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Nested allostery in scorpion hemocyanin (*Pandinus imperator*)

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The oxygen-binding behavior of the 24-meric hemocyanin of the scorpion *Pandinus imperator* and its dependence on allosteric effectors such as protons can be successfully described by the nesting model; the MWC model is not acceptable. The affinities of the four assumed conformations of the allosteric unit, the 12-meric half-molecule, are not dependent on pH whereas the three allosteric equilibrium constants decrease with decreasing proton concentration. Comparison with the oxygen-binding behavior of the 24-meric tarantula hemocyanin (*Eurypelma californicum*) reveals that the affinity values for the various conformations seem to be conserved for chelicerata hemocyanin.

1. Introduction

Hemocyanins are giant respiratory macromolecules responsible for oxygen transport in many species of arthropods and molluscs. Extremely high Hill coefficients ($n_H > 6$) were reported as well as strong sensitivities to protons [1–4]. Arthropod hemocyanins show obvious hierarchies in their structures. They consist of integral multiples of substructures containing six subunits, i.e., 1 · 6-mer, 2 · 6-mer, 4 · 6-mer, 6 · 6-mer and 8 · 6-mer. Each subunit (average molecular mass ~ 75 kDa) reversibly binds one molecule of oxygen by bridging two copper atoms.

With the exception of hexamers, arthropod hemocyanins cannot be described by the simple 'two-state' model of Monod, Wyman and Changeux [5]. In order to reveal the molecular mechanism of allosteric interactions, the nesting model with hierarchies of allosteric equilibria was developed [6,7]. This model successfully explains homo- and heterotropic interactions within two arthropod hemocyanins from the tarantula *Eurypelma californicum* and the lobster *Homarus americanus* [6–8].

The structure of the hemocyanin from the scorpion *Pandinus imperator* has recently been resolved [9]. Its molecular mass of 1.75 MDa results from the aggregation of 24 subunits. Seven to eight different subunit types can be distinguished by crossed immunoelectrophoresis. Electron microscopy reveals a structure similar to those of hemocyanins from the spider *E. californicum* and the scorpion *Androctonus australis* [10,11]. Two hexamers are closely connected. A deep cleft separates the two dodecameric half-molecules which are connected edge to edge only. The obvious hierarchical structure of *P. imperator* hemocyanin suggests this molecule as another test of the nesting model. I therefore measured oxygen-binding curves at different pH values in order to reveal homo- and heterotropic interactions. The effects are discussed with particular respect to the results obtained for *Eurypelma* hemocyanin [8].

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2. Theory

2.1. The nesting model

The nesting model and its application to the *Eurypelma* and *Homarus* hemocyanin were described previously [6–8,12]. In analogy, the half-molecule is considered to be the allosteric unit for the *Pandinus* hemocyanin (fig. 1). As in the MWC model, the allosteric units can adopt two conformations, r and t. Due to the interactions of the two nested half-molecules within the native 24-mer, each half-molecule is supposed to exist in four different conformations designated as rR, tR, rT and tT. In the absence of oxygen, the equilibrium between the concentrations of the half-molecules in the conformations rR and tR is described by $l_{R,0} = [tR_0]/[rR_0]$ and analogously by $l_{T,0} = [tT_0]/[rT_0]$. The parameter $L_0 = [T_0]/[R_0]$ $(= [tT_0] + [rT_0])/([tR_0] + [rR_0])$ represents the equilibrium of the concentrations of native molecules being in the R and T state. The intrinsic association constants for binding oxygen, which characterize the four different conformations, are k_{rR}, k_{tR}, k_{rT} and k_{tT}.

According to Wyman [13], the fractional saturation θ can be calculated from the binding polynomial of a given model:

$$\theta(x) = n_x^{-1} \cdot (P'x)/P \tag{1}$$

where n_x denotes the total number of ligand molecules X bound and P' the first derivative of the binding polynomial P with respect to the ligand activity x.

The binding polynomial for the whole (native) molecule, the $2xN$-mer, composed of two allosterically coupled half-molecules, the N-mers, is given by

$$P_{2xN\text{-mer}} = 1/(1 + L_0) \cdot P_R^2 + L_0/(1 + L_0) \cdot P_T^2 \tag{2}$$

where P_R and P_T represent the binding polynomials for the dodecamers when the native molecule is in the R or T state, respectively. The squaring of terms in binding polynomials is due to the presence of two dimerized half-molecules.

The binding polynomials for the dodecameric half-molecule are given by

$$P_R = 1/(1 + l_{R,0}) \cdot (1 + k_{rR}x)^{12} + l_{R,0}/(1 + l_{R,0}) \cdot (1 + k_{tR}x)^{12} \tag{3}$$

$$P_T = 1/(1 + l_{T,0}) \cdot (1 + k_{rT}x)^{12} + l_{T,0}/(1 + l_{T,0}) \cdot (1 + k_{tT}x)^{12} \tag{4}$$

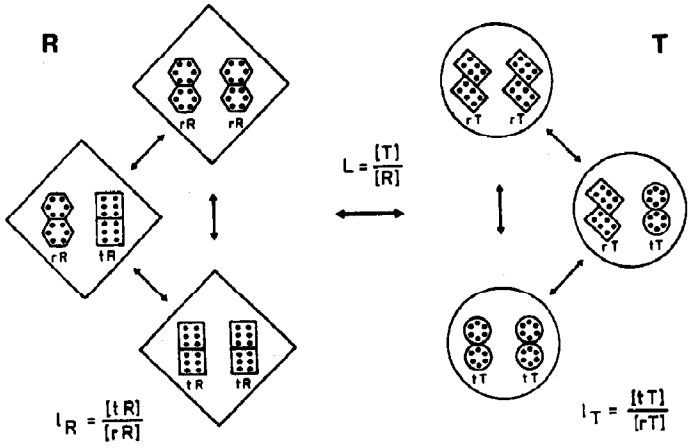

Fig. 1. The nesting model as applied to the scorpion hemocyanin: rR, tR, rT and tT denote the 'allosteric unit' being in the rR, tR, rT and tT conformation, respectively. (◇) The overall R state; (○) the overall T state of native hemocyanin. No functional heterogeneity is assumed for the individual subunits.

Heterotropic effectors are assumed to act on different levels, namely: (a) on the level of the 'allosteric units' due to the influence on the allosteric equilibrium constants $l_{R,0} = [tR_0]/[rR_0]$ and/or $l_{T,0} = [tT_0]/[rT_0]$; (b) on the level of the allosterically coupled 'allosteric units' due to their influence on the dodecamer-dodecamer interaction expressed by $L_0 = ([rT_0] + [tT_0])/([rR_0] + [tR_0])$.

Since negative cooperativity was not observed in our experiments, no functional heterogeneity was assumed to occur on the subunit level.

2. Materials and methods

2.1. Sample preparation

P. imperator hemolymph was obtained by puncturing the pericard. All samples were immediately diluted 1:2 with 0.1 M Tris buffer containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ at pH 8.0 and 20°C in order to stabilize the protein. The samples were then centrifuged for 10 min to remove blood cells. The supernatant was used as the stock solution and stored at 4°C. The protein concentration was between 20 and 30 mg/ml. The homogeneity was checked by analytical ultracentrifugation using a Beckman model E apparatus.

2.2. Oxygen-binding curves

Continuous oxygen-equilibrium curves were determined by the fluorimetric-polarographic method [14]. For the preparation of the sample, an aliquot of the stock solution was diluted in appropriate buffer to yield a protein concentration of about 0.1 mg/ml. The buffer had been adjusted to the desired pH. The binding curves of *Pandinus* hemocyanin were measured in 0.1 M Tris-HCl in the presence of 10 mM $CaCl_2$ and 10 mM $MgCl_2$. The oxygen partial pressure is calculated to a precision of 1 Torr and saturation to a precision of 0.5%. At all pH values the stability of the material was tested by analytical ultracentrifugation (Beckman model E).

2.3. Data analysis

The resulting continuous binding curves were digitized using a digitizing tablet (Cybernetic Research and Production, Freiburg) and an ATARI 1040 ST computer system. The data were analyzed by optimizing the parameter values via a nonlinear least-squares method based on Marquardt algorithms.

3. Results

Oxygen-binding curves were recorded at six different pH values ranging from pH 7.0 to 8.0, at 20.0°C. Divalent ions (10 mM $CaCl_2$ and 10 mM $MgCl_2$) were present in all samples (fig. 2).

Fig. 3 summarizes the results. The oxygen-binding affinities are strongly pH dependent. The oxygen affinities (values expressed as log dissociation constant (Torr)) increase from log $p_{50} = 1.126 \pm 0.061$ ($n = 5$) at pH 7.0 to 0.595 ± 0.062 ($n = 6$) at pH 8.0. The value for the normal Bohr effect $\Delta \log p_{50}/\Delta pH$ was calculated as -1.3 at pH 7.8.

In contrast to oxygen affinity, the cooperativity is almost pH independent. From pH 7.0 to 8.0, the

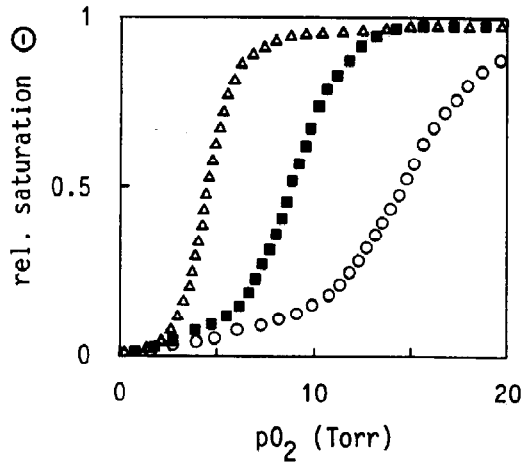

Fig. 2. Oxygen-binding curves obtained at three different pH values: pH 7.0 (○), 7.6 (■) and 8.0 (△) in 0.1 M Tris-HCl buffer and in the presence of 10 mM $CaCl_2$ and 10 mM $MgCl_2$; $T = 20$°C. Under these conditions the hemocyanin is stable and exists as an oligomeric molecule with 24 oxygen-binding sites. The oxygen affinities can be determined experimentally with an error of less than 1 Torr.

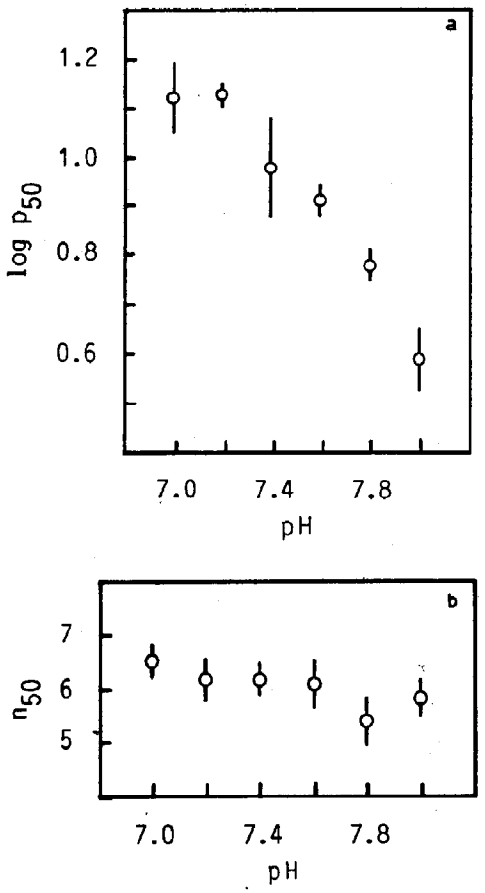

Fig. 3. pH dependence of the oxygen-binding behaviour of *Pandinus imperator* hemocyanin. (a) Oxygen affinities expressed, as log p_{50}; (b) Hill coefficient at half-saturation, n_{50} Number of experiments performed at different pH values in Tris buffer: pH 7.0, 5; pH 7.2, 3; pH 7.6, 3; pH 7.8, 3; pH 8.0, 6.

cooperativity slowly decreases from $n_{50} = 6.6 \pm 0.4$ to 6.0 ± 0.3.

3.1. Inapplicability of the MWC model

Before fitting the binding data by the MWC model its applicability has to be tested. A rapid graphical analysis [15] using binding curves at three different pH values shows that the classical MWC model cannot be used (fig. 4). For this method the binding data are transferred and plotted as $\log[\{1-(S/\alpha)\}/\{(S/\alpha)-c\}]$ vs $\log[\{1+(c\alpha)\}/(1+\alpha)]$, with $\alpha = p_{O_2}/p_{diss,N}$, $c = p_{diss,1}/p_{diss,N}$ and $S = \theta/(1-\theta)$. In order to obtain reliable values (Torr) for the initial and final oxygen binding constants, $p_{diss,1}$ and $p_{diss,N}$, two different graphical methods were used: one method calculates the values from the intersections of the asymptotes to the data transformed in a Hill plot with the ordinate $(\log(\theta/(1-\theta))$. The other, a modified Scatchard plot, was introduced by Loewe [14]. The data are depicted as a plot of $\log(\theta/(1-\theta)/p_{O_2})$ vs $N\theta$ (N: number of subunits of the oligomer). For the scorpion hemocyanin, N is equal to 24. The values determined from the two plots differ by less than 5%. If the binding data do fit the MWC model, they should yield a linear plot with slope $N-1$. This is not the case for oxygen-binding curves obtained from the scorpion hemocyanin.

3.2. Analysis in terms of the nesting model

The fitted parameters are summarized in fig. 5 (a,b). The four oxygen association constants which characterize the four conformations rR, tR, rT and tT are plotted vs pH. None of the oxygen affinity constants depends on the proton concentration. 20 oxygen-binding curves were analyzed. For the rR and the tR conformations, values of 2.1411 ± 0.1364 and 0.075 ± 0.0676 Torr^{-1}

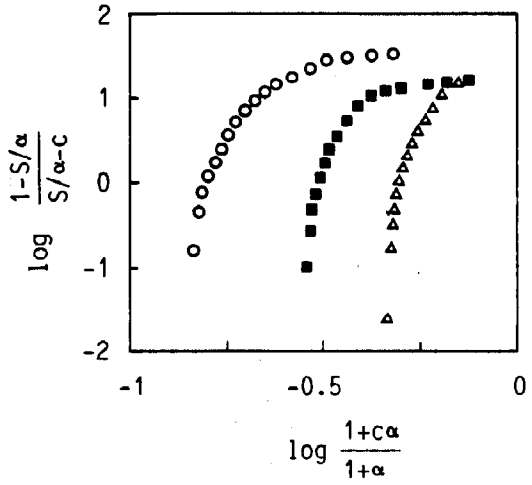

Fig. 4. Test of the applicability of the MWC model. The data of the oxygen-binding curves obtained at pH 7.0 (○), 7.6 (■) and 8.0 (△) were transformed according to Decker et al. [20].

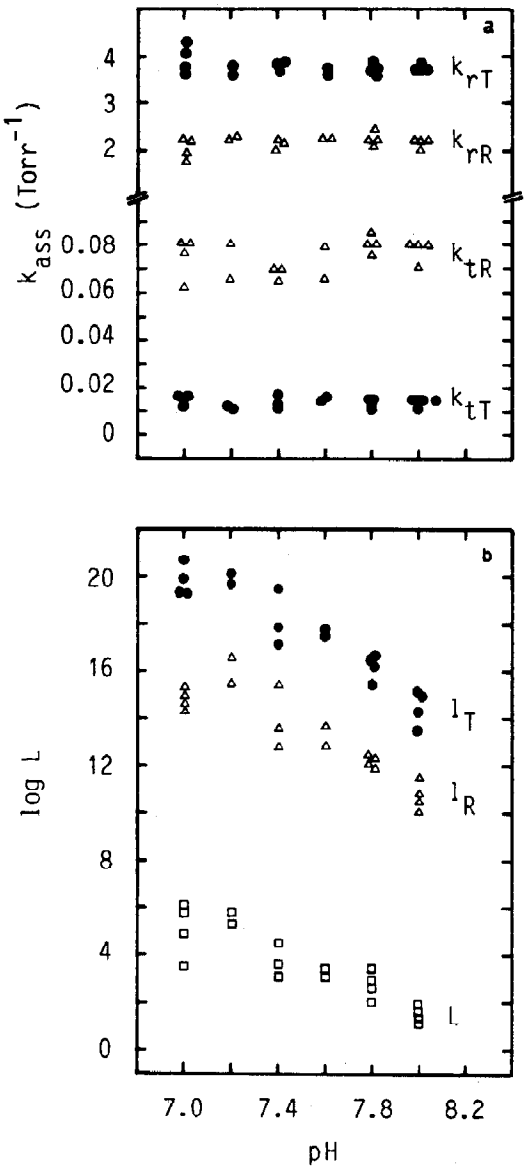

Fig. 5. Calculated parameters for the oxygen binding of *Pandinus* hemocyanin according to the nesting model at various pH values. (a) Oxygen affinity constants (Torr) k_{rR}, k_{tR}, k_{rT} and k_{tT}, characterizing the conformations rR, tR, rT and tT, respectively; (b) logarithms of the allosteric equilibrium constants $L = ([tT]+[rT])/([tR]+[rR])$, characterizing the interaction between the allosteric units, $l_R = [tR]/[rR]$ and $l_T = [tT]/[rT]$, characterizing the interactions within the allosteric unit. The fitting procedure is described in the text and elsewhere [10].

were calculated, those for the rT and tT conformations being determined as 3.766 ± 0.1835 and 0.0139 ± 0.002 Torr^{-1}, respectively.

There is a clearly distinct influence of pH on the allosteric equilibrium constants l_R, l_T and L (fig. 5b). Protons as heterotropic effectors shift the equilibria between the conformations. Between pH 7.0 and 8.0, the values of l_R, l_T and L drop by a factor of about 10^6, 10^7 and 10^5, respectively.

4. Discussion

The oxygen-binding behavior of the scorpion hemocyanin (*P. imperator*) was studied over a wide pH range. The hemocyanin belongs to the class of 24-meric hemocyanins which appear very similar to each other on electron micrographs: two hexamers are closely associated; two of the resulting dodecamers assemble edge to edge, being connected by two bridges. Other well-described examples of this type of macromolecule are hemocyanins from the scorpion *A. australis* and the tarantula *E. californicum* [10,11].

The oxygen-binding behaviour of these hemocyanins is characterized by high cooperativities ($n_{50} > 6$) and strong Bohr effects ($\Delta \log p_{50}/\Delta \mathrm{pH} < -0.7$ [2,8,14,16]). How can these functional properties be described on the basis of the structures of these proteins? The most popular model is the two-state model developed by Monod, Wyman and Changeux [5]. It assumes that the subunits of the macromolecule can exist in two conformations: these are characterized by a high and a low affinity to the ligand. The equilibrium between the concentrations of the two conformations is a property of the macromolecule. The allosteric unit consists of the set of associated subunits which are present in the same conformation and which take part in the conformational switch simultaneously. It makes sense to correlate the allosteric unit with obvious structural units. In the case of arthropod hemocyanins, the compact hexamers would appear to be suggested as the allosteric units. This assumption could be unambiguously confirmed for the hexameric hemocyanin of the spiny lobster *Panulirus interruptus* [17]. Its competitive binding of oxygen and carbon monoxide was satisfyingly

described by the two-state model with the allosteric unit containing six subunits. However, for 24-meric arthropod hemocyanins, the simple two-state model is not applicable as shown in the present study for *Pandinus* hemocyanin and as previously reported for that of *Eurypelma* [15].

The recently introduced nesting model correlates the obvious hierarchies in the structures of arthropod hemocyanins with the function of these molecules [6,7]. It has been shown in several cases [4,10,11,18] that the half-molecules of arthropod hemocyanins are the smallest structurally repeating units. They are regarded as allosteric units which are nested and allosterically coupled within the native molecule.

For the 24-meric *Pandinus* hemocyanin, the 12-meric half-molecule was assumed to be the allosteric unit (ref. 9; and Decker and Heimerl, unpublished observations), which can adopt four different conformations: rR or tR when the native molecule is in the R state, and rT or tT when the native molecule is in the T state. Comparison of the calculated values for the oxygen affinities and allosteric equilibrium constants obtained for *Pandinus* and *Eurypelma* hemocyanins reveals a common feature (table 1). The values for the affinities of the conformations appear to be conserved among cheliceratean hemocyanins. Additionally, allosteric effectors such as protons quantitatively influence the ratios between the concentrations of the conformations in the same way: when the concentration of protons is increased, the values of the allosteric equilibrium constants l_R, l_T and L also increase. The numerical values may be considered to be characteristic of the particular hemocyanin and may therefore be different. Although several arguments favor the nesting model [8], its validity can only be proved by the experimental elucidation of postulated conformations and conformational transitions. For *Eurypelma* hemocyanin, four different conformations have recently been detected after labelling the hemocyanin with a fluorescent dye [19].

Table 1

Oxygen affinity constants calculated for *Eurypelma californicum* and *Pandinus imperator* hemocyanins according to the nesting model

The data are expressed as the association constants ($Torr^{-1}$).

Conformation	*Eurypelma californicum* [a] ($n = 41$)	*Pandinus imperator* ($n = 20$)
rR	1.990 ± 0.070	2.141 ± 0.136
tR	0.049 ± 0.012	0.075 ± 0.068
rT	3.457 ± 0.056	3.766 ± 0.184
tT	0.014 ± 0.004	0.014 ± 0.002

[a] Values taken from ref. 8.

Acknowledgements

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