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SEDIMENTATION EQUILIBRIUM STUDIES OF A COMPLEX ASSOCIATION REACTION

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We have investigated the association of 17 S (monomer) particles of *Callianassa* hemocyanin by sedimentation equilibrium. The association proceeds in two steps: (1) a monomer–dimer association which is sensitive to Mg^{2+} concentration and insensitive to temperature, and (2) a dimer–tetramer association which is highly temperature dependent but insensitive to Mg^{2+}.

1. Introduction

The method of sedimentation equilibrium is now widely employed in the analysis of reversibly associating systems. A large number of protein association-dissociation reactions have been investigated, as the techniques for analysis have steadily improved. Much of this work is summarized in a very recent review [1].

While it has been observed that many such reactions proceed beyond the dimerization stage, to the formation of higher order aggregates, there have been few cases in which the individual steps in such processes could be isolated and studied individually. We have found, in the association of the hemocyanin from the ghost shrimp, *Callianassa*, a case in which this can be done. This paper describes the early stages of our investigation of this system.

In order to define the problem, we must describe in this introduction some results which have already been obtained and published [2,3]. *Callianassa* hemocyanin occurs, under the ionic conditions existing in the hemolymph, primarily in particles with sedimentation coefficient ($s^{\circ}_{20,w}$) of about 39 S. This component, when isolated from the hemolymph, and dialyzed against buffer containing less than 0.01 M divalent ions, dissociates reversibly into particles with $s^{\circ}_{20,w}$ = 17.1 S. It may be quantitatively reassociated, at room temperature, and pH 7.6, by raising the divalent ion concentration to 0.05 M or greater. Sedimentation equilibrium studies have shown (table 1) that the 39 S particles are tetramers of the 17 S particles. These latter, in turn, have been shown to consist of six polypeptide chains, each containing a single oxygen binding site.

One of the peculiarities of the *Callianassa* hemocyanin, as compared to other arthropod hemocyanin, has been the apparent absence of a stable dimeric intermediate between the 17 S monomer and the 39 S tetramer. Most arthropod hemocyanins exhibit a dimeric structure, with $s_{20,w} \approx 25$ S, under some circumstances. The reversible monomer–dimer association

Table 1
Observed associated states of *Callianassa* hemocyanin[a)]

$s^{\circ}_{20,w}$ (S)	$\bar{v}$ (ml/gm)	$M \times 10^{-5}$	Number of polypeptide chains	Conditions for stability
38.8	0.724	17.2	24	$[Mg^{2+}] \geq 0.05$ at room temp or above
17.1	0.724	4.31	6	$[Mg^{2+}] < 0.01$ M
~5	–	0.72	1	pH > 9.2, or in presence of SDS or 6 M Gn. HCl

a) Data of Roxby et al. [2].

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of lobster hemocyanin, for example, has been extensively investigated by Kegeles and co-workers [4–6]. It seems that in the case of lobster hemocyanin the dimerization, which is dependent upon divalent ions, represents the limit of association. A long range object of our researches with hemocyanins is to provide information which may help in understanding the nature of the special constraints which apply to the association reactions of different members of this class of proteins.

2. Experimental

2.1. Preparation of hemocyanin

Callianassa californiensis were collected from intertidal regions in Yaquima Bay, Oregon. The animals were bled, and the pooled blood passed over a Bio Gel A5 M column, equilibrated with 0.1 ionic strength Tris buffer, pH 7.65, containing 0.1 M $MgCl_2$, as previously described [2]. This procedure effectively isolates the "competent" hemocyanin [2] as a homogeneous 39 S component. Such material was used for all experiments. Most of the experiments described here were carried out at room temperature, in 0.1 ionic strength Tris-HCl buffers (pH 7.65), containing varying amounts of $MgCl_2$. The experiments in which temperature was varied employed a 0.1 ionic strength phosphate buffer containing 0.05 M $MgCl_2$. Buffer changes were made by dialysis. All chemicals employed were reagent grade, and water was doubly distilled.

2.2. Sedimentation equilibrium studies

All experiments were performed in Spinco Model E Ultracentrifuges, equipped with RTIC and Rayleigh optical systems. In all of the experiments described herein, the Yphantis method [7] was employed. Reference baselines were run on shaken-up cells, and subtracted in all cases. Plates were routinely read at about 20 r-values, reading 3–5 fringes at each point. A digitizer was used to punch coordinates directly onto paper tape for computer analysis.

3. Results

The sedimentation equilibrium data were analysed by the method developed by Dyson [8]. This technique utilizes the overdetermined set of equations

$$\frac{M_{wa}/M_1}{1-BM_{wa}c} - = \sum_{i\geq 2} c_{ai} \exp(-M_i B \Delta c)\, X_i, \qquad (1)$$

where

$$X_i = (M_i/M_1 - 1) \exp[M_i I(c)]/c, \qquad (2)$$

$$I(c) = \int_{\ln c_a}^{\ln c} (1/M_{wa})\, \mathrm{d} \ln c - B(c - c_a). \qquad (3)$$

In these equations M_{wa} is the apparent weight average molecular weight, M_1 the monomer weight, B the second virial coefficient and the c_{ai} are concentrations of the various molecular species at an arbitrary reference point.

The system is solved by the digital computer to yield the set of parameters (c_{ai}, B) which give the best least-squares fit to the M_{wa} versus c data. The number of species to be utilized, the monomer molecular weight and whether the system is to be considered ideal or non-ideal are input data. The program outputs the set of K_{1j}, defined by

$$K_{1j} = c_j/c_1^j, \qquad (4)$$

with the c_j expressed in fringes. A comparison of the predicted and observed weight average molecular weight, and the RMS error are also provided in output, as well as values of the fraction of each species at each point in the cell, corresponding to the best fit.

Experiments are summarized in table 2. In each case several possible stoichiometries were tried; these usually included monomer–tetramer and monomer–dimer–tetramer, both ideal and non-ideal. In a number of trials a trimer component was included; in no case did this significantly improve the fit, so it was eliminated. Since, we have never observed a component larger than the tetramer in the *Callianassa* system, no such components were included in the data analysis. The value of the monomer weight was taken to be 431 000; this is the result obtained previously [2].

In each case the stoichiometry giving the smallest RMS error was chosen. This turned out to be the non-ideal monomer–dimer–tetramer model, in every case

Table 2
Sedimentation equilibrium experiments

$[Mg^{2+}]$ (mol/ℓ)	T (°C)	Stoichiometry of best fit[a]	RMS M_W error	$B \times 10^9$ [b] (mole cm^3/gm^2)	K_{12} [b] $(mol/ℓ)^{-1}$	K_{14} [b] $(mol/ℓ)^{-3}$
0.020	20	M D T, n.i.	12950	2.6	c	2.0×10^{16}
0.020	20	M D T, n.i.	14291	1.0	c	1.5×10^{16}
0.025	20	M D T, n.i.	7430	4.3	4.6×10^5	2.9×10^{17}
0.030	20	M D T, n.i.	5189	1.8	7.8×10^5	5.8×10^{18}
0.035	20	M D T, n.i.	22399	5.2	1.3×10^6	2.2×10^{19}
0.040	20	M D T, n.i.	23957	1.4	2.6×10^6	1.6×10^{20}
0.05	4	M D T, n.i.	12893	2.2	2.5×10^7	1.8×10^{21}
0.05	10	M D T, n.i.	9280	2.2	2.4×10^7	4.1×10^{21}
0.05	15	M D T, n.i.	11280	2.2	3.1×10^7	1.2×10^{22}
0.05	30	M T, n.i.	21056	8.5	–	1.6×10^{23}

a) M = monomer, D = dimer, T = tetramer, n.i. = non-ideal.
b) Calculated from data expressed in fringes by the relation 1 mg/ml = 4.30 fringes [11].
c) In these two experiments the amount of dimer is *very* small and a very small *negative* value of K_{12} was obtained. In each case, M–T, n.i. fit gave virtually identical results, and nearly the same value of K_{14}.

but one (see table 2). Since the average molecular weight is on the order of 10^6 in most of these experiments, the RMS values correspond to only a few percent. The values found for the non-ideality parameter, B, are small, and of the order of magnitude expected for spherical particles [9]. Clearly, the B-values are not very precise, but they do represent a necessary correction. For example, fig. 1 shows the fitting of the data by both ideal and non-ideal monomer–dimer–tetramer models, for the experiment with the smallest RMS error in the M_{wa}. It is clear that the inclusion of the non-ideality term makes a small but significant improvement in fit.

In fig. 2, is shown the distribution of mass, corresponding to the best fit in fig. 1. It is clear from these and other data that under most circumstances appre-

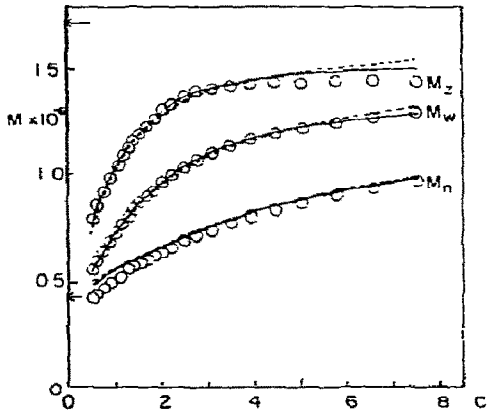

Fig. 1. Data obtained in an experiment at 20°C in the presence of 0.03 M Mg^{2+}. The points (circles) represents M_{na}, M_{wa}, and M_{za} respectively; the lines through the points are the best fit to M–D–T association. The broken line is for an ideal system (RMS = 11 216), the solid line for a non-ideal solution (RMS = 5189). Concentration values in fringes.

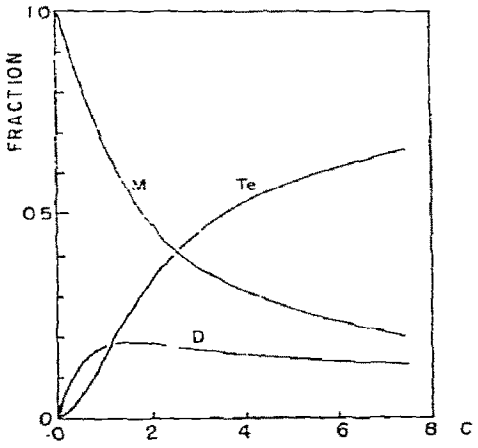

Fig. 2. The fraction of monomer, dimer, and tetramer at different total concentrations in the solution column for the experiment shown in fig. 1. The values are those predicted for the best-fit solution. Concentration values in fringes.

ciable amounts of dimer are present. As we shall subsequently see, the experiments define, in fact, certain conditions under which the system should be *largely* in the dimeric form. Thus, one of the main questions is answered immediately; *Callianassa* hemocyanin, like that of other arthropods, *does* form a stable dimer.

Since the data were (with one exception) fitted to monomer–dimer–tetramer equilibria, the program gave in each case a value of K_{12} and a value of K_{14}. However, these values are not always equally reliable, and in some cases one or the other may be wholly unreliable, even though the data are quite accurate and the curve fitting is quite satisfactory. As we shall see later, conditions can exist in this system in which either dimer or tetramer is present in very small amount; in this case calculation of the corresponding association constant is hazardous. Such situations can be spotted easily by examining the print-out of the kind of data shown in fig. 2.

The experiments which have been performed fall into two series; one (the *Mg-series*) in which the concentration of Mg^{2+} is varied at constant temperature, and the *T-series*, in which temperature is varied at constant Mg^{2+} concentration (0.05 M). The *T*-series were really one very long experiment, for the temperature of the rotor was simply changed after each equilibrium was attained. In order to keep the pH constant, a phosphate buffer was substituted for the Tris buffer we have usually employed.

The results of the Mg-series are summarized in fig. 3. In each case an approximately linear relationship between $\log K$ and $\log [Mg^{2+}]$ is observed, although the values of K_{12} are clearly not very accurate. (In most of the experiments we have done to data, dimer is a minor component.) The values of K_{14} appear to be far more reliable.

The simplest way to analyse curves like those in fig. 3 is to assume reactions of the form

$$2\,M + n\,Mg^{2+} \rightleftarrows D,$$

$$4\,M + m\,Mg^{2+} \rightleftarrows T,$$

where M, D, and T correspond to monomers, dimers, and tetramers, respectively. Then we may write

$$K_{12} = \frac{[D]}{[M]^2[Mg^{2+}]^n} = \frac{K_{12}^{obs}}{[Mg^{2+}]^n}, \qquad (5)$$

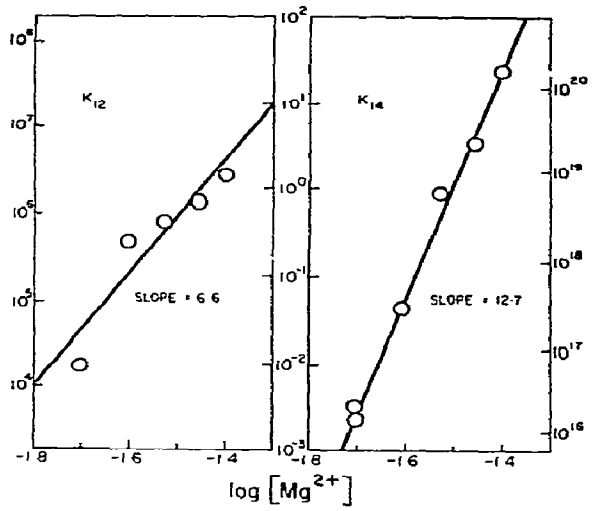

Fig. 3. Logarithmic graphs of K_{12} (left) and K_{14} (right) versus $\log [Mg^{2+}]$. The center scale expresses the equilibrium constants in fringe units, on the extreme left and right scales are given for K_{12} and K_{14}, respectively, in molar units $(mol/\ell)^{-1}$ and $(mol/\ell)^{-3}$ respectively. All experiments at 20°C, pH 7.65.

$$K_{14} = \frac{[T]}{[M]^4[Mg^{2+}]^m} = \frac{K_{14}^{obs}}{[Mg^{2+}]^m}, \qquad (6)$$

where K_{12}^{obs} and K_{14}^{obs} are the values we will calculate from observed concentrations of M, D, and T. This may be rewritten as

$$\log K_{12}^{obs} = \log K_{12} + n \log [Mg^{2+}], \qquad (7)$$

$$\log K_{14}^{obs} = \log K_{14} + m \log [Mg^{2+}]. \qquad (8)$$

So the slopes of the lines in fig. 3A and 3B yield n and m, respectively.

The interesting point is that $m \approx 2n$ in this case. That is, we are finding that it takes about twelve Mg^{2+} together with four monomers to make a tetramer. But since about six Mg^{2+} are required to form every dimer from two monomers, the *whole magnesium-dependence appears to be in the monomer → dimer step.* To put it another way: if we calculate the constant $K_{24} = K_{14}/(K_{12})^2$ for the dimer → tetramer step, we will find it to be nearly independent of $[Mg^{2+}]$ with the values of n and m given above.

The value of $n = 6.6$ is quite close to the value of $n = 5$ found by Morimoto and Kegeles [4] for the dimerization of lobster hemocyanin. Turning now to the temperature studies, we see from fig. 4 that K_{12}

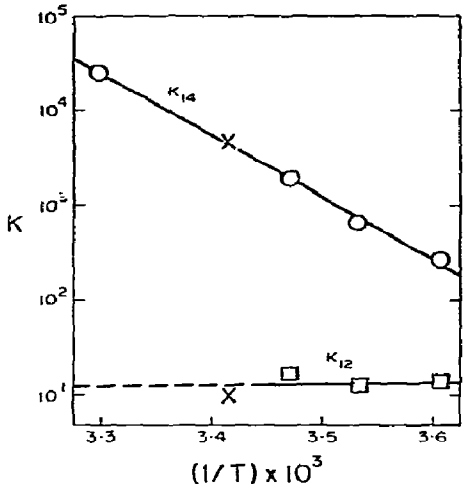

Fig. 4. A graph showing the variation with temperature of the logarithms of K_{14} (circles) and K_{12} (squares), both expressed in fringe units. All data are taken in phosphate buffer, pH 7.6, containing 0.05 M $MgCl_2$. The crosses denote values extrapolated to 0.05 M Mg^{2+} from the data in fig. 3. These two points should be treated with reservation however, because the pH and buffer conditions are slightly different.

and K_{14} behave very differently as the temperature is changed. Within the limits of error, K_{12} is independent of temperature; the reaction appears to have a very small enthalpy change. On the other hand, K_{14} is strongly temperature-dependent; calculation of ΔH° from the van't Hoff relationship yields 30 kcal for the formation of one mole of tetramer from four moles of monomer. But since ΔH° for the monomer–dimer step is virtually zero, *almost all of this enthalpy change must accompany the dimer → tetramer step.*

4. Discussion

The foregoing analysis has shown that, within the limitations due to error in these experiments, the two steps in the association of 17 S hemocyanin monomers to 39 S tetramers have the following characteristics:

2 M → D: requires about 6 Mg^{2+}; almost independent of temperature,

2 D → T : no Mg^{2+} required; strong temperature.

This suggest that the mechanisms of bonding between subunits in the first and second steps are quite different. Since the hemocyanins are very rich in aspartic and glutamic acid residues it may be that the 2M → D association involves bridging by Mg^{2+} between carboxylate groups on facing monomers. The "inverse" temperature dependence of the 2D → T step (high temperatures favoring association) suggests that hydrophobic interactions may play a major role in this process [10].

The information which has been gained in these preliminary studies will allow us to perform more accurate measurements, particularly on the monomer–dimer reaction. It is now clear that this reaction can be studied in the virtual absence of the complicating tetramer formation, simply by working at low temperature. On the other hand, if we carry out experiments at a very high Mg^{2+} concentration, and simply vary the temperature, we should be able to study a nearly pure dimer–tetramer reaction.

In any event, these experiments may give a first hint as to how the association is limited. It involves two stages, each of which is presumably stereochemically blocked from going past a dimerization, suggesting that in each case complementary surfaces of the molecules are involved. It is of interest that the association of monomers to dimers via the Mg^{2+}-dependent step is apparently a necessary prerequisite to the subsequent hydrophobic association. Note that in the absence of Mg^{2+}, no trace of *any* species larger than 17 S monomer is found even at room temperature. It is as if those surfaces involved in the dimer → tetramer association are either not large enough or rightly formed to cause the monomers to associate, or that the charge neutralization that must occur in binding Mg^{2+} removes an electrostatic barrier to association.

In any event, it is now clear that *Callianassa* hemocyanin is not so atypical as we has thought; it differs from other crustacean hemocyanins that have been studied principally in having the added dimer → tetramer reaction. Whether these two association–dissociation reactions, with their very specific and peculiar prerequisites, have any physiological function is unknown.

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