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Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor*. Oxygen Binding Studies of Major Components†

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ABSTRACT: Oxygen binding curves have been obtained for unfractionated hemocyanin from *Cherax destructor* and its major components, the 25S and 17S forms. In all cases the binding was characterized by positive cooperativity at pH 7.8 with a P_{50} of ~4 mmHg and a Hill coefficient, n_H, of ~3. There was no evidence of concentration dependence of the binding curves in the range 0.6–6 mg/mL, a finding which excludes a dynamic equilibrium between polymeric forms of different oxygen affinity as a source of the cooperative binding. A positive Bohr effect operates between pH 6.8 and pH 7.8 and removal of calcium ions from the 25S and 17S aggregates markedly reduces their affinities for oxygen. Cooperativity is retained in these circumstances though n_H drops to about 2.5 in the case of the 25S and 2.0 in the case of the 17S form. The two major monomers M_1 and M_2, from which the 25S and 17S complexes are constructed, may be reconstituted into the hexamers $(M_1)_6$ and $(M_2)_6$. These show oxygen binding behavior perfectly consistent with that expected of native hexamers as studied in the 17S fraction, a mixed population of hexamers. The monomer M_1 can also be studied in monomeric form and was found to show indistinguishable oxygen binding at pH 7.8 and pH 10, the curve being a rectangular hyperbola as expected. The oxygen binding curve of the single subunit hexamer $(M_1)_6$ was fitted adequately by a polynomial expression of order 6 as required for a molecule with six binding sites. Further interpretation in terms of a particular binding model was not attempted because available knowledge of the structures of arthropod hemocyanin aggregates and their oxygen binding sites does not yet justify it.

The protein hemocyanin functions in the hemolymph of arthropods to transport oxygen, and the properties of this system are of considerable interest because of the possibility of a complex response arising from the presence of several aggregated forms of the protein, often assembled from a heterogeneous subunit population. For this reason many workers have investigated the oxygen binding of arthropod hemocyanins, some of them using whole hemolymph (Chantler et al., 1973; Loewe & Linzen, 1975; Wajcman et al., 1977) and others using isolated components as well as hemolymph (Sullivan et al., 1974; Terwilliger et al., 1979).

As a result of earlier studies (Murray & Jeffrey, 1974; Jeffrey et al., 1976, 1978), the composition of *Cherax destructor* hemolymph is well understood, at least in terms of its major hemocyanin components, the 17S and 25S aggregates, and the major subunits from which they are constructed. We now report some of the oxygen binding characteristics of *C. destructor* unfractionated hemocyanin and of components isolated from it. The study was undertaken with the aim of relating the oxygen binding behavior of individual aggregated components to their subunit compositions and quaternary structure and ultimately of relating the oxygen binding behavior of the mixture, as it occurs in the animal, to the proportions and properties of the protein species of which it is composed. The present paper documents our progress in this direction to date and discusses our results with *C. destructor* hemocyanin in the context of previous similar studies of other arthropod hemocyanins.

Materials and Methods

Preparation of Hemocyanin Constituents. Pooled serum was prepared from *C. destructor* hemolymph as described previously (Murray & Jeffrey, 1974) and stored under toluene at 5 °C. Individual components were made from the serum by column electrophoresis using the LKB 7900 Uniphor apparatus. The monomer M_1, M_2-rich hexamers, and the 25S components were prepared as described before (Jeffrey et al., 1978). The 17S material was also obtained from Uniphor experiments designed to prepare the 25S component, the first peak from the elution profile consisting of 17S and the second of 25S material. It was found possible to prepare small quantities of the monomer M_2 by column electrophoresis in the same manner as for M_1 and M'_3: the fractions eluting between the M_1 and M'_3 peaks were collected, concentrated, reapplied to the column, and electrophoresed.

Polyacrylamide Gel Electrophoresis and Densitometry of Gels by Scanning. The techniques were carried out as described previously (Jeffrey et al., 1978). Polyacrylamide gel analysis was used to determine the purity and state of aggregation of the solutions used for oxygen binding and to ensure that there was no change in the gel pattern before and after the oxygen binding experiment.

Oxygen Binding Measurements. Oxygen binding experiments were carried out in a tonometer similar to that used by Konings et al. (1969). Since ionic strength has an appreciable effect on the state of aggregation of some hemocyanin components, we have, in general, dialyzed solutions for oxygen binding experiments at an ionic strength of 0.2, with one exception, as described below.

Computing. A nonlinear search routine using the program Fittem, devised by Hay (1968), was used with a Univac 1108

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Table I: Characteristic Parameters of Oxygen Binding by *C. destructor* Hemocyanin Components at $I = 0.2$ and 25 °C

component and conditions[a]	protein concn (mg/mL)	P_{50} (mmHg)	Hill coeff n_H[b]
unfractionated hemocyanin			
pH 7.8 and 0.03 M $CaCl_2$	5.91–0.64	4.25	2.8
pH 6.8 and 0.03 M $CaCl_2$	3.48	13.75	2.7
25S			
pH 7.8 and 0.03 M $CaCl_2$	3.50	3.25	2.8
pH 6.8 and 0.03 M $CaCl_2$	2.90	13.00	2.4
pH 7.8 and 0.1 mM EGTA	3.47	22.00	2.5
pH 7.8 and 0.1 mM EGTA → pH 7.8 and no EGTA	3.65	17.50	2.4
17S			
pH 7.8 and 0.03 M $CaCl_2$	3.55	3.4	2.7
pH 6.8 and 0.03 M $CaCl_2$	3.23	8.0	2.1
pH 7.8 and 0.1 mM EGTA	3.49	15.5	1.8
M_2-enriched 17S			
pH 7.8 and 0.03 M $CaCl_2$	1.28	3.5	1.4
hexamers reconstituted from			
M_1, pH 7.8, and 0.03 M $CaCl_2$	3.59	4.0	3.2
M_1, pH 7.8, and 0.03 M $CaCl_2$ → pH 7.8 and 0.1 mM EGTA	3.33	27.5	2.2
M_1, pH 7.8, and 0.1 mM EGTA	3.47 and 3.63 treated together	22.5	1.5
hexamers, corrected for presence of M_1 monomer,[c] reconstituted from		25.0	1.4
M_1, pH 7.8, and 0.1 mM EGTA → pH 7.8 and 0.03 M $CaCl_2$	3.63	5.3	2.7
M_2, pH 7.8, and 0.03 M $CaCl_2$ / M_2, pH 7.8, and 0.1 mM EGTA	1.06 / 1.26 treated together	11.0	1.5
monomeric M_1			
pH 10.1 and 0.1 mM EGTA	2.66	8.1	1.1
pH 7.8, 0.1 mM EGTA at $I = 0.035$	3.44	9.5	1.1

[a] See Figure 2 for polyacrylamide gel electrophoresis patterns of most of these solutions. [b] The value of n_H was obtained by drawing a tangent to the Hill plot at P_{50}. The error in the resulting slope, i.e., n_H, is assessed to be about ±0.2. [c] A new curve can be constructed to obtain these values. The curve for M_1 (Figure 4) is hyperbolic, and at any pO_2 value the contribution due to the known percentage of M_1 in the $(M_1)_6$ sample can be obtained. This is subtracted from the pO_2 value on the experimental curve, and the value obtained is corrected to that for 100% $(M_1)_6$.

computer for curve-fitting operations.

Results

It should be mentioned that we now believe that there are at least two more monomeric subunits and one dimeric subunit [Jeffrey et al. (1980) and unpublished observations] than those described in previous publications (M_1, M_2, and M'_3). Some or all of these may be important in the control of oligomer assembly, but they are present in very small amounts compared with the three subunits already characterized and, so far, have not been purified. In the oxygen binding experiments discussed here, the major proteins of native hemolymph, the 17S and 25S components, and the two major monomers, M_1 and M_2, were studied.

Unfractionated Hemocyanin. The oxygen binding curve for unfractionated hemocyanin is shown in Figure 1. The solution was dialyzed against 0.05 M Tris[1] buffer, pH 7.8, containing 0.03 M $CaCl_2$ with NaCl to bring the ionic strength to 0.2.

To investigate concentration dependence, we performed experiments at protein concentrations of 5.91, 3.64, 3.47, and 0.64 mg/mL in the same buffer. The differences in the curves were within experimental error, indicating that a dynamic equilibrium between hemocyanin species could be ruled out as a source of the cooperative oxygen binding behavior in *Cherax* hemocyanin in this concentration range. Unless otherwise stated, further experiments were performed with protein concentrations of ~3.5 mg/mL. It is worth remembering that this is only about one-tenth of the hemocyanin concentration in arthropod hemolymph, and, where supplies of protein and suitable techniques are available, it would be of interest to examine the binding under conditions approximating more closely those in the animal. The solid line curve shown in Figure 1 is the best curve drawn through the results for all the concentrations used with unfractionated hemocyanin and gives a value of 4.25 mmHg for P_{50}, the oxygen pressure at 50% oxygenation. The curve shows the sigmoid form characteristic of a cooperative binding response. From the same data, the Hill plot gave a value of n_H, the Hill coefficient evaluated from the slope at P_{50}, of 2.8 ± 0.2.

Bohr Effect in Unfractionated Hemocyanin. Also shown in Figure 1 is the binding curve for unfractionated *Cherax* hemocyanin at pH 6.8 in 0.05 M Tris buffer of ionic strength 0.2 containing 0.03 M $CaCl_2$. Again, the curve is sigmoidal, but the value of P_{50} has been shifted to 13.75 mmHg, showing a large positive Bohr effect over the pH range 6.8–7.8. The value of n_H is 2.7, indicating no change in the degree of cooperativity.

The results obtained with various *C. destructor* hemocyanin components, studied separately and now to be discussed, are summarized in Table I.

25S Component. The shape of the oxygen binding curve for the 25S component at pH 7.8, 0.03 M $CaCl_2$, and $I = 0.2$ was very similar to that for the mixture of components in unfractionated hemocyanin, and, as may be seen in Table I, the values of P_{50} and n_H were essentially indistinguishable from those of the unfractionated sample. A positive Bohr effect of similar magnitude to that in the unfractionated material was found for the 25S component. These results are perhaps not unexpected since the 25S component accounts for ~50% of the hemocyanin in serum.

Effect of EGTA[1] on the 25S Component. Removal of calcium by dialysis of a 25S hemocyanin solution against 0.05

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

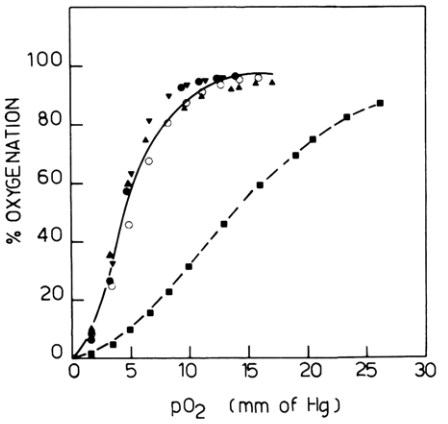

FIGURE 1: Oxygen binding curves for unfractionated *C. destructor* hemocyanin at 20 °C, $I = 0.2$, and 0.03 M $CaCl_2$. The upper, solid line and the points clustered about it are at pH 7.8 and (●) 0.65, (▼) 3.47, (▲) 3.64, and (○) 5.91 mg/mL hemocyanin. The lower, dashed line is for results at pH 6.8 and (■) 3.47 mg/mL.

M Tris buffer, pH 7.8, containing 0.1 mM EGTA with $I = 0.2$ also produced a marked change in the oxygen binding curve, but cooperativity was not lost, though the value of n_H dropped slightly to 2.5. Absence of calcium has therefore reduced the affinity of the molecule for oxygen, but cooperativity between the binding sites in the protein is retained. On polyacrylamide gels, the protein bands from solutions with calcium and with EGTA appeared identical.

It is possible that EGTA itself could have some intrinsic effect on the binding curve, acting either as an activator or inhibitor of oxygen binding. To test this, we dialyzed a solution of 25S hemocyanin first against 0.05 M Tris buffer, pH 7.8, containing 0.1 mM EGTA, with $I = 0.2$, and then against buffer at pH 7.8 with $I = 0.2$, containing no EGTA. This solution was used for an oxygen binding study. The curve obtained showed a small difference from the 25S plus EGTA curve but not enough to implicate an intrinsic effect of EGTA. It seems certain that the large change in the oxygenation curve produced by dialysis against EGTA is due to the absence of calcium and not the presence of EGTA.

17S Component. 17S material, consisting of M_1- and M_2-containing hexamers, isolated from unfractionated hemocyanin by column electrophoresis at pH 7.8 (see Figure 2a) gave sigmoidal oxygen binding curves in the $CaCl_2$-containing buffer, as shown in Figure 3. Although the value of P_{50} (Table I) is slightly different from that of unfractionated hemocyanin and 25S, no significance could be ascribed to this since the data, when plotted together, gave curves that were indistinguishable within experimental error.

Bohr Effect in the 17S Component. Again, a positive Bohr effect was obtained, but there seems to be a genuine difference from the unfractionated sample (which contains both 17S and 25S) and the 25S component in the values of both P_{50} and n_H at pH 6.8.

Effect of EGTA on the 17S Component. A marked effect of EGTA on oxygen binding is observed, as was the case with the 25S component. Further comment is made on this under Discussion. With the 17S component, as with the 25S, the gel patterns with and without EGTA were indistinguishable.

M_2-Enriched Hexamers. A higher proportion of M_2 in 17S material appears to alter the shape of the binding curve slightly, as shown in Figure 3, and the cooperativity is lower than in the native 17S fraction.

Reconstituted M_1 Hexamer. Hexameric 17S hemocyanin can also be reconstituted from monomeric 5S material (Jeffrey et al., 1976), and we have used this property to study the oxygen binding of a single hexameric form, $(M_1)_6$, produced from M_1 monomer. Dialysis of M_1 obtained at pH 10.1 against buffer (0.05 M Tris, pH 7.8, containing 0.03 M $CaCl_2$, $I = 0.2$) gives $(M_1)_6$ with only a trace of monomer M_1, as shown in the polyacrylamide gel photograph (Figure 2b), and such a solution was used for the oxygen binding experiment reported in Table I and shown in Figure 5. The oxygen binding characteristics of this $(M_1)_6$ hexamer are very similar to, but not identical with, those of the native hexameric 17S fraction, which is a mixture of M_1- and M_2-containing hexamers (Figure 2a).

Effect of EGTA on Reconstituted M_1 Hexamer. On dialysis of a solution of $(M_1)_6$, obtained as above, against EGTA-containing buffer with $I = 0.2$, the polyacrylamide gel pattern is indistinguishable from that before dialysis, indicating that no dissociation has taken place. Figure 5 shows that the oxygen binding curve is shifted to the right, the same effect of EGTA as in unfractionated hemocyanin, 25S, and 17S,

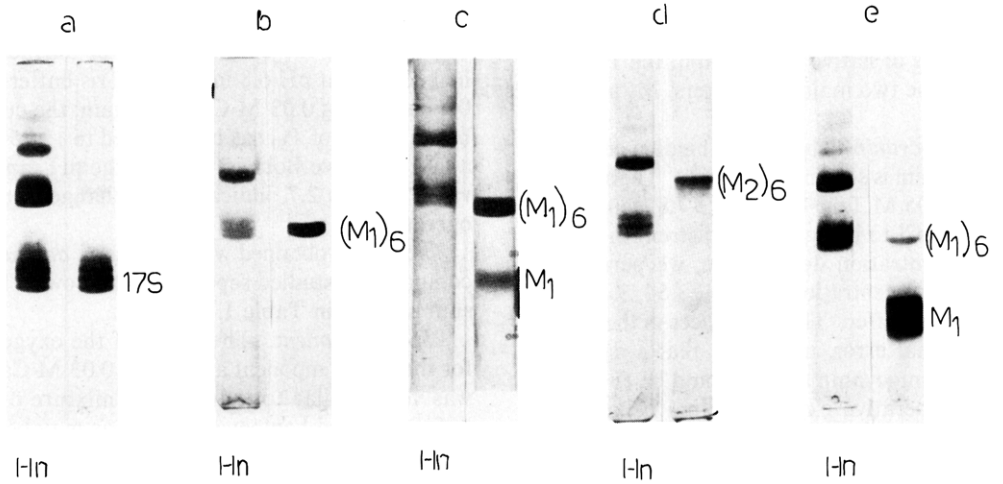

FIGURE 2: Polyacrylamide gel electrophoresis patterns for *C. destructor* hemocyanin fractions at pH 7.8. Each pattern is accompanied by one of unfractionated hemocyanin (Hn) run at the same time, to facilitate identification of the bands. The cathode is at the top and electrophoresis is from top to bottom. (a) 17S fraction. (b) The hexamer $(M_1)_6$ reconstituted from M_1 monomer by dialysis to pH 7.8 at $I = 0.2$ in the presence of 0.03 M Ca^{2+}. Only a trace of protein remains in monomeric form. (c) The hexamer $(M_1)_6$ reconstituted from M_1 monomer by dialysis to pH 7.8 in 0.1 mM EGTA at $I = 0.2$. About 10% remains in the monomeric form. (d) The hexamer $(M_2)_6$ reconstituted from M_2 monomer by dialysis to pH 7.8 in 0.1 mM EGTA at $I = 0.2$. Only a trace of protein remains in monomeric form. (e) The monomer M_1 dialyzed to pH 7.8 in 0.1 mM EGTA at $I = 0.035$. Virtually all of the protein remains monomeric.

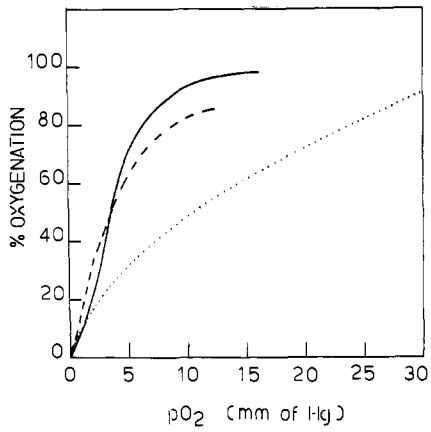

FIGURE 3: Oxygen binding curves for hexameric *C. destructor* hemocyanin fractions at pH 7.8, 20 °C, I = 0.2, and 0.03 M $CaCl_2$. The solid line is for the native 17S fraction (see Figure 2), the dashed line for a 17S fraction enriched with monomer M_2, and the dotted line for pure $(M_2)_6$ hexamers. See text.

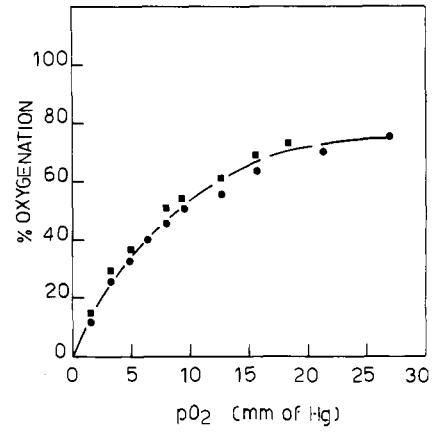

FIGURE 4: Oxygen binding results for monomeric *C. destructor* hemocyanin at 20 °C, 0.1 mM EGTA, (■) pH 10 and I = 0.2, or (●) pH 7.8 and I = 0.035. The protein concentration was 2.66 mg/mL at pH 10 and 1.26 mg/mL at pH 7.8, and the line is a mean through the points.

though the curve for $(M_1)_6$ is shifted even further to the right than in those cases.

Another way to convert monomer M_1 to the hexameric form is by dialysis to pH 7.8 at ionic strength 0.2 in the presence of 0.1 mM EGTA, that is, in the absence of calcium. Gel patterns (Figure 2c) show that the conversion to $(M_1)_6$ is not complete, M_1 to the extent of ~10% (as estimated by densitometry of gel patterns) remaining in the monomeric form. The values of P_{50} and n_H for such a solution are shown in Table I, together with those obtained by making corrections for the presence of M_1 as described in the footnotes of Table I. The curve obtained after applying these corrections more nearly corresponds to that for a solution of M_1 at pH 7.8 plus 0.03 M $CaCl_2$, with I = 0.2, dialyzed to pH 7.8 plus 0.1 mM EGTA, with I = 0.2, but the value of n_H is considerably lower.

Reconstituted M_2 Hexamer. Small amounts only of M_2 were obtainable from hemolymph, but it was possible to do an oxygen binding experiment at a concentration of 1.06 mg/mL in the usual $CaCl_2$-containing buffer at I = 0.2. A second experiment, also reported in Table I, at 1.26 mg/mL at pH 7.8 in buffer containing 0.1 mM EGTA, with I = 0.2, gave a curve so similar to the one from the solution containing $CaCl_2$ that they were considered indistinguishable, and the results were combined to obtain the reported values of P_{50} and n_H. The gel pattern for M_2 at pH 7.8 plus EGTA as used in the experiment is shown in Figure 2d and clearly indicates that the protein is in the hexameric form. The absence of any significant effect of EGTA on the oxygen binding behavior of $(M_2)_6$ is in marked contrast to the dramatic changes accompanying the removal of calcium which are seen in $(M_1)_6$, 17S, and 25S material. The binding curve of $(M_2)_6$ is shown in Figure 3, together with those of 17S and M_2-enriched 17S material in $CaCl_2$-containing buffers for comparison.

Subunit M_1. At pH 10.1 in buffer containing EGTA, with I = 0.2, the M_1 component of hemocyanin shows no tendency to hexamerize, so it was possible to do an oxygen binding experiment for M_1 alone under these conditions. The resulting curve is hyperbolic (Figure 4) with a P_{50} value of 8.5 mm of Hg, and the Hill plot gives a value of n_H of 1.09, as expected in view of the fact that arthropod hemocyanin monomers are known to contain only one oxygen binding site (van Holde & van Bruggen, 1971).

At low ionic strength (0.035) and in the absence of calcium ions, it is possible to obtain monomeric M_1 at pH 7.8. A small amount of $(M_1)_6$ is discernible on gels, as shown in Figure 2e, but estimations of the amount by scanning of the gels showed that this was <2%. The oxygen binding curve for monomeric M_1 at pH 7.8 shown in Figure 4 is very similar to that for M_1 at pH 10.1, being hyperbolic with P_{50} = 9.5 and n_H = 1.08.

Discussion

The oxygen binding experiments indicated that the *C. destructor* hemocyanin components used all showed positive cooperativity to some extent, with the exception of those which were in the monomeric state during the experiment, i.e., M_1 at pH 10.1 plus EGTA and M_1 plus EGTA at low ionic strength at pH 7.8. This is in agreement with the postulate of Klarman & Daniel (1977) that aggregated forms of arthropod hemocyanin have built-in cooperativity which is not dependent on the presence of divalent ions, unlike molluscan hemocyanins, where the presence of divalent ions is needed. In contrast, Miller & van Holde (1974) reported that 17S hemocyanin from *Callianassa californiensis* dialyzed against a divalent ion-free buffer exhibited a nearly noncooperative binding curve. We observed a slight reduction in the cooperativity of *Cherax* hemocyanin components when they were treated with EGTA to remove calcium ions. This may possibly be accounted for by the suggestion of Sullivan et al. (1974) that EGTA can bind to the copper in the active site. However, in the case of 25S hemocyanin, which was the only component we tested for an intrinsic effect of EGTA, n_H remained at about the same value even after the EGTA was removed by dialysis. The major effect of the removal of Ca^{2+} ions from aggregated components of *C. destructor* hemocyanin was to increase the P_{50} value dramatically as shown in Figure 5 for $(M_1)_6$ plus EGTA. This effect is not seen in the hexameric form of the M_2 monomer, and it seems likely that even in the native state this component does not contain calcium.

As noted under Results, removal of calcium ions with EGTA has a marked effect on the oxygen binding curves of both the 17S and 25S fractions, and, in general, a rather significant change in conformation accompanying the removal of calcium ions may be suspected. Actual dissociation of aggregates is apparently not involved since polyacrylamide gel electrophoresis patterns are identical in the presence and absence of EGTA at pH 7.8. Clearly, the effect of EGTA on the 17S and 25S components is quantitatively different as may be seen by comparing the magnitudes of changes in P_{50} and n_H (Table I). The 25S oligomers of *C. destructor* hemocyanin contain the dimeric subunit M'_3 (Jeffrey et al., 1978), and the effect of removal of calcium on their conformations would be expected to differ from that occurring in hexamers. However

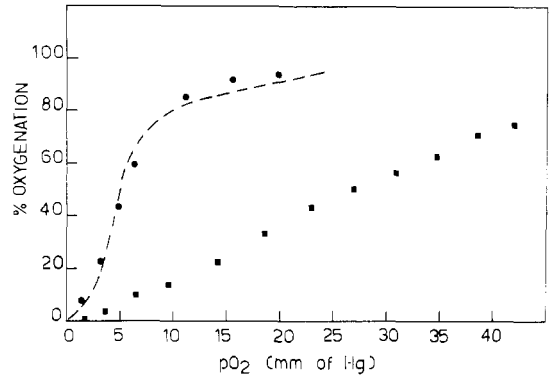

FIGURE 5: Oxygen binding curves for hexameric *C. destructor* hemocyanin at pH 7.8, 20 °C, and $I = 0.2$. Results for the reconstituted $(M_1)_6$ hexamer in 0.03 M $CaCl_2$ (3.6 mg/mL) are shown as filled circles, and those for the same material (3.3 mg/mL) after dialysis against 0.1 mM EGTA are shown as filled squares. The dashed line is a computer fit obtained as described in the text and was plotted from the equation $y = 1 + 0.00151x + 0.00264x^2 + 0.00976x^3 + 0.00473x^4 + 0.00032x^5 + 0.00002x^6$. The quantity x in the equation is the partial pressure of oxygen in millimeters of Hg, and the percentage oxygenation is obtained as $100y$/number of binding sites.

the presence of two different monomers, M_1 and M_2, in both hexameric and dodecameric oligomers must also play a role since their own aggregated forms respond so differently to the presence of calcium ions.

The 17S *C. destructor* hemocyanin aggregates formed from M_1 were obtained in two ways: by dialysis from pH 10.1 in EGTA to pH 7.8 in $CaCl_2$ or by dialysis to pH 7.8 in EGTA at an ionic strength of 0.2. These aggregates appeared identical on polyacrylamide gels. We found a difference in the n_H values in oxygen binding experiments for these two types of $(M_1)_6$ hexamers. The one formed in the presence of calcium ions has much higher oxygen affinity and a much higher degree of cooperativity than that formed without calcium (Table I).

Attempts were made at converting one form of the hexamer to the other at pH 7.8, either by dialyzing calcium into the preformed $(M_1)_6$ calcium-free hexamer or by removing it from the calcium-containing hexamer (by dialysis vs. EGTA). The oxygen binding characteristics of the calcium-containing hexamer formed by the two alternative routes (i.e., a one-step or a two-step procedure) are very similar, and the small differences observed can probably be attributed to differences in some subunits as a result of a different treatment, such as time of exposure to EGTA. The calcium-free hexamers show slightly greater differences, inasmuch as those formed by a one-step process, which have never been exposed to Ca^{2+}, show lower cooperativity than those from which the calcium has been removed by subsequent dialysis vs. EGTA. This may reflect a failure to remove all of the calcium ions by dialysis and/or modifications brought about by exposure to EGTA.

M_2 also forms hexameric aggregates, but much more readily than M_1, and is never seen on polyacrylamide gels as a monomer at pH 7.8, even at low ionic strength in the presence of EGTA, but is always hexameric. Hexamers formed from M_2 in the presence of calcium or of EGTA appeared identical on polyacrylamide gels and behaved so similarly with respect to oxygen binding that it is not possible to conclude with confidence that there is a real difference. Indeed, it may be that in the mixture of hexamers present as the 17S component in native hemolymph the $(M_2)_6$ oligomer does not contain Ca^{2+} ions. The preceding observations would all be consistent with such a postulate. It is known that the proportions of M_1- and M_2-containing hexamers in native serum can vary from animal to animal, some gels of serum from individuals showing quite high proportions of the M_2-containing hexamers and including a band corresponding to $(M_2)_6$ (Jeffrey et al., 1976). Hexamers reconstituted from M_2 certainly bind oxygen differently from those reconstituted from M_1 and are assumed to be similar to $(M_2)_6$ hexamers present in the native 17S material.

Thus, differences between the native 17S material and reconstituted $(M_1)_6$ may be qualitatively explained by the presence in native 17S material of M_2-containing hexamers. The n_H values for $(M_2)_6$ and M_2-enriched hexamers are lower than those for native 17S and $(M_1)_6$, and the binding curves show a consistent progression, as seen in Figure 3.

Investigation of the effect of pH on the oxygen binding behavior of *C. destructor* hemocyanin revealed that in all of the components studied a positive classical Bohr effect operates between pH 6.8 and 7.8 (Table I). This is characteristic of the majority of arthropod hemocyanins studied. A further effect of pH on oxygen binding has been reported by Miller & van Holde (1974) for *Callianassa* hemocyanin. In this protein the value of the Hill coefficient is seen to vary with pH, and the authors were able to explain their observations in terms of the Monod–Wyman–Changeux model (Monod et al., 1965) as extended by Rubin & Changeux (1966) and Buc et al. (1973) by postulating the presence of a significant proportion of hybrid RT molecules. We explored the possibility of observing the effect of pH on the binding characteristics of a *C. destructor* $(M_1)_6$ hexamer over an extended pH range to check for similar behavior in *C. destructor* hemocyanin. Polyacrylamide gel patterns showed, however, that the proportion of M_1 present in monomeric form, although negligible at pH 7.8 and below, increased with pH so that any oxygen binding curves obtained would not be directly comparable for the purpose of plotting a log P_{50}–n_H graph for $(M_1)_6$ alone. Monomeric M_1 has a very different binding curve from the hexameric form, and it would be possible in principle to correct the curves for the presence of monomer. In practice, the errors in the correction together with the experimental error in measuring P_{50} are comparable in size with the effect we would expect, and the study has not been pursued.

Direct comparison of the oxygen affinities of *C. destructor* hemocyanin components with those found by other workers for hemocyanins from other species is not always possible, since the conditions used by different workers vary. For example, the concentration of $CaCl_2$ is found to alter the P_{50} value (Miller & van Holde, 1974; Kuiper et al., 1975), and temperature also has an effect (Wajcman et al., 1977), while some workers have used a lower pH than that of 7.8 used in the present work [e.g., Redmond (1962)].

Choosing conditions as nearly equivalent as possible (20 or 25 °C and pH 7.55 or 7.8 in the presence of $CaCl_2$) it can be seen that P_{50} covers a large range of values from 2.5 mmHg for whole hemocyanin from *Homarus americanus* (Pickett et al., 1966) to 40 mmHg for *Cancer magister* hemolymph (Wajcman et al., 1977). *Penaeus setiferous* hemocyanin, consisting only of 17S material, with a P_{50} of 5.6 mmHg (Brouwer et al., 1978), *Cardisoma guahumi* hemocyanin (Redmond, 1962) with a P_{50} of 3.5 mmHg, and the monomeric subunits from *Eurypelma californicum* (Decker et al., 1979) with P_{50} values in the approximate range of 3–6 mmHg (0.4–0.8 kPa) show oxygen affinities in the same range as those for *C. destructor*.

While undoubtedly in some of these cases the hemocyanin used is homogeneous with respect to subunits, in others there is the possibility of microheterogeneity within the molecule, so that the properties observed must be considered as an overall

average for the several species of molecule present in the system.

The binding model proposed by Monod et al. (1965) provides a convenient framework for discussing the characteristics of the binding of oxygen to hemocyanin molecules and is often invoked in the literature, but it should be remembered that binding curves in themselves provide no information about the mechanism of the reaction being investigated. An alternative approach which is not restricted to a particular model is that described by Magar et al. (1971). The binding data are described in a quite general formulation by a polynomial relationship between the binding function J (= moles of ligand combined per mole of protein) and the concentration of ligand, [A], of the form

$$J = \frac{k_1[A] + 2k_1k_2[A]^2 + \ldots + nk_1\ldots k_n[A]^n}{1 + k_1[A] + k_1k_2[A]^2 + \ldots + k_1k_2\ldots k_n[A]^n}$$

The k's are the stepwise equilibrium constants for the binding of successive ligand molecules to the protein acceptor, and n is the number of binding sites. In terms of the parameters we have measured for the hemocyanin oxygen binding curves, namely the fractional saturation of sites, x, and the partial pressure of oxygen pO_2, $J = nx$, and $[A] \equiv pO_2$. In their paper Magar et al. (1971) describe how such data may be fitted by a single polynomial, and we have followed their procedure in fitting the binding curve of oxygen to a single *Cherax* hemocyanin component, the hexamer $(M_1)_6$ reconstituted at pH 7.8, and $I = 0.2$ in the presence of 0.03 M $CaCl_2$.

Several fitting protocols were tried with the computer program employed. The 25 data points were fitted to sixth-order polynomials with either the coefficients allowed to take positive and negative values or constrained to positive values only. The same procedure was followed with cubic polynomials. The result of the best fit gave the reconstructed binding curve shown in Figure 5, and it is reassuring to note that the polynomial was of sixth order with positive coefficients, since this is appropriate for binding to a molecule with six sites, as is to be expected for the $(M_1)_6$ hexamer. Further interpretation of the binding curves in terms of a specific model and microscopic binding constants is not justified at this time. As Magar & Steiner (1971) show, it may sometimes be possible to distinguish between models [for example, those of Koshland et al. (1966) and of Monod et al. (1965)] on the basis of certain relationships between the coefficients of the polynomial fitted to the binding data, but only when the errors in the coefficients are small and within well-defined limits. The conclusions of those workers were based on the available equations for tetrameric models, but comparable relationships have not been derived for hexamers as yet. It is clear, however, that the situation will be more difficult the more subunits are involved, since the complexity of the models increases and it becomes harder to fit satisfactorily the relevant high-order polynomials. Perhaps it is worth emphasizing that although discussion of binding results, such as those obtained with hemocyanins, in terms of a particular model can have heuristic value and may also be useful for comparative purposes, one should be careful that the model chosen does not become established by default. Definitive interpretation of binding events requires an immense amount of information as the analysis of the binding of oxygen by hemoglobin has shown. It seems that realistic postulates in terms of models described at the molecular level are generally feasible only in conjunction with knowledge of protein structure at the same level. We may expect that when such information is available for arthropod hemocyanins it can be combined with binding data of the type presented here to provide a coherent and detailed picture of how these complex systems carry out their physiological roles.

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