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Hexamers of Subunit II From *Limulus* Hemocyanin (a 48-mer) Have the Same Quaternary Structure as Whole *Panulirus* Hemocyanin Molecules

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ABSTRACT **Hemocyanins are copper-containing proteins that transport oxygen in a variety of invertebrates. Considerable evidence has accumulated that arthropodan hemocyanins are multimers of a fundamental hexameric unit. X-Ray crystallographic structure determination has revealed that the hemocyanin molecule from the spiny lobster *Panulirus interruptus* is a single hexamer having 32 point group symmetry. Using crystals of subunit II, one of 8 polypeptide types comprising the octahexameric hemocyanin of the horseshoe crab *Limulus polyphemus,* and the molecular replacement method for crystallographic phase determination we show that subunit II forms assemblies with the same hexameric quaternary structure as the whole *Panulirus* hemocyanin molecule. Observation of the same hexameric motif in two widely separated species provides strong additional evidence that this quaternary structural unit is a universal building block of arthropodan hemocyanins.**

Key words: hemocyanin, *Limulus, Panulirus,* horseshoe crab, spiny lobster, molecular replacement, X-ray, crystallography, protein structure

INTRODUCTION

Hemocyanins ("blue-bloods") are large, multisubunit proteins that transport oxygen in a variety of arthropods and mollusks. Hemocyanins circulate extracellularly in the hemolymph. Oxygen binding, which is usually cooperative, is thought to take place through a binuclear copper site in which the metals are covalently bound by protein side chains; no heme groups are involved.[1,2] Other ligands, such as CO,[3] NO_2^-,[4] CN^-,[5] N_3^-,[6] and O_2^{2-},[7] can also form complexes with hemocyanins at their oxygen-binding sites.

In the last 15 years there have been significant advances in knowledge of the primary, tertiary, and quaternary structures of various hemocyanins.[1,2] Although they have similar spectral and oxygen binding properties, hemocyanins of molluscan and arthropodan origin display major differences in molecular architecture.[1,2] Arthropod hemocyanin molecules are composed of subunits of molecular mass about 75,000 Da; each subunit comprises a single polypeptide chain containing one oxygen binding site. These molecules exist as assemblies of 6, 12, 24, 36, or 48 subunits, depending on the source species. Many distinct but related subunit types may be present in a single molecule. On the other hand, molluscan hemocyanins have subunits of molecular mass 350,000–450,000 Da, with 6–8 oxygen binding sites per subunit, and are assembled of multiples of 5 or 10 subunits.

The hemocyanin from the crustacean *Panulirus interruptus,* a spiny lobster, is an example of the smallest of the arthropodan molecules. It is a hexamer, containing 6 subunits of 3 types arranged with approximate point symmetry 32. A schematic of the hexamer structure, showing the relationships of the subunits in the hexamer and the locations of the three domains comprising a single subunit, appears in Figure 1. The *Panulirus* subunit atomic coordinates were used as the known structure in our crystallographic search procedures to determine the *Limulus* II subunit structure.

The hemocyanin molecule that circulates in the chelicerate *Limulus polyphemus,* the horseshoe crab, representative of the largest arthropodan hemocyanins, has a molecular mass of 3.2×10^6 Da,[8] and is believed to be composed of 48 subunits (8 hexamers). Electron micrographs of intact *Limulus*

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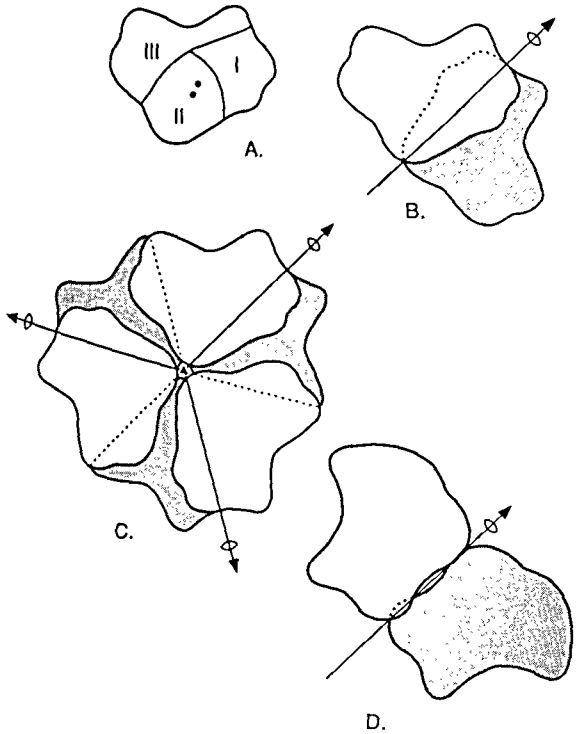

Fig. 1. The hemocyanin from the crustacean *Panulirus interruptus*, a spiny lobster, is a hexameric assembly with point symmetry 32 composed in vivo of 3 distinct types of polypeptide subunit chains. **(A)** An isolated hemocyanin subunit showing relationships of the three structural domains I, II and III. The copper atoms of the active site (●) are in domain II. **(B)** An isolated dimer. **(C)** Schematic of the *Panulirus* hemocyanin hexamer. The upper layer of three subunits (a trimer) is plain; the lower layer is cross-hatched. Alternately, one can view the structure as 3 copies of the dimer in **B**. Also shown are the 3-fold (▲) and 2-fold (→) rotation axes that relate subunit monomers in the hexamer. Orientation of the hexamer is the same as Figure 2. **(D)** View of the dimer rotated 90° degrees with respect to **B**.

hemocyanin reveal 8 morphological units per molecule, indicating that hexamers are important subassemblies of the whole hemocyanin molecular structure.

The *Limulus* hemocyanin molecule has been dissociated and purified into eight immunologically distinct subunits; each of these subunit types has a molecular mass of about 73,000 Da.[9] It is an important question whether all *Limulus* hemocyanin molecules have identical quaternary structure in terms of subunit composition, or whether there is microheterogeneity. There is evidence that a compositionally unique species of whole molecule exists in vivo. Hemocyanin molecules taken from different, individual horseshoe crabs and during different seasons display the same relative amounts of subunits I, II, IIa, IIIa, IIIb, IV, V, and VI. The exact relative amounts per whole hemocyanin molecule of some of the minor subunits are still being established, but it is clear the stoichiometry of the 48-subunit whole molecule is *not* 6 copies of each of the eight subunit types.

The various purified *Limulus* subunits display a variety of aggregation states in reconstitution experiments. Subunits II, IIa, IIIa, and IV, for example, can each form homohexamers; subunits V and VI make heterodimers; and subunits I and IIIb apparently do not self-aggregate.[12] Mixtures of subunits II, IV, V, and VI are sufficient to produce molecules of the same size as intact hemocyanin, about 60 S.[12] However, these aggregates cannot possess exactly the same microscopic arrangements of subunits as undissociated whole molecules. It is not currently known if correct assembly of 60 S hemocyanin occurs spontaneously or requires other proteins or energy.

Immunoelectron microscopic localizations of subunits within the whole *Limulus* molecule are reproducible,[13] also supporting the existence of a unique quaternary structure. Using such techniques, Lamy and Lamy and colleagues have built a model for the three-dimensional structure of the octohexameric *Limulus* hemocyanin.[13–15] The whole molecule appears to be composed of two layers, each a distorted square-planar array of 4 hexamers. One layer of 4 hexamers is rotated about 45° with respect to the other, so that in some electron micrographs whole *Limulus* hemocyanin appears pentagonal. Subunit II, the object of this crystallographic work, occupies positions within the intact *Limulus* molecule where hexamers interact. It is thus thought to be required for assembly of hexamers to the full-sized hemocyanin molecule, and possibly for cooperative oxygen binding.

The three-dimensional structure of spiny lobster hemocyanin has been determined at atomic resolution by Hol and his associates.[16,17] On the basis of studies utilizing X-ray absorption, UV spectroscopy, and EPR[18] to determine the extent of ligation, the hemocyanin in their crystals is thought to be primarily in its deoxygenated form. There is one hexameric molecule, containing two types of polypeptide chain, in the crystallographic asymmetric unit. (Molecules containing the third type of subunit mentioned above are apparently excluded during the crystallization process.) The tertiary structures of the two subunit types seen in the crystal structure are very much alike, unsurprising since their primary sequences are 97% identical.[19] Recently, restrained least-squares refinement of the 470,000 Da hexameric molecule has been completed at 3.2 Å resolution.[17]

The primary sequences of a variety of arthropodan hemocyanin subunits from diverse species have been determined and show strong similarity.[20] A close resemblance among the tertiary structures of all the known arthropodan hemocyanin subunits is thus suggested, and formed the basis for our use of the molecular replacement method for X-ray phase determination. Table I shows an alignment of the *Limulus* and *Panulirus* amino acid sequences.

The structure described here is of a hexameric ag-

gregate of purified subunit II of *Limulus* hemocyanin, in a primarily oxygenated form.[21] Subunit II by itself does not bind oxygen cooperatively.[3] This affords the possibility of producing an interesting variety of ligation states by direct chemical modification of the crystals. Unlike the situation occurring with hemoglobin crystals, which crack upon oxygenation, when crystals of *Limulus* II change their ligand state, they remain intact and isomorphous, and continue to diffract. It is our purpose here to show that the subunit II hexamer has the same tertiary and quaternary structure as the *Panulirus* hexamer, and to discuss the implications of the structure for the mechanism of cooperative oxygen binding in hemocyanins.

METHODS

Crystallization of *Limulus* II Hemocyanin

Purified subunit II of *Limulus polyphemus* hemocyanin was kindly provided by Drs. J. and C. Bonaventura of the Duke University Marine Laboratory. The existing crystallization protocol[21] has been modified so that the crystals of *Limulus* II in its oxygenated form are grown by the hanging-drop, vapor-diffusion method using polyethylene glycol 8000 as a precipitating agent.[22] Crystals grow to 1–2 mm in diameter in about 2 weeks from solutions containing 160 mM Bis Tris, 30 mM Tris, 30 mM glycine, 6 mM EDTA, 4% polyethylene glycol 8000, and 0.5 M NaCl, at pH slightly above 6. In solution, high concentrations of sodium chloride cause the aggregation of *Limulus* II monomers to hexamers and its presence in the crystallization medium is essential for good crystal formation. The blue crystals have the symmetry of the space group $R32$ with lattice constants $a = b = 117.2 \pm 0.6$ Å, $c = 286.9 \pm 0.9$ Å in the hexagonal setting, and contain one subunit of molecular mass 72,946 Da in the asymmetric unit. The average crystal volume per unit molecular mass is 2.6 Å^3/Da. The crystals routinely diffract to at least 2.7 Å resolution and have a useful lifetime during X-ray exposure of between 50 and 200 hours on a conventional generator. The lifetime of these crystals is improved by using single-wavelength radiation.

Data Collection

X-Ray reflections with Bragg spacings between 40 and 4 Å were collected from oxygenated *Limulus* II crystals on a Nicolet P3/F four-circle diffractometer at Johns Hopkins. The merging R-value* was 0.051 based on structure factor amplitudes.

Molecular Replacement

We carried out the molecular replacement procedures using a stand-alone version of the rotation function written by one of us,[24] and the translation function and ancillary programs in the package MERLOT,[25] an integrated and well-documented set of programs for implementing this method. We used the unrefined coordinates of the spiny-lobster hemocyanin subunit (1/6 of the hexamer) to generate calculated intensities for the molecular replacement procedure.[23] The two copper atoms were omitted from the test molecule. Also, alignment of the primary sequences of the two molecules[26] shows that in the region of helices 1.2 and 1.3 in the spiny lobster,[27] comprising residues 22–64, there is no apparent homology (see Table I) with the corresponding sequences in the horseshoe crab hemocyanin subunit, so these residues were omitted from the calculation. Finally, side chain atoms past Cβ were included in the test molecule model only when there was identity in the two primary sequences.

The translation function utilizes the complex Fourier transform of the test molecule electron density function. The sampling frequency in this transform is determined by the maximum distance from the electron density origin at which an atom is found. To reduce this frequency and thus save computer time, the spiny lobster hemocyanin subunit coordinates were translated so that the center of mass of the subunit lay at the origin of the coordinate system.

X-Ray diffraction data with Bragg spacings between 8.5 and 6.0 Å gave the clearest results in molecular replacement searches. The search results (summarized in Table II) were quite noisy: two false rotation function peaks were tested before finding one giving a consistent set of translation function peaks, and a reasonable R-value,† 0.45. Symmetry imposes rules relating the location of translation function peaks in the space group $R32$, and potential solutions inconsistent with theses rules can be rejected. Thus, the section $z = 0$ contains peaks corresponding to vectors between subunit pairs related by the crystallographic 3-fold rotation axis. Provisional values for x_c and y_c, and x- and y-coordinates of the origin of the hemocyanin subunit relative to the crystallographic origin, can be obtained from this section. Vectors between *Limulus* II subunits related by the crystallographic 2-fold rotation axes appear on the section of the translation function at $z = 2z_c$, where z_c is the z-coordinate of the subunit

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* $$R_{\text{merge}} = \frac{\sum_h \sum_j |F_j(h) - \langle F(h)\rangle|}{\sum_h \sum_j F_j(h)}$$

where the $F_j(h)$ are the individual observations of the structure factor amplitude at h in different scaling or observation groups, and $\langle F(h)\rangle$ is their average value.

† $$R = \frac{\sum_h |F_o(h) - F_c(h)|}{\sum_h F_o(h)}$$

where $F_o(h)$ and $F_c(h)$ are the observed and calcuated structure factor amplitudes at h.

TABLE I. Alignment of *Limulus II* and Panulirus Amino Acid Sequences*

```
     α1.1aaaaaaaaaa          α1.2aaa              α1.3aaaaaaaaaaa            α1.4aaaaaaaaaaa
       +-+  +  +  -                                - -++++ -+ +   +          + -+ -- ++  -
-----tlhdkqirvchlfeql----------------------ssatvigdgdkhkhsdrlknvgklqpgaifscfhpdhleearhlyevf
DALGTGNAQKQQDINHLLDKIYEPTKYPDLKEIAENFNPLGDTSIYNDHGAAVETLMKELNDHRLLEQRHWYSLFNTRQRKEALMLFAVL
1

a   α1.5aaaaaaaaa         α1.6aaaaaaaa    β1Abbbbbbb        α1 7aaaaaaaaa          β1Bb    bbbb
 -  -  -  -  +- +    -          -    ++-- +         -   -+          -  ++ + + - --          -  -
weagdfndfieiakeartfvneglfafaaevavlhrddckglyvppvqeifpdkfipsaaineafkkahvrpefdespilv     dvqdt
NQCKEWYCFRSNAAYFRERMNEGEFVYALYVSVIHSKLGDGIVLPPLYQITPHMFTNSEVIDKAYSAKM-----TQKQGTF     NVSFT
91

                α2.1aaaaaaaaaaaa                α2.2aaaaaaaaaaaaaaaaaaaa   β2Abbb   β2Bbbbb-b
    - - +    +--      +* + *          +    +++-++ -      *     + - -+     ++       --       +
gnildpeyrlayyredvginahhwhwhlvypstwnpkyfgkkkdrkgelfyymhqqmcarydcerlsngmhrmlpfnnfdeplagyaphl
GTKKNREQRVAYFGEDIGMNIHHVTWHMDFPFWWEDSY-GYHLDRKGELFFWVHHQLTARFDFERLSNWLDPVDELHWDRIIREGFAP-L
177

bbb  β2Cbbbbbbbbb          α2.3aaaaaaaaaaaaβ2Dbb   β2Ebb    α2.4aaaa              α2.5aaaa
 +    +    + -  + +-  -  -  -  + +-+  -  +       --  ++  -- +  -      -   -   +-     *   *
thvasgkyysprpdglklrdlgdi-eisemvrmrerildsihlgyvisedgshktldelhgtdilgalvessyesvnheyygnlhnwghv
TSYKYGGEFPVRPDNIHFEDVDGVAHVHDLEITESRIHEAIDHGYITDSDGHTIDIRQPKGIELLGDIIESSKKSSNVQYYGSLHNTAHV
267

aa aaa                          α2.6aaaaaa α2.7aaaa    α3.1aa  β3Abbbbbbbbbbb    bbb β3Bb
    + +-  - + +--     -      +-       *+  -   +- +   +  -+-     -    -   + + -       +   +-
tm arihdp dgrfheepgvmsdtstslrdpifynwhrfidnifheykntlkpydhdvlnfpdiqvqdvtlharvd    nvvhftmreq
ML GRQGDP HGKFNLPPGVMEHFETATRDPSFFRLHKYMDNIFKKHTDSFPPYTHDDLEFSGMVVNGVAIDGELI    TFFDEFQYSL
357

          β3Cbbbbb   β3Dbbbbbbbbbbbbb   β3Ebbbbbbbbbbb   β3F α3.2aaa b3Gbbbbbbb β3Hbbbbbbb
- - ++       +  + +  + -+-            - +   +      + --   - + -- ++   - -+ + - +  +    ++
elelkhginpgnarsikaryyhldhepfsyavnqnnsasdkhatvriflapkydelgneikadelrrtaieldkfktdlhpgkntvvrhs
INAVDTGESIEDVEINARVHRLNHKEFTYKITMSNNNDGERLATFRIFLCPIEDNNGITLTLDEARWFCIELDKFFQKVPKGPETIERSS
448

   β3Ibbbbbα3.3aaaaaaaa                α3.4aaaa     β3Jbbbbbbbbb α3.5a
 -    +     --  +     -++ -              +    +   +  - +      - -+-+ -   -     -       +-
ldssvtshqpptfedllhgvglnehkseyc-------scgwpshllvpkgnikgmeyhlfvmltdwdkdkvdg sesvacvdavsycgard
KDSSVTVPDMPSFQSLKEQADNAVNGGHDLDLSAYERSCGIPDRMLLPKSKPEGMEFNLYVAVTDGDKDTEGH NGGHDYGGTHAQCGVHG
539

β3Kbbbbb          α3.6aaaa β3Lbbb bbbbbbbb
-++   ++       -   + -+  -          + - + + +-
dhkypdkkpmgfpfdrpihtehisdfltnnmfik dikikfhe
GEAYPDNRPLGYPLERRIPDERVIDGVSNIKHVV VKIVHHLEHHD
629
```

*The primary sequences of a variety of arthropodan hemocyanin subunits from diverse species have been determined and show strong similarity. A close resemblance among the tertiary structures of all the known arthropodan hemocyanin subunits is thus suggested. The sequence of *Limulus II* hemocyanin is shown in lower case; the sequence of *Panulirus* a in capitals; residues marked in italics were omitted from the molecular replacement model model. Every tenth residue is marked with a boldface character. The residue number at the beginning of each row is also given. Residue numbers are for the *Panulirus* a sequence in the alignment of Bak et al.[26] Dashes represent gaps in both sequences needed to match other arthropodan sequences. Active site histidine residues are marked with an asterisk; charged residues with a +/−. α-Helices and β-strands of the *Panulirus interruptus* structure are labeled as previously described.[20] Thus, α1.2aaa labels the second helix in the first domain, which contains seven residues in all; β2Abbb labels the first β-strand in the second domain, which contains six residues.

origin. If x_c and y_c are already known, only specific locations on each z-section need be scanned. If no peak is found there, then the translation function does not provide a consistent solution. A model for the arrangement of the *Limulus* II subunits in our *R*32 crystals was generated by rotating the coordinates of the search molecule by an amount corresponding to the angles at the peak of the rotation function, and by translating these coordinates by an amount derived from the translation function peaks.[23] The positional and orientational parameters of this model were optimized using the rigid body refinement capacity of MERLOT.

The correctness of the proposed structure of *Limulus* II hemocyanin, based on molecular replacement, was evaluated using three criteria. First, atoms omitted from the test molecule should reappear in difference maps. In this case, both copper atoms of the active site were omitted from the structure factor calculation, as were the atoms in residues 22 to 64 (corresponding to helices 1.2 and 1.3) of *Panulirus* hemocyanin. A difference Fourier synthesis was

TABLE II. Search Results*

Procedure	Angles	Translations	R-value
	Angles given in units used by program and then as (α, β, γ)		
Rotation function/ translation function	$\theta_+ = 35, \theta 2 = 60, \theta_- = 80$ ($\alpha = 25, \beta = 60, \gamma = 45$) peak height 4.9$\sigma$	No consistent solution	—
Rotation function/ translation function	$\theta_+ = 84, \theta 2 = 75, \theta_- = 64$ ($\alpha = 58, \beta = 75, \gamma = 110$) peak height 4.2$\sigma$	No consistent solution	—
Rotation function/ translation function	$\theta_+ = 28, \theta 2 = 90, \theta_- = 28$ ($\alpha = 326, \beta = 90, \gamma = 90$) peak height 4.1$\sigma$ Subtract 30° from α to compensate for change in nul rotation convention $\alpha = 296, \beta = 90, \gamma = 90$	$x = .325, y = .150, z = .073$ fractional coordinates in crystal hexagonal cell	.67
Rigid body refinement	$\alpha = 302, \beta = 90.5, \gamma = 89$	$x = .33, y = .150, z = .075$	0.453
Direct superposition of spiny-lobster noncrystallographic symmetry axes onto *Limulus* crystal symmetry axes	$\alpha = 300, \beta = 90, \gamma = 90$ (*Panulirus* 3-fold is along *b*: *Limulus* 3-fold is along *c*)	$x = .326, y = .146, z = .077$ These value correspond to the offset applied to the coordinates before calculating the molecular transform	—
Difference Fourier synthesis reveals omitted copper atoms and density in region of deleted residues			

*The quasi-orthogonal Eulerian angles[29] θ_+, $\theta 2$, and θ_- are used by our rotation function, and yielded symmetrical and well-shaped peaks; the $\theta 1$, $\theta 2$, $\theta 3$ are conventional Eulerian angles as defined by Rossmann and Blow; the α, β, γ are an alternate definition of Eulerian angles used in the Crowther rotation function and in MERLOT. The various programs that use these angles have differing conventions about the alignment of axes for the null rotation. Care must be taken to obey these definitions and conventions. Angular steps of 5° were used in all rotation function runs. The asymmetric unit of rotation space is given by $0 \leq \theta_+ \leq 720$; $0 \leq \theta 2 \leq 90$; $0 \leq \theta_- \leq 120$. The following relations hold among the angles: $\theta_+ = \theta 1 + \theta 3$; $\theta_- = \theta 1 + \theta 3$; $\theta 2 = \theta 2$ and $\alpha = \theta 1 - \pi/2$; $\beta = \theta 2$; $\gamma = \theta 3 + \pi/2$.

made using coefficients $(F_L - F_P)\exp(i\alpha_P)$, where F_L is the observed structure factor amplitude from the *Limulus* crystals, and F_P and α_P are the phase and amplitude calculated from the molecular replacement model using the spiny-lobster coordinates. This difference map shows a large asymmetric peak corresponding to the expected di-copper location. In addition, a conventional electron density map using coefficients $F_L\exp(i\alpha_P)$ was calculated, to ensure that the molecular replacement structure actually reappeared. The $F_L\exp(i\alpha_P)$ map shows continuous density through the omitted region of the polypeptide chain, connecting the regions present in the model. Reappearance of the coppers and polypeptide at the predicted locations in the difference synthesis implies that the model and the phases calculated from it must be fairly accurate; a portion of the $F_L\exp(i\alpha_P)$ map is displayed is Figure 4.

Second, a model with valid packing of the subunits within the crystal has emerged. This is a critical check. In fact, the *Limulus* subunit in our model is placed at one of the relatively few possible positions which do not lead to significant interpenetration of molecules in the crystal lattice.

Third, a plausible quaternary structure has been generated. We employed a search procedure in which the orientation and position of a test subunit were unrestricted. It leads to a model of the *Limulus* II hemocyanin molecule comprising a hexameric assembly of subunits around the origin of the crystallographic unit cell—the same quaternary structure found in the *Panulirus* hexamer. This was in no way built into our procedure. Using our conventions, the rotation that superimposes the noncrystallographic 32 symmetry axes within the *Panulirus* hexamer onto the corresponding axes in our unit cell is described by the Eulerian angles $\alpha = 300, \beta = 90, \gamma = 90°$. Using the same conventions, the refined orientation angles from MERLOT are $\alpha = 302.0, \beta = 90.5, \gamma = 89.0°$.

RESULTS AND DISCUSSION

Description

Figures 2 and 3 summarize the structures involved. Figure 2 is a backbone drawing of a trimer (i.e., a half-hexamer) of *Panulirus* hemocyanin subunits, looking down the 3-fold axis. One such sub-

Fig. 2. ARTPLOT[28] α-carbon drawing of 3 *Panulirus* hemocyanin subunits, related by the noncrystallographic 3-fold axis. Helices in Domain I are represented as cylinders and are connected by arrows, Domain II α-carbons are connected with double lines, those of Domain III with single lines, and areas undefined in the crystal structure are dotted. The copper positions are marked by heavy dots. Helices are labeled as previously described[20]; helix 1.2, for example, is the second helix in the first domain of the subunit. Note that the helices 1.2 and 1.3, which do not have homologues in the *Limulus* subunit, are at the periphery of the trimer and play only a minor role in intra-molecular contacts. (Reproduced with permission from the Journal of Molecular Biology.)

unit represents the test structure used in the molecular replacement searches here. Figure 3 shows a view normal to this, looking down the 2-fold axis of a *Panulirus* dimer. Together these two view describe the *Panulirus* hexamer, the quaternary structural unit (Fig. 1) that has now been found in *Limulus* II subunit crystals.

Although the quaternary and tertiary structures of the *Panulirus* and *Limulus* II hexamers are surprisingly similar, they differ in details that may be implicated in their different cooperative oxygen binding properties and in their roles in the assembly of their respective whole hemocyanin molecules. There is little sequence identity in the region of helices 1.2 and 1.3 of *Panulirus a* hemocyanin (Table I). These helices lie at the periphery of the molecule (see Fig. 2) and only residues 62–64 are involved in inter-subunit contacts.[27] Another place in which the sequence agreement is poor lies in the middle of domain 3, and comprises roughly residues 420–470 and 545–570. With the exception of 3 residues in the short β-strand termed 3B,[20] none of these residues is implicated in intersubunit contacts.[27]

Refinement of this initial model for the *Limulus* structure is now in progress.

Discussion

The crystals of spiny-lobster hemocyanin contain a hexamer of point group symmetry 32 in the asymmetric unit. The unit cell dimensions and buoyant density of *Limulus* II crystals strongly indicated that they contained one monomer in the asymmetric unit. The 32 point group symmetry present at each lattice point in our crystals suggested the possibility that the quaternary structure of the *Panulirus* hexamer might be reproduced in them. We were reluctant to enforce such rigid conditions on the molecular replacement searches, however, for fear of locking ourselves into an incorrect solution. In addition, there would be no way of adjusting for small differences in the quaternary structures of the *Panulirus* and *Limulus* II hexamers if a rigid hexamer was used to search. Thus, full, three-dimensional rotational and translational searches were conducted.

The suggestion, emanating from solution studies and from immunoelectron microscopy, that arthropodan hemocyanins are multimers of a basic hexameric motif has received strong support from the work described here. Although hexamers of *Limulus* subunit II do not appear to be constituents of native, whole molecules, the ability of subunit II to poly-

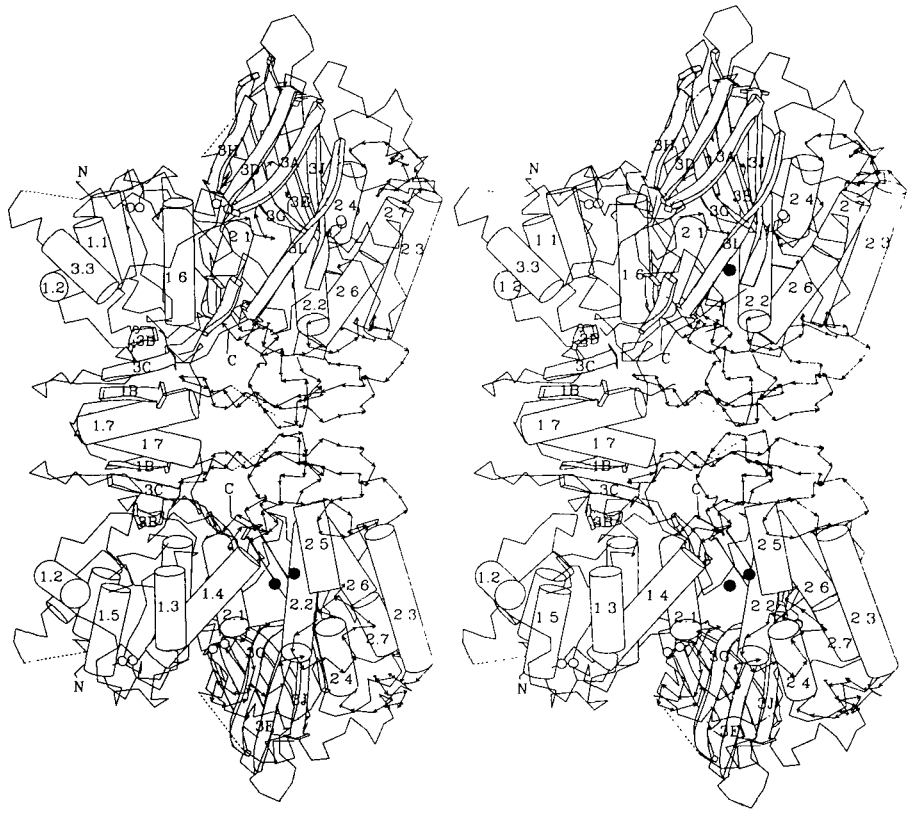

Fig. 3. ARTPLOT stereo α-carbon drawing of 2 *Panulirus* hemocyanin subunits, related by the noncrystallographic 2-fold axis. Helices are shown as cylinders, β-sheets as ribbons, and copper atoms as heavy dots. Because of the rather extensive intersubunit contacts, this pair of subunits is called a tight dimer, which is to be distinguished from a second type of dimer in the hexamer showing much less tight interactions. (Reproduced with permission from the Journal of Molecular Biology.)

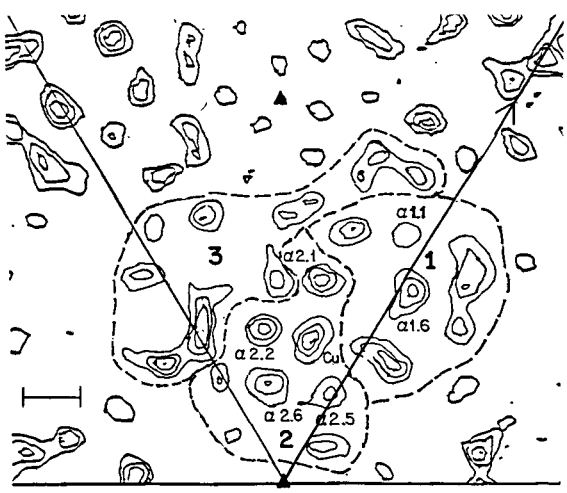

Fig. 4. Section normal to the 3-fold axis (▲) of an electron density map made using coefficients $F_L \exp(i\alpha_P)$. It shows the region containing the copper atoms omitted from the model. The dotted lines mark the boundaries of the three domains of the subunit containing the coppers., which is in the same orientation as the first panel of Figure 1. The predicted position of the copper site lies within the labelled peak. The direction of the two-fold axis, which does not lie in this section, is given by the marked (>) line. The scale bar is 10 Å. A few nearby features in the secondary structure are marked for reference.

merize into assemblies of the same size and appearance as in vivo molecules strongly suggests that the subunit II hexamer is closely representative of actual mixed-chain hexamers in the whole molecule. Thus, the evidence that the hexameric structure seen first in *Panulirus* and now in *Limulus* represents a universal hemocyanin building block is quite compelling.

A key feature of future work on hemocyanin will involve the elucidation of the quaternary structural changes that underlie cooperative ligand binding. Subunit II hexamers display no cooperativity, so that ligands can readily be diffused into existing crystals. The tertiary features of ligand binding should therefore be straightforward to clarify. Like hemoglobins, hemocyanins are believed to have high and low oxygen-affinity states. Although both *Limulus* II and *Panulirus* crystals were grown under standard atmospheric conditions, only the *Limulus* crystals clearly have the blue color characteristic of the oxygenated state. Differences exist in the ionic environment for crystallization, and in oxygen affinities, of these two hemocyanins, so that we are currently unable to determine if the two proteins are in

different oxygen affinity states. Knowing one structure in each of the two states would greatly aid understanding of the mechanism of cooperative behavior in hemocyanins.

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