J. Comp. Physiol. 140, 199–207 (1980)

Journal of Comparative Physiology · B

Hemocyanins in Spiders

XI. The Quaternary Structure of *Cupiennius* Hemocyanin

Jürgen Markl
Zoologisches Institut der Universität München, Luisenstrasse 14, D-8000 München 2, Federal Republic of Germany

Accepted September 22, 1980

Summary. 1. The hemocyanin of the lycosid spider *Cupiennius salei* was separated into its hexameric (16 S) and dodecameric (24 S) components, and analyzed quantitatively. The reassociation and topologic distribution of the subunits were studied.

2. There are two types of subunits. One is monomeric (5 S) and consists of 5 electrophoretically distinct bands which are, however, immunologically identical. The other is a disulphide bridged dimer (7 S) which yields 2 components upon electrophoresis or immunoelectrophoresis. The significance of this heterogeneity was not studied. The dimer is antigenically deficient with respect to the monomer.

3. Whereas the 16 S hemocyanin is composed of six monomers, 24 S hemocyanin contains 10 monomers and 1 dimer.

4. Alkaline dissociation of 24 S hemocyanin (dodecamer) into subunits passes through a heptameric state (18 S) which is composed of 5 monomers and the dimer. In the electron microscope, 16 S-like units with a seventh polypeptide attached can be distinguished.

5. Treatment of 24 S or 18 S hemocyanin with reducing agents to cleave the disulphide bridge leads to a second type of hexamer (16 S') which is electrophoretically distinct from native hexamers (16 S), and composed of 5 monomers and one constituent polypeptide chain of the dimer.

6. Upon dialysis of a monomer/dimer mixture against neutral buffer containing 40 mM calcium, 16 S, 18 S and 24 S particles are formed. The three reconstituted hemocyanins exhibit subunit compositions identical to the native hemocyanins and the 18 S component obtained during dissociation.

7. The results suggest that the 24 S hemocyanin particle consists of two identical hexamers linked by the disulphide bridge of a dimeric subunit shared by both hexamers.

Introduction

Among arthropods, the respiratory protein hemocyanin occurs in many Chelicerata and in the higher Crustacea. The basic subunit of M_r 75,000 is arranged in hexamers, showing an octahedral structure (Wibo 1966). Whereas the one-hexamer (16 S) is typical for a number of species, larger aggregates occur in others: two-hexamer (24 S), four-hexamer (35 S), and eight-hexamer (60 S) particles (Van Holde and van Bruggen 1971). Many authors have described arthropod hemocyanins as heterogeneous at the level of the polypeptide chains (for references, cf. Markl et al. 1979a, b). However, the extent of subunit heterogeneity, and its structural and functional significance are not yet clear; therefore it is difficult to recognize properties common to all arthropodan hemocyanins.

Whereas the functional significance of subunit heterogeneity is little understood, some information is available on its role in quaternary structure. This has been studied in the 60 S hemocyanin from the horseshoe crab *Limulus polyphemus* (Bijlholt et al. 1979), and the 35 S hemocyanin from the scorpion *Androctonus australis* (Lamy et al. 1981) and from the tarantula *Eurypelma californicum* (Markl et al. 1981). For example, the 35 S hemocyanin of *Eurypelma* is reassembled from a mixture of five different monomers and a heterodimer, all seven polypeptide chains being immunologically unrelated to each other. If certain chains are omitted, intermediate aggregates (10 S, 16 S, 24 S, 30 S) are formed, depending on the subunits present. In the hemocyanins of the three aforementioned species, a dimeric subunit plays a key structural role; if it is not present, the self-assembly process stops at the 16 S level. In some species having both 16 S and 24 S hemocyanins, dimeric subunits occur only in the two-hexamer component; this was found in the freshwater crayfish *Cherax destructor* (Murray and Jeffrey 1974) and *Astacus leptodactylus*

0174-1578/80/0140/0199/$01.80

(Markl et al. 1979b; Pilz et al. 1980), and in the spider *Cupiennius salei* (Markl et al. 1979a).

The present study investigates subunit composition of the 16 S and 24 S hemocyanins from *Cupiennius*, and the topologic arrangement of the dimer within the 24 S particle. For the first time a dimeric subunit can be clearly demonstrated to form the bridge in an hemocyanin aggregate, with one constituent polypeptide chain in each of two hexamers.

Materials and Methods

Isolation of Hemocyanins

Animal maintenance, withdrawal of hemolymph samples, isolation of 24 S and 16 S hemocyanin, and isolation of dissociation products by gel filtration have been described previously (Markl et al. 1979a).

Dissociation and Reassociation

Dissociation into subunits was performed by dialysis of 1–10 mg protein per ml against glycine/NaOH-buffer of pH 9.6, $I=0.05$ (overnight, room temperature). For reassociation, 1–10 mg protein per ml were dialyzed against 0.1 M Tris/HCl-buffer of pH 7.5, containing 40 mM calcium chloride (Schmid 1976).

Treatment with Reducing Agents

Protein solutions were treated for 1 h at 37° C with one of the following agents (final concentration): 20 mM cysteine, 10 mM dithiothreitol, 1% β-mercaptoethanol. Following this treatment the proteins were subjected to electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE)

For analytical purposes, slab gel electrophoresis in 5–10 per cent polyacrylamide gradients were employed throughout (Schneider et al. 1977). For polypeptide chains denatured by sodium dodecyl sulphate (SDS) the method of Laemmli (1970) was used; 'native' proteins were separated in the same system but omitting SDS. Micropreparative PAGE was carried out using the same sytem with 4.5 per cent gels in an analytical electrophoresis apparatus (Hölzel, Dorfen), modified and equipped with an elution chamber. For elution, the reassociation buffer was employed.

Immunoelectrophoresis

Crossed immunoelectrophoresis was performed according to Weeke (1973) in an LKB 2117 Multiphor apparatus using plastic plates (LKB) as support. The gel consisted of 1% Agarose M (LKB) in 0.12 M sodium barbital buffer pH 8.6, $I=0.02$. For the second dimension, 3 ml of antiserum were added per 100 ml. Antiserum against dissociated hemocyanin was prepared as described previously (Lamy et al. 1979a).

Analytical Ultracentrifugation

A Beckman Model E analytical ultracentrifuge equipped with scanner optics was used for sedimentation analysis. The experiments were performed by Mr. H. Decker.

Electron Microscopy

Electron microscopy was carried out by Mrs. W. Schutter in the laboratory of Prof. Dr. E. van Bruggen, University of Groningen, The Netherlands. Specimens were prepared by the spray-droplet technique using aqueous 1% unbuffered uranyl acetate as a negative stain (Siezen and van Bruggen 1974), and studied with a JEM 100 B electron microscope.

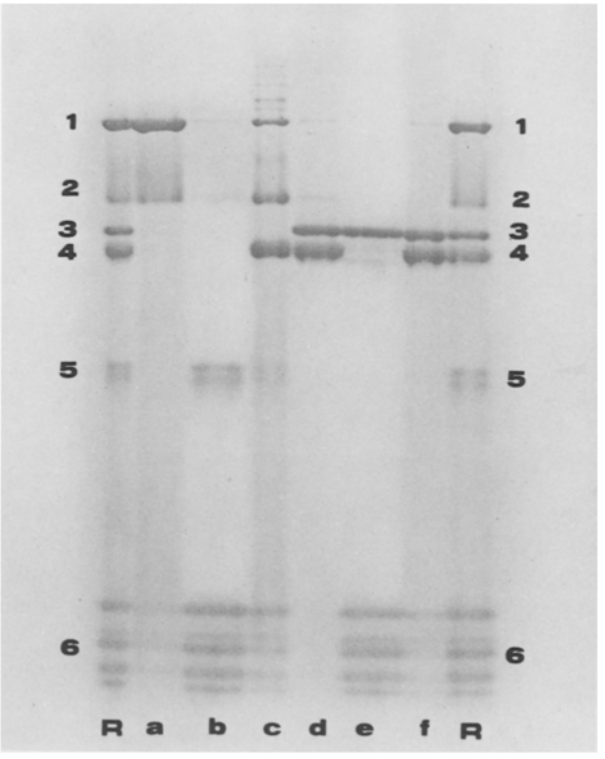

Fig. 1. Polyacrylamide gradient (5–10%) electrophoresis patterns of native (*a*), dissociated (*b*), and reassociated (*c*) 24 S hemocyanin (Hc), and of native (*d*), dissociated (*e*), and reassociated (*f*) 16 S hemocyanin from the spider *Cupiennius*. *R* reference samples, containing 24 S Hc =dodecamer (*1*), 18 S Hc =heptamer (*2*), 16 S non-respiratory protein (*3*), 16 S Hc =hexamer (*4*), 7 S Hc =dimer (*5*), and 5 S Hc =monomers (*6*). The two additional 16 S bands slightly visible in *e* are non-respiratory proteins (unpublished results). Note the aggregates larger than 24 S in *c*. Anode was at the bottom. The system of Laemmli (1970) was employed, omitting SDS

Quantitative Methods

Electrophoretic determination of molecular mass was done according to Hedrick and Smith (1968), using the system of Laemmli (1970) without SDS. Polyacrylamide gels from 4% to 6.5% were applied. 7 S, 16 S, and 24 S hemocyanin were used as standards.

For quantitative evaluation of subunit proportions, stained acrylamide gels were scanned in a Joyce-Loebl 'Chromoscan 200'. Peaks obtained by this technique, by gel filtration, and by crossed immunoelectrophoresis were planimetered using a Summagraphics digitalizing table connected to a Tektronix TK 31 table computer. The computer programme was developed by Mr. H. Decker.

Results

Dissociation of 24 S and 16 S Hemocyanin

The hemolymph proteins from *Cupiennius* were separated into two peaks by gel filtration (Markl et al.

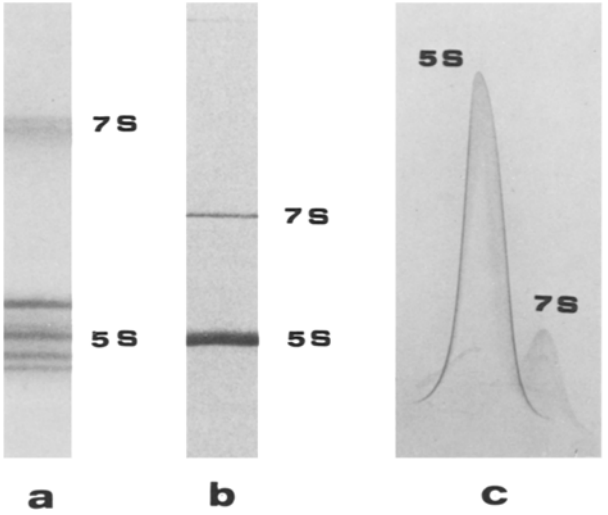

Fig. 2a–c. Patterns from regular PAGE (**a**), SDS-PAGE without reducing agents (**b**), and crossed immunoelectrophoresis (**c**) of dissociated 24 S hemocyanin from *Cupiennius*. 7 S = dimeric subunit, 5 S = monomeric subunits. Note the immunological identity within the monomers despite the marked electrophoretic heterogeneity, and the immunological deficiency of the dimer compared to the monomers (see also: Markl and Kempter 1980). The inhomogeneity of the dimer fraction visible in **a** and **c** is presently under study. From **a** and **b** a ratio of 10 monomers to 1 dimer was estimated by gel scanning. In **a** and **b** the anode is at the bottom, in **c** the anode in the first dimension is on the left. Systems of Laemmli (1970) (**a** and **b**) and Weeke (1973) (**c**)

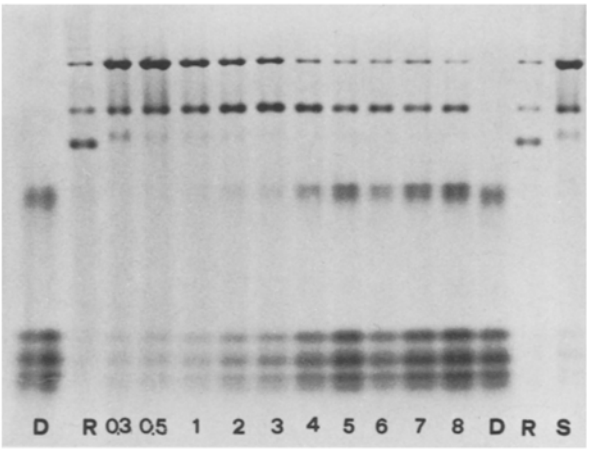

Fig. 3. Time course of dissociation of 24 S hemocyanin from *Cupiennius* studied by regular PAGE (system according to Laemmli 1970, but no SDS or reducing agent; anode is at the bottom). *S* starting material (containing 24 S, 18 S, and 16 S' hemocyanin) was dialyzed different time periods (0.3–8 h) against alkaline buffer. *D* completely dissociated material after 24 h dialysis, containing 7 S and 5 S hemocyanin. *R* reference samples containing 24 S, 18 S, and 16 S hemocyanin (from top to bottom). Note that 18 S hemocyanin shows the typical behavior of an intermediate product, whereas 16 S' (which is completely absent in very fresh samples) rapidly dissappears, and 16 S is not formed at all

1979a). The faster migrating component represents mainly 24 S hemocyanin. In polyacrylamide electrophoresis it showed a major and a somewhat diffuse minor band (Fig. 1). The second peak contained material sedimenting with ca. 16 S (see below). These bands have previously been observed by Czichos-Tiedt (1975). Analytical ultracentrifugation of the first peak revealed the presence of 85% 24 S molecules and 15% of 18 S material (sedimentation coefficients were not corrected). During aging, the percentage of the 18 S component in the sample increased slowly (after two weeks storage at 4 °C it was about 20% of the total); additionally, a considerable amount of 5 S material appeared.

Overnight dialysis against alkaline buffer (pH 9.6) dissociated the 24 S and 18 S molecules almost quantitatively into a number of monomeric (5 S) and dimeric (7 S) subunits (Fig. 1b). It has been demonstrated previously that the set of electrophoretically distinct monomeric bands is immunologically and functionally identical (Markl and Kempter 1981); the observed electrophoretic differences are caused by slight chemical differences (Markl et al. in preparation). The dimeric fraction was found to be antigenically deficient compared to the monomers (Fig. 2c). Both electrophoretically and immunologically it is not homogeneous, showing two components in each system but this has not been studied further. The time dependence of the dissociation of 24 S hemocyanin was investigated by PAGE (Fig. 3). Whereas the percentage of 24 S hemocyanin continually diminished, the proportion of 18 S material increased in the initial phase, remained high for several hours, and finally decreased when the 24 S hemocyanin had been broken down completely. This is the typical behaviour of a dissociation intermediate.

Electron microscopical analysis of 24 S material, dialyzed for 3 h against alkaline buffer in order to yield high amounts of 18 S material, showed typical one-hexamer and two-hexamer structures. However, many of the one-hexamer particles had a little knob attached to them (Fig. 4a). Obviously, the knob represents a seventh subunit sticking out of the hexamer, and the heptameric structure is identical with the 18 S component. The appearance of considerable amounts of 16 S-like structures is no contradiction of the results illustrated in Fig. 3 (where the 16 S band is nearly absent in the three-hour sample), since the attached subunit of the 18 S molecule should be visible only if the particle is oriented with the subunit sticking out sideways.

However, the absence of 16 S material from the

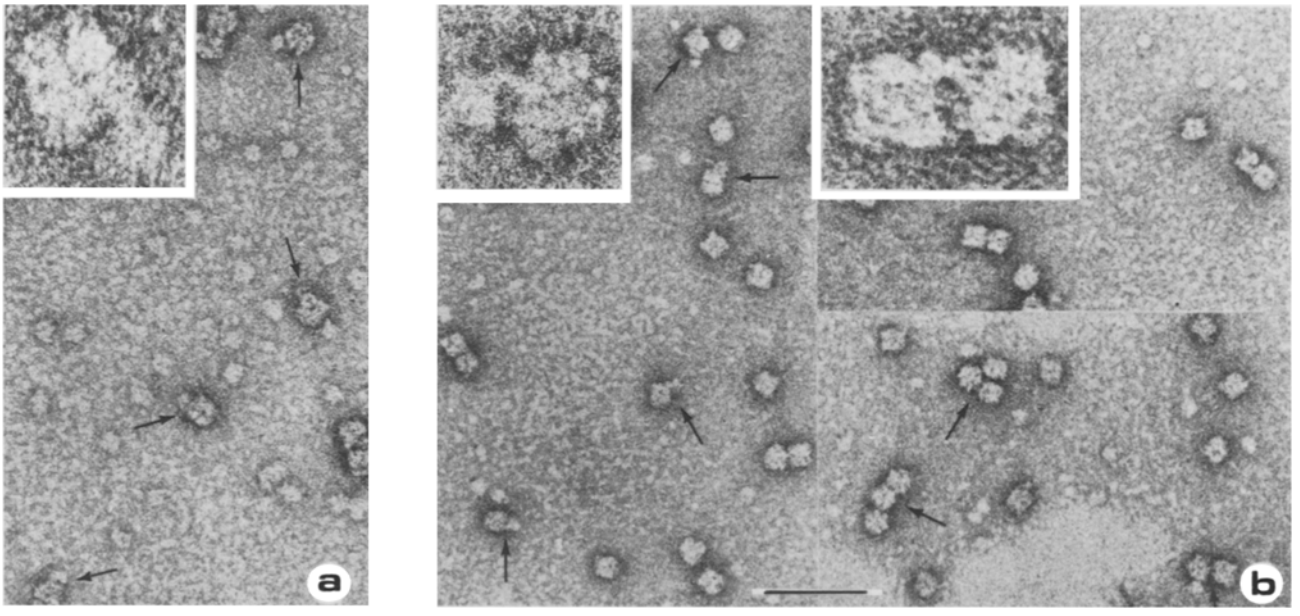

Fig. 4a, b. Electron microscopy of negatively stained *Cupiennius* hemocyanin aggregates. **a** 18 S structures (arrows) obtained by 3 h dialysis of 24 S hemocyanin against dissociation buffer. The inset shows a magnification of a characteristic profile. **b** 16 S, 18 S (arrows), 24 S, and larger (arrows) structures obtained by reassociation from a monomer/dimer mixture by dialysis against neutral buffer containing 40 mM Ca^{++} (three different areas from the same micrograph). The insets show magnified profiles of 18 S and 24 S particles; in both cases the small connecting bridge should be noted. The bar represents 50 nm; total magnification was 260,000×. Experiments were performed by Mrs. W. Schutter, University of Groningen

dissociation products of 24 S hemocyanin is only true for relatively fresh samples. After prolonged storage (about 2 weeks at 4 °C) a new band appeared in the electrophoresis pattern, migrating slightly slower than native 16 S hemocyanin (Fig. 3). After two months, it amounted to about 50% of the total (Fig. 5a). This component has been called 16 S'; it will be shown below that it is indeed a hexamer.

The native 16 S hemocyanin was present in the second peak of the initial gel filtration, contaminated by the 16 S 'non-respiratory protein' described previously (Markl et al. 1976). Overnight dialysis against alkaline pH led to complete dissociation into a number of monomeric bands which were electrophoretically identical with those produced by the 5 S fraction obtained from 24 S hemocyanin (Fig. 1e). No dimeric subunit was observed among the dissociation products of 16 S hemocyanin. The 16 S non-respiratory protein was composed of one major and two minor bands and was not dissociated at pH 9.6. The two minor bands have been mistaken for distinct 16 S hemocyanins in a recent paper (Markl and Kempter 1980).

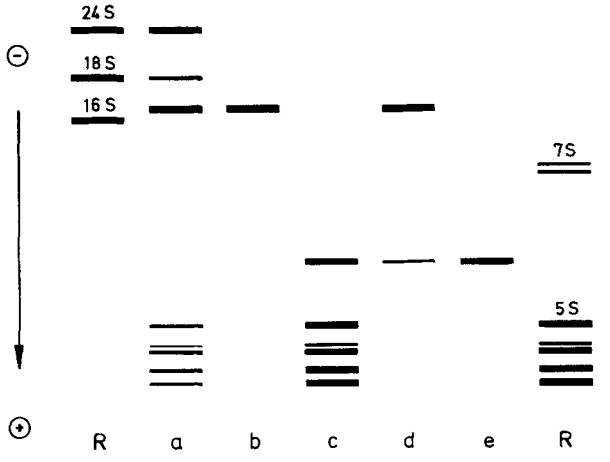

Fig. 5. Specific cleavage of the disulphide bridge of the dimeric subunit from *Cupiennius* hemocyanin. Schematical patterns of regular PAGE of various samples are shown. *a* Prolonged storage of 24 S Hc (2 months at 4 °C); 18 S Hc and 16 S' Hc are formed. *b* Treatment of 24 S Hc with reducing agents; 16 S' Hc is formed. *c* Sample *b* after alkaline dissociation, showing a new subunit. *d* Treatment of 18 S Hc with reducing agents; 16 S' Hc and the new monomeric subunit are formed. *e* Treatment of the dimer with reducing agents, showing the new monomer to be its constituent polypeptide chain. *R* reference samples

Reassociation of 16 S, 18 S, and 24 S Hemocyanins

Schmid (1976) and Loewe et al. (1977) have shown the importance of calcium ions for the reassociation of *Cupiennius* hemocyanin from its subunits. This was confirmed in the present study: without calcium the amount of aggregates obtained by dialysis against neutral pH was small, but the presence of 40 mM

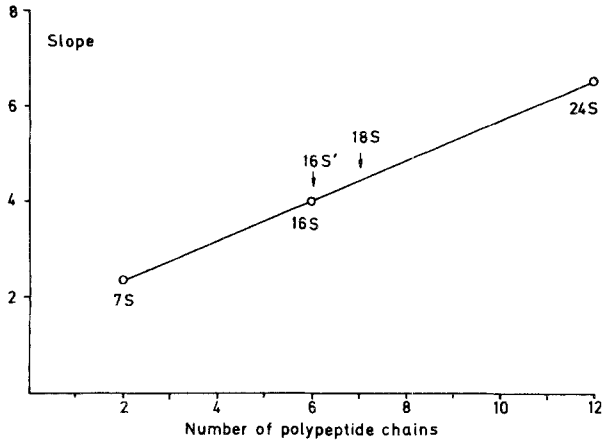

Fig. 6. Determination of the number of polypeptide chains contained in 16 S' and 18 S hemocyanin by the electrophoretic method of Hedrick and Smith (1968). The slope was calculated from the semilogarithmic plot of protein mobility relative to the dye front vs. acrylamide gel concentration. The following continuous gel concentrations were used: 4%, 4.5%, 5%, 5.5%, 6%, 6.5%. 7 S Hc (2 chains), 16 S Hc (6 chains), and 24 S Hc (12 chains) were employed as standards

calcium led to an almost quantitative reassociation of 16 S hemocyanin from monomers (Fig. 1f). If, in addition, the dimer was present, a mixture of 16 S, 18 S, and 24 S particles was obtained, accompanied by some still larger aggregates (Fig. 1c). Viewed by the electron microscope, typical one-hexamer and two-hexamer particles were visible, together with an appreciable amount of 16 S-like structures with a little particle attached to them (Fig. 4b). Judged by their shape, these molecules were identical with the heptamers (18 S) obtained during dissociation (Fig. 4a). Additionally, aggregates larger than 24 S were observed, especially three-hexamers and two-hexamers with an attached subunit (Fig. 4b).

Electron microscopy strongly suggests that the 18 S particle contains seven polypeptide chains. For an independent estimate of molecular mass, the electrophoretic method of Hedrick and Smith (1968) was applied, using 7 S hemocyanin (2 polypeptide chains), 16 S hemocyanin (6 polypeptide chains) and 24 S hemocyanin (12 polypeptide chains) as standards. The results show that there are seven subunits in the 18 S hemocyanin and six subunits incorporated in the ageing product 16 S' (Fig. 6).

Analysis of Subunit Proportions in the Various Aggregates (Table 1)

The various hemocyanin preparations were dissociated and the molar monomer-dimer ratios determined by several methods (Table 1): analytical ultracentrifugation, analysis of elution profiles from gel filtration (separation of monomers and dimers by Sephadex G 100 sf), and scan analysis of the electrophoresis patterns shown in Fig. 2 (regular PAGE, and SDS-PAGE, the latter without reducing agents). The native dodecameric 24 S molecule turned out to be composed of 10 monomeric subunits and 1 dimer. The immunoelectrophoresis pattern obtained from dissociated 24 S hemocyanin (Fig. 2) was calibrated using these values.

The 18 S component was isolated by preparative PAGE using 24 S material which had been dialyzed for 3 h against alkaline buffer. The eluted 18 S hemocyanin was dissociated and electrophoretically analyzed for subunit composition. The patterns from regular PAGE, SDS-PAGE, and crossed immunoelectrophoresis corresponded qualitatively to the results obtained from 24 S hemocyanin (Fig. 2), but the monomer-dimer proportion was different. Quantitative analysis revealed 18 S hemocyanin to be composed of 5 monomeric subunits and 1 dimer.

16 S' was isolated from aged samples of 24 S hemocyanin by preparative PAGE, and dissociated. Regular polyacrylamide electrophoresis showed a set of monomeric subunits and a new band migrating

Table 1. Quantitative proportions (per cent) of total protein in monomeric (first value) and dimeric (second value) subunit fractions contained in the various aggregates of *Cupiennius* hemocyanin. Amount of protein was calculated from the peak areas obtained by monitoring column effluents, scanning stained polyacrylamide electrophoresis gels, or by evaluating ultracentrifuge runs and crossed immunoelectrophoresis gels. *Diss* dissociation intermediates; *reass* reassociation products

Hemocyanin aggregate	No. of subunits	Analytical ultracentrifuge	Gel chromatogr.	Regular PAGE	SDS-PAGE	Immuno-electrophoresis
24 S native	12	82.5:17.5	80.0:20.0	83.2:16.8	81.5:18.5	84.8:15.2
24 S reass	12	–	–	84.3:15.7	82.8:17.2	–
18 S diss	7	–	–	74.0:26.0	72.2:27.8	73.4:26.6
18 S reass	7	–	–	70.5:29.5	70.2:29.8	–
16 S native	6	100 : 0	100 : 0	100 : 0	100 : 0	100 : 0
16 S reass	6	–	–	100 : 0	100 : 0	–
16 S' diss	6	–	–	81.6:18.4[a]	–	–

[a] Second value represents amount of 'artificial' monomer obtained by cleavage of the disulphide bridge in native 24 S hemocyanin

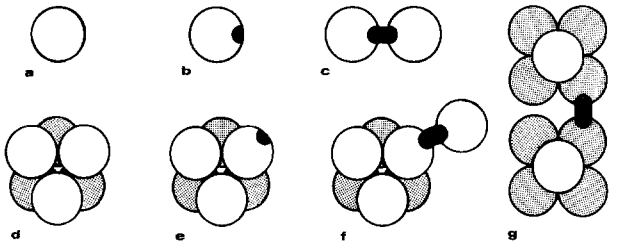

Fig. 7a–g. Model of quaternary structure of *Cupiennius* hemocyanin, showing the topologic distribution of the two distinct types of polypeptide chains. **a** Basic monomeric subunit (5 S), presumably possessing 4 intersubunit binding sites. **b** Constituent polypeptide chain of the dimeric subunit; the hypothetical fifth binding site, responsible for the intermolecular disulphide bridge, is indicated by a filled circle. **c** Dimeric subunit (7 S); the disulphide bridge is represented by a bar. **d** Native hexamer (16 S), composed of 6 identical monomers, and viewed from the direction of the common three-fold axis (hexagon projection). **e** Artificial hexamer (16 S'), obtained from 24 S hemocyanin after ageing or treatment with reducing agents. **f** Heptamer (18 S), obtained as intermediate product during dissociation of 24 S hemocyanin or during reassociation from a monomer/dimer mixture. **g** Native or reassembled dodecamer (24 S), composed of the central dimeric subunit and 10 monomers. The disulphide bridge connects the two juxtaposed hexamers. Square-square projection

close behind (Fig. 5c). This component was identified as a constituent chain of the dimer (Fig. 5e). By quantitative analysis of the gel scan a molar ratio of 5:1 between the regular monomers and the new component could be determined.

It was of special interest, then, to determine the subunit composition of the three reassociation products (16 S, 18 S, 24 S; see Fig. 1c) obtained by incubating a monomer/dimer mixture at neutral pH and 40 mM Ca^{++}. This should allow assessment of the specifity of the self-assembly process. The components were isolated by preparative PAGE and electrophoretically analyzed as described above. The 16 S fraction was exclusively composed of monomers, whereas the two larger particles also contained the dimer, the monomer-dimer mole ratios being 5:2 (18 S) and 10:2 (24 S), respectively. This agreed with the results obtained from the native molecules.

Cleavage of 24 S Hemocyanin into Half Molecules (16 S')

The two constituent polypeptide chains of the dimer are connected by disulphide bonds (Markl et al. 1976). To study the role of this bond in the quaternary structure, 24 S hemocyanin was treated with reducing agents under non-denaturing conditions. Whereas 20 mM cysteine was ineffective, incubation in 1% β-mercaptoethanol or 10 mM dithiothreitol (1 h at 37 °C) caused complete cleavage of the 24 S molecules into 16 S particles. Electrophoretically, the fragment was identical with the ageing product 16 S' (Fig. 5b). In the electron microscope, regular 16 S-like structures were visible. Dialysis against alkaline buffer established the identity of the fragment with 16 S': It revealed the same subunit composition (Fig. 5c), consisting of 'regular' monomers and the additional new monomeric component. The latter could be identified as a constituent polypeptide chain of the dimer by treatment of the dimer with reducing agents (Fig. 5e).

To establish the structural role of the disulphide bridge, 18 S hemocyanin was also incubated in dithiothreitol-containing buffer. Fragmentation into 16 S' and the half-dimer was observed by PAGE (Fig. 5d). This experiment proves unequivocally that the 'attached' subunit of the 18 S molecule is identical with one constituent chain of the dimer.

Discussion

Several 60 S and 35 S hemocyanins from cheliceratan species have been shown to contain 7–8 chemically and immunologically distinct polypeptide chains (Sullivan et al. 1976; Markl et al. 1979c; Hoylaerts et al. 1979; Lamy et al. 1979a, b, c). With respect to this complexity, the 24 S hemocyanin from the lycosid spider *Cupiennius salei* is of considerable interest, since this molecule is composed of only 2 immunologically different types of subunits – one is monomeric, the other is a disulphide bridged dimer (Markl et al. 1979a; Markl and Kempter 1981). Dimeric subunits have been described in a number of hemocyanins; generally they seem to play an essential role in the formation of aggregates larger than hexamers (Murray and Jeffrey 1974; Lamy et al. 1977; Markl et al. 1979a, b; Bijlholt et al. 1979; Markl et al. 1981). Loewe et al. (1977) demonstrated in the case of *Cupiennius* that subunits prepared from 16 S hemocyanin were not able to reassociate to 24 S particles. The absence of the dimeric subunit detected earlier (Markl et al. 1976) is a possible reason for this. In the present study it has been shown conclusively, that the dimer is essential for the formation of the 24 S structure. Quantitative analysis revealed that this particle is composed of 1 dimer and 10 monomers. The occurrence of well defined intermediate aggregation states between the dodecamer and the subunit level enabled the exact determination of the topologic position of the dimer within the 24 S molecule.

Fig. 7 shows schematically the proposed quaternary structure of *Cupiennius* hemocyanin. Based on electron microscopy (e.g. Van Holde and van Bruggen 1971) and ultracentrifugation data (e.g. Johnson and Yphantis 1978), 16 S hemocyanins from arthropods are generally accepted to be hexamers, and 24 S hemocyanins to be dodecamers of approximately spheri-

cal subunits (M_r=75,000). Recently, a more kidney-like shape has been reported for the subunits, based on X-ray crystallography data of *Panulirus interruptus* (a spiny lobster) 16 S hemocyanin (van Schaick et al. 1981). However, this has no implications for the conclusions drawn here.

The 6 polypeptides of a 16 S unit occupy the corners of a regular octahedron (Wibo 1966; Klarmann et al. 1979; Jeffrey 1979; Jeffrey and Andrews 1980; Pilz et al. 1980), or of a trigonal antiprism (Schepman 1975; Schutter et al. 1977; van Schaik et al. 1981). Lontie and Witters in their review (1973) pointed out that these structures can account for all contours seen in the electron microscope: hexagon, square, rectangle, and rhomb. The 16 S hemocyanin of spiders belongs to this general arthropodan scheme (Wibo 1966).

24 S hemocyanin is a dodecamer composed of two 16 S units. For *Cherax destructor* hemocyanin, Jeffrey (1979) proposed that the 2 hexamers are rotated 90° to each other to yield a close contact, in which 4 subunits are directly involved. For *Astacus leptodactylus* hemocyanin, an interhexamer contact involving 6 subunits was assumed (Pilz et al. 1980). In both cases hexagon-square projections are visible in the electron microscope. In contrast, in the contact region between the 2 juxtaposed hexamers of the 24 S hemocyanin from spiders, only 2 subunits are directly in contact, the hexamers showing a corner-to-corner attachment (Wibo 1966). In the present study, in many cases square-square projections have been observed in the electron micrographs, with a small bridge between the two 16 S units (Fig. 4). Such a picture is also clearly visible in *Tegenaria* hemocyanin (Fig. II 2.6 in Wibo's Thesis, 1966).

For *Cupiennius* it is concluded that the dimeric subunit occupies the central position, the disulphide bridge between the 2 constituent polypeptide chains connecting the two hexamers (Fig. 7g). Various arguments are in favour of this arrangement. A regular octahedron like the 16 S hemocyanin is an energetically favoured structure, stabilized by 12 inter-subunit binding sites. In contrast, there is only one single central binding site to connect two 16 S units to each other in a corner-to-corner arrangement (Fig. 7g). To obtain stability, this inter-hexamer linkage has to be very strong relative to the other bonds linking the subunits within the molecule. The disulphide bridge of the dimer fulfils this requirement.

The appearance, both as a dissociation and reassembly intermediate, of a stable hemocyanin heptamer (18 S), consisting of a regular hexamer and a seventh polypeptide attached to it, provides direct evidence of the pivotal role of the dimer and its disulphide bridge. A direct proof was achieved by treatment of isolated 18 S hemocyanin with reducing agents which specifically cleaved the bond between the hexamer and the attached subunit. The separated polypeptide was identified as a constituent chain of the dimer, whereas the hexamer (16 S') contained the same component and five native monomers in addition. Correspondingly, reducing agents were able to cleave the 24 S molecule into halves (16 S').

Immunochemical results fit well into the scheme. Theoretically, the 16 S structure could be constructed from 6 identical polypeptides, each possessing 4 binding sites for other subunits. The observation of a complete immunological identity of the 5 electrophoretically distinct monomers is in good accord with this hypothesis. The functional identity of the 5 bands has already been reported (Markl and Kempter 1981), and will be described in detail elsewhere (Markl et al. in preparation). The bridge between 2 hexamers requires an additional binding site at the surface of the 2 chains involved in this contact. This should be manifested in a different antigenic behaviour. The immunological identity of the monomers indicates their equivalent positions within the 24 S molecule and makes it unlikely that 1 or 2 of them function as inter-hexamer bridges. However, the dimeric subunit is immunologically distinct from the monomers, showing an antigenic deficiency compared to them. Presumably, the surfaces of the monomeric chains and of the dimer differ only with respect to the fifth binding site which is obviously sterically protected against antibodies.

The marked electrophoretical heterogeneity of the monomers of *Cupiennius* hemocyanin is only partially understood. It is based on slight chemical diversities which obviously do not influence the immunological, nor apparently, the functional properties (Markl et al. in preparation). The immunoelectrophoresis pattern of the dimer exhibited 2 components; this observation awaits independent confirmation. In various crustacean hemocyanins too the number of electrophoretically separated bands exceeds the number of immunologically distinct subunits (Markl and Kempter 1980). In dissociated hemocyanin from *Limulus*, 12 components were identified by PAGE (Markl et al. 1979a), whereas with immunological methods only 8 subunits could be detected (Hoylaerts et al. 1979; Lamy et al. 1979c). It appears doubtful that this 'micro-heterogeneity', which is not detectable by immunochemistry, should play a biological role.

Compared to *Cupiennius*, the localization of the dimer is less certain in other hemocyanins, although a dimeric bridge has been proposed in various instances. Jeffrey (1979) has published a preliminary model of the 24 S hemocyanin from *Cherax destructor* containing a dimer arranged symmetrically, but he

pointed out the possibility of an asymmetrical position. The 35 S hemocyanin of *Eurypelma californicum* appears to be composed of 2 dodecameric substructures with presumably identical subunit composition, each containing 1 dimer and 10 monomers (Markl et al. 1980). In reassembly experiments, the 24 S structure is not formed unless the dimer is present. It was assumed that the dimeric subunit is completely incorporated into one of the hexamers and specifically associated with particular subunits in the other hexamer to form the bridge (Markl et al. 1981). In contrast, in the case of the 35 S haemocyanin from *Androctonus autralis* a symmetrical position of the dimer within the 24 S substructure was proposed (Lamy et al. 1980). Since both hemocyanins are closely related to each other immunologically (Lamy et al. unpublished), presumably only one of these alternatives will be true. With respect to the present results obtained from *Cupiennius* hemocyanin, Lamy's hypothesis seems to be the more likely one. Experiments are in progress to investigate whether the model established for the 24 S *Cupiennius* hemocyanin is also applicable to the 35 S haemocyanin from *Eurypelma*.

Electron microscopy was carried out by Mrs. W.G. Schutter in the laboratory of Prof. Dr. E. F. J. van Bruggen, University of Groningen, The Netherlands. Mr. K. Gilissen prepared the photo documentation. Their interest and splendid cooperation is kindly appreciated.

This work was supported by the Deutsche Forschungsgemeinschaft (Li 107/20+21). The analytical ultracentrifuge is a gift of the Stiftung Volkswagenwerk. I thank Mrs. M. Brenzinger for her excellent technical assistance, Mr. H. Decker for supplementary experiments, and Prof. Dr. B. Linzen for his permanent interest and support, and critical discussion of the manuscript.

References

Bijlholt MMC, Bruggen EFJ van, Bonaventura J (1979) Dissociation and reassembly of *Limulus polyphemus* hemocyanin. Eur J Biochem 95:399–405

Czichos-Tiedt S (1975) Versuche zur Reinigung und Charakterisierung des Hämocyanins aus der Spinne *Cupiennius salei* Keyserling. Thesis, Universität München

Hedrick JL, Smith AJ (1968) Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch Biochem Biophys 126:155–164

Holde KE van, Bruggen EFJ van (1971) The hemocyanins. In: Timasheff SN, Fasman GD (eds) Subunits in biological systems. Dekker, New York, pp 1–53

Hoylaerts M, Préaux G, Witters R, Lontie R (1979) Immunological heterogeneity of the subunits of *Limulus polyphemus* haemocyanin. Arch Int Physiol Biochim 87:417–418

Jeffrey PD (1979) Hemocyanin from the Australian freshwater crayfish *Cherax destructor*. Electron microscopy of native and reassembled molecules. Biochemistry 12:2508–2513

Jeffrey PD, Andrews PR (1980) Application of calculated sedimentation ratios in the specification of models for protein dimers, trimers, tetramers and pentamers. Biophys Chem 11:61–70

Johnson ML, Yphantis DA (1978) Subunit association and heterogeneity of *Limulus polyphemus* hemocyanin. Biochemistry 17:1448–1455

Klarman A, Gottlieb J, Daniel E (1979) Quaternary structure and arrangement of subunits in hemocyanin from the scorpion *Leirus quinquestriatus*. Biochemistry 18:2239–2244

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T 4. Nature (London) 227:680–685

Lamy J, Lamy J, Baglin M-C, Weill J (1977) Scorpion hemocyanin subunits: properties, dissociation, association. In: Bannister JV (ed) Structure and function of haemocyanin. Springer, Berlin Heidelberg New York, pp 37–49

Lamy J, Lamy J, Weill J, Markl J, Schneider H-J, Linzen B (1979a) Hemocyanins in spiders. VII. Immunological comparison of the subunits of *Eurypelma californicum* hemocyanin. Hoppe-Seyler's Z Physiol Chem 360:889–895

Lamy J, Lamy J, Weill J (1979b) Arthropod hemocyanin structure. Isolation of eight subunits in the scorpion. Arch Biochem Biophys 193:140–149

Lamy J, Lamy J, Weill J, Bonaventura J, Bonaventura C, Brenowitz M (1979c) Purification and immunological identification of the dissociation products of native *Limulus polyphemus* and *Tachypleus tridentatus* hemocyanins. Arch Biochem Biophys 196:324–339

Lamy J, Lamy J, Sizaret P-Y, Weill J (1981) Quaternary structure of *Androctonus australis* hemocyanin. In: Lamy J (ed) Structure, active site, and function of invertebrate oxygen binding proteins. Dekker, New York, in press

Loewe R, Schmid R, Linzen B (1977) Subunit association and oxygen-binding properties in spider hemocyanins. In: Bannister JV (ed) Structure and function of haemocyanin. Springer, Berlin Heidelberg New York, pp 50–54

Lontie R, Witters R (1973) Haemocyanin. In: Eichhorn GL (ed) Inorganic biochemistry, vol 1. Elsevier, Amsterdam, pp 344–358

Markl J, Kempter B (1981) Subunit heterogeneity in arthropod hemocyanins. In: Lamy J (ed) Structure, active site, and function of invertebrate oxygen binding proteins. Dekker, New York, in press

Markl J, Schmid R, Czichos-Tiedt S, Linzen B (1976) Haemocyanins in spiders. III. Chemical and physical properties of the proteins in *Dugesiella* and *Cupiennius* blood. Hoppe-Seyler's Z Physiol Chem 357:1713–1725

Markl J, Markl A, Schartau W, Linzen B (1979a) Subunit heterogeneity in arthropod hemocyanins. I. Chelicerata. J Comp Physiol 130:283–292

Markl J, Hofer A, Bauer G, Markl A, Kempter B, Brenzinger M, Linzen B (1979b) Subunit heterogeneity in arthropod hemocyanins. II. Crustacea. J Comp Physiol 133:167–175

Markl J, Strych W, Schartau W, Schneider H-J, Schöberl P, Linzen B (1979c) Hemocyanins in spiders. VI. Comparison of the polypeptide chains of *Eurypelma californicum* hemocyanin. Hoppe-Seyler's Z Physiol Chem 360:639–650

Markl J, Savel A, Decker H, Linzen B (1980) Hemocyanins in spiders. IX. Homogeneity, subunit composition and the basic oligomeric structure of *Eurypelma californicum* hemocyanin. Hoppe-Seyler's Z Physiol Chem 361:649–660

Markl J, Decker H, Savel A, Linzen B (1981) Homogeneity, subunit heterogeneity, and quaternary structure of *Eurypelma* hemocyanin. In: Lamy J (ed) Structure, active site, and function of invertebrate oxygen binding proteins. Dekker, New York, in press

Murray AC, Jeffrey PD (1974) Hemocyanin from the Australian freshwater crayfish, *Cherax destructor*. Subunit heterogeneity. Biochemistry 13:3667–3671

Pilz I, Goral K, Hoylaerts M, Witters R, Lontie R (1980) Studies by small-angle X-ray scattering of the quaternary structure of

the 24 S-component of the haemocyanin of *Astacus leptodactylus* in solution. Eur J Biochem 105:539–543

Schaick EJM van, Schutter WG, Gaykema WPJ, Bruggen EFJ van, Hol WGJ (1981) The crystal structure of the hemocyanin hexamer from *Panulirus interruptus* at 5 Å resolution. In: Lamy J (ed) Structure, active site, and function of invertebrate oxygen binding proteins. Dekker, New York, in press

Schepman AMH (1975) X-ray diffraction and electron microscopy. Thesis, Rijksuniversiteit Groningen

Schmid R (1976) Chemisch-physikalische Untersuchungen an Spinnenhämocyaninen und ihren Untereinheiten. Thesis, Universität München

Schneider H-J, Markl J, Schartau W, Linzen B (1977) Hemocyanins in spiders. IV. Subunit heterogeneity of *Eurypelma (Dugesiella)* hemocyanin and separation of polypeptide chains. Hoppe-Seyler's Z Physiol Chem 358:1133–1141

Schutter WG, Bruggen EFJ van, Bonaventura J, Bonaventura C, Sullivan B (1977) Structure, dissociation and reassembly of *Limulus polyphemus* hemocyanin. In: Bannister JV (ed) Structure and function of haemocyanin. Springer, Berlin Heidelberg New York, pp 13–21

Siezen RJ, Bruggen EFJ van (1974) Structure and properties of hemocyanins. XII. Electron microscopy of dissociation products of *Helix pomatia* α-haemocyanin: Quaternary structure. J Mol Biol 90:77–89

Sullivan B, Bonaventura J, Bonaventura C, Godette G (1976) Hemocyanin of the horseshoe crab, *Limulus polyphemus*. Structural differentiation of the isolated components. J Biol Chem 251:7644–7648

Weeke B (1973) Crossed immunoelectrophoresis. Scand J Immunol 2:47–56

Wibo M (1966) Recherches sur les hémocyanines des arthropodes: constantes de sédimentation et aspects morphologiques. Thesis, Université Catholique de Louvain