Multiple Linkage in *Panulirus interruptus* Hemocyanin[†]

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ABSTRACT: The linkage between various ligands of *Panulirus interruptus* hemocyanin, namely, oxygen, proton, and calcium and sodium ion, has been investigated. The binding of calcium, followed by means of a calcium ion selective electrode, revealed that the number of binding sites is strongly pH dependent. The overall dissociation constant of calcium is ~0.05 mM. No difference between binding to oxygenated and deoxygenated protein could be detected. Binding of calcium or sodium ions is associated with a release of protons. There is a difference of approximately 10-fold in apparent affinity between calcium and sodium. A smaller number of protons are released from deoxygenated as compared to oxygenated hemocyanin. Moreover, competition between the two cations for the same sites is suggested. Bohr effect measurements show a strong dependence of the release of oxygen-linked protons on the calcium and sodium concentrations. Measurements of oxygen equilibrium as a function of calcium, sodium, and hydrogen ions reveal specific effects of these cations on the biological function of the protein. At pH 7.6 there are at least two types of oxygen-linked calcium-binding sites, with dissociation constants of approximately 0.1 and 20 mM. These sites affect preferentially either the lower (T state) asymptote or the upper (R state) asymptote. Under the same conditions, sodium affects only the upper asymptote. At pH 6.4 the set of low-affinity oxygen-linked calcium-binding sites seems to be inactive. The observed phenomena are analyzed in the context of general linkage theory, and two possible linkage maps are designed to illustrate the various reciprocal effects of these allosteric ligands. *Panulirus* hemocyanin may be taken as an example of an extended system with different types of binding sites which may interact in order to control its specific biological function.

The effects of hydrogen, magnesium, and calcium ions on the association–dissociation properties and the oxygen-binding behavior of hemocyanins are well established (Van Holde & van Bruggen, 1971). Experimental work on the role of these linked phenomena has been described very often in specialized literature, but frequently attention has been focused on the effect of only one of these allosteric effectors on either the molecular weight or the functional properties of hemocyanin. It is clear, however, that the various solvent components and oxygen interact with the macromolecule in a linked fashion, giving rise to a complex network of reciprocal effects. Unraveling the specific and reciprocal roles played by the various ligands in this strongly linked system presents a complex task and demands a complete experimental characterization of the binding phenomena for each set or subset of ligands.

In this paper we present a set of experiments bearing on the linkage between various ligands of the hemocyanin of *Panulirus interruptus*, namely, oxygen, proton, and calcium and sodium ion.

P. interruptus hemocyanin is a relatively small arthropod hemocyanin of hexameric structure (molecular weight 450 000 in the undissociated state and 75 000 for the subunit). Under the conditions of the present experiments, the protein was always in the undissociated state. Under Discussion an attempt is made to analyze the observed phenomena in the context of general linkage theory as developed by Wyman (1964, 1968, 1975). The subject is of wide interest since the behavior of *Panulirus* hemocyanin in response to a set of allosteric ligands may be taken as an example of a system in which different types of binding sites interact in order to achieve a higher order control of the specific function of a biological macromolecule.

Materials and Methods

P. interruptus hemocyanin was prepared and stored as described previously (Kuiper et al., 1975); in a few cases samples were kept in 50% glycerol at 4 °C. The protein was dialyzed initially against 0.02 M Tris-HCl, pH 7.2, and 3 mM EDTA and subsequently against several changes of deionized and distilled water at 4 °C. For oxygen equilibrium experiments a final dialysis was carried out in the appropriate buffer. Deoxygenated hemocyanin was prepared in a tonometer by evacuation and flushing with argon. All the experiments were carried out under conditions where the protein is in its aggregated state (M_r 450 000). Throughout the paper the protein concentration is expressed in terms of oxygen-binding sites/*l*, assuming 1 binding site/75 000 mol wt.

Calcium binding was measured with a calcium ion selective electrode connected to a digital voltmeter, Radiometer Type PHM 64. Approximately 2 mL of a protein solution was placed in a glass vessel surrounded by a water jacket (diameter 5 cm, height 8 cm). The solution was stirred and kept under a steady flow of oxygen or argon, previously bubbled through an alkaline solution and then through water. The amount of calcium bound to the protein was calculated as the difference between the concentration of free calcium in the protein solution and that in the solvent at each measured value of the calcium activity after addition of 0.5 and 2 M calcium chloride with a microsyringe. In the range of calcium concentrations used for the control experiments, the response of the calcium ion selective electrode was strictly linear.

Simultaneously, the number of protons released from the protein upon addition of calcium chloride was measured with a hydrogen ion electrode, connected to a Radiometer Potentiometer Type PHM 4C, by back-titration to the original pH after each addition of calcium chloride. The same procedure was followed in order to determine the proton release upon addition of sodium chloride to the protein.

Differential hydrogen ion titrations were performed as described by Brunori (1966). Oxygen-binding experiments were performed in the presence of 0.02 M Tris-HCl or 0.05 M Bistris and varying amounts of calcium or sodium chloride,

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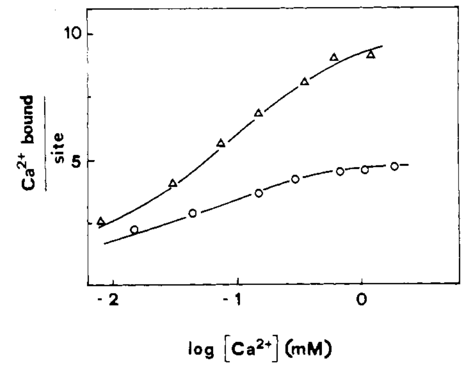

FIGURE 1: Calcium binding by *Panulirus* hemocyanin at pH 7.6 (△) and 6.0 (○) as measured with the calcium ion selective electrode. Protein concentration at pH 7.6 94 μM and at pH 6.0 131 μM.

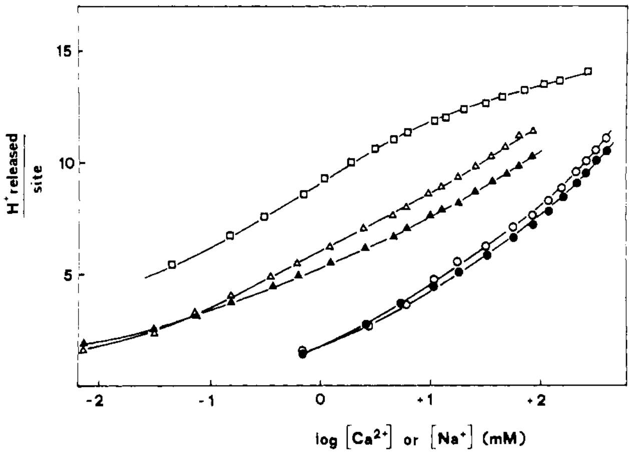

FIGURE 2: Number of protons released upon addition of calcium or sodium chloride to *Panulirus* hemocyanin as a function of the free calcium or sodium concentration. Results for (△) calcium binding to oxygenated hemocyanin at pH 7.6, protein concentration 94 μM, (▲) calcium binding to deoxyhemocyanin at pH 7.6, protein concentration 94 μM, (○) sodium binding to oxygenated hemocyanin at pH 7.6, protein concentration 52 μM, (●) sodium binding to deoxyhemocyanin at pH 7.6, protein concentration 52 μM, and (□) calcium binding to oxygenated hemocyanin at pH 6.0, protein concentration 131 μM.

essentially as described by Rossi Fanelli & Antonini (1958).

Results

(*A*) *Binding of Calcium.* Figure 1 shows the binding of calcium to unbuffered oxygenated *P. interruptus* hemocyanin at pH 7.6 and 6.0, as measured with a calcium ion selective electrode. It can be seen that the maximum number of calcium-binding sites per oxygen-binding site depends strongly on pH, being at least 10 at pH 7.6 and ~5 at pH 6.0. In both cases the overall dissociation constant is 0.05 mM. Below pH 5.4 no significant binding was observed. No difference between oxygenated and deoxygenated hemocyanin could be detected.

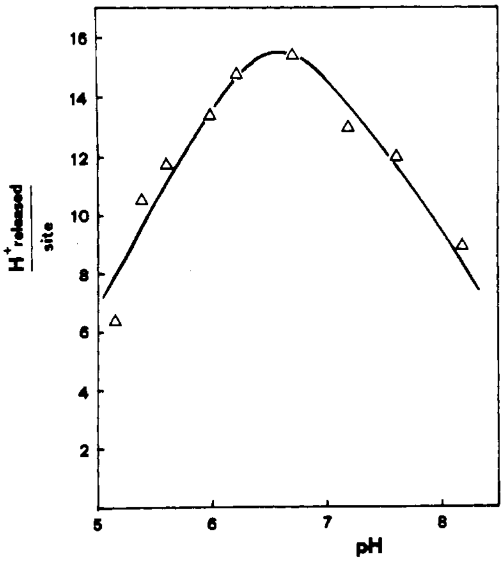

FIGURE 3: Number of protons released from oxygenated hemocyanin after addition of 0.1 M Ca^{2+} in the pH range 5–8.2. The protein concentration was 46.6 μM.

Table I[a]

NaCl (mM)	av Ca^{2+} affinity (mM)	no. of H^+ released per oxygen-binding site
100	14.3	5.1
20	4.5	8.5
0	1.0	12.1

[a] Affinity of calcium for *P. interruptus* hemocyanin and number of H^+ ions released upon binding of calcium as a function of NaCl concentration at pH 7.6.

(*B*) *Release of Protons upon Binding of Calcium or Sodium.* (*1*) *Proton Release at pH 7.6 and 6.0.* Figure 2 shows the number of protons released upon addition of calcium or sodium chloride to oxygenated and deoxygenated hemocyanin as a function of the free calcium or sodium concentration at pH 7.6 and 6.0. The free calcium concentration was measured electrometrically, and that of sodium was taken equal to the total amount of sodium chloride added in view of the low protein concentration employed. The extended shape of the titration curves indicates a heterogeneity of sites greater than that observed in the case of the direct calcium-binding measurements (see Figure 1). This is not surprising since in this case we may be dealing with additional nonspecific, ionic strength effects which do not show up in Figure 1. Figure 2 brings out the large difference in apparent affinity between sodium and calcium, approximately 10-fold.

At pH 7.6 a smaller number of protons are released from the deoxygenated protein as compared to the oxygenated one, the difference being dependent on the free calcium or sodium concentration. No measurements on the deoxygenated protein were made at pH 6.0. A comparison of the data reported in Figure 1 and 2 shows that the ratio of Ca^{2+} bound to protons released is strongly dependent upon pH.

(*2*) *Proton Release as a Function of pH.* Figure 3 shows the number of protons released from oxygenated hemocyanin after addition of 0.1 M Ca^{2+} in the pH range 5–8. Based on the experiments performed at 0.05 M Ca^{2+} and the results obtained from oxygen-binding experiments (see below), the specific binding sites should be saturated at a free calcium concentration of 0.1 M. Below the isoelectric point of 4.9 the proton uptake is reversed.

(*3*) *Competition between Calcium and Sodium.* The data presented in Table I suggest that the two cations (Ca^{2+} and Na^+) are competitors for the same set of binding sites. A more direct proof of this finding is afforded by recent ^{23}Na NMR relaxation studies (Norne et al., 1979).

(*C*) *The Bohr Effect as a Function of Calcium and Sodium Concentration at pH 7.6.* Figure 4 shows the number of protons released per oxygen-binding site at pH 7.6 upon complete saturation of deoxyhemocyanin with oxygen as a function of calcium or sodium ion concentration. The strong dependence of the release of the oxygen-linked protons (i.e., Bohr effect) on the cation concentration is apparent. Since in the absence of calcium 0.5 protons are released (as shown by the arrow), it follows that both curves extended back to

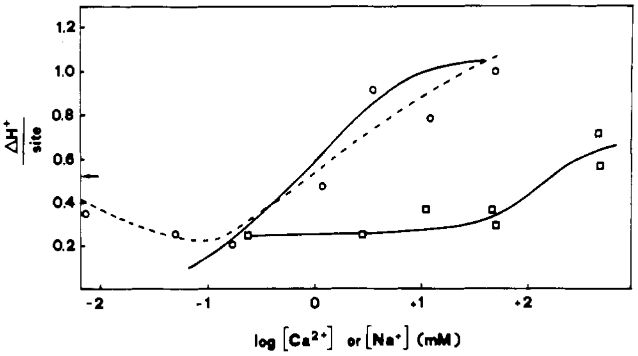

FIGURE 4: Release of Bohr protons determined by differential titration as a function of calcium (○, dotted curve) and sodium (□) activity at pH 7.6. Protein concentration employed in the case of calcium was 49 μM and for sodium 39 μM. Smooth curves for both calcium and sodium were drawn on the basis of the data presented in Figure 2. The arrow indicates the number of Bohr protons released in the absence of calcium or sodium.

zero ion activity must pass through a minimum. The smooth curves, based on the data reported in Figure 2, show that the two sets of experiments are in good agreement.

(*D*) *Oxygen Equilibrium as a Function of Calcium, Sodium, and Hydrogen Ion.* (*1*) Figure 5a shows oxygen binding in terms of Hill plots as a function of calcium concentration is 0.02 M Tris-HCl at pH 7.6. Inserts give the behavior of the two characteristic parameters log $p_{1/2}$ and $n_{1/2}$. It will be seen that both the position and the shape of the O_2-binding isotherms are strongly dependent on calcium. This may be compared with the somewhat different behavior previously observed in the presence of 50 mM Na^+ ions (Kuiper et al., 1975). In particular, this is true of both the upper and lower asymptotes, and as a result of this effect the total free energy of interaction (i.e., the difference between the free energy of binding of the first and the last ligand molecule) increases steadily with calcium concentration. It may be significant that the lower asymptote is most sensitive to calcium in the range of low concentrations and the upper asymptote in the range of high concentrations.

(*2*) Figure 5b shows a similar set of observations in the case of sodium. Here it will be seen that the lower asymptote is wholly insensitive to the cation concentration.

(*3*) Figure 5c shows a similar set of experiments in 0.02–0.05 M Bistris at pH 6.4 in the case of calcium. Here it is the upper asymptote which is insensitive to the cation concentration.

(*4*) *Functional Evidence Regarding the Competition between Sodium and Calcium.* In an experiment at pH 7.6 in 0.02 M Tris-HCl, 25 mM $CaCl_2$ was added to a protein solution already containing 500 mM NaCl. The oxygen affinity decreased from log $p_{1/2}$ = 0.90 to 1.02 as might be expected on the basis of the individual behavior of the two cations if they were competing for the same site(s).

(*5*) *Possible Anion Effect.* The contribution of the accompanying anion has been investigated in view of the reported chloride ion effect on the functional properties of *Limulus* and *Penaeus* hemocyanin (Brouwer et al., 1977, 1978).

The absence of any considerable effect of the anion was shown by replacing NaCl by Na_2SO_4 in an experiment in 0.02 M Tris-HCl at pH 7.6. The value of log $p_{1/2}$ = 0.74 in 0.2 M Na_2SO_4 is close to that observed in 0.5 M NaCl solution (log $p_{1/2}$ = 0.9).

Discussion

P. interruptus hemocyanin provides a good example of the complex linkage phenomena to be expected in any large polyfunctional macromolecule which binds a number of different ligands. The present observations on this hemocyanin embrace four ligands, namely, oxygen, proton, and calcium and sodium ion, all of them linked in a system which, in the absence of any appreciable dissociation of the macromolecule, may be regarded as allosteric. In trying to rationalize them, several questions present themselves.

(1) In the case of a system involving three or more ligands, it is necessary to distinguish between true and pseudolinkage (Wyman, 1968, 1975). Suppose we designate three ligands by X, Y, and Z, their chemical potentials by μ_X, μ_Y, and μ_Z, and the amounts of each bound by the macromolecule by $\bar{X}$, $\bar{Y}$, and $\bar{Z}$. If there is *true linkage* between all three of them, then the binding potential $\mathcal{L}$ which governs the situation will be a function in which all three chemical potentials occur together: $\mathcal{L} = \mathcal{L}(\mu_X,\mu_Y,\mu_Z)$. This gives rise to a set of linkage relations such as $(\partial\bar{X}/\partial \ln y)_{x,z} = (\partial\bar{Y}/\partial \ln x)_{y,z}$, in which the independent variables are the activities of the various ligands (denoted by the small italic letters). It may happen, however, that the potential breaks up into $\mathcal{L}(\mu_X,\mu_Z) + \mathcal{L}(\mu_Y,\mu_Z)$. In this case, although X and Z on one hand and Y and Z on the other remain truly linked, there is no true linkage between X and Y; nevertheless, there will be an interaction between X and Y binding mediated by Z. This is what is known as *pseudolinkage*, and it represents the fact that there are two kinds of Z-binding sites, one linked to the X-binding sites and the other to the Y-binding sites. When we change the activity of X, keeping $\bar{Z}$ constant, there will be a migration of Z from one set of sites to the other, with a corresponding effect on the properties of the Y-binding sites. Under these conditions, although

$$\left(\frac{\partial\bar{X}}{\partial \ln y}\right)_{x,z} = \left(\frac{\partial\bar{Y}}{\partial \ln x}\right)_{y,z} = 0$$

nevertheless

$$\left(\frac{\partial\bar{X}}{\partial \ln y}\right)_{x,\mathrm{Z}} = \left(\frac{\partial\bar{Y}}{\partial \ln x}\right)_{y,\mathrm{Z}} \neq 0$$

It is important to distinguish between these two types of linkage, one of which represents a true interaction of all sites as an inherent property of the macromolecule and the other of which arises as an indirect effect.

The question arises whether the various linkages observed in *Panulirus* hemocyanin are all true linkages or not. The answer to this question lies in the three linkage equations

$$\left(\frac{\partial\overline{O_2}}{\partial \ln Ca^{2+}}\right)_{O_2,H^+} = \left(\frac{\partial\overline{Ca^{2+}}}{\partial \ln O_2}\right)_{Ca^{2+},H^+} \quad (1a)$$

$$\left(\frac{\partial\overline{O_2}}{\partial \ln H^+}\right)_{O_2,Ca^{2+}} = \left(\frac{\partial\overline{H^+}}{\partial \ln O_2}\right)_{H^+,Ca^{2+}} \quad (1b)$$

$$\left(\frac{\partial\overline{H^+}}{\partial \ln Ca^{2+}}\right)_{H^+,O_2} = \left(\frac{\partial\overline{Ca^{2+}}}{\partial \ln H^+}\right)_{Ca^{2+},O_2} \quad (1c)$$

which may be transformed[1] respectively into

$$\left(\frac{\partial\overline{Ca^{2+}}}{\partial\overline{O_2}}\right)_{Ca^{2+},H^+} = -\left(\frac{\partial \ln O_2}{\partial \ln Ca^{2+}}\right)_{\overline{O_2},H^+} \quad (1a')$$

$$\left(\frac{\partial\overline{H^+}}{\partial\overline{O_2}}\right)_{H^+,Ca^{2+}} = -\left(\frac{\partial \ln O_2}{\partial \ln H^+}\right)_{\overline{O_2},Ca^{2+}} \quad (1b')$$

$$\left(\frac{\partial\overline{Ca^{2+}}}{\partial\overline{H^+}}\right)_{Ca^{2+},O_2} = -\left(\frac{\partial \ln H^+}{\partial \ln Ca^{2+}}\right)_{\overline{H^+},O_2} \quad (1c')$$

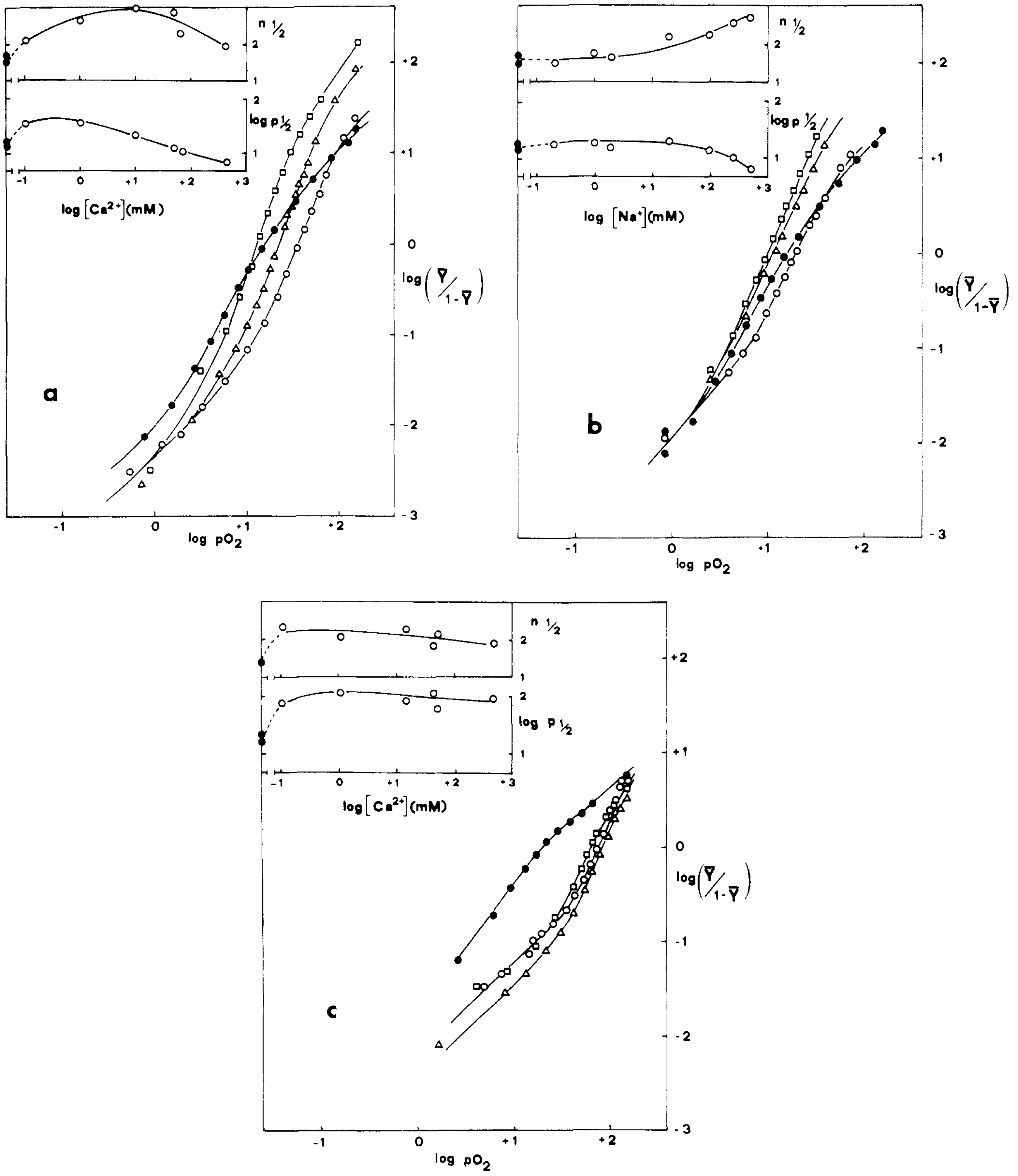

FIGURE 5: Oxygen binding as a function of calcium, sodium, and hydrogen ion concentration. Protein concentration was ~40 μM. (a) Hill plots of oxygen binding in 0.02 M Tris-HCl, pH 7.6, as a function of calcium concentration: (●) no Ca^{2+}, (○) 1 mM Ca^{2+}, (△) 10 mM Ca^{2+}, and (□) 50 mM Ca^{2+}. Inserts show the behavior of log $p_{1/2}$ and $n_{1/2}$ as a function of calcium activity (○); (●) no calcium present, log $Ca^{2+} \rightarrow -\infty$. (b) Similar set of observations in 0.02 M Tris-HCl, pH 7.6, as a function of sodium concentration: (●) no Na^+, (○) 0.02 M Na^+, (△) 0.1 M Na^+, and (□) 0.25 M Na^+. (c) Similar set of experiments in 0.02–0.05 M Bistris, pH 6.4, as a function of calcium concentration: (●) no Ca^{2+}, (○) 0.11 mM Ca^{2+}, (△) 14.7 mM Ca^{2+}, and (□) 50 mM Ca^{2+}.

In these equations a symbol with a bar denotes the amount of the given ligand bound per macromolecule, and the corresponding unbarred symbol denotes the activity of that ligand. If any one of either set of equations ceases to exist, it means that the corresponding true linkage does not exist.

It will be seen from Figures 1 and 2 that there is a true H^+–Ca^{2+} linkage (both members of eq 1c $\neq 0$, which of course implies that eq 1c′ exists), although there are no results for

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[1] For example, in order to pass from eq 1a to 1a′, we write the right-hand member of eq 1a as $-1/[(\partial \ln Ca^{2+}/\partial \overline{Ca^{2+}})_{O_2,H^+}(\partial \ln O_2/\partial \ln Ca^{2+})_{Ca^{2+},H^+}]$. If this vanishes, eq 1a′ reduces to 0 = 0.

intermediate values of O_2 (oxygen pressure) between zero and the very large values which give full oxygenation of the protein.

Likewise from Figures 2 and 4, we conclude that there is a true oxygen–proton linkage at every Ca^{2+} concentration (right-hand member of eq 1b $\neq 0$). The same conclusion is brought out by comparison of parts a and c of Figure 5 (left-hand member of eq 1b $\neq 0$), as indeed it must be since either equation implies the other.

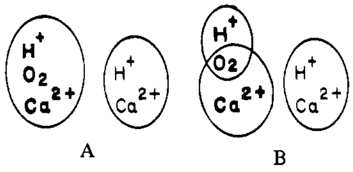
As to the oxygen–calcium linkage (eq 1a and 1a′), it is clear from the data of either Figure 5a or Figure 5c that this too must exist, although under the conditions of the experiments it is small. This may be inferred from the inserts ($\log p_{1/2}$ vs. $\log Ca^{2+}$) in parts a and c of Figure 5. From the slope of the right-hand portion of the curve in the lower insert of Figure 5a, we infer that the right-hand member of the expression

$$-\left(\frac{\partial \log p_{1/2}}{\partial \ln \mathrm{Ca}^{2+}}\right)_{\mathrm{O}_2,\mathrm{H}^+} = \left(\frac{\partial \overline{\mathrm{Ca}^{2+}}}{\partial \overline{\mathrm{O}_2}}\right)_{\mathrm{Ca}^{2+},\mathrm{H}^+}$$

is equal to ~0.3. This value, which is small in relation to the total number of sites (~10), would explain why it fails to show up in direct experiments with the Ca^{2+} ion selective electrode.

All the conclusions applicable to calcium are qualitatively valid also for sodium. The number of oxygen-linked sodium ions, as calculated from the right-hand portion of the curve in the lower insert of Figure 5b, is equal to that calculated in the case of calcium. Thus, we conclude that there is true linkage between all four ligands, taken two at a time.

(2) The next question is a more detailed one, concerning the individual sites. It might be assumed, as a first approximation, that the oxygen sites are all the same. Although not directly applicable to the polymer, it is significant that the subunits fractionated by ion-exchange chromatography (Kuiper, 1976) exhibit approximately the same oxygen-binding properties. As to proton sites, their number is very large and many of them are not linked at all, either to oxygen or to calcium. However, it is clear from Figure 2 that the number of proton sites linked to calcium greatly exceeds that of those linked to oxygen. (At pH 7.6, 10 proton sites are linked to calcium while only 1 is linked to oxygen.) Similarly, it seems likely that, as in the case of proton sites, some of the calcium sites may be wholly unlinked. The question arises whether the small number of "Bohr protons" are truly linked to the oxygen-linked calcium-binding sites or whether they form a separate set. The alternatives are pictured in the two accompanying linkage maps (A and B), from which for simplicity the wholly unlinked proton and calcium sites are omitted. Here each symbol stands for a set of sites which binds the corresponding ligand, and the loops indicate linkage. It is only in scheme A that there is a true linkage between the oxygen-linked calcium sites and the oxygen-linked proton sites. Scheme B represents pseudolinkage and would imply if applicable that there were two kinds of oxygen-binding sites, one truly linked to the Bohr proton sites and one truly linked to a set of calcium-binding sites. In both schemes there are additional pseudolinkage effects.



Unfortunately, the present data do not in themselves suffice to decide between scheme A and scheme B. However, if the linkages are of allosteric origin, as is suggested by the change of shape of the oxygen-binding curves with Ca^{2+} and H^+ (see parts a and c of Figure 5), alternative A would seem to be the correct one. But the answer to such detailed questions must await more searching experiments, associated with structural studies such as those currently carried out on hemoglobins.

(3) We now address ourselves to the questions of the specific influence of these allosteric effectors on the oxygen-binding behavior of the protein. It can be seen from the lower insert of Figure 5a that at pH 7.6 there must be at least two types of oxygen-linked calcium-binding sites, one of high affinity (K_{diss} on the order of 0.1 mM) responsible for the initial *decrease* of oxygen affinity with calcium activity and the other of much lower affinity (K_{diss} on the order of 20 mM) responsible for the later subsequent slower increase of oxygen affinity. The two effects are analogous to the reversed and normal proton–oxygen Bohr effect observed in human hemoglobin (Antonini & Brunori, 1971). It seems not unlikely that the high-affinity sites are the ones responsible for the behavior of the lower (T state) asymptote and the low-affinity sites for that of the upper (R state) asymptote.

It can be seen from Figure 4 (circles) that the proton Bohr effect as a function of calcium passes through a minimum at a free calcium ion concentration between 0.1 and 0.01 mM. This is consistent with the fact that in the range of low calcium ion concentration calcium shifts the oxygen-binding curve to the right, which implies the liberation of calcium ions from the high-affinity sites upon oxygenation. The replacement of these ions by protons would lead to the observed reduction of the proton Bohr effect. In the range of high calcium concentration the situation is reversed. The insert of Figure 5c shows that at pH 6.4 the sites with lower calcium affinity are no longer active and there is no effect on the upper asymptote. This may correspond to the diminished calcium binding at lower pH. It can be seen from the insert of Figure 5b that in the case of sodium the situation is different. There is no initial decrease in oxygen affinity, and the subsequent increase is greatly delayed. This is consistent with the fact that the lower asymptote is wholly unaffected by the sodium ion concentration. It will be recalled that in most allosteric systems the effect of the control ligand is on the lower (T state) asymptote (Imai & Yonetani, 1975; Brouwer et al., 1977; Zolla et al., 1978).

The results on the linkage between oxygen and calcium may have an interesting physiological implication. Since the calcium concentration of the hemolymph is in the range of 20 mM (Schlatter, 1941), we infer that the high-affinity calcium-binding sites in *Panulirus* hemocyanin are always saturated with calcium both in deoxy- and oxyprotein. These sites therefore are mainly involved in maintaining the molecule in its associated state, leaving to the low-affinity calcium-binding sites the role of control of biological function.

(4) One cannot but wonder whether the three ligands, calcium, sodium, and protons, are not competitors for the same sites. In this case we would have the two relations $\overline{\mathrm{Na}^+}/\overline{\mathrm{Ca}^{2+}} = m\mathrm{Na}^+/\mathrm{Ca}^{2+}$ and $\overline{\mathrm{H}^+}/\overline{\mathrm{Ca}^{2+}} = n\mathrm{H}^+/\mathrm{Ca}^{2+}$, where m and n are partition constants. Since $\overline{\mathrm{Na}^+} = \partial\mathcal{L}/\partial \ln \mathrm{Na}^+$, $\overline{\mathrm{Ca}^{2+}} = \partial\mathcal{L}/\partial \ln \mathrm{Ca}^{2+}$, and $\overline{\mathrm{H}^+} = \partial\mathcal{L}/\partial \ln \mathrm{H}^+$, these lead at once to the two equations $\partial\mathcal{L}/\partial \mathrm{Ca}^{2+} = m\partial\mathcal{L}/\partial \mathrm{Na}^+$ and $\partial\mathcal{L}/\partial \mathrm{Ca}^{2+} = n\partial\mathcal{L}/\partial \mathrm{H}^+$, whose general solution is $\mathcal{L} = \mathrm{f}(\mathrm{Ca}^{2+} + m\mathrm{Na}^+ + n\mathrm{H}^+)$. This shows that the three ligands may be treated as the activity of a single one.

Suppose we limit ourselves to the competition between protons and calcium, assuming no sodium to be present and assuming that they are competitors for a single site (i.e., are identically linked). Then if an amount of Ca^{2+} of a certain activity is added to the macromolecule already in equilibrium with protons at activity H^+, then there will be a displacement of a certain amount of H^+ given by $\Delta\overline{\mathrm{H}^+} = k_{\mathrm{H}^+}\mathrm{H}^+/(1 + k_{\mathrm{H}^+}\mathrm{H}^+) - k_{\mathrm{H}^+}\mathrm{H}^+/(1 + k_{\mathrm{H}^+}\mathrm{H}^+ + k_{\mathrm{Ca}^{2+}}\mathrm{Ca}^{2+})$, where k_{H^+} and $k_{\mathrm{Ca}^{2+}}$ are the affinity constants of H^+ and Ca^{2+} for the macromolecule. This equation describes a bell-shaped curve, and $\Delta\mathrm{H}^+$ will approach 0 as $\mathrm{H}^+ \rightarrow 0$ or $\mathrm{H}^+ \rightarrow \infty$ and will be a maximum at some intermediate value of $\mathrm{H}^+ = \mathrm{H}^+{}_{\mathrm{max}}$, given by $k_{\mathrm{H}^+}\mathrm{H}^+{}_{\mathrm{max}}(1 + k_{\mathrm{Ca}^{2+}}\mathrm{Ca}^{2+})^{1/2} = [1/(1 - \overline{\mathrm{Ca}^{2+}})]^{1/2}$, where $\overline{\mathrm{Ca}^{2+}}$ is the fractional saturation of the site with Ca^{2+}, which would be realized if no protons were present (at high pH). It can be seen that as $\mathrm{Ca}^{2+} \rightarrow 0$, $\log \mathrm{H}^+{}_{\mathrm{max}} \rightarrow \mathrm{p}K_{\mathrm{H}^+}$, but in this case the displacement will be too small to be seen. Upon increasing Ca^{2+}, $\mathrm{H}^+{}_{\mathrm{max}}$ will move farther and farther from $\mathrm{p}K_{\mathrm{H}^+}$. For example, when $\overline{\mathrm{Ca}^{2+}} = {}^8/_9$, $\log \mathrm{H}^+{}_{\mathrm{max}} = \mathrm{p}K_{\mathrm{H}^+} + 0.5$. The curve which describes the number of protons released upon addition of calcium as a function of pH (Figure 3) exhibits qualitatively the behavior to be expected on the basis of this analysis. However, since the shape of the curve is asymmetrical and since the proton uptake is reversed below pH 5, the situation cannot be as simple as this and it would seem that more than one type of binding site is involved. It is not unreasonable to suppose that in *Panulirus* hemocyanin a set of binding sites with a pK of 6–6.5 is involved. Whether the three ligands, proton, sodium, and calcium ions, are all competitors for the same site could be tested by performing the same experiments shown in Figure 3 in the presence of varying amounts of sodium both with deoxy- and oxyhemocyanin. Measurements performed at pH 7.6 (see Table I) already point in this direction. It may be noted that a similar phenomenon has been observed for *Dolabella* hemocyanin, where a pK of 6.25 was calculated for the calcium-chelating group (Makino, 1972).

In conclusion it should be noted that we have considered the two cationic ligands Na^+ and Ca^{2+} singly, neglecting the accompanying anions which might also be expected to show binding as in the case of hemoglobins (Chiancone et al., 1972, 1975). Our only justification for this lies in the fact that we have observed no large effect in the replacement of chloride by sulfate ions. But in spite of such shortcomings in the attempted analysis and the various unanswered questions to which it gives rise and which call for further, more detailed experiment, we may feel confident that in its overall tenor the picture presented by *P. interruptus* hemocyanin is representative of a large group of respiratory proteins and enzymes.

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