**Limulus polyphemus** Hemocyanin: 10 Å Cryo-EM Structure, Sequence Analysis, Molecular Modelling and Rigid-body Fitting Reveal the Interfaces Between the Eight Hexamers

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The blue copper protein hemocyanin from the horseshoe crab ***Limulus polyphemus*** is among the largest respiratory proteins found in nature (3.5 MDa) and exhibits a highly cooperative oxygen binding. Its 48 subunits are arranged as eight hexamers (1×6mers) that form the native 8×6mer in a nested hierarchy of 2×6mers and 4×6mers. This quaternary structure is established by eight subunit types (termed I, IIA, II, IIIA, IIIB, IV, V, and VI), of which only type II has been sequenced. Crystal structures of the 1×6mer are available, but for the 8×6mer only a 40 Å 3D reconstruction exists. Consequently, the structural parameters of the 8×6mer are not firmly established, and the molecular interfaces between the eight hexamers are still to be defined. This, however, is crucial for understanding how allosteric transitions are mediated between the different levels of hierarchy. Here, we show the 10 Å structure (FSC₁/2-bit criterion) of the oxygenated 8×6mer from cryo-electron microscopy (cryo-EM) and single-particle analysis. Moreover, we show its molecular model as obtained by DNA sequencing of subunits II, IIIA, IV and VI, and molecular modelling and rigid-body fitting of all subunit types. Remarkably, the latter enabled us to improve the resolution of the cryo-EM structure from 11 Å to the final 10 Å. The 10 Å structure allows firm assessment of various structural parameters of the 8×6mer, the 4×6mer and the 2×6mer, and reveals a total of 46 inter-hexamer bridges. These group as 11 types of interface: four at the 2×6mer level (II–II, II–IV, V–VI, IV–VI), three form the 4×6mer (V–V, V–VI, VI–IIIB/IV–V), and four are required to assemble the 8×6mer (IIIA–IIIA, IIIA–IIIB, II–IV, IV–IV). The molecular model shows the amino acid residues involved, and reveals that several of the interfaces are intriguingly histidine-rich and likely to transfer allosteric signals between the different levels of the nested hierarchy.

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**Keywords:** hemocyanin; cryo-electron microscopy; 3D reconstruction; quaternary structure; amino acid sequence

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**Introduction**

arthropod hemocyanins are extracellular oxygen-transport proteins that exhibit a complex allosteric behaviour during oxygen binding, with Hill coefficients up to 9 in some Chelicerata, such as the horseshoe crab ***Limulus polyphemus***. The oxygen-binding site of hemocyanin is formed by two copper ions that are directly bound to the protein, causing the blue colour of blood in the oxygenated state.**
very different hemocyanin family occurs in the phylum Mollusca; however, these are not considered here.  

Arthropod hemocyanins are hexamers (1×6mers) or oligohexamers (n×6mers) of bean-shaped 75 kDa polypeptide subunits (~650 amino acid residues). The 3.2 Å resolution crystal structure of the deoxygenated 1×6mer of the spiny lobster (Panulirus interruptus) hemocyanin is known.  

The crystal structures of the deoxygenated and oxygenated subunit II of L. polyphemus hemocyanin became available at 2.4 Å resolution, and 1×6mers of subunit II have the same quaternary structure as the native 1×6mer of Panulirus hemocyanin.  

The subunit comprises three structural domains: The N-terminal domain 1 is rich in α-helices, the central domain 2 contains a four-helix bundle with the copper active site, and the C-terminal domain 3 is rich in β-sheets. Each structural domain plays a specific functional role. To build the hexamer, the six subunits are arranged as two trimers in D3 point-group symmetry. As deduced from sequence analysis of hemocyanin subunits from very distantly related arthropods, the secondary structure elements are highly conserved, and therefore, the published crystal structures are likely to hold true for arthropod hemocyanin subunits in general.

Some arthropods, notably spiny lobsters such as P. interruptus, possess a hemocyanin that is a single hexamer. Also, the various copper-free hemocyanin homologues (“storage proteins”) that occur in the hemolymph of many crustaceans and insects are single hexamers. Other arthropods, such as the brachyuran crab Carcinus maenas or the ctenid spider Cupiennius salei, possess 2×6mer hemocyanin. A tetrahedral 4×6mer hemocyanin occurs in thalassiid shrimps, and a different 4×6mer is typical for many arachnids, such as tarantulas and scorpions. A peculiar 6×6-mer hemocyanin has been detected in scutigeromorph centipedes. The largest arthropod hemocyanin known is the 8×6mer of horseshoe crabs, and has been particularly well studied in L. polyphemus. It shows cooperative oxygen binding and heterotropic allosteric regulation by protons, chloride ions and divalent cations, and its quaternary structure is stabilized by hydrophilic and polar forces. As deduced from immunoelectron microscopy and biochemical analyses, this 8×6mer consists of two arachnid-type 4×6mers assembled face-to-face.

Whereas 1×6mer hemocyanin can be built from a single subunit type, the 2×6mers require at least two subunit types, with the second type forming the inter-hexamer bridge. In the chelicerate 4×6mer hemocyanins, seven or eight different subunits are required; they follow a fixed stoichiometry and occupy defined positions within the molecule. This heterogeneity has been correlated with the specific oxygen-binding properties of the respiratory protein and as prerequisite for the assembly beyond the hexamer.  

In L. polyphemus hemocyanin, eight different subunit types are present with a different number of copies: six of type I; two of type II A; eight of types II, IIIA, IIIB, and IV; and four of types V and VI. Immunological correlations of these subunits with the subunit complements of the scorpion Androctonus australis and the tarantula Eugypelma californicum are available, and the topological position of each subunit type within the 4×6mers of these three hemocyanins is known from immunoelectron microscopy. That each subunit type indeed fulfils a specific role in the oligomeric architecture has been confirmed by reassembly and hybrid reassembly experiments.

Although a plethora of arthropod hemocyanin subunit sequences are known, the complete subunit set of a particular hemocyanin has been sequenced in only a few cases. Among the chelicerates, this is the case for the tarantula E. californicum, the golden orb web spider Nephila inaurata (with one type missing) and the ctenid spider Cupiennius salei.

Thus, many details of the primary and quaternary structure of arthropod hemocyanins are known. However, sequence data of several functionally well-studied hemocyanins are incomplete or lacking, and the exact mode of the higher-ordered assembly of hexamers remains obscure. With respect to the 4×6mer/8×6mer chelicerate hemocyanins, different image analysis procedures were used to solve the quaternary structure of Androctonus, Eurypelma and Limulus hemocyanins, starting from electron microscope images of negatively stained preparations. In a different approach, using small-angle X-ray scattering (SAXS), structural differences between the oxy and the deoxy state of Eurypelma hemocyanin have been claimed. A 3D reconstruction from cryo-electron microscope images was performed for the Androctonus 4×6mer, and the Limulus 8×6mer, but the resolution limit was about 40 Å in both cases. These approaches revealed various basic parameters of the 4×6mer and the 8×6mer, but a reliable molecular fitting of the crystal structure was not possible due to the low resolution obtained.

Previously, we presented a cryo-EM structure of Palinurus elastis (spiny lobster) hemocyanin below 10 Å, solved by single-particle analysis (8 Å according to the Fourier shell correlation (FSC) criterion; 10 Å according to the FSC criterion). A 3D reconstruction allowed a convincing fit with the crystal structure of Panulirus interruptus 1×6mer, and molecular modelling of some parts. This result encouraged us to study, by cryo-EM, the 8×6mer hemocyanin of L. polyphemus.

Results and Discussion

3D cryo-electron microscopy

A total of 58 focal pair cryo-electron micrographs (Figure 1) were recorded under low-dose conditions, and image analysis was performed as described in Materials and Methods. The resulting 3D reconstruction was calculated with 11,633 single particles...
averaged in 766 classes and achieved a resolution of 10.8 Å (FSC1/2-bit criterion) and 8.8 Å (FSC3σ criterion). This reconstruction (not shown) was used for the molecular fitting as described below and in Materials and Methods. The resulting molecular model of the whole 8×6mer was converted into a density map, low-pass filtered to a nominal 5 Å and used as a reference for the final refinement round, applying projection matching. The final cryo-EM structure was calculated with the whole data set (12,069 single particles) in 1140 class averages (~11 particles per class). Examples of these classes and their reprojections are presented in Figure 1.

As shown in Figure 2(a), the FSC curve crosses the FSC1/2-bit noise curve at 9.6 Å and the FSC3σ noise curve at 8.1 Å, whereas the FSC0.5 cut-off is at 12.2 Å. Assuming 0.844 Da/Å3, the threshold was set at 0.01, corresponding to a molecular mass of ~3.5×106 Da (48 subunits of 73,000 Da each). The resolution of the final cryo-EM structure of the 8×6mer is almost the same as in our previous 3D reconstruction of the native 1×6mer hemocyanin from P. elephas.39 Indeed, the eight hexamers are clearly defined and exhibit molecular detail; notably, the 48 individual subunits are discernible (Figure 3(a)–(c)). The handedness of the 1×6mer was confirmed by X-ray analysis of P. interruptus hemocyanin crystals;4 this allowed unambiguous assignment of the correct handedness to the cryo-EM density map of the 8×6mer.34

The topology of the eight subunit types of the Limulus hemocyanin 8×6mer is known.20 This allowed us to identify, in the present 10 Å cryo-EM structure, the individual subunit types and to label them with their correct designations (Figure 3(a′)–(c′)). In the case of subunit types I, II, IIIA, IIIB and IV that each occur in each hexamer as a single copy, this assignment was clearly defined. The sixth position in each hexamer is occupied by subunit V or subunit VI. These two subunits form a central heterodimeric bridge within the basic 2×6mer and a central tetrameric ring within the 4×6mer (see Figure 3(c′)). This leaves two possibilities for their orientation: that shown in Figure 3(c′), or the alternative one with the labels V and VI exchanged. In the case of subunit I, the picture is complicated because in two hexamers this subunit type is substituted for a structural variant termed subunit IIA;20 this subunit is incorporated in Figure 3(a)–(c) but is of no relevance for the present study, because within the Limulus 8×6mer, subunits I/IIA occupy the outer edges of the eight hexamers and are not involved in any inter-hexamer contact (see Table 2). The known subunit topology of the 8×6mer provided us with a firm basis for molecular fitting (see below).

**Architecture of the 8×6mer**

The architecture of the 8×6mer has been described.20,32–34,38,40 It consists of two identical...
4×6mers that correspond structurally to the native 4×6mer hemocyanins of some arachnids, such as *E. californicum* and *A. australis*. The 4×6mer is assembled from two identical 2×6mers (dodecamers) lying side by side in an antiparallel orientation, but shifted and skewed significantly with respect to each other. Within each 2×6mer, the two 1×6mers (hexamers) are rotated ∼105° with respect to each other; they differ slightly in their subunit composition (see Figure 3(a)). Because of the D2 point-group symmetry of the 8×6mer structure, a repeating 2×6mer is the basic building block of the 3D reconstruction. Some previously published parameters of 4×6mer and 8×6mer hemocyanins are summarized in Table 1, together with the values obtained in the present study.

The most detailed 3D reconstruction of an 8×6mer previously published has a resolution of ∼40 Å. Compared to this, the present 10 Å structure allows, with superior accuracy, the assignment of the different positions, shifts and rotations of the two hexamers within the basic 2×6mer, and of the four 2×6mers within the whole molecule. Measurements were performed by two different approaches. First, densities of 1×6mers, 2×6mers, and 4×6mers were extracted from the total density map, and volume alignments were calculated. Second, the generated and fitted models were aligned against each other. The measurements were assessed within the ResolveRT software package and yielded the following results (see also Table 1):

**The basic 1x6mer**

The hexamer is composed of two staggered layers of three bean-shaped subunits (staggering angle 120°; D3 point-group symmetry). It appears as a hexagon when viewed from the top (along its 3-fold axis) and as a square when viewed from the side (see Figure 4(a)). Although the crystal structures of a native crustacean hemocyanin hexamer and of a hexamer assembled from subunit II of *Limulus* hemocyanin are available, and show the shape and arrangement of the six subunits at high resolution,4,5 and, significantly, yielded a perfect fit with our recent 10 Å cryo-EM structure of a native 1×6mer,39 it cannot be *a priori* taken for granted that this arrangement is copied exactly in the various higher-order assemblies. Indeed, a slight but significant deformation was observed by molecular fitting of the subunit II homo-hexamer crystal structure and the present cryo-EM density map (see below). The degree of this twisting depends on the subunit position within the whole molecule and the contacts with subunits in neighbouring hexamers. Therefore, this is not a reconstruction artefact, but a consequence of the structural interactions between the eight hexamers. For the determination of parameters of the different hierarchy levels of *Limulus* hemocyanin, and interpretations of inter-hexamer interfaces, it is therefore crucial to deal with a density map determined from the whole 8×6mer,38 instead of a map determined from a combination of sub-structures.35,36

**Parameters of the 2x6mer**

In the 10 Å structure, the 2×6mer measures 146 Å (x-axis) × 230 Å (y-axis) × 168 Å (z-axis), and the local 3-fold axes of the constituent hexamers are 100(±3) Å apart. With respect to the lower hexamer, the upper one shows three rotations: counterclockwise 8(±2)° around its local 3-fold axis, counterclockwise 105(±5)° around the dodecamer longitudinal axis, and 7° towards the observer (Figure 4(a); Table 1). This yields a close contact between the two hexamers and explains why biochemically isolated *Limulus* 2×6mers appear in the electron microscope as a combination of a hexagon and a square (e.g. see Figure 1b of Bijlholt et al.40). Note that the 7° rotation is indeed 7+180°, which brings the two hexamers into an antiparallel position with respect to their subunit topology.
Parameters of the 4×6mer, flip-flop shift, and rocking effect

Within the 4×6mer half-molecule, which measures 249 Å (x-axis) × 244 Å (y-axis) × 168 Å (z-axis), the two 2×6mers are associated in an antiparallel arrangement, thereby showing a local 2-fold symmetry (Figure 4(b)–(e)). Their axial distance is 102(±3) Å, and the constituent 2×6mers are shifted 14(±1) Å with respect to each other (see Figure 4(b) and (b′); and Table 1). This so-called flip-flop shift gives the 4×6mer, when viewed from the top, the shape of a parallelogram. As a consequence, two different top views are possible, depending on which face of the molecule is exposed to the observer when the 4×6mer rests on a support (see Figure 4(b) and (b′)). These two orientations have been termed flip view and flop view, and define the two surfaces of the 4×6mer.

Table 1. Structural parameters of the 4×6mer and 8×6mer hemocyanins

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Data</th>
<th>1×6mers in 2×6mer</th>
<th>2×6mers in 4×6mer</th>
<th>4×6mers in 8×6mer</th>
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<tr>
<td>4×6mer</td>
<td>3D (40 Å)</td>
<td>- 105</td>
<td>- 11</td>
<td>- 14</td>
</tr>
<tr>
<td>4×6mer</td>
<td>2D</td>
<td>- 120</td>
<td>- 5</td>
<td>5±1</td>
</tr>
<tr>
<td>4×6mer</td>
<td>SAXSsyn</td>
<td>105±1.3</td>
<td>104.4±1.2</td>
<td>18±3</td>
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<tr>
<td>4×6mer</td>
<td>SAXSdeoxy</td>
<td>105±1.3</td>
<td>103.5±0.8</td>
<td>4±2</td>
</tr>
<tr>
<td>8×6mer</td>
<td>2D</td>
<td>102±5</td>
<td>108±5</td>
<td>17±4</td>
</tr>
<tr>
<td>8×6mer</td>
<td>3D (40 Å)</td>
<td>102±5</td>
<td>108±5</td>
<td>12±3</td>
</tr>
<tr>
<td>8×6mer</td>
<td>3D (10 Å)</td>
<td>100±3</td>
<td>102±3</td>
<td>14±1</td>
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</table>

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<thead>
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<th></th>
<th></th>
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<tr>
<td></td>
<td>37</td>
<td>35</td>
<td>36</td>
<td>34</td>
<td>38</td>
</tr>
</tbody>
</table>

* Androctonus hemocyanin.

** Eurypelma hemocyanin.

** Limulus hemocyanin.

Figure 3. Views of the 3D reconstructed 8x6mer. (a) and (a′) Lateral view (along the x-axis). (b) and (b′) Cross-view (along the y-axis), with the distance of the two 4×6mers indicated. (c) and (c′) Ring view (along the z-axis), with the staggering angle between the two 4×6mers indicated. In (a)–(c), the two hexamers within each 2×6mer are highlighted by light grey and anthracite, respectively. In (a′)–(c′), the eight subunit types as biochemically defined and topologically localized20 are indicated by different colours (red, type I; pink, type IIA; green, type II; yellow, type IIIA; blue, type IIIIB; turquoise, type IV; white, type V; black, type VI). Note in (b) and (b′) the prominent, oblique IIIA–IIIA bridge between the two associated 4×6mers (for molecular details, see Figure 10(a) and (a′). Note that in (c) and (c′), the flop face of the upper 4×6mer is visible (for comparison, see Figure 4(b) and (b′).
molecule as flip face and flop face. Each face shows a V-shaped longitudinal cleft that separates the two assembled 2×6mers; the two clefts differ in their contours (see Figure 4(c)). The four hexamers are not coplanar, but the longitudinal axes of the 2×6mers are skewed with respect to each other by the so-called rocking angle (see Figure 4(d)). In the 10 Å structure, this rocking angle is 16(±2.5)°, which is comparable to published values (see Table 1). The rocking angle is responsible for the rocking behaviour of the 4×6mer. Due to the non-coplanar nature of the 4×6mer, only three of the four hexamers have contact to a plane support. To change between the two stable positions, the molecule has to rock on this support around a diagonal “rocking axis”. The resulting tilt angle of the 4×6mer (and the 8×6mer) between the two stable positions is the rocking angle (Figure 4(e)).

Parameters of the 8×6mer

In the 10 Å structure, the 8×6mer measures 284 Å (x-axis)×271 Å (y-axis)×282 Å (z-axis). The two 4×6mers have an axial distance of 106(±3) Å and are clearly assembled flip-to-flip, thereby exposing their flop faces; their staggering angle is 42(±2)° (see Figures 3(b) and (c) and 7(c)). Other workers have collected evidence for a flip-to-flip assembly, and predicted similar shifts and staggering angles (see Table 1). In the electron microscope, associations of two or more 8×6mers (flop-to-flop assembly), or of additional 4×6mers to the native 8×6mer (flop-to-flop or flip-to-flop assembly) are not observed; they would require molecular interfaces that apparently do not exist.

Primary structure and modelling of Limulus hemocyanin subunits

Sequence analysis

A cDNA library was constructed from the inner organs of a single adult specimen of L. polyphemus. This library was screened with two distinct polyclonal antibodies that were raised in rabbits against total Limulus hemocyanin. More than 100 positive
clones were identified, of which about 40 contained inserts that encode hemocyanin subunit cDNA. The hemocyanin sequences were assembled and completed by primer walking. On the basis of known N-terminal sequences, the sequences were assigned to subunit types II, IIIA, IV and VI. The cDNA sequences have been deposited in the EMBL/GeneBank database (see Materials and Methods). The primary structure of subunit II was already known from protein sequence analysis.

The cDNA sequences comprise 2054–2252 bp, which include 28 to 50 bp of the 5′ untranslated regions and open reading frames of 1875–1917 bp. The 3′ untranslated regions comprise 118–294 bp, include the standard polyadenylation signals (AATAAA) and are followed by poly(A) tails of variable lengths. The open reading frames of the hemocyanin subunits translate into distinct polypeptides of 624–638 amino acid residues (Figure 5), with calculated molecular masses in the range of about 72–73.5 kDa. As in other chelicerate hemocyanins, no signal peptide required for transmembrane transport has been found in the Limulus subunit sequences. Thus, the nascent proteins do not pass through the Golgi apparatus, and the putative N-glycosylation sites (NXT/S) in the primary structures are probably not used.

Sequence comparison and phylogenetic tree

A multiple alignment of chelicerate hemocyanin amino acid sequences was constructed (available from the authors on request). Comparisons of the Limulus hemocyanin sequences with those of the arachnids E. californicum and N. inaurata allowed an unambiguous interspecific assignment of most, but not all, distinct subunit types. In phylogenetic analyses, which were carried out by Bayesian
Inference on the basis of the WAG model of amino acid substitutions, *Limulus* hemocyanin subunit II groups with the a-type subunits of the spiders, IIIA with the g-type subunits, IV with the d-type subunits and VI with the b/c-type subunits (see Figure 5). This assignment (Table 2) fits the results previously derived from immunological and reassembly studies.1

**Molecular modelling and rigid-body fitting**

Because of the high degree of sequence similarity (>75%) between chelicerate hemocyanin subunits (see Figure 5), ternary structure modelling was straightforward on the basis of the published crystal structure of oxygenated subunit II.6 For modelling of the subunits IIIA, IV and VI, their authentic sequences were applied. Because the sequences of subunits I, IIA, IIIB and V are not known, structurally equivalent subunit sequences were used for modelling. In the case of subunit V, the sequence of subunit VI was taken. In the case of subunits I, IIA and IIIB, the orthologous sequences of *Euryptelma* hemocyanin were applied (see Table 2). This is uncritical in the case of subunits I and IIA, because these subunit types (which play equivalent roles in the oligomeric architecture) are not involved in any inter-hexamer contact (see Table 3). In the case of subunits V and IIIB, however, these circumstances should be kept in mind. Also, the complete sequence of the subunit II was modelled for fitting into the density map of the cryo-EM structure. The amino acid residues 22–29, 134–138, 527–530 and 569–572, which are missing from the published crystal structure of *Limulus* subunit II, were modelled by means of spatial restraints.

The fitting procedure as described in Material and Methods resulted in a molecular model of the entire 8×6mer (Figure 6). Its quality was assessed by FSC (Figure 2(b)), and by calculating standard and Laplacian cross-correlations against the 10 Å cryo-EM structure, which yielded values of 0.810 and 0.802, respectively. Most of the amino acid residues in the molecular model of the 8×6mer do not overlap. Although a few side-chains overlap in some inter-hexamer interfaces, the backbones are invariably at proper distances. Since the sequences were modelled by means of spatial restraints on a single subunit (the crystal structure of the oxy-form of *Limulus* subunit II),6 the MODELLER procedure ignored the presence of any neighbouring subunit. Furthermore, the orientation of the side-chains is not necessarily optimal. Therefore, when necessary, an additional pair-wise fitting of subunits was performed, which eliminated the overlaps completely without moving the backbones (see below). Although further work might improve the present

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**Table 2.** Topological correspondencies of subunit types

<table>
<thead>
<tr>
<th>Subunit type</th>
<th>Contribution to interfaces</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Limulus</em></td>
<td><em>Euryptelma</em></td>
</tr>
<tr>
<td></td>
<td>2×6mer</td>
</tr>
<tr>
<td>I&quot; , IIA b</td>
<td>e c</td>
</tr>
<tr>
<td>II c</td>
<td>a c</td>
</tr>
<tr>
<td>IIIA c</td>
<td>g c</td>
</tr>
<tr>
<td>IIIB c</td>
<td>F c</td>
</tr>
<tr>
<td>IV c</td>
<td>d c</td>
</tr>
<tr>
<td>V b /VI c</td>
<td>b c /c c</td>
</tr>
</tbody>
</table>

* a Complete amino acid sequence is available.
* b Complete amino acid sequence is not available.
* c Complete amino acid sequence is available.

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**Table 3.** Amino acid residues involved in inter-hexamer interfaces

<table>
<thead>
<tr>
<th>Interface</th>
<th>Residues in first subunit</th>
<th>Residues in second subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. between the two 2×6mers of the 2×6mer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VV</td>
<td>Both subunits: 402 HARVD406(393–403), H410(407), F412(409), R414(411), 436 YHYLDHEFPFSY446(433–443), H456(503), K466(625), H468(627)</td>
<td></td>
</tr>
<tr>
<td>VVI</td>
<td>K406(380), E403(387), E405(443), H435(445)</td>
<td>140 HK413(139–140)</td>
</tr>
<tr>
<td>VI-IV</td>
<td>Both subunits: 26KFALKAHDPK63(25–35), 73FEEFLDCHQVRDP60(672–85), Y90(91)</td>
<td>272 FEEFLDCHQVRDP60(672–85), Y90(91)</td>
</tr>
<tr>
<td>VI-IV</td>
<td>Both subunits: 436 YHYLDHEFPFSY446(433–443), H456(503), K466(625), H468(627)</td>
<td></td>
</tr>
<tr>
<td>C. between the two 4×6mers of the 8×6mer</td>
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<td></td>
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<tr>
<td>IIIA-IIIAB</td>
<td>140 DK411(136–137), H413(134), H435(423)</td>
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<tr>
<td>IIIA-IIIB</td>
<td>454 SGS456(447–451), K459(567)</td>
<td>140 DK411(136–137), H413(134), H435(423)</td>
</tr>
<tr>
<td>IV-IV</td>
<td>Both subunits: F435(385), 430 DTVKH434(447–451), 492 FHHGKHK498(484–492), 566 VE570(558–559), H570(567), F574(566)</td>
<td></td>
</tr>
</tbody>
</table>

Superscript, relative residue numbers as in the text and in Figure 5. Parentheses, absolute residue numbers as in the crystal structure of *Limulus* II,6 and in the present molecular models of the different subunits.
molecular model, it proved to be very suitable for assessing the molecular structure of the different inter-hexamer bridges.

Analysis of the inter-hexamer bridges

The crystal structure of arthropod hemocyanin subunits shows three different structural domains, here designated as #1, #2 and #3 (see Figure 5).

Domain #2 contains the copper active site that is attached to four $\alpha$-helices; this domain is usually not involved in any inter-hexamer contact, in contrast to the flanking domains #1 and #3. The N-terminal domain #1 is rich in $\alpha$-helices, whereas the C-terminal domain #3 is dominated by a seven-stranded anti-parallel $\beta$-barrel and a long $\beta$-hairpin that reaches towards domain #1. As already mentioned, the molecular fitting revealed that in the molecular model of the 8×6mer, the subunits are not exactly arranged as in the isolated spiny lobster 1×6mer, but slightly deformed, notably those that are involved in inter-hexamer contacts. From the observation that *Limulus* 8×6mers can be dissociated into 4×6mers, 2×6mers and subunits by changing the ionic conditions, hydrostatic bonds should be primarily expected to establish the higher-order quaternary structure. Details about the domains and amino acid residues involved in the inter-hexamer bridges to be discussed now are summarized in Table 3, the positions of the bridges are shown in Figure 7, and the molecular interfaces are illustrated by Figures 8 x10. The $\alpha$-helices and $\beta$-strands as predicted from the crystal structure of *Limulus* subunit II are indicated in the present sequence (see Figure 5). The residue numbers given below conform to the numbering in this alignment, to allow the reader their clear identification. In addition to this relative numbering, the absolute residue numbers as obtained from molecular modelling are given in Table 3.

Bridges between the two 1×6mers of the 2×6mer

The 10 Å cryo-EM structure shows four different types of interface that yield six bridges (Figure 7(a)): a II–II interface with two sub-regions; a V–VI interface; two equivalent II–IV interfaces with two sub-regions; and a IV–V interface (on the flop face of the 4×6mer) with the equivalent IV–VI interface (on the flip face). Bridges between II–II, II–IV, and V–VI have been described, whereas the IV–V (respectively IV–VI) bridge is new.

In the molecular model, the prominent II–II bridge is formed by complementary association of domain #3. In particular, both copies of the $\beta$-3A→$\beta$-3B loop (402HARVD406) and the $\beta$-3C→$\beta$-3D loop (440DHEPFSY446) are assembled into a rather dense structure to which other amino acid residues also contribute. This is in contrast to the kind of association predicted in the earlier studies.
Initially, we thought that there might be some general overlapping at this interface, but we did indeed discover that the two subunits fit perfectly (apart from residues R404 and H506/D406, see below), and we revealed an intriguing contact apparatus (Figure 8(a) and (a')). The upper part is formed by two polypeptide backbones that run antiparallel and thus form a gap of 4–5 Å between eight residues (A403, V405, E442 and P443). This motif might connect the two subunits via direct backbone interaction. There is an interesting association of four histidine residues (H402, H506), two serine residues (S445) and two aspartic acid residues (D406) arranged in a ring. (Initially, this association yielded a slight overlap of H506 and D406, which could be corrected by pair-wise remodelling.) This

**Figure 7.** Topography of the inter-hexamer bridges. The respective hierarchy levels (2×6mer, 4×6mer, 8×8mer) are cut through their inter-hexamer bridges, and the cut surfaces exposed to the reader. Areas forming a joint interface (bridge) are marked by identical symbols. There exist eleven different types of interface that form a total of 46 inter-hexamer bridges within the 8×6mer. (a) Interfaces connecting the 1×6mers within the basic 2×6mer. Note that there are six bridges but only four types of interface, symbolized by ellipse (V–VI bridge), triangle (II–II bridge), rhombus/square (IV–V and IV–VI bridge that are structurally equivalent), and double field (II–IV bridge). (b) Interfaces connecting the 2×6mers within the 4×6mer. Note that there are five bridges but only three types of interface, symbolized by circle (V–V bridge), pentagon (VI–IIIB/IV–V bridge), and hexagon (V–VI bridge). (c) Interfaces connecting the 4×6mers within the final 8×6mer. Note that there are twelve bridges but only four types of interface, symbolized by circle/ellipse (II–IV bridge), triangle (IIIA–IIIA bridge), pentagon (IV–IV bridge), and hexagon (IIIA–IIIB bridge). Comparison to Figure 4(b) and (b') shows that the 4×6mers are assembled at their flip faces.
ring is associated with four aromatic residues (F444 and Y446) that flank S445 in the sequence. The lower part of the II–II interface is composed of three aromatic residues (Y446, Y448) that form a tripartite screen around the peptide bond of R414 (see Figure 8(a')). Even more striking is the central part of the II–II interface. It is a chamber about 10 Å in diameter formed by six histidine residues (H410, H441, H632), two phenylalanines residues (F412), and four acidic residues (D440, E442). Within this chamber, the nitrogen atoms of two basic residues (R414) are brought together. Two additional arginine residues (R404) and two lysine residues (K630) flank the chamber. (Initially, the long residue R404 had not been modelled correctly, and overlapped with other atoms; this required remodelling). The chamber has two wide openings, and it is reasonable to presume that it permits the binding of an allosteric ligand, and that the acidic and basic residues form salt-bridges. Indeed, its components are also conserved in the orthologous subunit a of *Eurypelma* hemocyanin.29 Allosteric interaction within the 8×6mer has somehow to be transferred between the hexamers. As deduced from low-resolution SAXS analyses, within the 2×6mer the rotation angle between the two hexamers is increased significantly upon deoxyge-

Figure 8. Molecular structure of the inter-1×6mer bridges in the 2×6mer. (a) and (a') Views of the II–II interface; for a detailed description, see the text. (b) The IV–VI interface; note the position of the bridge (arrow) in the cryo-EM structure (grey), indicating that the 140HK141 turn might be modelled incorrectly. (c) The II–IV interface. Note the histidine/phenylalanine clusters that are associated with the contact zone shown on the right. (d) The V–VI interface; some residues are displayed with 0.25 atomic volume. The structure of this interface is ambiguous, because the authentic sequence of subunit V is still unknown.
the histidine-rich C terminus of subunit IV is also in the neighbourhood. Whether this is of any functional significance remains to be investigated.

The II-IV interface is localized between domain #1 of subunit II and domain #3 of subunit IV, and is subdivided into two contact points: In the first, the N-terminal end of subunit II (2TLHD3) is connected to the β-3A→β-3B loop of subunit IV, indicating the presence of hydrostatic bonds between a lysine (K404) of subunit IV and a threonine/glutamic acid of subunit II (Figure 8(c)). Interestingly, this site is surrounded by various histidine and phenylalanine residues and therefore might contribute to allosteric interaction between hexamers. At the second contact point, the acidic α-1.7→α-1B loop of subunit II (140EFDE143) binds electrostatically to the basic-to-polar C-terminal α(H32). In the wall of the cavity, four phenylalanine residues (F73, F76) beneath the cavity surround four glutamic acid residues (74EE75). We think that, like the II–II bridge, the V–VI interface might be involved in transferring allosteric interaction between the two hexamers. Since many residues forming this interface are conserved in *Eurypelma* subunits b and c (except H32), there is a good chance that *Limulus* subunit V possesses them, and modelling with the sequence of subunit VI might be justified to describe this bridge. Nevertheless, further analysis of this interface requires the authentic sequence of subunit V.

**Bridges between the two 2×6mers of the 4×6mer**

As shown in Figure 7(b), three types of interface between the two 2×6meric halves of a 4×6mer can be identified in the 10 Å cryo-EM structure, yielding a total of five bridges: two equivalent VI–IIIB/IV/V interfaces at the flip face, two equivalent V–VI interfaces at the flop face, and a small V–V interface at the flop face. It should be kept in mind that the designations V and VI are interchangeable: It remains unknown as to which is which, and consequently, we do not know which of these two positions corresponds to the amino acid sequence of subunit VI.

On the flop face of the 4×6mer, a tiny bridge crosses the narrow cleft between the two copies of subunit V. As shown in Figure 9(a), this bridge is due to interaction of the two N termini (2VL3); these residues are identical in subunits V and VI, as known from N-terminal sequencing.20 but the bridge cannot be formed between subunits VI because of their larger distance (see Figure 7(c)). The bridge is not visible in the cryo-EM density map, at the mass-correlated threshold, but it is detectable if the threshold is decreased slightly.

The V–VI bridge is subdivided into two adjacent contact points (Figure 9(b)). In one, the β-2D→β-2E loop of subunit VI (284DEN297) joins the β-3D→α-3E loop of subunit V (345TSK459), which indicates hydrostatic interaction; however, the sequence of subunit V is unknown and the sequence of VI has been used instead. At the other contact point, the situation is not well defined, because the large loop following α-helix 3.5 in subunit V has apparently been modelled incorrectly and protrudes from the cryo-EM structure. To form the second contact point, this loop seems to interact with its region 568HDQVDGH574 to the region 190EVIGKY195 of the α-2.1→α-2.2 loop of subunit VI. An interaction between D573 and K194 is indicated, but in the case of subunit V it is not the authentic primary structure. Thus, the details of this second contact remain obscure, and the whole interface requires further research. It should be noted, however, that the sequence of subunit VI provides a number of histidine residues in this area.

The analogous β-3D→α-3E loop in subunit VI does not point directly towards the β-2D→α-2E loop of subunit V, but is closer to the central hole of the opposing hexamer. There, it also joins the two other subunits of the flip face of this hexamer; namely, IV and IIIB (Figure 9(c)). This VI–IIIB/IV/V
interface could not be predicted from the earlier 40 Å resolution structure, but is highly significant with respect to our search for centres of allosteric transmission: There is evidence from X-ray crystallography that within the 1×6mer, the two subunit trimers behave as rigid bodies that can rotate 3.2° with respect to each other around their 3-fold axis upon oxygenation. Such a rotation would dislocate the VI–IIIB/IV/V bridge, and by domain #3 of subunit VI acting as a lever, a power transmission to the opposing 2×6mer is possible. The β-3D→β-3E loop of subunit VI (454-TSKTK458) joins β-strand 2E of subunit IV at 284KE295, and electrostatic bonding is likely to occur between them (Figure 9(c')). To this site, β-strand 2E of subunit V contributes 292NGS294 (although this might not hold true in the authentic sequence of V), and subunit IIIB (with the orthologous sequence of Eurypelma subunit f applied) delivers residues 292EGQE295 in β-strand 2E (see Figure 9(c')). Most interestingly, this central cluster of charged residues is surrounded by 12 histidine residues (see Figure 9(c)) brought together from all four subunits (IIIB, H287, H291; IV, H287, H289, H292; V, H284, H287, H295; and VI, H453, H497, H500, H568). Therefore, the VI–IIIB/IV/V interface is indeed a promising candidate for transferring allosteric interaction from one 2×6mer to the other. It differs from the V–VI bridge in two aspects: the joining of four different subunits in a single small site, and the lack of the second anchor point (see Figure 9(b)).

Bridges between the two 4×6mers of the 8×6mer

In the 10 Å cryo-EM structure, four different types of interface can be identified that together provide 12 bridges between the two 4×6mers (Figure 7(c)). Most prominent are two central, structurally equivalent IV–IV bridges. According to reassembly experiments, subunit IV is stringently required for 8×6mer formation. The other contacts are arranged in two peripheral arcs around the central IV–IV bridges (see Figure 7(c)). We identified four equivalent II–IV bridges, four equivalent IIIA–IIIB bridges, and two equivalent IIIA–IIIA interfaces. It is interesting to note that in reassembly experiments, in addition to subunit IV, subunit IIIA proved to be indispensable for higher-order assembly beyond the 4×6mer. Except for the IIIA–IIIA bridge, the contact regions are invariantly located on the edges of the V-shaped cleft between the two 2×6mers (see Figure 4(c)). A IV–IV bridge as well as a IIIA–IIIB bridge
have been described; the other two interface types (II–IV and IIIA–IIIA) are new.

The IIIA–IIIA bridge is easily detected in the cross-view representation of the 10 Å cryo-EM structure (see Figures 3(c) and 6(c)) and is formed between domains #1 and #3. The fitted ternary structures revealed a most interesting interface (Figure 10(a)). It is formed by two copies of $^{140}$DK$^{141}$ in the $\alpha$-1.7→$\beta$-1B loop, which suggests the presence of two strong salt-bridges between the two subunits. The distance between the two potential binding atoms in the molecular model is 4.5 Å. This arrangement is closely associated with four histidine residues (H138 in the same loop and H430 in the $\beta$-3B→$\beta$-3C loop); their aromatic rings occupy the corners of a tetrahedron with edge length of 8 Å. Viewed from the top, the four histidine residues expose a wide opening, probably for a ligand (Figure 10(a')). Due to its peripheral position and oblique orientation between the two 4×6mers (see Figures 3(b) and 6(c)), chemical changes within the two IIIA–IIIA bridges (that are localized at opposite surfaces of the 8×6mer) might result in a slight rotation of the two 4×6mers with respect to each other around their staggering axis. This could transmit forces over long distances and thereby mediate allosteric interaction between the two 4×6mers.

The intricate molecular structure of the IIIA–IIIA interface is very promising with respect to this role. The small IIIA–IIIB bridges are not visible in the cryo-EM density map at the mass-correlated threshold, but they appear if the threshold is decreased slightly. In the case of IIIB, the orthologous sequence of Eurypelma hemocyanin (subunit f) was applied; however, since Eurypelma hemocyanin is a native 4×6mer, it could be expected that regions involved in inter-4×6mer bridges differ in the two sequences. Other workers have described a IIIA–IIIB bridge, but their predicted sites differ completely from those found in the present study. We detected potential linkages between both domains #3, and between domain #3 of subunit IIIA and domain #2 of subunit IIIB (Figure 10(b)). To the #3→#3 contact, IIIA contributes lysine K574 in the loop following helix $\alpha$-3.5; to the #3→#2 contact it delivers the sequence $^{454}$SGS$^{456}$ in the $\beta$-3D→$\beta$-3E loop. This indicates the presence of hydrostatic bonds in both cases. Indeed, with the orthologous Eurypelma type f sequence applied for IIIB, at a proper distance acidic residues (D454 in the $\beta$-3D→$\beta$-3E loop, E301 in the $\beta$-2.E→$\alpha$-2.4 loop) are available for bonding (see Figure 10(b)). However, the correct description of this bridge requires the authentic primary structure of subunit IIIB.
The II–IV bridge is localized, in both subunits, at exactly the same position in the α-1.4→α-1.5 loop. Ionic bonding is possible between residue D72 of subunit II and residue K71 of subunit IV (Figure 10(c)); all other possibilities can be excluded on the basis of the distances. As can be seen in Figure 10(c), the lysine residue points in the wrong direction, but this might be due to incorrect modelling. The backbone distance between the two amino acid residues is ~10 Å, which is easily bridged if the two residues were oriented towards each other.

In the prominent, somewhat oblique, IV–IV bridge, three regions of domain #3 are involved (see Table 3), which, in principle, were traced in the earlier 40 Å structure.36 Now, they could be more firmly identified as 455DTVKH459 in the β-3D→β-3E loop, 492HHIHKHG490 from β-strand 3C to β-strand 3H, probably phenylalanine F391, and several amino acid residues in α-helix 3.5 and the following loop (560WE567, δ250, and E574). Although this loop is missing from the X-ray structure of Limulus II hemocyanin, we found very few overlaps of side-chains in this region. These overlaps could be eliminated by a joint remodelling of the two subunits (see above), which did not influence the backbone, but re-oriented several side-chains and resulted in a perfect fit at the interface. Despite the numerous amino acid residues involved in this site, a clear and fascinating picture emerges (Figure 10(d) and (d’)). This interface contains the following elements: The relevant secondary structures from both subunits are arranged in an antiparallel manner, and their equivalent turns 455DTVKH459 join each other at 4 Å distance. This assembles the two hydrophobic valine residues (V457) that apparently help to bind the two turns together (see Figure 10(d)). This binding might be reinforced by hydrostatic interaction between the charged residues D455, T456 and K458 delivered from both subunits. More peripheral at either flank of the interface, bonding is likely to occur between K498 and E567, as well as between H500 and E574 (see Figure 10(d)). The clue to this interface, however, is two equivalent residue clusters that resemble minute “wheelhouses”; they are located at either side of the joined turns (see Figure 10(d’)). The “wheel” is the central C-6 ring of a tryptophan (W566), and the “house” is four closely associated histidine residues (H459, H493, H497 and H570) that surround the tryptophan. An additional histidine (H494) and two phenylalanine residues (F391 and F492) are in the neighbourhood (at 8–10 Å from the tryptophan), but it is not clear whether they contribute to this site. It is probably more significant that one of the four central histidine residues (H497), with a ring-to-ring distance of only 2–3 Å to the tryptophan, comes from the opposite subunit. If the tryptophan was dislocated with respect to H497 by an allosteric effect, this could transmit forces between the opposite backbones to change their distance. This, in turn, would change the length of the IV–IV bridge and, due to its oblique orientation, this would change the staggering angle between the two 4×6mers. It appears that the two IV–IV bridges (together with the two IIIA–IIIA bridges between the alternating hexamers; see Figure 7(c)) have the potential to transmit allosteric interaction between the 4×6mers.

**Concluding remarks**

In conclusion, 11 types of inter-hexamer interface have been identified in the present 10 Å structure; which, in the native 8×6mer sum up to 46 inter-hexamer bridges: there are 24 within the four 2×6mers, ten to establish the two 4×6mers, and 12 to assemble the two 4×6mers into an 8×6mer. From advanced molecular modelling and rigid-body fitting, each of these interfaces has been assigned to very few amino acid residues that now serve as candidates for the chemical bonds between the eight hexamers. In chelicerate hemocyanins, all hierarchical levels of the protein are involved to establish the oxygen-binding properties, and reveal a stepwise decrease of cooperativity from 4×6mers to 2×6mers to 1×6mers;36,42,43 consequently, allosteric interaction has to be somehow transferred via the inter-hexamer bridges. Several intriguing structures have now been unravelled as possible mediators of allosteric signals between the different levels of the nested hierarchy: the II–II interface, the II–IV interface and the V–VI interface between the 1×6mers, the VI–IIIB/IV/V interface between the 2×6mers, the IIIA–IIIA interface and the IV–IV interface between the 4×6mers. The present *Limulus* hemocyanin preparation has been studied under oxygen saturation conditions, in the presence of 25% oxygen, to produce vitrification of the protein molecules in their fully oxygenated state (under physiological conditions usually the R-state). Indeed, the retrieved parameters of the *Limulus* 4×6mer convincingly fit the SAXS model of the oxy-4×6mer of *Eurypelma*, whereas the deoxy-4×6mer SAXS model is significantly different (see Table 1). We are optimistic that the same procedure, carried out under oxygen-free conditions (i.e. in 100% nitrogen), will produce the hemocyanin molecule in its fully deoxygenated state (usually the T-state). A 3D reconstruction and analysis at a comparable resolution should then show the different inter-hexamer bridges in the deoxygenated 8×6mer, which might assist the understanding of how, during oxygen binding, allosteric signals are structurally transferred between the different levels of the oligomeric hierarchy. Thus, the present 10 Å structure opens the door to a fundamental understanding of the function of this highly cooperative protein.

**Materials and Methods**

**Animals**

Several *L. polyphemus* were obtained from the Marine Biolab (Woods Hole, USA). They were kept in artificial seawater at 18 °C (12 h light /12 h dark cycle); and were fed on fish.
Hemocyanin purification

Hemolymph was withdrawn from living animals at the prosoma-opisthosoma joint by inserting the needle of a syringe into the dorsal sinus. The blue hemolymph was centrifuged for 30 min at 3500g. The cell-free supernatant was then centrifuged for 2 h at 130,000g. The hemocyanin pellets were resuspended in a low-salt buffer, (100 mM Tris–HCl (pH 7.8), 10 mM CaCl₂, 10 mM MgCl₂).

Preparation and cryo-electron microscopy

Rapid freezing/vitrification was performed as described. A droplet of the purified protein at a concentration of 0.1–0.3 mg/ml was applied to a glow-discharged holey carbon support film. After removing excess fluid by blotting, the grid was frozen by plunging it in liquid ethane under a controlled oxygen-containing atmosphere in a plunging tube. A constant stream of gas from the top to the bottom of the tube provided a stable and controlled condition during plunging. For the oxygenated state of the hemocyanin, a gas stream of 25% (v/v) O₂/75% (v/v) N₂ was used, and the sample was equilibrated for 2 min (exchange rate of gas volume: 23 tube volumes/min). The hemocyanin cryo-transfer was performed using a Gatan model 626 single-tilt cryo-transfer system (Gatan, Munich, Germany). Electron micrographs were recorded to low-dose conditions at an instrumental magnification of 59,000×. The underfocus was set between 1.0 μm and 5.0 μm. The negatives (Kodak SO 163 film) were developed for 12 min in full-strength Kodak D19 developer.

Image processing

Drift- and astigmatism-free electron micrographs were scanned and digitized using a PRIMESCAN drum scanner (Heidelberger Druckmaschinen AG, Heidelberg, Germany). The micrographs were scanned with 1.8 Å and 1.0 Å step size corresponding to 10.6 μm and 5.9 μm, respectively, on the negative. Image processing was performed mainly with the IMAGIC-5 software package. Images of single particles were selected semi-automatically with the module BOXER from the EMAN 1.7 software package, which also allowed the processing of focal pair micrographs. Estimation of the defocus and astigmatism parameters was performed with the program CTFFIND3, and CTF correction with the module TRANSFER from IMAGIC-5. CTF-corrected images were band-pass filtered and normalized.

The first data set (focal pairs) with a scanning step size of 1.8 Å comprised 8984 single particles (for both high and low defocus), the second data set with a scanning step size of 1.8 Å comprised 3085 single particles. Analysis started with the exact filter back-projection method with imposed D2 symmetry. Refinement steps were performed iteratively with calculated 3D reconstructions. The final projection-matching step was performed with the modelled PDB file of the 8×6mer as reference. The resolution was assessed by FSC of two halves of the data set. The final reconstruction was filtered to a nominal 10 Å, thus eliminating structural details beneath this cut-off.

Molecular modelling and fitting

The models of the different sequences were built using the MODELLER 7x7 software and the 3D structure of the L. polyphemus subunit type II hemocyanin (RSCB PDB code 1NOL) as template. Since the sequences for the subunits type I, IIa, IIb and V were not available, the orthologous sequences of E. californicum subunit type e (Ila) and type f (IIb) were used. Because subunit types V and VI are immunogenically very similar, subunit type VI was used instead of subunit type V. For each sequence, 20 different models were calculated. The alignments of the sequences for modelling were calculated with the CLUSTALW 1.81 software. The cryo-EM densities of the 24 subunits of the 4×6mer (one-half of the whole molecule) were isolated with the ResolveRT software (TGS Europe, Düsseldorf, Germany). A generous and roughly subunit-shaped mask was placed around every subunit and then multiplied with the cryo-EM density maps of the isolated subunits. The latter were exported into the CCP4 file format for the molecular fitting procedure. All of the 20 models for each sequence were fit preliminarily into their respective cryo-EM subunit density with the MOLREP 9.2 software. The model with the highest score was then chosen. We considered this an objective approach of selecting a model from a number of possibilities.

The eight chosen molecular models (one for each subunit type) were then fit independently in the cryo-EM density map of the 8×6mer that was low-pass filtered to a nominal 8 Å but left unmasked. Fitting was done with a newly developed rigid-body fitting tool, creating eight homo-8×6mers. Given the very large dataset (170³ voxels), we have implemented a novel optimized docking program termed eliquos (J.H. & W.W., unpublished) that processes large datasets efficiently while maintaining compatibility with the exhaustive search methodology of the colores tool of the Situs fitting package. The improved efficiency is achieved by an extensive use of computational geometry algorithms (J.H. & W.W., unpublished). Laplacian filtering of both the cryo-EM density
map and the molecular models was employed. For each subunit, a six-dimensional grid-based exhaustive search was performed using an angular step size of 2° and a grid spacing of 1.8 Å. A distance-dependent pruning algorithm (J.H. & WW, unpublished) then reduced the number of solution candidates to 48. The resulting subunit positions were optimized individually at sub-voxel accuracy using an off-lattice Powell optimization algorithm. The final subunit fitting was assembled with the CHIMERA version 1 Build 2199 software by choosing the molecular modelled subunit types according to their respective topological positions within the 8×6-mer.

Cloning and sequencing of hemocyanin cDNAs

Hematopoiesis was induced by bleeding L. polyphemus individuals about one week before RNA preparation. Animals were immobilized for several hours on ice and killed by cutting the ventral nerve cord. The inner organs were immediately removed and shock-frozen in liquid nitrogen. Total RNA was extracted using the guanidine thiocyanate method. Poly (A)+ RNA was purified by means of the PolyATract kit (PROMEGA). A directionally cloned cDNA expression library was established using the Lambda ZAP-cDNA synthesis kit from STRATAGENE. The library was amplified once and screened with various specific anti-Limulus-hemocyanin antibodies. Positive phage clones were converted to pBK-CMV plasmid vectors with the material provided by STRATAGENE according to the manufacturer’s instructions and sequenced on both strands by the commercial GENterprise (Mainz, Germany) sequencing service. Complete hemocyanin cDNA sequences were obtained by the primer walking method using specific oligonucleotides.

Sequence analyses and molecular phylogenetic studies

The cDNA sequences were assembled by hand with the aid of GeneDoc 2.6. The web-based tools provided by the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics were used for analyses of DNA and amino acid sequences. The amino acid sequences of the L. polyphemus hemocyanin subunits were added by hand to published alignments of chelicerate hemocyanin sequences. The chelicerate hemocyanin subunit alpha, beta, delta, epsilon, and gamma sequences were:

- E. californicum (AJ122749, AJ122750),
- Nephila inaurata madagascariensis (AJ290429),
- Homarus americanus (AJ277491, AJ277492),
- Euryphantus tachypleus (P04254),
- Leontopanulirus interruptus (P80476),
- Nautilus pompilius (P90476),

Four crustacean hemocyanin subunit sequences, AM260213 (subunit II), AM260214 (subunit IIIA), AM260215 (subunit IV), and AM260216 (subunit VI), have been deposited in the EMD database (EMBL-EBI) under the accession number EMD-1304.

Bayesian phylogenetic analyses were performed with MrBayes 3.1. The WAG model of amino acid substitution with gamma distribution of rates was applied. Metropolis-coupled Markov chain Monte Carlo sampling was performed with four chains that were run for 100,000 generations. Prior probabilities for all trees were equal, starting trees were random, trees were sampled every tenth generation. Posterior probability densities were estimated on the final 5000 trees (burn-in = 5000).

Database accession numbers

The cryo-EM density map of the L. polyphemus 8×6-mer has been deposited in the EMD database (EMBL-EBI) under the accession number EMD-1304. The L. polyphemus cDNA sequences have been deposited in the EMBL/GenBank databases under the accession numbers AM260213 (subunit II), AM260214 (subunit IIIA), AM260215 (subunit IV), and AM260216 (subunit VI).

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