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Subunit Heterogeneity in Crustacean Hemocyanins as Deduced by Two-Dimensional Immunoelectrophoresis *

Jürgen Markl and Bernhard Kempter

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

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Summary. 1. Multiple subunits of hemocyanins from 3 brachyuran crabs (*Carcinus maenas, Cancer pagurus, Hyas araneus*), a spiny lobster (*Palinurus vulgaris*), a freshwater crayfish (*Astacus leptodactylus*), and a lobster (*Homarus americanus*) were isolated by preparative polyacrylamide gel electrophoresis (PAGE), and compared by two-dimensional immunoelectrophoresis.

2. In the 24 S hemocyanin isolated from each of the 3 crabs, two of the four subunits separated are immunologically identical; the third subunit is antigenically deficient compared to the first two. The fourth chain is immunologically unrelated to the other three.

3. In the 16 S hemocyanin of *Palinurus* two of the four subunits are immunologically identical; the third is closely related. The fourth chain is partially identical with the other three, but is antigenically deficient.

4. The 16 S hemocyanin of *Astacus* is composed of two immunologically unrelated subunits. The 24 S hemocyanin of this species contains, in addition, a dimeric subunit which is partially identical with one of the former, but not related to the other.

5. In the 24 S hemocyanin from *Homarus,* five subunits were separated; a group of 2, and a group of 3 subunits which are immunologically identical, but which are not related to each other.

6. At the level of quaternary structure, a common principle is suggested for crustacean hemocyanins: 24 S hemocyanins are composed of three types of subunits, while 16 S hemocyanins contain only two types.

* A preliminary account of this work was presented at the EMBO-workshop at Tours, 20–24 August, 1979, and will be published in the Proceedings (Markl and Kempter 1981)

Introduction

The arthropod respiratory protein, hemocyanin, is structurally variable between species, having a one-, two-, four-, or eight-hexamer structure, the monomeric polypeptide chains having an M_r of about 75,000. The aggregation state is generally identified by the sedimentation coefficient: 16 S, 24 S, 35 S, and 60 S. Dissociation into monomers (5 S) occurs at alkaline pH (Van Holde and van Bruggen 1971). In some species dimers (7 S) were observed after dissociation (e.g. Murray and Jeffrey 1974). Recently it has been established that arthropod hemocyanins are composed of more than one type of polypeptide chain, although there are large differences in the extent and significance of subunit heterogeneity. In previous studies 18 cheliceratan and crustacean species have been investigated for subunit heterogeneity by polyacrylamide gradient slabgel techniques. In all these species the minimum number of subunits found was 4; the maximum number was 12 (Markl et al. 1979a, b, where earlier literature is also quoted).

In order to investigate the specific role of the different hemocyanin chains in the multihexameric particle it is important to determine whether the components observed by electrophoresis represent functionally distinct types of subunit, or whether subunits with similar function can be classified into groups. Three dimensional structure is responsible for the specific function of proteins, but since no primary structure (which would ultimately determine the conformation) has yet been determined for an hemocyanin, the quickest method to obtain an understanding of the differences between hemocyanins is immunochemistry: in globular proteins antibodies are preferably developed against conformational determinants (Crumpton 1974; Baty and Lazdunski 1979). The reaction of antibodies with proteins is so specific that

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that it is even possible to distinguish between oxy- and deoxy-hemoglobin (Reichlin et al. 1974). Even the exchange of single amino acids can be detected in special cases (e.g. Nisonoff et al. 1970). Although the antigen conformation determines the specificity of an antiserum, a correlation between immunological relationship and functional relationship does not necessarily exist and must be established independently. For some cheliceratan hemocyanins which are composed of a number of immunologically distinct subunits (Lamy et al. 1979a, b, c), it has been demonstrated that these subunits play different roles in the self-assembly process of the multi-hexamer (Bijlholt et al. 1979; Markl et al. 1981; Lamy et al. 1981). In contrast, a uniform reassociation behavior has been described for the 5 electrophoretically distinct subunits of the hemocyanin from the spider, *Cupiennius salei,* which were found to be immunologically identical (Markl and Kempter 1981). It is not yet clear if this observed similarity is also true for the oxygen binding function; nevertheless, immunochemistry has proved to be an efficient tool for determining the number of conformationally different types of polypeptide chains in cheliceratan hemocyanins.

In order to extend our knowledge of such relations to the Crustacea, an immunological comparison of the hemocyanins from six species was undertaken.

Materials and Methods

Blood Samples

The European crabs *Cancer pagurus* (fam. Cancridae), *Carcinus maenas* (fam. Portunidae), and *Hyas araneus* (fam. Majidae) were obtained from the Biologische Anstalt Helgoland. The European freshwater crayfish *Astacus leptodactylus* (fam. Astacidae), the American lobster *Homarus americanus* (fam. Homaridae), and the European spiny lobster *Palinurus vulgaris* (fam. Palinuridae) were obtained from a local seafood dealer.

Blood was sampled by puncturing the base of a walking leg. After clotting, the clot and the debris were removed by centrifugation. Most experiments were performed on fresh samples; otherwise the serum was stored at 4 °C.

Hemocyanin Isolation and Subunit Separation

Hemolymph was centrifuged for 12 h at 130,000 × g in a Beckman model L preparative ultracentrifuge, the supernatant discarded, and the blue-green hemocyanin pellet redissolved in 10 mM EDTA containing glycine/NaOH buffer of pH 9.6, $I=0.05$ M (final protein concentration 10 mg/ml). After overnight dialysis (room temperature) against the same medium, most of the hemocyanin was dissociated into its subunits (Markl et al. 1979b). Gel chromatography, analytical and preparative PAGE were performed as previously described (Markl et al., 1979a).

Preparation of Antisera

After dissociation, and without further purification, 0,5 ml of the hemocyanin solution (10 mg/ml), and 0.5 ml of Freund's adjuvant (Difco Bacto Complete Freund adjuvant) were mixed and injected beneath the dorsal skin of a rabbit. Two and four weeks later, booster injections were given (the second one without adjuvant). Two weeks after the last injection, blood was collected from the lateral ear vein and allowed to clot overnight at 4 °C. The serum was separated, any remaining cells removed by centrifugation, and used directly. Blood was repeatedly collected over a period of 3 months. The antibody titer was controlled by immuno-diffusion according to Ouchterlony (1958, 1962).

Immunoelectrophoresis

Crossed immunoelectrophoresis was performed according to Weeke (1973); tandem-crossed and crossed-line immunoelectrophoresis as described by Krøll (1973a, b). 1% Agarose M (LKB) in 0.12 M sodium barbital buffer (pH 8.6) was used for gel preparation. For the second dimension, 2–4 ml of antiserum were used per 100 ml. Experiments were carried out in an LKB 2117 Multiphor apparatus using plastic plates (LKB) as support.

Results

Whole Subunit Mixtures

In the methods described above the hemocyanins did not completely dissociate into subunits and, in the case of *Homarus, Hyas* and *Cancer,* were still contaminated by an additional high molecular weight protein (Markl et al. 1979b). As the presence of these contaminants led to unclear crossed immunoelectrophoresis patterns, the hemocyanin subunit mixtures were first purified by gel chromatography through Sephadex G-100 sf (Markl et al. 1979b). Relatively clear patterns were then obtained with high reproducibility (Fig. 1). Several immunologically distinct components were present in the hemocyanins of each of the six species, indicating that subunit heterogeneity also appears in the antigenic behaviour.

Isolation of Individual Subunits

In order to determine whether each peak documented in Fig. 1 corresponded to one or more electrophoretic subunits, and to assign the electrophoretic components (Markl et al. 1979b) to those obtained by immunoelectrophoresis, individual subunits were isolated by preparative PAGE (Markl et al. 1979a) and identified by analytical PAGE (Markl et al. 1979b). They were then checked for purity by crossed immunoelectrophoresis (Fig. 2). If more than one component was present in a fraction, crossed-line immunoelectrophoresis was used for identification. Examples are shown in Fig. 3. A number of subunits were almost pure; others, although contaminated, could clearly be identified by being the main component. Even if the fractions contained two subunits in comparable amounts, the experiments described below led to unambiguous results, the only exception being subunits 3 and 4 of *Palinurus* hemocyanin, which could not be separated. Identification of the subunits in the peaks ob-

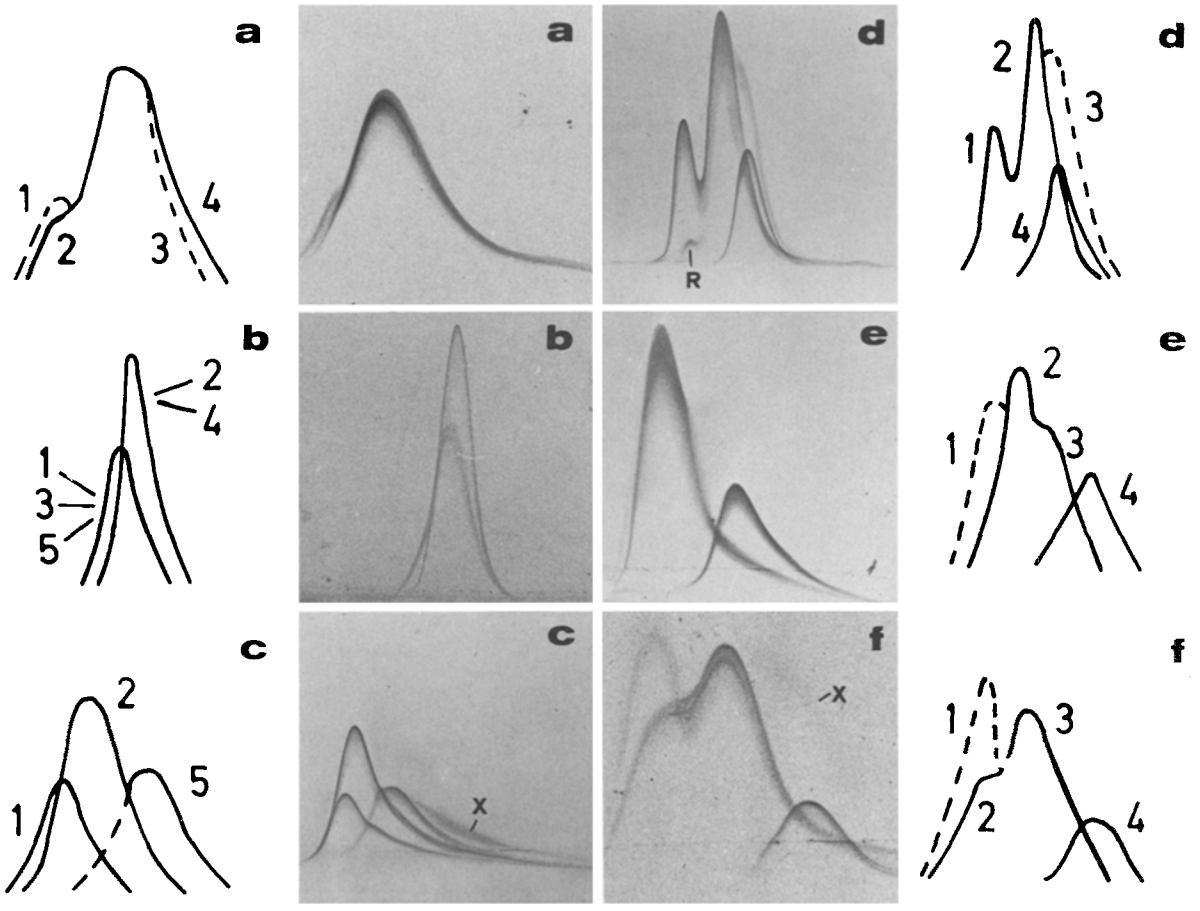

Fig. 1a–f. *Central plates:* Crossed immunoelectrophoresis patterns of whole dissociated hemocyanin from *Palinurus* (**a**), *Homarus* (**b**), *Astacus* (**c**), *Hyas* (**d**), *Cancer* (**e**), and *Carcinus* (**f**). Antisera prepared against unpurified dissociated hemocyanin from the same species were used. In the first dimension, the anode was on the left. *R* 16 S reassembly product. *X* not identified contaminant observed only in this particular experiment. Note: component *Astacus*-5 is a heterodimer composed of subunits 3 and 4. All other visible components from the six species are monomeric subunits. *Outer figures:* The same patterns, drawn schematically, and interpreted with the aid of data obtained from individual subunits to demonstrate more clearly the diverse cross-reactivities. The labelling of the subunits is according to the patterns obtained by PAGE (Markl et al. 1979b)

tained by immunoelectrophoresis of the whole mixtures (Fig. 1) was achieved by comparison of electrophoretic mobility, by co-electrophoresis, tandem-crossed-, and crossed-line immunoelectrophoresis. Some examples are presented in Fig. 3.

Immunological Relationship Between Subunits

To find the number of immunologically distinct subunits in the various hemocyanins, we compared the antigenic cross-reactivity between all subunits from one species according to the criteria of Bock and Axelsen (1973). Conclusions from Fig. 1 were, for example, that there was no identity between *Cancer*-3 and *Cancer*-4, partial identity between *Astacus*-2 and *Astacus*-5 (dimeric subunit), and complete identity between *Hyas*-1 and *Hyas*-2. More conclusive evidence was obtained for each of the species by comparison of the individual subunits by tandem-crossed immunoelectrophoreses. Examples are documented in Fig. 3. From the combined results, schematic patterns for the six hemocyanin subunit mixtures were drawn (Fig. 1), to indicate the cross-reactivities.

In each of the brachyuran crabs, and also in the spiny lobster, two of the four electrophoretically distinct hemocyanin subunits were completely immunologically identical, and a third was antigenically deficient compared to them. The fourth subunit did not show any immunological relationship to the other 3 components in case of *Cancer, Carcinus* and *Hyas*. In the case of *Palinurus,* however, all 4 components were clearly related immunologically. 16 S hemocyanin from *Astacus* showed two immunologically distinct subunits (1 and 2), in agreement with earlier electrophoretic data (Markl et al., 1979b). 24 S hemocyanin from *Astacus* contained, in addition, a dimeric sub-

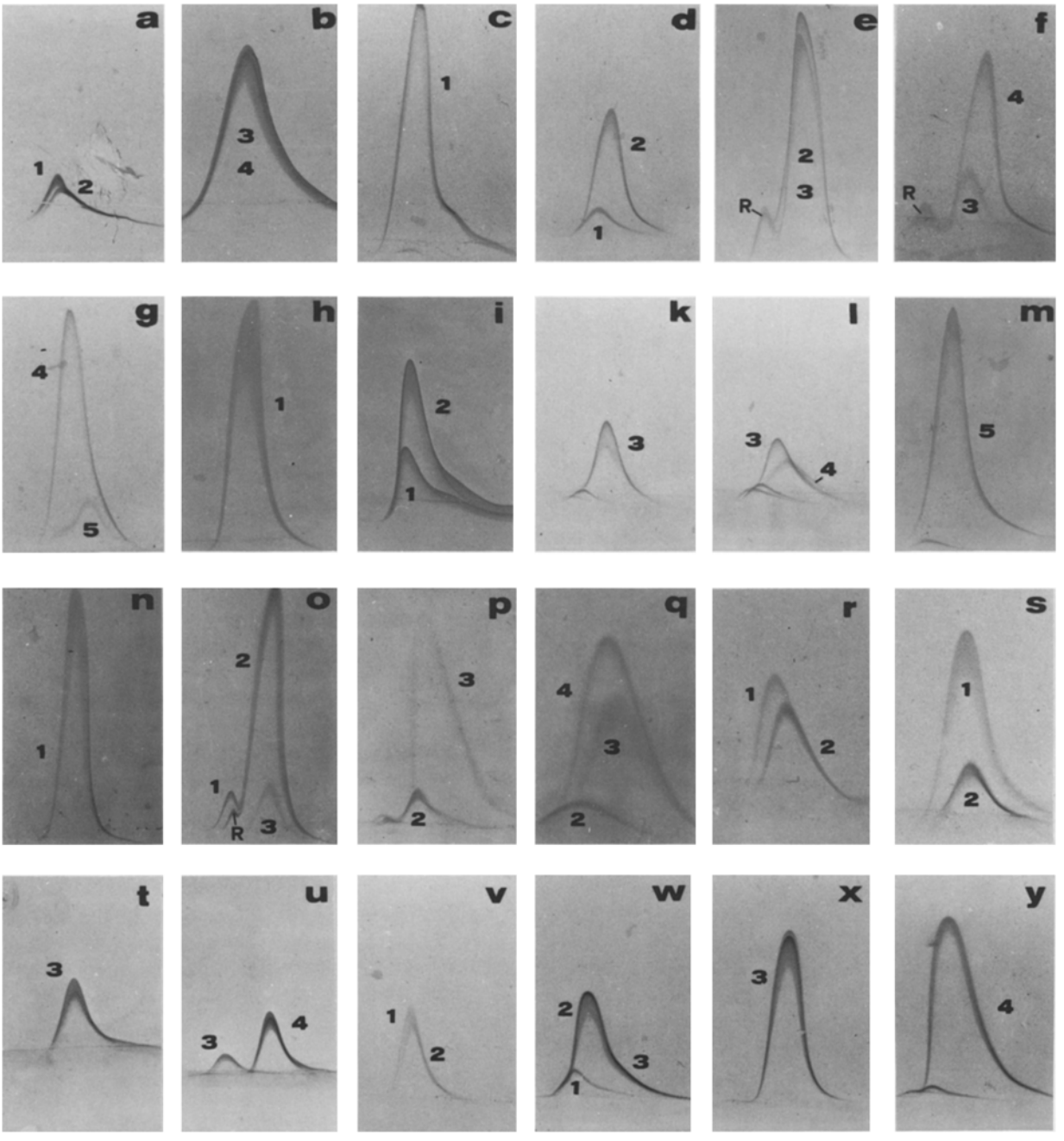

Fig. 2a–y. Crossed immunoelectrophoresis patterns of isolated hemocyanin subunits from *Palinurus* (**a–b**), *Homarus* (**c–g**), *Astacus* (**h–m**), *Hyas* (**n–q**), *Cancer* (**r–u**), and *Carcinus* (**v–y**). The homologous antisera were used. In the first dimension, the anode was on the left. *R* 16 S reassembly product. The subunits were isolated by preparative PAGE (Markl et al. 1979b)

unit 5 which turned out to be partially identical with subunit 2, but not related to subunit 1. Very small amounts of two further subunits (3 and 4) were always present in the dissociated 24 S hemocyanin from *Astacus*. It was shown previously (Markl et al. 1979b) that these represented the monomeric components of the heterodimer. Indeed, both subunits were immunologically related to the dimeric subunit 5 and also to each other, but showed additional, specific antigenic reactions. Because they were present in very small amounts their antigenic behaviour could not be established with certainty.

The five electrophoretically distinct hemocyanin subunits from the lobster *Homarus americanus* were subdivided into two immunologically unrelated groups (subunits 1, 3, 5, and 2, 4, respectively). Within

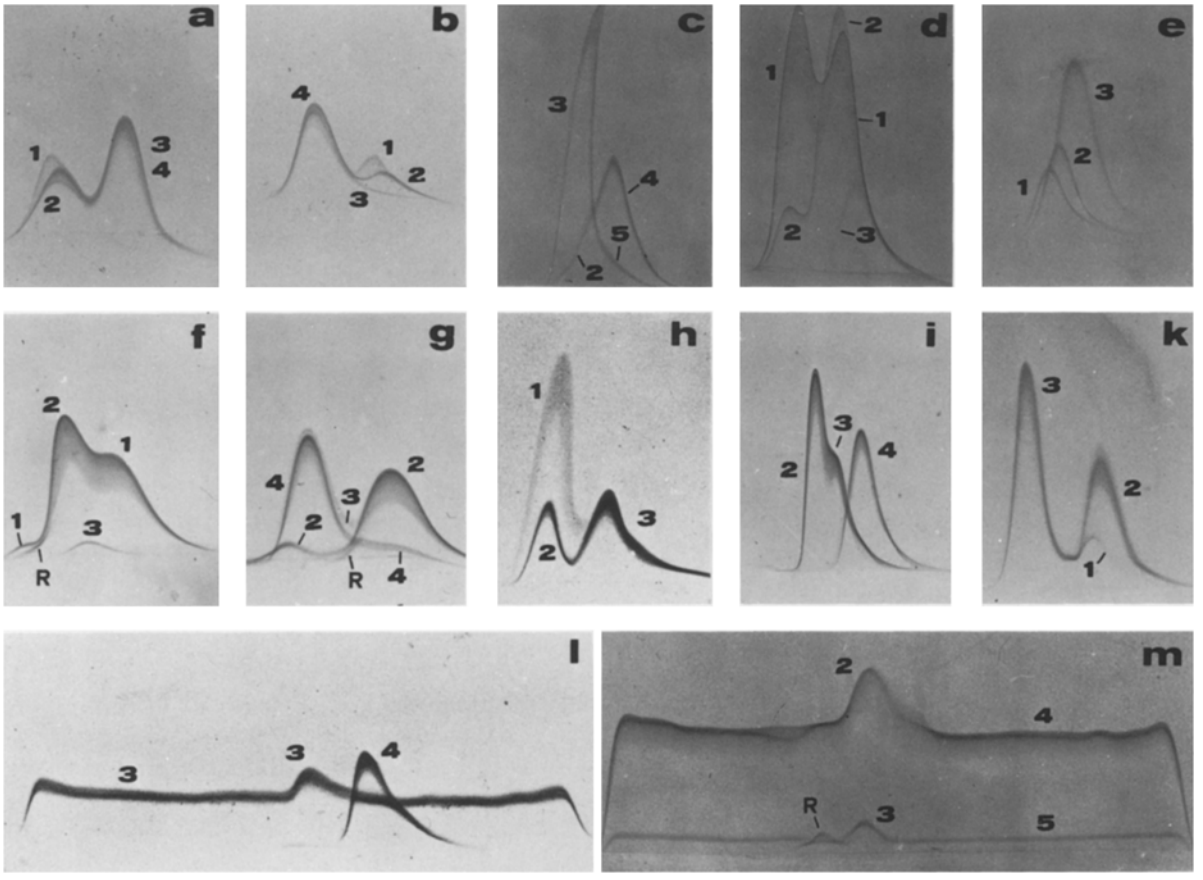

Fig. 3a–m. Tandem-crossed (**a–k**) and crossed-line (**l, m**) immunoelectrophoresis patterns taken from a total of more than 100 experiments with hemocyanin subunits of six crustacean species, carried out in order to clarify the cross-reactivities within each system. In general, the subunit fractions shown in Fig. 2 were employed. *Palinurus* (**a, b**), *Homarus* (**c, m**), *Astacus* (**d, e**), *Hyas* (**f, g**), *Cancer* (**h, l**), *Carcinus* (**i, k**). *R* 16 S reassembly product. The homologous antisera were used. In the first dimension, the anode was on the left

these groups the immunological identity was complete. The dissociation products obtained from 24 S material of *Homarus* blood contained, in addition, a copper-free dimeric 7 S component (removed by gel chromatography as shown in Fig. 1). It has been suggested previously that 24 S hemocyanin is accompanied by a non-respiratory protein of similar size (Markl et al. 1979b). However, the 7S protein shares some properties with the hemocyanin subunits: its appearance after alkaline dissociation, and a M_r of 80,000 for the constituent polypeptide chain (previously labelled α: see Fig. 3 in Markl et al. 1979b). It was suspected that this protein (which corresponds to about one third of the total) represents apo-hemocyanin. Figure 4 shows that the undissociated 24 S protein from *Homarus* blood is immunologically homogeneous, i.e. it consists exclusively of hemocyanin. Upon dissociation, three immunologically unrelated components of the same electrophoretic mobility appeared, two of them corresponding to the hemocyanin subunits 1–5, the third corresponding to the 7 S protein in question (Fig. 4). Thus the 7 S protein must originally have been incorporated in the 24 S hemocyanin molecule and therefore seems to be a copper-free hemocyanin subunit. However, this has still to be proved independently.

Discussion

The Problem of Subunit Heterogeneity

During the last decade, rapid development of electrophoretic techniques has greatly increased the number of identifiable protein chains. Polyacrylamide gradient slabgel electrophoresis and isoelectric focusing can be sensitive enough to separate proteins differing by only a single amino group (e.g. α- and β-aldolase; Midelfort and Mehler 1972). It is not surprising therefore that with high resolution techniques, many proteins turn out to be heterogeneous, at least at the subunit level. With respect to hemocyanins the number of subunits distinguished by PAGE has been

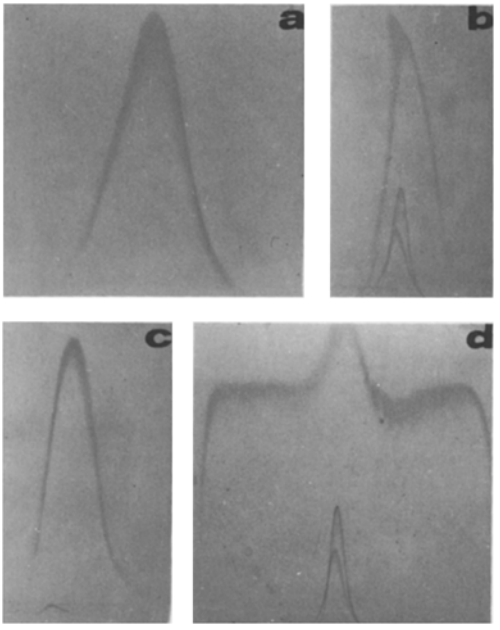

Fig. 4a–d. Crossed immunoelectrophoresis patterns of *Homarus* hemocyanin, using the homologous antiserum. **a** Undissociated 24 S material, isolated by gel chromatography; **b** dissociated 24 S material; **c** 7 S component, isolated by PAGE; **d** crossed IE of dissociated 24 S material with the 7 S component in the line. In the first dimension, the anode was on the left

increased in the case of *Panulirus* hemocyanin from 3 (van den Berg et al. 1977) to 4 (Markl et al. 1979b) and 5 (van Eerd and Volkerts 1981), and in *Limulus* hemocyanin from 5 (Sullivan et al. 1974) to 8 (Sullivan et al. 1976) and 12 (Markl et al. 1979a). However, as deduced from immunochemistry, *Limulus* hemocyanin is composed of only 8 types of subunit (Hoylaerts et al. 1979), so that the occurrence of polypeptide chains with very similar conformation must be assumed. Even more striking is the immunological identity of the 5 subunits which compose the 16 S hemocyanin from the spider *Cupiennius salei* (Markl and Kempter 1981). Thus, prior to an investigation of the functional significance of different hemocyanin subunits from a given species, one should try to classify them into groups of similar conformation.

Subunits Showing Complete Immunological Identity

As in cheliceratan species, in crustacean hemocyanins some subunits with clearly distinct electrophoretic mobilities yield completely fused precipitation lines in two-dimensional immunoelectrophoresis. Moreover, several of those immunologically identical chains from *Homarus, Hyas, Cancer* and *Carcinus* not only differed in charge but also in M_r as determined by SDS-PAGE (Markl et al. 1979b). The observed differences (between 2,000–3,000) correspond to about 15–25 amino acid residues (or perhaps to a number of carbohydrate residues). On the other hand, differences in the number and nature of amino acids would not be detected by immunochemical analysis of native subunits if their conformation is similar, which is obviously the case. Those immunologically identical hemocyanin chains might also behave similarly, at least in the aggregation to the hexamer, as has been reported for *Cupiennius* hemocyanin (Markl and Kempter 1981), and thus would represent, functionally, one type of subunit.

Subunits Showing Partial Immunological Identity Or No Cross-Reactivity

Compared to all other components, the subunits *Palinurus*-1, *Hyas*-3, *Cancer*-1, and *Carcinus*-1 showed weak precipitation lines which fused completely with certain other peaks. This behaviour reveals a partial immunological identity corresponding to an antigenic deficiency. In the case of the dimeric subunit *Astacus*-5, the precipitation line crossed that of *Astacus*-2 (which is not incorporated in the dimer) but was slightly weakened by it (Fig. 1c), indicating an immunological deficiency but, at the same time, also the presence of specific antigenic determinants.

Rochu et al. (1978) have described subunits with antigenic deficiencies in the hemocyanins from 5 brachyuran crabs (including *Cancer pagurus* and *Carcinus maenas*) and from an African spiny lobster. The possibility of enzymatic cleavage and the existence of different types of subunits was discussed. A clear distinction between these alternatives was, however, not possible. In the present study, enzymatic cleavage can be excluded since, according to earlier data (Markl et al. 1979b), the deficient chains have the same, or even a higher, M_r compared to immunologically complete chains. The antigenic deficiency must thus be the result of a different conformation which may stem from a difference in amino acid composition, as the components also differ in electrophoretic mobility. Compared to immunologically complete chains, the deficient chains might represent a second type of subunit.

No immunological identity between certain hemocyanin subunits was found to occur in five of the six crustacean species investigated, the only exception being *Palinurus*. The present data for this spiny lobster do not agree with data reported previously (Markl and Kempter, 1981).

For hemocyanin from *Limulus polyphemus* and *Eurypelma californicum* it has been demonstrated that immunologically unrelated chains (Lamy et al. 1979b, c) show marked chemical differences (Sullivan et al. 1976; Markl et al. 1979c). Protein chains with this

degree of structural diversity clearly represent distinct types of subunits which should differ also in their reassembly behaviour (e.g. Markl et al. 1981), and perhaps also in their specific oxygen binding properties (Sullivan et al. 1974).

Number of Types of Subunits

A similarity was found between the five 24 S hemocyanins investigated: each one was composed of 3 immunologically different types of subunits. In *Cancer, Carcinus* and *Hyas* the two immunologically identical chains represent the first group; the chain which is antigenically deficient compared to the former represents the second type, and the third type in each species is subunit 4 which shows no cross-reactivity with other chains. The presence of 2 types of subunits without cross-reactivity in the hemocyanin from *Homarus* and *Astacus* is similar to the situation found in brachyuran hemocyanins. In contrast to the latter, however, *Homarus* and *Astacus* hemocyanin possesses a dimeric component which is not (*Homarus*), or only slightly (*Astacus*), immunologically related to other subunits.

The presence of only two distinct types of subunits could be a characteristic of 16 S hemocyanins from crustacean species. In this respect our data for *Astacus* agree with electrophoretic results obtained for *Cherax destructor* (Murray and Jeffrey 1974) which were confirmed by immunochemistry (Lamy and Jeffrey unpublished). Also the North American spiny lobster *Panulirus interruptus* possesses 16 S hemocyanin composed of 2 immunologically distinct groups of subunits (van Eerd and Volkerts 1981). The data obtained for *Palinurus vulgaris* are the least well established due to the failure to isolate all the subunits. The present study indicates only four electrophoretically distinct polypeptide chains, in contrast to six reported previously (Markl et al. 1979b). Two of these components, appearing only in SDS-gels, are presumably products of enzymatic cleavage and have not been taken into consideration in the present study. *Palinurus*-1, being antigenically deficient compared to the other chains, should represent a distinct type of subunit, although it represents only a very small fraction of the total (Fig. 1a). At the moment it is not clear whether *Palinurus*-3 is immunologically distinct from chains 2 and 4 (which are immunologically identical). In this respect the diversity described in our preliminary report (Markl and Kempter 1981) could not be confirmed here.

Our main conclusions from the present data are summarized in Fig. 5 and compared to various cheliceratan hemocyanins. It is interesting to note that in the 24 S- and 16 S-hemocyanin from the spider

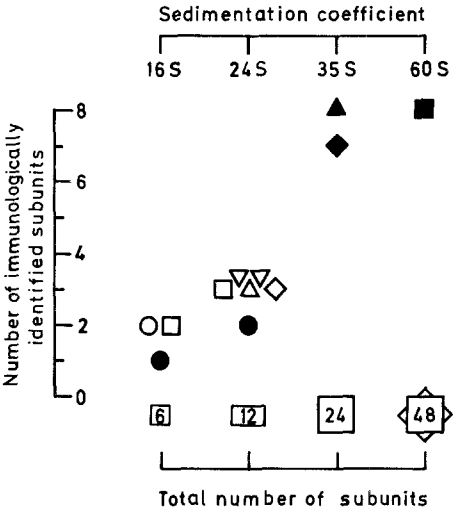

Fig. 5. Number of immunologically identified subunits in various cheliceratan (closed symbols) and crustacean (open symbols) hemocyanins. ■ *Limulus* (Hoylaerts et al. 1979); ▲ *Androctonus* (Lamy et al. 1979a); ◆ *Eurypelma* (Lamy et al. 1979b); ● *Cupiennius* (Markl and Kempter 1981); □ *Astacus;* ▽△ *Hyas, Cancer; Carcinus;* ◇ *Homarus;* ○ *Palinurus*

Cupiennius, only two, respectively one, type of subunit is involved (Markl and Kempter 1981). The significance of a higher degree of subunit heterogeneity in the corresponding aggregation states of crustacean hemocyanins is an important question to be answered in the future.

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References

Baty D, Lazdunski C (1979) An anti-(signal peptide) antibody: purification, properties and use as a conformational probe. Eur J Biochem 102:503–507

Berg AA Van den, Gaastra W, Kuiper HA (1977) Heterogeneity of *Panulirus interruptus* hemocyanin. In: Bannister JV (ed) Structure and function of haemocyanin. Springer, Berlin Heidelberg New York, pp 6–12

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

Bock E, Axelsen NH (1973) Comparison of antigens: The reaction of partial identity. Scand J Immunol 2, Suppl 1 95–99

Crumpton MJ (1974) Protein antigens. In: Sela M (ed) The antigens vol II. Academic Press, Oxford, pp 1–79

Eerd JP van, Folkerts A (1981) Isolation and characterization of the five subunits from the hemocyanin of the spiny lobster *Panulirus interruptus*. In: Lamy J (ed) Structure, active site, and function of invertebrate oxygen binding proteins. Dekker, New York, in press

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

Krøll J (1973a) Tandem-crossed immunoelectrophoresis. Scand J Immunol 2, Suppl 1:57–59

Krøll J (1973b) Crossed-line immunoelectrophoresis. Scand J Immunol 2, Suppl 1:79–81

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

Lamy J, Lamy J, Weill J, Markl J, Schneider H-J, Linzen B (1979b) 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, 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

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, 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, 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

Midelfort CF, Mehler AH (1972) Deamination in vivo of an asparagine residue of rabbit muscle aldolase. Proc Natl Acad Sci USA 69:1816–1819

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

Nisonoff A, Reichlin M, Margoliash E (1970) Immunological activity of cytochrome c. J Biol Chem 245:940–946

Ouchterlony Ö (1958) Diffusion-in-gel methods for immunological analysis. Prog Allergy 5:1–78

Ouchterlony Ö (1962) Diffusion-in-gel methods for immunological analysis II. Prog Allergy 6:30–154

Reichlin M, Bucci E, Wymann J, Antonini E, Rossi-Fanelli A (1964) The immunological difference between horse oxy- and deoxyhemoglobin. J Mol Biol 9:785–788

Rochu D, Lambin P, Ghidalia W, Fine JM (1978) Hemocyanin subunits and their polymeric forms in some decapod Crustacea. Comp Biochem Physiol 59B:117–122

Sullivan B, Bonaventura J, Bonaventura C (1974) Functional differences in the multiple hemocyanins of the horseshoe crab, *Limulus polyphemus L.* Proc Natl Acad Sci USA 71:2558–2562

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, Suppl 1:47–56