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Architecture of *Limulus polyphemus* Hemocyanin[†]

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ABSTRACT: The architecture of the 48-meric hemocyanin of the horseshoe crab *Limulus polyphemus* has been determined from electron micrographs of whole (48-mer) molecules and half- (24-mer) molecules. The assembly of hexamers of kidney-shaped subunits can produce two dodecameric enantiomorphs, designated as right and left. The assembly of 24-mers can again result in two enantiomorphs. By taking into account the rocking effect described by Van Heel and Frank [Van Heel, M., & Frank, J. (1981) *Ultramicroscopy* 6, 187-194], we deduced that the 24-meric half-molecule is made up of two copies of the left dodecameric enantiomorph. In addition, the two constituent dodecamers of the half-molecule are shifted with respect to a symmetric head-to-tail arrangement, which makes it possible to distinguish two different faces of the 24-mer, termed flip and flop. A model of the whole molecule

was built from two copies of the 24-meric half-molecule. This model presents the four distinct views observed in the electron microscope (pentagon, ring, cross, and bowtie). In addition, the model shows the pentagonal view to exist in two varieties: symmetric and asymmetric. An analysis of electron micrographs presenting the pentagonal view by image processing using the statistical technique of correspondence analysis confirmed the existence of two types of pentagonal view, representing projections of a molecule built from two copies of the left 24-meric enantiomorph. In addition, the best fit between the averaged molecule images and the possible models was observed with a flop-flop inter 24-mer contact. The final model is shown in a series of stereo views produced by computer graphical techniques.

The hemocyanin of the horseshoe crab *Limulus polyphemus* is the biggest representative of this class of oxygen carriers in the Arthropod phylum (van Bruggen et al., 1981). It is generally considered to be composed of 48 monomeric subunits assembled in eight groups of six subunits, also called hexamers. The whole molecule is therefore termed an (8 × 6)-meric hemocyanin.

Native hemocyanin can be successively dissociated into 24-mers, 12-mers, and monomers, each form possessing its own domain of stability (Johnson & Yphantis, 1978). The monomers resulting from the dissociation of the native hemocyanin are highly heterogeneous. At least eight different polypeptide chains differing in their chromatographic, electrophoretic, and antigenic properties (Lamy et al., 1979; Brenowitz et al., 1981) and their oxygen affinities (Sullivan et al., 1974) have been found in the dissociation products of the whole molecules.

While the final aim of this work is the determination of the quaternary structure of *L. polyphemus* hemocyanin, we must

first determine the architecture of the whole molecule in which the 48 individual subunits are to be localized. A recently developed technique suggested a reasonable approach to this goal. This is the analysis of electron micrographs by computer alignment and the statistical technique of correspondence analysis (Van Heel & Frank, 1981; Frank et al., 1982; Frank & Van Heel, 1982). The application of this technique to the hemocyanin half-molecule (24-mer) of *L. polyphemus* (Van Heel & Frank, 1981) revealed two very interesting structural aspects. The first result was that the molecule in its top view is not a flat structure, as had been generally assumed, but rather a noncoplanar structure resting on three hexamers, with the fourth hexamer suspended above the plane of the support film. Thus, the molecule is able to rock between two stable positions. The second feature discovered in this study was that the molecule in its top view is not perfectly square but rather is slightly rhombic, due to a slight shift of the two dodecameric halves with respect to one another. Therefore, two different top views, called flip and flop, can be distinguished.

The same observations were subsequently made on the 24-meric hemocyanin molecule of the scorpion *Androctonus australis* (Bijholt et al., 1982; Van Heel, 1981). More recently, Sizaret et al. (1982) used the evidence of the rocking effect and the rhombic arrangement to choose between the two possible enantiomorphs of the dodecamer and thus to determine the absolute configuration of the molecule in terms of the positions of all 24 subunits. The aim of this paper is to describe the architecture of the 48-meric native hemocyanin of *L. polyphemus* by the methods successfully employed for

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the elucidation of *A. australis* hemocyanin.

Materials and Methods

Hemocyanin. Crude native 48-mer hemocyanin drawn from the horseshoe crab *L. polyphemus* was purified as previously published (Lamy et al., 1979) and was stored at 4 °C after overnight dialysis against 0.05 M Tris-HCl¹ buffer, pH 7.5, containing 10 mM CaCl₂. As published by Schutter et al. (1977), a 15-min dialysis of the 48-mer hemocyanin against 0.05 M Tris-HCl buffer, pH 7.5, plus 10 mM EDTA yielded the 24-mer half-molecule.

Electron Microscopy. Specimens for electron microscopy were prepared by negative staining with 2% uranyl acetate. The specimens were observed in a Jeol 100 B electron microscope at an accelerating voltage of 80 kV. Electron images were taken on sheets. In the microscope, the grid was oriented upside down with the carbon film facing the electron beam and the molecules facing the emulsion side of the photographic film. This procedure restores the correct orientation of the molecule on the print.

Image Processing. Molecule images corresponding to certain views of the molecule recorded in the electron microscope were quantitatively analyzed by image processing. The electron micrographs were scanned with a Perkin-Elmer flatbed microdensitometer at 50 μ m, corresponding to a sampling resolution of 0.62 nm on the object scale. For each view, roughly 100 images were selected and subjected to computer alignment (Frank et al., 1978). Correspondence analysis (Van Heel & Frank, 1981; Frank et al., 1982; Frank & Van Heel, 1982) was used to determine the presence of systematic trends in the interimage variation. Subsets of molecule images selected by this procedure were averaged and displayed with a limiting resolution of 1.7 nm.

Computer Graphical Techniques. The graphics system at the National Institutes of Health uses spheres as the primitive for representing space occupancy. Any shape can be obtained by composing an arbitrary collection of spheres. We found that twenty spheres in two planar arrays could adequately represent the shape of the protomer from the information available. Assembly of the protomer into hexamers and dodecamers was done by using rotation and translation operations to produce symmetrical copies. The rocking effect that related two dodecamers in the formation of a 24-mer was achieved by using the graphics system under human control to produce rather more arbitrary rotations and translations. In the same way, the relationship between two 24-mers in the complete assembly was achieved by various translations and rotations. The end point of graphical modeling was achieved at each stage when the space-filling representation of particular protomers were in contact but not interpenetrating.

Results

Various EM Views of *L. polyphemus* Hemocyanin. Typical views of the half-molecule and the native molecule of *L. polyphemus* are shown in Figure 1. In the half-molecule two types of views are observed, which are respectively denoted as top view and side view according to previous nomenclature (Lamy et al., 1981). In its top view, the molecule is not perfectly square but has a rhombic shape with a long and a short diagonal. The top view occurs in two mirror-related versions; if the longitudinal cleft between the dodecamers is oriented vertically, then one version has its long diagonal running from the lower left to the upper right (Figure 1a) and

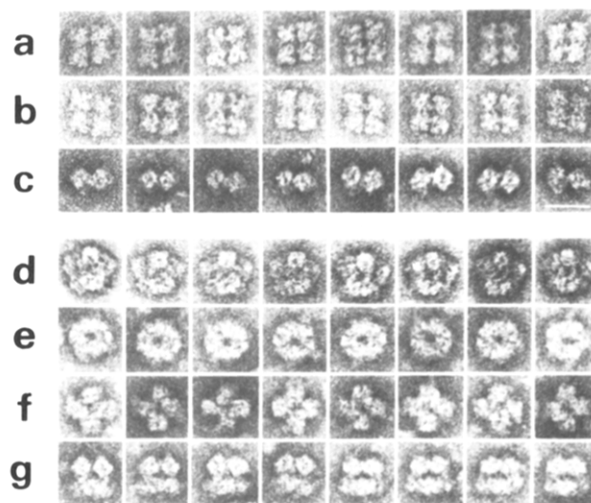


FIGURE 1: Selected views of *L. polyphemus* hemocyanin: (a–c) 24-mer hemocyanin; (a) top view of the subunit resting on its flip face; (b) top view of the subunit resting on flop face; (c) side view; (d–g) 48-mer hemocyanin; (d) pentagonal view; (e) ring view; (f) cross view; (g) two different forms of the bowtie view. The length of the bar is 25 nm.

the other from the lower right to the upper left (Figure 1b). The four types of views of the whole 48-meric native molecule are respectively denoted as pentagonal, ring, cross, and bowtie views (Figure 1c–f).

If one starts from these elements, the problem of determining the architecture can be summarized as follows. First, the architecture of the half-molecule must be characterized as precisely as possible. Specifically, the right or left enantiomer must be chosen, and the flip and flop faces [i.e., the sides of the half-molecule facing the support film in the flip and flop positions defined by Van Heel & Frank (1981)] must be characterized. Second, the relative positions of the two 24-mers in the 48-meric whole molecule must be determined. In particular, it must be determined whether the two faces of the half-molecules in contact in the 48-mer are two flop faces, two flip faces, or one flip and one flop face. Finally, a model that can account for all four distinctive views of the whole hemocyanin molecule must be built.

Choice of an Enantiomorph. Before determining which enantiomer is present in *L. polyphemus* hemocyanin, it is necessary to determine the symmetries of the different intermediates of the aggregation process. The native molecule is usually considered to be constructed from kidney-shaped subunits having one concave, one convex, and two flat faces. This subunit structure has been observed by X-ray crystallography for one subunit of *L. polyphemus* hemocyanin (Magnus, 1980) and for hemocyanin of the spiny lobster (van Schaick, 1981). The six subunits of the hexamer are considered to be in a trigonal antiprism arrangement (Figure 2) as previously shown for hemocyanin of *Panulirus interruptus*. It is clear that due to the existence of a 3-fold axis, no enantiomorph of the hexamer occurs, at least as long as the six subunits are assumed to be identical. When two hexamers are brought into contact in such a way that one of the hexamers derives from the other by translation, there is still no handedness in the resulting dodecamer. However, if one of the hexamers is rotated by 90° to the right or to the left, as indicated in Figure 2, two enantiomorphic dodecamers appear. As previously defined for *A. australis* hemocyanin (Sizaret et al., 1982), we designate as the *left enantiomorph* the structure resulting from a 90° rotation of the upper hexamer to the left. As this stage, the definition is purely conventional,

¹ Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy.

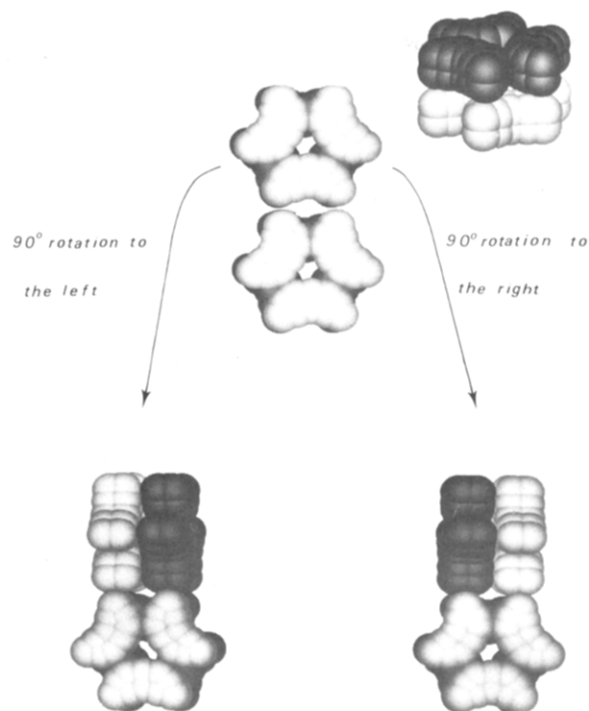


FIGURE 2: Schematic representation of the building of two enantiomorphic dodecamers.

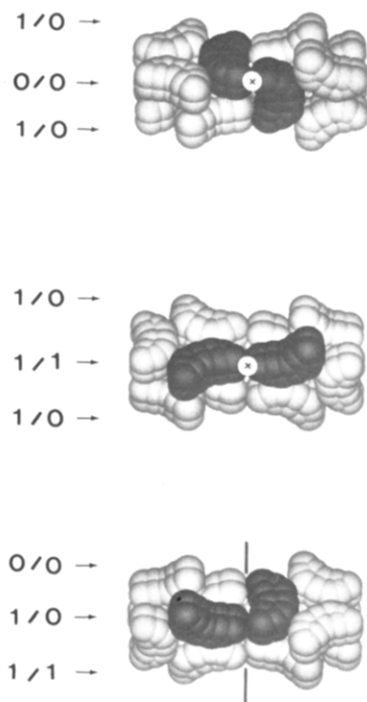


FIGURE 3: Schematic views of the four different edges in the right dodecameric enantiomorph. The basis for our nomenclature is the concave accessible faces of the two subunits located in the middle of the edge. "1" and "0" respectively mean that the considered subunit does or does not have a concave accessible face. The 2-fold axis of the dodecamer passes through the middle of the 1/1 and 0/0 edge.

but it will take on a physical significance after the formation of the 24-mer.

The dodecamer is roughly inscribed in a parallelepiped having six faces (two square and four rectangular) and twelve edges (eight short and four long). As shown in Figure 3, each long edge is closely related to four subunits, each of which has one flat accessible face. In addition, some subunits have a

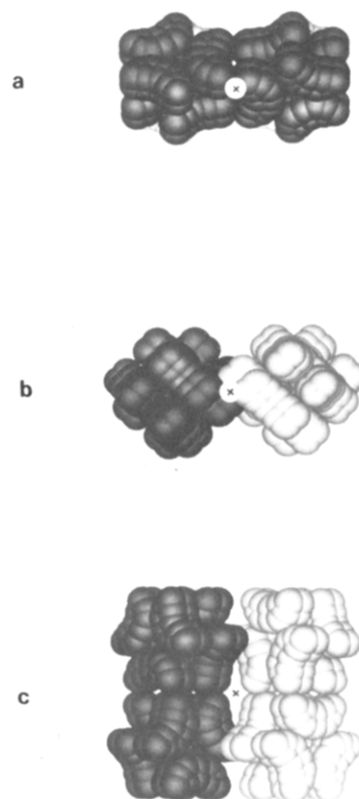


FIGURE 4: Definition of the three 2-fold axes of the 24-mer model previous to introduction of a shift between the dodecamers. The molecule is viewed (a) from the 2-fold axis of the dodecamers, (b) from the 2-fold axis passing through the bridges, and (c) from the 2-fold axis passing through the central hole.

concave accessible face. Each of the four long edges can be characterized by considering the two subunits located near the middle of this edge. The first edge, which has two central subunits with concave accessible faces, will be denoted as the 1/1 edge. The opposed long edge has no such faces in its central subunits and is denoted as the 0/0 edge. Finally, the remaining long edges both have one subunit with, and one without, a concave accessible face and will be called 0/1 edges. Since these two 0/1 edges are lying opposed in the parallelepiped, the dodecamer has a 2-fold axis, as evident from Figure 3, which passes through the middle of the 0/0 and 1/1 edges. The existence of such an axis of symmetry leads us to consider each of the enantiomorphs as a meso derivative, if we ignore the differences among the twelve subunits.

Right and left 24-meric enantiomorphs are formed when two copies of the same dodecameric enantiomorph are oriented and combined in a head-to-tail position. In this arrangement, the 1/1 edge of one dodecamer comes to lie side by side with the 1/1 edge of the other dodecamer, and the flat faces of the two central subunits of the two 1/1 edges are in contact. This arrangement is the only one leading to a symmetrical central hole and two external crevices in the neighborhood of the cleft, in good agreement with electron microscopy (Figure 1a) and other data that will be discussed later. The model now has eight long faces and four short faces. The eight faces are classified into four external and four internal faces. The external faces are those containing a 0/0 edge, and the internal ones are those containing a 1/1 edge. As shown in Figure 4, such a molecule, in either of the two enantiomeric forms,

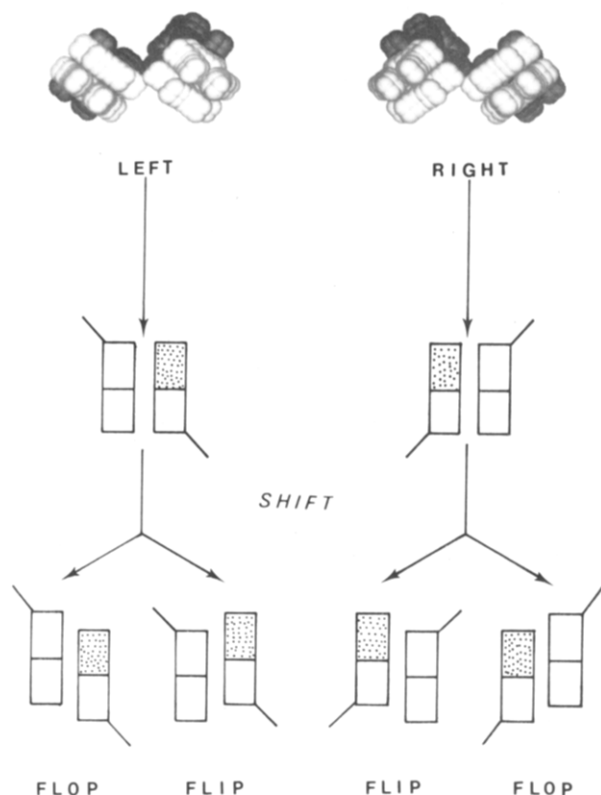


FIGURE 5: Schematic view of right and left enantiomorphs of *L. polyphemus* 24-mer hemocyanin: (upper part) analogy with the double helix for the nomenclature of the 24-mer; (lower part) orientations of the molecule's points of contact with the supporting plane. The dotted areas correspond to parts of the molecule suspended above the support plane. The diagonal lines correspond to the rocking axes.

possesses three 2-fold axes; the first one is the 2-fold axis common to the two dodecamers; the second axis passes through the two bridges between the dodecamers; and the third one, which is perpendicular to the plane of the molecule when it is in its top view, passes through the middle of the central hole. Such a model is perfectly stable when it rests on the four hexamers of which the molecule is composed.

Now we introduce into the model the rocking effect described by Van Heel & Frank (1981). For this purpose, two displacement steps are applied to one of the dodecamers. In the first step, one dodecamer is separated from the other by a translation along the 2-fold axis common to the two dodecamers. In the second step, it is rotated in order to again bring into contact the flat faces of the subunits constituting the bridges. These operations only slightly change the orientation of the three 2-fold axes of symmetry. However, the four hexamers are no longer coplanar, which thus allows the molecule to move: Of the three points of contact with the support, two are diagonally opposed. Thus the molecule can rotate around the diagonal joining these two points, alternatively bringing the third and the fourth hexamers into contact with the support plane. According to Van Heel & Frank (1981), this rocking movement explains the observed variation in the degree of staining between opposed hexamers.

The crucial point is that for the two dodecameric enantiomorphs, the orientation of the flat accessible faces of the subunits involved in the interdodecamer bridges is such that the rotation step that brings the dodecamers into contact occurs in opposite directions. As shown schematically in Figure 5, each 24-meric enantiomorph can thus be considered as a small segment of a double helix whose axis coincides with the 2-fold axis going through the bridges. The designation of enan-

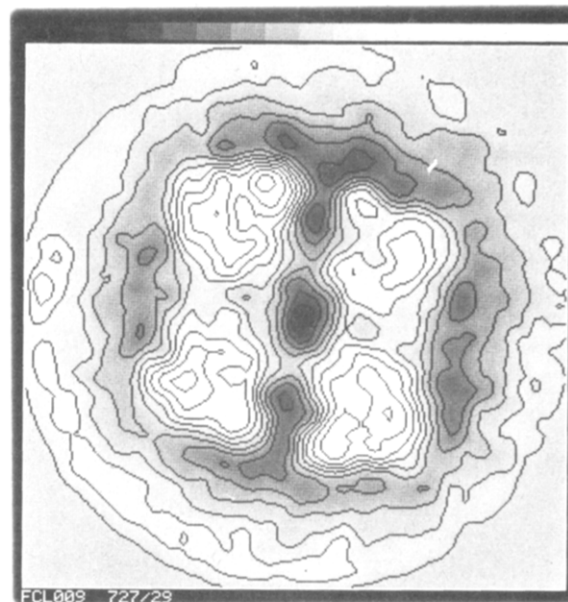


FIGURE 6: Averaged image of 24-mer hemocyanin produced by the correspondence analysis method of image processing of Van Heel & Frank (1981). The rocking axis passes through the lower right and upper left hexamers.

tiomorphs is such that the right dodecameric enantiomorph leads to a right-handed double helix and the left enantiomorph to a left-handed double helix. Figure 5 also shows that the rocking axis (the diagonal of the 24-mer around which the rocking occurs) of the left-handed derivative is the diagonal passing through the upper left and the lower right hexamers, while the rocking axis of the right-handed derivative is the diagonal passing through the lower left hexamer and the upper right hexamer. The problem of the choice of the correct enantiomorph of the 24-mer is therefore simplified to the determination of the rocking axis or to the determination of those hexamers that are permanently in contact with the support film.

Before we turn to the experimental determination of the enantiomer present in *L. polyphemus* hemocyanin, a second improvement must be introduced into the model. As mentioned earlier, the 24-mer hemocyanin is not perfectly square, due to a shift of the dodecamers with respect to one another. The shift results in the appearance of a short and a long diagonal, destroying part of the symmetry. The common 2-fold axes of the dodecamers as well as the axis passing through the bridges disappear, leaving only the 2-fold axis passing through the central hole. The existence of two different diagonals, together with the rocking effect, makes it possible to define two different faces of the molecule. We define the *flop face* as the side of the molecule in contact with the support when the rocking axis is the long diagonal. Conversely, the *flip face* is the face in contact with the support when the rocking axis is the short diagonal. Thus the molecule presents its flop view, in Van Heel and Frank's terminology, when it faces the support with its flop faces.

The choice of the correct enantiomer was based on a synthesis of data from three different sources: first, published data in the literature; second, visual examination of electron micrographs; and third, investigation of a new image set with correspondence analysis.

Because the problem was essentially to choose one of the four patterns drawn at the bottom of Figure 5, we first expected that the data published by Van Heel & Frank (1981) would have enabled us to make this discrimination. In fact,

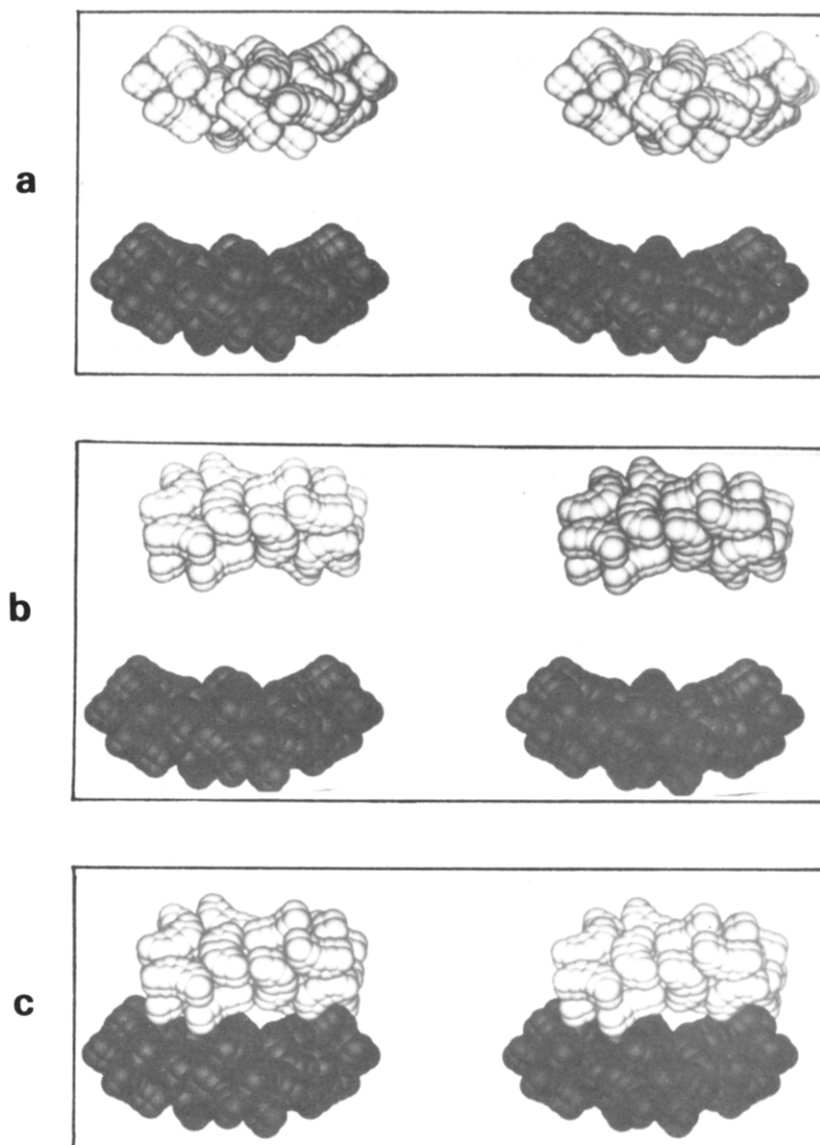


FIGURE 7: Stereo pairs of the building process of a model of 48-mer hemocyanin: (a) two 24-mer molecules are superimposed so that their 2-fold axes are aligned and their longitudinal clefts are parallel; (b) the upper molecule has been rotated to the left around the 2-fold axis in order to align its lower rocking axis with the upper cleft of the lower molecule; (c) the upper molecule has been transposed down until it comes into contact with the lower molecule.

these data clearly show the rocking axis of the 24-mer hemocyanin from *L. polyphemus* to be oriented from the lower left to the upper right, a pattern that corresponds to a right enantiomorph. However, in the same year Bijlholt et al. (1981) published images of the same molecule that showed the rocking axis to be oriented from the lower right to the upper left. There is only one probable explanation for this discrepancy: that there had been different numbers of image inversions between the object and the published print in the two experiments. Since the steps of photographic reproduction follow a general convention, the discrepancy was most likely to have been produced by the different orientations of the specimen grid in the electron microscope. Therefore, we carefully examined micrographs taken in our laboratory with the grid upside down and found that the pattern of stain behavior agreed with Bijlholt's et al. orientation of the rocking axis. To confirm these observations, we repeated Van Heel & Frank's (1981) statistical analysis with our new data. The result of this experiment (Figure 6) definitely shows that the rocking axis goes from the lower right to the upper left, meaning that the dodecameric derivative used in the assembly of the 24-mer is the left enantiomorph in our nomenclature.

Now that the enantiomorph has been determined without ambiguity, the definition of the flip and the flop faces can be restated in the following way: The flip and flop faces respectively are in contact with the support when the long diagonals are oriented to the upper right and to the upper left.

Building a Model of 48-mer Hemocyanin. The method used to build a model of the 48-mer hemocyanin is as follows. First, a model of the left 24-mer enantiomorph is positioned on a support so that it presents the top view. On this is placed another copy of the 24-mer model, so that the 2-fold axes of the two 24-mers are aligned. The upper molecule is rotated in order to align its (lower) rocking axis with the cleft of the lower molecule. Finally, the upper molecule is translated along the common 2-fold axis until it comes into contact with the lower molecule, and then it is slightly adjusted to give the best fit. This procedure, illustrated in Figure 7, yields a compact model of the 48-mer molecule perfectly consistent with ultracentrifugation data showing that the 12-, 24-, and 48-mers are successive dimers of an unobserved hexamer (Johnson & Yphantis, 1978; Jeffrey & Andrews, 1980). However, because of the difference between the flip and the flop faces, there are three ways of assembling the two halves: with a flop-flop, a

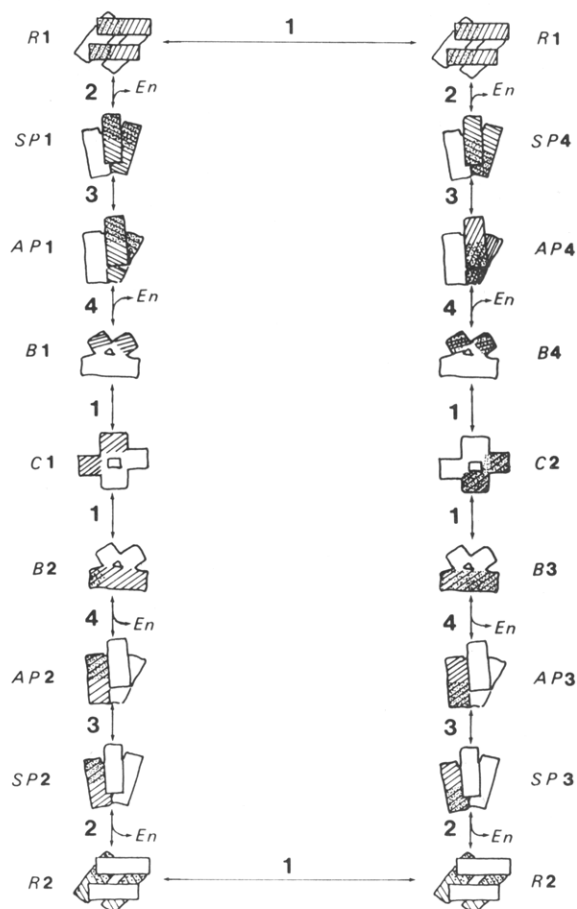


FIGURE 8: Schematic representation of different views of the model of 48-mer hemocyanin: (R) ring view; (SP) symmetric pentagon; (AP) asymmetric pentagon; (B) bowtie view; (C) cross view. The type of movement required to interconvert the views are numbered: (1) rocking; (2) rotation around 0/1 edge; (3) rotation around 0/0 edge; (4) rotation around two opposed hexamers. "En" means that the molecule must receive a push to cross the energy barrier.

flip-flip, and a flip-flop contact. We will return to this complication in a later section.

Let us consider the model resulting from the assembly of two copies of the left 24-meric enantiomorph. Figure 8 shows a schematic representation of the different possible views and the different movements of the molecule that convert one view into another. So that we could make the scheme easier to understand, one of the two half-molecules has been hatched, and two hexamers of one half-molecule have been dotted.

The view resulting from projecting the 48-mer in a direction corresponding to the top view of the 24-mer (i.e., the view that becomes immediately visible when the model has been assembled in the way described above) is a ring view, which we will call the R1 view. The rocking of the lower 24-mer on which the molecule is resting produces two closely related versions of this view. From either rocking position it is possible, by lightly pushing the model, to obtain a pentagonal view, which we will denote as the symmetrical pentagonal (SP) view. The axes of rotation that lead to the SP views are the 0/1 edges of the face on which the molecule is resting. In its SP views the molecule is standing on a single external face belonging to a single dodecamer. This is a rather unstable position, which may lead the molecule either to return to the original ring view (R1) or to further rotate around the 0/0 edge of the external face in contact with the support.

In this latter case, we obtain a new pentagonal view, with the molecule standing on three hexamers. We will call this

view the asymmetric pentagonal (AP) view, because of certain asymmetric features to be discussed later. When the molecule is in this position, it can of course return to the original SP view with which this view equilibrates, or else, it can further tilt, as when a new push is given to the model to convey it over the energy barrier.

The new position of the molecule reached from the AP position is characterized by the model standing on three square faces belonging to three dodecamers. The corresponding view is the bowtie (B1) view. From this position, the molecule is able to tilt around an axis passing through the two opposed hexamers (one from each 24-mer) in contact with the support, attaining successively positions presenting the cross (C) view and another bowtie (B2) view. This tilting movement is very similar to the rocking of the 24-mer. Indeed, of the four possible points of contact, two are permanently in contact with the support, thereby defining a rocking axis, while the other two are alternately above and in contact with the support. The intermediate position, which corresponds to the cross view, is relatively unstable because the molecule rests only on two hexamers and tips over easily, giving rise to either one of the two bowtie views.

From the B2 view to the second ring view (R2), the pathway is exactly the reverse of that leading from the first ring view to the bowtie view B1. The R1 and R2 views are exactly equivalent, but with the molecule resting on different halves. Finally, there is a return pathway from the R2 view to R1 passing through a series of views similar to those occurring between R1 and R2 but with six opposite half-molecules being upside down.

The different views of the 48-mer model were generated by computer graphical techniques (Feldmann et al., 1978). Stereographic pairs relating to each view are presented in Figure 9. These always depict the side of the molecule opposed to the side that is in contact with the support film.

When comparing the model as represented in Figure 9 with the experimentally obtained views, one should bear in mind that the representation of the model shows only the surface of the molecule, while the electron micrograph renders a projection of the stain-excluding protein material. Partial stain immersion may leave part of the molecule (the side facing away from the support) invisible or obscure. The comparison should therefore focus on the outline of the particle and on the identification of regions with exceptionally high- or low-projected protein densities. The comparison between electron micrographs (Figure 1) and views of the model (Figure 9) indicates good agreement for the ring, the cross, and the bowtie views.

In the case of the two pentagonal views expected from examination of the model (SP and AP), it was more difficult to verify the agreement, due to their close similarity. However, the existence of a distinct asymmetric view postulated by our model and linked to the choice of enantiomer makes such verification particularly important. We therefore used the method of multivariate image classification (Van Heel & Frank, 1981) to seek a definite answer. One-hundred molecule images showing a pentagonal view were scanned, automatically aligned by the computer, and subjected to correspondence analysis (Figure 10). We found a continuous distribution of the images along the two most prominent factors (with each factor corresponding to a statistically independent component of interimage variation). Molecule images falling into the four quadrants of the factorial map were separately averaged and displayed in contour representations (Figure 10).

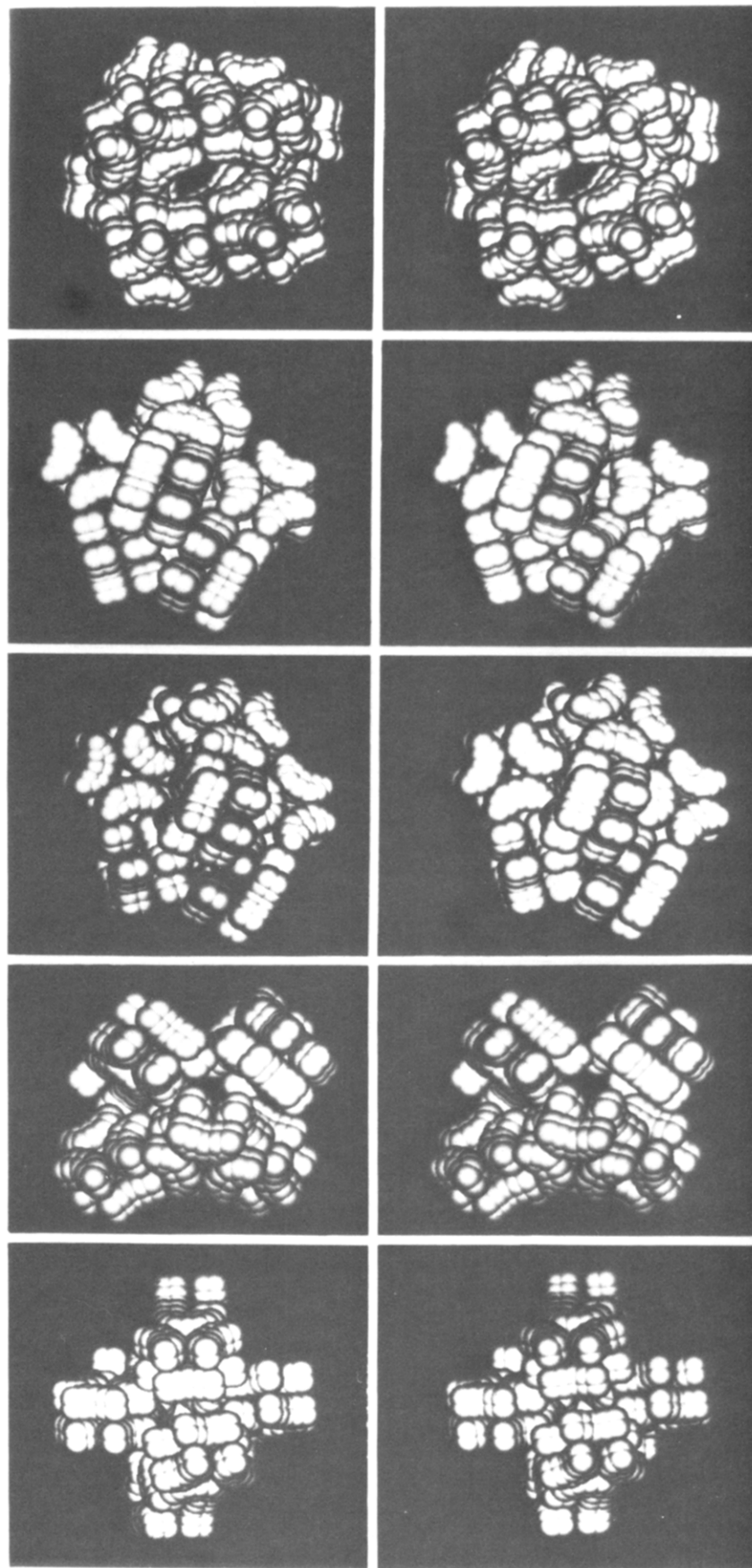


FIGURE 9: Stereo pairs of different views of a model of 48-mer hemocyanin built using four copies of the left enantiomorph: (from top to bottom) ring view, symmetric pentagon view, asymmetric pentagon view, bowtie view, and cross view.

While the meaning of the second axis (in terms of structural differences between averages on the left and the right) is not immediately obvious, the first axis clearly describes a con-

tinuous transition between the SP and the AP forms of the pentagonal view. The images showing the SP view fall in the upper part of the map, whereas those showing the AP view

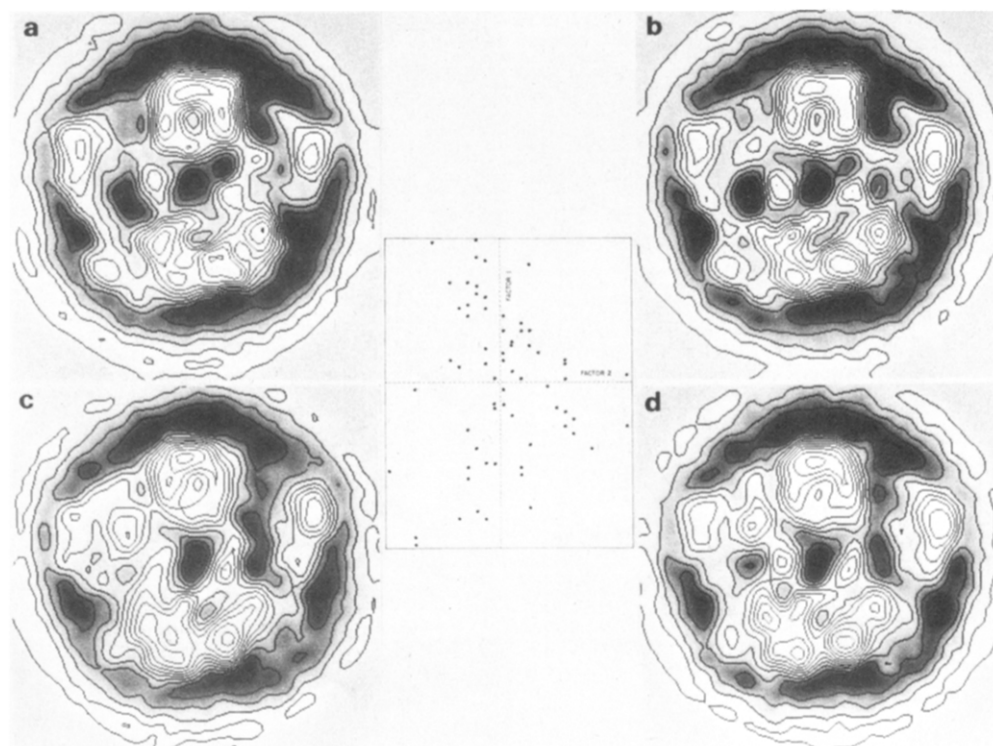


FIGURE 10: Averaged images of different pentagonal views of 48-mer hemocyanin from *L. polyphemus* hemocyanin, selected by the correspondence analysis method of image processing of Van Heel and Frank. In the center is the map showing the distribution of images along the first two factors. Only 66 of the original 103 images are shown here, 37 having been eliminated as outliers in preliminary runs of the correspondence analysis program. Along axis 1 there is a continuous transition from molecules presenting the symmetric pentagonal view (a and b) to those presenting the asymmetric view (c and d). The four averages were obtained by averaging images falling into the four quadrants of the map.

fall in the lower part. Indeed, the averaged molecules corresponding to the upper part of the map are roughly symmetrical (Figure 10a,b). Specifically, the nuclei of stain exclusion located in the upper right and upper left corners of the pentagon are almost equal in size and intensity. In contrast, the averaged images from the lower part of the map show distinctly different stain-density distributions. Among other noticeable differences, the nucleus of stain exclusion located in the right upper corner is now much more intense than that of the left one and a vertical cleft appears on the right, separating a narrow border region of the molecule from its center.

Both features make the molecule view asymmetric. It is important to note that among all molecules that we studied showing the pentagonal view, no asymmetric pentagonal view with the opposite handedness (i.e., nucleus of stain exclusion in the upper left corner stronger than that in the upper right, appearance of a cleft on the left) was ever observed. The exclusive occurrence of one type of AP view confirms our choice of enantiomer and overall assembly procedure as being correct. Indeed, the only AP view that can be obtained by tilting the model from its SP position corresponds to a position of the molecule in which the hexamer that forms its upper right corner is situated so as to produce stronger stain exclusion than its counterpart on the left (Figure 9). The fact that the stain exclusion experimentally observed is stronger on the right side therefore demonstrates that the enantiomer present is the left one (the one chosen in the building of the model). For further verification of this point, we built a model of the 48-mer using four copies of the right dodecameric enantiomer. This derivative in its AP view led to a strong stain exclusion on the left side of the pentagon, a pattern, as we noted above, that was never observed.

Examination of the average images for SP and AP views made it much easier to visually identify these views among

experimental images, once the important features had been established. For example, the first four molecules in Figure 1d are SP views, whereas the last four molecules present AP views. They can be distinguished by the difference between the stain nuclei in the left and right upper corners and by the presence or absence of the cleft formed by stain on the right-hand side.

Inter 24-mer Contact and Assembly of Full Molecule. As was pointed out before, the existence of the flip and flop faces introduces three new possibilities of isomerism. The assembly of two copies of the left 24-mer enantiomorph brings into contact the flip faces of the halves, or the two flop faces, or a flip face with a flop face. It is very difficult to discriminate between these possibilities, since the distinction between the flip and flop faces themselves results from relatively minor translations and rotations of the dodecamers constituting the 24-mer, at least as long as all subunits are assumed to be identical.

In an attempt to solve this problem, we built the three possible models of the 48-meric hemocyanin using two copies of the left enantiomorph 24-mer and compared their SP views with parts a and b of Figure 10. As shown in Figure 8, four possible views of the symmetrical pentagon may occur. They are denoted as SP1–SP4 in Figure 8 and correspond to the whole molecule standing up on the four different external faces of dodecamers, two belonging to each 24-mer.

All three models lead to very similar stain distributions in the SP views; with the molecule viewed as from underneath the support plane, the two dodecamers of the lower half, as well as the central hole that they enclose, always appear on the right side as in Figure 9b. (Note that the averages in Figure 10a,b show this hole in the expected position on the left-hand side, because here the vantage point is above the support film.) Both flip–flip and flop–flop models have in

common that the four SP views are identical. On the other hand, the flip-flop model presents two different types of SP view (SP1 = SP4 and SP2 = SP3 in Figure 8), since the accessible face of the lower 24-mer can be a flip or a flop face. The fact that little structure diversity occurs among the molecules in SP view apart from the transition into the AP view (note the close similarity between parts a and b of Figure 10 with respect to the second factor in the statistical analysis!) makes it likely therefore that we have exclusively flop-flop or flip-flop association.

Either of these possibilities is more likely than the flip-flop association for another reason, which has to do with the need for terminating the self-assembly of the molecule at the stage of the 48-mer. A flip-flop arrangement would lead to a long stack of 24-mers. However, if, as suggested by the model, one of the two faces is self-complementary, then the highest expected polymer would be composed of two copies of the 24-mer. Nevertheless the self-complementarity of one face of an homogeneous population of 24-mer molecules is not absolutely required to explain that the assembly process stops at the 48-mer level. The existence of 24-meric halves with identical architectures but different subunit compositions and/or different intramolecular locations would explain as well the termination of the polymerization, provided that some critical subunit(s) is (are) not simultaneously present in equivalent positions in both faces of the molecule.

This view is also supported by the fact that in the 24-mer hemocyanin of *A. australis*, it has been recently shown that the two copies of subunits 3A, 3B, 3C or 5B have the same orientation with respect to the flip and flop faces (Sizaret et al., 1982). For instance, the flat accessible faces of the two copies of subunit 3B of *A. australis* hemocyanin are both located on the flip face. If the subunits in *L. polyphemus* are arranged according to the same rules as in *A. australis* hemocyanin, then it is likely that homologous faces are in contact.

Distinguishing between the possibilities of flip-flop and flop-flop associations requires a detailed comparison between the alternative models in their various views and the electron micrographs. Again, we found the averaged SP views (Figure 10a,b) very informative. Focusing on the slanting of the upper two sides of the pentagon, we found a much better agreement between model and experiment with flop-flop association than with flip-flop association.

It is clear that additional evidence must be obtained to establish the flop-flop model beyond doubt. This may be found in a detailed examination of the other views, in the use of computer modeling of staining, and in the investigation of the significance of the second and higher factors in the multivariate image analysis.

Conclusions

Our present model of the quaternary structure of the *Limulus* hemocyanin molecule explains all experimental views obtained in the electron micrographs. Further refinement, and a quantitative match with experimental images, could be achieved through the introduction of a model of negative staining that takes into account the partial staining effects observed for large macromolecular structures (Frank et al., 1982).

Ultimately, a tilt series of electron micrographs presenting the molecule in a continuous range of views should be analyzed: first, to demonstrate the interconvertibility of the five distinct views that have been examined in the present study and, second, to enable a three-dimensional reconstruction of the molecule. Finally, the identification of the subunits making up the 48-mer, which have been assumed to have an identical structure at the level of resolution we have been dealing with, is expected to be accomplished soon by immunolabeling methods (Lamey et al., 1981).

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