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Allostery in very large molecular assemblies ☆

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Abstract

In contrast to small allosteric systems (like hemoglobin) those containing very large numbers (n) of binding sites never exhibit cooperativity (as measured by the Hill coefficient, n_H) even approaching the potential limit, n. The reason for this appears to be that in such macromolecules the cooperative unit always represents some sub-structure of the entire structure. On the other hand, it is frequently observed that such sub-structures, when isolated, do not exhibit cooperativity at all. This paper describes studies of some molluscan hemocyanins that explore this apparent anomoly. It is concluded that it is the higher order structure of the molecule that provides a framework within which the sub-structures may exhibit their allosteric behavior.

Keywords: Allostery; Cooperative binding; Hemocyanin; Oxygen binding; Quaternary structure

1. Introduction

Descriptions and discussions of allostery usually take for their examples one or two small proteins, vertebrate hemoglobin being the archetype. This is not unreasonable for the evolution of the concepts of cooperative binding and allosteric effects have been closely coupled to attempts to understand the transport of oxygen in vertebrate blood. Indeed, the first hints of these concepts are to be found in the work of Hill [1], nearly a century ago. The problem continued to occupy many scientists during the entire first half of the century. Finally, in the 1960s there emerged detailed models which explained both homotropic and heterotropic allostery in a broad conceptual framework. The most useful of these have been based on the 'concerted' model of Monod et al. [2]. Although these models and their later derivative forms can be applied to structures of any size (see [3] for example), textbooks and reviews, even to this day, tend to illustrate discussion of al-

☆ We would like to dedicate this paper to Dr Henryk Eisenberg. Over many decades his research has set standards for the rest of us to emulate. Heinie's qualities as a person and a friend have been equally important to a whole generation of scientists.

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Table 1
Examples of invertebrate oxygen-transport proteins

Protein	n[a]	n_{Hmax}[b]	q[c]	Reference
Arthropod hemocyanins				
Panulirus interruptus	6	2	6	[5]
Homarus americanus	12	4	6	[6]
Eurypelma californicum	24	7	12	[6]
Androctanus australis	24	9	ND	[7]
Limulus polyphemus	48	5	6 + 12	[8]
Molluscan hemocyanins				
Octopus dofleini	70	4	7	[9,10]
Sepioteuthis lessoniana	80	2	8	This work
Archachatina marginata	160	1	NA	[11]
Helix pomatia (β)	160	7	16	[12,13]
Hemoglobins				
Homo sapiens	4	3	4	[14]
Lumbricus terrestris	≥ 144	6	ND	[15]
Hemerythrins				
Lingula reevi	8	2	4	[16]

[a] The number of O_2 binding sites in the functional molecule.
[b] The largest value of the Hill coefficient reported, rounded to one figure.
[c] The allosteric unit. ND, not determined; NA, not applicable.

losteric effects by vertebrate hemoglobin, or relatively small multi-subunit enzymes such as aspartate carbamoyltransferase.

2. Very large allosteric systems

There exist, however, many much larger structures in which allosteric effects are observed, and these can exhibit features not clearly shown by small molecules. The most extreme examples are found among the oxygen transport proteins of invertebrates. Table 1 lists a number of these large multi-subunit proteins. Some of these proteins are truly immense; for example, the hemocyanins found in gastropod molluscs commonly contain 160 oxygen binding sites, or even multiples of this number [4].

Nevertheless, these very large numbers of binding sites are not reflected by correspondingly high values of the Hill coefficient, n_H. It will be recalled that the theoretical range for n_H in a positively cooperative molecule with n sites is

$$1 \le n_H \le n \tag{1}$$

Small allosteric molecules often exhibit Hill coefficients approaching n, e.g. vertebrate hemoglobins, with $n = 4$, show maximum Hill coefficients in the range of 3–3.5. By analogy, the *Helix pomatia* hemocyanin listed in Table 1 could be expected to exhibit a Hill coefficient of over 100. In fact, values approaching this have never been observed. To our knowledge, the largest values of n_H ever reported are approximately 9, in certain spider hemocyanins containing 24 oxygen binding sites (see Table 1). For most such large molecules, the values are spectacularly lower than might be expected. For example, even the giant hemoglobin of *Lumbricus* and the extremely large molluscan hemocyanins do not exhibit Hill coefficients exceeding approximately 8. Indeed, some of the latter show almost no cooperativity. As an example, consider the hemocyanin of the mollusc *Archachatina* (Table 1); with 160 oxygen binding sites; the molecule remains almost wholly non-cooperative.

The fact that n_H is generally much less than n we term the 'Hill coefficient anomoly'. One might wonder if there is some *intrinsic* reason, inherent in the nature of cooperativity, for this peculiar result. However, examination of theories of allostery reveals no fundamental reason why quite substantial values of n_H should not in principle be attained with large macromolecular structures. Following Zhou et al. [17], it can be easily shown that the MWC model [2] yields for a molecule with n sites

$$n_H = 1 + \frac{L(n-1)(1-c)^2\alpha Y}{(1+\alpha)(1+c\alpha)(1+LY)(1+cLY)} \tag{2a}$$

where $Y = [(1 + c\alpha)/(1 + \alpha)]^{n-1}$ (2b)

Here $\alpha = k_R X$, where X is ligand activity and k_R the affinity constant for the R state, $c = k_T/k_R < 1$, and $L = [T]/[R]$, the T/R equilibrium constant for unliganded molecules.

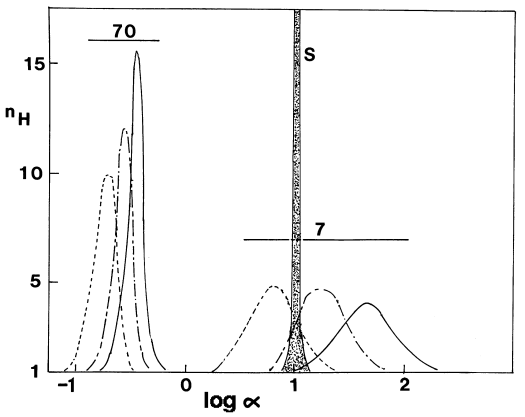

Fig. 1. The Hill slope, n_H, as a function of the logarithm of reduced ligand activity ($\alpha = k_R X$), for molecules containing either 7 or 70 binding sites in the allosteric unit. The parameters $c = 10^{-2}$ and $L = 10^6$–10^{10} have been chosen to be in the range frequently observed for allosteric oxygen transport proteins. Code: dotted line, $L = 10^6$; broken line, $L = 10^8$; solid line, $L = 10^{10}$. All curves are for $c = 10^{-2}$. The curve (S) enclosing the shaded area is for $n = 70$, with $c = 10^{-2}$ and L having the very large value of 10^{70}. Its maximum value (off graph) is $n_H = 47.2$. For any of these curves, decreasing c to 10^{-4} or 10^{-6} only slightly increases the maximum value of n_H.

Eq. (2a) gives the slope of the Hill plot as a function of α. Calculation of n_H yields the kind of curves represented in Fig. 1. In each case the slope increases from unity to a maximum value and then decreases again to unity as α increases. Assuming values of L and c in the range often found experimentally we observe, for a 70-site molecule, the kind of curves shown to the left in Fig. 1; even these exhibit a maximum n_H value considerably larger than any actually observed. Furthermore, if we allow L to become very large (i.e. $L = 10^{70}$), very high values of the maximum n_H (i.e. $n_H^{max} = 47$) can be predicted. We might in fact expect L to attain such values if the entire molecule were involved in the $T \rightarrow R$ transition; there is expected to be a linkage between L and size. In any event, the results of such calculations tell us that there is no *intrinsic* limit to account for the much lower Hill slopes actually observed.

A distinguishing feature of Hill plots for very large n values is the sharpness of the $T \rightarrow R$ transition. As can be seen in Fig. 1, the entire transition in all cases where $n = 70$ occurs over a very small range of α values. In contrast, where n is much smaller (i.e. $n = 7$, Fig. 1) the $T \rightarrow R$ transition is spread over a much broader range of α values.

The parameters used in the calculations depicted in Fig. 1 were chosen to relate the calculations to a molecule like *Octopus* hemocyanin, which carries 70 oxygen binding sites. The native protein is made of 10 subunit polypeptide chains each carrying seven sites (Fig. 2). Thus, for the *Octopus* hemocyanin *subunit* we would expect to find $n_H \approx 3$–5 under most circumstances (see Fig. 1). This is in fact the range of values observed for the *whole* hemocyanin molecule ($n = 70$) under experimental conditions [9,10]. Oxygen-binding data for the whole molecule can be fitted to the MWC model, but the value required for the number of cooperating sites is seven, not 70. In other words, the giant hemocyanin molecule behaves as if the *subunit*, containing seven binding sites were the allosteric entity. This kind of behavior has been observed in many cases (Table 1) and has led to the concept of the *allosteric unit* within a larger macromolecular assembly. Operationally, the allosteric unit is defined by the number of sites (q) required to adequately fit the binding data to an appropriate model for allosteric behavior. In fact, an effort has usually been made to relate the size of the allosteric unit to some recognizable sub-structure in the giant macromolecule. This is true of for examples in Table 1.

The idea of small functional allosteric units within large (even infinite) aggregates was suggested in the 1969 paper by Wyman [3]. However, that paper is not primarily concerned with the Hill coefficient anomoly, but rather the possibility of allosteric phase transitions in infinite aggregates.

3. Examples: structure and oxygen binding by cephalopod hemocyanins

The hemocyanins of the cephalopods, such as octopi and squids, array their 10 subunits in open cylindrical structures with fivefold symmetry ([4], see Fig. 2). These subunits are themselves very large polypeptide chains, each containing seven

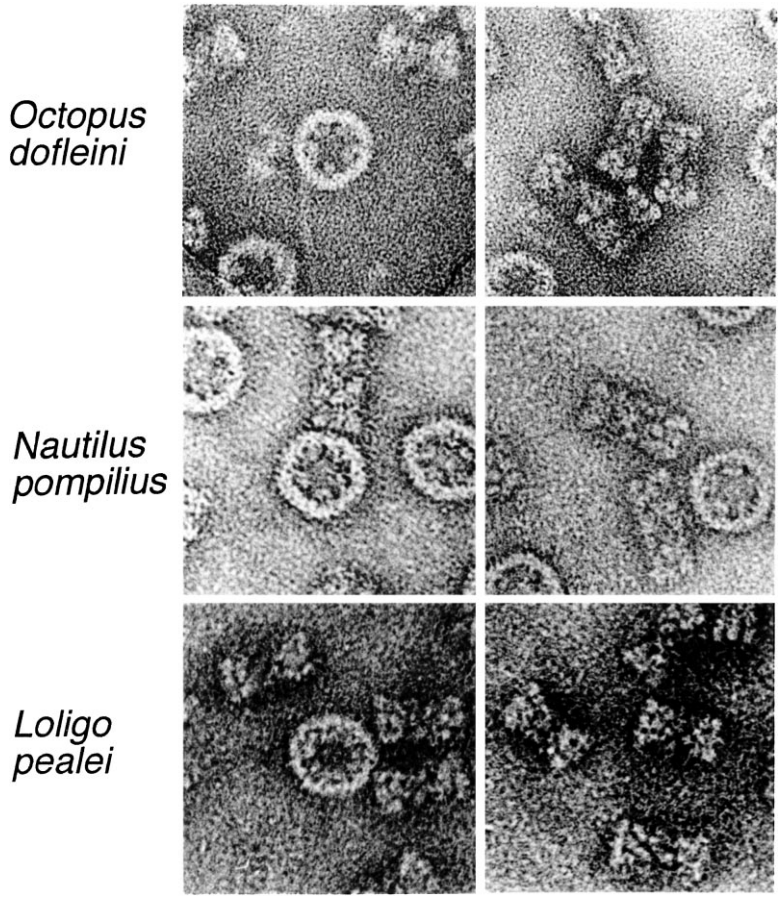

Fig. 2. Electron micrographs of representative cephalopod hemocyanins. The molecules are hollow cylinders, approximately 30 nm in diameter and 15 nm high, and exhibit an axis of fivefold symmetry. Each contains 10 polypeptide chains. The *Octopus* (and probably *Nautilus*) hemocyanin molecules contain 70 oxygen binding sites, those from the squid *Loligo* have 80.

(octopus, nautilus) or eight (squid) functional units. Each functional domain carries one oxygen binding site. The sequence of the seven-unit subunit of *Octopus dofleini* hemocyanin has been determined; the polypeptide chain contains 2896 residues [18]. The sequences of the individual functional units are highly conserved (approx. 40% identity) and are connected by less well-conserved 'linker' regions. The structure of one of the functional units has been determined by X-ray diffraction [19], and tentative models for the entire structure have been proposed from imaging studies ([20]; also Cuff and van Heel, private communication).

Oxygen binding by *Octopus dofleini* hemocyanin has been carefully studied [9,10]. As Fig. 3 shows, the binding curves can be fitted quite accurately by the standard MWC model with an allosteric unit of seven sites. The same allosteric unit serves to fit the data over a wide range of pH and temperatures [9]. No evidence for higher order interactions (as in 'nesting', in which each allosteric unit forms an element in a higher order allosteric array [21]) was found [10].

This might lead one to expect that the individual polypeptide chain, containing seven oxygen binding sites, could by itself exhibit cooperative binding. This is not the case; when the *Octopus*

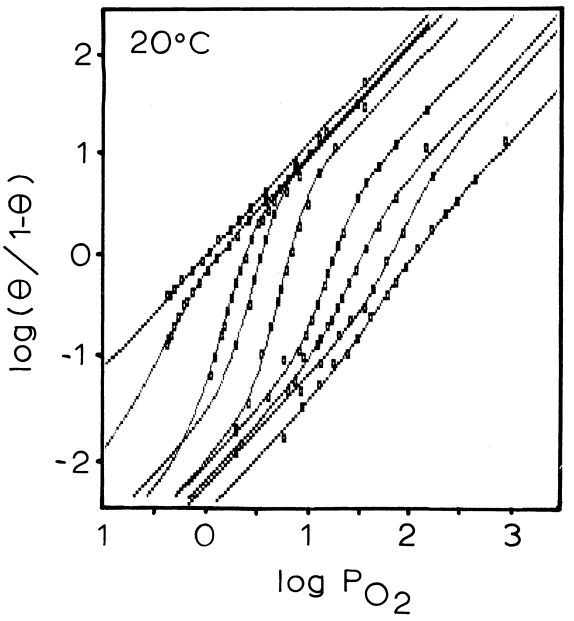

Fig. 3. Hill plots for oxygen binding by *Octopus dofleini* hemocyanin at a variety of pH values at 20°C. The curves have been fitted by the MWC model [2], using a value of $q = 7$ for the allosteric unit. The symbol θ represents fraction saturation. (From Miller [9].)

hemocyanin is dissociated into subunits by removal of divalent cations at pH 8.0, wholly noncooperative binding is observed [9,10]. This observation is in fact general for molluscan hemocyanins; whatever cooperativity may be observed for the intact structures, the multi-site subunits, when isolated, are not cooperative (see [4,22]).

4. What is the source of cooperativity?

Most molluscan hemocyanins exhibit cooperativity only in the native aggregate, but with a cooperative unit corresponding to the multi-site polypeptide chain (see Table 1). The fact that the isolated chains are *not* cooperative poses a puzzle: What then is the source of the cooperativity?

Answering this question is complicated by the possibility that the presence of divalent ions, necessary for the preservation of the native aggregated state of these molecules, might coincidentally also be essential for cooperativity. This possibility is diminished, but not eliminated, by the observation of Miller [9] that either moderate concentrations of divalent cations or much higher concentration of NaCl preserved *both* the decameric form of the *Octopus* hemocyanin and the cooperativity in oxygen binding. Thus, divalent cations per se are not essential for either. Although the studies do not entirely rule out coincidental effects, their existence is rendered less likely.

If the individual multi-site subunit is not capable of cooperative binding, it may be that contacts with other subunits somehow establish constraints that permit the formation of distinct T and R states. If so, how extended must such contacts be? Is the whole decameric structure required, or will more limited contacts (as within dimers of subunits, for example) suffice? Indeed, there is some experimental support for the latter idea; Klarman et al. [23] reported that dimers of the subunit of the hemocyanin of the gastropod *Levantina hierosolima* exhibited cooperative O_2 binding. Is this a general phenomenon?

5. Cooperativity in oxygen binding by *Sepioteuthis* hemocyanin

We wanted to examine the question as to the minimal structure for allosteric behavior using the well-studied *Octopus* hemocyanin. Analysis of the oxygen binding by dimers of the subunits should provide a critical test. Electron micrographs of molluscan hemocyanin dimers reveal a highly organized structure, quite unlike the irregular 'beads on a string' morphology shown by monomeric subunits [24]. Unfortunately, *Octopus* hemocyanin exhibits only two well-defined quaternary structural states: the monomeric polypeptide chain and the decamer of these chains; intermediate structures such as dimers are found only as transient species during association or dissociation [24,25]. We had observed, however, that the hemocyanin of the squid *Sepioteuthis lessoniana* could form, under appropriate solution conditions, a very stable dimer as the sole structural

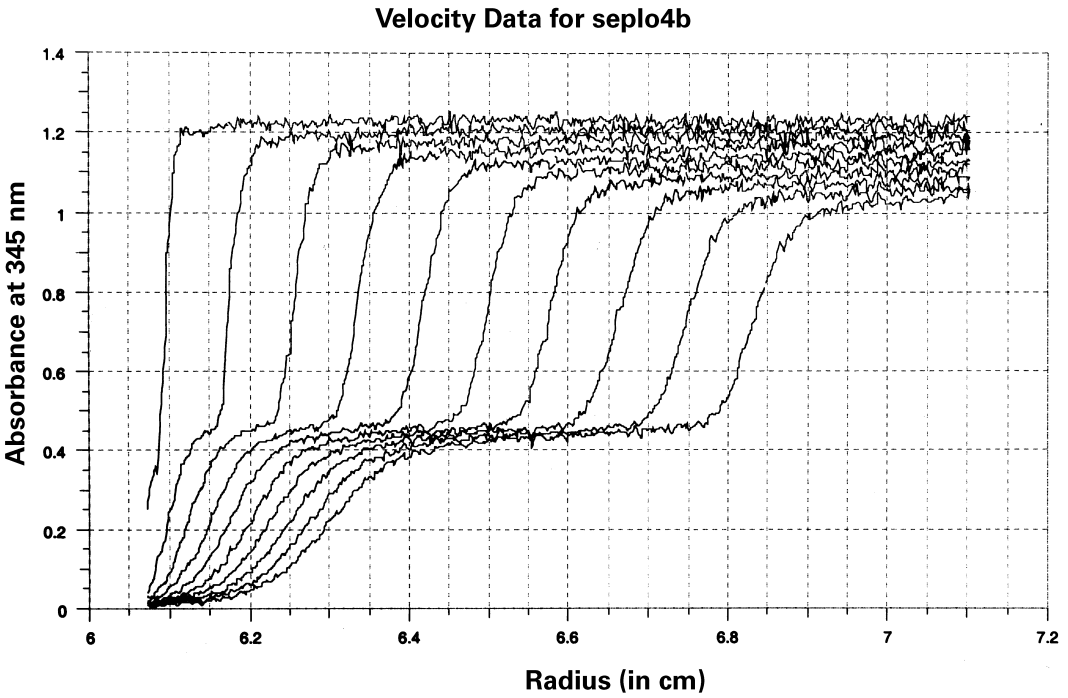

Fig. 4. Sedimentation velocity analysis of *Sepioteuthis* hemocyanin at pH 7.65, 15 mM Mg^{2+}. The two well-resolved components are the 19S dimer and the 60S decamer [26]. The proportion of the two components is sensitive to [Mg^{2+}]; at 5 mM all is in the 19S form, at 50 mM all is 60S. Experiments were performed in a Beckman XL-A ultracentrifuge, at 20 000 rev./min.

form [26]. Therefore, we decided to compare the oxygen binding behavior of *Sepioteuthis* hemocyanin in well-defined dimeric and decameric states. At any given pH value near neutrality, the state of association of this protein is determined specifically by the divalent cation concentration. At 5 mM Mg^{2+} the only component present is the 19S dimer; at 50 mM Mg^{2+}, all of the protein is in the 60S decameric form. At intermediate concentrations of Mg^{2+}, mixtures of these two forms are found that can be clearly resolved by sedimentation velocity studies (Fig. 4). This association–dissociation reaction is (slowly) reversible [26].

Fig. 5 contrasts the oxygen-binding by the decameric and dimeric states of the protein at pH 7.9. Clearly, the dimer exhibits completely non-cooperative binding (the Hill coefficient is 1.02), whereas the decamer is moderately cooperative. However, is the cooperative binding exhibited by the decamer a consequence of the quaternary

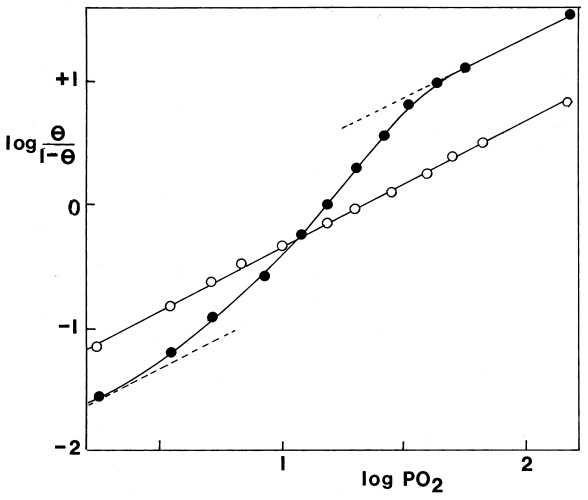

Fig. 5. Hill plots for oxygen binding by *Sepioteuthis* hemocyanin at pH 7.9, 20°C, in the dimeric state (open circles) or decameric state (filled circles). In the former case, the slope of the line is 1.02; in the latter case the maximum slope is 2.4. Experiments were carried out as described previously [9]. Symbols as in Fig. 3.

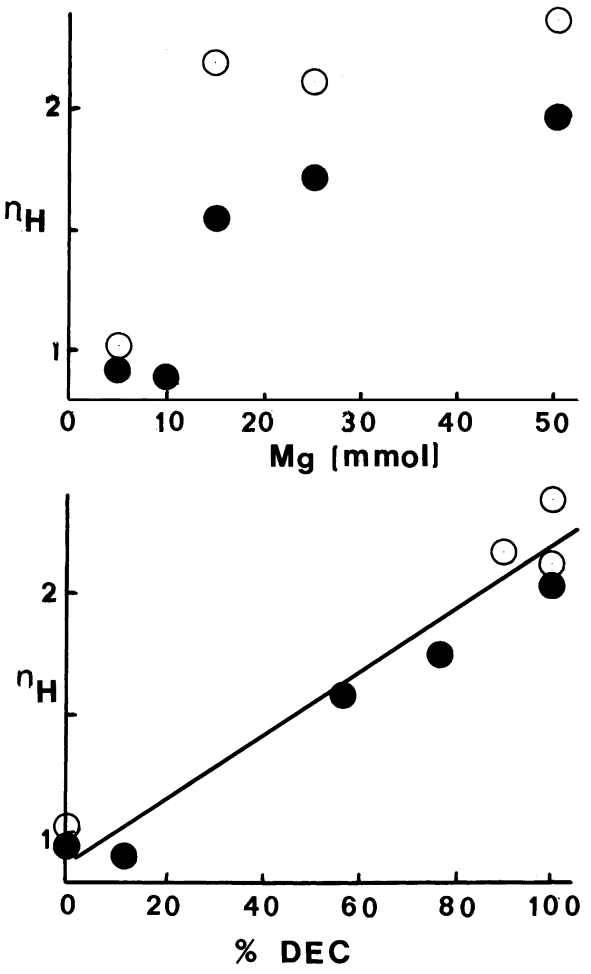

Fig. 6. The dependence of the Hill coefficient for oxygen binding by *Sepioteuthis* hemocyanin on $[Mg^{2+}]$ (top), or on the fraction decamer (bottom). Data are from two sets of oxygen binding studies — one at pH 7.65 (filled circles) the other at pH 7.9 (open circles). Very little correlation is shown in the top figure, whereas a simple linear correlation is found in the bottom figure, indicating that it is the fraction of decamer, rather than $[Mg^{2+}]$ per se, that determines the level of cooperativity.

structure of the molecule or of the higher concentration of Mg^{2+} needed to stabilize this structure?

A provisional answer to this question is provided by carrying out the oxygen binding studies at more than one pH value. The dependence of decamer stability on Mg^{2+} concentration is different at different pH values, so we can ask — does the cooperativity depend upon $[Mg^{2+}]$ per se, or does it depend upon the existence of the decamer, rather than the dimer? Fig. 6 (top) shows the variation of the Hill coefficient, n_H, as a function of Mg^{2+} concentration, at two different pH values. In contrast, Fig. 6 (bottom) depicts the dependence of n_H upon the fraction decamer, regardless of $[Mg^{2+}]$. It seems clear from these data that cooperativity is correlated with the presence of the decameric quaternary structure, rather than with Mg^{2+} concentration in itself.

We find then, in this example at least, that even the multiple contacts between functional units in the dimer are not sufficient to impose cooperative oxygen binding; rather, the entire cyclic structure is necessary. Yet the entire molecule does not act as an allosteric unit; it only *enables* the multi-site monomers within the molecule to behave as allosteric units. How this 'enabling' is achieved is still unclear. One might hypothesize that in the cyclic decameric structure two states (R and T) are possible for each of the 10 subunits, whereas these states do not exist (or are of equal oxygen affinity) when the subunits or their dimers are free of decameric constraints. Alternatively, the behavior could be explained by the possibility, at the decameric level, of stronger interactions between units in the R state than in the T state.

However, the necessity for a decameric framework does not necessarily mean that there exist favorable or unfavorable allosteric interactions between pairs of subunits in the decamer. It may be that we are encountering here an example of one special case of the DiCera [13] model for allostery in cyclic structures. DiCera writes the partition function for binding in terms of 'morphological units' (which can be supposed, in the case of molluscan hemocyanins, to be the polypeptide chains). Each unit can exist in a T or R state, and adjacent morphological units interact with energies W_{RR}, W_{TT}, and $W_{TR} = W_{RT}$. In the special case where $W_{TT} = W_{TR} = W_{RT}$, the units are insensitive to one another's state. In that case, the allosteric unit, q, becomes equal to the morphological unit.

From the limited data available (Table 1), it would seem that although the above model may perhaps be applicable to molluscan hemocyanins in general, it does not suffice in other cases. The arthropod hemocyanins, for example, do not exhibit cyclic structures, and in general require a 'nesting' model [21] to adequately describe their behavior. Note that in this case, the typical allosteric unit, which is a hexamer of polypeptide chains, can both exhibit cooperative binding in the isolated state and can function as the allosteric unit in larger structures. Thus, the mechanisms of allostery appear to be fundamentally different in the molluscan and arthropodan hemocyanin.

The question remains as to *why*, in a functional sense, these very large molecules should have such small allosteric units. A suggestion by a reviewer of this paper seems intruiging to us: As Fig. 1 shows, if the allosteric unit were very large, the $T \rightarrow R$ shift would be very abrupt. Indeed, over most of the range of ligand concentrations, the molecule would be binding *non*-cooperatively, in either the T or R state. If there are physiological reasons for having these molecules so large, the division into smaller allosteric units may be essential to spread the allosteric transition over a useful range of ligand concentration.

Acknowledgements

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