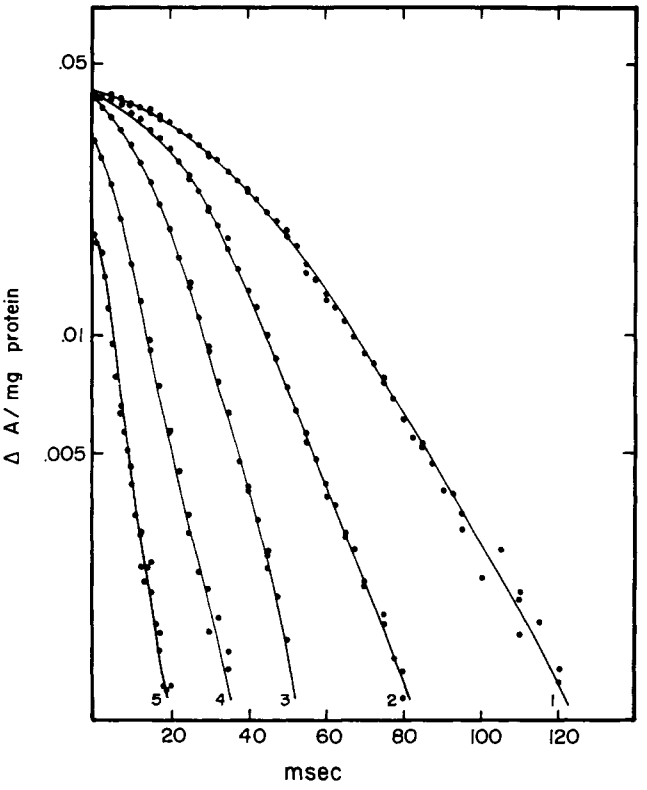

FIGURE 3: Time course of the deoxygenation reaction of oxyhemocyanin in the presence of dithionite at pH 8.0 and various sodium chloride concentrations. Conditions as in Figure 1. The change in absorbance at 390 nm was followed. ΔA (absorbance observed at time t − absorbance after completion of the reaction) per mg of protein is plotted as a function of time. (1) 0.07 M Cl; (2) 0.22 M Cl; (3) 0.52 M Cl; (4) 1.48 M Cl; (5) 3.77 M Cl. Curves at 0.12, 1.02, 1.96, and 2.91 M Cl have been omitted for clarity.

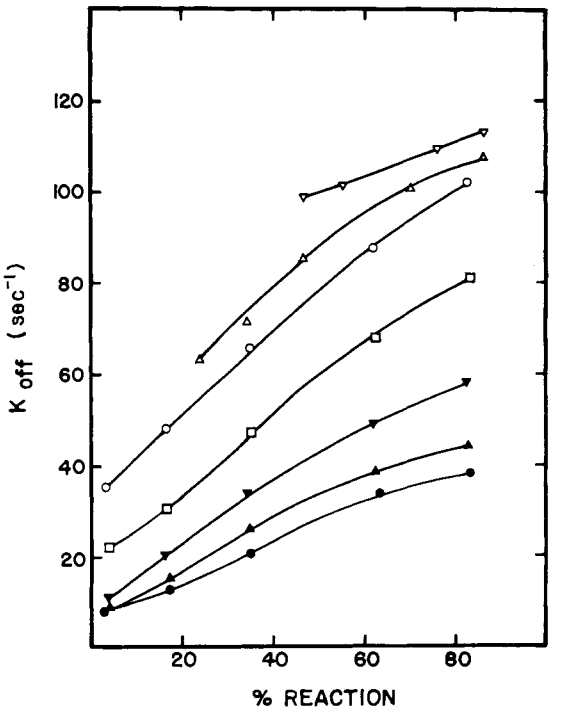

FIGURE 4: Apparent oxygen dissociation rate constants plotted as a function of the percentage reaction. Conditions were as in Figures 1 and 3. (●) 0.07 M Cl; (▲) 0.12 M Cl; (▼) 0.22 M Cl; (□) 0.52 M Cl; (○) 1.02 M Cl; (△) 1.48 M Cl; (▽) 1.96 M Cl.

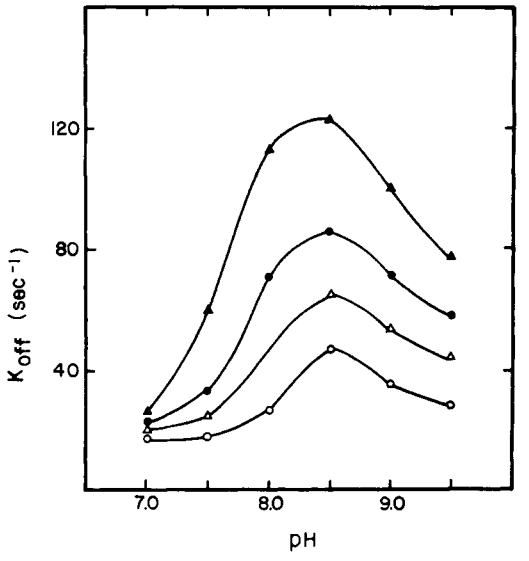

FIGURE 5: Effect of chloride on the apparent oxygen dissociation rate at 50% of the reaction. $k_{off,50\%}$ calculated from the slope of the first-order plot between 60 and 40% of the deoxygenation reaction. Experimental conditions were as in Figure 2. (○) 0.12 M Cl; (▽) 0.52 M Cl; (●) 1.02 M Cl; (▲) 1.96 M Cl.

mixing experiments is the same as the dissociation rate at 50% saturation in the equilibrium experiment. Figure 5 shows that NaCl increases the oxygen dissociation rate at all the pH values studied, with a maximum at pH 8.5, for all the NaCl concentrations examined.

Discussion

The foregoing results show that the oxygen-binding properties of *Limulus* hemocyanin can be modulated by NaCl. The effect of NaCl on 60S *Limulus* hemocyanin may be due to (1) ionic strength, (2) sodium ions, (3) competition between sodium and calcium, or (4) chloride ions.

We observe that the $p_{1/2}$ of *Limulus* hemocyanin in a 50 mM Tris buffer, pH 8, containing 10 mM $CaCl_2$, made up to ionic strength 3 with sodium sulfate or sodium chloride, is 19 and 38 mmHg, respectively. Thus, the first possibility can be ruled out.

The $p_{1/2}$ of *Limulus* hemocyanin in a 50 mM Tris buffer, pH 8, containing 10 mM $CaCl_2$ plus 1 M Na_2SO_4 or 2 M NaCl is 19 and 30 mmHg, respectively. Though both hemocyanin solutions have the same sodium concentration, their oxygen affinities are clearly different. This makes the second possibility very unlikely.

Oxygen-binding affinities, measured in 50 mM Tris buffers (pH 7.5, 8, and 8.5) at constant Ca/Na ratios, still decrease with increasing sodium chloride concentrations. This rules out the possibility that Na^+ is competing with Ca^{2+} as an allosteric effector, leaving us with hypothesis 4 as the most likely explanation for the observed results. We wish to emphasize that other anions, e.g., sulfate and acetate, act qualitatively in the same way as chloride. Direct measurement of ^{36}Cl binding by equilibrium dialysis was not successful. Due to the high chloride concentrations, the differences in ^{36}Cl counts between buffer and protein compartment were too small to be statistically significant.

In order to explain the observed chloride effects, let us first consider oxygen binding by a hypothetical solution of functionally equivalent noninteracting hemocyanin subunits in the presence of chloride. In this case, if we assume one oxygen- and one chloride-binding site per subunit, the ligand binding can

be described by the following square:

$$\begin{array}{ccc} \text{Hcy} & \overset{K_1}{\longrightarrow} & \text{Hcy-O}_2 \\ {\scriptstyle \text{Cl}}\uparrow\downarrow{\scriptstyle K_2} & & {\scriptstyle K_3}\uparrow\downarrow{\scriptstyle \text{Cl}} \\ \text{Hcy-Cl} & \overset{K_4}{\longrightarrow} & \text{Hcy-Cl-O}_2 \end{array}$$

in which Hcy stands for unliganded hemocyanin, K_1, K_2, K_3, and K_4 are the equilibrium association constants and $K_3/K_2 = K_4/K_1$.

The Hill plot of the oxygen binding is then given by

$$\log \frac{Y}{1-Y} = \log \frac{K_1 + K_1K_3(\text{Cl})}{1 + K_2(\text{Cl})} + \log p\text{O}_2 \qquad (1)$$

Plots of $\log [\bar{Y}/(1 - Y)]$ vs. $\log p\text{O}_2$ at different chloride concentrations will give a set of straight lines, characterized by slope 1 and an intercept with $\log \bar{Y}/(1 - \bar{Y}) = 0$, equal to the reciprocal of the apparent oxygen equilibrium association constant

$$K_{\text{app}} = \frac{K_1 + K_1K_3(\text{Cl})}{1 + K_2(\text{Cl})} \qquad (2)$$

This means that K_{app} is a function of K_1, K_2, K_3, and the chloride concentration. K_{app} will equal K_1 and K_4 only if $K_3 = K_2$, i.e., when Hcy and Hcy-O_2 have the same affinity for chloride. Only in this case will one straight line be observed in plots of $\log \bar{Y}/(1 - \bar{Y})$ vs. $\log p\text{O}_2$ at different chloride concentrations.

A plot of K_{app} vs. chloride concentration should give a hyperbola (if $K_3 \neq K_2$). Its asymptote, when the chloride concentration approaches infinity, would be

$$K_{\text{app}} = \frac{K_1K_3}{K_2} = K_4 \qquad (3)$$

At a chloride concentration of zero:

$$K_{\text{app}} = K_1 \qquad (4)$$

Differentiation of K_{app} with respect to chloride gives

$$\frac{dK_{\text{app}}}{d(\text{Cl})} = \frac{K_1K_3}{1 + K_2(\text{Cl})} - \frac{\{K_1 + K_1K_3(\text{Cl})\}K_2}{\{1 + K_2(\text{Cl})\}^2} \qquad (5)$$

This relation indicates that the initial slope of the hyperbola would be

$$\frac{dK_{\text{app}}}{d(\text{Cl})} = K_1(K_3 - K_2), \text{ for } (\text{Cl}) \rightarrow 0 \qquad (6)$$

Equations 3, 4, and 6 enable us to calculate K_1, K_2, K_3, and K_4, respectively.

Inspection of Figure 1 clearly shows the presence of a number of deoxy asymptotes. All of these, obtained at varied chloride concentrations, have unit slope. This means that under these conditions the subunits in the 60S molecule are equivalent and noninteracting. Therefore, the treatment given above for individual subunits should apply to the oxygen binding of 60S hemocyanin molecules in the initial $n = 1$ part of the binding curves. Figure 6 shows a plot of K_{app} vs. chloride concentration. Values for K_{app} at the varied chloride concentrations were obtained by extrapolating the $n = 1$ curves to half saturation. The four equilibrium constants were calculated from this graph using eq 3, 4, and 6: $K_1 = 3.56 \times 10^4\ \text{M}^{-1}$; $K_2 = 8.68\ \text{M}^{-1}$; $K_3 = 3.73\ \text{M}^{-1}$; and $K_4 = 1.50 \times 10^4\ \text{M}^{-1}$. Equation 2 can be rewritten as:

$$y' = \frac{\{1/K_2\ (K_1 - K_4)\}}{x'} \qquad (7)$$

in which $y' = K_{\text{app}} - K_4$ and $x' = 1/K_2 + (\text{Cl})$.

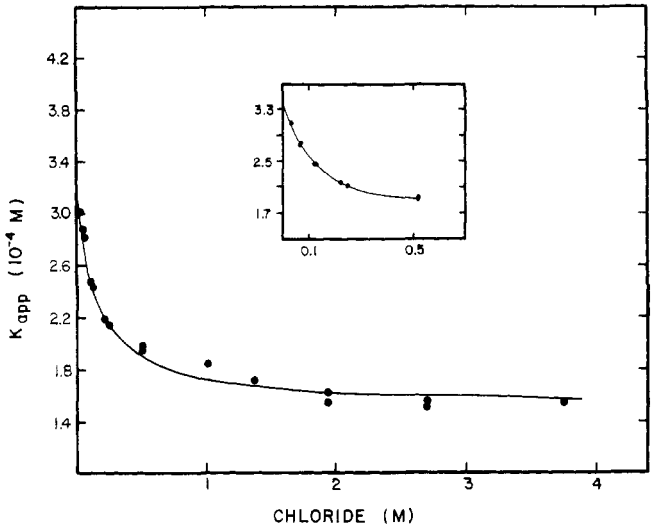

FIGURE 6: K_{app} (calculated from the intercepts of the deoxy asymptotes in Figure 1 with $\log Y/(1 - Y) = 0$) as a function of chloride concentration. (●) Experimental results. The line is calculated by linearizing eq 7 in the text, using the experimental points given in this figure, and then transforming the obtained linear regression line back into a hyperbola. The insert shows the first part of the K_{app} vs. Cl plot.

This equation can be linearized by plotting $K_{\text{app}} - K_4$ vs. $[1/K_2 + (\text{Cl})]^{-1}$, with $K_2 = 8.68\ \text{M}^{-1}$. Such a plot is a straight line with a correlation coefficient of 0.985. This confirms that the experimental data as given in Figure 6 indeed represent a hyperbolic function.

We now wish to examine if the oxygen-binding properties of *Limulus* hemocyanin can be described by the MWC model (Monod et al., 1965), with the extensions of the model as given by Rubin and Changeux (1966) and Blangly et al. (1968). To do so we must take into account the observation of different deoxy asymptotes which represent apparent T states with different oxygen affinities. Conventional notation will allow us to define $p_{1/2,\text{R}}$ as the $p_{1/2}$ of the R state determined from the oxy asymptote in Figure 1. To provide for the multiplicity of apparent T states we define $p_{1/2,\text{T}}$ as equal to the $p_{1/2}$ of the apparent T states, determined from the deoxy asymptotes of Figure 1. According to theory, the Hill plot can be described by

$$\frac{\bar{Y}}{1 - \bar{Y}} = \frac{\alpha(1 + \alpha)^{n-1} + L'\alpha c(1 + \alpha c)^{n-1}}{(1 + \alpha)^{n-1} + L'(1 + \alpha c)^{n-1}} \qquad (8)$$

in which $\alpha = p\text{O}_2/p_{1/2,\text{R}}$, c = the nonexclusive binding coefficient = $p_{1/2,\text{R}}/p_{1/2,\text{T}}$, n is the number of interacting sites, and L' is the apparent allosteric equilibrium constant, which is given by

$$L' = \frac{(\alpha_{1/2} - 1)}{(1 - \alpha_{1/2}c)}\left(\frac{1 + \alpha_{1/2}}{1 + c\alpha_{1/2}}\right)^{n-1} \qquad (9)$$

in which $\alpha_{1/2} = p_{1/2}/p_{1/2,\text{R}}$. The different values for $\alpha_{1/2}$, L', and c are given in Table I.

Using these values of L' and c we have calculated Hill plots at different chloride concentrations with eq 8. The fit between the experimental results and the calculated Hill plots is satisfactory as shown in Figure 7A using $n = 6$. For other values of n no reasonable fits can be obtained. This is illustrated in Figure 7B. This suggests that the hexamer is the cooperative unit and that interactions between the oxygen-binding sites in different hexamers in the native protein do not occur.

From the foregoing considerations, we can say that the oxygen equilibrium curves of *Limulus* hemocyanin, containing 48 oxygen-binding sites, can, as a first approximation, be de-

TABLE I: Effect of Chloride on the Allosteric Parameters of *Limulus polyphemus* Hemocyanin.

Cl(M)	$\alpha_{1/2}$[a]	$L' \times 10^{-7}$[b]	$c \times 10^{2}$[a]
0.07	11.74	0.1064	3.63
0.12	14.79	0.3740	3.16
0.22	18.20	1.163	2.82
0.52	25.71	7.854	2.51
1.02	32.37	32.81	2.40
1.48	35.43	54.19	2.13
1.96	44.68	327.5	1.99
2.91	51.30	∞	1.95
3.77	53.02	∞	1.89

[a] $\alpha_{1/2}$ and c were calculated from Figure 1. [b] L' was calculated with eq 9.

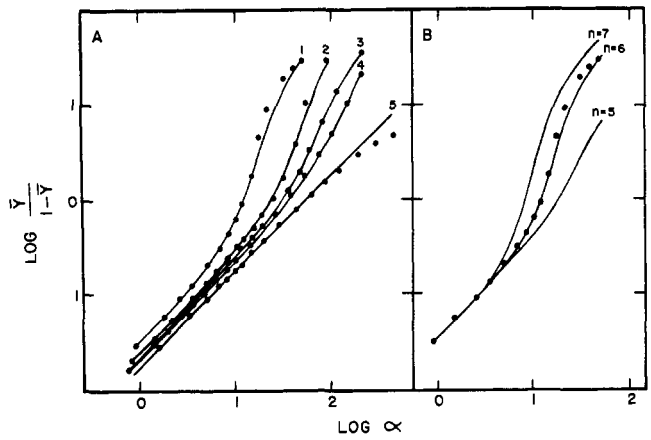

FIGURE 7: (A) Theoretical Hill plots computed based on eq 8 of the text, with the values of L' and c as given in Table I, taking $n = 6$. Lines are calculated and circles are experimental results. Conditions were as in Figure 1. (1) 0.07 M Cl; (2) 0.52 M Cl; (3) 1.02 M Cl; (4) 1.48 M Cl; (5) 3.77 M Cl. (B) Theoretical Hill plots computed based on eq 8 of the text, with the values of L' and c given in Table I for 0.07 M Cl, taking $n = 5$, 6, and 7.

scribed by a concerted model in which one R state and more T states are present. A detailed analysis of other models, such as described by Adair (1925) and Koshland et al. (1966), was not carried out in this study, because the analysis based on the more simple model of Monod et al. (1965), with the modification as described in the text, gave a satisfactory explanation of the observed binding behavior.

The effect of Cl on the oxygen binding of *Limulus* hemocyanin is similar to the effect of 2,3-diphosphoglycerate on human adult hemoglobin. It has been reported that 2,3-diphosphoglycerate not only changes the allosteric constant L, but also the nonexclusive binding coefficient, c, by preferentially changing the oxygen affinity of the T state (Imai, 1973).

An additional value which can be obtained from the Hill plot is the free energy of interaction among the binding sites. The free energies of the oxygen-binding reaction of the R state and the apparent T state in 0.07 M Cl (Figure 1) are −7920 and −5960 cal/mol, respectively. The difference in free energy of oxygenation of both states represents the change in free energy associated with the T → R transition, and equals the total free energy of interaction per site realized in saturating hemocyanin with oxygen: $\Delta F_I = -1960$ cal/mol. Additional chloride decreases the free energy of interaction. Interactions are completely abolished at very high chloride concentrations. The value $\Delta F_I = -1960$ cal/mol is lower than that reported for the tetrameric horse and sheep hemoglobins, −2600 and −3000 cal/mol, respectively, and similar to the −1830 cal/mol reported for the high-molecular-weight *Spirographis* chlorocruorin (Wyman, 1964). It is considerably higher than the −910 cal/mol found for hemocyanin from the mollusc *Levantina hierosolima* (Er-El et al., 1972).

As mentioned in the introduction, *Limulus polyphemus* subunits can be fractionated into at least five major chromatographic zones, with different ligand-binding properties. However, the studies reported in this paper strongly suggest that the binding sites in the undissociated molecule are identical. This might be an indication that the functional properties of the different subunits change upon reassociation. We must keep in mind, however, that the functional differences between the zones are small, except for zone V, which occurs in small amounts (Sullivan et al., 1974).

The time course of oxygen dissociation from *Limulus* hemocyanin is autocatalytic (Figures 3 and 4). This is generally interpreted as being the kinetic reflection of cooperativity, i.e., the homotropic interactions between oxygen-binding sites. Figure 3 shows that in 1.48 and 3.77 M Cl 20 and 50% of the reaction is lost in the 2.4-ms dead time of the apparatus. The 20% loss in 1.48 M Cl can be completely accounted for by the dead time of the stopped-flow apparatus. The observed 50% loss in 3.77 M Cl is only partially due to this dead time, indicating that a very fast process is present. This is not surprising in view of the observed Hill plot under these conditions (Figure 1).

The occupation of the R and T state at different values for α can be calculated with the R-state function (Monod et al., 1965):

$$R = \frac{(1+\alpha)^n}{(1+\alpha)^n + L'(1+\alpha c)^n} \qquad (10)$$

Calculations show that air equilibrated hemocyanin solutions in 0.52, 1.02, and 1.96 M Cl have 95, 85 and 60%, respectively, of their molecules in the R state. At low oxygen pressures, 6 mmHg or lower, only the T states are populated, irrespective of the chloride concentration. With this in mind, let us look at the plot of k_{off} vs. % reaction in Figure 4. At low chloride concentrations, 0.07, 0.12, and 0.22 M, these curves have the same intercept with the 0% reaction axis: 8 s^{-1}. This is interpreted as an estimate of the oxygen dissociation rate of the R state. Our data agree very well with the results of van Driel et al. (1974), who showed the oxygen dissociation of *Helix pomatia* hemocyanin, in a Tris buffer, pH 8.2, plus 10 mM $CaCl_2$, to be autocatalytic, having a K_{off}^R of 10 s^{-1}.

The curves in Figure 4 have different intercepts with the 100% reaction axis. This suggests that there are several apparent T states which differ in their oxygen dissociation rates. Therefore, the kinetic experiments seem to support our equilibrium experiments, which also showed the presence of apparent T states, with different oxygen affinities. A comparison of Figures 2 and 5 clearly shows that the change in $p_{1/2}$ of *Limulus* hemocyanin with chloride concentration at constant pH, or with pH at constant chloride concentration, is kinetically reflected in a change in the oxygen dissociation rate.

Summarizing, we can say that chloride ions lower the oxygen affinity of the huge extracellular hemocyanin of *Limulus polyphemus*, an evolutionary relict of the Devonian age, by binding preferentially to the unliganded state of this protein. In this respect it is rather remarkable that chloride ions also lower the oxygen affinity of the small intracellular human hemoglobin by binding preferentially to its deoxy conformation (Chiancone et al., 1972). This shows us that the oxygen binding by two completely different proteins, which are evolutionary

very remote, can be regulated by the same small anion in a very similar way.

Acknowledgments

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