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Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor*. Characterization of a Dimeric Subunit and Its Involvement in the Formation of the 25S Component†

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ABSTRACT: The molecular weight of a dimeric subunit, M_3', isolated from *Cherax destructor* hemocyanin has been measured by sedimentation equilibrium to be 144 000. Peptide mapping and end-group analysis together with gel electrophoresis show that the dimer consists of two very similar or identical monomers, cross-linked by disulfide bridges. Dissociation of the 25S component of the hemocyanin shows that it contains the dimer and two previously identified monomers, M_1 and M_2. Its molecular weight is 900 000 by sedimentation equilibrium, and reconstitution studies show that the dimer is essential for its formation. Analysis of the results of polyacrylamide disc gel electrophoresis experiments with the 25S component indicates that it consists of a population of 11 compositional isomers. These all contain one dimeric subunit and ten monomeric subunits, the latter being present in all the combinations of M_1 and M_2.

Evidence for the existence in *Cherax destructor* hemocyanin of a subunit of about twice the molecular weight generally accepted for the monomer of arthropod hemocyanins has been presented previously (Murray and Jeffrey, 1974). It was reported that dissociation of aggregated forms in whole serum at high pH, or in sodium dodecyl sulfate, gave rise to three different subunits. These were detected by polyacrylamide disc gel electrophoresis and were denoted M_1, M_2, and M_3'. The molecular weights of M_1 and M_2 were estimated by gel electrophoresis to be in the range 70 000–80 000, a more accurate value of 74 700 being measured by sedimentation equilibrium for M_1, which could be isolated in pure form. The molecular weight of M_3' was found by gel electrophoresis to be 132 000 in the absence, and 190 000 in the presence, of sodium dodecyl sulfate. Further dissociation of M_3' could be effected by the inclusion of dithiothreitol in the incubation with sodium dodecyl sulfate to produce a subunit, denoted M_3, of molecular weight about 84 000. It was concluded that M_3' was likely to be a dimer of this subunit which, as a subsequent study showed (Jeffrey et al., 1976), differed in amino acid composition from M_1 and M_2.

The dimeric subunit M_3' was observed following dissociation of the 25S component of *C. destructor* hemocyanin; it was not observed when the hexameric 17S component was dissociated (Murray and Jeffrey, 1974). Further, reconstitution studies showed (Jeffrey et al., 1976) that the hexameric component was the largest aggregate produced when only the monomer M_1 or M_2 was present. These findings suggested that M_3' was necessary for the formation of the higher aggregates, notably the 25S component. We present here the results of our studies on some properties of M_3' and its role in the formation and composition of the 25S component, which accounts for about 50% of the hemocyanin present in the native serum of *C. destructor*.

Experimental Section

Preparation of Hemocyanin Components. Serum was prepared from *C. destructor* hemolymph as described previously (Murray and Jeffrey, 1974) and stored under toluene at 5 °C. All of the hemocyanin components to be discussed were isolated from serum pooled from several animals.

The monomer, M_1, was separated on Sephadex G-200 as described before (Jeffrey et al., 1976) or by column electrophoresis on polyacrylamide gel using the LKB 7900 Uniphor apparatus. In the latter method, a 10-cm column of 5% polyacrylamide gel in 0.025 M glycine at pH 10.1 containing 0.1 mM EGTA[1] was used. The reservoir contained 1.5 L of the

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

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same buffer and crosswise pumping of anode and cathode buffers was employed. After 20 min of preelectrophoresis at 250 V, up to 70 mg of serum protein in a volume of up to 2 mL or more, usually 1 mL of serum, dialyzed overnight against the buffer, was applied to the column, the voltage was applied gradually until 250 V was reached, and the emerging protein was eluted with buffer pumped at 20 mL/h. The component M_3' was isolated by column chromatography on Sephadex G-200 in buffer of pH 10 containing 0.1 mM EGTA–0.05 M glycine as reported previously (Murray and Jeffrey, 1974). As was shown, the resulting elution profile contains three peaks, the second of which can be separated and rechromatographed to yield electrophoretically pure M_3' (Murray and Jeffrey, 1974, Figures 3B and 5). The 25S component was prepared by column electrophoresis on polyacrylamide gel using a 4% gel in 0.025 M Tris, pH 7.8, containing 0.1 mM EGTA, and similar conditions of voltage and elution to those specified above for the preparation of monomer M_1.

Polyacrylamide Disc Gel Electrophoresis. The technique, carried out on 7-cm gels in a water-cooled Buchler apparatus at 20 °C, was exactly as previously described (Jeffrey et al., 1976). The acrylamide concentration in gels run at pH 10 (0.05 M glycine–0.1 mM EGTA) was routinely 6% while in those at pH 7.8 (0.05 M Tris) it was 4%, and electrophoresis was continued for about 2 h. In some experiments at pH 7.8 with the 25S component where more resolution of bands was sought, 3.5% gels were employed and electrophoresis was continued for 3 h. Usually gels were stained for half an hour with 0.05% Coomassie brilliant blue in 25% isopropyl alcohol–10% acetic acid and then covered with 7% acetic acid and left to stand overnight before destaining in a Canalco horizontal destainer. When estimates of the proportion of protein present in bands observed on gels were required, the gels were scanned with a Schoeffel spectrodensitometer SD 3000. The protein samples which were scanned in this way were the dissociation products of the 25S component. The starting material was a protein solution from a tube near the maximum of the peak representing the 25S component in the column electrophoresis of serum at pH 7.8 and was shown to run as a single band on disc electrophoresis in 4% polyacrylamide gels at pH 7.8. Two procedures were used for estimating the proportions of M_1, M_2, and M_3' resulting from the dissociation of this 25S component at pH 10. In the first, the protein solution obtained from the column electrophoresis at pH 7.8 was concentrated to about 1 mg/mL and dialyzed overnight to pH 10 (0.05 M glycine–0.1 mM EGTA). Samples of the dialyzed solution of volume 5 and 20 μL were applied to 7-cm columns of polyacrylamide gel for electrophoresis at pH 10. The conduct of the experiments and the staining of the gels were as described above, and the stained gels were scanned at a wavelength of 540 nm in the spectrodensitometer. In the second procedure, the protein samples were concentrated to about 3 mg/mL before dialysis to pH 10, and following the disc gel electrophoresis the gels were transferred to quartz tubes and scanned at 280 nm. Estimates of the proportions of the proteins resolved on the gels were made by tracing the appropriate areas under the scans and cutting out the resulting peaks and weighing them. It was hoped that comparison of the results from the two methods would make it possible to allow for any errors arising from deficiencies in them. Chiefly, these are likely to be nonlinear uptake of dye in the method employing stained gels, and difficulties in establishing a reliable baseline in the gels scanned in the ultraviolet region of the spectrum.

Sedimentation Equilibrium. Experiments were performed at 20 °C in a Spinco Model E analytical ultracentrifuge fitted with electronic speed control. The temperature was controlled to within ±0.1 °C by means of the RTIC unit, and Rayleigh interference patterns were recorded on Kodak IIG photographic plates. In both of the experiments, 1-mL volumes of protein solution, in the appropriate buffer, were dialyzed overnight at about 20 °C in washed Visking 18/32 dialysis tubing against 500 mL of buffer. A volume of 0.1 mL of protein solution was placed in the solution sector of the double-sector ultracentrifuge cell after the introduction of 0.01 mL of the fluorocarbon FC 43, which acted as an inert base fluid. This volume of solution gives a solution column height of just under 3 mm. The solvent sector of the cell contained 0.12 mL of the buffer equilibrated with the protein solution by dialysis. In the experiment with the component M_3' the buffer was 0.05 M glycine, 0.2 M NaCl, 0.1 mM EGTA, pH 10.0; the initial concentration of the protein solution was 1.05 mg/mL; the speed was 17 000 rpm; and a photograph of the sedimentation equilibrium concentration distribution after 12 h was measured for use in computing the molecular weight of the sample. In the experiment with the 25S component, the corresponding parameters were: buffer 0.025 M Tris, 0.2 M NaCl (pH 7.8); initial concentration 0.96 mg/mL; speed 6400 rpm; equilibrium photograph taken after 24 h at the set speed. The protocol of the experiments and the measurement of the photographic records were exactly as described by Yphantis (1964). The concentration distributions, in terms of displacement in microns on the photographic plate vs. radial distance from the axis of rotation in centimeters, were found to be invariant over the last 2 h of the experiments, and it was concluded that sedimentation equilibrium had been attained in the stated times. These times were consistent with those which can be estimated by the method suggested by Yphantis (1964). A value of 0.73 mL/g was used for the partial specific volume of both hemocyanin components (Ellerton et al., 1970).

Amino Acid Analysis, End-Group Analysis, and Peptide Maps. Amino acid analyses were performed with a Beckman 120C analyzer by the method of Spackman et al. (1958). Samples were hydrolyzed for 22 h at 110 °C in evacuated sealed tubes containing 2–4 mL of 6 M HCl (Crestfield et al., 1963) and 1% (v/v) aqueous phenol; HCl was removed by rotary evaporation. Methionine and half-cystine were determined as methionine sulfone and cysteic acid, respectively, on samples which had been oxidized with performic acid (Hirs, 1956).

The N-terminal amino acid was determined by the dansylation method (Gros and Labouesse, 1969). After conversion to the dansyl derivative, the protein was precipitated with 13% trichloroacetic acid, washed twice with 1 M HCl, and then hydrolyzed for 4 h at 110 °C with 6 M HCl in an evacuated, sealed tube. The HCl was then removed by evaporation and the residue dissolved in 50% pyridine. Identification of the dansyl amino acid was carried out by thin-layer chromatography on Baker-flex 1 B-F silica gel using chloroform–ethanol–acetic acid in the ratio 38:4:3 (v/v) as the solvent (Deyl and Rosmus, 1965).

Tryptic peptide maps were prepared as follows: the protein was denatured by oxidation with performic acid (Hirs, 1956) and then suspended in 0.5% ammonium bicarbonate, pH 8, for digestion by trypsin, 2% (w/w) at 37 °C for 4 h. Peptides with limited solubility at pH 4 were precipitated by the dropwise addition of acetic acid to the digest in water and removed by centrifugation. The soluble peptides were subjected to high-voltage electrophoresis (40 V/cm, 1.3 h) on Whatman 3MM paper followed by ascending chromatography in butanol–acetic acid–pyridine–water (15:3:10:12, v/v) for 18 h. The maps were stained with ninhydrin and residue specific reagents (Easley, 1965).

TABLE I: Amino Acid Composition of the Dimeric Subunit.

amino acid	no. of residues[a]
Lys	33
His	38
Arg	35
Asp	95
Thr	33
Ser	35
Glu	66
Pro	32
Gly	36
Ala	40
Cyst(e)ine	2–3
Val	40
Met	5
Ile	31
Leu	58
Tyr	28
Phe	40
Trp	Nd[b]

[a]The values are the mean of two determinations calculated on the basis of aspartic acid equal to 95 for comparison with previous results. This leads to a molecular weight of about 75 000 for the above composition. [b]Not determined.

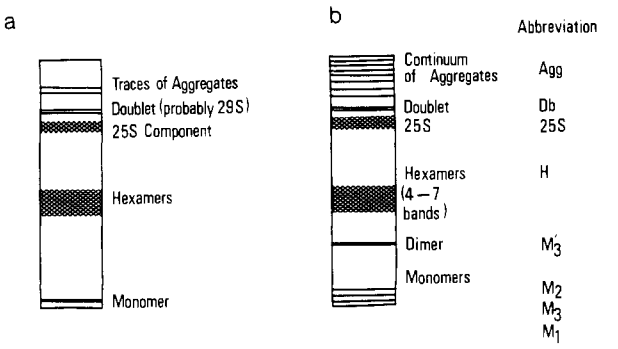

FIGURE 1: Diagrammatic representations of banding patterns observed in polyacrylamide disc gel electrophoresis experiments with *C. destructor* hemocyanin. (a) The pattern observed with whole serum at pH 7.8. The band labeled monomer is not normally visible, this component being present in too low an amount; it is included in the diagram to indicate its mobility relative to the other components. (b) A composite pattern indicating the relative mobilities of components observed in any of the experiments reported in the text. The diagram is to be regarded only as a key, providing a system of nomenclature for discussion in the text. The abbreviations are employed in subsequent diagrams, in which capital letters imply large proportions and lower-case letters small proportions, of the various components.

Results and Discussion

The Dimeric Subunit. The plot of the logarithm of total concentration vs. the square of the radial distance, obtained in the sedimentation equilibrium experiment with M_3', was linear, indicating homogeneity with respect to molecular weight. Its slope corresponded to a molecular weight of 144 000 with an estimated error of about ±4000. The values estimated previously by gel electrophoresis and given in the introduction are in satisfactory agreement with this figure, but the latter should be regarded as the most accurate determination because of the rigorous thermodynamic basis of sedimentation equilibrium theory and the more precise nature of the measurement of records of sedimentation equilibrium concentration distributions. Thus, in molecular weight this subunit is characterized by a value twice that for a typical arthropod monomer (molecular weight 75 000). The copper content of M_3' was found to be, by the method of atomic absorption spectroscopy, 3.5 ± 0.5 copper atoms/144 000 daltons and is the same as for a typical arthropod hemocyanin monomer on a weight of copper/weight of protein basis (two copper atoms/75 000 daltons). The subunit has accordingly been designated a dimeric subunit. It was shown previously (Murray and Jeffrey, 1974) that M_3' could be dissociated in sodium dodecyl sulfate in the presence of dithiothreitol, the resulting single band on polyacrylamide gel in sodium dodecyl sulfate having a mobility equivalent to a molecular weight of about 84 000 and the corresponding particle being, therefore, of a similar size to the two monomers of *C. destructor,* M_1 and M_2. Evidence for differences in amino acid composition between M_3' and the monomer M_1 of *C. destructor* hemocyanin has also been presented (Jeffrey et al., 1976), but the amino acid composition of M_3' is repeated here in Table I for ease of reference. It may be noted that the presence of half-cystine residues in M_3' together with the findings cited above are consistent with the notion that it consists of two monomeric units joined by 2–3 disulfide bonds. The analyses of M_1- and of M_2-enriched hexamers had shown M_1 and probably M_2 to be devoid of cyst(e)ine.

The question arises as to whether the two monomers which comprise M_3' are the same or different. Amino-terminal analysis gave a single residue, aspartic acid. Peptide maps of a tryptic digest of M_3' were also prepared. Inspection of Table I shows that if the dimeric subunit consists of two identical monomers each of molecular weight about 75 000 one would expect, on a tryptic peptide map, 69 spots representing the sum of the number of lysine and arginine residues plus one, a maximum of 28 spots containing tyrosine, and a maximum of 38 spots containing histidine. If the two monomers were different, the maximum number of spots expected from the peptides containing the respective amino acids would be double the figures cited. The number of spots observed on the peptide map stained with ninhydrin was 66, an additional 5 staining for tyrosine but not with ninhydrin, bringing the maximum to 71. The total number of spots counted for tyrosine was 25, 18 strongly and 7 more weakly staining, while 20 strong and 13 weak histidine spots for a total of 33 could be observed. The tryptic peptide material with limited solubility at pH 4 was less than 10% of the total protein and therefore the observations are inconsistent with the possibility that the dimeric subunit is made up of two different monomers, but rather that the monomers are very similar or identical. The single band observed in polyacrylamide gel electrophoresis following dissociation by dithiothreitol in sodium dodecyl sulfate is the strongest evidence for identical monomers.

The Composition of the 25S Component. The molecular weight of a sample of the 25S component of *C. destructor* hemocyanin isolated by column electrophoresis on polyacrylamide gel at pH 7.8 was measured by sedimentation equilibrium to be 900 000 ± 30 000. The plot of the logarithm of total concentration vs. the square of the radial distance was linear except for some small, but apparently real, upward deviation, which may be significant, at the highest concentrations measured. This suggested the presence of some more highly aggregated material. The amount or size of such material could not be calculated because it was not possible to continue measurement of the sedimentation equilibrium pattern right to the cell bottom. It was possible to conclude that it formed only a small proportion of the total protein in solution. In agreement, a disc gel electrophoresis experiment, performed subsequently on the sample used in the sedimentation equilibrium experiment, did show a trace of more slowly migrating material when a heavy loading was used. However, the value

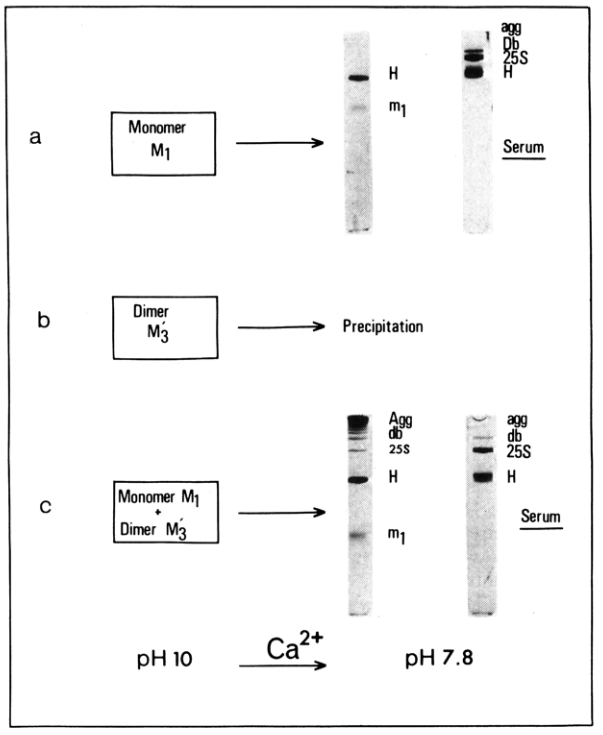

FIGURE 2: Results of reconstitution experiments in which monomer M_1 and dimer M_3' initially at pH 10 in 0.05 M glycine–0.1 mM EGTA were dialyzed to pH 7.8 against 0.05 M Tris containing 0.03 M Ca^{2+}. The polyacrylamide gel electrophoresis patterns of the resulting solutions are shown together with those of *C. destructor* serum (20-μg loading) run under the same conditions for comparison. The nomenclature employed is given in Figure 1. (a) Formation of the hexamer $(M_1)_6$ from M_1 (5 μg of protein loaded). (b) No bands were observed on gels loaded with solutions of the dimer dialyzed to pH 7.8 in the presence of calcium; apparently, the formation of large aggregates results in the precipitation of all of the protein. (c) A mixture of M_1 and M_3' in the ratio 3.7:1 (close to that found by dissociating the 25S component) was dialyzed to pH 7.8 and a volume containing 20 μg of protein was loaded on the gel. The result shows that some 25S component is formed together with many higher molecular weight species.

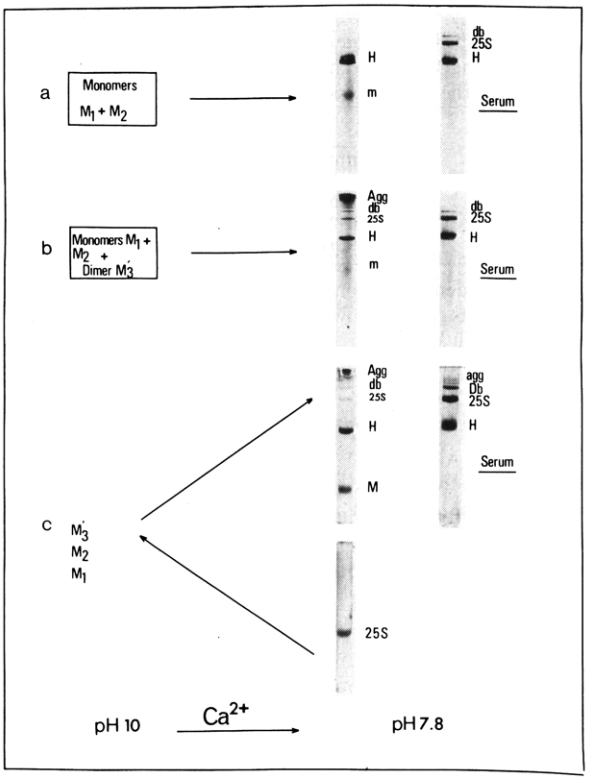

FIGURE 3: Results of reconstitution experiments in which the monomers M_1 and M_2 and dimer M_3' were dialyzed from pH 10 (0.05 M glycine–0.1 mM EGTA) to 7.8 in the presence of calcium ions (0.025 M Tris–0.03 M Ca^{2+}). The nomenclature employed is given in Figure 1. (a) Formation of hexamers from a mixture of M_1 and M_2 in the ratio 7:2 obtained by dissociation of the hexameric component of serum. About 30 μg of protein was loaded while the serum pattern was obtained with a loading of 5 μg. (b) A mixture of the two monomers as in (a) with the dimer, in the ratio monomers/dimer = 3.7:1. The total protein loaded was 20 μg and comparison with the serum pattern (5 μg) shows that some 25S component is formed together with many higher molecular weight species. (c) A sample of the 25S component obtained by column electrophoresis at pH 7.8 was dialyzed to pH 10, with removal of calcium ions, to produce the mixture of subunits M_1, M_2, and M_3' shown in the gel-electrophoresis pattern on the left. The loading was 13 μg. The same solution (of concentration 0.48 mg/mL) was then dialyzed back to pH 7.8 with the addition of calcium ions, and the resulting gel-electrophoresis pattern (13 μg) is shown on the right. The pattern resembles that in (b) but is different from that of the starting material and of serum (20 μg).

of 900 000 is evidently that of the molecular weight of the 25S component and is in good agreement with values obtained for this component of other arthropod hemocyanins (Morimoto and Kegeles, 1971; Carpenter and van Holde, 1973; Hamlin and Fish, 1977). It is concluded that the 25S component of *C. destructor* hemocyanin is equivalent in size to 12 monomeric subunits, a consideration relevant to attempts to define its composition. Studies of the dissociation of the 25S component, and on the reconstitution of the various aggregated forms of hemocyanin from the monomers and the dimer, are also relevant to this endeavor and are summarized in Figures 1–3.

Figure 1a is a diagrammatic representation of the polyacrylamide disc gel electrophoresis pattern obtained when *C. destructor* serum is run at pH 7.8, while Figure 1b shows the relative positions of all bands representing hemocyanin components obtained separately in all of the experiments performed. The latter figure is presented to establish a nomenclature which will allow discussion of the results of electrophoretic experiments with *C. destructor* hemocyanin components and should therefore be regarded only as a key. The system of nomenclature employed utilizes previously established designations wherever possible but is necessarily arbitrary with respect to bands representing components whose size and/or composition is unknown. Figure 2 summarizes the results of reconstitution experiments in which monomer M_1 or dimer M_3', isolated as previously described, was dialyzed from pH 10 to 7.8 in the presence of 0.03 M calcium. As reported before (Jeffrey et al., 1976), such a process with monomer M_1 results in almost complete conversion to the hexamer $(M_1)_6$, although traces of some material usually remain in monomeric form (Figure 2a). The important point is that no 25S component is formed. When dimer M_3' is dialyzed in the same way, the result is the formation of very large aggregates and precipitation of most of the protein. It was also found that when dimer and hexamer freed of calcium were mixed at pH 7.8 and calcium was added by dialysis all of the dimer precipitated and hexamer remained as hexamer; no larger aggregates were seen on the gels. However, when M_1 and M_3' are mixed prior to dialysis to pH 7.8, hexamer and 25S component are formed (Figure 2c) (together with some more slowly moving bands), indicating the requirement of the presence of the dimeric subunit for the formation of the 25S component. The bands of more slowly migrating components may represent aggregates of this component, and, as may be seen by reference to Figure 1, some of them correspond with bands present in the *C. destructor* serum itself.

In Figure 3 the results of similar experiments are reported,

TABLE II: Weight Percentages Obtained by Spectrodensitometry at Two Wavelengths of the Three Subunits Resulting from Dissociation of the 25S Component at pH 10.

subunit	Results at 540 nm[a]	at 280 nm[b]	Mean
monomer M_1	62.0 (58–64)	68.5 (61–74)	65.5
monomer M_2	19.2 (18–22)	13.2 (11–19)	16.2
dimer M_3'	18.8 (18–20)	18.3 (14–23)	18.6

[a] Mean of six determinations. [b] Mean of nine determinations. The figures in brackets show the lowest and the highest values, respectively, recorded for each subunit.

but here the second monomer, M_2, is included. As noted before (Jeffrey et al., 1976), attempts to isolate this monomer have so far been unsuccessful, but it is possible to obtain mixtures of the two monomers M_1 and M_2 by dissociating the total hexamer isolated from the serum. As in the reconstitution experiments discussed above, no 25S component is obtained unless the dimer M_3' is included in these experiments, with the two monomers giving rise to electrophoretic patterns very similar to those observed when only monomer M_1 was mixed with dimer. Figure 3c shows the result of dissociating the 25S component at pH 10 then dialyzing the resulting mixture of M_1, M_2, and M_3' back to pH 7.8 in the presence of calcium ions. A pattern very similar to that produced by dialyzing the mixture of M_1, M_2, and M_3' referred to in Figure 3b is obtained. Evidently the conditions used to effect dissociation alter the subunits in some way, which prevents the native association pattern from being correctly reestablished. Such alterations may reflect changes in the conformation of the subunits, their partial degradation, or both, and probably account for the inability of some monomer M_1 material to form hexamer noticed in Figure 2a. The possible induction of conformational changes in the subunits resulting from exposure to pH 10 and removal of calcium remains to be investigated, but it is known that degradation of monomer M_2 (rapid) and monomer M_1 (slow) occurs at pH 10 (Jeffrey et al., 1976). The results of the experiments reported in Figures 2 and 3 do show that the 25S component is formed only when the dimer M_3' and one, or both, monomers are present. They also show that, once the aggregated components of *C. destructor* hemocyanin have been dissociated by exposure to pH 10 and removal of calcium, they are altered in a way which seems to predispose toward formation of larger aggregated forms when the dimeric subunit is present.

Attention can now be directed to the question of the composition of the 25S component of *C. destructor* hemocyanin, and to this end the results of experiments designed to determine the proportions of M_1, M_2, and M_3' in this component are reported in Table II. The mean values are consistent with the existence of a single species of composition $(M_3')_1\cdot(M_2)_2\cdot(M_1)_8$ which has theoretical values of 16.7% for M_3', 16.7% for M_2, and 66.7% for M_1. However, when a sample of serum was subjected to polyacrylamide disc gel electrophoresis using a 3.5% gel concentration and a running time of 3 h, resolution of the 25S component into four bands was observed. These consisted of two moderately staining bands flanking a heavy band and followed by a very faint band of lower mobility which is visible on the actual gels but which photographs poorly (Figure 4). This resolution, which was not seen either in serum or in purified samples of the 25S component subjected to electrophoresis on polyacrylamide gels run under standard conditions (4% gel concentration, 2-h running time), is reminiscent of that seen with the hexameric component of *C. destructor* hemocyanin (Jeffrey et al., 1976). It seemed likely that the 25S component, like the hexameric, consisted of a population of species of the same size but with varying proportions of the two monomers and the dimer.

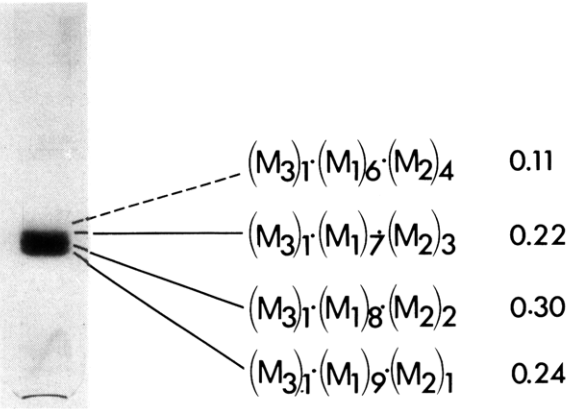

FIGURE 4: Polyacrylamide disc gel electrophoresis of the 25S component of *C. destructor* hemocyanin at pH 7.8. The cathode is at the top and electrophoresis is from top to bottom on a 3.5% gel run for 3 h with about 10 μg of the 25S component. The compositions and proportions indicated for the bands are based on the discussion in the text. A faint band, visible on the gels, but which could not be reproduced photographically, is indicated by the dotted line.

It is possible, using the previously established weight percentages of the subunits M_3', M_1, and M_2, to calculate the proportions of species of size equivalent to 12 monomers which could be formed by their combinations under various models. The resulting species are designated $(M_3')_i\cdot(M_1)_j\cdot(M_2)_k$, where $2i + j + k = 12$ and the individual values of i, j and k depend on the model under consideration. For example, if all combinations of the three subunits were formed with equal probability, i would take the values 0–6 and j and k the values 0–12, for a total of 49 different stoichiometries in the species comprising the 25S component. This proposal can be rejected on the grounds that it includes species containing no dimeric subunits ($i = 0$) and no monomeric subunits ($i = 6$), and it has been demonstrated that the 25S component is formed only when both are present. A modified scheme in which i has values 1–5 and j and k values 0–10 would imply the existence of 35 different 25S species, but this is also rejected as being incompatible with the electrophoretic pattern shown in Figure 4 which exhibits only a small number of resolved bands.

A simpler hypothesis which may be explored is that the species comprising the 25S component have only one stoichiometry with respect to one of the subunits, but that within that restriction all combinations of the other two subunits are allowed. Three cases arise for consideration; those in which M_1, M_2, or M_3', respectively, has a fixed stoichiometry. In all three the appropriate stoichiometry can be decided by reference to the measured weight percentage of the relevant subunit obtained upon dissociation of the 25S component. Thus, for M_1 the measured weight percentage was 65.4 and the only compatible value of j is 8, which is equivalent to a weight percentage of M_1 of 66.7. Only two 25S species are possible within the restriction imposed that $2i + j + k = 12$ and these are $(M_1)_8\cdot(M_3')_1\cdot(M_2)_2$ and $(M_1)_8\cdot(M_3')_2$. The model involving fixed stoichiometry with respect to M_1 can be rejected, since at least four species of the 25S component are indicated by the gel-electrophoresis pattern shown in Figure 4.

Similar reasoning, and reference to the figures given in Table II, shows that for a model with fixed stoichiometry with respect to M_2 only that with $k = 2$ is relevant and for that with fixed stoichiometry with respect to M_3' only that with $i = 1$ is relevant, even when the maximum spread of the measured values

is taken into account. However, the model in which all the species comprising the 25S component contain two molecules of M_2 is obviously not appropriate because it does not allow for species containing only M_1 and M_3'. Reconstitution experiments have shown that the combination of these two subunits does result in the formation of the 25S component (Figure 2). The species generated under the remaining scheme considered, and their expected proportions, are listed in Table III. These results show that four species containing high proportions of M_1 account for 85% by weight of the total population of 11 species relevant to the scheme under discussion. Thus, if this were the correct interpretation of the observed heterogeneity in the 25S component, the pattern seen on polyacrylamide gel electrophoresis would be expected to exhibit, in order of descending mobility toward the anode, a faint band followed by three heavier bands and another faint band. These conclusions are based on the fact that the electrophoretic mobility of M_1 toward the anode is greater than that of M_2 (Jeffrey et al., 1976) and that with the protein loadings employed in disc gel electrophoresis the lower limit observable as a stained band is about 10% of the total material loaded. Reference to Figure 4 shows that the three readily visible bands correspond in sequence and proportion with the species designated $(M_3')_1 \cdot (M_1)_9 \cdot (M_2)_1$, $(M_3')_1 \cdot (M_1)_8 \cdot (M_2)_2$, and $(M_3')_1 \cdot (M_1)_7 \cdot (M_2)_3$, respectively, in Table III. On the actual gels a very faint slower band, which would represent $(M_3')_1 \cdot (M_1)_6 \cdot (M_2)_4$, is also discernible. Since, as the last column of Table III shows, this would only represent 10% of the original material, it would be expected to be at the very limit of detectability, and the fastest band, corresponding to $(M_3')_1 \cdot (M_1)_{10}$, would not be visible. It is felt that the very good correlation between the observed disc gel electrophoretic pattern and the expected proportions of the various species reported in Table III provides evidence strongly in favor of the postulated scheme as the correct description of the heterogeneity of the 25S component of *C. destructor* hemocyanin.

TABLE III: Calculated Weight Percentages of Compositional Isomers Comprising the 25S Component of *C. destructor* Hemocyanin.[a]

composition	wt %
$(M_3')_1 \cdot (M_1)_{10}$	8.9
$(M_3')_1 \cdot (M_1)_9 \cdot (M_2)_1$	24.3
$(M_3')_1 \cdot (M_1)_8 \cdot (M_2)_2$	30.0
$(M_3')_1 \cdot (M_1)_7 \cdot (M_2)_3$	21.9
$(M_3')_1 \cdot (M_1)_6 \cdot (M_2)_4$	10.5
$(M_3')_1 \cdot (M_1)_5 \cdot (M_2)_5$	3.4
$(M_3')_1 \cdot (M_1)_4 \cdot (M_2)_6$	2.1
$(M_3')_1 \cdot (M_1)_3 \cdot (M_2)_7$	0.1
$(M_3')_1 \cdot (M_1)_2 \cdot (M_2)_8$	0.01
$(M_3')_1 \cdot (M_1)_1 \cdot (M_2)_9$	<0.01
$(M_3')_1 \cdot (M_2)_{10}$	<0.01

[a] The 25S component, of molecular weight 900 000, is taken to consist of a population of species formed with equal probability from all possible combinations of ten molecules of the two monomers, M_1 and M_2 (molecular weight 75 000), with one molecule of dimer, M_3' (molecular weight 150 000). The total weight percentages employed in the calculation were: M_1, 65.5; M_2, 17.9; M_3', 16.7; and the resulting species are listed in the expected order of decreasing electrophoretic mobility toward the anode (see text).

Conclusions

It is believed that our previously published work (Murray and Jeffrey, 1974) was the first report of the existence of a dimeric subunit in an arthropod hemocyanin. The further characterization of this dimer, M_3', which is obtained upon dissociation of the 25S component, indicates that it is composed of two identical disulfide-linked monomers of approximately the same size as, but of different composition from M_1 and M_2. In a recent study, Ellerton et al. (1977) have presented evidence for the presence of a dimeric subunit in the hemocyanin of the spiny lobster *Jasus edwardsii*. It also seems that the hemocyanin of the swimming crab *Ovalipes catharus* contains a dimeric subunit (Ellerton, 1977, personal communication).

In our experiments with *Cherax destructor* hemocyanin, the 25S component is formed only in the presence of the dimeric subunit and one or both monomers. It is equivalent in size to 12 monomers (molecular weight 900 000) and appears to consist of a population of 11 species formed by the combinations of ten molecules of the two monomers M_1 and M_2 with one molecule of dimer. It is of interest that in the reported study of *Jasus edwardsii* hemocyanin (Ellerton et al., 1977) no 25S component was observed even though dimer had been shown to be present. However, Ellerton (personal communication) has since observed a small percentage of 25S component in *Jasus* hemocyanin. In general, the spiny lobsters seem to have little or no 25S component, and it is intriguing to speculate that this observation may reflect the absence or very low proportion of a dimeric subunit in the hemolymph of such animals. It should be added that the presence of a component of molecular weight near 900 000 and a sedimentation coefficient near 25S in the hemolymph of an arthropod species does not necessarily imply that it is formed only with the aid of a dimeric subunit—there seems little doubt that the 25S component in some arthropod species (perhaps most) is formed by dimerization of hexameric (17S) particles (Morimoto and Kegeles, 1971; Carpenter and van Holde, 1973; Hamlin and Fish, 1977).

The attempts in the present work to reconstitute 25S particles from the constituent subunits are similar in outcome to those reported by Carpenter and van Holde (1973) with *Cancer magister* hemocyanin. In both studies, although some 25S component was obtained, there were other species present as well. The explanation advanced by Carpenter and van Holde invoked heterogeneity in the subunits which could lead, upon reassociation to the hexamer, to a large population of hexamers of random composition, only a few of which would be capable of dimerizing to form 25S species. This hypothesis is not contradicted by our results with *Cherax destructor* hemocyanin where, however, the requirement of a dimeric subunit for the formation of 25S species and the inability of hexamers to dimerize provides, apparently, a different criterion for the formation of stable 25S species. In general terms, it is felt that the results of the experiments with *Cherax destructor* hemocyanin support the hypothesis advanced by Carpenter and van Holde because it has been possible, in this arthropod, to demonstrate the existence of compositional isomers of both the 17S and 25S components. We would add only that alteration in the subunits accompanying dissociation of aggregates is also a possibility requiring further study.

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Circular Dichroism and Resonance Raman Studies of Cytochrome b_{562} from *Escherichia coli*†

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ABSTRACT: A modified procedure for the isolation of cytochrome b_{562} from *Escherichia coli* is described. The preparation obtained has the same spectral properties as the twice-crystallized preparation [E. Itagaki and P. L. Hager (1966) *J. Biol. Chem. 241,* 3687] moves as a single band on gel electrophoresis and has an amino acid composition, except for the lysines, of that of the known sequence. The CD spectra of the ferric and ferrous protein in the region 185–600 nm and the resonance Raman spectra in the region 1300–1700 cm^{-1} upon excitation with 5145 Å are reported. Resonance Raman spectra of both the ferric and ferrous forms exhibit features characteristic of the low-spin electronic configuration of heme iron. The intrinsic CD spectrum is typical of α-helical proteins, 52% α helix, no β structure, and 49% random form in the case of the ferric protein and 49% α helix and no β structure in the reduced form. The aromatic CD spectrum of the ferric form exhibits a band at 260 nm, whereas the ferrous form exhibits bands at 274, 288, and 299 nm. The Soret spectra of the two forms are distinct; the ferric form has a large negative Cotton effect at 421 nm and a small positive peak at 398 nm, and the ferrous form has only two negative Cotton effects at about 423 and 435 nm. The visible spectrum of the ferric form is negative. A low-spin electronic configuration of heme iron in both valences of the metal atom and at neutral pH is established. From a consideration of the CD spectrum it is concluded that (1) heme is localized in an asymmetric environment, (2) heme undergoes a conformational alteration upon change of valence state of the metal atom, and (3) the oxidation–reduction process involves an alteration of the environment of the aromatic chromophores.

Optical activity and resonance Raman spectroscopy provide a unique combination of probes for the elucidation of the protein conformation of hemoproteins (Myer and Pande, 1978; Myer, 1978; Blauer, 1974) and heme stereostructure and the spin and oxidation states of the metal atom (Spiro and Loehr, 1975), two complementary aspects of structure–conformation–configuration relationships. Among the *b*-type cytochromes (Hagihara et al., 1975), circular dichroism (CD)[1] studies of a variety of proteins have been reported: b_5 (Huntley and Strittmatter, 1972), b_2 (Sturtevant and co-workers, 1969; Iwatsubo and Risler, 1969), and b_{555} and b_{563} (Okada and Okunuki, 1970a,b). Comparative resonance Raman (RR) studies of cytochromes *b* and b_5 have been reported by Adar and Erecinska (1974) and Kitagawa et al. (1975). These studies have led to a better comprehension of interrelationships between protein conformation, the presence or absence of prosthetic groups, the oxidation state of the metal atom, etc. (Myer and Pande, 1978; Myer, 1978), and the mode of differentiation among these proteins (Adar and Erecinska, 1974; Kitagawa et al., 1975), but a detailed elucidation of structural–conformational or structure–heme configuration relationships has been difficult to obtain. This is because most of these proteins are poorly characterized, i.e., they are of unknown sequence, amino acid composition, spin state of heme iron, etc., for example, cytochromes b_2, b_{555} and b_{563}. Since a question has been raised regarding the integrity of membrane-solubilized preparations, e.g. cytochrome b_2 (Jacq and Lederer, 1974), the long-term significance of findings for studies of these systems is uncertain. In this report we present CD and RR spectroscopic studies of a water-soluble and non-membrane-localized cytochrome *b*, b_{562} from *Escherichia coli* (Itagaki and Hager, 1966), which is devoid of all the problems mentioned above. Its amino acid sequence is known (Itagaki and Hager, 1968), the molecular site of the prosthetic group, a single iron protoporphyrin IX, has been elaborated (Warme and Hager, 1970), and recently preliminary results from X-ray diffraction have also been reported (Czerwinski et al., 1972). The suggestion that the three-dimensional structure of this protein is similar to that of myoglobin (Warme and Hager, 1970), on the one hand, and that the protein possibly functions as a soluble electron carrier, on the other (Hagihara et al., 1975; Itagaki and Hager, 1968; Warme and

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[1] Abbreviations used: CD, circular dichroism; RR, resonance Raman; DM, debye magneton, unit for rotatory strength of Cotton effects, R_k, 1 DM = 0.927×10^{-38} cgs units.

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