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Association–Dissociation Equilibria of *Octopus* Hemocyanin†

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ABSTRACT: The equilibria between the native (decameric) *Octopus* hemocyanin and its subunits were studied by analytical sedimentation. Equilibrium is obtained slowly, but the reaction is thermodynamically reversible. The mass action law for a monomer–decamer reaction is obeyed. The reassociated hemocyanin is virtually identical in its sedimentation behavior and oxygen binding with the native protein. The association–dissociation equilibria are mediated by cations; Mg^{2+}, Ca^{2+}, Na^{+}, and H^{+} are all effective in stabilizing the decameric form at appropriate concentrations. About three to four cations per monomer must be bound for association to occur. Under some conditions, dimers of the subunits can be observed, but formation of this dimer does not depend on cation concentration, and it does not appear to be an obligate intermediate in the association to decamer.

In a previous publication (Miller & van Holde, 1982), we have characterized the subunit structure of the hemocyanin from *Octopus dofleini*. In this paper, we shall describe studies of the association–dissociation equilibria of this protein.

The *Octopus* hemocyanin exists in the hemolymph as a 51S decamer of 11S subunits. Such decameric structures are common among the molluscan hemocyanins (van Holde & Miller, 1982; Ellerton et al., 1983). However, in cases investigated so far, attempts to reassociate the subunits to create decamers have not been quantitatively successful. For example, in his extensive studies of the *Helix pomatia* hemocyanin, Siezen (1974) found that 11S subunits reassociated very poorly, with the formation of numerous intermediate structures. Even with the relatively well-behaved *Loligo* hemocyanin, van Holde & Cohen (1964a) achieved no more than 75% reassociation. Similar results were obtained by Brouwer et al. (1978) with *Murex* hemocyanin. The reasons for these limitations are obscure but may relate to subunit heterogeneity (Siezen & van Driel, 1973; Brouwer et al., 1978). In any event, they signify that the dissociation of these hemocyanins is not wholly reversible, which invalidates most studies of its thermodynamics. This is unfortunate, for thermodynamic analysis of equilibria involving such large subunits and their aggregates would be of considerable general interest. Furthermore, fundamental studies of ligand binding are of uncertain validity if the thermodynamic state of the system is not well-defined.

Therefore, we were extremely pleased to note in the earlier studies that *Octopus dofleini* hemocyanin could be *quantitatively* reassociated from subunits by such simple treatments as the addition of divalent cations to the solution. This has prompted us to undertake a detailed study of the association states of this hemocyanin and the equilibria connecting these. This paper describes these experiments. The results provide a defined framework for the oxygen binding studies described in the following paper (Miller, 1985).

Materials and Methods

Preparation of the Hemocyanin Solutions. Hemolymph was taken from live, ethanol-anaesthesized octopi either by cannulation of the vena cava or by hypodermic extraction from the afferent branchial vein. The hemolymph was kept sterile and on ice for transportation to the laboratory, at which point it was centrifuged at low speed and 0.1% phenylmethanesulfonyl fluoride was added as a protease inhibitor. It was then stored at 4 °C; such samples appeared, from sedimentation studies, to be stable for at least 2 months.

For purification of the hemocyanin, the hemolymph was passed over an A5-M column, equilibrated with I = 0.1

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tris(hydroxymethyl)aminomethane (Tris)[1] (pH 7.65) containing 50 mM $MgCl_2$ and 10 mM $CaCl_2$. Fractions eluted near the void volume showed only the 51S hemocyanin in analytical sedimentation velocity experiments. All of the experiments described in this paper utilized material purified in this way. For many experiments, we used I = 0.1 Tris buffers prepared according to Long (1961) with added salts or EDTA. However, at pH values below 7.5, HEPES or PIPES buffers were employed; these were prepared by titrating 0.1 M HEPES or PIPES to the appropriate pH with NaOH.

Sedimentation Experiments. These were all carried out in a Beckman Model E analytical ultracentrifuge utilizing scanner optics. Wavelengths in the vicinity of either 280 or 345 nm (the absorption maximum for oxyhemocyanin) were employed. Most experiments were at 20 ± 2 °C, the temperature being controlled during each run to at least ±0.05 °C.

Sedimentation coefficient data were corrected to $s_{20,w}$ values by the conventional method. For some of the buffers used (HEPES, PIPES), relative viscosities were not available and were measured as required by using an Otwald viscometer.

In some experiments, relative amounts of components were measured from plateau levels on scanner traces. This was done only when boundaries were clearly resolved (see below). In each case, results from several scans were individually corrected for radial dilution, and the results were averaged. The Johnson–Ogston effect was judged to be negligible with the dilute solutions used (1–3 mg/mL), the large separation in sedimentation coefficient (11 S vs. 51 S), and the small dependence of s on C (K. I. Miller and K. E. van Holde, unpublished results).

Since the extinction coefficients of 11S and 51S components are different, because of differences in scattering and (at 345 nm) in the absorbance of the Cu band, it was necessary to correct for this in determining the fraction of 51S component from the scanner traces. We have measured the extinction coefficient ratio at pH 8.0 for decamer/monomer ($R = e_D/e_M$) at 345 and 280 nm and find these to be R_{345} = 1.24 and R_{280} = 1.10, respectively. The latter value is almost identical with that reported by van Holde & Cohen (1964b) for the comparable *Loligo* hemocyanin. With these values, the fraction of decamer is calculated as

$$f_D = \frac{A^*_D}{A^*_D + RA^*_M} \quad (1)$$

where A^*_D and A^*_M are the absorbances of decamer and monomer in the ultracentrifuge cell corrected for radial dilution.

In cases where we wished to express the hemocyanin concentration on an absolute basis, we have used as an extinction coefficient at 280 nm the value of 16.7 $(cm \cdot mg/mL)^{-1}$ reported for *Octopus vulgaris* by van Holde (1967). None of the substantive conclusions of this paper are significantly affected by any inaccuracy in this value.

Oxygen Binding. The O_2 binding was measured by the spectrophotometric tonometer technique described elsewhere (Miller & van Holde, 1974; Miller, 1985.)

Results

Association States of Octopus Hemocyanin. We begin with a series of sedimentation studies which describe those states of aggregation of *Octopus* hemocyanin which can be detected at various pH values and at high and very low concentrations of cations. As will be shown below, divalent cation concentrations in excess of 20 mM or sodium chloride solutions more concentrated than 500 mM will completely stabilize the associated form of the protein. All of these experiments have been conducted with the solution in equilibrium with air. This means that we are dealing with saturated or nearly saturated oxyhemocyanin in all association–dissociation reactions.

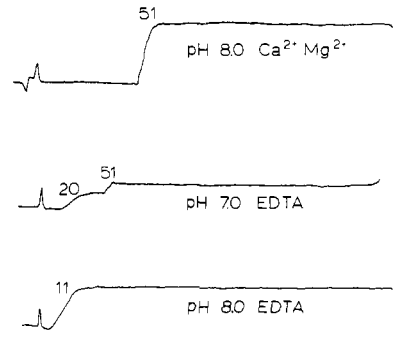

FIGURE 1: Representative scanner traces from sedimentation studies of *Octopus* hemocyanin under a variety of conditions. The pH and ionic conditions are indicated: Ca^{2+} Mg^{2+} corresponds to 10 mM Ca^{2+} and 50 mM Mg^{2+}. Those designated EDTA were performed in 10 mM EDTA. All experiments in I = 0.1 Tris-HCl.

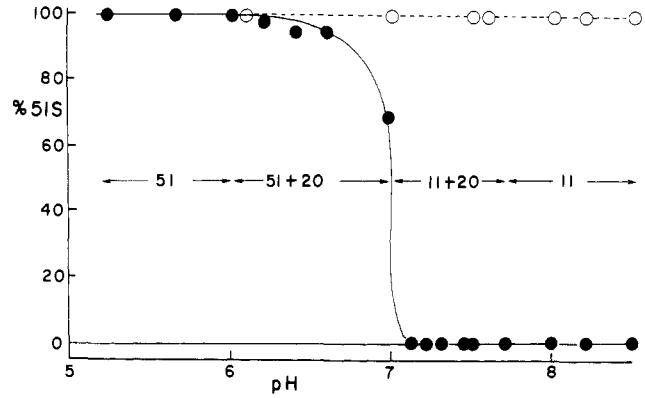

FIGURE 2: Percent of 51S hemocyanin as a function of pH. Open circles are data obtained in the presence of 10 mM Ca^{2+} and 50 mM Mg^{2+}. Closed circles correspond to solution dialyzed against 10 mM EDTA. The numbers between arrows indicate the components present in different pH ranges.

Figure 1 depicts representative scanner traces from these experiments. These show that under most conditions, the oxyhemocyanin exists either as the 11S subunit or as the 51S decamer of these units [see Miller & van Holde (1982)]. As is illustrated in Figure 2, the 51S particle is stable over the entire pH range from below 7.0 up to 9.5 when 10 mM $CaCl_2$ and 50 mM $MgCl_2$ are present. On the other hand, when divalent ion concentrations are made very low by dialysis of solutions against 10 mM EDTA, the behavior is more complex. Below pH 6, we find only the 51S component, but between pH 6 and ~pH 7.6, a second, more slowly sedimenting boundary is resolved as well (see Figures 1 and 2). This component has $s_{20,w} \cong 20$ S. We believe it to be comparable to the 19S form of *Loligo* hemocyanin, which was shown to be a dimer of 11S units (van Holde & Cohen, 1964b). Comparable aggregation states have been detected and characterized in other Molluscan hemocyanins [see van Holde & Miller (1982)]. We have not further characterized the 20S component from *Octopus*, for we have not as yet found conditions under which it is pure and uncontaminated by either 51S or 11S material.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Table I: Dissociation–Reassociation Experiment[a]

pH	$s_{20,w}$ (S): purified native hemocyanin in 50 mM Mg^{2+} and 10 mM Ca^{2+}	$s_{20,w}$ (S): after dialysis vs. 10 mM EDTA	$s_{20,w}$ (S): after redialysis vs. 50 mM Mg^{2+} + 10 mM Ca^{2+}
7.0	50.7	49.4 + 22.3 (resolved components)	50.3
7.5	50.1	20.7 (reaction boundary)	49.9
8.0	49.8	11.1	49.8

[a] All experiments were carried out in I = 0.1 Tris buffers at ~20 °C using a hemocyanin concentration of about 2.5 mg/mL.

Above pH 7.0 (in 10 mM EDTA), all traces of the 51S component vanish. Scanner traces obtained for hemocyanin dialyzed against buffers in the range 7.0 < pH < 8.0 typically show broad, partially resolved boundaries, suggestive of a fairly rapid equilibrium between 20S and 11S particles. As the pH increases, the fraction of more rapidly sedimenting material decreases; by pH 8.0, only a homogeneous 11S boundary can be detected (Figure 1). This remains as the stable component at higher pH (Figure 2).

Hemocyanin Subunits Can Be Quantitatively Reassociated by Addition of Divalent Ions. As we have reported (Miller & van Holde, 1982), addition of Mg^{2+} or Ca^{2+} in excess of about 10 mM results in complete reassociation of the subunits. As Figure 2 of that paper shows, there is no evidence for residual subunits after reassociation; all of the hemocyanin now sediments in a sharp, fast boundary. We have now compared the reassociated material with the native hemocyanin by two criteria: sedimentation coefficient and oxygen binding. In one set of experiments, purified 51S hemocyanin was dialyzed against 10 mM EDTA to produce partial or complete dissociation at pH values of 7.0, 7.5, and 8.0. The results are shown in Table I and as scans (for pH 7.0 and 8.0) in the lower two traces in Figure 1. At pH 7.5, a broad reaction boundary was observed. Upon dialysis of these solutions back to the original magnesium and calcium levels, boundaries like that shown in the top trace of Figure 1 were obtained in all three cases. As Table I shows, the sedimentation coefficients obtained after reassociation were within 1% of the original values. The values also agree to the same accuracy with our earlier studies of the concentration dependence of sedimentation coefficient for the 51S component. As a further check on the fidelity of reassociation, we have compared the oxygen binding of native and reassociated 51S components. As Figure 3 shows, the curves are nearly identical. The cooperativity in binding has been fully restored; the Hill coefficient is 1.7 in both cases. There does seem to be a small, reproducible difference in *affinity*, but this difference is close to the limit of reproducibility in separate binding experiments. It is possible that this difference is real and results from the loss of some low molecular weight allosteric modulator bound to the native molecule. The question requires further investigation. The main point, however, is that reassociation produces a molecule fully capable of the homeoallostery typical of the native structure.

Association of 11S Subunits To Form 51S Particles Is a Relatively Slow Process at pH 8.0. It is not necessary to dialyze solutions back to high divalent ion concentrations to cause reassociation. Direct addition of sufficient Ca^{2+} or Mg^{2+} to complex the EDTA and leave an excess of divalent ions suffices. Figure 4 depicts the time course of reassociation of 11S subunits into 51S decamers upon addition of magnesium ion. Under the conditions chosen, the reaction is slow enough that it can be followed, at least in part, by sedimentation velocity experiments. This is an important point, for it justifies our use, in subsequent experiments, of the sedimentation velocity technique to study the association–dissociation equilibria at this pH. In a rapidly associating–dissociating system,

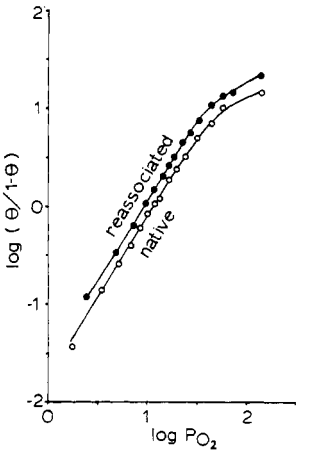

FIGURE 3: Hill plots of oxygen binding by purified native (○) and dissociated–reassociated (●) hemocyanin. Experiments were at 20 °C in 0.1 M HEPES buffer, pH 7.6, containing 370 mM NaCl, 45 mM $MgCl_2$, 10 mM K_2SO_4, and 8 mM $CaCl_2$ ("physiological saline"). The hemocyanin had been completely dissociated to 11S subunits by extensive dialysis against I = 0.1 Tris, pH 8.05, containing 10 mM EDTA before reassociation by dialysis against physiological saline. The binding by the subunits is noncooperative (Miller, 1985).

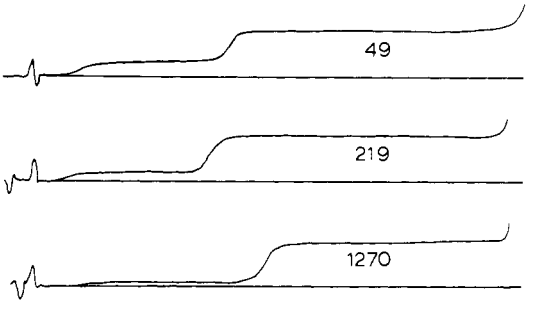

FIGURE 4: Slow reassociation of hemocyanin subunits in 8 mM Mg^{2+}. To dissociated hemocyanin in EDTA was added enough Mg^{2+} to yield this concentration. Sedimentation experiments were started at 49, 219, and 1270 min after addition of Mg^{2+}. The final scan (1270) corresponds to the equilibrium mixture under these conditions.

reassociation of material left behind in the slower monomer boundary can produce artifactual results, whereas if the reassociation reaction is slow, a preestablished equilibrium can be analyzed by determining the relative amounts of material in the two boundaries. It will be noted that in Figure 4 there is a nearly flat plateau region between the slower and faster boundary, indicating insignificant reequilibration during the run. This conclusion is reinforced by the observation that the slow and fast boundaries exhibit the appropriate sedimentation coefficients expected for the pure monomer and decamer at the given concentration. As indicated by Figure 4, hours are required for equilibrium to be established. Therefore, all solutions used in the equilibrium studies described below were allowed to equilibrate at 20 °C for at least 12 h before measurements were made.

Association–Dissociation Equilibrium Obeys the Law of Mass Action. At pH values in the vicinity of 8.0, the presence

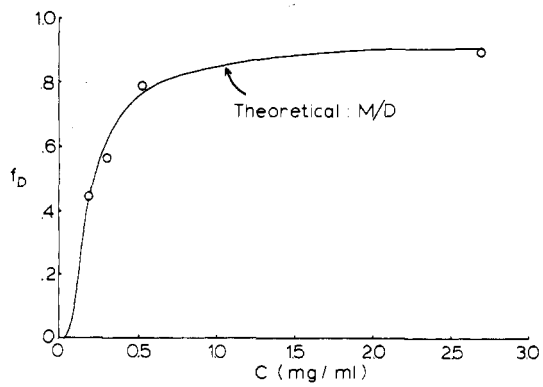

FIGURE 5: Hemocyanin dissociation–association equilibrium obeys the mass action law. The equilibrium fraction of 51S decamer (f_D) was determined at various total hemocyanin concentrations in 8 mM Mg^{2+}. The curve is a theoretical graph for a monomer–decamer equilibrium.

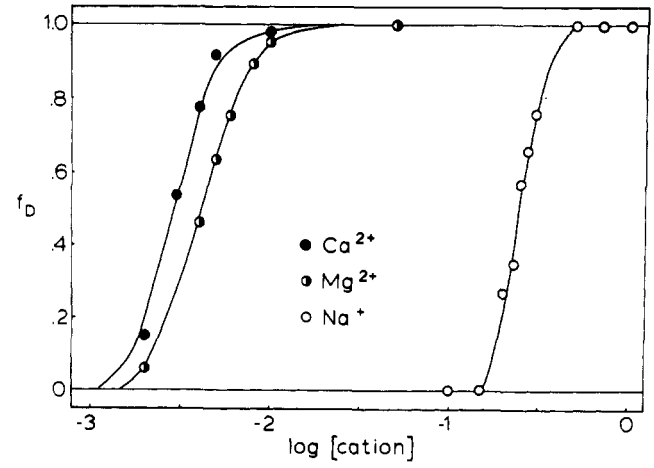

FIGURE 6: Equilibrium fraction of decamer at 20 °C as a function of ion concentration at a fixed hemocyanin concentration.

of low (1–10 mM) concentrations of Mg^{2+} yields mixtures of monomer and decamer which can be easily resolved in sedimentation velocity experiments. To test whether these are equilibrium mixtures, obeying the law of mass action, solutions containing 8 mM Mg^{2+} at several different hemocyanin concentrations were allowed to equilibrate and then analyzed by the sedimentation velocity method. Figure 5 depicts the results: As expected, dilution of the protein leads to increased dissociation, and the data can be fitted well by the theoretical curve for a monomer–decamer equilibrium. This result assures us that we may consider such mixtures to be in thermodynamic equilibrium.

Variation of the Monomer–Decamer Equilibrium with Ion Concentrations. Having established that we are dealing with a measurable, reversible process, we were able to carry out meaningful studies of the effects of various ions on the equilibrium. To this end, hemocyanin solutions were dialyzed against various concentrations of $CaCl_2$, $MgCl_2$, and NaCl. The divalent ion studies were conducted in solutions buffered with $I = 0.1$ Tris, at pH ≅8.2, a value at which the protein, in 10 mM EDTA, is entirely in the monomeric form. In the course of studies of oxygen binding by this hemocyanin [see Miller (1985)], it was discovered that high concentrations of NaCl would also stabilize the 51S form. We have therefore also included studies of the effect of NaCl on the monomer–decamer equilibrium at pH ≅7.7, the range in which the relevant O_2 binding studies were carried out.

The results of all of the association equilibrium experiments are presented in Figure 6. It is seen that each of the salts, $CaCl_2$, $MgCl_2$, and NaCl, modifies the monomer–decamer equilibrium over a particular concentration range. Since the Cl^- ion is common to all three, and the ranges of concentrations of the various salts at which the equilibrium is shifted are so very different, we conclude that the *cations* must mediate the association process, presumably via binding to the protein.

Since we are dealing with a reversible process, it is meaningful to calculate equilibrium constants from the decamer fractions obtained at each cation concentration by using eq 1. For a monomer–decamer equilibrium, the appropriate equation is

$$K = \frac{1}{C_0^9} \frac{f_D}{(1 - f_D)^{10}} \tag{2}$$

where C_0 is the total protein concentration. Because of the strong power dependence, the values of K are very sensitive to small errors, especially when $f_D \rightarrow 1$. We consider the general set of reactions in which a monomer (M) can bind m ligands with affinity constant k_M and a polymer (P) composed of n monomers can bind mn of the same ligands with affinity k_P. The unliganded monomer and polymer are assumed to be in equilibrium, with equilibrium constant K_0. The system can be wholly defined by the set of reactions:

$$n\mathrm{M} \overset{K_0}{\rightleftharpoons} \mathrm{P}$$

$$\mathrm{M} + \mathrm{L} \overset{k_M}{\rightleftharpoons} \mathrm{ML} \qquad \mathrm{P} + \mathrm{L} \overset{k_P}{\rightleftharpoons} \mathrm{PL}$$

$$\mathrm{ML} + \mathrm{L} \rightleftharpoons \mathrm{ML_2} \qquad \mathrm{PL} + \mathrm{L} \rightleftharpoons \mathrm{PL_2}$$

$$\mathrm{ML}_{m-1} + \mathrm{L} \rightleftharpoons \mathrm{ML}_m \qquad \mathrm{PL}_{mn-1} + \mathrm{L} \rightleftharpoons \mathrm{PL}_{mn}$$

If the binding to both monomer and polymer is assumed to be noncooperative, we can readily obtain an expression for the apparent equilibrium constant for association, K:

$$K = K_0 \left(\frac{1 + k_P[\mathrm{L}]}{1 + k_M[\mathrm{L}]} \right)^{mn} \tag{3}$$

This is the general result for such a system. Certain special cases are of interest. For example, if $k_M = k_P$, then $K = K_0$; there is no effect of ligand binding on the equilibrium. If $k_M = 0 \neq k_P$ and $k_P[\mathrm{L}] \gg 1$, we obtain

$$K = K_0 k_P{}^{mn} [\mathrm{L}]^{mn} \tag{4}$$

or

$$\log K = \log K_0 + mn \log k_P + mn \log [\mathrm{L}] \tag{5}$$

which is the expression most often used to describe such processes. The restrictive assumptions should be noted. This corresponds to a situation in which binding is possible only to the associated state and is strong. In such a situation, a straight line of slope nm will be obtained when log K is plotted vs. log [L]. However, more general situations will give approximately straight lines over a considerable region in such a plot. The question, then, is the following: What is the meaning of its slope? Differentiating eq 3 twice and taking the maximal slope (S_{max}), we find

$$S_{max} = mn \frac{k_M{}^{1/2} - k_P{}^{1/2}}{k_M{}^{1/2} + k_P{}^{1/2}} \tag{6}$$

Thus, if $k_P = 10^4 k_M$, we have $S_{max} = 99/101$ mn. Therefore, in such cases there is only a small error in taking the slope as mn. However, if k_P is not much greater than k_M and $k_P[\mathrm{L}] \gg 1$, a graph of log K vs. log [L] will be curved. Therefore, we conclude that in a system which yields a straight line for

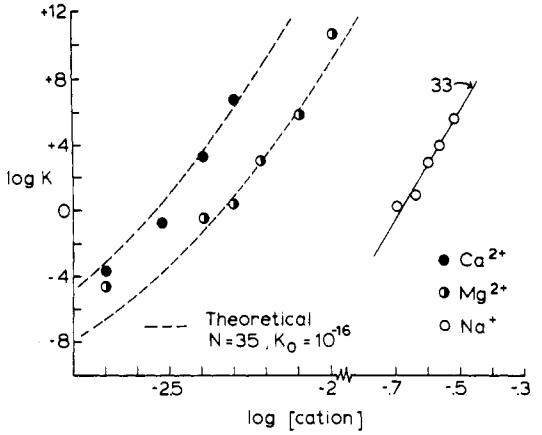

FIGURE 7: Equilibrium constants for the monomer–decamer reaction calculated from the data shown in Figure 5. The broken curves correspond to eq 8 with parameters as given. The slope of the Na^+ line (33) is also indicated.

log K vs. log [L] over a wide range, the slope quite accurately estimates nm, and hence m. Furthermore, the error will be one of *under*estimation.

In practice, it is very difficult to obtain reliable data over a range of [L] values sufficient to allow determination of all of the parameters in eq 3. This is particularly true with a monomer–decamer reaction, since the value of K is extremely sensitive to small errors in f_D, especially in the limit where $f_D \rightarrow 1$.

In Figure 7, we present graphs of log K_{obsd} vs. [L], where [L] is the molar concentration of Ca^{2+}, Mg^{2+}, or Na^+. At least for Ca^{2+} and Mg^{2+}, for which the most extensive data are available, it is clear that the lines are curved, indicating that eq 5 is not sufficient.

Therefore, we ask if a less restrictive model will fit the data. For example, suppose we assume that $k_M \ll k_P$. This corresponds to a situation in which the binding of cations is very much stabilized by the decameric structure. Then, in a range where $k_M[L] \ll 1$, eq 3 becomes

$$K \cong K_0(1 + k_P[L])^{nm} \quad (7)$$

$$\log K = \log K_0 + nm \log (1 + k_P[L]) \quad (8)$$

We have attempted to fit the data shown in Figure 7 to eq 8. First, we note that the maximum slopes of the three graphs in Figure 6 range between 30 and 33. Since, according to eq 6, mn should be slightly greater than this slope, we have estimated $mn = 35$. We then find that both the Ca^{2+} and Mg^{2+} data can be fitted by using a single value of K_0. This generates the curves drawn in Figure 6. We have not attempted to fit the Na^+ data in the same way, for they do not cover a sufficient range of values to reveal the expected curvature at low [L].

We do not claim that this fitting of the data is unique but only that it shows that the data are consistent with the uptake of 30–40 cations per decamer in the association process. This corresponds to three to four cations per monomer.

Discussion

The monomer–decamer equilibrium of the *Octopus dofleini* hemocyanin provides an unusual model system for the study of macromolecular association reactions. The dissociation of this hemocyanin is fully reversible and obeys the law of mass action. Salvato et al. (1979) have reported that dissociation of the *Octopus vulgaris* hemocyanin at pH >10.8 is not wholly reversible upon lowering the pH. We have not attempted dissociation and reassociation in this manner, for we feel that dissociation by removal of divalent ions at moderate pH is a much gentler treatment. Salvato et al. also report that the molecule can be reversibly dissociated in 3 M urea, although they do not give a quantitative measure of the efficiency of reassociation. Even if this method of dissociation does allow complete reassociation upon removal of the urea, simple cation removal would appear preferable to the use of a denaturant in studies of the functional behavior of subunits.

Our data indicate that approximately 35 cations are bound to the decamer that are only very weakly bound to the monomers. Since titration to a low pH (<7.0) also leads to association, we must conclude that hydrogen ions can play a role similar to that of Ca^{2+}, Mg^{2+}, or Na^+. Furthermore, the hydrogen ion induced transition is also very sharp (Figure 2), indicating that a substantial number of titratable groups are involved. We cannot, at this point, analyze the titration data as we have the cation binding data, for the association equilibria near pH 7 are complicated by the presence of dimer.

There are at least two ways in which cation binding might promote association of a macromolecule. The first is by formation of "bridges" between anionic groups (presumably carboxylates) on adjacent subunits. Alternatively, neutralization of anionic groups by cation binding could reduce electrostatic repulsion between subunits, allowing other forces to promote association. While the efficiency of Ca^{2+} and Mg^{2+} in stabilizing the decamer structure might seem to implicate bridging, the fact that protons or high concentrations of Na^+ ions can accomplish the same effect suggests that neutralization alone may be sufficient. If the groups are in fact carboxylates, the apparent pK_a (~7.0) is abnormally high. However, such an anomalous value might be anticipated in an environment such as the interface between strongly interacting subunits.

It is noteworthy that the 20S component, which we believe corresponds to a dimer, can be formed in EDTA solution at low ionic strength. It is only upon addition of cations that the decamer is formed. Therefore, it would seem at first glance reasonable to postulate that this dimer is an intermediate in the process of decamer formation. However, kinetic studies (unpublished results) indicate that the dimeric species observed at pH <8.0 is an *alternative* structure, not on the path to decamer.

Acknowledgments

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Oxygen Equilibria of *Octopus dofleini* Hemocyanin†

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ABSTRACT: Oxygen binding by *Octopus dofleini* hemocyanin was examined under very nearly physiological conditions. The effects of pH, ionic composition, temperature, and aggregation were controlled so that the role each plays in modulating oxygen binding can be isolated. There is a very large effect of pH on affinity, the Bohr effect ($\Delta \log P_{50}/\Delta pH = -1.7$), which is the same at 10 and 20 °C. However, cooperativity is substantially altered over the same range of pHs at the two temperatures. The allosteric properties were examined by comparing the experimental data points to curves generated by use of the Monod-Wyman-Changeux model. A computer-fitting process was developed which allowed the individual allosteric parameters to be varied independently until the best fit could be determined. The relationship between k_R and k_T is responsible for the effect of pH on cooperativity. A change in the allosteric properties of the T form is primarily responsible for the differences due to temperature. Changing cation concentrations when the molecule is in the fully aggregated 51S form alters affinity without influencing cooperativity. The effect of Mg^{2+} is much greater than that of Na^+. If the 51S decamer is dissociated to 11S monomers by removing divalent cations, oxygen binding is noncooperative. There is evidence for negative cooperativity, indicating heterogeneity of function within the subunit which contains seven oxygen binding domains. Association into decamers generates conformational change which results in a much wider range of allosteric function.

Until now, our work on *Octopus dofleini* hemocyanin has been limited to physical studies. We have characterized the subunit structure (Miller & van Holde, 1982), and in the preceding paper, we have described the association behavior of this respiratory protein (van Holde & Miller, 1985). This paper describes oxygen binding by *Octopus* hemocyanin.

Oxygen equilibria have been examined in considerable detail for *Octopus dofleini* by Lenfant & Johansen (1965), and recently for *Octopus vulgaris* by Houlihan et al. (1982). In both cases, the Bohr effect was controlled by using CO_2, and oxygen binding curves were measured using whole blood. In neither case were Hill plots prepared, so the allosteric behavior of the protein could not be evaluated. At the other extreme, Salvato & Tallandini (1977) and Tallandini & Salvato (1981) have carried out extensive studies on allosteric modulations in oxygen binding of *Octopus vulgaris* hemocyanin. However, much of this work was done under conditions in which the aggregation state was unknown or in which mixtures of different aggregation states were present. No attempt was made to duplicate physiological conditions. For this reason, we decided to examine the allosteric behavior of *Octopus dofleini* hemocyanin in a series of oxygen binding equilibria under very nearly physiological conditions, but with knowledge of and control over pH, temperature, ionic composition, and aggregation state.

Materials and Methods

Hemocyanin was collected and purified by gel filtration on an A5-M column as described in the preceding paper (van Holde & Miller, 1985). The column buffer was $I = 0.1$ tris(hydroxymethyl)aminomethane (Tris),[1] pH 7.65, containing 50 mM $MgCl_2$ and 10 mM $CaCl_2$. Oxygen binding curve studies were performed by using Tris buffers [prepared according to Long (1961)] with added salts; however, we found the temperature sensitivity of Tris buffers to be a liability when we wished to control pH very precisely. In these cases, we used 0.1 M HEPES or 0.1 M PIPES buffers, titrated to the desired pH with NaOH. Hemocyanin purified by gel filtration was used for most oxygen binding experiments, but for the two Bohr effect series, we used a concentrated hemocyanin solution obtained by sedimenting the hemolymph and removing the serum. A small amount of the resulting soft pellet of highly concentrated hemocyanin was added to buffer to obtain the desired concentration. This did not compromise the precision of these binding curves, since there is only one hemocyanin species present in the hemolymph (Miller & van Holde, 1982). For oxygen binding experiments under close to physiological conditions, the buffers were made up in a physiological saline which we prepared from published values of the major ions present in *Octopus* blood (Potts & Todd, 1965) and from a physiological saline developed for squid (Prosser, 1973). The composition of this saline was as follows: 370 mM NaCl, 45 mM $MgCl_2$, 10 mM K_2SO_4, and 8 mM $CaCl_2$.

Oxygen equilibria were measured by the tonometric method as described previously (Miller & van Holde, 1974). In order

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MWC, Monod-Wyman-Changeux.

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