Quaternary Structure of *Limulus polyphemus* Hemocyanin†

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ABSTRACT: *Limulus polyphemus* (*Lp*) hemocyanin, a high molecular weight copper protein which serves as an oxygen carrier, is a 48-subunit ensemble containing eight immunologically distinct types of subunits. Determination of its quaternary structure was approached in four steps. (1) The heterogeneity of the subunits present after dissociation was reexamined by determining the N-terminal sequences of isolated subunits *Lp* I, II, IIA, IIIA, IIIB, IV, V, and V-VI. Eight different polypeptide chains could be identified. Subunit *Lp* IIA, which had been previously considered as a possible degradation product of subunit *Lp* IIIA, is definitely a distinct polypeptide chain as evidenced by its N-terminal sequence and its antigenic properties. (2) The number of copies of the various polypeptide chains was determined by rocket immunoelectrophoresis using subunit-specific antibodies purified by immunoadsorption. The number of copies per (8 × 6)-mer is 5.3 (*Lp* I), 7.8 (*Lp* II), 1.5 (*Lp* IIA), 7.2 (*Lp* IIIA), 8.6 (*Lp* IIIB), 7.8 (*Lp* IV), 5.1 (*Lp* V), and 4.6 (*Lp* VI). The values obtained for subunits *Lp* I and *Lp* IIA rule out the possibility that the native molecule be composed of four identical dodecamers. (3) Intramolecular location of the subunits in the (4 × 6)-meric half-molecule was achieved by a modification of a method involving electron microscopy of immunologically labeled subunits [Lamy, J., Bijlholt, M. M. C., Sizaret, P.-Y., Lamy, J. N., & van Bruggen, E. F. J. (1981) *Biochemistry 20*, 1849–1856]. In the top view, subunit *Lp* I was found in the corners, subunit *Lp* II in the middle of the lateral edges, subunit *Lp* IIIA near the end of the dodecamers, subunit *Lp* IIIB in the immediate neighborhood of the cleft, and subunits *Lp* V and VI in the interdodecamer bridge area. Subunit *Lp* IV was observed only in the side view near the top/bottom edges. Subunit *Lp* IIA was found in positions corresponding to those of subunits *Lp* I and/or *Lp* IIIA. Labeling of the whole molecule led to immuno complexes in perfect agreement with those of half-molecules. (4) Hybrid oligomers were reassembled from isolated subunits of *A. australis* and *L. polyphemus* hemocyanins, and the structural roles of the various subunits were studied. Subunits *Lp* V-VI and *Aa* 3C-5B, as well as subunits *Lp* IV and *Aa* 5A, are interchangeable for the (4 × 6)-mer reconstruction. Subunit *Lp* II is capable of replacing subunits *Aa* 3A and 3B, but the reverse is not true. By electron microscopy of labeled subunits, we demonstrated that subunit *Lp* II actually occupies the positions of subunits *Aa* 3A/3B in the *A. australis* system. These results are discussed in terms of their evolutionary implications.

The largest form of arthropod hemocyanin is undoubtedly the (8 × 6)-mer found in horseshoe crabs (Van Holde & Miller, 1982; van Bruggen et al., 1981). During the last 3 years, two major technical breakthroughs made it possible to determine the quaternary structure of (4 × 6)-meric arthropod hemocyanins. First, Van Heel & Frank (1981) introduced the use of correspondence analysis for image analysis of molecules visualized by electron microscopy, a technique which allowed recognition of two important features of (4 × 6)-meric hemocyanins, the rocking and the shift. The other major technical advance was the ability to localize subunit positions in electron micrographs by use of subunits labeled with subunit-specific Fab fragments (Lamy et al., 1981b). This method led to the elucidation of the relative positions of the 24-constituting subunits of *Androctonus australis* hemocyanin (Sizaret et al., 1982) and to a first approach of the quaternary structures of *Eurypelma californicum* (Markl et al., 1981) and *Limulus polyphemus* (Lamy et al., 1983a) hemocyanins.

The quaternary structure of *L. polyphemus* (8 × 6)-mer[1] hemocyanin is much more difficult to solve than those of (4 × 6)-meric hemocyanins. The homogeneity of the native population of molecules is not definitely established, and the heterogeneity and the stoichiometry of the various constituting subunits are still a matter of controversy. The architecture of the (8 × 6)-meric structure with its five EM views (Lamy et al., 1982) is much more complex than that of (4 × 6)-meric hemocyanins which present only three views (Lamy et al., 1981b; Van Heel et al., 1983), and moreover, the intermediate (4 × 6)-meric and (2 × 6)-meric dissociation products of *L. polyphemus* hemocyanin proved to be rather unstable upon binding of Fab fragments.

This paper brings at least partial resolution to all these difficulties. Our results demonstrate that *L. polyphemus* hemocyanin cannot be composed of four identical dodecamers. We find that the eight antigenically distinct subunits possess eight different N-terminal amino acid sequences and describe an accurate assay method for the various subunits. The (4 × 6)-meric half-molecule was stabilized by cross-linking with dimethyl suberimidate (DMS)[2] and, in this state, aided us in determining the topology of the eight immunologically distinct subunits by immunoelectron microscopy.

Materials and Methods

Hemocyanins. L. polyphemus hemocyanin was prepared as previously reported (Lamy et al., 1979b). Dissociation of

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[1] The aggregation states of arthropod hemocyanins are typically multiples of six subunits. For *L. polyphemus* hemocyanin, the 48-subunit complex is referred to as a (8 × 6)-mer, the half-molecule as a (4 × 6)-mer, and the quarter molecule as a (2 × 6)-mer or dodecamer.

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DMS, dimethyl suberimidate; EM, electron microscopy; *Lp*, *Limulus polyphemus*; *Aa*, *Androctonus australis*; Tris, tris(hydroxymethyl)aminomethane.

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the native (8 × 6)-mers into (4 × 6)-meric half-molecules was completed in an overnight dialysis against an $I = 0.1$, Tris-HCl buffer, pH 7.0, containing 10 mM EDTA (Brenowitz, 1982). *L. polyphemus* hemocyanin is composed of eight immunologically distinct polypeptide chains termed *Lp* I, II, IIA, IIIA, IIIB, IV, V, and VI, respectively. All of these, except chain *Lp* VI, which has not yet been isolated and is present in dissociated hemocyanin as dimeric subunit *Lp* V-VI, were prepared in a highly purified form according to the method of Lamy et al. (1979b).

A. australis hemocyanin is also composed of eight polypeptide chains termed *Aa* 2, 3A, 3B, 3C, 4, 5A, 5B, and 6, which have all been isolated. The subunits used in this work, namely, the monomeric subunits *Aa* 3A, 3B, 4, and 5A and the dimeric subunit *Aa* 3C-5B, were prepared according to a previously reported method (Lamy et al., 1979a).

All the subunits were pure as judged by crossed immunoelectrophoresis using antisera prepared against dissociated hemocyanin. In order to prevent any confusion, the subunits from *L. polyphemus* and *A. australis* are respectively prefixed *Lp* and *Aa* throughout this paper.

Cross-Linked Hemocyanins. Cross-linking of (8 × 6)-mer and (4 × 6)-mer forms of *L. polyphemus* hemocyanin were carried out by using DMS according to the method of Davies & Stark (1970). For whole (8 × 6)-meric hemocyanin, incubation conditions were as follows: 40 μM hemocyanin, 170 mM triethanolamine, pH 8.5, 9 mM DMS, and 10 mM $CaCl_2$. Cross-linking of half-molecules required slightly different conditions: 40 μM hemocyanin, 300 mM triethanolamine, pH 8.5, 15 mM DMS, and 25 mM EDTA. All other conditions were those described by Davies and Stark.

N-Terminal Amino Acid Sequences. All reagents for the sequencer were obtained from Pierce, Merck, or SDS (Marseille).

Automated Edman degradation was carried out in a Beckman Sequencer 890 C by the 1 M quadrol simple cleavage method (Edman & Begg, 1967). Double coupling was used for the first cycle. The thiazolinones were converted into phenylthiohydantoin (PTH-) amino acids by treatment with 20% trifluoroacetic acid at 80 °C for 12 min (Sladic-Simic et al., 1977).

The PTH-amino acids were identified by thin-layer chromatography (Jollès et al., 1974) and by high-performance liquid chromatography (Waters chromatograph, Model ALC GPC 204) (a) on a Waters μBondapak C 18 column [buffer A, 40 mM sodium acetate, pH 4.4, mixed with methanol (9:1 v/v); buffer B, 40 mM sodium acetate, pH 4.4, mixed with methanol (1:9 v/v); gradient from 5 to 45% of buffer B] and (b) on a Waters fatty acid column [solvent A, 0.085% propionic acid adjusted to pH 3.8; solvent B, 90% methanol; gradient from 20 to 55% of solvent B]. Val and Met or Phe and Ile could thus be separated.

Antibodies. (*1*) *Definition and Preparation Procedure.* Antisera were prepared as previously reported (Lamy et al., 1979b) by injecting either pure subunits or unfractionated dissociated hemocyanin into rabbits. For example, anti-*Lp* I antiserum was raised against immunologically pure subunit *Lp* I while anti-*Lp* Hc antiserum was raised against unfractionated dissociated hemocyanin. Starting from crude antisera subunit-specific antibody preparations were prepared by the immunoadsorption procedure described below. For example, *Lp* I specific antibodies were purified from anti-*Lp* I antiserum. In contrast to the crude anti-*Lp* I antiserum, which was capable, albeit weakly, of precipitating all the subunits but *Lp* V and *Lp* VI and producing soluble immuno complexes with all the subunits (Lamy et al., 1983b), the *Lp* I specific antibodies did not bind to any other subunit except *Lp* I.

The subunit-specific antibody preparations were obtained as follows. First, 75 mg of dissociated hemocyanin were covalently bound to 1 g of activated CH-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala) according to the method recommended by the manufacturer. This product is further designated as *immobilized dissociated hemocyanin.* Second, subunit-specific antisera were incubated with small amounts of immobilized dissociated hemocyanin for 2 h at 37 °C, and the immobilized immuno complexes were removed by centrifugation at 5000 rpm for 10 min. The partially purified antiserum was then tested in crossed immunoelectrophoresis with unfractionated dissociated hemocyanin. When more than one precipitation peak was obtained, the second step was repeated until complete disappearance of the undesired precipitation peaks. In a third step, the antibody preparation was adsorbed for 2 h at 37 °C on the corresponding pure subunit covalently bound to CH-Sepharose 4B. After the nonadsorbed material was washed out with 0.05 M Tris-HCl, pH 7.5, buffer, containing 0.15 M NaCl, the subunit-specific antibodies were eluted with a 0.53 M formic acid–0.15 M NaCl solution. Immediately after the elution, the pH was raised to 7.5, and the solution was dialyzed overnight against the 0.05 M Tris-HCl, pH 7.5, buffer, containing 0.15 M NaCl. The ability of the antibody preparation to produce soluble immuno complexes with other subunits was then investigated by the screening technique described under Screening of Soluble Immuno Complexes. When soluble immuno complexes were produced, responsible antibodies were removed as much as possible from the preparation through an additional immunoadsorption step. For example, to remove the antibodies capable of forming soluble immuno complexes with subunit *Lp* IIIA from an *Lp* I specific antibody preparation, the preparation was incubated with pure subunit *Lp* IIIA immobilized on CH-Sepharose. Because of the unavailability of pure subunit *Lp* VI, antibodies capable of reacting with this subunit were removed from the *Lp* V specific antibody preparation by incubation with immobilized subunit *Lp* V-VI, but the yield of the step was rather low because of the trapping of anti-*Lp* V antibodies by the dimeric subunit.

Subunit-specific antibodies purified by this procedure were further used to determine the number of copies of the various subunits in native hemocyanin and to prepare Fab fragments for intramolecular localization of the subunits.

(*2*) *Characterization of the Antibodies.* The various subunit-specific antibody preparations were characterized as follows. First, all the preparations were capable of precipitating their homologous subunit in immunoelectrophoresis. In the specific case of subunits Lp V and *Lp* VI, the *Lp* VI specific antibody preparation did precipitate the dimeric subunit *Lp* V-VI but did not precipitate subunit *Lp* V, and the *Lp* V specific antibody preparation precipitated subunits *Lp* V and *Lp* V-VI.

Second, all the antibody molecules of each preparation were capable of rebinding to their homologous subunit (*Lp* V-VI for *Lp* VI specific antibodies) immobilized to CH-Sepharose upon a second immunoadsorption step. No antibody could be detected in the filtrate of the column by the screening test for soluble immuno complexes described below.

Third, the inability of all the antibody preparations to bind to heterologous subunits was also demonstrated by the screening protein A–Sepharose test for soluble immuno complexes. However, in the case of the *Lp* V specific antibody preparation which strongly cross-reacted with subunit *Lp* VI,

antibodies capable of forming soluble complexes with subunit *Lp* VI could not be completely removed from the preparation due to a lack of pure subunit *Lp* VI (required both for the immunoadsorption step and for testing the cross-reactivity).

Screening of Soluble Immuno Complexes. To detect soluble immuno complexes, the following three-step radioimmunological test was devised. In a first step, 5 μL (35×10^{-15} mol) of [^{125}I]iodine-labeled pure subunit, prepared according to Greenwood et al. (1963), was incubated at 37 °C for 1 h with 20 μL of the corresponding subunit-specific antibodies diluted with 500 μL of 0.05 M Tris-HCl, pH 7.5, buffer, containing 0.15 M NaCl and 1 g of bovine serum albumin/L. In a second step, 40 μL of a protein A–Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala) suspension, containing 4.6 mg of dry material swollen in the same buffer, was added to the incubation mixture to trap free IgG and immuno complexes. The mixture was stirred at 4 °C for 30 min. Finally the insoluble material was washed 3 times with the buffer to remove the unadsorbed subunit, and the radioactivity of the gel was determined. The background was obtained by replacing the subunit-specific antibodies with a preparation of total IgGs, from nonimmunized rabbits at the same concentration. The purity of the subunit-specific antibody preparations was assayed by measuring gel radioactivity-to-total antibody concentration ratios when the suspected cross-reacting subunits were radiolabeled and used in step 1. Antibody concentrations were estimated by spectrophotometry at 210 nm (Malinowski & Manski, 1981).

Immunoelectrophoreses. To determine the number of copies of the various subunits in native hemocyanin, rocket immunoelectrophoreses were done according to the technique of Weeke (1973a), making use of subunit-specific antibody preparations. Calibration curves were obtained from pure subunit solutions of which the protein concentration had been determined by the biuret reaction (Goa, 1953), with the mixture of subunits present in dissociated *L. polyphemus* hemocyanin as a standard. The *L. polyphemus* standard was determined spectrophotometrically at 280 nm, assuming an extinction coefficient value $E^{1\%}_{1cm}$ of 13.9 (Nickerson & Van Holde, 1971).

Crossed immunoelectrophoreses and crossed-line immunoelectrophoreses were performed according to the methods of Weeke (1973b) and Krøll (1973), respectively.

Immunolabeling. Immunolabeling experiments were carried out as previously reported (Lamy et al., 1981b) with the following modifications. First, 200 μg of (8 × 6)-meric or (4 × 6)-meric cross-linked hemocyanin was incubated at 37 °C for 1 h with approximately eight Fab molecules per copy of the subunit to be labeled. For example, for the (8 × 6)-mer molecule containing approximately 8 copies of subunit *Lp* IIIB, the incubation mixture contained 64 Fab molecules per (8 × 6)-mer molecule. Second, the purification of soluble immuno complexes was performed by gel filtration on a Bio-Gel A-1.5m microcolumn (30 cm in height and 5 mm in diameter) buffered with 0.05 M Tris-HCl, pH 7.5. One-milliliter fractions were collected and examined in the electron microscope.

Reassembly. The subunit mixtures used in reassembly experiments were prepared by remixing isolated subunits. The traces of undesirable subunits found in the various components of the mixture were removed by immunoadsorption to their specific antibodies immobilized on protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala). For this purpose, 5 mL of an antiserum raised against the subunit to be removed was first incubated with 1 g of protein A–Sepharose swollen in 0.05 M Tris-HCl, pH 7.5, buffer, containing 0.15 M NaCl and 0.02% sodium azide, for 1 h at 0 °C. Then the insoluble material was extensively washed with the buffer until complete removal of serum proteins was accomplished as judged by the decrease in absorbance at 280 nm. In a second step, the subunit mixture was incubated for 1 h at 37 °C with enough IgGs immobilized on protein A–Sepharose to trap the contaminating subunit. Insoluble immuno complexes (protein A–Sepharose–immunoglobulin–hemocyanin) were subsequently removed by filtration, and the mixture of free subunits was concentrated by vacuum dialysis up to a final concentration of about 25 mg/mL. The concentration of the unwanted subunit in the reassembly mixture was finally determined by rocket immunoelectrophoresis; it never exceeded 0.1%.

After the removal of traces of contaminating subunits, the reassembly mixture was subjected to the following two-step reassembly procedure: (1) the subunit mixture, initially in a 0.05 M Tris-HCl, pH 8.9, buffer, containing 10 mM EDTA, was dialyzed overnight against a 0.05 M Tris-HCl buffer, pH 7.5, containing 10 mM EDTA; (2) the dialysis bag was transferred to a 0.05 M Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$, for 24 h. The mixture was then submitted to a gel filtration on Bio-Gel A-1.5m in the same buffer, and the fractions were examined in the electron microscope.

Electron Microscopy. The samples were applied to carbon-coated copper grids and negatively stained with 3 drops of 1.5% (w/v) of uranyl acetate successively blotted on filter paper. The support was previously glow discharged. The specimens were viewed in a Jeol 100B or Jeol 1200Ex electron microscope at an accelerating voltage of 80 kV.

Results

The determination of the quaternary structure of a complex protein such as *L. polyphemus* hemocyanin requires answers to the following questions. Is the whole material composed of a single population of molecules? How many different subunits comprise the whole molecule? What is the number of copies of each of them in the whole molecule? What are the overall shapes of the various subunits? What is the architecture of the whole molecule? What is the intramolecular location of each copy of each subunit in the whole molecule? All these questions are now being answered at least partially.

Subunit Heterogeneity in Dissociated Hemocyanin. Since the initial demonstration by Sullivan et al. (1974) of the presence of five chromatographic zones in dissociated hemocyanin of *L. polyphemus*, the number of "true" subunits has been a perpetual matter of controversy. Two years after the initial paper, Sullivan et al. (1976) proposed on the basis of heterogeneity within the chromatographic zones that *L. polyphemus* hemocyanin is composed of at least eight different kind of subunits. Then Hoylaerts et al. (1979) and Lamy et al. (1979b) independently confirmed the existence of eight different polypeptide chains on the basis of their immunological properties. In 1979, Markl et al. used a combination of polyacrylamide gel electrophoreses in the presence and in the absence of SDS and found 12 different electrophoretic fractions, and Brenowitz et al. (1981) observed up to 15 fractions by ion-exchange chromatography and SDS gel electrophoresis. However, some of the chromatographically purified fractions were found to be functionally and antigenically similar and were therefore denoted subunits I, I′, and I″ or subunits II, II′, and II″ (Brenowitz et al., 1981). Therefore, it was interesting to determine the N-terminal amino acid sequence of the various subunits or fractions. It was expected that if the various antigenically similar but chromatographically distinct fractions observed by Brenowitz et al. (1981) had

Table I: N-Terminal Amino Acid Sequences of the Eight Subunits of *Limulus polyphemus* Hemocyanin[a]

Subunit	Cycle -2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Lp I			T	I	K	K	K	Q	A	S	I	L	A	L	F	E	H	L	T	S	L	P	K	Q	H	I
Lp II			T	L	H	D	K	Q	I	R	V	V	H	L	F	E	Q	L	S	S	A	T	V	F	H	
Lp IIA			T	V	K	E	K	Q	S	R	L	L	P	L	F	(E)	H	L	T	S	L	(P)				
Lp IIIA			T	V	K	E	K	Q	S	R	L	L	P	L	F	(K)	H	L	T	S	L	(T)	T	D	Q	L	P
Lp IIIB			T	I	Q	E	K	Q	N	H	I	L	S	L	L	E	H	L	N	N	L	T	K	H	Q	L	
Lp IV			T	L	K	E	K	Q	D	R	I	L	V	L	F	E	H	L	T	S	L	T	K	H	Q	L	P	E
Lp V	V	L	(S)	(V)	L	(Q)	K	Q	L	R	V	L	P	L	F	E	(E)	A	(T)	I	P	T	K	E		
Lp VI	V	L	(G)	(A)	L	(E)	K	Q	L	R	V	L	P	L	F	E	(Y)	A	(S)	I	P	T	K	E		

[a] Rectangles surround invariant residues. Differences between amino acids in the same positions are circled in subunit *Lp* IIA and *Lp* IIIA and in subunits *Lp* V and *Lp* VI.

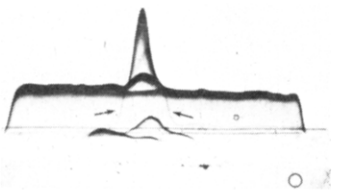

FIGURE 1: Crossed-line immunoelectrophoresis of subunit IIA (deposit well) and subunit IIIA (trough) against an anti-*Lp* HC antiserum.

different polypeptide chains, then some differences might appear in their N-terminal sequences. However, if different amino acid sequences demonstrate that two subunits have distinct polypeptide chains, identical N-terminal sequences bring at most additional evidence of homogeneity without definitely ruling out the possibility of heterogeneity.

Table I shows the N-terminal sequences of subunits *Lp* I, II, IIA, IIIA, IIIB, IV, V, and VI according to the nomenclature of Lamy et al. (1981b). These subunits were preferred for this study to those prepared according to Brenowitz et al. (1981) for two reasons: Brenowitz's fractions were very difficult to prepare in a high state of purity (e.g., subunit I′ free of subunits I and I″), and a difference in the sequence of only one constituent of the mixture would be easily detectable, since several amino acids would be expected at some degradation steps.

Two major features are obvious in Table I. First, the first 20 amino acids of the 8 subunits present differences, and second, the 8 subunits exhibit strong similarities, demonstrating that they evolved from a common ancestral polypeptide chain.

These two statements have important consequences. Specifically, the fact that the N-terminal amino acid sequence of subunit *Lp* IIA is different from those of the seven other subunits definitely demonstrates that subunit *Lp* IIA is a true subunit and that it cannot be considered any longer as a degradation product of subunit *Lp* IIIA or of any other subunit. Moreover, the crossed-line immunoelectrophoresis of Figure 1 shows that subunit *Lp* IIA possesses enough antigenic determinants, not present in subunit *Lp* IIIA, to cause the precipitation feet under the line of subunit *Lp* IIIA. As shown in a further section, the fact that subunit *Lp* IIA is definitely a distinct polypeptide chain has important consequences for the (2 × 6)-mer heterogeneity.

Subunits *Lp* I through *Lp* V appeared chemically pure under the conditions of automatic Edman degradation. Though this result does not rule out the possibility that the fractions denoted by superscript primes by Brenowitz et al. (1981) are true polypeptide chains, this possibility becomes less probable in that they have exactly the same first 22 amino acids. When subunit *Lp* V-VI was submitted to the sequencing procedure, a single amino acid was found in all positions, except in positions 1, 2, 4, 15, and 17. In all the positions where a single amino acid was found, this amino acid was the same as in subunit *Lp* V. In the five positions where two amino acids were characterized, one of them was always the same as in subunit *Lp* V, exactly as expected for a *Lp* V-VI dimer or for a mixture of subunits *Lp* V and *Lp* VI. These results explain how the N-terminal sequence of subunit *Lp* VI was deduced from a comparison of the sequences of subunits *Lp* V and *Lp* V-VI, even though subunit *Lp* VI has not yet been isolated in a chemically pure form.

Table II: Frequency of Invariant Residues between Positions −2 and 20 in the Subunits of *Limulus polyphemus* Hemocyanin

subunit	*Lp* I	*Lp* II	*Lp* IIA	*Lp* IIIA	*Lp* IIIB	*Lp* IV	*Lp* V	*Lp* VI
Lp I		36.4	63.6	54.5	50.0	63.6	31.8	27.3
Lp II	36.4		40.9	40.9	31.8	50.0	36.4	40.9
Lp IIA	63.6	40.9		81.8	45.5	68.2	45.5	40.9
Lp IIIA	54.5	40.9	81.8		45.5	68.2	45.5	40.9
Lp IIIB	50.0	31.8	45.5	45.5		54.5	27.3	31.8
Lp IV	63.6	50.0	68.2	68.2	54.5		40.9	40.9
Lp V	31.8	36.4	45.5	45.5	27.3	40.9		77.3
Lp VI	27.3	40.9	40.9	40.9	31.8	40.9	77.3	

Table I also shows that all the constituting subunits of *L. polyphemus* hemocyanin are strongly structurally related. For example, subunits *Lp* I through *Lp* IV begin with a threonine and subunits *Lp* V and VI with a valine followed by a leucine. All the subunits possess a lysine in position 5, a glutamine in position 6, and a leucine in position 12. Positions 9, 10, 12, and 13 are all occupied by hydrophobic residues. Position 16 contains a leucine in subunits *Lp* I through *Lp* IV and an alanine in subunits *Lp* V and VI. Position 17 contains a hydroxy amino acid in all the subunits except *Lp* IIIB. The percentage of invariant residues between positions −2 and 20 is given in Table II. The greatest degrees of homology are between subunits *Lp* IIA and *Lp* IIIA and between subunits *Lp* V and *Lp* VI and the least are between subunits *Lp* I and *Lp* VI and between subunits *Lp* IIIB and *Lp* V. As we shall discuss in another section, although these frequencies were obtained on about 3% of the total sequences, they are in good agreement with immunological cross-reactivities determined on the whole subunits (Lamy et al., 1983b).

Table III: Number of Copies of the Various Subunits in Dissociated *Limulus polyphemus* Hemocyanin Determined by Ion-Exchange Chromatography and Rocket Immunoelectrophoresis

subunit	M_r[a]	ion-exchange chromatography: calculated from Lamy et al. (1979b)[b]	ion-exchange chromatography: calculated from Bijlholt et al. (1979)[b]	ion-exchange chromatography: calculated from Brenowitz (1982)	rocket immunoelectrophoresis
Lp I	68 000	5.6	4.3	6.4	5.3
Lp II	67 333	10.2	10.9	9.8 } 10.6	7.8 } 9.3
Lp IIA	67 000			0.8 }	1.5 }
Lp IIIA	71 000	15.6	16.2	7.7 } 15.1	7.2 } 15.8
Lp IIIB	65 000			7.4 }	8.6 }
Lp IV	65 000	8.1	7.8	8.4	7.8
Lp V	65 000	8.5	8.7	7.6	5.1 } 9.7
Lp VI	59 000				4.6 }

[a] M_r values are from Brenowitz et al. (1981). [b] Calculated from elution profiles assuming equal extinction coefficient at 340 nm for all the subunits.

Determination of the Number of Copies of the Various Subunits in Dissociated Hemocyanin. The knowledge of the number of copies of the various subunits is of prime importance (a) to determine the presence or absence of heterogeneity within the population of (8 × 6)-meric whole molecules, (4 × 6)-meric molecules, or (2 × 6)-mers and (b) to establish the quaternary structure. Two methods may be employed for this purpose, a densitometry of the various chromatographic or electrophoretic elution peaks and a quantitative immunoassay such as rocket immunoelectrophoresis.

Table III shows the results of both methods. Ion-exchange chromatography elution profiles on DEAE-Sepharose previously published (Lamy et al., 1979b) were used to calculate the number of copies of the various subunits by assuming that the extinction coefficients of all the subunits are identical at 340 nm. However, the five chromatographic zones of ion-exchange chromatography were heterogeneous so that the peaks of subunit *Lp* II and *Lp* IIA overlapped as well as those of subunits *Lp* IIIA and *Lp* IIIB or of subunits Lp V and *Lp* V-VI. Table III also shows the number of copies calculated from analogous elution curves published by Bijlholt et al. (1979) and Brenowitz (1982). Except for subunit *Lp* I, the agreement between the three sets of results is good. The number of copies of the eight subunits determined by rocket immunoelectrophoresis was also in acceptable agreement with the results of ion-exchange chromatography. However, in the case of subunits *Lp* V and VI, the number of copies determined by rocket immunoelectrophoresis may be overestimated. Actually, the *Lp* VI specific antibody preparation, though perfectly specific in crossed immunoelectrophoresis, still produced soluble immuno complexes with subunit *Lp* V. The lack of pure subunit *Lp* VI prevented testing the cross-reactivity of the *Lp* V specific antibodies with subunit *Lp* VI, the dimeric subunit *Lp* V-VI being useless under these circumstances.

In contrast to previously reported results with *A. australis* hemocyanin (Lamy et al., 1981a), the subunit composition of *L. polyphemus* hemocyanin obtained by rocket immunoelectrophoresis was not compatible with the existence of a single population of (2 × 6)-mers. This possibility was ruled out by the number of copies of subunit *Lp* IIA (1.5/48). Furthermore, except for subunits *Lp* II and *Lp* IV which were found in approximately eight copies per (8 × 6)-mer, the proportions of most of the other subunits were not stoichiometric. These results will be discussed in detail in a further section.

Topological Localization of the Subunits in L. polyphemus Hemocyanin. After the quaternary structure of *A. australis* hemocyanin was solved by labeling the whole molecule with subunit-specific Fab fragments, we expected that a repetition of this experiment with half-molecules of *L. polyphemus* hemocyanin would give similar results. Unfortunately, when Fab fragments were bound, the (4 × 6)-mers underwent strong structural changes, leading to a complete dissociation of the molecules (Billiald et al., 1983) so that in most cases the few stable immuno complexes obtained were unhelpful in localizing the subunits. To prevent a deformation or a dissociation of the molecules, whole molecules and half-molecules were cross-linked by DMS. This bifunctional agent bridges amino groups exposed on the external surface of the oligomer, thus freezing the molecule in its native form. As shown in the electron micrographs of Figure 2, cross-linked hemocyanins retained their architecture under stabilizing (Figure 2b) as well as under dissociating conditions (Figure 2d). Notice that the various views of cross-linked hemocyanins shown in the galleries of Figure 2b,d are respectively perfectly similar to the corresponding views of native hemocyanin (Figure 2a). However, if architecture preservation were a prerequisite, it would also be necessary that cross-linked molecules retain their ability to bind antibodies. The fact that they do retain this ability, is demonstrated by the single precipitation peak, shown in Figure 3b, produced by (8 × 6)-meric cross-linked hemo-

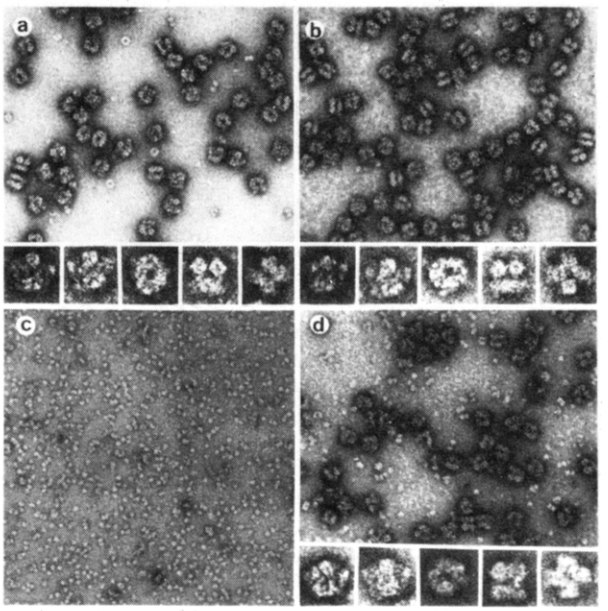

FIGURE 2: Electron micrographs of native and cross-linked hemocyanin from *Limulus polyphemus*. Native (a and c) and cross-linked (b and d) hemocyanins were dialyzed overnight in the presence of 0.05 M Tris-HCl buffer, pH 7.5, containing 10 mM $CaCl_2$ (a and b) and 10 mM EDTA (c and d). From left to right, the views shown in the galleries are termed symmetric pentagon, asymmetric pentagon, ring, bow tie, and cross.

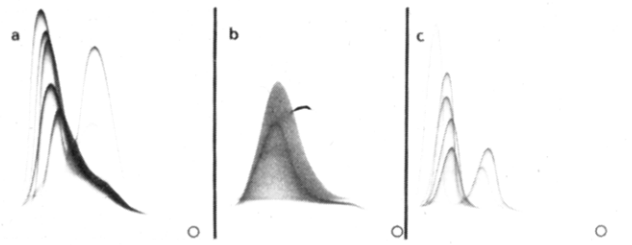

FIGURE 3: Crossed immunoelectrophoreses of *Limulus polyphemus* hemocyanin using an antiserum raised against dissociated hemocyanin (anti-*Lp* Hc antiserum). (a) Native hemocyanin; (b) cross-linked hemocyanin; (c) dissociated hemocyanin.

FIGURE 4: Immunolabeling of half-molecule of *Limulus polyphemus* hemocyanin by Fab fragments specific for the various subunits. Galleries A–G, respectively, correspond to subunits *Lp* I, II, IIA, IIIA, IV, V-VI. In rows A, B, E, F, and G, hemocyanin was cross-linked by DMS, prior to labeling. The bar is 25 nm.

cyanin against an anti-*Lp* Hc antiserum under the conditions of crossed immunoelectrophoresis.

(*1*) *Subunit Localization in (4 × 6)-meric Half-Molecules.* The immunoelectron microscopy Fab labeling procedure of Lamy et al. (1981b) was carried out by using native or cross-linked (4 × 6)-mers and subunit-specific Fab fragments. The results of this experiment are shown on Figure 4 and summarized in Table IV. Table IV also shows which subunits occupy the corresponding positions in *A. australis* hemocyanin.

All the immuno complexes except those shown in Figure 4C,D were produced by Fab fragments bound to cross-linked (4 × 6)-mers. However, no anti-*Lp* IIA or anti-*Lp* IIIA Fab fragments pointing out the outline of any of the three views of cross-linked (4 × 6)-mers could be observed, although a large range of Fab/hemocyanin ratios were investigated. A likely explanation would be that the cross-linking procedure destroyed the specific antigenic determinants of these subunit. Indeed subunits *Lp* IIA and *Lp* IIIA being strongly antigenically related, most of the antibody molecules present in each subunit-specific antibody preparation were removed during the purification. The few antibodies present in the preparation may therefore have been prevented from binding their determinants, either because of steric hindrance or of destruction of the determinant by the cross-linking procedure. Because cross-linked hemocyanins were useless here, the experiment was repeated with native (4 × 6)-mers, and the complexes were observed at various times after mixing Fabs fragments and hemocyanin. Though very few immuno complexes could be observed under these conditions, they unequivocally indicate the intramolecular location of subunits *Lp* IIA and *Lp* IIIA (Figure 4C,D). Furthermore, these locations are supported by the presence of many incomplete molecules of the type shown on the right part of Figure 4C.

Table IV: Topology of the Various Subunits in *Limulus polyphemus* (4 × 6)-mer Hemocyanin[a]

subunits	location	subunit located in the same position in *A. australis* hemocyanin
Lp I	corners in the top view	*Aa* 6
Lp II	middle of the lateral edges in the top view	*Aa* 3A + *Aa* 3B
Lp IIA	corner and/or end of (2 × 6)-mers in the top view	*Aa* 6
Lp IIIA	middle of the end of the (2 × 6)-mers in the top view	*Aa* 4
Lp IIIB	end of the (2 × 6)-mer in the top view in the immediate neighborhood of the cleft	*Aa* 2
Lp IV	near the top/bottom edges in the side view	*Aa* 5A
Lp V + VI	interdodecamer bridge area	*Aa* 3C, *Aa* 5B

[a] For the designation of the various parts of the EM views, see Figure 5.

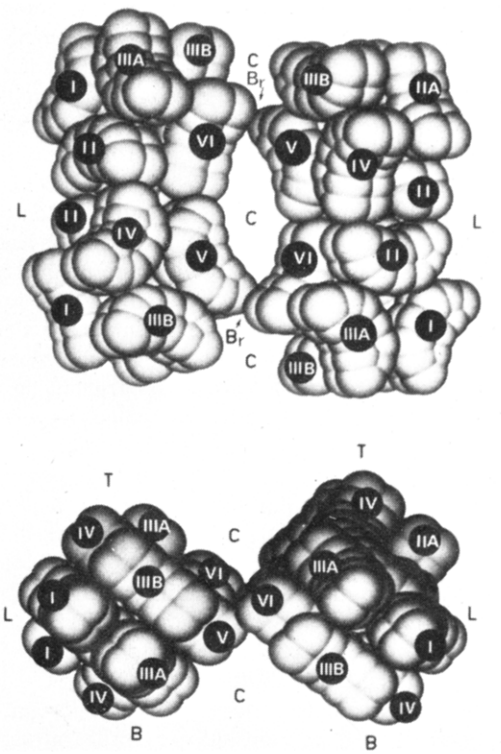

FIGURE 5: Model of quaternary structure of half-molecule of *Limulus polyphemus* hemocyanin, drawn by computer graphical techniques: (top) top view; (bottom) side view. C = cleft; T = top edge; B = bottom edge; L = lateral edge; Br = bridge. The architecture of this model is composed of two copies of the left enantiomorphic (2 × 6)-mer (Lamy et al., 1982). The rocking axis is oriented toward the upper left in the top view and from the front right hexamer toward the back left hexamer in the side view. The face seen in the top view is the flip face.

A similar complication occurred with *Lp* V and *Lp* VI specific antibody preparations. Indeed, as described (see Antibodies) due to the lack of pure subunit *Lp* VI required for the immunoadsorption step, each antibody preparation was still capable of forming soluble immuno complexes with the other subunit. Therefore, both preparations were considered as anti-*Lp* V/VI antibodies in immunolabeling experiments, and in fact, they produced the same type of immuno complexes (Figure 4G).

The model, shown in Figure 5, composed of two copies of the left dodecameric enantiomorph (Lamy et al., 1982), pro-

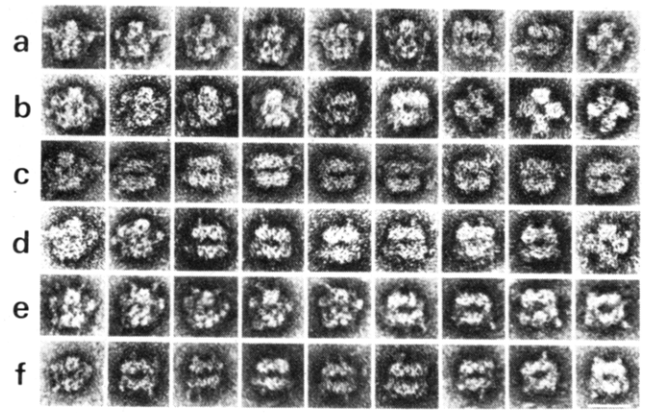

FIGURE 6: Immunolabeling of whole *Limulus polyphemus* hemocyanin by Fab fragments specific for various subunits. Galleries a–f, respectively, show molecules labeled by Fab specific for subunits *Lp* I, II, IIA, IIIA, IIIB, and IV. All the molecules were cross-linked by DMS prior to the labeling. The bar is 25 nm.

vides an interpretation to the location of the subunits in the (4 × 6)-mer. The model also supposes that the number of copies of all the subunits are integers with one copy of subunit *Lp* IIA, two copies each of subunits *Lp* V and VI, three copies of subunit *Lp* I, and four copies each of subunits *Lp* II, IIIA, IIIB, and IV in a half-molecule. The same locations have been assigned to subunits *Lp* I and *Lp* IIA, essentially because the sum of their number of copies reaches 4, but the labeling of subunit *Lp* IIA was also in agreement with a location in place of subunit *Lp* IIIA. The changes in intramolecular location that could be expected in other models, for example, in a model built from right enantiomorphs of the dodecamer, are discussed below.

(*2*) *Subunit Localization in the Whole Molecule.* In cross-linked (8 × 6)-mer hemocyanin with subunit *Lp* I labeled, the Fab fragments were essentially seen on the outline of the pentagonal views. As shown in Figure 6a, all the Fab fragments are bound to the corners of the pentagons with several molecules bearing two labels. For example, the first molecule on the left is labeled on its left and right upper corners. Similarly, the sixth molecule is labeled on its upper left and on its lower right corners. Some cross views also bear Fab fragments on their external angles, a location in good agreement with that observed in the pentagonal views.

Subunit *Lp* II is clearly located in the middle of the right and left side of the pentagons (Figure 6b, views 1–4). This location is in perfect agreement with that observed in the half-molecule (Figure 4B). The positions of the Fab fragments bound to the ring view (Figure 6b, views 5 and 6) and to the cross view (Figure 6b, views 7–9) also support this location.

Anti-*Lp* IIA Fab fragments were also capable of binding to (8 × 6)-mers. The label was mainly visible on the ring and bow tie views (Figure 6c, views 2–9). However, these views were of poor help for the localization of subunit *Lp* IIA, as well as for other subunits, because of a lack of asymmetry. Figure 6c also shows one pentagonal view labeled on its upper right corner, a disposition in agreement with the location observed in the half-molecule, near the end of the dodecamer (Figure 4C).

The soluble immuno complexes shown in Figure 6d were produced by anti-*Lp* IIIA Fab fragments. The label is bound to the upper left corner in the pentagonal view, to the right and left, or both, top edges in the bow tie view, and to the external edge in the cross view. The structure of these complexes suggests that subunit *Lp* IIIA is located near the top and bottom edges in the half-molecule in the neighborhood

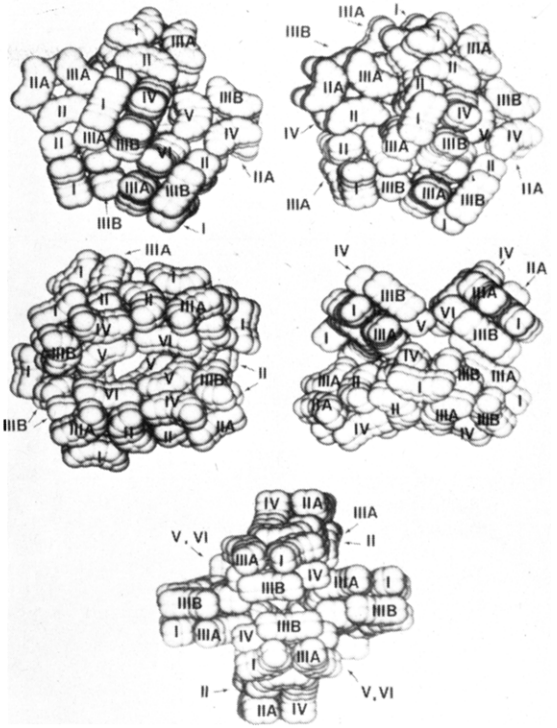

FIGURE 7: Model of quaternary structure of whole molecule of *Limulus polyphemus* hemocyanin, drawn by computer graphical techniques. Views are respectively designated (top left) symmetric pentagon, (top right) asymmetric pentagon, (middle left) ring, (middle right) bow tie, and (bottom) cross. Because of the mechanism of the negative stain, only the outline of the model can be directly compared to the immuno complexes shown in Figure 6.

of the dodecameric end. Once again, the agreement with the locations observed in Figure 4D is good, although Fab fragments bound to the upper left corner of the pentagonal view do not perfectly fit the model of Figure 7. The reason for this unique disagreement may be related to the enantiomorph conflict (see Architecture of Native Hemocyanin).

As shown in Figure 6d, anti-*Lp* IIIB Fab fragments are seen in the pentagonal view on the right and left upper sides close to the upper central corner and in the bow tie view on the lower right corner of the lower rectangle. All these images are in agreement with the subunit topology of Figure 4 as well as with the architecture model of Figure 7.

Fab fragments bound to subunit *Lp* IV are visible in Figure 6f on the two top edges of the upper half-molecule and on the length of the rectangle near the lower corners. Once again this disposition is in good agreement with the location of subunit *Lp* IV in the half-molecule.

No anti-*Lp* V/VI Fab fragments could be observed in any of the cross-linked (8 × 6)-mer views despite the fact that in the model these subunits are located near the outline in the cross and bow tie views.

The model of Figure 7 composed of two copies of the (4 × 6)-mer model of quaternary structure (Figure 5) shows the respective locations of the various subunits. A comparison with the EM views of Figure 6 shows that, except for subunit *Lp* IIIA, the results are consistent, provided it is remembered that the EM view is a hollow print in the stain corresponding to the bottom of the protein while the model is a three-dimensional structure viewed from the top. In other words, the face of the molecule in contact with the stain and the face observed in Figure 7 are not the same.

Assembly of Hybrid Oligomers from Isolated Subunits of Androctonus australis and Limulus polyphemus. (*1*) *Prin-*

Table V: Reconstruction of Hybrid Hemocyanins Using Highly Purified Subunits from *Limulus polyphemus* and *Androctonus australis*

subunit mixture[a]	higher oligomer obtained: (1 × 6)-mer	(2 × 6)-mer	(4 × 6)-mer	(8 × 6)-mer	aggregates higher than (8 × 6)	remarks[b]
complete *Lp*				+		
complete *Aa*			+			
complete *Lp*-*Lp* II	+					
complete *Aa*-*Aa* 3A, 3B						
complete *Lp*-*Lp* II + *Aa* 3A, 3B	+		?	?		
complete *Aa*-*Aa* 3A, 3B + *Lp* II			+			
Aa 3A, 3B						monomers only
Lp II	+					
complete *Lp*-*Lp* V-*Lp* V-VI	+					
complete *Aa*-*Aa* 3C-5B	+					
complete *Lp*-*Lp* V-*Lp* V-VI + *Aa* 3C-5B				+		
complete *Aa*-*Aa* 3C-5B + *Lp* V-VI			+			
Aa 3C-5B					2-D crystal	
Lp V-VI						dimer only
complete *Lp*-*Lp* IV			+		± regular	
complete *Aa*-*Aa* 5A	+					
complete *Lp*-*Lp* IV + *Aa* 5A			+			
complete *Aa*-*Aa* 5A + *Lp* IV			+			
Aa 5A						no oligomer
Lp IV	+					
complete *Lp*-*Lp* IIIA				+	high M_r irregular	
complete *Aa*-*Aa* 4			+			
complete *Lp*-*Lp* IIIA + *Aa* 4					3 × 6 to irregular	
Lp IIIA	+					
Aa 4	+					

[a] Subunits have the nomenclature of Lamy et al. (1979b). For example, subunit *Lp* II is equivalent to a mixture of subunits II + II′ + II″ according to Brenowitz et al. (1981). [b] Also see results of isolated subunits reassembly in the *L. polyphemus* system by Schutter et al. (1977), Bijlholt et al. (1979), and Brenowitz (1982) and in the *A. australis* system by Lamy et al. (1977a,b, 1980, 1981b).

ciple of the Method. The intramolecular location of the subunits in specific positions strongly suggests that the various subunits play special structural and/or functional roles. Architecturally speaking, critical subunits are engaged in inter(1 × 6)-mer, inter(2 × 6)-mer, and inter(4 × 6)-mer junctions. A detailed examination of Figures 5 and 7 shows that these roles are held by subunits *Lp* II, IIIA, IV, V, and VI. To clarify the structural roles of these strategic subunits, a series of hybrid oligomers composed of subunits belonging to *L. polyphemus* and *A. australis* hemocyanins was prepared. Since detailed information about quaternary structure and autoassembly of subunits of *L. polyphemus* (Bijlholt et al., 1979; Brenowitz, 1982) and of *A. australis* hemocyanins (Lamy et al., 1977a,b, 1980) is available, it was expected that subunits with identical intramolecular location in both species would be capable of replacing each other.

Various mixtures of isolated subunits were submitted to the two-step procedure of reassembly and were examined in the electron microscope. An important point is that all the subunits used in these experiments had been highly purified and that the traces of the omitted subunits were carefully removed from the various reassembly mixtures by binding them to their specific IgGs linked to protein A–Sepharose.

The results shown in Table V clarify and complete previous observations by van Bruggen et al. (1980).

(2) Intra(2 × 6)-mer Contacts. As shown in Figure 5, the subunits of each hexamer which may be involved in intradodecamer contacts are subunits *Lp* II, *Lp* V-VI, and *Lp* IV in the *L. polyphemus* system and *Aa* 3A, *Aa* 3B, *Aa* 3C-5B, and *Aa* 5A in the *A. australis* system.

Omission of subunit *Lp* II in the *L. polyphemus* system or of subunit *Aa* 3A and *Aa* 3B in the *A. australis* system limited aggregation to the hexameric level (Figure 8a). These observations confirm previous results of Bijlholt et al. (1979) and Lamy et al. (1981a) and support the hypothesis that subunits *Lp* II and *Aa* 3B play important specific roles in their respective dodecamers. Furthermore, Figure 8b shows that, in the *A. australis* system, subunit *Lp* II was capable of replacing subunits Aa 3A and *Aa* 3B. Finally, Figure 8c shows a gallery of selected views of cross-linked reassembled (4 × 6)-mers labeled with anti-*Lp* II Fab fragments. The subunit mixture which produced these molecules contained subunit *Lp* II and all the *A. australis* subunits except *Aa* 3A and *Aa* 3B. It is clear that subunit *Lp* II is located exactly in the position of subunits *Aa* 3A and *Aa* 3B or both, in the middle of the lateral edges. This is the first *direct* demonstration that in a reassembled oligomer a subunit actually replaces the subunit which it is supposed to do. Table V also shows that, unexpectedly,

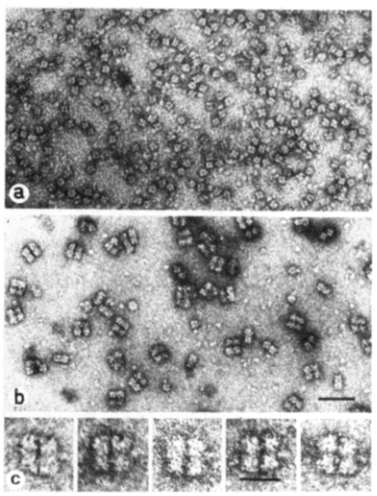

FIGURE 8: Demonstration of the role of subunit *Lp* II in the reassembly of hybrid oligomers from *A. australis* and *L. polyphemus* subunits. (a) Complete mixture of *A. australis* subunits with *Aa* 3A and *3B* omitted. (b) Same mixture plus subunit *Lp* II. (c) Selected views from the same mixture as in (b) submitted to the cross-linking procedure and then labeled with *Lp* II specific Fab fragments. The bar is 50 nm in (a) and (b) and 25 nm in Figure (c).

subunits *Aa* 3A and *Aa* 3B or both could not play in the *L. polyphemus* system the same role as subunit *Lp* II in the *A. australis* system.

Omission of the dimeric subunits *Lp* V-VI and *Aa* 3C-5B also limited the self-assembly to the hexameric level in both systems. This behavior demonstrates that the dimeric subunits play some critical role in the cohesion of the (2 × 6)-mers.

The case of subunits *Lp* IV and *Aa* 5A is less clear. While in the *A. australis* system the assembly of oligomers higher than hexamers required the presence of subunit *Aa* 5A, in the *L. polyphemus* system subunit *Lp* IV was not necessary for (4 × 6)-mer assembly. However, it is highly relevant that subunits *Lp* IV and *Aa* 5A could be interchanged to make up (4 × 6)-mers in the *A. australis* system.

(*3*) *Interdodecamer Contacts in (4 × 6)-mer Molecules.* As described above, the subunits involved in interdodecamer junctions are exclusively the dimeric subunits *Lp* V-VI and *Aa* 3C-5B. This double role of the dimeric subunits in the (2 × 6)-mer and (4 × 6)-mer assembly is in perfect agreement with the reassembly experiment of Table V. Indeed, on the one hand, omission of the dimeric subunit prevents the formation of (4 × 6)-mers and of (2 × 6)-mers. On the other hand, the dimeric subunits of both species are perfectly interchangeable, leading to hybrid (4 × 6)-mers in both systems.

(*4*) *Inter(4 × 6)-mer Contacts in (8 × 6)-mer Molecules.* Subunits involved in the inter(4 × 6)-mer contacts, deduced from the subunit topology, are subunits Lp IV and *Lp* IIIA (Lamy et al., 1982). The role of subunit *Lp* IV in the (4 × 6)-mer-to-(8 × 6)-mer association, originally suggested by Schutter et al. (1977), was confirmed by Bijlholt et al. (1979) and van Bruggen et al. (1980). As shown in Table V, it is especially interesting that subunit *Aa* 5A was incompetent to promote a (8 × 6)-mer autoassembly while it was fully competent in (4 × 6)-mer reconstruction. This behavior is in agreement with an absence of (8 × 6)-mer from *A. australis* hemocyanin and with the suggestion of Lamy et al. (1983b) that the ability of subunit *Lp* IV to promote (8 × 6)-mer assembly appeared in the merostome ramification.

The role of subunit *Lp* IIIA in (8 × 6)-mer assembly is less clear. While subunit *Lp* IIIA seemed to limit the aggregation at the (8 × 6)-mer level, omission of subunit *Aa* 4 did not change the aggregation level in the *A. australis* system. On the other hand, subunit *Aa* 4 was not capable of replacing subunit *Lp* IIIA in the *L. polyphemus* system.

Discussion

Heterogeneity of Native Hemocyanin. L. polyphemus hemocyanin, a heteropolymer with 48 subunits and a molecular weight of more than 3 MDa, is certainly one of the more complex proteins. Though great progress has been made in subunit characterization, isolation, and intramolecular location, the question of heterogeneity of the native population of (8 × 6)-mers is still unresolved. Usually, a population of molecules is considered as homogeneous when all available tests fail to demonstrate heterogeneity. In the case of *L. polyphemus* hemocyanin, all chromatographic, electrophoretic, and isoelectrofocusing attempts to show the existence of several subpopulations of (8 × 6)-mers have, thus far, been unsuccessful.

Heterogeneity at the level of the (2 × 6)-meric dissociation products of *L. polyphemus* hemocyanin is now clear. The best argument for heterogeneity is certainly the nonstoichiometric proportions of the subunits. For example, the number of copies of the less abundant polypeptide chain, namely, subunit *Lp* IIA, was found to be 1.5 by rocket immunoelectrophoresis (this work) and 0.8 by densitometry of ion-exchange chromatography (Brenowitz, 1982). Though the agreement between the two methods is not perfect, it nevertheless appears that subunit *Lp* IIA can hardly exceed two copies in the (8 × 6)-mer. This amount implies the existence of at least two different (2 × 6)-mers, one with and one without subunit *Lp* IIA. Such a result is not formally in conflict with the hypothesis of homogeneous populations of whole molecules. Indeed, there are two and only two ways for homogeneous populations of (8 × 6)-mers to contain two copies of subunit *Lp* IIA. The first way is to be composed of two identical (4 × 6)-mers, each containing one dodecamer with and one without subunit *Lp* IIA. The second way is to contain one dodecamer with two copies of subunit *Lp* IIA and another one devoid of subunit *Lp* IIA. If the (8 × 6)-mer contains subunit *Lp* IIA in only one copy, there must be different (4 × 6)-mers. All of the other possibilities require the existence of several subpopulations of (8 × 6)-mers containing one or two copies of subunit Lp IIA.

With respect to an eventual heterogeneity of the (8 × 6)-mer population of molecules, an important point is the determination of the subunit preparations used as standards in rocket immunoelectrophoreses. The best method would certainly be a spectrophotometric assay at 340 or 280 nm. Unfortunately, though all the subunits, but subunit *Lp* VI, are now available in an immunologically and chemically pure form, their extinction coefficients have not yet been definitely established. Therefore, the only correct solution is presently to use a method independent of the spectral properties of hemocyanin, such as the biuret reaction or the absorbance at 210 nm, two methods generally considered as more or less specific for the peptide bond. Furthermore, in order to reduce the differences of behavior of the standard or the sample with the reagent, the standard must be as chemically related as possible to the sample. From this point of view, the mixture of monomeric and dimeric subunits present in dissociated hemocyanin is certainly the best possible standard for the calibration of individual subunits.

The proportion of subunit *Lp* I in dissociated hemocyanin, like that of subunit *Lp* IIA, is not compatible with the existence of a simple type of dodecamer. Actually, an average number of five or six copies cannot fulfill the requirement of a multiple of four copies for homogeneous populations of (2 × 6)-mers.

The average proportions found for the other subunits were not incompatible with a homogeneous population of (8 × 6)-mers; however, the difference between the observed and the theoretical numbers of copies may be due to experimental imprecision.

The line of discussion assumes that *L. polyphemus* hemocyanin is composed of only eight different polypeptide chains. The chromatographically separable but antigenically identical subunits such as *Lp* I, I′ and I″ (Brenowitz, 1982) are regarded as a single kind of subunit. The justification for this simplification is that these antigenically equivalent subunits were also found to be equivalent in terms of their oxygen binding, reassembly properties and N-terminal amino acid sequences (this paper). In the absence of this simplification concerning the subunits denoted by primes, we must, of course, regard the *Limulus* (8 × 6)-mers as a heterogeneous population.

Resemblances and Differences between the Subunits. The study of Tables I and II completes and clarifies recent results of Lamy et al. (1983b) on the immunological relatedness of the subunits of *L. polyphemus* hemocyanin. These authors found that some subunits were antigenically related and others were not. Subunits *Lp* V and VI formed a very homogeneous group, very distant from all the other subunits. A second group

containing subunits *Lp* I, IIA, and IIIA was also very homogeneous, although subunit *Lp* IIA resembled subunit *Lp* IIIA more than subunit *Lp* I. This group and a third group composed of subunits *Lp* IIIB and *Lp* IV, two related subunits, were very similar. Finally, subunit *Lp* II was distant from the other three groups. A comparison of these results with the frequency of homologies shown in Tables I and II reveals strong similarities. Thus, the N-terminal sequences of subunits Lp V and VI are remarkably similar to one another and different from those of all the other subunits. The sequence of subunit *Lp* II was not strongly related to any of the other subunits. The N-terminal sequences of subunits *Lp* IIA and IIIA had the highest content of invariant residues while in the group composed of subunits *Lp* I, IIA, IIIA, IIIB, and IV, the lower rate of homologies was 50.1% (45.5 × 24/22).

The results are in good agreement with those of immunological relatedness, although they were calculated on only 22 residues. This agreement encourages us to predict that the still unknown part of the sequences will exhibit comparable frequencies of homologies with the N-terminal fragments and reinforce the evolutionary conclusions of Lamy et al. (1983b) drawn from immunological relatedness between the subunits.

Architecture of Native Hemocyanin. Our knowledge of the architecture of *L. polyphemus* hemocyanin essentially comes from electron microscopy. Half-molecules produce three views respectively termed top view, side view, and 45° view. The 45° view, recently identified by Van Heel et al. (1983), is named after the orientation of the general plane of the molecule with respect to the carbon film plane. The (8 × 6)-meric whole molecules lead to five views called symmetric pentagon, asymmetric pentagon, ring, bow tie, and cross (Lamy et al., 1982).

Starting from all these views, except the 45° view which was not yet recognized, and from additional information such as the orientation of the rocking axis to the upper left (Van Heel & Frank, 1981) when the molecule is seen lying on the carbon film, the kidney shape of the subunits deduced from X-ray crystallography (Magnus, 1980; van Schaick et al., 1981; Fearon et al., 1983), and a correspondence-analysis, image processing of the pentagonal views, Lamy et al. (1982) proposed a model of (8 × 6)-mer architecture. This model, used in Figure 5, is composed of four copies of the left dodecameric enantiomorph, a molecule resulting from the superposition of two hexamers followed by a 90° rotation of the upper hexamer to the left. However, the model is in conflict with the recently reported data of Van Heel et al. (1983) on the 45° view. On the other hand, the EM data of Van Heel et al. being inconsistent with their crystallographic data, the only reasonable conclusion is that additional experiments are required to solve the enantiomorph controversy.

Since the choice of the proper (2 × 6)-mer enantiomorph is controversial, this raises the important problem of the interpretation of the immunolabeling experiments shown in Figures 4 and 6. Compared with the model of (4 × 6)-mer, shown in Figure 5, the subunit topology, in a model composed of right (instead of left) dodecameric enantiomorphs, would not change with respect to the contour line and the rocking axis, but the orientation of the subunits toward the flip and flop faces would be inverted.

A (8 × 6)-mer model built from the right enantiomorph could be substantially different from the model shown in Figure 7: however, the five main views would be similar, and the topology of the subunits with respect to the outline would not dramatically change. The subunits engaged in contacts responsible for the cohesion of the (2 × 6)-, (4 × 6)- and (8 × 6)-mers would also be unchanged. For example, subunits *Lp* V and VI would still be involved in the (2 × 6)-mer-to-(4 × 6)-mer transition and subunits *Lp* IV and IIIA in the (4 × 6)-mer-to-(8 × 6)-mer transition.

Evolutionary Preservation of the Subunits. There is little doubt that all the arthropodan hemocyanins descended from a unique ancestral polypeptide chain. According to a recent theory, the ancestral subunit may have evolved from an ubiquitous oxygen-binding copper protein such as tyrosinase through a series of gene rearrangements around the original copper-containing active site (Van Holde & Miller, 1982). The presently available data also indicate that some subunits evolved rapidly while others were strongly preserved. Usually the recognition of an evolutionary preservation implies the identity of sequence homology or antigenic relatedness. In the case of hemocyanin, all the subunits are more or less structurally similar. Their common phylogenic origin could make difficult the discrimination between vestigial and preserved homologies; however, the task is considerably simplified if the intramolecular location and the role in reassembly are considered.

For example, it is now perfectly clear that the dimeric subunits in the chelicerate hemocyanin have been highly preserved. Actually, in addition to the antigenic and N-terminal sequence homologies, they occupy the same intramolecular location in *A. australis* and in *L. polyphemus* and are perfectly interchangeable in reassembly experiments. Their preservation clearly results from their intramolecular location in a critical position for the (2 × 6)-mer and (4 × 6)-mer cohesion.

There is little doubt that subunits *Lp* IV and *Aa* 5A have been preserved by evolution. Indeed they are strongly antigenically related, they occupy the same intramolecular positions, and they are capable of replacing each other in (4 × 6)-mer reassembly. However, it is not clear why subunit *Aa* 5A is critical for (2 × 6)-mer reassembly while subunit *Lp* IV is not, nor why subunit *Lp* IV is involved in the calcium-dependent (4 × 6)-mer-to-(8 × 6)-mer transition while subunit *Aa* 5A is not. Therefore, it is possible that the reason for the evolutionary preservation of these subunits is that they confer some critical functional advantage. With respect to that hypothesis, Brenowitz (1982) recently established that homohexamers of subunit *Lp* IV reassembled in the presence of calcium exhibit a cooperative oxygen binding. Unfortunately, this behavior could not be confirmed or inferred in *Aa* 5A because this subunit does not produce hexamers under the same conditions.

It could be expected, due to their important position at the interhexamer junction area, that subunits *Lp* II and *Aa* 3A/3B would have been preserved by evolution. In fact these subunits exhibit many common features. Specifically, the N-terminal sequence of subunit *Lp* II exhibits a higher frequency of homologies with subunit *Aa* 3B than with any other subunit from *L. polyphemus* and *A. australis* hemocyanin [57% of homologies in the first 14 residues after Jollès et al. (1979)]. Omission of both of them from the reassembly mixture limits the aggregation at the hexameric level, and subunit *Lp* II can replace subunits *Aa* 3A/3B. However, they are antigenically distant from each other (Lamy et al., 1983b), and subunit *Aa* 3A/3B cannot replace subunit *Lp* II. Therefore, it seems that the evolutionary preservation was only partial. This hypothesis is also supported by the fact that the positions occupied by subunit *Lp* II in *L. polyphemus* and by subunit *Ec* a in *E. californicum* (Markl et al., 1981) are occupied by two different polypeptide chains in *A. australis* hemocyanin (*Aa* 3A and

3B). The fact that a single subunit occupying two positions could be replaced in the scorpion hemocyanin by two subunits each occupying a single position means that the structure of the ancestor had not been completely frozen by structural or functional constraints.

The sequence homologies and antigenic relatedness observed in other subunits occupying similar positions in both hemocyanins are also indicative of an evolutionary preservation. However, since most of them are capable of replacing each other, it is more difficult to decide whether the observed homologies are the expression of vestigial or of preserved structures.

Conclusion

The intramolecular localization of the subunits in the whole *L. polyphemus* hemocyanin molecule allows us to interpret in terms of evolution the observed N-terminal sequence homologies as well as the antigenic identities between isolated subunits. The method has become so precise that we can make predictions on the intramolecular location of the subunits in new hemocyanins. For example, the observed structural homologies between the subunits of *L. polyphemus* and *Tachypleus tridentatus* (Lamy et al., 1979b) allow us to predict that subunits *Tt* I, II, III, IV, V, VIA, and VIB [respectively renamed α, δ, γ, β, ϵ, ζ, and ζ' by Takagi & Nemoto (1980)] have the same respective locations as subunits *Lp* I, IIIA, IIIB, IV, II, V, and VI. In the future, the same method should help us to understand the evolution of both hemocyanin and arthropods.

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