THE ROLE OF STRUCTURALLY DIVERSE SUBUNITS IN THE ASSEMBLY OF THREE CHELICERATAN HEMOCYANINS

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Received 23 May 1980

1. Introduction

Hemocyanins are high molecular mass respiratory copper proteins occuring in many arthropods and molluscs [1]. With the discovery of subunit heterogeneity in arthropod hemocyanins has come the question of how the presence of different subunits may influence the assembly and the functional properties of these proteins [2]. Certain subunits play specific roles in the assembly of the higher ordered structures [3–8]. The generality of these studies was tested for Cheliceratan hemocyanins.

The results obtained lead to the conclusion that there are structural correspondences between subunits of the hemocyanins from the horseshoe crab *Limulus polyphemus* (eight-hexamer), the scorpion *Androctonus australis* (four-hexamer) and the tarantula *Eurypelma californicum* (four-hexamer). In all cases we can distinguish 'hexamer-formers' and 'linkers' needed to obtain the 'multi-hexameric' aggregates. It is even possible to form hybrid four-hexamers or a hybrid eight-hexamer from a mixture of subunits combining hexamer-formers from one species with a linker from another species. Electron microscopy and thin-layer gel chromatography were used to monitor the assembly processes.

2. Materials and methods

Hemocyanin and purified subunit fractions from the three Cheliceratan species *L. polyphemus*, *A. australis* and *E. californicum* were isolated and prepared as in [2–4,9,10].

Reassembly of structures more complicated than monomers (6 monomers form 1 hexamer) was carried out using a single-step and/or two-step procedure. The single-step procedure involves dialysis of subunits, or subunit mixtures for ~8 h against 50 mM Tris · HCl, 5 mM $CaCl_2$ and 10 mM $MgCl_2$ (pH 7.5). The two-step procedure consists of an 8 h dialysis against 50 mM Tris · HCl, 10 mM EDTA (pH 7.5) followed by a second period of dialysis against 50 mM Tris · HCl, 5 mM $CaCl_2$ and 10 mM $MgCl_2$ (pH 7.5).

Assembly processes were monitored by two techniques. The structures of the assembly products were analyzed by electron microscopy using negative staining methods as in [3]. The sizes of the assembly intermediates and products were studied by thin-layer gel chromatography [7]. In cases where hybrid oligomers were created, proof of their hybrid nature was obtained by isolation of the spots, followed by dissociation and slab-gel electrophoresis [9,11].

3. Results and discussion

Fig.1 generally illustrates the morphology of structures designated as monomers, hexamers, etc. This designation does not imply complete knowledge of the number or type of subunits present in the reconstituted molecules.

L. polyphemus hemocyanin (M_r 3.3 × 10^6) occurs as an eight-hexamer [3]. It can be dissociated via four-hexamers and two-hexamers into monomers [12]. Stable hexamers have not been observed. In the reassembly experiments reported here and in earlier studies it should be noted that *Limulus* fractions I and IV are pure monomers, II and III each contain two types of monomers (II and II_A, III_A and III_B) and V contains two components (V_A, a monomer, and V_B, a heterodimer) [13]. Hexameric structures can be formed by fractions II, III or IV [6]. Fraction V and possibly fraction II are needed to form 'structures larger than hexamers'. Absence of fraction III in a mixture of all other fractions results in irregular aggregation of 'hexamers'. Fraction IV and perhaps fraction II are needed to make 'eight-hexamers' from 'four-hexamers'. No structural role for fraction I has been found.

A. australis hemocyanin (M_r 1.7 × 10^6) is observed as a four-hexamer. The native molecule can be dissociated into 6 antigenically different monomeric subunit fractions designated as 2, 3A, 3B, 4, 5A, 6 and a very stable heterodimer (fraction 1). This heterodimer dissociates in the presence of 3 M urea into two other monomers called 3C and 5B [10]. The subunit fractions play specific roles in reassembly experiments. Only fraction 4 is able to form homohexamers. The monomeric fractions 2, 3B, 4, 5A, 6, but not fraction 1 or the monomeric fraction 3A, are easily incorporated into hexamers from binary and ternary mixtures like 2 + 4, 4 + 6, 2 + 6, 2 + 4 + 6 [7]. Without the heterodimer (fraction 1) and the monomeric fractions 3B and 5A it is impossible to assemble two-hexamer or four-hexamer-like structures [4].

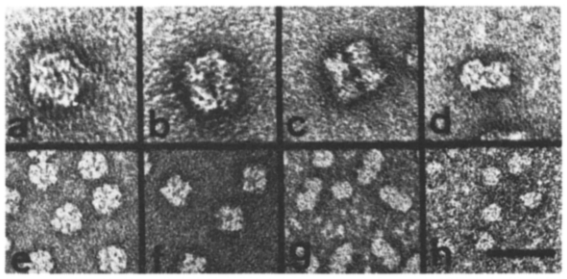

Fig.1. Morphology of structures designated as: eight-hexamer (a,b); four-hexamer (c); two-hexamer (d); hexamers (e,f); dimers (g); and monomers (h). The different hemocyanin molecules are negatively stained with uranyl acetate. The bar is 30 nm.

E. californicum hemocyanin (M_r 1.7 × 10^6) also occurs as a four-hexamer [14,15]. Alkaline dissociation yields four monomeric (1,2,3,4 M) and two dimeric (4_D,5) fractions. Fraction 4_D is a heterodimer which can be dissociated into monomers in 4 M urea. Fraction 5 is a dimer of fraction 3. Its dissociation is promoted by 5 mM cysteine, although intermolecular disulphide bridges are absent [9]. Further studies resolved fraction 2 into two monomeric fractions. Recent chemical and immunological studies have established that this hemocyanin contains 7 distinct polypeptide chains [11,16]. When unfractionated *Eurypelma* hemocyanin is dissociated and then dialyzed according to the single-step reassembly procedure, there is a high yield of reassembled four-hexamers. Dialysis of individual fractions against the same buffer shows that they are incapable of reforming native-like structures. If the monomer mixture is incubated in reassembly buffer, hexamers are obtained with a high yield. If, furthermore, all monomer fractions and fraction 4_D are mixed, reassembly proceeds to yield four-hexamers [8].

Thus within the three Cheliceratan hemocyanin systems we observe a considerable specificity in intersubunit interactions. While certain monomeric subunit fractions can self-assemble to form hexamers, other subunit fractions cannot. The assembly of structures larger than hexamers requires the presence of specific components which we have called linkers. The linkers of the three species are *Limulus* fraction V, *Androctonus* fraction I and *Eurypelma* fraction 4_D. These fractions share the property that they are commonly isolated in dimeric form.

The interaction between *Limulus, Androctonus* and *Eurypelma* hemocyanin subunit fractions were investigated by the two-step reassembly procedure. About 50 combinations were analyzed. The results of the most successful reassembly experiments are summarized in table 1. The morphologies of the hybrids are illustrated by the electron micrographs of fig.2.

Hybrid four-hexamers can be formed using mixtures of *Limulus* + *Androctonus, Limulus* + *Eurypelma* and *Androctonus* + *Eurypelma* hemocyanin fractions. In all cases the formation of hybrid four-hexamers

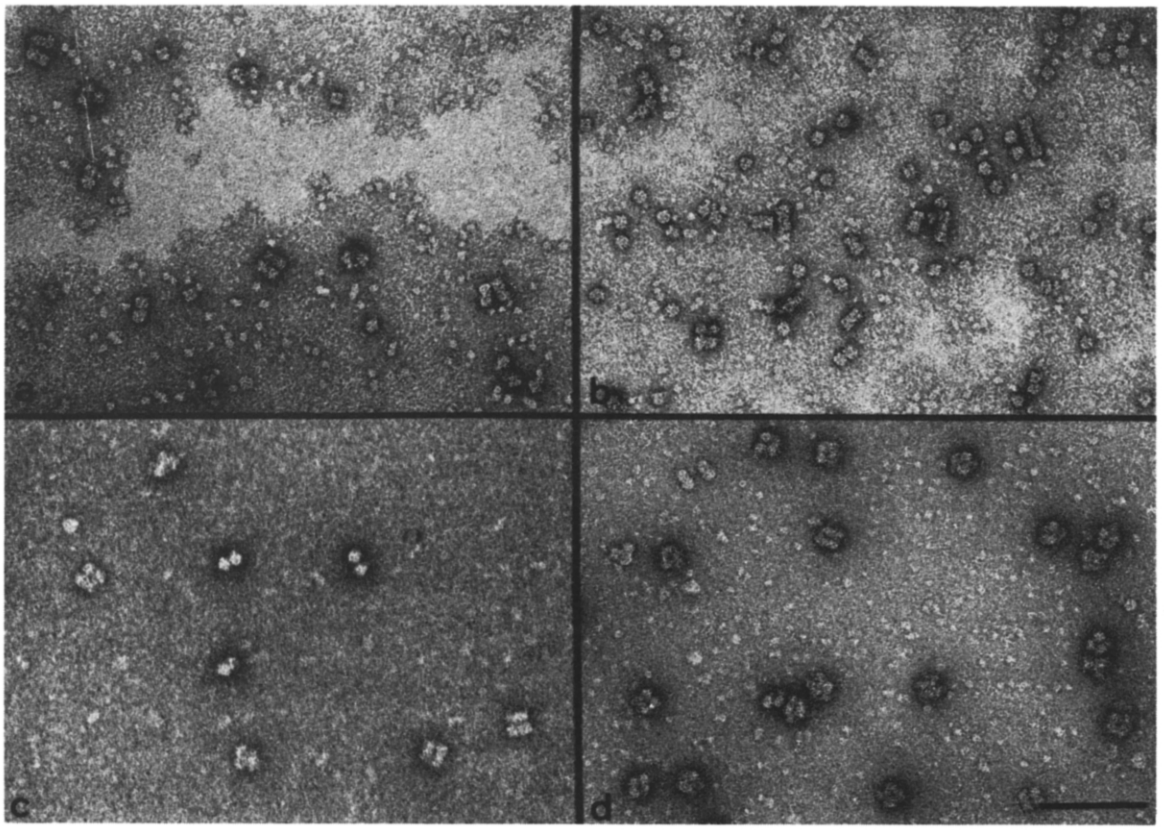

Fig.2. Morphology of some interCheliceratan hybrid hemocyanin molecules negatively stained with uranyl acetate: four-hexamers obtained from a combination of *Limulus* fraction V and *Androctonus* fraction 4 (a); *Androctonus* fraction 1 and *Limulus* fraction III (b); *Androctonus* fraction 1 and *Eurypelma* fraction 1 + 2 + 4 M (c). Eight-hexamers were obtained from *Androctonus* fraction 1 and *Limulus* fractions II + III + IV (d). The bar is 100 nm.

requires a combination of hexamer-formers from one species and a linker from another species. The latter are underlined in table 1.

After high molecular weight aggregates were formed from hexamer-formers and linkers, they were purified by thin-layer gel chromatography. The eluted spots were dissociated and then analyzed by gradient polyacrylamide slab-gel and immuno-electrophoresis. The reconstituted aggregates were found to contain the subunits which had been mixed for reassembly.

A hybrid eight-hexamer was prepared using *Limulus* fraction II + III + IV and *Androctonus* fraction I. The reassembly product is indistinguishable by electron microscopy from the native *Limulus* hemocyanin molecule. Clearly *Androctonus* fraction I is the linker needed to reach the four-hexamer state, while the presence of *Limulus* fraction IV permits the assembly of eight-hexamers. Chromatographic and electrophoretic analysis of this hybrid revealed that the isolated aggregate was indeed made up of both *Limulus* and *Androctonus* hemocyanin components.

In most cases electron microscopy visualized four-hexamers as the maximum attainable aggregate with two-hexamers and occasionally three-hexamers as assembly intermediates. Less distinct assembly products and misfit aggregates occur, depending on the composition of the mixture and on the reassembly procedure. The presence of Ca^{2+} and Mg^{2+} in the reassembly mixture generally promotes the formation of misfit structures. These misfits are apparent from electron microscopy and from flocculence and precipitates in the vials. We therefore used a two-step procedure in which calcium was absent in the first phase and present in the second. Also we noted that the formation of four-hexamers does not necessarily require the presence of divalent cations.

With the knowledge obtained from these pilot studies, it will be possible to make larger amounts of

Table 1
Ultimately obtainable reassembly products resulting from two-step dialysis of hemocyanin components and specific mixtures of components

Mixture				Products		
Limulus fraction	*Androctonus* fraction	*Eurypelma* fraction	Mono and/ or dimer	1 × 6	4 × 6	8 × 6
V			+			
	4			+		
V	4				Hybrid	
		1 + 2 + 4 M		+		
V		1 + 2 + 4 M			Hybrid	
	1				Ordered	
II				+	aggregate	
II	1				Hybrid	
III				+		
III	1				Hybrid	
	1	1 + 2 + 4 M			Hybrid	
		4D	+			
	4	4D			Hybrid	
II + III + IV				+		
II + III + IV	1					Hybrid

See text for details of assembly and analysis. Components which act as linkers and permit assembly of multi-hexamer aggregates are underlined

hybrid four-hexamers and eight-hexamers. Since the functional properties of the native molecules differ widely, it is anticipated that analysis of these hybrids will provide further information on the way in which the diverse subunits contribute to their functional properties in vivo.

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