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The quaternary structure of four crustacean two-hexameric hemocyanins: immunocorrelation, stoichiometry, reassembly and topology of individual subunits*

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Summary. Two-hexameric (2 × 6) hemocyanins from the brachyuran crabs *Cancer pagurus* and *Callinectes sapidus*, the freshwater crayfish *Astacus leptodactylus* and the lobster *Homarus americanus* were isolated and dissociated into native subunits.

The subunits of each hemocyanin were analyzed by electrophoresis and immunology. Three immunologically distinct subunit types, which were termed α, β and γ, could be identified in each case. They were isolated preparatively, and interspecifically correlated. Subunit α is subdivided into several electrophoretically distinct isoforms which are immunologically closely related (*Astacus*) or identical (other species). In *Astacus* and *Cancer* one of these isoforms was shown to dimerize and to act as inter-hexamer bridge. It represents a fourth subunit type termed α′. A fifth, 'diffuse' component, which in PAGE migrated at the position of a dimer, was identified in the crossed immunoelectrophoretic patterns as denatured hemocyanin.

A common feature of the four hemocyanins is the presence of 4 copies of β and 8 copies of α/γ within the 2 × 6 particles. The α:γ ratio is 4:4 in the two Astacidea and 6:2 in the two Brachyura. α′ exists in 2 copies in *Astacus* and *Cancer* which means that a single dimer α′−α′ is present in a two-hexamer. This leaves 2 monomeric α copies in *Astacus* and 4 in *Cancer*.

Every subunit from the four species except of *Astacus* α′–α′ was capable to form hexamers in reassembly experiments. If subunit combinations were tested, hetero-hexamers were formed preferentially. Two-hexamers were reconstituted only in the presence of all subunit types and the native subunit stoichiometry was required to obtain two-hexamers in considerable yields. Factors limiting 2 × 6 reassembly are discussed.

Authentic 2 × 6 molecules of *Astacus*, *Homarus* and *Cancer* hemocyanin were immunolabeled with subunit-specific antibody fragments (F_{ab}) or IgG molecules, and the resulting immuno complexes were studied in the electron microscope. A topological model of the quaternary structure of decapod 2 × 6 hemocyanins is derived, showing the position of each copy of the four subunit types. In this model, the inter-hexamer bridge α′–α′ is surrounded by two β and two γ subunits forming the central core of the dodecamer. Two additional β and two additional γ subunits form the periphery together with one α subunit occupying the peripheral short edges of each hexameric half structure. The model is discussed with respect to the current literature.

Abbreviations: PAGE polyacrylamide gel electrophoresis; *SDS* sodium dodecyl sulfate

This paper is dedicated to Professor Dr. Bernt Linzen, whose sudden death on August 5, 1988, leaves a deeply felt void.

* Preliminary accounts of this work have been presented in the proceedings of a symposium at Tutzing 1985. Linzen B (ed) (1986) Invertebrate oxygen carriers. Springer, Berlin Heidelberg New York. This also includes: Stöcker et al. 1986; Markl et al. 1986) and in a review article (Markl 1986)

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Introduction

The hemolymph of many Chelicerata and Crustacea (Phylum Arthropoda) contains the blue oxygen carrier hemocyanin, an allosteric multi-subunit protein. In vivo, arthropodan hemocyanins occur

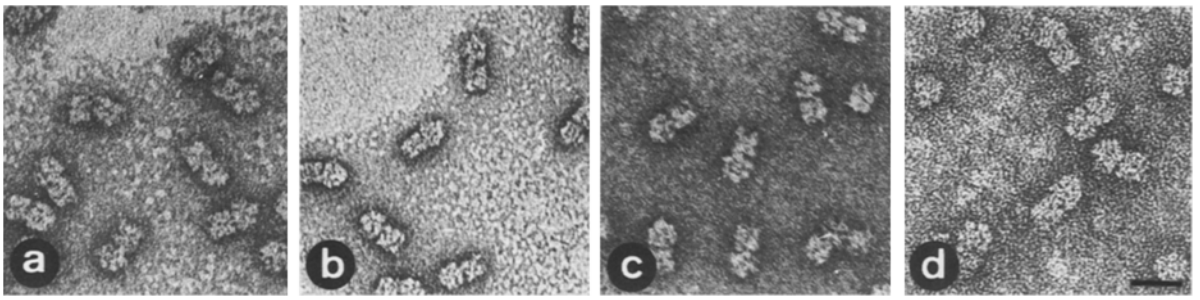


Fig. 1. Electron micrographs of hemocyanin 2 × 6 particles from *Astacus leptodactylus* (**a**), *Homarus americanus* (**b**), *Cancer pagurus* (**c**), and *Callinectes sapidus* (**d**), negatively stained with 1% unbuffered uranyl acetate. The primary magnification was 60000-fold. The bar represents 25 nm

as oligomers with sedimentation coefficients of 16 S, 24 S, 35 S or 60 S, composed of 6, 12, 24 or 48 subunits, respectively. Each polypeptide (subunit) carries a single binuclear copper active site and has a molecular mass close to 75000 Da.

The primary structures of several hemocyanin subunits of both crustacean and cheliceratan origin are known and have been found to be homologous. To date, the only known tertiary structure of an arthropodan hemocyanin is that for the spiny lobster *Panulirus interruptus* (Gaykema et al. 1984). All of the known primary structures fit this tertiary structure, confirming that all arthropod hemocyanins are homologous proteins (Linzen et al. 1985). Despite this homology, the variety of subunits can be identified by pronounced immunological differences (reviews: Linzen 1983; Lamy 1983; Markl 1986).

Six of these monomeric subunits combine to form a hexamer in the shape of a trigonal antiprism (van Holde and van Bruggen 1971; van Holde and Miller 1982; Gaykema et al. 1984). These hexamers can aggregate to form the oligomeric superstructures. It has been shown for cheliceratan hemocyanins that the different monomeric building blocks differ in their structural contribution to oligomer formation (review: Markl 1986). So far, detailed topological models are available for some 2 × 6, 4 × 6 and 8 × 6 hemocyanins from Chelicerata (Markl 1980, 1986; Lamy et al. 1981, 1983; Markl et al. 1981 a). The localization of the different subunits within the quaternary structure of crustacean hemocyanins, however, has not been elaborated. Most Crustacea, except for the thallassinid shrimps which possess a tetrahedrally arranged 4 × 6 hemocyanin, have either simple hexameric or 2 × 6 hemocyanin or both (van Holde and van Bruggen 1971; van Holde and Miller 1982). In the electron microscope, 2 × 6 hemocyanin from decapod Crustacea appears as a combination of a square and a hexagon with a total length of approximately 220 Å and a width of 110 Å (Fig. 1). The square exhibits two sharp and two fuzzy edges (van Bruggen et al. 1980). These structures are interpreted as two hexamers rotated at 90 degrees relative to each other. The small cleft between the two hexamers is interrupted by the point-shaped contact zone which is formed by a corner of the hexagon and a corner of the fuzzy edge of a square (van Bruggen et al. 1981; van Bruggen 1983).

Although the structural roles of individual subunit types have not yet been defined for crustacean hemocyanins, it was deduced from these electron micrographs that a specific subunit might be required as a link between the two hexamers. As candidates for such crosslinking subunits, certain disulfide-bridged dimeric components have been proposed which have been observed in the 2 × 6 hemocyanins of freshwater crayfishes (*Cherax destructor, Astacus leptodactylus:* Jeffrey 1979; Markl et al. 1979; Markl and Kempter 1981 a).

This assumption was confirmed by Markl et al. (1981 b), who obtained heptameric (18 S) intermediates after partial dissociation of *Astacus* 2 × 6 molecules (24 S). The same authors reported that during dissociation of 2 × 6 hemocyanins from the crab *Cancer pagurus* and the lobster *Homarus americanus* hexameric (16 S) dissociation intermediates occur, and that these hemocyanins do not contain covalent dimers. The latter is at variance with the data of Rochu and Fine (1984a, b) who observed a SDS-stable dimeric hemocyanin component in *Cancer*.

A major drawback for a better understanding of crustacean hemocyanin quaternary structure was that the identification of subunit counterparts among the species was still unclear. In this study we present our interpretation of the role of individual subunits in crustacean hemocyanins. We first identify hemocyanin subunits from four different

crustaceans (two Astacidea and two Brachyura), and define subunit types which share immunological similarity. We then reconstitute aggregates from these subunits and find that certain subunit types from different species play similar roles in the 2×6 structure. Finally, we present a model of the quaternary structure of crustacean 2×6 hemocyanins based on direct localization of individual subunits by immuno electron microscopy.

Materials and methods

Chemicals. All chemicals were of analytical grade and purchased from Merck/Darmstadt, Serva/Heidelberg or Sigma/Deisenhofen.

Hemocyanin sources. The European freshwater crayfish *Astacus leptodactylus* (Fam. Astacidae) and the North American lobster *Homarus americanus* (Fam. Homaridae) were purchased from a local seafood dealer. The European crab *Cancer pagurus* (Fam. Cancridae) was obtained from the Biologische Anstalt Helgoland. The American blue crab *Callinectes sapidus* (Fam. Portunidae) was collected at Beaufort, North Carolina, USA. Blood was withdrawn by puncturing the ventral abdominal sinus (*Astacus, Homarus*) or the base of a walking leg (*Cancer, Callinectes*) after cooling the animal on ice. The blood was allowed to clot, and the clotted material and cell debris were removed by centrifugation (12000 *g*). Hemocyanin was separated from most of the other serum proteins by sedimentation in a preparative ultracentrifuge (200000 *g*) as described elsewhere (Markl et al. 1979). The blue hemocyanin pellet was redissolved in 0.1 *M* Tris/HCl buffer, pH 7.5, containing 10 m*M* $CaCl_2$, to a final concentration of 25–100 mg/ml and stored deep frozen (−25° C or liquid air). 2×6 hemocyanin was purified on a column of Biogel A5m (Biorad) as described previously (Markl et al. 1979).

Isolation of hemocyanin subunits. For dissociation, hemocyanins were diluted to 4–6 mg/ml, and dialyzed overnight versus 0.05 *M* Tris/HCl buffer, pH 8.8, containing 1 m*M* EDTA (in the case of *Homarus* hemocyanin), or glycine/NaOH-buffer, pH 9.6, $I = 0.05$, containing 10 m*M* EDTA. This yields 100% monomers in the case of *Homarus, Cancer*, and *Callinectes* hemocyanin, and about 80% monomers and dimers in *Astacus* hemocyanin. Dissociated *Astacus* hemocyanin was concentrated to 20 mg/ml by dialysis against 25% polyethylene glycol (PEG 20000, Serva, Heidelberg) and submitted to gelfiltration through Sephadex G 100 (Pharmacia, Uppsala, Sweden) which, according to Markl et al. (1979), separated undissociated material from a monomeric and a dimeric subunit fraction. The monomeric subunit mixtures were fractionated either by semi-preparative PAGE as described by Markl et al. (1984) or by ion exchange chromatography on a 20×1 cm column of DEAE-Sepharose Cl-6B (Pharmacia, Uppsala, Sweden) equilibrated with 0.05 *M* Tris/HCl-buffer of pH 8.8, containing 1 m*M* EDTA. Elution was performed with a linear gradient from 0.1–0.6 *M* NaCl in equilibration buffer.

Reassociation conditions. Reassembly experiments were performed by dialyzing single hemocyanin subunit types or mixtures of different subunits, at 4° C for seven days against 0.1 *M* Tris/HCl buffer, pH 7.5, containing 10 m*M* $CaCl_2$. The protein concentration was maintained at 1 mg/ml.

Analytical techniques. Protein concentration of hemocyanin solutions was determined at 280 nm in a Cary 118 spectrophotometer (Varian) as described by Loewe and Linzen (1973), in combination with the method of Lowry (1951).

Polyacrylamide gel electrophoresis (PAGE) was performed in slab gels using the system No. 6 of Maurer (1968) for oligomers, the system of Laemmli (1970) for SDS-denatured subunits, and the Laemmli system without sodium dodecyl sulfate (SDS) and β-mercaptoethanol, for native subunits. The acrylamide concentration varied from 3.7% to 20%.

Analytical ultracentrifugation was performed in a Beckman model E ultracentrifuge as previously described (Decker et al. 1980).

Specimens for transmission electron microscopy were prepared according to Siezen and van Bruggen (1974) by the spray droplet technique. For negative staining, 1% (w/v) uranyl acetate was used. Specimens were studied in a JEM 100B electron microscope at 80 kV as described by van Bruggen et al. (1980).

Immunochemistry. Rabbit antisera were raised as described elsewhere (Markl et al. 1984; Kempter et al. 1985). The specificity of antisera for single subunit types ('monospecific antisera') was checked by crossed immunoelectrophoresis. Unspecific reactivities were removed by immunoprecipitation with adequate amounts of pure subunits or subunit mixtures (Markl et al. 1981 a).

IgG-fractions were isolated by affinity chromatography as previously described (Markl et al. 1981 a). In order to generate F_{ab}- and F_c-fragments, IgG-material was incubated with papain (Putnam et al. 1962). The formation of F_{ab}- and F_c-fragments and the purity of IgG-preparations were tested by SDS-PAGE in the absence of reducing agents. F_c-fragments were not removed prior to the immunolabeling experiments. The titer of antisera, IgG-solutions and solutions of F_{ab}-fragments was determined using either the spot-technique of Elwing et al. (1977) or the method of Loft (1975), modified according to Lamy et al. (1983). Alternatively, the whole serum IgG-fraction was isolated by Na_2SO_4-precipitation (Kekwick 1940).

F_{ab}-fragments of the whole IgG-fraction were prepared as described above.

Immunolabeling experiments. Samples of the native dodecameric hemocyanins (3.5 mg/ml) were incubated for 30 min at 37 °C with a ten-fold molar excess of F_{ab}-fragments in 0.1 *M* Tris/HCl-buffer, pH 7.5, containing 10 m*M* $CaCl_2$ and then stored in solution at 4° C. To prepare hemocyanin-IgG-complexes, the conditions were the same, except that the molar ratio of hemocyanin:IgG was 1:1 to avoid precipitation. F_c-fragments, uncoupled F_{ab}-fragments or uncoupled IgG-molecules were removed by thin layer gel chromatography (TLG) on Sephadex G 200sf or G 150 (Pharmacia, Uppsala, Sweden) in 0.1 *M* Tris/HCl buffer, pH 7.5, containing 10 m*M* $CaCl_2$ (Markl et al. 1981 a; Markl et al. 1982). The soluble hemocyanin-immuno complexes were observed in the electron microscope.

Results

Analysis of subunit compositions

Hemocyanin of *Cancer pagurus, Callinectes sapidus, Astacus leptodactylus* and *Homarus americanus* consists of 2×6 aggregates, accompanied by small amounts of hexamers (Fig. 1). All four hemocyanins could be dissociated into native subunits at alkaline pH, which is in agreement with

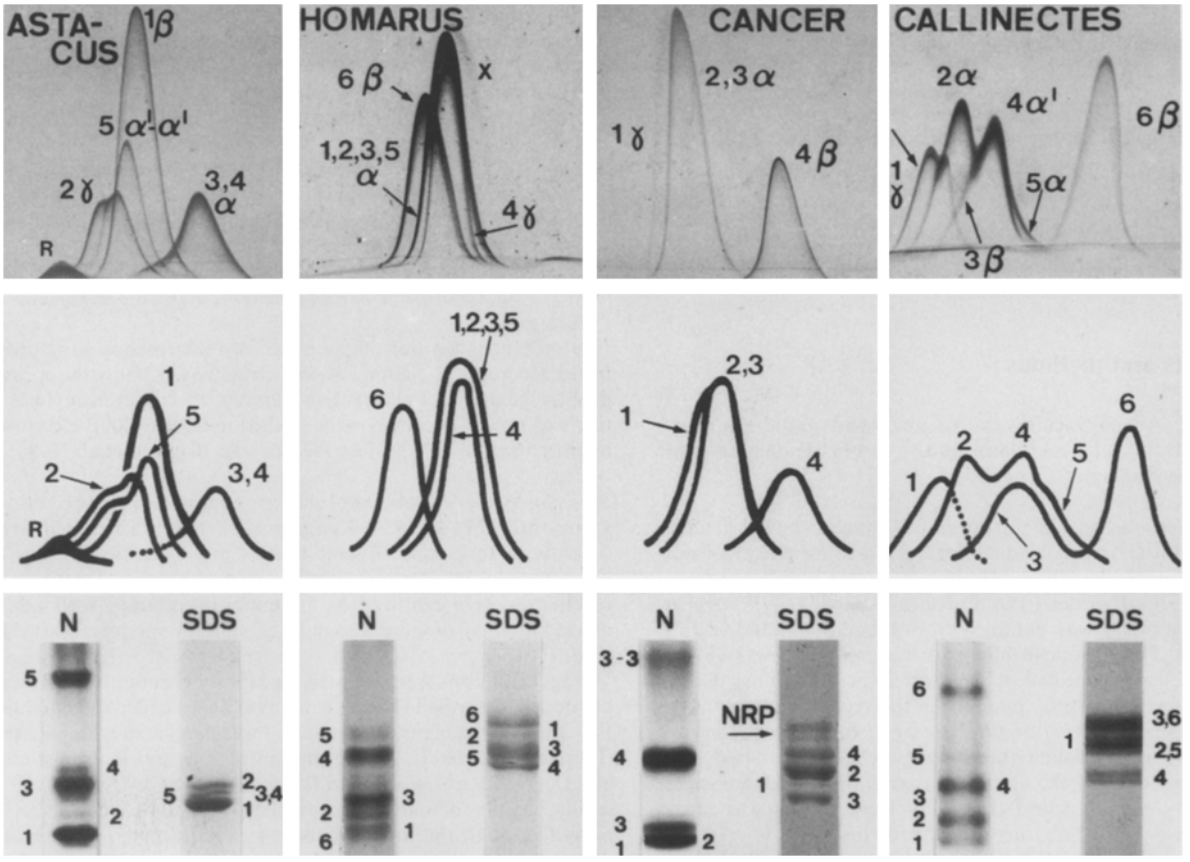


Fig. 2. Upper lane: Crossed immunoelectrophoresis patterns of the dissociated hemocyanins from *Astacus, Homarus, Cancer,* and *Callinectes*, against their respective homologous antisera. In the first dimension, the anode was on the left. Middle lane: Schematical drawings of the above patterns, demonstrating the diverse cross-reactivities as deduced from several experiments. Lower lane: Corresponding PAGE patterns of the native (*N*) and the denatured (*SDS*) hemocyanin subunits at pH 8.8. An acrylamide gradient 5–10% in the system of Laemmli (1970) was employed; for native PAGE, SDS and β-mercaptoethanol were omitted. The anode was to the bottom. Subunits are numbered according to their mobility in native PAGE. *Astacus* 5 and *Cancer* 3-3 are dimers. Immunologically corresponding subunits are classified with greek letters, according to the data described in the text. Molecular masses as deduced from SDS-PAGE: *Astacus* 75000–80000; *Homarus* 76000–82000; *Cancer* 73000–78000; *Callinectes* 76000–83000. *R*=reassociated hemocyanin; *X*=denatured hemocyanin; *NRP*=non respiratory protein

earlier data (Markl et al. 1979; B. Johnson, personal communication). Subsequent to alkaline dissociation, the subunit compositions were analyzed by native PAGE, SDS-PAGE and crossed immunoelectrophoresis (Fig. 2). The subunits were defined according to their mobility in native PAGE, in the context of earlier data (Markl et al. 1979; Markl and Kempter 1981 a).

Cancer and *Callinectes* 2×6 hemocyanin could be completely dissociated into subunits by glycine/NaOH, pH 9.6 (I=0.05). In this buffer, however, the dissociation of *Astacus* hemocyanin was incomplete and at least 20% of the material resisted disassembling below the hexamer level. As verified by SDS-PAGE (not shown), this material is composed of the same set of subunits as the dissociated material. *Astacus* hemocyanin could be completely dissociated in bicarbonate buffer, pH 10.1 (I= 0.05), but then additional diffuse precipitates in crossed immunoelectrophoresis patterns were observed. As will be discussed below, this is an indication that denaturation is occurring.

After dissociation in glycine/NaOH buffer, *Homarus* hemocyanin displayed two distinct subunits plus a diffuse precipitate in crossed immunoelectrophoresis. This diffuse component has been described previously as "copper-free dimer" because in native PAGE it migrates as a fuzzy band with the mobility of a dimer, and it lacks copper as shown by atomic absorption spectroscopy (Markl and Kempter 1981 a).

When 0.05 *M* Tris/HCl buffer, pH 8.8, containing 1 mM EDTA was used as the dissociation medium, the formation of the diffuse component was

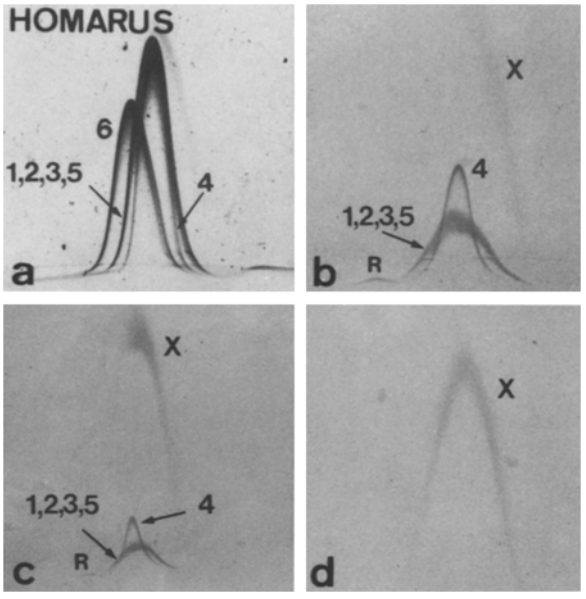


Fig. 3. a Crossed immunoelectrophoresis of *Homarus* hemocyanin, dissociated into subunits at pH 8.8, versus anti-(*Homarus*) antiserum. **b–d** The same preparation, incubated 1 h (**b**), 6 h (**c**), and 24 h (**d**) at room temperature in 4 *M* urea. *X*= denatured hemocyanin, corresponding to the 'copper-free dimer' of Markl and Kempter (1981a)

prevented and a previously unobserved third, immunologically distinct subunit appeared in the crossed immunoelectrophoresis pattern (Fig. 3a). Incubation of this preparation in 4 *M* urea completely transformed the crossed immunoelectrophoresis pattern into the 'copper-free dimer' within 24 h (Fig. 3b–d). Similar results were obtained by 4 *M* urea treatment of *Astacus* hemocyanin. These observations are probably due to partial denaturation, since 'diffuse' peaks were observed frequently in crossed immunoelectrophoresis patterns of aged subunit samples of all four hemocyanins studied here. Sometimes, 'fuzzy' dimeric bands also were seen in native PAGE of fresh samples (Fig. 4b, 'P'; Fig. 5b, 'R'). The proportion of the diffuse peaks increased with time of storage.

Isolation of single subunit types

In contrast to earlier studies (Markl et al. 1979; Markl and Kempter 1981a) in which crustacean hemocyanin subunits were purified by preparative PAGE, we here applied gel chromatography, ion exchange chromatography and immunoprecipitation. This is exemplified for *Cancer* hemocyanin (Fig. 4) and also documented for *Astacus* hemocyanin (Fig. 5). *Homarus* and *Callinectes* hemocyanin subunits were purified in the same way or according to Markl et al. (1984).

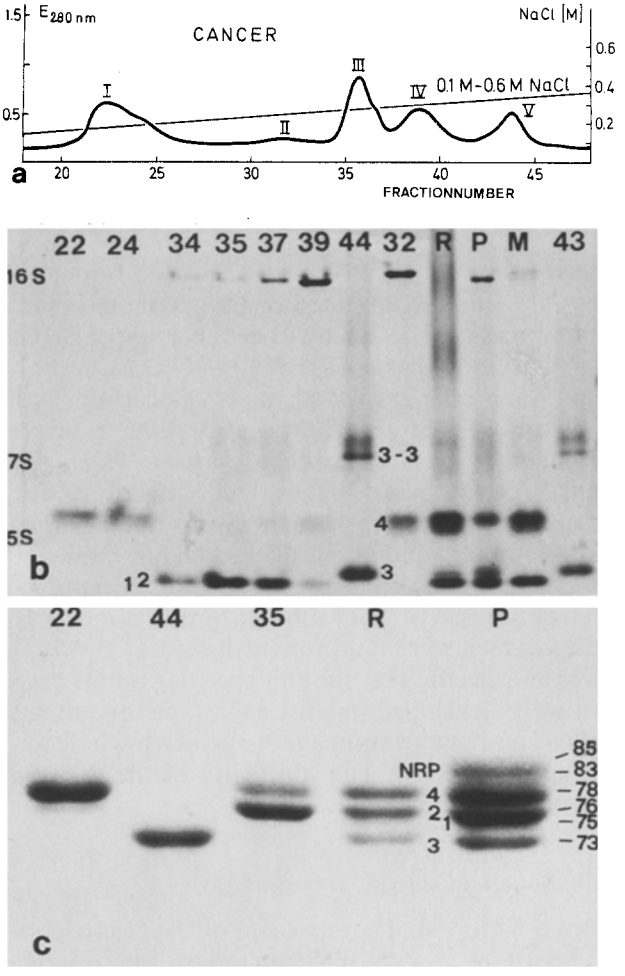


Fig. 4. a Ion exchange chromatography of 32 mg dissociated *Cancer* hemocyanin on DEAE-Sepharose Cl-6B at pH 8.8. **b** Analysis of fractions from **a** by native PAGE. **c** Analysis of fractions from **a** by SDS-PAGE, with the molecular masses of the components indicated in kD. In **b** and **c** the anode was at the bottom and a 5–10% acrylamide gradient was employed in the system of Laemmli (1970) at pH 8.8. **b** is without SDS and β-mercaptoethanol. The fuzzy bands in **b** are probably due to partially denatured hemocyanin. *R*=*Cancer* subunits from 2×6 hemocyanin which was isolated from hemolymph by gel chromatography on Biogel A5m and served as starting material in **a**. *P*=*Cancer* whole hemolymph proteins. 2×6 hemocyanin completely dissociates under the conditions of native PAGE (pH 8.8). *M*=*Cancer* subunits from 1×6 hemocyanin which was isolated from hemolymph by gel chromatography on Biogel A5m (note that subunit 3 is absent). *NRP*=non-respiratory serum protein

Cancer hemocyanin is composed of 4 distinct subunits, designated 1 through 4 (Markl et al. 1979). Anion-exchange chromatography of the subunit mixture exhibits a profile of five peaks (Fig. 4a). Subunit 4 was eluted both in the monomeric form (Peak I) and as a hexameric (16 S) aggregate (Peak II) as verified by sedimentation analysis. Peak III contained the monomeric subunits 1

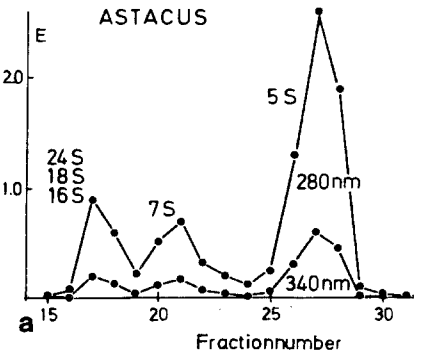
and 2, which are not separated by PAGE (Fig. 4b), but which were clearly discernible by crossed immunoelectrophoresis (Fig. 2). A further separation of 1 and 2 was not achieved in *Cancer*, but their immunological counterparts in *Callinectes* could be isolated (Fig. 6k, l). Peak IV contained hexameric aggregates of the subunits 1, 2 and 4, but lacked subunit 3. The material of Peak V represented immunologically pure subunit 3. Interestingly, it could be separated by electrophoresis into a monomer and a dimer. The dimer appeared as a well-defined band (Fig. 4b, '44'). Moreover, Peak V material displayed an UV-spectrum with a 340 nm/280 nm absorption-ratio of 0.2 which is typical for intact native hemocyanins. Thus, this dimer is certainly not the product of denaturation. An ability to dimerize in this way was not observed with the other subunits of *Cancer* hemocyanin.

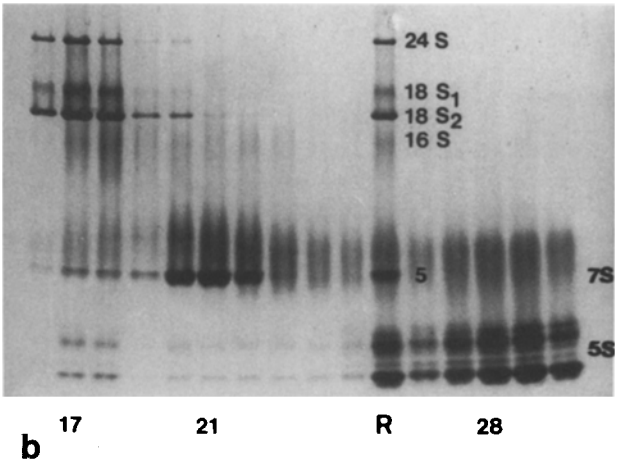
Figure 6 shows the two-dimensional immunoelectrophoresis of the subunit preparations obtained, which were used for further study.

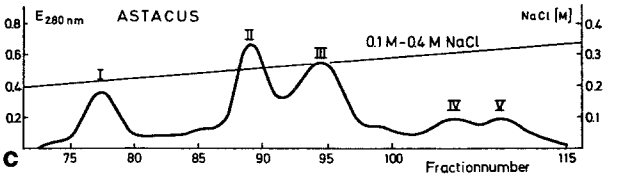
Monospecific (i.e. subunit specific) rabbit antisera were developed against each type, except for *Callinectes* hemocyanin, against which a whole antiserum was raised. The specificity of the antisera is documented in Fig. 7.

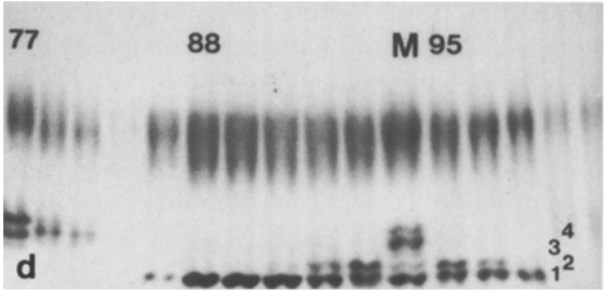
Immunological subunit correspondence

From a mere visual comparison of the electrophoretic patterns, it is difficult or impossible to judge









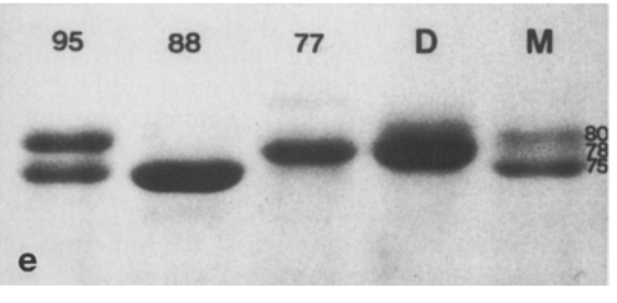


Fig. 5. **a** Gel filtration of 100 mg dissociated *Astacus* 2×6 hemocyanin on Sephadex G 100sf. The sedimentation coefficients of the peak fractions are indicated. **b** Analysis of fractions from **a** by native PAGE. **c** Ion exchange chromatography of material from the 5S-monomer-peak (30 mg) from **a** on DEAE-Sepharose Cl-6B at pH 8.8. **d** Analysis of fractions from **c** by native PAGE. **e** Analysis of fractions from **c** by SDS-PAGE; the molecular weights of the components are indicated in kD. In **b**, **d** and **e** the anode was at the bottom and a 5–10% acrylamide gradient was employed in the system of Laemmli (1970) at pH 8.8, in **b** and **a** without SDS and β-mercaptoethanol. In **b**, the monomeric bands in the fractions 17–22 are due to partial dissociation during electrophoresis in the alkaline gel-system. The fuzzy bands in **b** and **d** are probably due to partially denatured hemocyanin which displays an electrophoretic mobility like a dimeric component. As verified by sedimentation analyses prior to native PAGE, these fuzzy bands are an effect of the PAGE conditions. *R* = dissociated *Astacus* 2×6 hemocyanin = starting material in **a**. *M* = *Astacus* hemocyanin subunits from the 5 S (monomer) peak in **a**. *D* = *Astacus* dimer (subunit 5) from the 7 S peak in **a**. 24 S = 2×6 hemocyanin (dodecamer); 18 S_1 and 18 S_2 = heptameric hemocyanin, dissociation intermediates during the breakdown of *Astacus* 2×6 hemocyanin (Markl et al. 1981b); it is not yet clear, why two 18 S bands occur; 16 S = native 1×6 hemocyanin; 7 S = dimeric, 5 S = monomeric hemocyanin

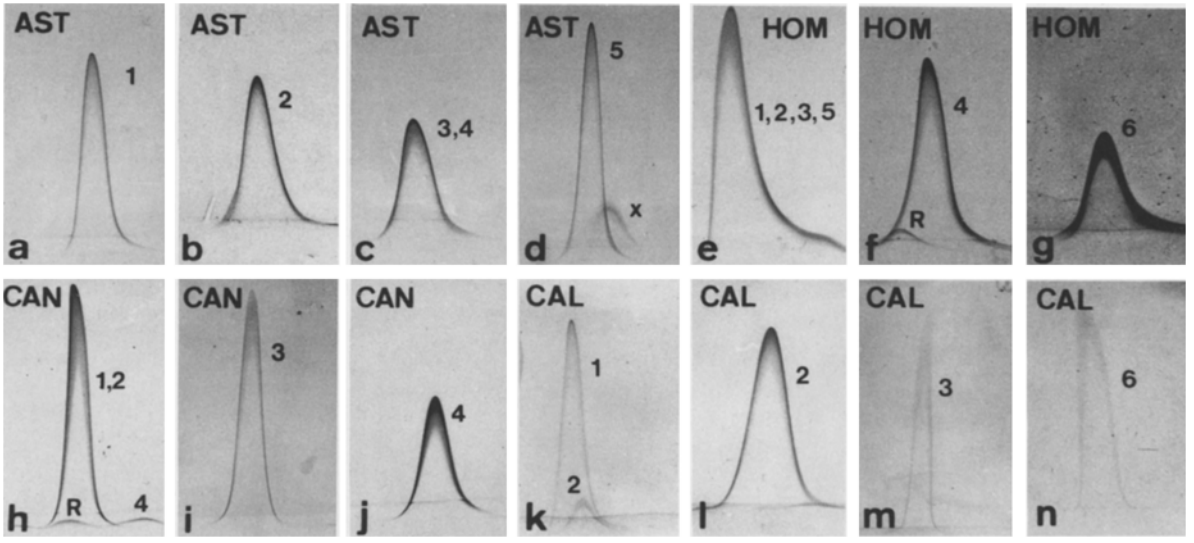


Fig. 6a–n. Examination of the purity of subunit fractions obtained in the present study, by crossed immunoelectrophoresis against the respective homologous anti-(whole hemocyanin) sera. *AST = Astacus; HOM = Homarus; CAN = Cancer; CAL = Callinectes; R* = reassociated hemocyanin; *X* = denatured hemocyanin

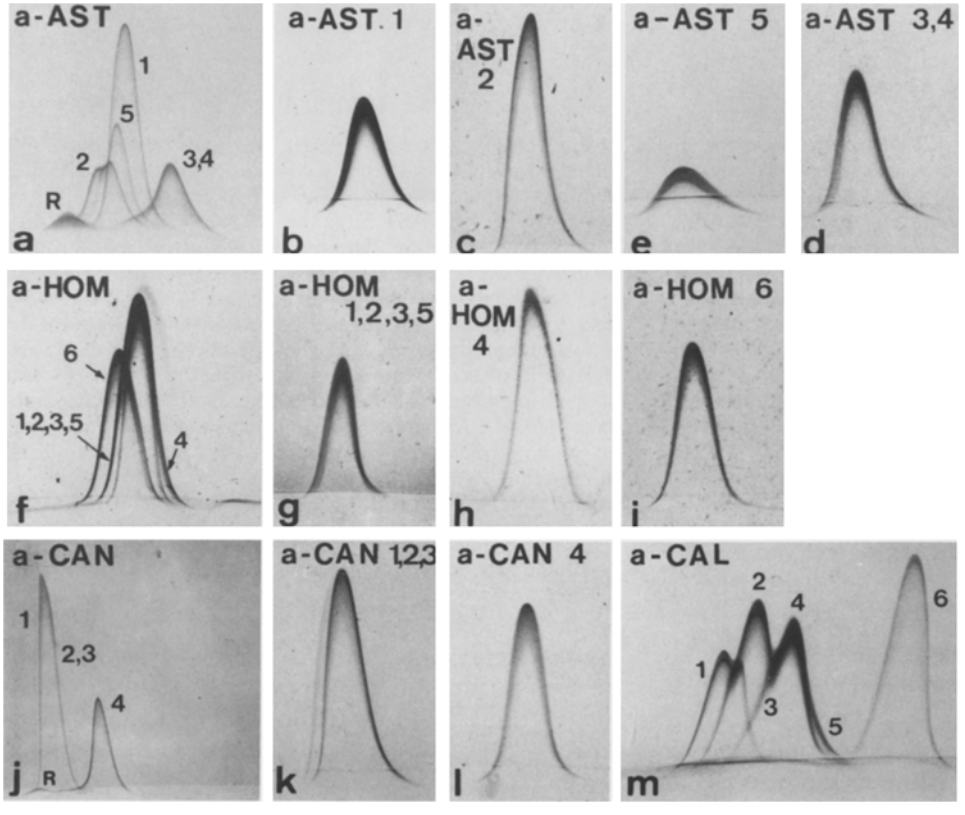


Fig. 7a–m. Specificities of antisera (*a* = anti) raised against individual subunits (see Fig. 5) of *Astacus* (**b–e**), *Homarus* (**g–i**), and *Cancer* (**k–l**). In each crossed immunoelectrophoresis, the whole subunit mixture of the respective hemocyanin was administered, but only the target subunit was precipitated. In **a, f**, and **j**, the respective anti-(whole hemocyanin) antiserum was applied; in **m**, the pattern of *Callinectes* is shown

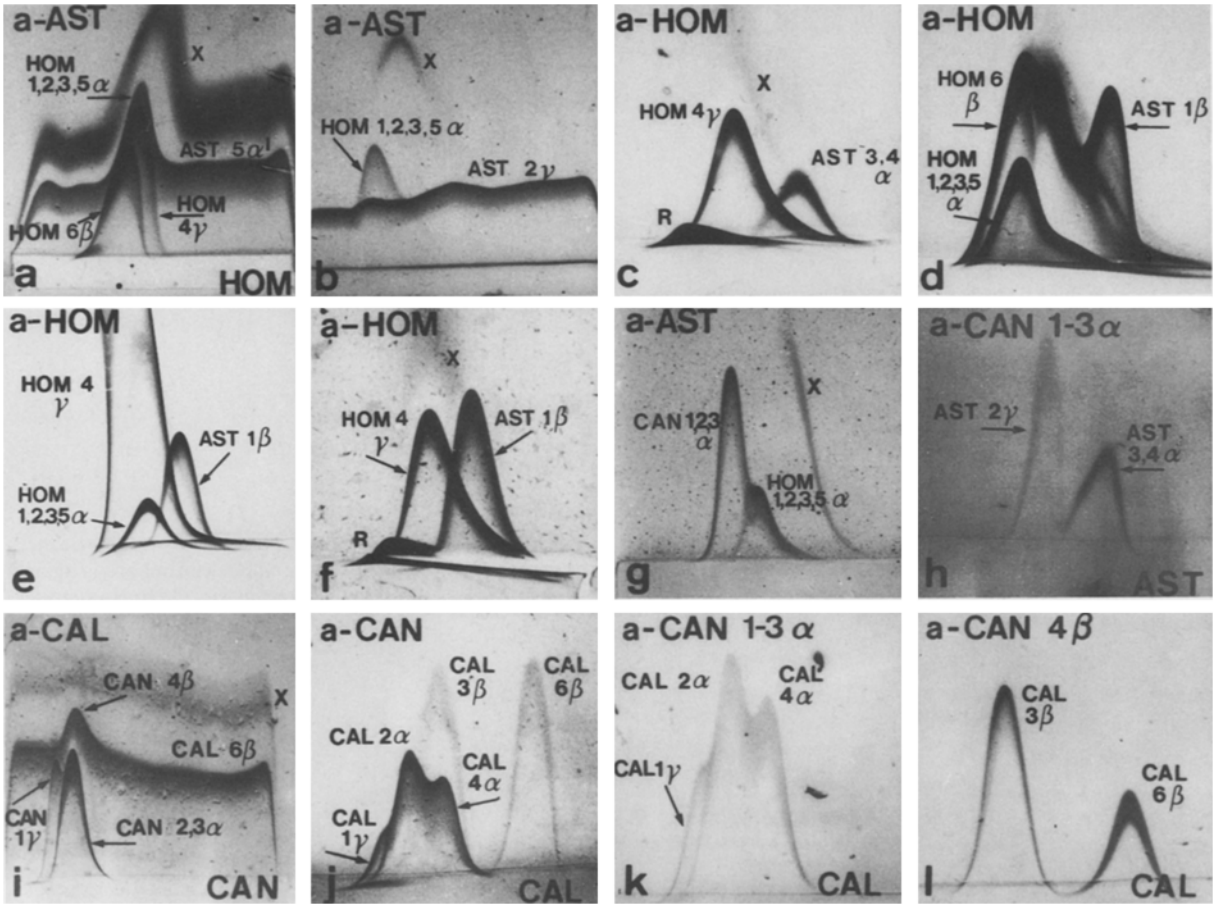


Fig. 8a–l. Immunological correspondences between hemocyanin subunits, demonstrated by crossed (**h, j, k, l**), crossed-line (**a, b, i**), and tandem-crossed (**c–g**) immunoelectrophoresis. *AST = Astacus; HOM = Homarus; CAN = Cancer; CAL = Callinectes*. In **a** and **h–l**, the whole subunit mixtures were applied in the lower right corner of the pictures; in the other experiments, isolated subunits were used. The antiserum employed (a = anti) is indicated in the upper left corner. R = reassociated hemocyanin; X = denatured hemocyanin. **a** AST 5 ($\alpha'-\alpha'$) in the line cross-reacts with HOM 1,2,3,5 (α). Denatured hemocyanin (X) of both species reacts immunologically identical, which is consistent with cheliceratan data (Kempter et al. 1985). **b** AST 2 (γ) in the line cross-reacts with HOM 1,2,3,5 (α). **c** Cross-reactivity between HOM 4 (γ) and AST 3,4 (α). **d** Cross-reactivity between HOM 6 (β) and AST 1 (β). **e** No cross-reactivity between HOM 1,2,3,5 (γ) and AST 1 (β). **f** No cross-reactivity between HOM 4 (γ) and AST 1 (β). **g** CAN 1,2,3 ($\gamma/\alpha/\alpha'$) cross-reacts with HOM 1,2,3,5 (α); the anodic 'foot' of HOM α indicates that this component is recognized better than CAN 1, 2, 3 ($\gamma/\alpha/\alpha'$) by the anti-AST antiserum. **h** Cross-reactivity between AST 2 (γ) and AST 3, 4 (α) with an anti-CAN $\gamma/\alpha/\alpha'$ antiserum. The reaction with AST α is stronger than with AST γ; AST 1 (β) is not precipitated. AST 5 (α') had been removed in this sample. **i** CAL 6 (β) in the line cross-reacts with CAN 4 (β). **j** Anti-CAN antiserum precipitates the whole subunit pattern of CAL, but the reactivity towards CAL 3,6 (β) is weak, in correspondence with the hypothesis that β varies interspecifically (Markl and Kempter 1981 b). The cathodic foot of CAL 1 (γ) is not precipitated, which corresponds to the antigenic deficiency of CAN 1 (cf. Fig. 2). **k** Anti-CAN α antiserum is unable to precipitate CAL 3,6 (β) from the whole subunit mixture. **l**, Anti-CAN β antiserum is unable to precipitate CAL 1 (γ) and CAL 2,4 (α) from the whole subunit mixture

which hemocyanin subunit of one species is the counterpart of a given subunit from another species. Therefore, a large number of crossed immunoelectrophoresis experiments were conducted to correlate the subunits of the four hemocyanins according to their antigenic specificities (examples are shown in Fig. 8 and a summary is presented in Table 1).

The native PAGE bands 1, 2, 3 and 5 of *Homarus* hemocyanin form a completely fused peak in crossed immunoelectrophoresis and are thus – by definition – immunologically identical. This cluster of isoforms was defined as 'subunit α' (Fig. 2).

The immunological counterpart of *Homarus* α in *Astacus* (an isoform cluster consisting of PAGE bands 3 and 4) was consequently termed *Astacus* α. This scheme was complicated by the observation

Table 1. Immunological relations among hemocyanin subunits

	α	α′	β	γ
Astacus	3,4	5	1	2
Homarus	1,2,3,5		6	4
Cancer	2	3	4	1
Callinectes	2,5	4	3,6	1

The hemocyanin subunits are numbered according to their mobility in native PAGE (Fig. 2) and classified into the three categories α/α′, β and γ. Subunits of the same category share more common epitopes than subunits from different categories. To define α′, the ability to form dimers was used as criterion

that the disulfide-bridged dimer of *Astacus* (PAGE band 5) could not be distinguished from 3 and 4 with anti-(*Homarus*) antisera. This means that *Astacus* 5 is also a counterpart of *Homarus* α. The cross-reaction between *Astacus* 3, 4 and 5 when anti-*Astacus* antiserum is used (Markl and Kempter 1981a) underlines this relationship. Therefore *Astacus* 5 was designated as subunit α′ (Fig. 2), which takes into account that the dimer, in contrast to monomeric 3 and 4, plays a specific structural role as the inter-hexamer link (see below). In the case of *Homarus*, however, there was no evidence allowing to distinguish a particular component of α in analogy to *Astacus*, because no functional differences could be observed between the four isoforms 1, 2, 3 and 5.

PAGE bands 2 and 3 of *Cancer* hemocyanin correspond to astacidean α (Fig. 8g, h). Both are immunologically identical, but differ in function since, in contrast to band 2, band 3 is absent in native 1×6 hemocyanin (Fig. 4b, 'M') (Markl et al. 1979); it is capable of dimerization (Fig. 4b, '44'). It very probably acts as inter-hexamer bridge (see below), and therefore corresponds in function to *Astacus* 5. The term α′ also was chosen for this component (Fig. 2).

Corresponding properties were detected in the hemocyanin of *Callinectes*, although by means of native PAGE six different bands were observed as compared to only four in *Cancer* (Fig. 2). Subunits 2, 4 and 5 are immunologically identical and correspond to *Cancer* α (Fig. 8k). Band 5 is present only in very minor quantities and exhibits the same molecular mass in SDS-PAGE as component 2 (Fig. 2). Thus, it might be a derivative of band 2 rather than an independent type of subunit. Band 4 most probably corresponds to *Cancer* 3 (α′) with respect to its electrophoretic and immunological behavior, and therefore was designated accordingly (Fig. 2; Fig. 8k).

Cancer 4 is immunologically completely distinct from bands 1 through 3 and, as outlined earlier, varies considerably among brachyuran species (Markl and Kempter 1981a, b). We defined this subunit as 'β'.

In *Callinectes*, β is represented by two electrophoretically very distinct, but nevertheless immunologically identical components, namely PAGE bands 3 and 6 (Fig. 2; Fig. 8l). Despite the unusually slow mobility of band 6 in native PAGE (Fig. 2; Fig. 8l), the method of Hedrick and Smith (1968) demonstrated that it is monomeric like the other subunits, and not a dimer.

Brachyuran β could be correlated with *Astacus* 1 and with *Homarus* 6. However, this cross-reaction was not visible by crossed immunoelectrophoresis, but could be demonstrated by immunoblotting after transfer of the native PAGE patterns onto nitrocellulose (Markl et al. 1986; details will be presented in a subsequent paper). This relationship was confirmed within the Astacidea. *Astacus* 1 was immunologically completely distinct from the other *Astacus* subunits (Fig. 2; Fig. 8a, e, f), as was *Homarus* 6 from other *Homarus* subunits (Fig. 2; Fig. 8a). Interspecifically, however, both exhibited a strong cross-reaction when compared directly by tandem-crossed immunoelectrophoresis (Fig. 8d). Consequently, *Astacus* 1 and *Homarus* 6 were classified as β subunits.

The remaining crayfish subunits, *Astacus* 2 and *Homarus* 4, turned out to represent a third group of immunologically related components, which we have termed 'γ'. Although it was not immediately obvious, γ is partially related with α, as demonstrated with heterologous antisera (Fig. 8b, c, h).

Components *Cancer* 1 and *Callinectes* 1 are immunological counterparts as well and they are immunologically related with α. This is obvious in *Cancer* because *Cancer* 1 is immunologically deficient compared to *Cancer* 2 (=α) or 3 (=α′) (Fig. 2). On the other hand, *Callenectes* 1 appears to be distinct from α as long as anti-*Callinectes* antiserum is used (Fig. 2). With anti-*Cancer* antiserum, however, the crossed immunoelectrophoresis pattern of *Callinectes* closely resembles that obtained with *Cancer* hemocyanin (Fig. 8j, k). If precipitated with an anti-brachyuran antiserum, *Astacus* γ appears to be antigenically deficient compared to *Astacus* α (Fig. 8h). In the reverse experiment with anti-astacidean antiserum, *Cancer* 1 is indistinguishable from *Cancer* alpha (Fig. 8g). Thus, it seemed to be justified to classify *Cancer* 1 and *Callinectes* 1 as γ, although it remains unclear whether γ from crayfishes and γ from crabs evolved from a common ancestor, or whether they branched from α independently.

Analysis of subunit stoichiometries

In order to quantitatively analyze the proportions of the various subunit types, the techniques described by Markl et al. (1981c) were used. Single subunits, as well as subunit mixtures of known stoichiometry, were used to calibrate crossed immunoelectrophoresis patterns of the whole subunit mixtures. This provided correction factors for each subunit type in combination with a particular antiserum and thus eliminated both the effect of variations in subunit-specific antibody titers and the effect of cross-reaction between subunits. As a second approach, we analyzed the elution profiles obtained during the preparative isolation of subunits. This was based on the absorbance at 280 nm (Loewe and Linzen 1973). As to denaturation or loss of copper, the absorbance ratio 280 nm/340 nm was taken into account. It turned out that in *Astacus* 2×6 hemocyanin 2α and 2α′ subunits are combined with 4 copies each of β and γ. Correspondingly, *Homarus* hemocyanin contains the three subunit classes α, β and γ in a ratio of 4:4:4 (α′ could not be defined as discussed above). *Cancer* 2×6 particles yielded the same proportions as *Astacus*, except that there was some uncertainty in the α:γ ratio of 4:2. It is certain, however, that the ratio of α/γ:β:α′ is 6:4:2. Analyses of *Callinectes* hemocyanin fit well into this scheme.

Hexamer and dodecamer formation in reassembly experiments

To study their reassembly capability, subunits were dialyzed for 7 days at 4° C against 0.1 *M* Tris/HCl buffer, pH 7.5, containing 10 m*M* $CaCl_2$ and then analyzed by native PAGE, analytical ultracentrifugation, and electron microscopy. A survey of the yields of reconstituted molecules is presented in Tables 2 and 3.

Purified monomers of *Astacus* hemocyanin were able to form homo-hexamers (Table 2). Co-reassembly of *Astacus* α, β and γ excluding α′−α′ yielded 77% hexamers, but no dodecamers (Fig. 9a; Table 2). Considerable amounts of native-type 2×6 molecules (20%) could be formed

Table 2. Reassembly products of purified subunit types and of subunit mixtures of equimolar stoichiometry. Yields were calculated from sedimentation analyses on a percent basis

	24 S (2×6)	20 S–22 S (ill defined products)	16 S (1×6)	7 S (dimers)	6 S (monomers +dimers)	5 S (monomers)
Astacus						
α			67			33
α′		11[a]		89		
β			69			31
α′+β		31	31		38	
α′+α		30	22		48	
β+γ			78			22
α′+β+γ		20	33		47	
α′+α+β		15	40		45	
α+β+γ			77			23
Homarus[b]						
α			30			70
γ			25			75
α+β			75			25
α+γ			56			44
Cancer[c]						
α′			47	53		
β			80			20
α′+β			55			45
α+γ			80			20
α+β+γ			95			5
α′+α+γ			85			15

[a] Heterogeneous material (presumably denatured portion)
[b] In *Homarus* no specific inter-hexamer linker has been identified
[c] Comparable results were obtained with *Callinectes* hemocyanin. In particular, *Callinectes* γ yielded 1×6 molecules

Table 3. Reassembly products of whole dissociated hemocyanins, and of all subunit types artificially mixed according to the native stoichiometry (values in brackets). Yields were either calculated from sedimentation analyses (% values), or estimated from native PAGE patterns and electron micrographs (** = many, * = some)

		Astacus	*Homarus*	*Cancer*	*Callinectes*
> 24 S	undefined products	12 (22)	#		
24 S	2 × 6	20 (20)	70 (55)	33 (18)	*
20 S–22 S	undefined products	30 (16)			
18 S	heptamers	18 (24)			
16 S	1 × 6		20 (27)	50 (76)	**
6 S	dimers + monomers	20 (18)		17 (6)	*
5 S	monomers		10 (18)		

Under the conditions used in the sedimentation studies, the large 8 × 6 and 4 × 6 aggregates, which are visible on electron micrographs of reassembled *Homarus* hemocyanin (Fig. 9), have not been detected

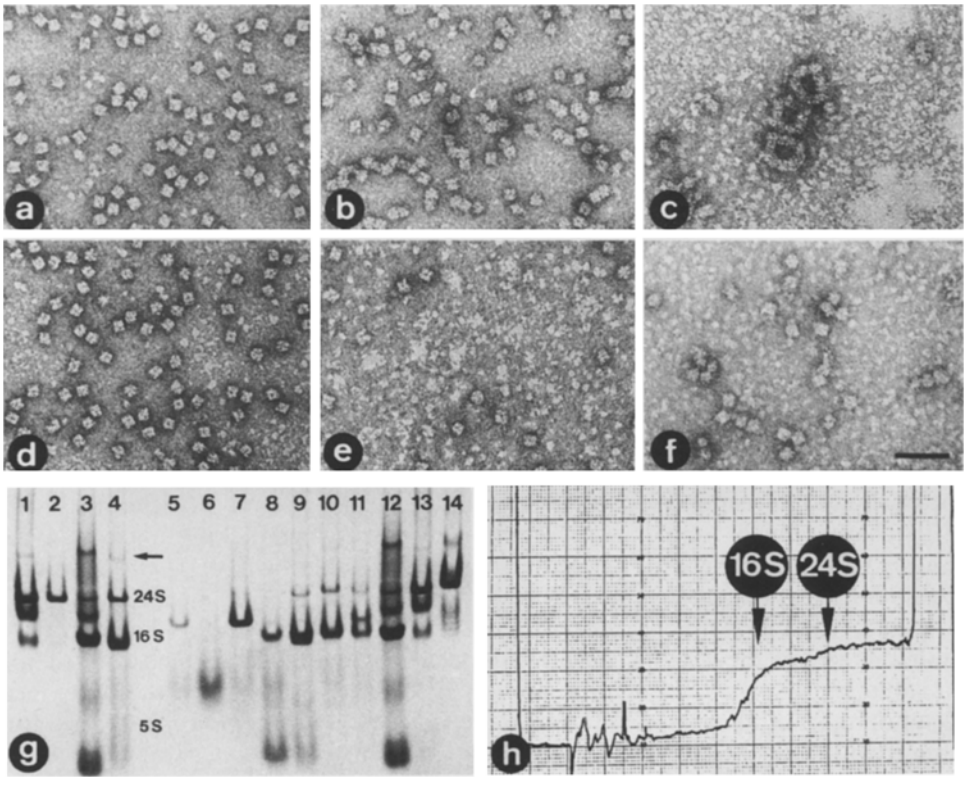


Fig. 9. a–f Electron micrographs of reassociated hemocyanin particles after negative staining with 1% unbuffered uranyl acetate. The primary magnification was 60000-fold. The bar represents 25 nm. **a** Hexamers obtained by co-dialysis of *Astacus* subunits 1–4, excluding the dimer 5 (α'). **b** 1 × 6 and 2 × 6 particles of *Astacus* hemocyanin, reconstituted from a mixture of all subunit types. **c** *Homarus* hemocyanin molecules reassembled from a mixture of all subunit types. **d** Hexamers obtained by co-dialysis of *Cancer* hemocyanin subunits 1–3, excluding subunit 4 (β). **e** Hexamers, reassembled from subunit *Cancer 4* (β). **f** 1 × 6 and 2 × 6 particles of *Callinectes* hemocyanin, reassembled from a mixture of all subunit types. **g** Native PAGE of reassembled *Cancer* hemocyanin at pH 7.5 in the system No. 6 of Maurer (1968) with 5–10% acrylamide. The anode was at the bottom. (1) Crude *Cancer* hemolymph proteins. (2) Purified *Cancer* 2 × 6 hemocyanin. (3) Sample (1), after 1 h dialysis at pH 9.6. (4) Sample (2), after quantitative dissociation, and reassociation. The arrow indicates a 33 S component probably corresponding to a 3 × 6 or 4 × 6 structure. (5) Homo-hexamer, assembled from *Cancer* 4 (β). (6) *Cancer* 4 (β). (7) = (5). (8) Hexamer, assembled from *Cancer* 1–3 (α/γ); the same sample as in **d**. (9) Reassembly products from *Cancer* 1–4, with 1–3 in excess*. (10) Reassembly products from *Cancer* 1–4 in native proportions; the same sample as in **e***. (11) Reassembly products from *Cancer* 1–4, with 4 (β) in excess*. (12) = (3); (13) = (1). (14) Crude *Astacus* hemolymph. * Note that homo-hexamers are formed from a heterogeneous mixture only if one subunit type is present in excess. **h**, Sedimentation analysis (analytical ultracentrifuge) of the *Cancer* sample in **e**, sample (10) in **g**. Original trace after 32 min at 34000 rpm. Calculated values are 10% monomers, 70% 1 × 6 (16 S) and 20% 2 × 6 (24 S) molecules

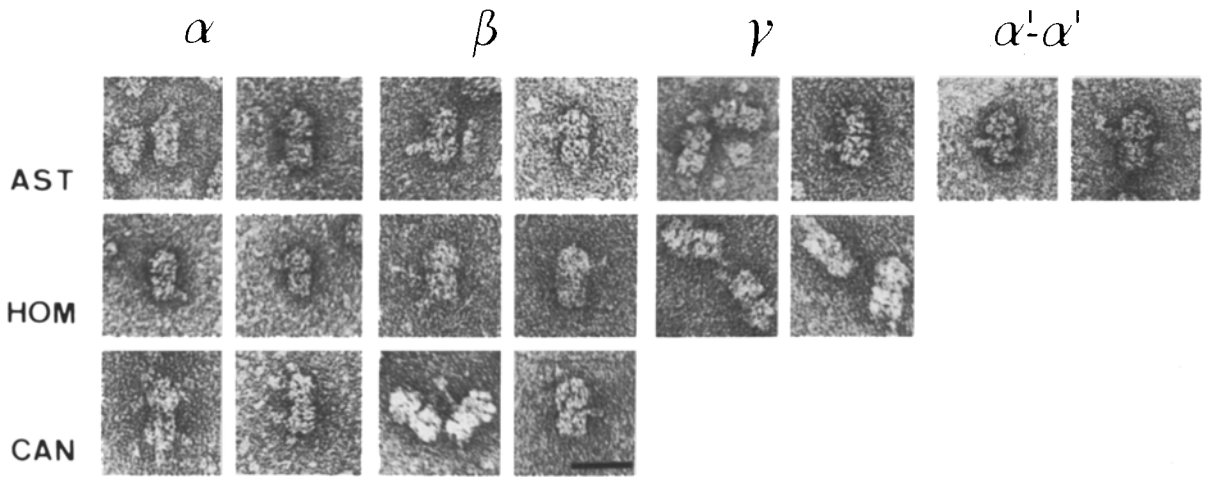


Fig. 10. Electron micrographs of immuno-labeled hemocyanin negatively stained with 1% unbuffered uranyl acetate. Upper lane: *Astacus* hemocyanin; middle lane: *Homarus* hemocyanin; lower lane: *Cancer* hemocyanin. The hemocyanin molecules were incubated with subunit specific F_{ab} fragments or whole IgG molecules (see below) against the immunologically distinct types of subunits (α, β, γ and α'), as indicated at the top. In two cases whole IgG was used to label hemocyanin dodecamers: Both the left image of *Cancer* β and the left image of *Astacus* γ show two dodecamers linked by an antibody molecule. The primary magnification was 60000-fold. The bar represents 25 nm

only if all three monomers plus the dimer were present (Fig. 9b; Table 3). Another observation was that these samples always contained heptamers (18 S; Table 3). Those are hexamers with an attached particle of the size of a subunit, which have already been predicted upon electrophoretic evidence (Markl et al. 1981b). Two additional fractions sedimenting with 20–22 S, and beyond 24 S, respectively, were detected by analytical ultracentrifugation (Table 3). The latter should correspond to oligohexamers arranged in chains which were observed by electron microscopy (Fig. 9b), whereas the 20–22 S material corresponds to oligomers of varying size, which could be observed by PAGE, if the dimer $\alpha'-\alpha'$ was included in any of the subunit reassociation mixtures (Table 2).

Like in *Astacus*, isolated subunits from *Homarus* were capable of forming regular hexameric structures (homo-hexamers; 16 S); combinations containing two of these subunits also did not aggregate to structures larger than hexamers (Table 2). For the formation of dodecamers all three components were required (Table 3). It could not be decided, which component functions as interhexamer bridge. Under the conditions used here, in addition to the dodecamers, circles composed of eight hexamers and semicircles of four hexamers were also formed (Fig. 9c). *Homarus* hemocyanin provides no stable dimeric subunit, and accordingly, heptameric intermediates were observed neither in earlier dissociation experiments (Markl et al. 1981b) nor in the present reassembly study.

In *Cancer*, the β subunit easily formed homohexamers (Table 2). In correspondence to earlier results (Markl and Kempter 1981b), α and γ (the latter was only available in a pure form from *Callinectes*) also formed homo-hexamers. Based upon sedimentation analyses, the proportion of hexamers increased up to 80% of the total protein material if α and γ were co-reassembled (Fig. 9d; Table 2). However, the highest yield of hexamers (95%) was obtained if α, β and γ (without $\alpha'-\alpha'$) were combined, thereby mimicking a native-type hexamer (Table 2). Subunit α' in *Cancer* yielded a mixture of dimers (53%) and hexamers (47%) as reassembly products. In *Cancer* and also in *Callinectes*, dodecamers could be formed only if all four subunit types were present and the best yield (18%, as verified by sedimentation analysis for *Cancer* hemocyanin) was obtained by combining the subunits in their native proportions (Fig. 9e, f, g, h; Table 3).

Immuno electron microscopy

Monovalent, subunit specific F_{ab}-fragments were prepared from the monospecific antisera documented in Fig. 6, and incubated with the native 2×6 hemocyanins. The resulting soluble immunocomplexes were purified by TLG and analyzed in the electron microscope. In the case of *Cancer* β and *Astacus* γ, labeling with whole IgG molecules also was performed. The results are shown in Fig. 10.

In *Astacus* hemocyanin, where α and α′ are somewhat immunologically distinct, a specific antiserum could be developed against each of them. This was not possible for *Cancer* α and α′, due to the immunological identity of these components. Figure 10 shows F_{ab}-fragments against *Astacus* α′ decorating the contact region between the two hexameric half-structures. In contrast, F_{ab}-fragments against monomeric *Astacus* α most frequently occupied the peripheral short edge of each hexamer. Anti-*Homarus*-alpha F_{ab}'s were attached to both peripheral positions and positions near the inter-hexamer cleft. In the case of *Cancer*, other than anti-β, only an anti-α/α′/γ antiserum was available due to the immunological identity of α and α′ and the antigenic deficiency of subunit γ compared to α. F_{ab}-fragments derived from this antiserum were found in nearly every position on the surface of the dodecamer except on the long edge opposite to the bridge. This long edge could be labeled with anti-β F_{ab}-fragments. In *Astacus* and *Homarus* hemocyanin, β subunits are localized in the same position. In all three species, occasionally anti-β F_{ab}-fragments were observed which were bound to the peripheral short edge. For the reasons discussed above, it was not possible to definitely locate *Cancer* γ, but this was possible in the cases of *Astacus* and *Homarus*. In these hemocyanins there was anti-γ labeling at the side of the inter-hexamer bridge, but also at the peripheral short edges of the dodecamer.

Discussion

Subunit heterogeneity has been observed in many crustacean hemocyanins (for references, cf. Markl et al. 1979; Markl and Kempter 1981a, b; Linzen 1983; van Holde and Miller 1982; Ellerton et al. 1983; Markl 1986), but until now a common scheme could not be derived, due to marked differences in electrophoretic subunit patterns. The advantage of the immunological correspondence analysis presented here is that purified subunits, as well as subunit-specific antisera are employed. Although being only semiquantitative, the employed immunological techniques are advantageous since they facilitate the distinction of the different native subunits of a given sample from denatured hemocyanin and from oligomeric material, as already discussed (Markl et al. 1986). It turned out that 2 × 6 hemocyanins from *Astacus*, *Homarus*, *Callinectes* and *Cancer* are composed of four classes of subunits, which could be interspecifically correlated. These subunits occur in all four species in similar stoichiometric proportions, have a corresponding reassociation behavior, display comparable immunolabeling patterns, and in the quaternary structure, are arranged according to a common topological scheme. Phylogenetic implications of the present findings will be discussed in a subsequent paper which provides a survey of a larger number of crustacean hemocyanins.

Reassembly behaviour

The dissociation of crustacean 2 × 6 hemocyanins is a process that is not fully reversible. Many investigators failed to reconstitute 2 × 6 structures, particularly in the case of brachyuran crabs (van Holde and Miller 1982). In contrast, the larger 4 × 6 hemocyanins from arachnids and the 8 × 6 hemocyanin from *Limulus polyphemus* could be reconstituted from their subunits at full scale (Decker et al. 1980; Bijlholt et al. 1979), and even 4 × 6 and 8 × 6 hybrid reassembly was possible (van Bruggen et al. 1980). Interestingly, reassembly of 2 × 6 hemocyanin particles of the hunting spider *Cupiennius salei* is more difficult than reconstitution of cheliceratan 4 × 6 and 8 × 6 hemocyanins, and was achieved to a certain extent only under rather drastic conditions (e.g. presence of 20 m*M* calcium), yielding 3 × 6 and 4 × 6 misfits as by-products. This was interpreted as inproper incorporation of a dimeric subunit which connects hexamers (Markl 1980). It appears that in spider 2 × 6 hemocyanin, the reassembly process is less specific than in spider 4 × 6 hemocyanins (e.g. *Eurypelma californicum:* Markl et al. 1982).

This has interesting parallels in crustacean hemocyanins. The more complicated, tetrahedrally assembled 4 × 6 hemocyanin of the thallassinid shrimp *Callianassa californiensis* could be reconstituted from its subunits, although a portion of 'incompetent' 1 × 6 material was always formed in addition (Roxby et al. 1974; Miller et al. 1977).

In the case of *Astacus* hemocyanin, where a dimeric inter-hexamer linker (subunit 5) is present, 2 × 6 hemocyanin could be reconstituted, but oligohexameric misfits were also observed (Table 3). Successful 2 × 6 reconstitution was also reported for the hemocyanin of *Cherax destructor*, another freshwater crayfish (Jeffrey 1979; Marlborough et al. 1981). For *Homarus americanus* hemocyanin, Morimoto and Kegeles (1971) described a reversible calcium-dependent equilibrium between 2 × 6 particles and their 1 × 6 half-structures. However, dissociation into monomers followed by reassociation yielded hexamers not competent of dimeriza-

tion. In our study, *Homarus* 2 × 6 hemocyanin could be reconstituted in high yield, but ring-like 4 × 6 and 8 × 6 structures were formed in addition.

A similar situation is encountered in crabs, where, to our knowledge, our data provide the first example of a successful 2 × 6 reconstitution.

One limitation for crustacean hemocyanin association beyond the hexameric stage might be the incorporation of partially denatured subunits. Indeed, reassociation of mixtures of isolated subunits, which have endured certain purification processes, generally leads to lower yields of 2 × 6 products, as compared to the reassembly of freshly dissociated hemocyanins. A similar observation was reported for spider hemocyanin (Markl et al. 1982). Since under our buffer conditions, the crustacean hemocyanins appeared to be less stable than hemocyanins from chelicerates, this could be an important factor, which prevents high 2 × 6 yields.

The efficiency of hexamer formation appears to be an argument against this hypothesis. However, within the hexameric half structure the subunits are linked by specific non-covalent interactions. The amino acid residues involved in these interactions have been conserved during evolution as has been shown for various crustacean and cheliceratan hemocyanins (Linzen et al. 1985). Thus, hexamer forming subunits probably can substitute for each other because they contain homologous binding sites. This might be responsible for the heterogeneity of hemocyanins at the hexamer level, e.g. as reported for *Cherax destructor* hemocyanin (Murray and Jeffrey 1974). In contrast, the inter-hexamer link is more variable among species. E.g. in *Homarus* hemocyanin it is an ionic bond involving calcium ions, whereas in *Astacus* hemocyanin a disulfide linked dimer serves as the bridge. One might assume that the inter-hexamer linkers are highly specific and cannot be substituted by other subunits.

Suboptimal reassociation conditions should also decrease the yield of reassociated dodecamers. For example, in tarantula hemocyanin the presence of 40 m*M* $CaCl_2$ caused formation of a 1 × 6 misfit product incapable of further aggregation (Decker et al. 1980), but for the formation of 2 × 6 *Cupiennius* hemocyanin high calcium levels were necessary. Another observation was that with *Eurypelma* hemocyanin, at pH 7.5 the reassembly process is more efficient than at pH 8.0 (Markl et al. 1982). In earlier reassembly experiments by overnight dialysis of crab hemocyanin subunits, the reorganization did not exceed the level of hexamers (Markl and Kempter 1981 b). It was deduced that reassembly of crustacean 2 × 6 molecules needs relatively long incubation times and low protein concentrations in order to lower the percentage of misfit products.

Apart from these arguments, the reconstitution of 2 × 6 is probably limited by kinetic considerations, and the major drawback for in vitro 2 × 6 assembly of crustacean hemocyanins could be irregular hexamer formation. With the exception of *Astacus* α', all subunit types investigated by us have the inherent ability to form homo-hexamers. Combinations of different subunit types yield hetero-hexamers with varying stoichiometries, depending on the proportion of the individual components. In reassembly experiments with *Cancer* subunits there was, for example, a strong tendency to form $\alpha/\beta/\gamma$ hetero-hexamers, and homo-hexamers were formed from heterogeneous subunit mixtures only if one subunit type was present in excess (Fig. 9 g, samples 9–11). Thus, the formation of 2 × 6 molecules in higher yield might be prevented simply by the fact that our buffer conditions (as well as in the work of previous authors) favored hexamer formation. Only by chance might then inter-hexamer linkers be incorporated at the correct position. Individual subunits of the 4 × 6 hemocyanin from the tarantula *Eurypelma* are incapable of forming homo-hexamers at low (1 mg/ml) concentrations under reassembly conditions (Markl et al. 1982) while the complete mixture reassembles readily. Similarly, this ability was rarely observed for other cheliceratan hemocyanins (Bijlholt et al. 1979; Brenowitz et al. 1983). In contrast, homo-hexamer formation is common in experiments with *Cupiennius* hemocyanin and difficulties arise in trials to obtain reconstituted 2 × 6 particles (Markl 1980; Markl and Kempter 1981 b).

The only component that did not self-aggregate to homo-hexamers in the four crustacean hemocyanins was *Astacus* $\alpha'-\alpha'$. This makes sense if one assumes that the reassembly experiment had started from a dimer stabilized by disulfide bonds. The disulfide bond locks the two constituents into a particular orientation relative to each other which is not suitable for a correct arrangement of the trigonal antiprismatic structure of the hexamer. Unlike *Astacus* $\alpha'-\alpha'$, *Cancer* α' combines the two properties of a tendency to be incorporated into hexamers and a tendency to dimerize. However, unlike in *Astacus* hemocyanin, *Cancer* α' starts the reassembly process as a monomer. Indeed we observed the formation of 47% of homo-hexamers along with 53% of dimers incompetent of further aggregation. If this also occurs during co-assembly with the other subunits, the $\alpha'-\alpha'$ portion not incorporated into hexamers would be

lost for 2×6 formation along with α' homo-hexameric material.

Theoretically, it should be possible to form 2×6-mers, long chains, or even infinite layers, from *Cancer* α' homohexamers, but this was never observed. Subunits β and γ, which also are localized at the inter-hexamer cleft, probably mediate in 2×6 formation. This hypothetical 'helper' function is underlined by the observation that the co-reassembly of *Cancer* α, α' and γ (in the absence of β) stops at the hexamer level (Table 3). It cannot be excluded that the low levels of 2×6 reconstitution might in fact be a consequence of the involvement of more than one subunit type in the bridge, which would complicate the path of reassembly. However, the data from electron microscopy do not support this suggestion.

At the sites of crustacean hemocyanin biosynthesis, in the cyanocytes proliferating from lymphocytogenic nodules of the outer gizzard wall (Ghiretti-Magaldi et al. 1977), either the microenvironment or additional regulators might control the assembly process of newly synthesized hemocyanin polypeptides. This hypothetical control might not be a prerequisite in the case of *Eurypelma*-like hemocyanins, in which the subunits are capable of a highly specific self-assembly. An important process in the biosynthesis of all hemocyanins could be that dimeric linkers are presented to the other subunits and could direct the assembly towards particles larger than hexamers. Indeed, in *Eurypelma* 4×6 reconstitution experiments, a mixture of the constituents *b* and *c* was not effective compared to the intact *bc* heterodimer (Markl et al. 1982). Post-translational processing might also be a control mechanism in the in vivo assembly of hemocyanin oligomers. However, the arrangement and the regulation of the genes encoding hemocyanin have not yet been investigated that far.

Despite the obvious possibility of constructing a 2×6 hemocyanin from only two subunit types as is the case in *Cupiennius* (Markl 1980) and despite the inherent ability of every monomeric crustacean subunit to form homo-hexamers, the architecture of crustacean 2×6 hemocyanins requires four specialized subunit types, which can not substitute for one another. The underlying structural constraints may have been the stimulating forces for the evolution of immunologically distinct subunit classes within the Decapoda.

Model of the quaternary structure

Our data from immuno-labeling experiments are more difficult to interpret than the corresponding work with cheliceratan hemocyanins (Lamy et al. 1981, 1983, 1985; Markl et al. 1981a), because certain crustacean subunits which were immunologically identical or related turned out to have distinct functional properties. In particular, with the exception of *Astacus* hemocyanin, inter-hexamer linkers could not be distinguished immunologically from certain non-linkers, and thus, a linker-specific antiserum was not available. This made it impossible, for example, to directly locate *Cancer* subunit 3 (α') due to its cross-reactivity toward subunits α and γ. Fortunately, the role of subunit *Cancer* α' as inter-hexamer linker could be derived from reassociation experiments, and F_{ab}-labeling experiments of *Astacus* hemocyanin unambiguously identified *Astacus* α' as the bridge between the two hexameric halves.

Despite their immunological relatedness, it was possible to distinguish between labeling patterns for astacidean α and γ, but a large number of labeled 2×6 molecules had to be analyzed first. Anti-α and anti-γ F_{ab}'s both decorated the peripheral short edges of *Astacus* and *Homarus* 2×6 particles. In the case of γ, however, the long edge on the side of the inter-hexamer bridge also was labeled. For the corresponding situation in *Cancer*, there was no direct approach due to the antigenic deficiency of γ compared to α, but at least the topologic position of *Cancer* β is clearly defined, and corresponds to the position of astacidean β.

For *Callinectes* hemocyanin, no separate immunolabeling experiments have been performed because its subunit composition and reassociation behavior fit completely into the scheme derived for *Cancer* hemocyanin.

In our model (Fig. 11) the different subunits are symmetrically arranged within the 2×6 particle. Each of the four trimeric layers contains one of each subunit types, α (α'), β and γ, respectively. The inter-hexamer bridge is formed by an $\alpha' - \alpha'$ dimer. The dimer, two β subunits and two γ subunits build up a central cluster. Additional β and γ subunits ($2+2$) form the distal parts of the molecule, and one α subunit occupies the peripheral short edge of each hexamer. This model is consistent with the situation in *Astacus* hemocyanin and also applicable to the other three hemocyanins which are composed slightly differently. It takes into account that in contrast to our preliminary report (Stöcker et al. 1986), the more recent results have shown immunolabeling of β at the peripheral short edges in addition to positions near to the inter-hexamer cleft. The model conforms with the idea of Jeffrey (1979) who placed, upon evidence from reassembly studies, the disulfide-bridged

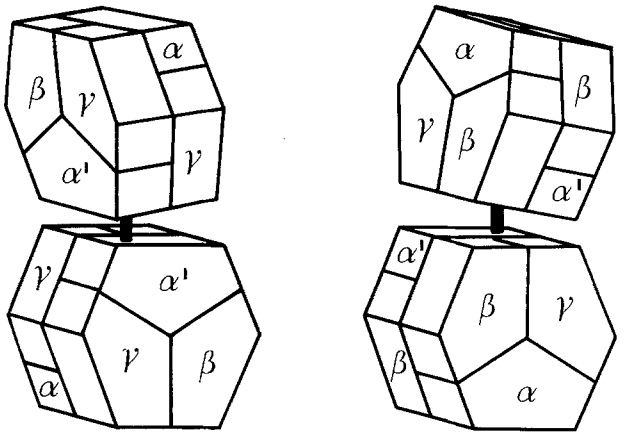


Fig. 11. Topographical model of the quaternary structure of *Astacus* 2×6 hemocyanin. Except for an exchange of two γ subunits for two α subunits in crabs, it is fully applicable to the other 2×6 hemocyanins of this study. The morphology of the hexamer is based on X-ray data (Gaykema et al. 1984). The orientation of the two hexamers relative to each other is based on analyses of electron micrographs (van Bruggen et al. 1981; van Bruggen 1983). Of two possible enantiomers the one was chosen that fits the data from computer correspondence analysis (Bijlholt, unpublished). The intramolecular subunit topography was derived from the combined results of four different approaches: (i) determination of subunit composition and stoichiometry, (ii) analysis of dissociation intermediates (Markl et al. 1981 b), (iii) reassembly experiments and (iv) immuno labeling of native hemocyanin dodecamers using F_{ab} fragments or whole antibody molecules. α (α'), β and γ subunits are proposed to be in a clockwise arrangement when viewed along the trigonal axis of the hexamer since only in this constellation the β subunits are located opposite to the interhexamer bridge as deduced from immunolabeling patterns

dimer of *Cherax destructor* hemocyanin in the same position as α'−α' in the present model. For *Astacus leptodactylus* hemocyanin, a dimeric subunit was discovered by Markl et al. (1979) and Pilz et al. (1980), and its bridging function was studied by Markl et al. (1981 b, 1983). The previous (Markl et al. 1983) designation of β' for this subunit is changed here based upon the new immunological evidence.

Within each hexamer, the subunits are arranged in a trigonal antiprism as suggested by Wibo (1966) and proven by Gaykema et al. (1984). In our 2×6 hemocyanin model, the morphology of the inter-hexamer contact is based on electron microscopic observations (van Bruggen et al. 1981; van Bruggen 1983) which indicate that in decapodan 2×6 hemocyanin particles, one corner of a hexagonal 1×6 image is connected to the fuzzy edge of a rectangular 1×6 image. The two hexamers are somewhat tilted with respect to each other in the direction of the contact region. The choice between the two possible enantiomers was made on the basis of preliminary data from computer image analysis (Bijlholt, unpublished). Since anti-β F_{ab}'s almost exclusively decorated positions opposite to the inter hexamer bridge, the decision was allowed that within each trimeric layer the subunits α (α'), β and γ are arranged in a clockwise pattern. A counter-clockwise arrangement would require β subunits at the side of the bridge. From the third possibility, a hexamer consisting of a clockwise trimer and a counter-clockwise one, a "trimer of homodimers" would result as the most probable constellation. This idea is intriguing, however, it would be in contradiction to the localization of *Astacus* α at the peripheral short edges of the 2×6 particle.

In contrast to our model, Pilz et al. (1980) and Bernhard et al. (1983) favored the idea of a three-point contact as deduced from small-angle X-ray scattering experiments with 2×6 hemocyanins from *Astacus leptodactylus, Cancer pagurus* and *Homarus vulgaris*. However, their interpretation is not fully covincing, because they use spherical subunits with a radius of 31 Å, which is not consistent with observations by X-ray crystallography. It has been demonstrated to 3.2 Å resolution that crustacean hemocyanin subunits are bean-shaped, measuring 45×55×80 Å (Gaykema et al. 1984, 1985). In this context, Bernhard et al. (1983) also replaced each of the twelve 31 Å spheres by 13 smaller spheres with a radius of 13 Å, but this gave no improvement in the fit. The use of spheres instead of beans is also a drawback in the theoretical approaches of Klarman and Daniel (1981) and Sculley et al. (1984), and of the topological models of Jeffrey (1979) and Rochu and Fine (1984a). Nevertheless, we cannot completely exclude the possibility that a three-point (or a two-point) contact exists because this was also derived by Herskovits et al. (1984) from the effects of ureas and neutral salts on the dissociation process of *Homarus americanus* 2×6 hemocyanin. If this holds true, the only candidates for additional bridges are the two β and the two γ subunits next to the inter hexamer cleft and consequently β−β and α'−γ interactions might be involved additionally (Fig. 11).

Except for our present model (Fig. 11) and its preliminary scheme (Markl et al. 1983), the only detailed, topological model of a crustacean 2×6 structure stems from Rochu and Fine (1984a). Since they used the same hemocyanin (*Cancer pagurus*) and did analyses by crossed immunoelectrophoresis (Rochu and Fine 1984b), the results can be compared. Their resulting patterns correspond to the data published earlier by our group (Markl and Kempter 1981a, b) and are the extension of even earlier immuno double-diffusion experiments

(Rochu and Fine 1980). The French group designates their components as I through V. In our terminology, I = *Cancer* 2 (α), II = *Cancer* 4 (β), and III = *Cancer* 1 (γ). Components IV and V are not detectable in our pattern of *Cancer* hemocyanin (Fig. 2). Rochu and Fine (1984b) interpreted V to be a product of proteolytic degradation. Since their band V provides a darkly contrasted, small immunoelectrophoresis peak migrating at the anodic edge of the α/γ cluster, it is in our opinion, 1×6 material reassembled from α/γ rather than a degradation product. A degradation product should display a large, diffuse peak. Indeed, in Fig. 5 of Rochu and Fine (1984b) the presence of 1×6 material among the dissociation products was documented.

IV was identified as a dimer and, at first glimpse, seems to correspond to our $\alpha' - \alpha'$ dimer, due to its stability in electrophoresis buffer (a buffer which also enabled the formation of 1×6 particles!). However, IV appears to be stabilized by an intermolecular disulfide bridge, and it is obviously immunologically distinct from I/III (as well as from II), although the authors stress a relatedness with I/III. This does not fit the properties of α'; we did not observe a dimer like component IV in our study. Our opinion is that IV represents denatured, dimerized α material (cf. Fig. 4b), but we can not positively exclude other possibilities.

Rochu and Fine (1984a, b) provide evidence that subunit III ($=\gamma$) is absent in native 1×6 hemocyanin, and therefore might be the candidate for the inter-hexamer bridge. This is interesting, particularly because our own data are not clear in this respect, since in native PAGE and in SDS-PAGE, subunit 1 (γ) migrates together with *Cancer* 2, and in crossed immunoelectrophoresis patterns it is not always discernible from 2. Moreover, the small percentage of naturally occurring 1×6 particles in *Cancer* (less than 10%, and lacking subunit 3 as shown in Fig. 4b), is frequently obscured by 1×6 halves of 2×6 material which are artifacts of in vitro dissociation. This process increases considerably during storage of the samples, or by freezing and thawing, and is a special feature of *Cancer* hemocyanin as reported by Markl et al. (1979). Rochu and Fine (1984b) refer to 1×6 and 2×6 particles as the 'two main polymeric forms' of *Cancer* hemocyanin, but since they were isolated under the severe conditions of preparative PAGE, which in our experience promotes dissociation, a significant contamination of naturally occurring 1×6 with artifactual 1×6 might well have happened. Thus, the results derived from this 1×6 material remain debatable. Future experiments of both groups should concentrate on this topic.

Rochu and Fine (1984a) calculated the molar ratio of the various subunits by planimetry of the patterns of crossed immunoelectrophoresis. This is not reliable without having developed calibration factors by an independent method, because the reactivity of antisera towards each subunit class might vary. Moreover, it remains unclear how the proportion of the antigenically deficient component III ($=\gamma$) could have been determined by planimetry. Nevertheless, the data conform with the stoichiometry of our model insofar as 4 copies of subunit β (= II) and 2 copies of subunit γ (= III) were proposed for the 2×6 particle. As in our earliest (Markl et al. 1983) and our recent model (Stöcker et al. 1986; Markl 1986) but not in the present study, Rochu and Fine (1984a) placed all four copies of subunit II (β) in a position close to the inter-hexamer cleft, without attributing a bridging function. Unfortunately, experimental evidence or arguments for this specific topology were not given. In their model, a γ (= III) homodimer connects the two hexamers as already discussed. A component corresponding to our proposed bridge, *Cancer* 3 (α'), was also described by Rochu and Fine (1984b), but interpreted as a degradation product of subunit I (= *Cancer* 2 α). This idea is interesting, because in SDS-PAGE *Cancer* 3 has the lowest molecular weight (Fig. 2) and its ability to dimerize could be the result of degradation. On the other hand, in reassembly experiments, subunit 3 was clearly required for aggregation above the hexamer level and could not be replaced by subunit 2. Moreover, it is present also in *Carcinus maenas*, *Hyas aranaeus*, and *Callinectes sapidus* 2×6 hemocyanin (Markl and Kempter 1981a; and present paper), and its proportion remained constant from preparation to preparation, and during aging of the samples. Therefore, we have no reason to believe that this component is a degradation product. In our opinion α' is the most likely candidate for the inter-hexamer bridge, although we concede that the model of Rochu and Fine (1984a) in which γ is the bridge in crab hemocyanins should be kept in mind. The solution should come from immuno electron microscopy with monoclonal antibodies, which have proved to be most suitable for refining topological models of hemocyanin quaternary structures (Lamy et al. 1985).

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