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Aggregation Patterns in *Cherax destructor* Hemocyanin: Control of Oligomer Distribution by Incorporation of Specific Subunits†

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ABSTRACT: Recent polyacrylamide gel electrophoresis studies on *Cherax destructor* hemocyanin have demonstrated the presence of three further constituent fractions in the alkaline dissociation product in addition to the three subunits reported in earlier work. Two of these recently discovered subunits are monomeric with molecular weights around 75 000, while the third subunit is of similar size to the previously identified dimeric subunit M3′ with a molecular weight near 150 000. The aggregation process is influenced by the presence of calcium ions, particularly in the distribution of hybrid hexameric species. However, the relative proportions, as well as the types of subunits present initially, are of primary importance in determining the oligomer distribution pattern obtained upon reconstitution from alkaline pH to pH 7.8 of selected mixtures of subunits. An additional significant factor in the assembly process has been proposed: the operation of different relative rates of aggregation between different types of subunits. Reconstitution experiments based on these findings substantially explain the complex distribution of oligomeric forms in *C. destructor* hemolymph.

Several recent publications have discussed the subunit heterogeneity of arthropod hemocyanins. Commonly, these hemocyanins are composed of several different subunits of similar molecular weight. For example, hemocyanin from the scorpion *Androctonus australis* has been shown to contain eight different polypeptide chains of molecular weight ~75 000 (Jollès et al., 1979; Lamy et al., 1979a; Markl et al., 1979), and hemocyanin from the spider *Eurypelma californicum* (Lamy et al., 1979a; Markl et al., 1979) seven constituent polypeptide chains. Some other arthropods, studied in less detail, are known to have five or six subunits in their hemocyanins (Markl et al., 1979).

In earlier studies of *Cherax destructor* hemocyanin, three subunits were identified, two monomers of molecular weight about 75 000 and a dimer of molecular weight ~150 000 (Murray & Jeffrey, 1974). Subsequently the role of these subunits in formation of the polymeric aggregates was studied (Jeffrey et al., 1976, 1978). More recently the existence of a fourth subunit has been demonstrated (Jeffrey et al., 1980), while in the present paper we report the identification of two more subunits. The involvement of certain specific subunits in the control of oligomer formation in arthropod hemocyanins is already clear in some instances. Thus, it is known in *Limulus polyphemus* hemocyanin that fraction V is required to form structures larger than 16 S (equivalent to 6 monomers) and fraction IV to form 60 S (equivalent to 48 monomers) from 34 S (equivalent to 24 monomers) (Bijlholt et al., 1979), in *Androctonus* hemocyanin that subunit 1—a heterodimer—is required to form 34 S (Lamy et al., 1977), in *Eurypelma* hemocyanin that subunit 4D—also a heterodimer—is required to form 34 S (Markl et al., 1979), and in *Cherax* hemocyanin that M3′—a homodimer—is required to form 25 S (equivalent

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to 12 monomers) (Jeffrey et al., 1978). In the case of *Cherax* the detection of more subunits implies that some amplification of the details of the previous discussion of the aggregation behavior is necessary. The major emphasis of the present work, though, is on understanding the roles of the *C. destructor* hemocyanin subunits in the formation of its various oligomers. In considering factors which could give rise to the observed distribution of the oligomers, the experiments to be reported were designed to take into account possible differences in the rates of assembly of different subunits as well as their compositional heterogeneity.

Materials and Methods

Preparation of Hemocyanin Constituents. Serum was prepared from *C. destructor* hemolymph as described previously (Jeffrey et al., 1976) and stored under toluene at 5 °C. All of the hemocyanin components discussed here were isolated from serum pooled from several animals.

The 17S and 25S components were separated by preparative electrophoresis on an LKB 7900 Uniphor apparatus using a 4% polyacrylamide gel in 0.025 M Tris,[1] pH 7.8, buffer, with crosswise pumping of anode and cathode buffers from a reservoir containing 1.5 L of the same buffer. Up to 70 mg of protein in 1.5 mL, dialyzed against buffer, was applied to the column and electrophoresis performed for ~8 h at 230 V for a 10-cm column.

The $M1_1$, $M2_1$, $M2_2$, and M3′ subunits were separated similarly on the LKB 7900 Uniphor apparatus but by using a 5% polyacrylamide gel in 0.025 M glycine, pH 10, buffer, containing 0.1 mM EGTA. The hemocyanin sample had been predialyzed for 24 h against the 0.025 M glycine, pH 10, buffer containing 0.1 mM EGTA before being applied to the column. The $M1_1$ subunit could be obtained essentially pure from one run on the column, but enriched $M2_1$ and $M2_2$ fractions and pure M3′ subunit could only be obtained on a second run. The $M2_1$ and $M2_2$ subunits could not be obtained as completely homogeneous preparations.

Reassembly Procedure. The reconstitution to oligomers of different mixtures of the various components in 0.025 M glycine, pH 10, buffer containing 0.1 mM EGTA was effected either in a one-step procedure directly into 0.05 M Tris buffer, pH 7.8, containing 0.03 M $CaCl_2$ (I = 0.2 with NaCl) or a two-step procedure into the pH 7.8, 0.1 mM EGTA–Tris buffer (I = 0.035) and then to pH 7.8, 0.03 M $CaCl_2$ (I = 0.2 with NaCl) with 24-h dialysis at each step.

Polyacrylamide Gel Electrophoresis. All experiments were carried out at 20 °C in a water-cooled Buchler apparatus using columns 7 cm in length and continuous buffer systems. The acrylamide to *N,N′*-methylenebis(acrylamide) ratio was 40:1, and the gels were polymerized with 0.1% *N,N,N′,N′*-tetramethylenediamine and 0.067% ammonium persulfate. At pH 7.8 the buffer was 0.05 M Tris, adjusted to pH 7.8 with HCl, and gels of 4% acrylamide were used. At pH 10 the buffer was 0.05 M glycine adjusted to pH 10 with NaOH and containing 0.1 mM EGTA. In the latter case gels of 6% acrylamide were used. Protein (5–20 μg) in 10% glycerol was loaded on the gels, and electrophoresis carried out at 2 mA/gel for 10 min, followed by 5 mA/gel for 1.5 h in the case of pH 10 electrophoresis or 2 h for pH 7.8 electrophoresis. Gels were stained for 1 h with 0.05% Coomassie Brilliant Blue in 25% 2-propanol–10% acetic acid and then covered with 7% acetic acid overnight. The gels were destained in a Canalco horizontal destainer.

Molecular weight determinations were made at pH 10.1 in buffer containing 0.1 mM EGTA with the use of Ferguson (1964) relationship according to the procedure of Hedrick and Smith (1968):

$$\log R_f = \log (R_f)_0 + K_R T$$

R_f is the mobility of a protein relative to that of a tracking dye, T is the total gel concentration, K_R is the retardation coefficient, and $(R_f)_0$ is the R_f value at a gel concentration of zero. Gels of acrylamide concentration between 5.5 and 8.5% were used. The standard proteins used for calibration were bovine serum albumin monomer, dimer, and trimer.

Results

Subunit Composition of C. destructor Hemocyanin. Whole serum *C. destructor* hemocyanin consists predominantly of the 17S and 25S components with small traces of 29S and possibly 35S bands in the gel electrophoresis pattern. Alkaline dissociation of the whole serum hemocyanin results in a number of electrophoretically distinguishable subunits (Figure 1). Of these subunits, three are in high amount and are identifiable with the M_1, M_2, and M_3' subunits described in previous papers (Murray & Jeffrey, 1974; Jeffrey et al., 1976, 1978), now denoted as $M1_1$, $M2_1$, and M3′, respectively. The three new subunits reported here, $M1_2$, $M2_2$, and M4′, are present in smaller amounts. From the molecular weight determinations of ~70 000 for the former two and ~135 000 for the latter, it can be concluded that $M1_2$ and $M2_2$ are monomers and that M4′ is a dimer. The values obtained for $M1_1$ and M3′ in these experiments were 75 000 and 160 000, respectively, while previous equilibrium sedimentation determinations gave values of 74 700 for $M1_1$ (Murray & Jeffrey, 1974) and 144 000 for M3′ (Jeffrey et al., 1978). In view of the agreement for these subunits, the values given above for the newly reported subunits are regarded as being in the appropriate ranges.

There is good evidence that the subunits newly discussed in this paper are genuine constituents of *C. destructor* hemocyanin, not artifacts of the preparative procedure or products of proteolysis. Thus, first, they always appear in polyacrylamide gel electrophoresis patterns of freshly prepared serum. Second, immunoelectrophoresis experiments (Jeffrey et al., 1980) have characterized $M1_1$, $M2_1$, $M2_2$, and M3′ as being antigenically distinguishable species. This finding would not be expected if some constituents were simply degradation products of others. Signs of a fourth monomeric constituent (which could correspond to $M1_2$) were also seen in the immunological studies. Third, M4′ is not obtained when the 17S fraction is dissociated and is evidently not generated by any breakdown and subsequent recombination of monomeric subunits. Its presence in the 25S fraction is consistent with the known assembly roles of dimeric components of several other hemocyanins.

The 17S and 25S components can be separated from whole serum hemocyanin by preparative electrophoresis. Subsequent alkaline dissociation of the individual components gives the different subunit compositions shown in Figure 1. As demonstrated previously (Jeffrey et al., 1976), the 17S component shows no dimeric subunits on dissociation. It dissociates to produce predominantly $M1_1$, a trace of $M1_2$, and small, almost equal amounts of $M2_1$ and $M2_2$. Dissociation of the 25S component results in production of the same subunits as above but now with $M2_1$ in significantly greater proportion than $M2_2$ and with the notable addition of the dimeric subunits M3′ and M4′.

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

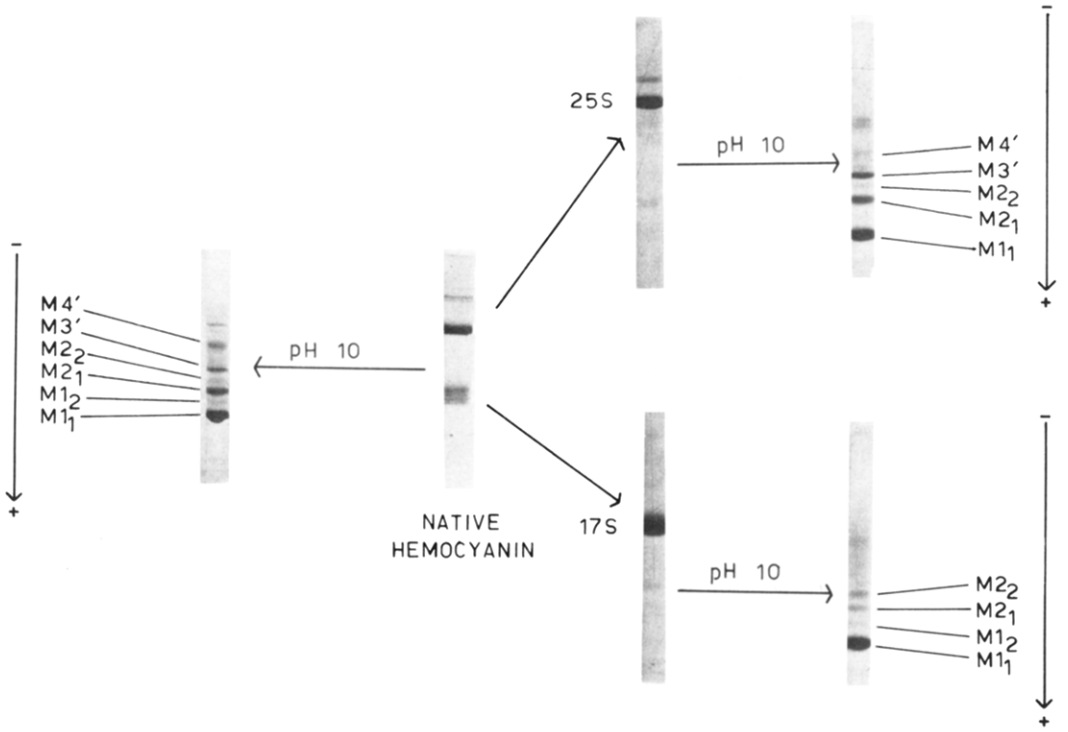

FIGURE 1: Polyacrylamide gel electrophoresis patterns of native *C. destructor* hemocyanin and the individually separated 17S and 25S components run in 0.05 M Tris, pH 7.8. The gel patterns of the respective alkaline dissociation products of hemocyanin serum, 17S components and 25S components run in 0.05 M glycine, pH 10, containing 0.1 mM EGTA are also shown. The individual subunits $M1_1$, $M1_2$, $M2_1$, $M2_2$, M3′, and M4′ that have been identified are marked. The direction of electrophoresis is indicated by the vertical arrows.

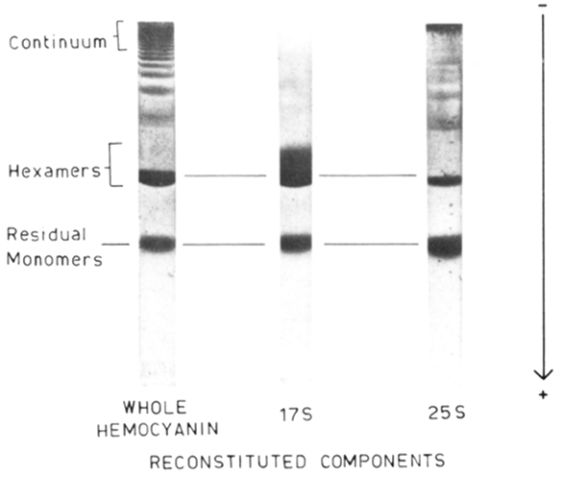

FIGURE 2: Polyacrylamide gel electrophoresis patterns of the one-step reconstitution (0.03 M Ca^{2+}, pH 7.8) products following alkaline dissociation of the whole serum, 17S components and 25S components of *C. destructor* hemocyanin. The direction of electrophoresis is indicated by the vertical arrow. It is noteworthy that reconstitution of these unfractionated mixtures of subunits produces oligomer distribution patterns notably different from the original patterns (cf. Figure 1).

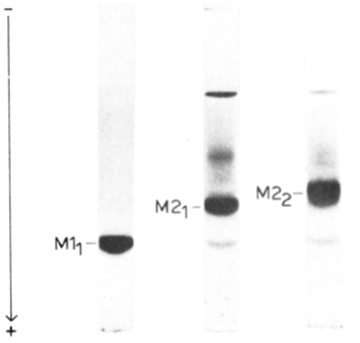

FIGURE 3: Polyacrylamide gel electrophoresis patterns at pH 10 in 0.05 M glycine buffer containing 0.1 mM EGTA of the fractionated monomeric subunits $M1_1$, $M2_1$, and $M2_2$. Equal loadings of 13 μg were used in each case. The slight contamination of the M2 fractions with $M1_1$ and the resulting presence of traces of aggregates do not affect the interpretation of reconstitution experiments with these fractions. The direction of electrophoresis is indicated by the vertical arrow.

Whole serum, the 25S component, and the 17S component were reconstituted via the one-step method after alkaline dissociation. The gel electrophoresis results for the three different reaggregation patterns are shown in Figure 2. Reconstitution of alkali-dissociated whole serum hemocyanin results in hexamers corresponding to the 17S component but strongly biased to the fastest running hexamer, that is, $(M1_1)_6$ (Jeffrey et al., 1976), and a series of discrete bands cathodic to the 25S position, forming a continuum to the origin [see also Jeffrey et al. (1978)]. The pattern is very similar to that obtained upon reconstitution of alkali-dissociated 25S component, except in the former case there is a stronger representation of the slower bands in the continuum. By contrast, in the case of reconstitution of 17S component, there are no higher aggregates than the hexamer bands, as would be expected from the absence of dimeric M3′ or M4′.

Reconstitution Experiments with Monomeric Subunits. Figure 3 shows the polyacrylamide gel electrophoresis patterns of the separated monomeric subunits, $M1_1$, $M2_1$, and $M2_2$. Dialysis of $M1_1$ solutions or $M1_1/M1_2$ mixtures from pH 10 to pH 7.8 at low ionic strength in the absence of divalent cations results in very little reaggregation to hexameric components. However, upon subsequent dialysis into pH 7.8 buffer containing Ca^{2+} ions or upon direct dialysis from pH 10 to pH 7.8 buffer containing Ca^{2+} ions (one-step procedure), ~90% of the subunit is polymerized to the hexamer (as estimated from the relative areas of Sephadex G-200 elution patterns of the monomer and hexamer after reconstitution experiments).

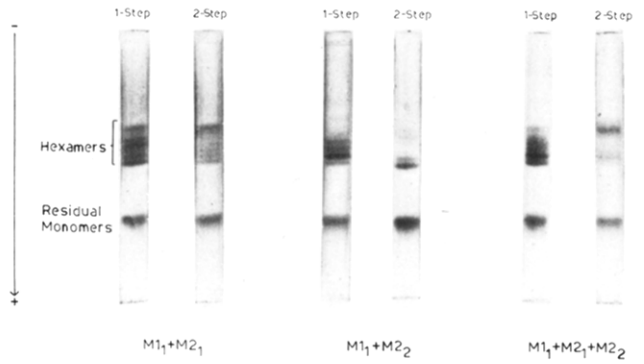

FIGURE 4: Polyacrylamide gel electrophoresis patterns of one-step and two-step reconstitutions into 0.05 M Tris, pH 7.8, containing 0.03 M $CaCl_2$ of *C. destructor* hemocyanin $M1_1$ and M2 subunit mixtures. In the mixtures with two types of subunit, the weight concentration ratio of $M1_1$ to $M2_1$ and $M2_2$, respectively, was 2:1. The ratio of weight concentrations $M1_1$:$M2_1$:$M2_2$ was 4:1:1. The direction of electrophoresis is indicated by the vertical arrow.

In contrast, by use of $M2_1$ or $M2_2$, any lowering of the pH below 9, even at low ionic strength and in the absence of divalent cations, results in polymerization to hexamers. Thus, it was expected that reconstitution of mixtures of $M1_1$ with M2-type monomers would give different hexamer distributions dependent upon whether the one-step or two-step dialysis is performed. That this is true can be seen in Figure 4 where gel electrophoresis patterns are shown for reconstitutions of $M1_1$ with $M2_1$, $M1_1$ with $M2_2$, and $M1_1$ with both $M2_1$ and $M2_2$. In all three cases, the one-step reconstitution gives a more even distribution of hybrid $M1_1$–$M2_1$–$M2_2$ hexamers than the two-step reconstitution. In the latter procedure the hexamer distribution is more sharply divided into the faster and the slower running hexamers. This distribution arises naturally in the two-step procedure because the first step would result in the formation of hexamers of $M2_1$ or of $M2_2$ or of hybrid hexamers of both, while $M1_1$ retains its integrity as a monomer. Only in the second step, where Ca^{2+} ions are introduced, does $M1_1$ form a hexamer.

By comparison of the results in Figure 4, it is apparent that, in the one-step procedure using $M2_1$, the pattern shows rather even distribution of hexamer components whereas with $M2_2$ (Figure 4) the hexamer distribution is biased toward the faster components. This point is dealt with at more length under Discussion. The hexamer distribution after the one-step reconstitution of the $M1_1$, $M2_1$, and $M2_2$ mixture (Figure 4) is similar to that of native 17S component.

Reconstitution Experiments with the Dimeric Subunit M3′ Included. When a mixture of $M1_1$ and M3′ is submitted to the one-step procedure, the resultant aggregation pattern is dependent upon the initial ratio of $M1_1$ to M3′ (Figure 5). If the ratio of the amount of $M1_1$ to M3′ is lower than 10, the polyacrylamide gel electrophoresis pattern of the resultant reconstitution in pH 7.8 buffer with Ca^{2+} ions gives a heavy band corresponding to $(M1_1)_6$ and then a series of band cathodic to the 25S position merging into a continuum toward the origin, analogous to the pattern obtained from reconstituted whole serum. If, however, the ratio of $M1_1$ to M3′ is raised above 10, the continuum is much less evident in the reconstitution product (Figure 5) and a polyacrylamide gel electrophoresis pattern much closer to that of native serum is obtained. The doublet corresponding to 29 S is reproduced in all of these reconstitution experiments, as is a single sharp band at the 25S position. The bands in the continuum region become progressively fainter as the ratio of $M1_1$ to M3′ increases. Again in these experiments some incompetent $M1_1$ remains, as was also seen in reconstitutions without dimer

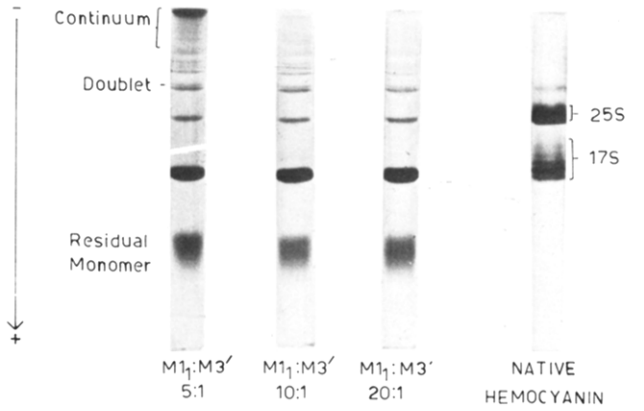

FIGURE 5: Polyacrylamide gel electrophoresis patterns of one-step reconstitutions into 0.05 M Tris, pH 7.8, containing 0.03 M $CaCl_2$ of *C. destructor* hemocyanin $M1_1$–M3′ subunit mixtures with varying ratios of $M1_1$ to M3′. The pattern of native hemocyanin serum run in 0.05 M Tris, pH 7.8, is shown for comparison. The direction of electrophoresis is indicated by the vertical arrow.

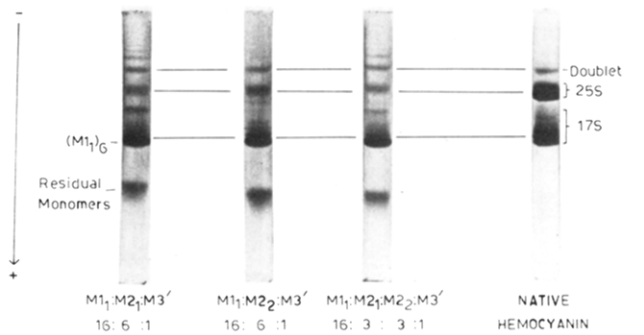

FIGURE 6: Polyacrylamide gel electrophoresis patterns of one-step reconstitutions into 0.05 M Tris, pH 7.8, containing 0.03 M $CaCl_2$ of various mixtures of the *C. destructor* subunits $M1_1$, $M2_1$, $M2_2$, and M3′. The pattern of native hemocyanin serum run in 0.05 M Tris, pH 7.8, is shown for comparison. The direction of electrophoresis is indicated by the vertical arrow.

present (Figures 2 and 4). The amount of incompetent $M1_1$ does not appear to vary significantly with the ratio of $M1_1$ to M3′.

Inclusion of M2-type subunits in the reconstitution experiments with $M1_1$ and M3′ gives a more diffuse 25S band than is the case for reconstitutions with mixtures containing only $M1_1$ and M3′, presumably because of the inclusion of $M2_1$–$M2_2$-containing 25S species. The polyacrylamide gel electrophoresis patterns resulting from these reconstitution experiments with mixtures of $M1_1$ and M3′ with M2-type monomers (Figure 6) show significant differences only in the hexamer distributions, where the differences are similar to those observed without the dimer; that is, the presence of $M2_1$ results in the formation of slow and fast hexamers with less of those having mobilities between these extremes.

Discussion

As we have reported above, the number of subunits now known to constitute *C. destructor* hemocyanin has increased from three to six. The demonstration of more extensive subunit heterogeneity in *C. destructor* hemocyanin brings it more into line with the patterns observed for other intensively studied hemocyanins (Lamy et al., 1979b).

From the results of reconstitution experiments with $M1_1$, it is evident that its hexamerization is strongly influenced by the presence or absence of Ca^{2+} ions. Thus, in the absence of Ca^{2+} ions and in buffers of low ionic strength, very little, if any, formation of $(M1_1)_6$ is observed. At higher ionic

strength and/or in the presence of Ca^{2+} ions, $M1_1$ does form hexamers at pH 7.8, although never to completion. On the other hand, the formation of hexamers from $M2_1$ and/or $M2_2$ proceeds in the absence of Ca^{2+} ions and at low ionic strength. While there is no direct evidence as to whether the rate constants for $M2_1$–$M2_2$ hexamerizations are affected by the presence of Ca^{2+}, it has been found that the oxygen binding properties of $M2_1$–$M2_2$ hexamers are unaffected by the presence of Ca^{2+}, implying that structures with the same conformation are assembled whether Ca^{2+} is available or not. This may be contrasted with the oxygen binding properties of $M1_1$ hexamers, which are distinctly different in the absence and presence of Ca^{2+} (Jeffrey & Treacy, 1980).

We noted earlier that in one-step reconstitution experiments with mixtures of $M1_1$ and $M2_2$ more faster running hexamers were formed than in $M1_1$–$M2_1$ mixtures, even though, individually, $M2_1$ monomers are more mobile than $M2_2$ monomers (compare Figures 3 and 4). This behavior may be explained by postulating that the hexamerization rate for $M2_2$ in the presence of Ca^{2+} ions is lower than that of $M2_1$. The consequence would be the observed tendency to form relatively more hybrid $M1_1$–$M2_2$ hexamers than $M1_1$–$M2_1$ hexamers in comparable one-step experiments. The finding that the subunit composition of fractionated 17S component shows a higher $M2_2$ to $M2_1$ subunit ratio than whole serum hemocyanin is perfectly consistent with this hypothesis. In the fractionation the cut is made toward the faster running side of the 17S peak which, as we have seen, is expected to contain more $M2_2$ than $M2_1$ hybrid hexamers.

The distributions of hexamers resulting from the two-step reconstitutions with mixtures of $M1_1$ and $M2_1$ or $M1_1$ and $M2_2$ are just what would be expected from the properties of the individual subunits. Thus, since both M2 monomers can form hexamers in the absence of Ca^{2+}, they tend to do so in the first step of the process, while the introduction of Ca^{2+} ions in the second step leads to the hexamerization of $M1_1$. The resulting distributions inevitably are largely composed of the two types of homogeneous hexamers relevant to each mixture. Since $(M2_2)_6$ has lower mobility than $(M2_1)_6$, the hexamer distribution from the $M1_1$–$M2_2$ mixture shows even wider divergence in electrophoretic mobility between the homogeneous hexamers than is the case for $M1_1$–$M2_1$ mixtures.

Further, examination of the results of reconstitution experiments with the monomers indicates that the $M1_1$ hexamerization rate with M2 monomers in the absence of Ca^{2+} ions is not zero, as the two-step procedure does give some hybrid hexamers. As would be predicted from the experiments discussed above with mixtures of two monomers, it is evident (Figure 4) that with three types of monomer present the one-step procedure results in a more even distribution of hybrid hexamers than the two-step procedure.

Inclusion of M3′ subunits in the reconstitution experiments gives rise to even more complex aggregation patterns. As may be seen in Figure 5, all of the faster running components obtained from reconstitution experiments on $M1_1$–M3′ mixtures, even in the absence of $M2_1$ or $M2_2$, correspond to components present in the native serum. Thus, $(M1_1)_6$ corresponds to the fastest moving band in 17 S, and a band corresponding to 25 S is obtained, as is a doublet corresponding to the 29S doublet in native serum. A slower running band than this doublet also corresponds to the slowest running band visible in the polyacrylamide gel electrophoresis pattern of whole serum hemocyanin at higher concentrations. However, as is the case with reconstituted whole serum, a series of slower running bands merging into a continuum toward the origin is obtained in the mixed $M1_1$–M3′ reconstitution experiment, its exact composition being dependent upon the $M1_1$ to M3′ ratio.

The distribution between 17S- and 25S-like components in these reconstitutions is quite dissimilar to that of native serum hemocyanin, but, as one might anticipate, reconstitution experiments in which $M2_1$ and/or $M2_2$ monomers are added to the mixtures of $M1_1$ and M3′ result in oligomer distribution patterns more like those in native serum (Figure 6). When these patterns are compared, it should be remembered that the 25S component is taken to consist of a series of virtual dodecamers made up of one dimeric subunit (M3′) and ten monomeric subunits (Jeffrey et al., 1978). As Figure 6 shows, the 25S-like band resulting from the reconstitution is more diffuse and slower running when the (slower) M2 monomers are included, implying that these are incorporated into hybrid dodecamers faster than M1 monomers. Preferential incorporation of M2 monomers into the 25S component in this way leaves an excess of $M1_1$ for the formation of hexamers, and this is seen as a disproportionately heavy band of $(M1_1)_6$.

The reconstitution experiments we have been able to do so far are not entirely successful in producing an oligomer distribution pattern identical with that in native *C. destructor* serum. Clearly, there is uncertainty in equating experimental conditions of relative and total protein concentration, pH, ionic conditions, etc. with those in the animal. In addition to such considerations, differences from the native pattern probably result in part from structural damage to subunits as a result of the alkaline conditions used in the dissociation, destroying in some molecules determinants necessary for regulation of assembly. On the other hand, the reported experiments do demonstrate that all of the individual components seen in serum can be re-formed on the bench from the subunits discussed here. Failure to duplicate the native composition of hemolymphs by reconstitution experiments with mixtures of arthropod hemocyanin subunits is often reported, for example, with *Cancer magister* (Carpenter & van Holde, 1973), *Callinectes sapidus* (Hamlin & Fish, 1977), *Limulus polyphemus* (Bijlholt et al., 1979), and *Ligia exotica* (Terwilliger et al., 1979), and probably has a variety of causes, including that mentioned above. The present series of experiments with *C. destructor* hemocyanin subunits has led to the recognition of another factor that can introduce control in the assembly of arthropod hemocyanin oligomers, namely, the importance of the relative rates of combination of different subunits in mixtures that reequilibrate very slowly. The consequence we wish to emphasize is that quite different distributions of hybrid oligomeric forms can be produced by adding particular subunits at different stages of the assembly process. It is conceivable that some such control is available in vivo. In any event, it is clear that the possibility of such mechanisms provides an additional reason for the difficulties experienced in matching native distributions in artificial reconstitution experiments.

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Effect of Macromolecular Crowding upon the Structure and Function of an Enzyme: Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The specific activity of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPD) has been measured as a function of GAPD concentration in the absence and presence of 18 g/dL ribonuclease A. The specific activity of GAPD at fixed concentration has been measured as a function of the concentration of added ribonuclease A, β-lactoglobulin, bovine serum albumin, and poly(ethylene glycol) (M_r 20000) at additive concentrations of up to 30 g/dL. All of the data may be semiquantitatively accounted for by a simple model based upon the following qualitative assumptions: (1) Under the conditions of the reported experiments, GAPD exists primarily as an equilibrium mixture of monomers and tetramers of GAPD subunits. (2) The monomers have a much larger specific activity than do the tetramers. (3) The addition of high concentrations of unrelated globular proteins does not affect the activity of either monomer or tetramer but does promote the formation of tetramer due to space-filling properties of the added species, as proposed by Minton [Minton, A. P. (1981) *Biopolymers* (in press)].

Studies of enzyme structure and function in solution are traditionally carried out in solutions which are as dilute in protein as accurate measurement will allow. Measurements of the dependence of a measured property upon protein concentration are most frequently employed as a means of extrapolation to infinite dilution. The properties thus measured are characteristic of an isolated enzyme molecule in a bath of solvent containing salts, substrates, and cofactors as required. Such an environment differs in one very important respect from the fluid medium in which an enzyme ordinarily performs its biological task. Such a medium may indeed be dilute in the particular enzyme of interest but will contain a large variety of other mobile macromolecules, which, taken together, occupy a substantial fraction of the total volume. Such a medium will be described as *volume occupied* or *crowded*.

The effect of volume occupancy upon the thermodynamic activity of globular proteins in solution has been the subject of several studies in this laboratory (Minton, 1977, 1980; Ross & Minton, 1977, 1979; Ross et al., 1978). Recent calculations suggest that crowding may have a substantial effect upon the structure and catalytic activity of an enzyme which is itself present at very low concentrations. Several possible mechanisms for such an effect have been advanced (Minton, 1981), among them the following. Consider an enzyme which may exist in more than one state of self-association (e.g., monomer, dimer, trimer, . . .). If the catalytic activity of the enzyme varies with the degree of self-association and then if crowding alters the average degree of self-association, it will also alter the average catalytic activity of the enzyme.

The study presented here was undertaken in order to answer two questions: (1) Can crowding affect the catalytic activity of an enzyme by the mechanism outlined? (2) If so, how well can the phenomenon be described by the approximate theory of Minton (1981)?

The enzyme D-glyceraldehyde-3-phosphate dehydrogenase (GAPD)[1] catalyzes the reaction

$$\text{D-glyceraldehyde 3-phosphate} + NAD^+ + P_i \rightleftharpoons \text{1,3-diphosphoglycerate} + NADH + H^+ \quad (1)$$

GAPD consists of a tetramer of identical M_r 36000 subunits, which can reversibly dissociate to dimers and/or monomers, depending upon experimental conditions (Hoagland & Teller, 1969; Constantinides & Deal, 1969; Lakatos et al., 1972; Lakatos & Zavodszky, 1976). The specific activity of GAPD has been shown to vary with GAPD concentration (Jancsik et al., 1979) in a manner which suggests that the specific activity of the subunit depends upon the degree of self-asso-

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[1] Abbreviations used: GAPD, glyceraldehyde-3-phosphate dehydrogenase; GAP, 3-phospho-DL-glyceraldehyde (or DL-glyceraldehyde 3-phosphate); βLG, β-lactoglobulin, RNase, ribonuclease A; PEG, poly(ethylene glycol); Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.