Oxygen Binding by Hemocyanin from *Levantina hierosolima*. I. Exclusion of Subunit Interactions as a Basis for Cooperativity†

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ABSTRACT: The effect of oxygen on the distribution of hemocyanin from *Levantina hierosolima* among the three sedimenting species 20, 60, and 100 S was determined under two sets of experimental conditions: (a) at pH 7.63 in the absence of Ca^{2+}, where oxygen binding is noncooperative; (b) at pH 8.20 in the presence of 2×10^{-3} M Ca^{2+}, where oxygen binding is cooperative. A comparison of the results in the two cases eliminates the possibility that equilibrium between species with different oxygen affinities is responsible for the cooperative behavior. Cooperative oxygen binding was demonstrated for the 20S subunits at pH 8.80 and 1×10^{-3} M Ca^{2+}. Under these conditions, the concentration of calcium is sufficient to affect the oxygen affinity, but the concentration of calcium plus proton is not sufficient to bring about association. The findings exclude interactions among 20S subunits as a basis for cooperativity in hemocyanin.

The very high molecular weight of molluscan hemocyanin and the readiness with which it dissociates into smaller subunits have prompted investigators to study the relationship between oxygen binding and subunit association in these molecules (for a recent review of the literature, see Van Holde and Van Bruggen, 1971). A study of the structure–function relationship in this system must take into consideration that (a) the molecule can exist in different states of aggregation: wholes (100 S), halves (60 S), tenths (20 S); (b) oxygen binding can be either of the noncooperative type or cooperative; (c) protons bring about association but no cooperativity; (d) alkaline earth cations, calcium and magnesium, bring about association and cooperative oxygen binding. DePhillips *et al.* (1970) found that oxygenation affected the state of aggregation of *Busycon* hemocyanin. Their conclusion was that the 60 S ↔ 100 S equilibrium alone cannot account for the cooperativity. Van Driel (1973) studied the correlation in *Helix pomatia* hemocyanin between 20 S → 100 S association and the appearance of cooperative oxygen binding. He concluded that cooperativity cannot be expressed at the level of the 20S subunit and that association to the 100S or at least the 60S molecule was essential. One possibility of interpreting the results of both studies is to assume that interactions among 20S subunits in the assembled 100S (or 60S) molecule are needed for cooperative oxygen binding. In this paper, we shall present new findings which contradict such an interpretation and show that subunit association cannot serve as a basis of cooperativity.

Materials and Methods

Hemocyanin was prepared from the snail *Levantina hierosolima* as described by Shaklai and Daniel (1970). Oxygen saturation curves were obtained by fluorometric titration according to the procedure of Er-el *et al.* (1972). Calcium concentration was measured at the beginning and end of each titration using a Varian Techtron AA-5 atomic absorption spectrometer. Absorption spectra were determined in a Cary 14 spectrophotometer. Sedimentation was performed in a Model E Beckman analytical ultracentrifuge, using phase-plate schlieren optics. All chemicals were of analytical grade.

In experiments designed to find out the effect of oxygen on the sedimentation pattern, a solution of oxyhemocyanin was deoxygenated and reoxygenated, and the oxy-, deoxy-, and reoxyhemocyanin were sedimented at the same temperature and the same speed. Hemocyanin was deoxygenated by flushing with pure nitrogen and transferred to a centrifuge cell in a nitrogen box specially designed for this purpose. That the protein was still in the deoxygenated state was ascertained by determination of the absorption spectrum of the protein in the centrifuge cell at the end of the sedimentation experiment. Complete deoxygenation was inferred from the disappearance of the 345-nm peak. The distribution of the protein among the different sedimenting species was determined from the areas under the schlieren sedimentation peaks after correction for radial dilution.

Results

Effect of Ca^{2+} on Oxygen Binding at pH 8.20. Figure 1 shows the binding behavior at two calcium concentrations at pH 8.20. It is seen that at a calcium concentration of 1×10^{-3} M, the binding curve is noncooperative and the half-saturation pressure equals 4.6 mm. The latter value is the same as the one determined in calcium-free solution (Er-el *et al.*, 1972). Increasing the calcium ion concentration to 2×10^{-3} M brings about cooperativity (Figure 1b). Cooperative oxygen binding at pH 8.20 and 2×10^{-2} M Ca^{2+} has already been demonstrated (Er-el *et al.*, 1972).

Effect of O_2 on the Sedimentation at pH 8.20. No effect of oxygen on the sedimentation pattern can be detected at pH 8.20 when the calcium ion concentration is 1×10^{-3} (Figure 1a, insert) or 2×10^{-2} M (Figure 2). The effect of oxygen on the sedimentation pattern at pH 8.20 and 2×10^{-3} M Ca^{2+} is shown in Figure 3. Under these conditions, oxyhemocyanin shows three sedimentation boundaries with

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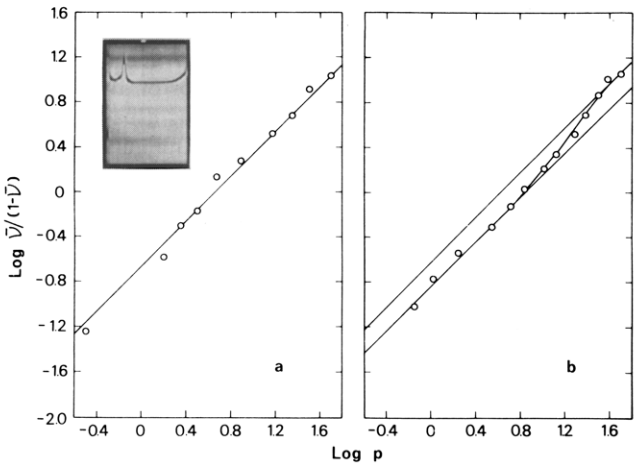

FIGURE 1: Hill plots of oxygen binding by hemocyanin from *L. hierosolima* at pH 8.20 and 25°, in solutions containing 0.1 M Tris-HCl buffer and 1×10^{-3} (a) or 2×10^{-3} (b) M Ca^{2+}. Insert: Schlieren sedimentation pattern of solution a, centrifuged for 12 min at 44,000 rpm, showing the presence of 20S subunits; deoxygenation did not change the sedimentation pattern.

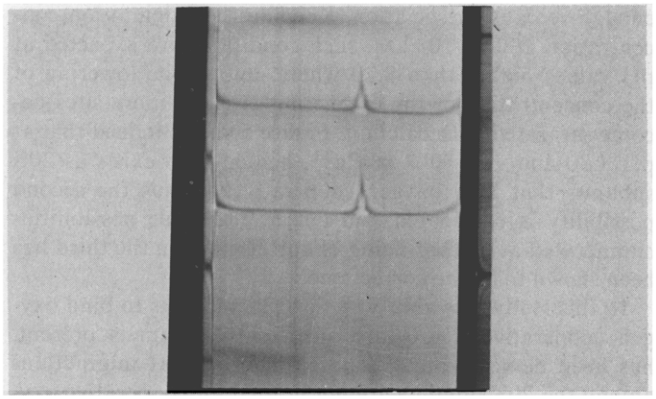

FIGURE 2: Schlieren sedimentation pattern of *L. hierosolima* hemocyanin at pH 8.20 and 19°, in a solution containing 0.1 M Tris-HCl buffer and 2×10^{-2} M Ca^{2+}, in the presence (lower curve) and absence (upper curve) of oxygen. The photograph, taken 6 min after reaching an operating speed of 52,000 rpm, shows the presence of 100S molecules. Two single-sector cells, one with a plane and another with a wedged upper window, were used for this experiment.

sedimentation coefficients 20, 60, and 100 S. Deoxygenation causes an increase in the 60S species and a concomitant decrease of the 20- and 100S species (Table I). A comparison of the sedimentation pattern of oxyhemocyanin with that of reoxygenated hemocyanin shows that the changes brought about by oxygen are reversible.

Effect of O_2 on the Sedimentation in Calcium-Free Solution at pH 7.63. The sedimentation patterns of oxy-, deoxy-, and reoxyhemocyanin in calcium-free solution at pH 7.63 are shown in Figure 4. Here, too, the protein is distributed among the three species 20, 60, and 100 S. The effect of oxygen on the distribution of protein among the three sedimenting species is presented in Table I.

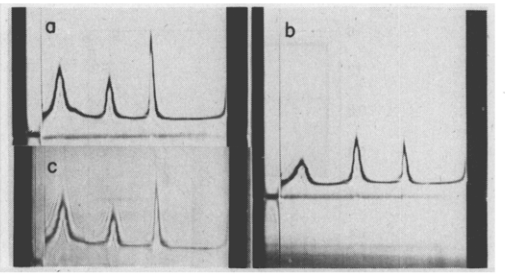

FIGURE 3: Schlieren sedimentation patterns of *L. hierosolima* hemocyanin, centrifuged about 16 min at 28,000 rpm, in 0.1 M Tris-HCl buffer and 2×10^{-3} M Ca^{2+}, pH 8.20 and 20°: (a) oxyhemocyanin; (b) deoxyhemocyanin; (c) reoxyhemocyanin.

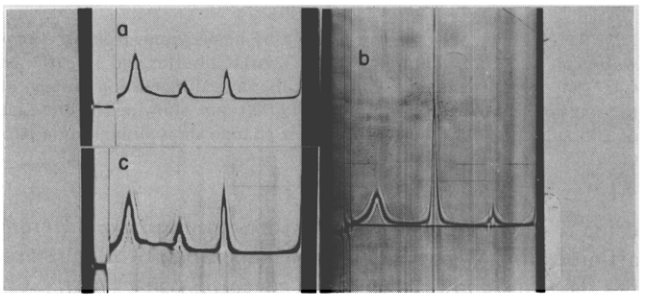

FIGURE 4: Schlieren sedimentation patterns of *L. hierosolima* hemocyanin, centrifuged about 16 min at 28,000 rpm, in 0.1 M Tris-HCl buffer, pH 7.63 and 20°: (a) oxyhemocyanin; (b) deoxyhemocyanin; (c) reoxyhemocyanin. In b, solvent was placed in a separate cell and run simultaneously to yield a base line for comparison purposes.

Oxygen Binding and State of Aggregation at pH 8.80 and 1×10^{-3} M Ca^{2+}. Figure 5 shows the binding behavior at pH 8.80 in the presence of 1×10^{-3} M Ca^{2+}. Oxygen binding is seen to be cooperative. Under these conditions, hemocyanin exists as 20S subunits (Figure 5, insert).

Discussion

Previous work has definitely shown that Ca^{2+} (or Mg^{2+}) is needed for the cooperative oxygen binding of hemocyanin. On the other hand, it is well known that Ca^{2+} brings about association of 20S dissociated subunits into 100S molecules. This dual effect of Ca^{2+} raises the question to what extent is the reaction 20 S ↔ 100 S involved in the mechanism of cooperativity. The following possibilities

TABLE I: Effect of Oxygen on the Distribution of *L. hierosolima* Hemocyanin among Different Sedimenting Species.[a]

Sedimenting Species	No Ca^{2+}; pH 7.63[b]			2×10^{-3} M Ca^{2+}; pH 8.20[b]		
	Oxyhemocyanin	Deoxyhemocyanin	Reoxyhemocyanin[c]	Oxyhemocyanin	Deoxyhemocyanin	Reoxyhemocyanin[c]
20 S	64	35	59	40	30	38
60 S	14	54	14	23	42	26
100 S	22	11	27	37	28	36

[a] Determined by measuring the area under the corresponding peak in the schlieren pattern and expressed as per cent of total sedimenting protein. [b] Other conditions: 0.1 M Tris-HCl, 25°. [c] Obtained by reoxygenation of deoxyhemocyanin.

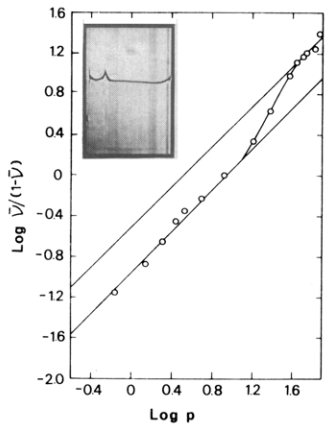

FIGURE 5: Hill plot of oxygen binding by hemocyanin from *L. hierosolima* in a solution containing 0.1 M Tris-HCl buffer and 1×10^{-3} M Ca^{2+}, at pH 8.80 and 25°. Insert: Schlieren sedimentation pattern of the solution, centrifuged for 8 min at 48,000 rpm, showing the presence of 20S subunits; deoxygenation did not change the sedimentation pattern.

come to mind. (1) The 20- and 100S species have different affinities for oxygen and are noncooperative by themselves, but the transition between them generates cooperativity. (2) The 20S species is not cooperative but the 100S molecule is cooperative. (3) Both 20- and 100S species are cooperative.

The first of the three possibilities can be examined by studying the effect of oxygen on the sedimentation under conditions where the binding of this ligand is cooperative. Cooperative oxygen binding by hemocyanin has previously been reported at pH 8.20 and 2×10^{-2} M Ca^{2+} (Er-el *et al.*, 1972). The sedimentation pattern at these conditions and in the presence and absence of oxygen is shown in Figure 2; it is seen that hemocyanin exists exclusively as 100S molecules in both oxygenated and deoxygenated states. Evidently, this result does not favor an explanation of cooperativity in terms of a transition between 20- and 100S species. A clear-cut conclusion can be obtained by examining the effect of deoxygenation under experimental conditions where the ratio of 20- to 100S molecules is close to unity, and any change in the relative abundance of the sedimenting species is easily detectable. A situation where whole molecules and subunits coexist while oxygen binding remains cooperative can be realized by reduction of the calcium ion concentration to 2×10^{-3} M, keeping the pH at 8.20 (Figures 1b and 3). Under these conditions, deoxygenation is not found to affect the 20:100S ratio (Table I). By the theory of linked functions (Wyman, 1964), this result means that the oxygen affinities of the 20- and 100S species are identical. Thus, an explanation of cooperativity on the basis of different oxygen affinities for the 20S subunits and 100S whole molecules is ruled out.

The change brought about by oxygen in the relative abundance of the 60S species (Figure 3 and Table I) implies a different oxygen affinity for the latter relative to that of the 20- or 100S molecules. To what extent is the difference in affinity of the 60S species involved in the cooperativity? To answer this question, the effect of oxygen on the distribution of protein among 20-, 60-, and 100S species under conditions of noncooperative oxygen binding was examined. In calcium-free solution, at pH 7.63, deoxygenation brings about changes in the relative abundance of the 60S species (Figure 4 and Table I). The fact that under these conditions no cooperativity can be detected (Er-el *et al.*, 1972) shows that the difference in oxygen affinity of the 60S species, relative to that of the 20- or 100S molecules, is too small in magnitude to serve as a basis for explaining cooperative oxygen binding. A similar conclusion has been reached by DePhillips *et al.* (1970) for hemocyanin from *Busycon*.

The second possibility, *viz.* that the 20S is not cooperative but the 100S is cooperative, has been proposed by Van Driel (1973). The experiments carried out in this study at pH 8.20 (Figures 1 and 3) show indeed that, at this pH, cooperativity and appearance of 100S molecules go together. This finding may not, however, be taken as a proof that 20S subunits cannot under any conditions bind oxygen cooperatively, since the possibility exists that the calcium concentrations necessary for association and cooperativity happen to coincide at this pH. It should be borne in mind that H^+ or Ca^{2+} is effective in bringing about association (Klarman *et al.*, 1972), while Ca^{2+} alone can bring cooperativity (Er-el *et al.*, 1972). We therefore looked for conditions such that the concentration of Ca^{2+} is sufficient to bring about cooperativity, but the concentration of Ca^{2+} *plus* H^+ is not enough to cause association. Keeping the calcium ion concentration at 1×10^{-3} M, such conditions are expected at pH values higher than 8.20 where, due to the lowering of the concentration of the competing protons, more sites become saturated with calcium. Figure 5 shows indeed that at pH 8.80 and 1×10^{-3} M Ca^{2+}, hemocyanin exists as 20S subunits that bind oxygen cooperatively. Thus the second possibility is eliminated and out of the three possibilities enumerated at the beginning of our discussion, the third has been shown to be the correct one.

In this study, the ability of the 20S subunits to bind oxygen cooperatively, provided sufficient calcium is present, has been demonstrated.[1] The realization that interactions among the 20S subunits are not needed for cooperative oxygen binding by hemocyanin raises the question of the role played by calcium in the mechanism of cooperativity, a subject which will be taken up in the following paper.

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[1] Van Driel (1973) studied the oxygen binding of 100S hybrid molecules of *Helix pomatia* hemocyanin, which contained native one-tenth subunits as well as decopperized subunits. He found that the incorporated native 20S subunits were unable to bind oxygen cooperatively, even in the presence of a sufficient concentration of calcium. This observation does not, however, contradict our conclusions, since the 20S subunit in Van Driel's experiment was constrained by the matrix of the apoprotein which, being devoid of oxygen binding sites, is unable to undergo the conformational change associated with the low-to-high oxygen affinity transition.