Oxygen Binding by Hemocyanin from *Levantina hierosolima*. II. Interpretation of Cooperativity in Terms of Ligand–Ligand Linkage†

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ABSTRACT: Oxygen binding by hemocyanin from *Levantina hierosolima* was studied at pH 7.30, in solutions containing calcium in the concentration range 0–1 M. The binding was found to be cooperative, the degree of cooperativity being calcium concentration dependent. The dependence on calcium concentration of the affinity toward oxygen for both deoxygenated and oxygenated hemocyanin was interpreted in terms of two oxygen-linked calcium ions, one promoting and the other opposing oxygen binding. The results show that cooperativity may be fully explained on the basis of a coupling of the free energy of binding between calcium and oxygen.

The mechanism of cooperativity of oxygen binding by hemocyanin is a subject of current interest (Wyman, 1969). In spite of the early realization that manifestation of cooperative behavior needed the presence of Ca^{2+} (or Mg^{2+}), the role of the latter remained unsolved. The readiness with which hemocyanin dissociates into subunits and the ability of Ca^{2+} to bring about their reassociation made it plausible to propose subunit interaction as a basis for cooperativity. In the preceding paper (Klarman *et al.*, 1975) this explanation of the role of Ca^{2+} was ruled out. What is needed then is an interpretation which will retain its validity down to the level of a subunit. The presentation of such an interpretation and the examination of its ability to account quantitatively for the experimental binding behavior are the purposes of the present paper.

Experimental Section

Hemocyanin was prepared from the snail *Levantina hierosolima* as described previously (Shaklai and Daniel, 1970). The protein solutions were buffered with 0.1 M Tris-HCl (pH 7.30) throughout. At this pH, hemocyanin exists as 100S molecules. Oxygen saturation curves were obtained by fluorometric titration according to the procedure of Er-el *et al.* (1972). Calcium concentration was measured using a Varian Techtron AA-5 atomic absorption spectrometer. All chemicals were analytical grade.

The relative solubility of oxygen was measured in the following way. A series of solutions containing different concentrations of the desired salt was prepared and left overnight in a shaking thermostat to equilibrate with air at atmospheric pressure. The dissolved oxygen was determined according to MacArthur (1916). For each solution, the results were expressed as a ratio of oxygen concentration relative to that in double-distilled water equilibrated with air under the same conditions.

Results

Binding Behavior in NaCl Solutions. Since in this study we were to carry out oxygen binding titrations at high concentrations of calcium, we felt the need to check the influence of ionic strength *per se* on the binding reaction. Na^+ does not bind to hemocyanin (unpublished results) and thus NaCl constitutes a suitable salt for investigating this point. Oxygen binding titrations were conducted in the presence of 0–3 M NaCl concentrations, covering the ionic strength of $CaCl_2$ used in this study. The oxygen binding was always noncooperative (Figure 1), but the half-saturation pressure, $p_{1/2}$, was found to be dependent on the NaCl concentrations (Figure 2, lower curve). This variation of $p_{1/2}$ means that the activity coefficients γ_{Hcy} and γ_{HcyO} of deoxy- and oxyhemocyanin in the expression of the equilibrium constant $K_a = ([Hcy]\gamma_{Hcy}/[HcyO]\gamma_{HcyO})p_{O_2}$, where p_{O_2} is the partial oxygen pressure, do not cancel out. Since data on γ_{Hcy} and γ_{HcyO} are unavailable, we decided to present binding constants in terms of concentrations, rather than in mixed units of activities and concentrations (see Tanford, 1961). In each case we calculated $K_{obsd} = ([Hcy]/[HcyO])[O_2]$. Oxygen concentrations were obtained using solubility data given in Figure 3. From Figure 2, it is seen that K_{obsd} is practically independent of NaCl concentration in the range examined.

Binding Behavior in $CaCl_2$ Solutions. Oxygen titrations at calcium ion concentrations covering the range 0–1 M were carried out. Binding data, presented as Hill plots, are given in Figure 4. At calcium concentrations below 3.2×10^{-3} M, no cooperativity can be detected, as evidenced by linear Hill plots with a slope $n = 1$. The half-saturation pressure is found to be independent of calcium concentration in this range. At calcium ion concentrations equal to 3.2×10^{-3} M and above, we find slopes $n > 1$ at intermediate saturations $\bar{\nu}$, and $n = 1$ at $\bar{\nu} \rightarrow 0$ and $\bar{\nu} \rightarrow \infty$. This behavior is typical for cooperative binding (Wyman, 1964). Following a nomenclature introduced in a previous work (Er-el *et al.*, 1972), the asymptote to the Hill plot at $\bar{\nu} \rightarrow 0$, which is the one with the lower affinity, will be referred to a noncooperative hypothetical L form, and the asymptote at $\bar{\nu} \rightarrow 1$, with the higher affinity, to an H form. From Figure 4, it is seen that, in the range 3.2×10^{-3} to 1 M Ca^{2+}, the affinities of both L and H forms depend on the concentration of calcium. Loss of oxygen binding ability with time precluded the determination of the binding behavior at calcium concentrations higher than 1 M. For each calcium concentration, values of the oxygen dissociation constants for the L

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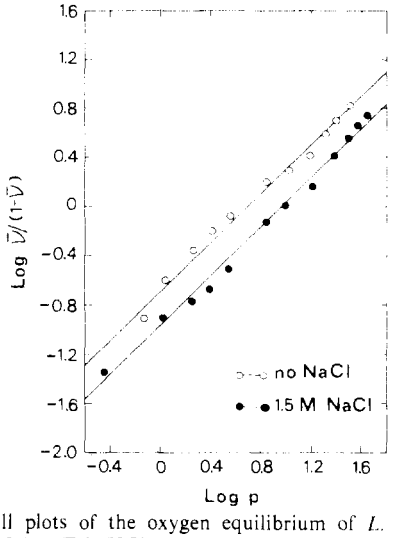

FIGURE 1: Hill plots of the oxygen equilibrium of *L. hierosolima* hemocyanin in 0.1 M Tris-HCl and 1.5 M NaCl (lower curve) and 0.1 M Tris-HCl alone (upper curve), at pH 7.30 and 25°.

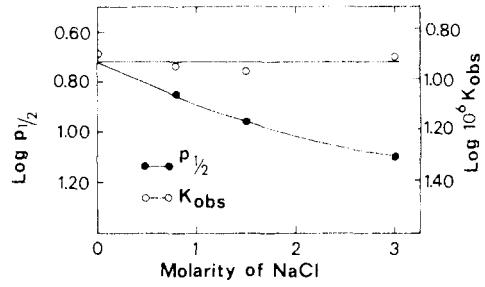

FIGURE 2: The dependence of the oxygen half-saturation pressure on NaCl concentration for *L. hierosolima* hemocyanin in 0.1 M Tris-HCl, pH 7.30 and 25°: (●) measured half-saturation pressure, $p_{1/2}$; (○) observed dissociation constant, K_{obsd}.

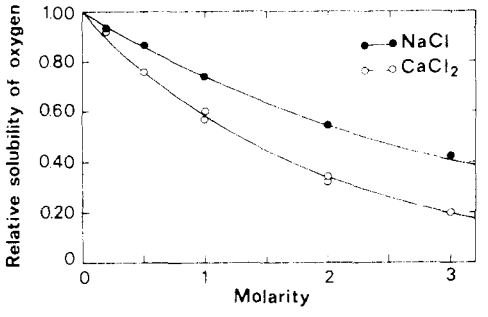

FIGURE 3: The dependence of the relative oxygen solubility on the molarity of salt in solutions of NaCl (●) and $CaCl_2$ (○) in water at 25°.

and H forms were obtained from the asymptotes to the Hill plot, using data for the solubility of oxygen (Figure 3). The dependence on calcium ion concentration of K_{obsd} of the L and H forms is presented in Figure 5.

Discussion

In a previous publication (Er-el *et al.*, 1972), we have shown that the presence of calcium ion affects the oxygen binding affinity of hemocyanin. The results of this study indicate that the effect is calcium concentration dependent. In a previous study, we have shown that hemocyanin binds Ca^{2+} (Klarman *et al.*, 1972). Taken together, these findings show that the changes in oxygen affinity observed in the presence of Ca^{2+} must be attributed to the calcium ions bound to the protein. Thus, hemocyanin constitutes a system in which the binding of two ligands, calcium and oxygen, is linked. The theory of linked functions has been developed by Wyman (1964). The interaction between two ligands may be one of two types, an opposing or an enhancing one. The variation of the oxygen affinity with the calcium concentration can be used, in the same way as the Bohr effect in hemoglobin, to determine the nature of the interaction between the two ligands. It can be seen from Figure 5 that the L branch of the log K_{obsd} *vs.* log $[Ca^{2+}]$ curve passes through an extremum. This means that at least two calcium sites must interact with the oxygen binding sites—one opposing and the other enhancing oxygen binding.

The generation of cooperativity through the interaction of two ligands has been treated by Wyman (1967) and by Weber (1972). Weber derived equations for the case where two identical sites for one ligand interact with one site for another. He showed that the interaction gives rise to cooperative effects whether the two types of ligand oppose or enhance each other's binding. We wish to apply Weber's treatment to the case where two identical sites for one ligand interact with two nonidentical sites for another. Let us consider a model of the hemocyanin–calcium–oxygen system having two identical sites for the binding of oxygen and two oxygen-linked calcium binding sites different from one another in their affinity. The equilibria and the dissociation constants involved are shown in Figure 6. In this model K is the dissociation constant of the oxygen sites, K_1 and K_2 are the dissociation constants of the two calcium sites, and β_1 and β_2 are related to the free energy of interaction by the relation $\beta = \exp(-\Delta F^\circ_1/RT)$. For the model considered, the oxygen saturation $\bar{\nu}$ at a given concentration of calcium ion $[Ca^{2+}]$ and oxygen $[O_2]$ can be written as (*cf.* eq 1.12, Weber, 1972)

$$\bar{\nu} = \frac{\frac{[O_2]}{K}\frac{B}{A} + \frac{[O_2]^2}{K^2}\frac{C}{A}}{1 + 2\frac{[O_2]}{K}\frac{B}{A} + \frac{[O_2]^2}{K^2}\frac{C}{A}} \quad (1)$$

where

$$A = 1 + \frac{[Ca^{2+}]}{K_1} + \frac{[Ca^{2+}]}{K_2} + \frac{[Ca^{2+}]^2}{K_1K_2}$$

$$B = 1 + \frac{[Ca^{2+}]}{\beta_1K_1} + \frac{[Ca^{2+}]}{\beta_2K_2} + \frac{[Ca^{2+}]^2}{\beta_1\beta_2K_1K_2}$$

$$C = 1 + \frac{1}{\beta_1^2}\frac{[Ca^{2+}]}{K_1} + \frac{1}{\beta_2^2}\frac{[Ca^{2+}]}{K_2} + \frac{1}{\beta_1^2\beta_2^2}\frac{[Ca^{2+}]^2}{K_1K_2}$$

Equation 1 corresponds to a system with two dissociation constants for oxygen, K_I and K_{II}, given by

$$K_I = \frac{1 + \frac{[Ca^{2+}]}{K_1} + \frac{[Ca^{2+}]}{K_2} + \frac{[Ca^{2+}]^2}{K_1K_2}}{1 + \frac{[Ca^{2+}]}{\beta_1K_1} + \frac{[Ca^{2+}]}{\beta_2K_2} + \frac{[Ca^{2+}]^2}{\beta_1\beta_2K_1K_2}} K \quad (2)$$

$$K_{II} = \frac{1 + \frac{[Ca^{2+}]}{\beta_1K_1} + \frac{[Ca^{2+}]}{\beta_2K_2} + \frac{[Ca^{2+}]^2}{\beta_1\beta_2K_1K_2}}{1 + \frac{1}{\beta_1^2}\frac{[Ca^{2+}]}{K_1} + \frac{1}{\beta_2^2}\frac{[Ca^{2+}]}{K_2} + \frac{1}{\beta_1^2\beta_2^2}\frac{[Ca^{2+}]^2}{K_1K_2}} K \quad (3)$$

K_I is the dissociation constant of the deoxy form of the model and K_{II} that of the oxy form. Equations 2 and 3[1]

[1] Equation 2—and a similar statement applies to eq 3—may be cast into the form

$$\log K_I = \log \beta_1\beta_2 K + \log \frac{([Ca^{2+}] + K_1)([Ca^{2+}] + K_2)}{([Ca^{2+}] + \beta_1K_1)([Ca^{2+}] + \beta_2K_2)} \quad (4)$$

which clearly brings out its identity with the relation derived by Wyman (1948) for the dependence of oxygen affinity on proton concentration (Bohr effect) in hemoglobin.

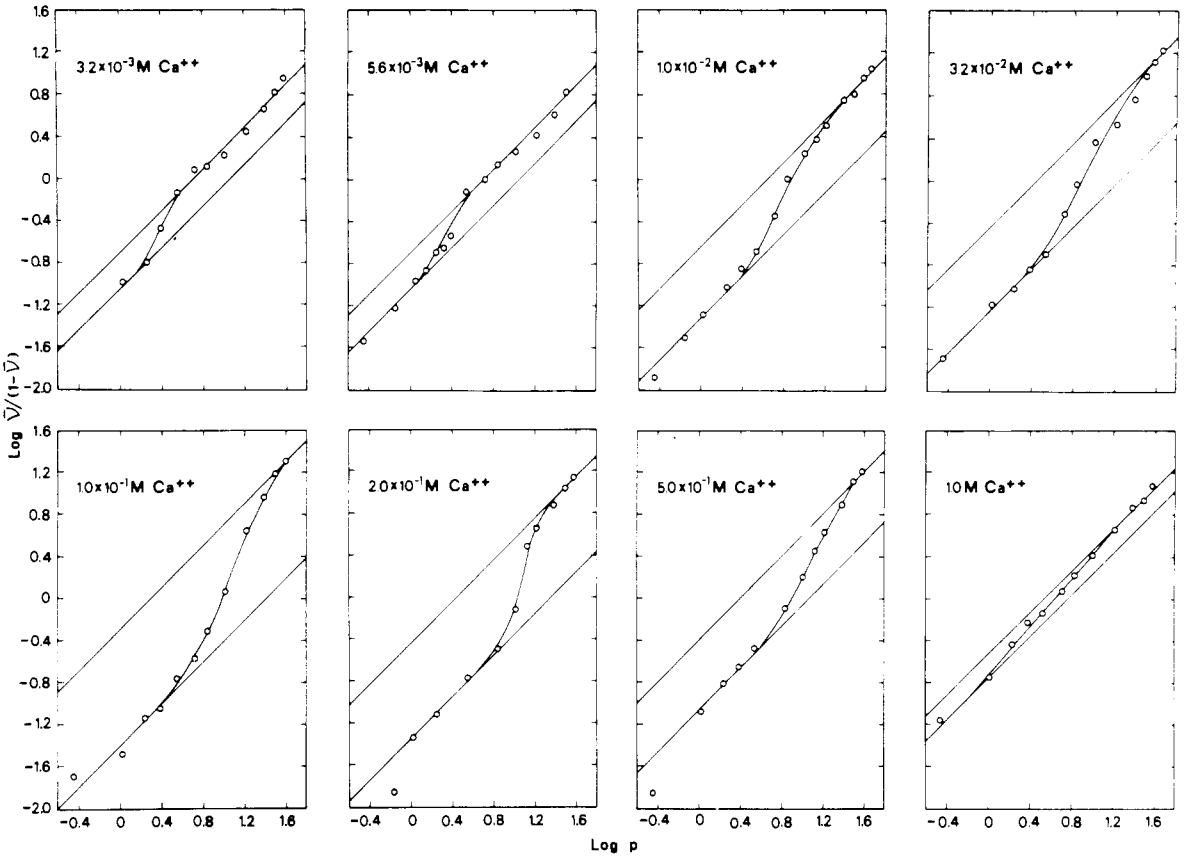

FIGURE 4: Hill plots of the oxygen equilibrium of *L. hierosolima* hemocyanin in solutions containing 0.1 M Tris-HCl at pH 7.30 and 25°. Indicated are the total calcium concentrations. Due to the weak binding of calcium to hemocyanin and the low concentration of protein, the free concentration of calcium is practically equal to the total concentration of this ion. The linear asymptotes represent the binding of the L and H forms.

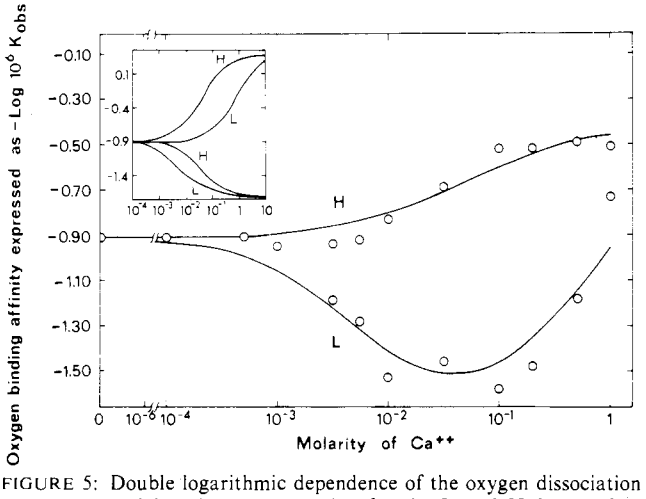

FIGURE 5: Double logarithmic dependence of the oxygen dissociation constant on calcium ion concentration for the L and H forms of *L. hierosolima* hemocyanin; conditions, 0.1 M Tris-HCl, pH 7.30 and 25°; (O) observed values, each point being an average value of two experiments, one of which is shown in Figure 4; (—) calculated best fit curve for the model discussed in the text. Insert: Resolution of the best fit curve to show the contribution of each of the two oxygen-linked calcium ions. The lower two curves, given by $K(1 + [Ca^{2+}]/K_1)/(1 + [Ca^{2+}]/\beta_1 K_1)$ and $K(1 + [Ca^{2+}]/\beta_1 K_1)/(1 + [Ca^{2+}]/\beta_1^2 K_1)$, describe, respectively, the expected behavior of the L and H forms in the presence of the opposing Ca^{2+}. The upper two curves, obtained by substituting K_2, β_2 for K_1, β_1 show the expected behavior in the presence of the enhancing Ca^{2+}.

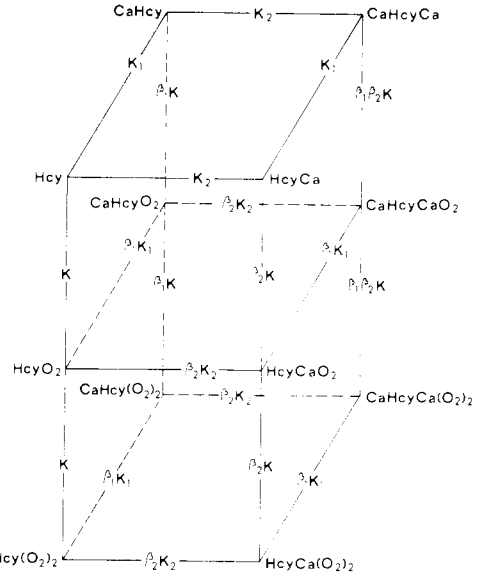

FIGURE 6: Ligand equilibria for the model of the hemocyanin-calcium-oxygen system proposed in the text. Two identical oxygen sites (intrinsic dissociation constant K) and two different oxygen-linked calcium sites (dissociation constants K_1 and K_2) are assumed. The bound calcium has been written to the left or to the right, as CaHcy or HcyCa, to distinguish between the two calcium binding sites. The interaction between calcium and oxygen is expressed in terms of the parameters β_1 and β_2.

make possible the calculation of K_I and K_{II} as a function of K_1, K_2, β_1, β_2, and K. Using a computer, we looked for values of the five parameters that will give the best fit of K_I and K_{II} with the experimentally determined values K_{obsd} of the L and H forms, respectively. The following values were obtained: $K_1 = 1.99 \times 10^{-3}$ M, $K_2 = 3.07$ M, $\beta_1 = 6.25$

($\Delta F^\circ_1 = -1080$ cal), $\beta_2 = 5.20 \times 10^{-2}$ ($\Delta F^\circ_1 = 1750$ cal), and $K = 8.10 \times 10^{-6}$ M. The best fit curve is drawn in Figure 5. The fit of the L form is within twice the estimated experimental error; that of the H form is better, within experimental error.

Let us compare the dissociation constants found for the oxygen-linked calcium ions with the dissociation constant for Ca^{2+} determined experimentally. In a previous work (Klarman *et al.*, 1972), it was found that a unit which carries one oxygen site accommodates 20 calcium sites with a dissociation constant of the order of 10^{-2} M. At pH 7.30, a dissociation constant $\sim 3 \times 10^{-2}$ M was measured (unpublished data). A comparison of the latter value with $\beta_1 K_1 = 1.24 \times 10^{-2}$ and $\beta_2 K_2 = 16 \times 10^{-2}$ M indicates that the two oxygen-linked calcium sites may not be distinguishable experimentally, as far as their affinity for calcium is concerned, from the totality of the calcium sites in hemocyanin.

Let us consider next the contribution of each of the two linked Ca^{2+}. It is seen (Figure 5, insert) that the effect of the opposing Ca^{2+} is to lower, and that of the enhancing one to increase, the affinity of both deoxy and oxy forms. At very high calcium concentrations, the affinity of the deoxy and oxy forms reaches the same limiting value—lower than the calcium-free affinity in the case of the opposing, and higher in the case of the enhancing, calcium. At $\sim 10^{-2}$ M Ca^{2+}, the effect on the affinity of the oxy form of the opposing Ca^{2+} is practically cancelled by that of the promoting one. In our previous work (Er-el *et al.*, 1972), which was carried out at a single calcium concentration, 2×10^{-2} M, we found that the affinity of the deoxy form of hemocyanin was lowered by the presence of Ca^{2+}, while that of the oxy form was unaffected. It is thus seen that the implications of the present model encompass the binding behavior found before.

The results obtained in this study show that the cooperative nature of oxygen binding by hemocyanin in the presence of Ca^{2+} can be fully explained on the basis of a coupling of the free energy of binding of the two ligands, calcium and oxygen. The finding (Klarman *et al.*, 1975) that cooperativity can be expressed at the level of the 20S subunit means that the number of oxygen binding sites needed for cooperative behavior does not exceed 18, a number much smaller than the 180 sites present in a 100S molecule. In the present study, we find that a simple two-oxygen site model can account for the binding behavior of the two limiting states of the molecule, the deoxy and oxy states. Certainly, a more refined model will be needed to cover the binding behavior at intermediate degrees of saturation.

The idea that linkage between two ligands may *modify* preexisting cooperativity has been discussed by Weber (1972) in the context of the action of diphosphoglycerate on oxygen binding by hemoglobin. What is unique in the hemocyanin case is that the linkage between calcium and oxygen *generates* cooperativity in an otherwise noncooperative system.

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