Oxygen Binding Properties of Stripped (Calcium Ion and Magnesium Ion Free) Hemocyanin from the Scorpion *Leirus quinquestriatus*†

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ABSTRACT: The binding of oxygen to stripped (Ca^{2+},-Mg^{2+}-free) hemocyanin from the scorpion *Leirus quinquestriatus* was studied over a wide range of pH and temperature. Oxygen binding was cooperative in the entire range of pH where the native hemocyanin structure is preserved. Probably 12, or even the totality of the 24 oxygen binding sites of the molecule, act as a cooperative unit. The oxygen binding affinity and the degree of cooperativity were both affected by pH. The dependence on pH of the half-saturation pressure—Bohr effect—was interpreted in terms of two oxygen-linked protons, one opposing and the other promoting oxygen binding. The effect of pH on the maximal slope of the Hill plot indicates that proton dissociation and cooperativity are linked phenomena. Analysis of the binding data using linkage theory shows that an efficient coupling of the homotropic (oxygen–oxygen) and the heterotropic (proton–oxygen) free energies of interaction can provide a satisfactory interpretation of the cooperative behavior. Our findings suggest that in arthropod hemocyanin, as in molluskan hemocyanin, cooperativity of oxygen binding can be attributed to ligand–ligand linkage. In molluskan hemocyanin, however, cooperativity is generated solely by alkaline earth ion–oxygen linkage. In arthropod hemocyanin, both alkaline earth ion– and proton–oxygen linkage can generate cooperative behavior.

Hemocyanins, the copper-containing multisubunit proteins that serve as oxygen carriers in many species of mollusks and arthropods, are functional analogues of vertebrate hemoglobin and, like the latter, show cooperativity in their oxygen binding behavior under physiological conditions. Until recently, it has been widely accepted that calcium and magnesium ions normally present in the hemolymph are necessary for the expression of cooperativity in hemocyanin of both molluskan origin and arthropod origin (Van Holde & Van Bruggen, 1971). As a result, studies aiming at an understanding of the mechanism of cooperativity were conducted, as a rule, in the presence of one or the other of the two ions (DePhillips et al., 1970; Er-el et al., 1972; Van Driel, 1973; Miller & Van Holde, 1974; Shaklai et al., 1975; Bannister et al., 1977; Colosimo et al., 1977; Salvato & Tallandini, 1977; Brouwer et al., 1977, 1978). In a recent communication (Klarman & Daniel, 1977), we have shown that, unlike molluskan hemocyanins, arthropod hemocyanins are capable of cooperative oxygen binding in the absence of calcium and magnesium ions. These findings raise the question of the source of the built-in cooperativity in hemocyanins of arthropod origin. In this paper, we report on a study of the oxygen binding properties of stripped (Ca^{2+},-Mg^{2+}-free) arthropod hemocyanin from the scorpion *Leirus quinquestriatus*. Structural properties of this hemocyanin were previously reported (Klarman et al., 1977, 1979).

Experimental Section

Materials. Hemocyanin was prepared from the scorpion *L. quinquestriatus*. Hemolymph, extracted as previously described (Klarman et al., 1977), was centrifuged, first at 27000*g* for 20 min to remove particulate matter and then at 226000*g* for 90 min, whereupon hemocyanin sedimented. The sediment was dissolved in buffer, 0.1 M Tris-HCl at pH 7.3, and the solution was centrifuged again for 90 min under the same conditions. The pellet was redissolved in the same buffer as before, and the solution was dialyzed against buffer containing 10^{-3} M EDTA to remove Ca^{2+} and Mg^{2+}. The EDTA was subsequently removed by exhaustive dialysis against buffer. Examination by atomic absorption of sample solutions containing 2–3 mg/mL stripped hemocyanin showed less than 10^{-6} M Ca^{2+} or Mg^{2+}.

Solutions of hemocyanin (~0.3 mg/mL) for oxygen binding studies were prepared by dilution (100–200-fold) of a stock solution with the appropriate buffer. Buffers, 0.1 M, were used in the following pH ranges: acetate, 3.8–6.0; cacodylate, 6.0–7.2; Tris-HCl, 7.2–8.5; glycine–NaOH, 8.5–10.0.

Methods. Oxygenation of hemocyanin was measured spectrophotometrically by using the 339-nm absorption band. This band is absent in deoxyhemocyanin and is gradually built up as oxygenation proceeds. Oxygen titrations were carried out in tonometers according to the procedure previously described (Er-el et al., 1972), except that in the present study the absorption instead of the fluorescence was measured. The oxygen partial pressure, p, and the degree of saturation, $\bar{Y}$, were determined at each step of the titration. Calculation of p was made according to Spoek et al. (1964); $\bar{Y}$ was obtained from the relation

$$\bar{Y} = (A - A_0)/(A_\infty - A_0) \quad (1)$$

where A is the measured absorbance and A_0 and A_∞ are the absorbances of deoxy- and oxyhemocyanin, respectively. For A_0, the value of the absorbance of the deaerated solution was used, and for A_∞, that of the solution in equilibrium with air or oxygen at atmospheric pressure. In cases where the oxygen affinity of hemocyanin was too low to enable direct determination of A_∞, its value was obtained by extrapolation (DePhillips et al., 1969).

The partial pressure of oxygen required to produce 50% saturation of oxygen binding sites, $p_{1/2}$, is simply related to the oxygen association constant and free energy of binding for a molecule containing a single binding site, like the 5S subunit of hemocyanin. For a molecule containing several interacting sites, like the 36S whole molecules of hemocyanin, the median saturation pressure, p_m, introduced by Wyman (1964) is a more fundamental quantity and is the one to be used in calculations of thermodynamic parameters. The value of p_m was computed for some of our data, and it was found that the difference between p_m and $p_{1/2}$ did not exceed, in any case, 10% (0.04 on a logarithmic scale) and hardly warrants the effort needed to extract p_m in every experiment. Half-satu-

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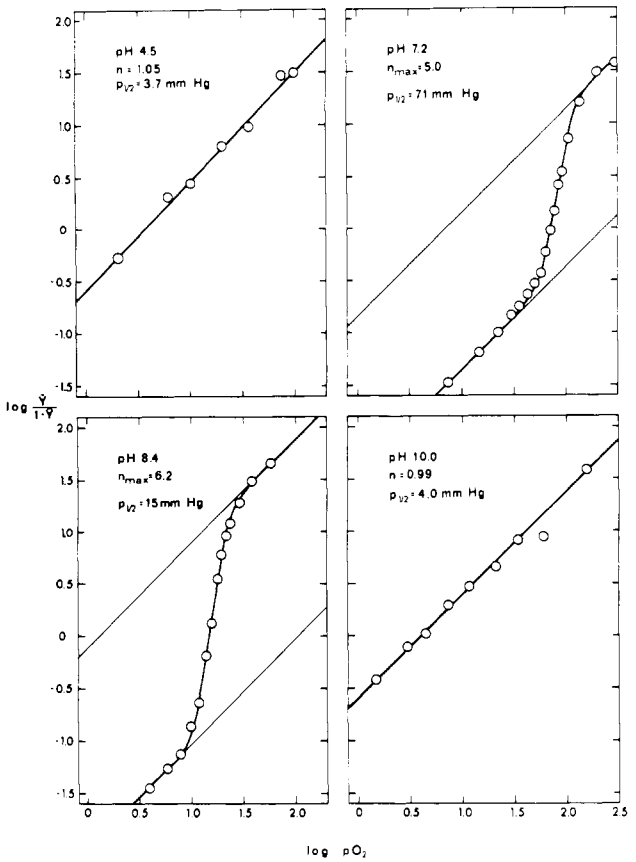

FIGURE 1: Hill plots of the oxygen equilibrium of *L. quinquestriatus* stripped (Ca^{2+},Mg^{2+}-free) hemocyanin at 20 °C. Indicated are the pH, the maximum slope n_{max}, and the half-saturation pressure $p_{1/2}$. Calculation of the median saturation pressure (Wyman, 1964) at pH 7.2 and 8.4 gave p_m = 65 and 14.5 mmHg, respectively. The lower and upper linear asymptotes with n equal unity represent the binding of the L (deoxy) and H (oxy) forms.

ration pressures were accordingly used throughout in our calculations: log $p_{1/2}$ vs. pH instead of log p_m vs. pH in the analysis of the Bohr effect; log $p_{1/2}$ vs. $1/T$ instead of log p_m vs. $1/T$ in the obtention of the thermodynamic parameters of the oxygenation reaction.

Absorption spectra were obtained with a Cary 118 C spectrophotometer. Atomic absorption measurements were done with a Varian Techtron AA-5 instrument. All chemicals were of analytical grade.

Results

Oxygen Binding Behavior of Stripped (Ca^{2+},Mg^{2+}-Free) Hemocyanin. Oxygen binding of stripped *L. quinquestriatus* hemocyanin was studied over a wide range of pH, covering the stability range 5.0 ≤ pH ≤ 9.7 where hemocyanin exists as a 36S molecule containing 24 oxygen binding sites and extending to more acid or alkaline pH values where the molecule is dissociated into 5S subunits carrying 1 oxygen binding site each (Klarman et al., 1979). Figure 1 shows representative binding data, presented as Hill plots. In acid (4.5) and in alkaline (10.0) pH, the binding was of the simple type and the Hill plot had a slope, n, of unity. Within the stability range of the 36S molecule, the binding was cooperative and the Hill plot had the characteristic shape predicted by Wyman (1964), $n = 1$ at $\bar{Y} \rightarrow 0$ and $\bar{Y} \rightarrow 1$ and $n > 1$ at intermediate saturations $0 < \bar{Y} < 1$. In the pH range investigated, the half-saturation pressure showed a variation of almost 2 orders of magnitude, from ~4 mmHg at pH 4.5 and 10.0 to ~100 mmHg at pH 7. In fact, the affinity of hemocyanin toward oxygen is so weak at neutral pH that the protein is only ~90% saturated when exposed to air at atmospheric pressure. To obtain the complete saturation curve under these conditions, it was necessary to substitute oxygen for air as the titrating gas. It should be pointed out that the extrapolation procedure used to obtain A_∞ in such cases (see Experimental Section) may introduce some errors in the calculated values of $\bar{Y}$. Such errors are magnified in the plot of log $\bar{Y}/(1-\bar{Y})$ vs. log p when $\bar{Y} \rightarrow 1$. The position of the upper asymptote of a Hill plot obtained at near neutral pH should accordingly be viewed with reservation (e.g., the plot at pH 7.2, Figure 1). The same considerations lead us to expect that the plot will be very little affected at $\bar{Y} = 0.5$ and that the error in $p_{1/2}$ will be negligible.

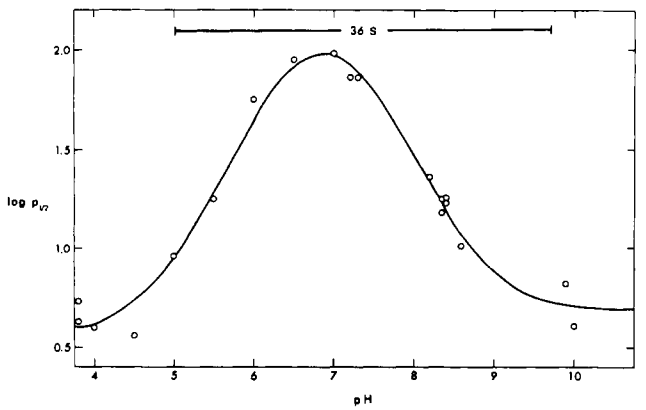

FIGURE 2: Dependence of log $p_{1/2}$ on pH for *L. quinquestriatus* stripped hemocyanin at 20 °C. (O) Observed values; (—) calculated best-fit curve using eq 2 and setting $K_1' = 1.60 \times 10^{-9}$ M, $K_1'' = 7.23 \times 10^{-8}$ M, $K_2' = 1.55 \times 10^{-5}$ M, $K_2'' = 2.61 \times 10^{-7}$ M, and $C = 0.56$. The stability range of the 36S structure is indicated by a line segment drawn parallel to the abscissa.

Table I: Heterotropic Free Energies of Interaction of the Oxygen-Linked Acid Groups in *L. quinquestriatus* Hemocyanin[a]

type of interaction	pK' of deoxyhemocyanin	pK'' of oxyhemocyanin	$\Delta G^\circ_{H^+-O_2}$ (kcal/mol)
opposing	8.80	7.14	2.2
promoting	4.81	6.58	−2.4

[a] In buffers of 0.1 ionic strength at 20 °C.

The variation of the oxygen binding affinity—represented by −log $p_{1/2}$—with pH (Figure 2) brings out the existence of a normal (above pH 7) and a reverse (below pH 7) Bohr effect. The finding that the oxygen binding affinity passes through a minimum means that at least two different proton binding sites interact with each oxygen binding site, one opposing and the other promoting oxygen binding. The variation of the oxygen binding affinity with pH can be used in the same way as is done for the Bohr effect in hemoglobin to determine the pK values of the oxygen-linked protons. The data were fitted to the equation[1]

$$\log p_m = C + \log \frac{([H^+] + K_1')([H^+] + K_2')}{([H^+] + K_1'')([H^+] + K_2'')} \quad (2)$$

[1] In the original treatment of the Bohr effect of hemoglobin (Wyman, 1948), it was assumed that the oxygen equilibrium curves were symmetrical and shape invariant with pH and the derived relation involved the variation of log $p_{1/2}$ with pH. In a later publication (Wyman, 1964), it was indicated that the derived relation acquires general validity when expressed in terms of p_m. The fact that log p_m depends only on the binding constants of H^+ to fully oxygenated and deoxygenated structures was recently emphasized in a review by Baldwin (1975) (eq 2 of this text is a special case of eq 4.3 of Baldwin). It is assumed that there is no interaction between the proton sites within the same subunit or in different subunits (Baldwin, 1975).

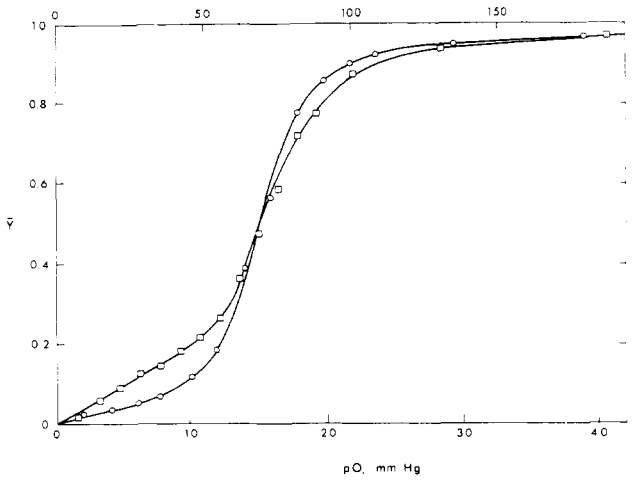

FIGURE 3: Oxygen saturation curves for *L. quinquestriatus* stripped hemocyanin at 20 °C, pH 7.2 [(□) upper abscissa] and 8.4 [(○) lower abscissa]. The experimental data are the same as in Figure 1 and the change of scale of p has been made to bring the two curves into coincidence at $\bar{Y} = 0.5$.

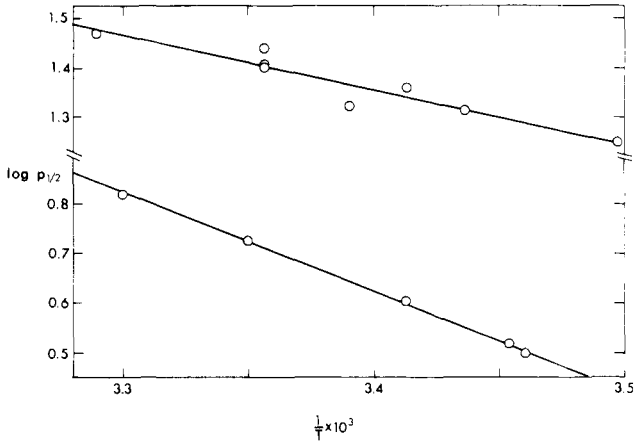

FIGURE 4: Logarithmic plot of the half-saturation pressure vs. the reciprocal absolute temperature for *L. quinquestriatus* stripped hemocyanin at pH 10.0 (lower plot) and 8.2 (upper plot). The pH was brought to the specified value at the temperature of the experiment.

where K_1' and K_2' are the proton ionization constants of deoxyhemocyanin, K_1'' and K_2'' are the corresponding values of oxyhemocyanin, $[H^+]$ is the molar concentration of protons, and C is a constant. The best fit with the experimental data was obtained by using the parameters given in Table I.

The shape of the saturation curve—$\bar{Y}$ vs. log p—was found to vary with pH within the pH range where the integrity of the 36S structure is preserved. This point is illustrated by a comparison of the binding data for the two pH values, pH 7.2 and 8.4 (Figure 3).

Thermodynamics of Oxygen Binding Reaction. The thermodynamics of the oxygen equilibrium were studied for both the 5S subunits (pH 10.0) and the 36S whole molecules (pH 8.2). The free energy of oxygenation was obtained from the value of the half-saturation pressure, and the enthalpy change was obtained from the dependence of log $p_{1/2}$ on $1/T$ (Figure 4). The free energy, enthalpy, and entropy of the oxygen binding reaction at 25 °C are listed in Table II.

Additional information about the oxygenation reaction at pH 8.2 where oxygen binding is cooperative was obtained from a consideration of the Hill plot over the entire range of pressures covered. The binding behavior of hemocyanin under these conditions may be viewed, formally at least, as a transition from a low-affinity state of the protein, the L form, to a state with a high affinity toward oxygen, the H form. The

Table II: Thermodynamic Parameters for the Oxygenation of *L. quinquestriatus* Hemocyanin at pH 10.0 and pH 8.2

hemocyanin	reaction	$\Delta G°$ [a] (kcal/mol)	$\Delta H°$ [b] (kcal/mol)	$\Delta S°$ (cal deg^{-1} mol^{-1})
	pH 10.0 [c]			
5S subunits	oxygenation	−7.0	−5.9	3.6
	pH 8.2 [d]			
36S molecules	oxygenation	−6.0	−1.9	13.7
L form	oxygenation	−5.0	3.1	27.4
H form	oxygenation	−7.4	−7.4	0
	O_2–O_2 interaction	−2.4	−10.5	−27.4

[a] Obtained from the expression $-RT \ln A$, where A is the association equilibrium constant given by $A = (\kappa p_{1/2})^{-1}$, $p_{1/2}$ is the half-saturation pressure, and κ is a scaling factor relating the concentration of dissolved oxygen to the partial pressure of the gas above the solution. At 25 °C, $\kappa = 1.6 \times 10^{-6}$ M/mmHg. [b] Obtained from the slope of the van't Hoff plot—log $p_{1/2}$ vs. $1/T$—by taking into account the heat of solution of oxygen (−3.1 kcal/mol at 25 °C). [c] In 0.1 M glycine–NaOH buffer at 25 °C. [d] In 0.1 M Tris-HCl buffer at 25 °C.

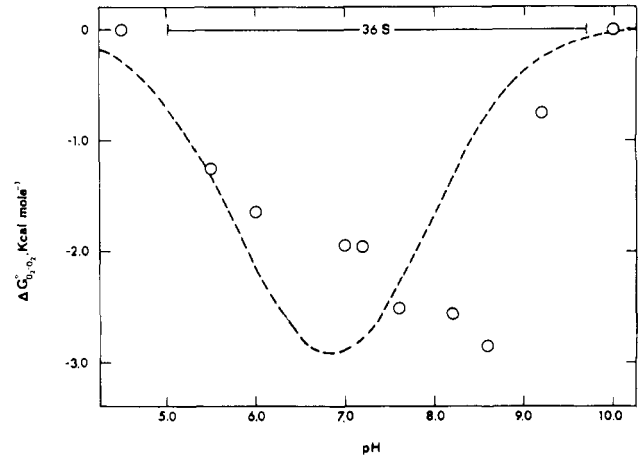

FIGURE 5: Dependence on pH at 20 °C of the homotropic energy of interaction (○) of *L. quinquestriatus* stripped hemocyanin. (---) Theoretical curve for an energetically equivalent system, calculated as explained in the text.

two asymptotes of the Hill plot at $\bar{Y} \rightarrow 0$ and $\bar{Y} \rightarrow 1$ having slopes of unity represent the binding behavior of these two hypothetical forms (Er-el et al., 1972). The thermodynamic parameters of the binding of oxygen by the L and H forms of *L. quinquestriatus* hemocyanin at pH 8.2 were determined in the usual manner from the corresponding asymptotes to the Hill plots, and the results are given in Table II. The homotropic energy of interaction $\Delta G°_{O_2\text{–}O_2}$—being the difference between the free energies of interaction on binding the first and last ligands (Saroff & Minton, 1972)—and the enthalpy and entropy contributions $\Delta H°_{O_2\text{–}O_2}$ and $\Delta S°_{O_2\text{–}O_2}$ were obtained from the difference of the corresponding thermodynamic parameters of the L and H forms and are also presented in Table II. The variation of $\Delta G°_{O_2\text{–}O_2}$ with pH is shown in Figure 5.

Discussion

In a recent communication, we reported cooperative oxygen binding by stripped *L. quinquestriatus* scorpion hemocyanin (Klarman & Daniel, 1977). The results of the present study indicate that both oxygen binding affinity and cooperativity are pH dependent. Thus, it is clear that in this hemocyanin (a) both homotropic interactions among oxygen binding sites and heterotropic interactions involving protons and oxygen

binding sites do occur and (b) proton dissociation and cooperativity are linked phenomena.

The thermodynamic parameters of the homotropic interaction were determined at pH 8.2 (Table II). From Figure 5 it is seen that maximal cooperativity in oxygen binding is manifested in the vicinity of this pH. The free energy of interaction, $\Delta G^\circ_{O_2\text{-}O_2}$ = 2400 cal, is comparable to values reported for other hemocyanins: 2000 cal for *Homarus americanus* (Wyman, 1967), 910 cal for *Levantina hierosolima* (Er-el et al., 1972), 1800 cal for *Helix pomatia* (Colosimo et al., 1977), and 2320 cal for *Murex trunculus* (Bannister et al., 1977). However, it should be borne in mind that, whereas the value for scorpion hemocyanin was obtained in Ca^{2+},-Mg^{2+}-free solution, the values for other hemocyanins were obtained in solutions which contained Ca^{2+}, Mg^{2+}, or both of these ions. As with snail hemocyanin (Er-el et al., 1972), the free energy of interaction in scorpion hemocyanin is composed partly of an enthalpic and partly of an entropic change. This may be taken as an indication that the L → H transition involves, besides conformational changes, the formation of new noncovalent bonds or the rupture of existing ones, as was shown to be the case for mammalian hemoglobin (Perutz, 1970).

Examination of the thermodynamics of oxygenation under conditions of cooperative binding, though for obvious practical reasons limited in this study to a single pH, reveals the remarkable finding that there is a change of sign of ΔH upon going from the L (deoxy) to the H (oxy) form (Table II). This may be viewed as a reflection of the profound difference in the properties of the unliganded and liganded states of the molecule. Comparison of the entropy term is not less striking. While oxygenation of the L form is associated with appreciable change in entropy, the free energy of oxygenation of the H form is entirely enthalpic and involves no change in entropy. A likely interpretation of these findings is that the first oxygen molecule has to surmount a conformational barrier before it can bind to hemocyanin, in contrast to the last one which can bind with little need for conformational adjustment of the protein. In other words, deoxyhemocyanin is in a conformationally unfavorable state for binding oxygen, and oxyhemocyanin is in a conformationally favorable one. The conformations of hemocyanin that predominate in the deoxy and oxy forms partake of the attributes postulated for the "tense" and "relaxed" states in the allosteric model (Monod et al., 1965). Very similar results were previously reported for *L. hierosolima* snail hemocyanin (Er-el et al., 1972).

The minimal number of interacting sites can be estimated from the slopes of the Hill plots. This procedure is valid provided the oxygen binding sites can be shown to have the same intrinsic affinity toward oxygen. To examine this point, we studied the oxygen binding properties of a noninteracting site by carrying out experiments at such high (10) or low (4.5) pH that the 36S molecule was dissociated into 5S subunits carrying one oxygen binding site each. The fact that values of n close to unity were obtained under these conditions (Figure 1) indicates that the heterogeneity in the constituent polypeptide chains (Klarman et al., 1979) does not reflect in the affinity of the oxygen binding sites. In the pH range where the 36S structure is stable, values of n_{max} as high as 7 have been obtained, indicating that the cooperative domain encompasses more than the six oxygen binding sites contained in a hexameric unit. Probably 12, or even the totality of the 24 sites in the 36S molecule, act as a cooperative unit in *L. quinquestriatus* hemocyanin. From this aspect, scorpion hemocyanin seems to be very similar to spider hemocyanin, where the allosteric unit extends beyond the confines of the 16S hexameric unit (Linzen et al., 1977; Loewe et al., 1977). In contrast, a hexamer has been postulated as the allosteric unit in *Callianassa californiensis* (Miller & Van Holde, 1974) and *Limulus polyphemus* (Brouwer et al., 1977) hemocyanins.

The heterotropic interaction between proton and oxygen binding sites is manifested in the variation of the oxygen binding affinity with pH. An analysis using Wyman's theory of linked functions (Wyman, 1964) shows that the Bohr effect can be interpreted in terms of two oxygen-linked protons, one opposing and the other promoting oxygen binding. The magnitudes of the free energy of interaction $\Delta G^\circ_{H^+\text{-}O_2}$ = 2200–2400 cal (Table I) are similar to the free energy of interaction of protons and oxygen responsible for the alkaline Bohr effect in human hemoglobin, 1800 cal (Wyman, 1948). On the basis of the pK values found, it is possible to assign likely candidates for the groups responsible for the Bohr effect. The promoting oxygen-linked proton responsible for the acid Bohr effect may be assumed to be that of a carboxyl side chain. The opposing proton responsible for the alkaline Bohr effect may likewise be attributed to a cysteinyl, histidyl, or terminal amino group.

The findings of the present study permit an evaluation of the factors involved in the cooperativity of oxygen binding to scorpion hemocyanin. The fact that oxygen binding curves obtained at different pH values cannot, as is the case in mamalian hemoglobin, be brought into coincidence by a mere shift in the ligand concentration scale (Figure 3) indicates that linkage between oxygen and proton sites makes a contribution to the observed cooperativity. The extent of the ligand–ligand contribution can be assessed through a comparison of the free energy of interaction experimentally observed with that computed from linkage theory (Wyman, 1967; Weber, 1972, 1975). Computations of this kind are, however, complicated by the lack of our knowledge of the actual coupling pattern in as complex a system as hemocyanin, where a large variety of coupling schemes are a priori possible among the 24 oxygen sites and twice that number of oxygen-linked protons, half of which promoting and half opposing oxygen binding. To surmount this difficulty, we have considered a simplified but energetically equivalent system in which a molecule of protein binds two molecules of oxygen and two coupled protons, each proton equally affecting both oxygens, one proton promoting and the other opposing oxygen binding [a representation of the coupling scheme in the equivalent system is obtained by substituting protons for the calcium ions in Figure 6 of Shaklai et al. (1975)]. It should be stressed that the equivalent system with its only two oxygen binding sites is clearly unrealistic as a model and is introduced for the sole purpose of gaining insight into the interplay between homotropic and heterotropic interaction energies brought about by the coupling of proton and oxygen sites. The equivalent system has the apparent dissociation constants for oxygen (K_I for the deoxy form; K_{II} for the oxy form) given by the equations (Weber, 1972, 1975; Shaklai et al., 1975)

$$K_I = \left(\frac{1 + \epsilon_1}{1 + \epsilon_1\beta_1^{-1}}\right)\left(\frac{1 + \epsilon_2}{1 + \epsilon_2\beta_2^{-1}}\right)K \qquad (3)$$

$$K_{II} = \left(\frac{1 + \epsilon_1\beta_1^{-1}}{1 + \epsilon_1\beta_1^{-2}}\right)\left(\frac{1 + \epsilon_2\beta_2^{-1}}{1 + \epsilon_2\beta_2^{-2}}\right)K \qquad (4)$$

where K is the intrinsic unperturbed dissociation constant of the oxygen sites, $\epsilon_1 = [H^+]/K_1'$, $\epsilon_2 = [H^+]/K_2'$, $\beta_1 = K_1''/K_1'$, and $\beta_2 = K_2''/K_2'$. Values of the homotropic energy of interaction $RT \ln (K_I/K_{II})$ were calculated for given proton concentrations $[H^+]$, and the results were plotted as a function

of pH in Figure 5. The plot is seen to simulate the general behavior of the observed dependence of $\Delta G^{\circ}_{O_2\text{-}O_2}$ on pH, tending to be zero at high and low pH and reaching a maximal value of ~3000 cal, and indicates that an efficient coupling between the heterotropic and homotropic energies of interaction can provide a satisfactory interpretation of the cooperative behavior. In other words, it is possible to account for the cooperativity of oxygen binding by scorpion (arthropod) hemocyanin in terms of ligand–ligand linkage alone, as was done in the case of snail (molluskan) hemocyanin (Shaklai et al., 1975).

Molluskan and arthropod hemocyanins show cooperative oxygen binding under physiological conditions. Our findings suggest that in arthropod, as in molluskan, hemocyanins, cooperativity is generated by ligand–ligand linkage. In molluskan hemocyanin, however, cooperativity is generated solely by alkaline earth (calcium or magnesium) ion–oxygen linkage. In arthropod hemocyanin, both alkaline earth ion– and proton–oxygen linkages can generate cooperative behavior.

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