Quaternary Structure of Scorpion (*Androctonus australis*) Hemocyanin. Localization of Subunits with Immunological Methods and Electron Microscopy†

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ABSTRACT: The quaternary structure of the hemocyanin from the scorpion *Androctonus australis* has been determined by using a method of labeling of the subunits in situ with specific Fab fragments, followed by an observation of the soluble immunocomplexes in the electron microscope. A model of the native (4 × 6)-mer molecule was built from four hexamers of kidney-shaped subunits of the type described in *Panulirus interruptus* hemocyanin [van Schaick, E. J. M., Schutter, W. G., Gaykema, W. P. J., van Bruggen, E. F. J., & Hol, W. G. J. (1980) in *Invertebrate Oxygen Binding Proteins, Structure, Active Site, and Function* (Lamy, J., & Lamy, J., Eds.) Marcel Dekker, New York (in press)]. The half-molecule [(2 × 6)-mers] was made from two *Panulirus*-type hexamers with their threefold axes perpendicular to each other and to the length direction. The 34S model contained two half-molecules in a head-to-tail disposition. The centers of the four hexamers were, however, not coplanar so that the model was able to rock perpendicular to one of the diagonal [van Heel, M., & Frank, J. (1980) in *Pattern Recognition in Practice* (Gelsema, E. S., & Kanal, L. N., Eds.) North-Holland, Amsterdam (in press)]. When antisera specific for the eight subunits of which *Androctonus* hemocyanin is built were used to precipitate the native molecule, it appeared that the subunits can be classified in two categories: internal subunits, which hardly led to precipitation, and external subunits, easily accessible to antibodies. This method was improved in two ways. Monovalent Fab fragments were used instead of divalent IgG to label the subunits, and the label was localized in the native molecule by observation of the soluble Fab–hemocyanin immunocomplexes by electron microscopy. The eight subunits were localized by this method. These results were still supported by the fact that a dimeric subunit located inside the molecule was able to self-assemble, giving a two-dimensional crystallizing network. This observation confirmed that two of these dimeric subunits constituted a central ring to which all the 20 other monomeric subunits were linked.

Hemocyanin is a copper-containing oxygen carrier found in Arthropods and Molluscs. However, this definition does not show the great difference in structure between hemocyanins from these two phyla. Mollusc hemocyanins are large cylindrical molecules, 350 Å in diameter, built from polypeptide chains with M_r 450000. On the other hand, Arthropod hemocyanins consist of one, two, four, or eight hexameric subunits, each made up of polypeptide chains with M_r ranging from 70000 to 94000. The size and shape of the different Arthropod hemocyanins have been extensively reviewed in the past decades (Wibo, 1966; Van Holde & van Bruggen, 1971; Antonini & Chiancone, 1977).

Arthropod hemocyanins can be dissociated into a mixture of monomeric and dimeric subunits by various procedures in the absence of divalent cations, such as increasing the pH or dialysis against 1 M urea. These subunits are highly heterogenous with respect to their N-terminal amino acid sequence, antigenic specificity, and electrophoretic and chromatogrphic behavior. They also have different oxygen-binding and structural properties. Thus, it is definitively demonstrated that the N-terminal sequences of three subunits of *Panulirus interruptus* (van Eerd & Folkerts, 1980) hemocyanin and of seven subunits of *Androctonus australis* hemocyanin are different (Jollès et al., 1979). Such a subunit heterogeneity probably also occurs in other Arthropod species, such as *Cherax destructor* (Murray & Jeffrey, 1974), *Jasus edwardsii* (Robinson & Ellerton, 1977), *Eurypelma californicum* (Schneider et al., 1977), and *Limulus polyphemus* (Sullivan et al., 1974; Sugita & Sekiguchi, 1975; Lamy et al., 1979b). This high degree of heterogeneity causes a considerable complication in the subunit–subunit interactions. The identification of these interactions is of great importance to understand the functional properties, in particular, the exceptionally high degree of cooperativity observed in the hemocyanin of Chelicerata. Thus, in the scorpion *A. australis* (Lamy et al., 1980c) and the spider *E. californicum* (Loewe, 1978), the Hill coefficient reaches a very high value. The first step in studying these subunit–subunit interactions is the refinement of the quaternary structure of the whole molecule and the determination of the exact position of each subunit.

Until now, the only information available on the intramolecular localization of subunits results from the reassembly of isolated subunit mixtures. It is shown, for instance, that a dimeric subunit plays a central role in the formation of structures larger than 16 S in *Cherax destructor* (Jeffrey et al., 1978), *Androctonus australis* (Lamy et al., 1977a), *Eurypelma californicum* (van Bruggen et al., 1980), and *Limulus polyphemus* (Bijlholt et al., 1979). However, it is not possible to give a more precise location of these subunits from these experiments.

High-resolution X-ray crystallography can solve this problem. This is a tremendous task. Only the structures of *Panulirus interruptus* hemocyanin (van Schaick et al., 1980) and of one subunit of *Limulus polyphemus* hemocyanin are presently studied (Magnus & Love, 1977). We therefore used a less powerful but easier method of labeling with subunit specific antibodies. This technique has previously been used with success in the localization of subunits in ribosomes (Wabl, 1974; Lake, 1978) and some other proteins (Stannard &

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Lennon, 1978; Bowien & Mayer, 1978).

In the case of *Androctonus australis* hemocyanin, the results with antibody labeling were not good enough, mainly due to instability or the large size of the formed complexes. We therefore modified the technique by using monovalent Fab fragments instead of divalent IgG (Craig & Offer, 1976; Aebi et al., 1977). This paper describes the results of this technique which lead to a considerable improvement of our knowledge about the intramolecular location of the hemocyanin subunits.

Materials and Methods

Isolation and Purification of Hemocyanin Solutions

Blood from the scorpion *Androctonus australis garzonii* was collected by cardiac puncture and centrifuged for 10 min at 800*g* to remove blood cells (Goyffon & Lamy, 1973). Hemocyanin [34 S; (4 × 6)-mer)] was isolated by gel filtration on Bio-Gel A-5m by using 50 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$. Complete dissociation of the purified hemocyanin was achieved by dialysis against 50 mM Tris-HCl, pH 8.9, and 10 mM EDTA during 24 h.

The unfractionated dissociation products are a mixture of seven subunits. Six of them are single polypeptide chains, also called "monomeric subunits". They are designated according to their mobility in polyacrylamide gel electrophoresis: 2, 3A, 3B, 4, 5A, and 6. The seventh subunit found in the mixture is made from two polypeptide chains (3C and 5B) and is therefore called "dimeric subunit" or fraction 1 according to its electrophoretic mobility (Lamy et al., 1973).

As recently reported (Lamy et al., 1980b), the 34S native molecule contains four copies of subunits 2, 4, 5A, and 6 and two copies of subunits 3A, 3B, 3C, and 5B.

The eight subunits were isolated in a chemically and antigenically pure form according to a previously published method (Lamy et al., 1979a).

Immunological Techniques

Preparation of Antisera, Immunoglobulins, and Fab Fragments. The eight subunit-specific rabbit antisera were prepared by using isolated subunits as antigen, as previously published (Lamy et al., 1979a). The specificity of the crude antisera was tested by crossed immunoelectrophoresis (Weeke, 1973) by using the unfractionated mixture of dissociation products as antigen. In most cases, the crude antisera had to be purified because they precipitated minute quantities of undesired subunits. There are two possible explanations for this phenomenon. First, the antigen given to the rabbit could be not perfectly pure, and, second, active cross-reactivity could occur due to the presence of identical antigenic determinants on different subunits. Anyhow, purification was achieved by incubating the crude antisera for 30 min at 37 °C with the smallest quantity of unfractionated mixture of dissociation products able to remove the undesired antibodies. After the incubation, the mixture was allowed to stand overnight at 4 °C, whereafter the immunoprecipitate was removed by centrifugation.

The purified subunit-specific antisera were used for the preparation of the purified IgG. For preparation of the Fab fragments, the purified IgG was treated with papain to remove the Fc fragment of the immunoglobulin. The Fab fragments were purified according to the method of Porter (1959). Finally they were dialyzed against 50 mM Tris-HCl and 15 mM NaCl, pH 7.5, and stored in the same buffer.

Determination of the Equivalency between Hemocyanin and Its Specific Fab and IgG. An estimation of the equivalency between a subunit and its specific Fab fragments or IgG was made by using a modification of the method of Loft (1975). A gel plate was covered with a layer of an agarose gel containing the antiserum specific for a given subunit. Then a trough was cut on the cathodic side of the plate as in a line immunoelectrophoresis and filled with a gel containing a known quantity of the corresponding isolated subunit. A series of wells was drilled parallel to the trough and filled with dilutions of the Fab or IgG solution. Finally, the electrophoretic migration of the subunit was started. After the completion of the migration, the soluble proteins were washed out of the gel, and the precipitate was stained with Coomassie Brilliant Blue R250. At the level of the wells, the retardation of the precipitation line was proportional to the concentration of the Fab fragments or the IgG in the deposit well. The lowest concentration of Fab or IgG able to interrupt the precipitation line was considered to be equivalent to the subunit concentration in the trough.

Line Immunoelectrophoreses. As a first approach to the intramolecular location of the subunits, line immunoelectrophoreses were performed according to the method of Kroll (1973). Native hemocyanin was put in the left half of the trough while the unfractionated mixture of the dissociation products with the same total protein concentration and previously dialyzed against Tris-HCl buffer (pH 7.5) was put in the right half. A gel containing a subunit-specific antiserum in 0.12 M barbital–barbital sodium buffer, pH 8.6, was poured in the whole plate (Lamy et al., 1980a).

Preparation and Purification of Soluble Immunocomplexes. The binding of Fab or IgG to hemocyanin was achieved by a 30-min incubation at 37 °C in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 10 mM $CaCl_2$. The reaction mixture contained 350 μg of native hemocyanin and twice the corresponding quantity of IgG or Fab fragments required to abolish the precipitation of an equivalent amount of subunit. After the incubation, the reaction mixture was concentrated by vacuum dialysis to a total hemocyanin concentration of 1.7 mg/mL. After standing overnight at +4 °C, the whole mixture was submitted to preparative thin-layer gel filtration, using an apparatus from Pharmacia. The migration proceeded in 50 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.5, in Sephadex G-150 SF at room temperature, at an angle of 15°. After separation, the excluded zone containing the soluble immunocomplexes was scraped from the gel, eluted with buffer, and examined in the electron microscope.

Reassembly of Fraction 1. For the reassembly of fraction 1, a two-step procedure was used. This involved a dialysis for 8 h vs. 50 mM Tris-HCl and 10 mM EDTA, pH 7.5, followed by a second dialysis for 8 h vs. 50 mM Tris-HCl, 5 mM $CaCl_2$, and 10 mM $MgCl_2$, pH 7.5.

Electron Microscopy. EM specimens of fraction 1 were prepared by negative staining by using the spray–droplet technique as described previously (Siezen & van Bruggen, 1974). Specimens of the soluble immuno complexes were prepared by negative stainng with 2% uranyl acetate by using the single-layer technique described by Valentine et al. (1968). The specimens were viewed in a JEOL 100 B electron microscope at an accelerating voltage of 80 kV. Electron images were taken on Kodak 4463 sheets.

Results

Architecture of the Native Molecule. For the intramolecular localization of the subunits, the architecture of the molecule must be known. Therefore, we build a model on the basis of the information available. Our best knowledge of *Androctonus* hemocyanin structure results from electron microscopy. Figure 1 presents an EM picture of *Androctonus*

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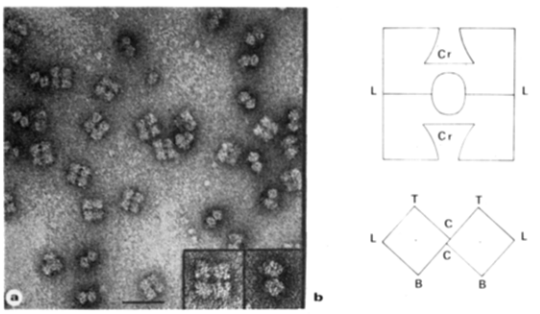

FIGURE 1: (a) Electron micrograph of *Androctonus australis* hemocyanin. The bar represents 50 nm. (b) Schematic views of *Androctonus australis* hemocyanin showing the salient features of the molecule. C = cleft; Cr = crevice; B = bottom edge; T = top edge; L = lateral edge.

hemocyanin and a drawing giving the salient features of the molecule, which should be taken into account when building the model.

Two kinds of images are seen. One is roughly square and flat and corresponds to the top view of the molecule, while the other consists of two squares attached to each other at a corner and is surrounded by a thick layer of metal stain. This represents a side view of the molecule. The other side view is never observed, probably because the molecule is unable to stand up on the carbon film in this orientation and falls down to give the flat square structure.

The tope view consists of two halves separated by a cleft. These two halves are linked by two bridges across the cleft, delimiting a symmetrical central hole and a small crevice on each side of the molecule. These crevices are more narrow near the side of the molecule than near the bridge. A half-molecule in turn is divided into two substructures, each corresponding to a hexamer. The side view also shows the two halves, now being presented by a square. Notice that each square has a small central hole.

Recent progress in the analysis of EM images has been reported by van Heel & Frank (1980) for the 34S form [(4 × 6)-mers] of hemocyanin from the horseshoe crab *Limulus polyphemus*, a molecule closely related to *Androctonus* hemocyanin. Firstly, they were able to distinguish two "top views", which they called "flip" and "flop". These two "top views" can be distinguished because their profiles are slightly rhombic due to skewing of the two halves parallel to the cleft. This skewing occurs in two ways with manual mirror symmetry. Their second important result is that the centers of the four hexamers are not coplanar. Since the (4 × 6)-mers of *Androctonus* and *Limulus* hemocyanin look very similar, we will, to a first approach, integrate the data observed by van Heel and Frank in our model.

Another important element is the shape of the subunits and of the hexamer. All the previously published models used spherical subunits (Klarman et al., 1979; Lamy et al., 1980b; Markl & Kempter, 1980). The fit of these models with electron microscopy is poor. Recently, van Schaick et al. (1980) reported on the basis of X-ray crystallography that the subunits of *Panulirus interruptus* hemocyanin are asymmetric, with a shape looking like a kidney or a bean (Figure 2a).

Such a kidney shape has also been observed by X-ray crystallography on subunit II of *Limulus polyphemus* hemocyanin (K. A. Magnus and W. E. Love, personal communication). Thus, the kidney shape of a subunit is probably a general character of Arthropod hemocyanins and will therefore be incorporated in our model of *Androctonus* hemocyanin. Van Schaick et al. (1980) also reported that the hexamers of *Panulirus* hemocyanin occur as a triangular antiprism with

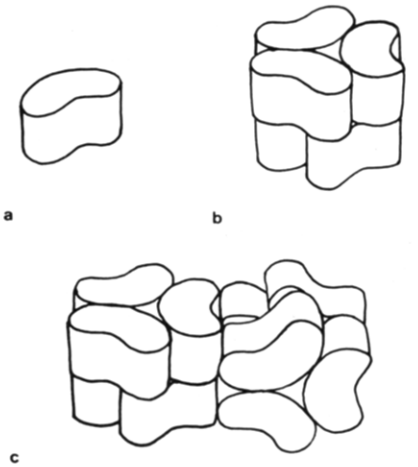

FIGURE 2: Schematic view of the hypothetic constituents of native *Androctonus australis* hemocyanin. (a) Subunit, (b) hexamer [(1 × 6)-mers], and (c) (2 × 6)-mers.

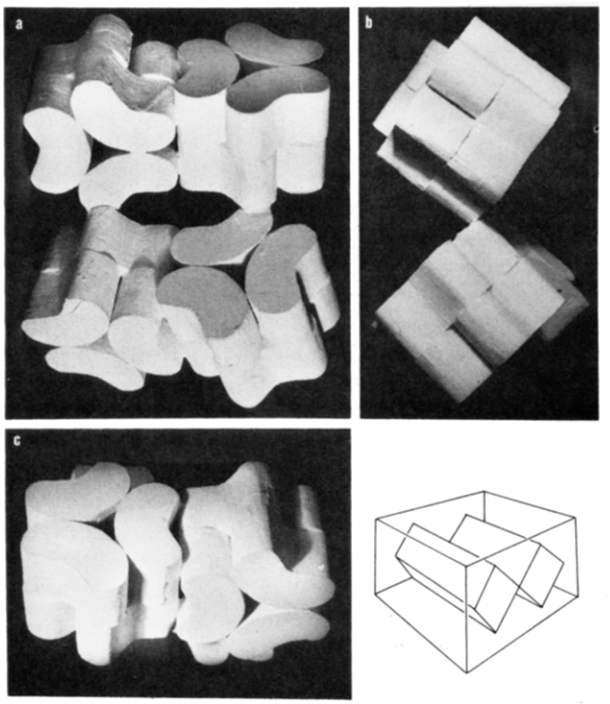

FIGURE 3: Model of the architecture of native hemocyanin from *Androctonus australis*. (a) Top view, (b) side view, and (c) side view never observed in EM.

point group symmetry 32 (Figure 2b).

All these observations are incorporated in our models for the 24S and 34S molecules.

The 24S model is made from two *Panulirus*-type hexamers with their threefold axes perpendicular to each other and to the length direction. This (2 × 6)-meric model (Figure 2c) fits the electron microscopy nicely, which usually shows a combination of a rosette and a rectangle for 24S hemocyanin. This still leaves us with two ways for the assembly of two hexamers into a half-molecule. These two types of half-molecules are enantiomorphic.

The 34S model must contain two of these (2 × 6)-mers. From the many possibilities of assembling the different enantiomers, only the arrangement shown in Figure 3 seems to fit all our requirements. Two identical (2 × 6) enantiomers are parallel to each other, with the threefold axes of the hexamers at angles of about 45° with respect to their support. The centers of the hexamers are not coplanar; the model can rock perpendicular to one of the diagonals.

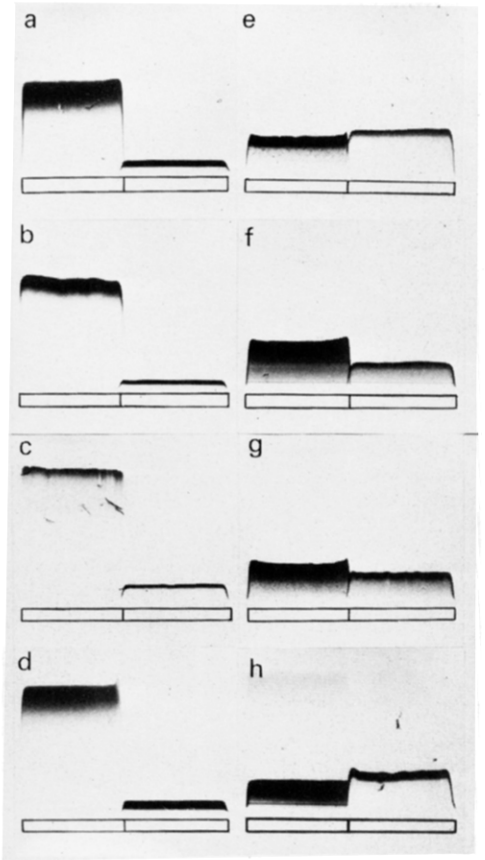

FIGURE 4: Line immunoelectrophoresis of native hemocyanin (left trough) and of subunit mixture (right trough) using antisera raised against the eight subunits of *Androctonus australis* hemocyanin. (a) Anti-3A serum, (b) anti-3B serum, (c) anti-3C serum, (d) anti-5B serum, (e) anti-2 serum, (f) anti-4 serum, (g) anti-5A serum, and (h) anti-6 serum.

We are presently unable to decide which of the two enantiomorphic whole molecules corresponds to native hemocyanin. All the work presented in this paper has been done with one model, but it is clear that all the results can equally well be applied to its enantiomorphic form.

For indication of the IgG and Fab binding sites, we have to distinguish between the different areas and edges of the model. We therefore define two lateral edges, two top edges, and two bottom edges. These edges correspond to the corners marked L, T/B, and B/T in the drawing of the electron microscopic side view of the 34S molecules (Figure 1). On the electron microscopic top view, only the lateral edges are seen running parallel to the cleft.

Labeling of the Subunits. (*a*) *Line Immunoelectrophoresis.* The principle of the method was to precipitate the native hemocyanin by an antiserum specific for a single subunit. It was expected that subunits buried inside the molecule would not easily precipitate while the external subunits would. The free subunits (unfractionated mixture of the dissociation products) were taken as a reference. Line immunoelectrophoresis was chosen to test the precipitation because it allows for a quantitative analysis of the precipitation patterns. For each subunit specific antiserum, the result thus appears as the comparison of the migration distance of two line patterns: one for the native and one for the dissociated molecule. Figure 4 shows that the subunits of *Androctonus australis* hemocyanin can be classified into two groups, one with comparable migration distances (2, 4, 5A, and 6) and one with a very high migration distance of native hemocyanin compared to that of dissociated hemocyanin (3A–C and 5B). This phenomenon

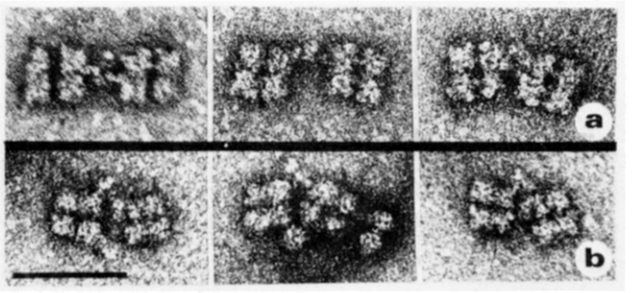

FIGURE 5: Electron micrographs of soluble immunocomplexes between *Androctonus australis* hemocyanin and antibodies specific for (a) subunit 3B and (b) subunit 4. The bar represents 50 nm.

was interpreted by supposing an external localization of the subunits 2, 4, 5A, and 6 and an internal localization of the other subunits. This explanation is supported by the fact that subunits 3C and 5B, that constitute a very stable dimer designated as fraction 1, are in the same group.

(*b*) *Labeling with Antibodies.* The line immunoelectrophoresis experiments shown in the last paragraph demonstrate that the immunoprecipitation occurs differently when antisera specific for different subunits are used. This clearly indicates that the antibody molecules bind to subunits differing in their accessibility.

It was therefore expected that different immunocomplexes between hemocyanin and antibodies could be seen in the electron microscope and that the precise location of the binding site of the antibody would indicate the location of the corresponding subunit. Experiments were performed to visualize such complexes with the eight specific antisera.

With antisera raised against subunits 3B and 4, we obtained (Figure 5) weak indication that subunit 4 is located near the corner of the molecule and that subunit 3B is located near the middle of the lateral edges of the molecule. This result seems not to be in agreement with the results of immunoprecipitation experiments. No clear-cut results were obtained with the other six antisera. This was partly due to the distortion or disruption of the complexes formed and partly to the fact that the technique using rabbit antisera in antigen excess leads to the formation of large insoluble immunocomplexes, while EM needs small soluble complexes.

(*c*) *Labeling with Fab Fragments.* After we saw that the method of labeling hemocyanin with antibody was practicable, we tried to improve the results by suppressing the precipitation. We decided to use monovalent Fab fragments instead of divalent IgG, which has the advantage that it retains the ability to bind to antigenic determinants while the capacity of precipitation is lost. The results are shown in Figure 6.

As a general conclusion, we can say that the different Fab fragments bind to specific areas of the molecule and that in many cases these areas can be identified with great precision. A second conclusion is that within the group of the four external subunits (2, 4, 5A, and 6), the bound Fab fragments always project from the periphery of the molecule. This is seen on the top view for subunits 2, 4, and 6 and on the side view for subunits 5A and sometimes 4. It is most evident in the case of subunit 6 where several Fab fragments are seen at the four corners of the same molecule (Figure 6a,f). This is a direct proof that the native molecule contains four copies of subunit 6, a result in perfect agreement with previous experiments (Lamy et al., 1980b). Figure 6c clearly demonstrates that two of the four copies of subunit 2 are localized just in front of each other at the end of each half-molecule near the cleft. In a few cases, a third Fab molecule is visible on the opposite side near the cleft. However, we never observed Fab

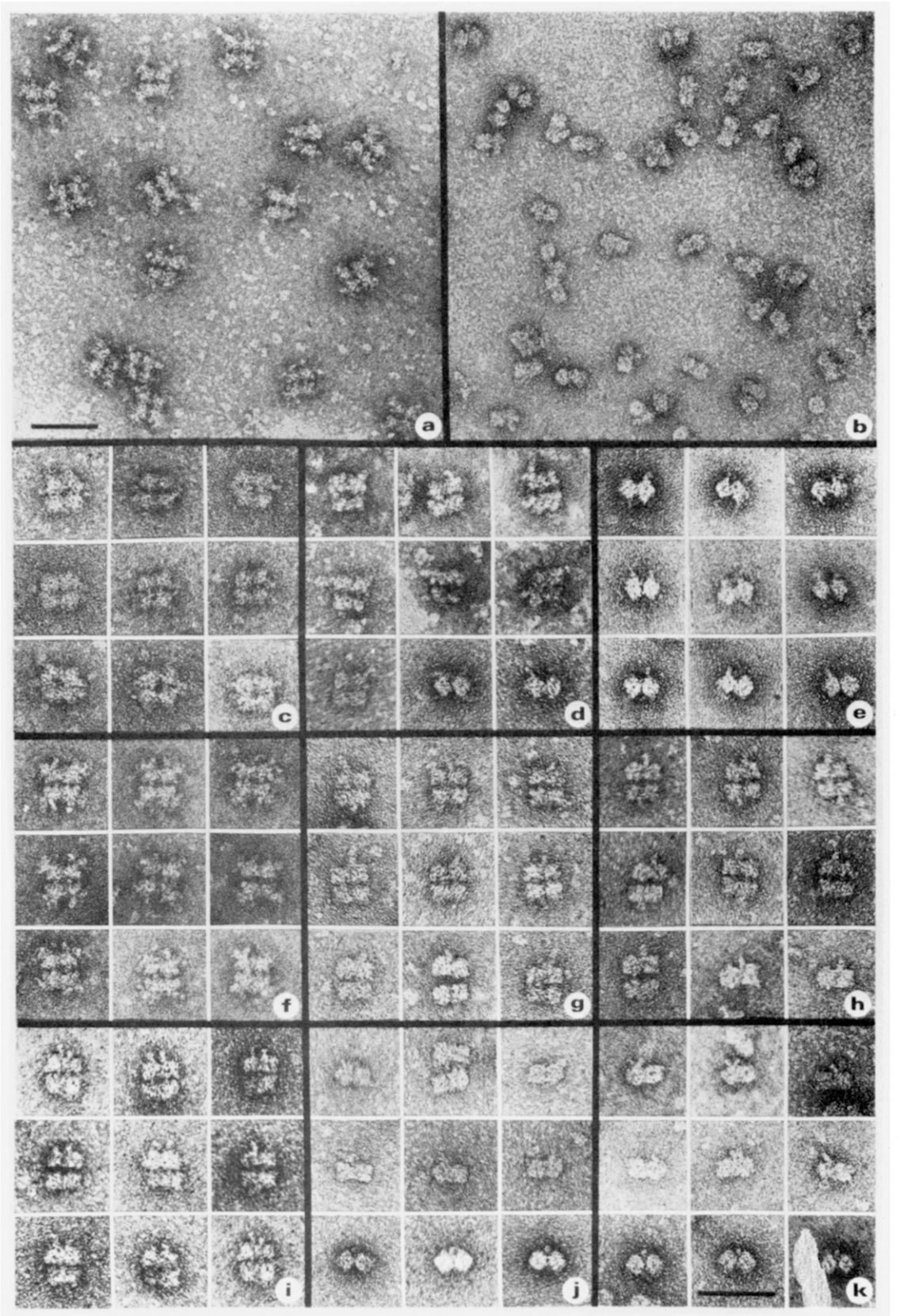

FIGURE 6: Electron micrographs of soluble complexes resulting of the binding of Fab fragment specific for isolated subunits to *Androctonus australis* hemocyanin. The bar is 50 nm. General view of hemocyanin molecules labeled with (a) anti-6 Fab and (b) anti-5B Fab. Photogallery of hemocyanin molecules labeled with (c) anti-2 Fab, (d) anti-4 Fab, (e) anti-5A Fab, (f) anti-6 Fab, (g) anti-3A Fab, (h) anti-3B Fab, (i) anti-3A Fab + anti-3B Fab, (j) anti-3C Fab, and (k) anti-5B Fab.

fragments on four different sites of the molecule simultaneously. A likely explanation is that, because of the fact that the four hexamers are not coplanar, the fourth Fab fragment is out of the stain and therefore not visible. That Fab 2 is attached to both sides of the molecule is also supported by the absence of side views on electron micrographs, an observation also made for Fab 6 (Figure 6a,f). In the case of Fab 4 (Figure 6d), both the side view and the top view are present, meaning that the Fab 4 fragments are not bound to the end of the half-molecule. It is rather surprising that in most cases only

one Fab 4 fragment is seen on the same molecule, either on the side view or on the top view. This may bear a relation to the observation that in the group of the so-called external subunits, subunit 4 leads to the highest ratio of migration distances (see Figure 4f). With subunit 5A (Figure 6e), the Fab fragments are mostly seen pointing out from the top edges on the side view of the molecule. In the order of decreasing frequency, we observe one Fab fragment bound, two Fab fragments bound on one side, two fragments on opposite sides, and rarely three fragments bound on two sides of the molecule. It is also frequently seen that the cleft is filled with material which is probably a Fab fragment.

With the so-called internal subunits (3A–C and 5B), it was expected that Fab either would not bind to the molecule due to masked antigenic sites or would bind to internal areas such as the bridges or the cleft.

In fact, such patterns were observed frequently only in the case of subunit 3C (Figure 6j). With Fab 3C, two characteristic images were observed. The most frequent one is the filling of one side of the cleft in the side view of the molecule. Many of these kind of images have been observed, but we never found one in which both sides of the cleft were filled. This is another argument in favor of the existence of two different faces of the molecule, as was already suggested by the flip–flop theory. Subunit 3C is accessible from only one side of the molecule. The second frequently encountered pattern is a 24 S [(2 × 6)-mers] with an Fab fragment bound to the rosette. In some cases we observed two half-molecules, each with a Fab fragment bound, lying close together. These two halves clearly resulted from the dissociation of the same molecule. The position of the Fab fragments clearly indicates that the two copies of subunit 3C are located in different halves. Turning now to subunit 5B, the second component of the dimeric fraction 1, we observed the same images (Figure 6b,k). A great difference with subunit 3C is the high frequency of half-molecules with an Fab attached to it. These two results demonstrate that the bridges between the half-molecules consists of a link between a subunit 3C belonging to one half and a subunit 5B belonging to the other half of the molecule.

In the case of subunits 3A and 3B, the situation is very different (Figure 6g,h). Contrary to what was expected, the Fab fragments are seen pointing out from the lateral edge on the top view. In many cases, two Fab fragments were seen slightly aside from the middle on each lateral edge. The patterns from 3A and 3B were exactly the same, except that many more complexes were observed with 3B than with 3A. Such patterns could result from the occurrence in the anti-3A serum of an antibody specific for a single determinant of subunit 3B. As discussed later, such a contamination is not unlikely due to the method of antiserum preparation. In order to test this possibility, we repeated the experiment by using a mixture of anti-3A and anti-3B sera. It was expected and indeed observed (Figure 6i) that two Fab fragments would bind near the middle of the same lateral edge. This experiment disproves the possibility of a contamination of both anti-3A and anti-3B sera.

Two-Dimensional Crystallization of Fraction 1. Fraction 1, consisting of subunits 3C and 5B, appears to play a central role in this hemocyanin molecule. Application of the two-step reassembly procedure to fraction 1 alone resulted in a two-dimensional crystalline network (Figure 7). This phenomenon was not observed for other subunits. This result demonstrates that fraction 1 is capable of self-reassembly and that at least three binding sites for another fraction 1 molecule occur on each molecule. In the model of the next paragraph, the bridges

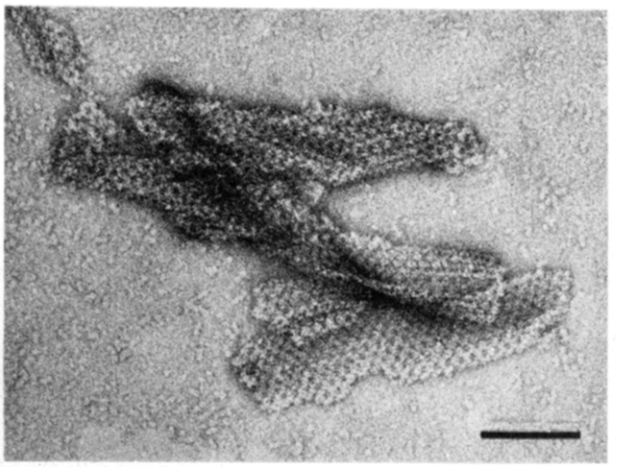

FIGURE 7: Electron micrograph of fraction 1 after two-step reassembly procedure. The bar is 100 nm.

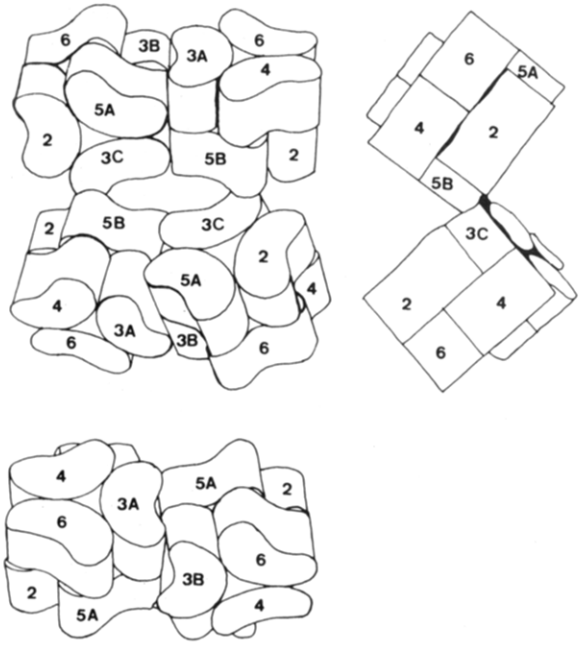

FIGURE 8: Model of the intramolecular localization of the subunits of *Androctonus australis* hemocyanin. The numbers refer to the subunit numbering described under Materials and Methods.

result from the binding of two fraction 1 molecules to each other. This requires for each fraction 1 molecule two sites of self-reassembly. The capability of two-dimensional crystallization shows that this condition is fulfilled.

The Model of Androctonus Hemocyanin. In a previous section, we presented a model for the structure of *Androctonus* hemocyanin. We will now attribute a name to each individual subunit of this model (Figure 8).

The core of the molecule is a ring made from two molecules of the dimeric fraction 1 (3C + 5B) in a head-to-tail orientation which is leading to two different faces. To explain this two-sidedness, let us define the four faces of each subunit. We distinguish two flat faces, the upper face and the lower face, and two curved faces, one convex and one concave (Figure 2a). For every subunit, one flat face and the convex face are engaged in the contact with other subunits of the same hexamer. In addition, for subunits 3C and 5B, a part of the second flat face is used to form the bridge. Thus, each copy of the subunits 3C and 5B has only the concave face and part of a flat face accessible. In the head-to-tail disposition, the concave faces of the two copies of, for instance, subunit 3C are ac-

cessible from the same side of the molecule and the partially free flat face from the other side. The reverse situation holds for subunit 5B. This implies that an antigenic determinant located on a specific face, e.g., the concave face of a given subunit, will be accessible only from one side of the molecule. This is exactly what we see in the side views of Figure 6j,k. The head-to-tail association is also clear from Figure 6j, showing two halves of one molecule lying close together, each with an Fab fragment bound. This direct demonstration of the existence of two different faces of the hemocyanin molecule is a confirmation of the flip–flop theory of van Heel & Frank (1980). However, it is not yet known which subunit is 3C and which is 5B.

The four corners each contain one subunit 2, one subunit 4, and one subunit 6. Subunit 6, the most external, is situated on the lateral edge. Subunits 2 and 4 are located close to the cleft, subunit 2 being at the end of the half-molecule while subunit 4 is near the top or bottom edges.

The central part of each half-molecule contains four subunits, two copies of 5A and one copy each of 3A and 3B. The two copies of 5A are situated, one in each hexamer of the half-molecule, on opposite top and bottom edges, respectively. The two remaining places in the middle of the half-molecule are occupied by subunits 3A and 3B.

The model has a twofold axis of rotation perpendicular to the plane of the four hexamers.

Discussion

Comparison of the Results from Immunoprecipitation and Immunoelectron Microscopy. Immunoelectron microscopy provides a good explanation to the patterns observed with line immunoelectrophoresis and vice versa. The precise location of some subunits by immunoelectron microscopy allows the following general conclusion to be drawn from the results of Figure 4. Incorporation of a subunit in a native molecule reduces the number of antigenic determinants accessible for hemocyanin binding antibodies. The possible causes of this decrease are the masking of the determinants by neighboring subunits or a disappearance as a result of a conformational change due to the incorporation. Further clustering of the different copies of a subunit in the same molecule can strongly reduce the number of antigenic sites *simultaneously* accessible. Finally, the higher the number of antigenic sites simultaneously accessible on the same native hemocyanin molecule, the lower is the migration distance of the precipitation line in line immunoelectrophoresis. This effect reaches a critical degree when the number of simultaneously accessible antigenic sites falls below three (or two in the case of long linear immunocomplexes).

The examination of the migration distances of the line with the antisera specific for subunits 3A, 3B, 3C, and 5B, respectively (Figure 4), shows that they are all very high compared to the free subunit line. It appears now that these four lines are high for different reasons. In the case of 3C and 5B, it is clear that the subunits are located in the cleft and thus not easily accessible. They are therefore really internal subunits. This explains that the ratio of the migration distances of the precipitation lines was only slightly modified when a mixture of anti-3C and anti-5B sera was used instead of anti-3C or anti-5B serum, as reported in a previous paper (Lamy et al., 1980a). On the contrary, subunits 3A and 3B occupy rather external positions in the middle of the half-molecule. However, as there are only two copies of each of them per hemocyanin molecule, it is likely that only two molecules of IgG can bind simultaneously and thus prevent the growth of a precipitate. This is supported by the fact that, in many cases, only one Fab fragment is seen on each lateral edge of the molecule.

With the external subunits (2, 4, 5A, and 6), the best fit between the immunoprecipitation and the immunoelectron microscopy was observed with subunit 6. In a previous paper, it was suggested on the basis of immunoelectrophoresis that "the four copies of subunit 6 occupy external positions, i.e., one copy at each corner of the whole molecule" (Lamy et al., 1980a). Immunoelectron microscopy confirms this conclusion, showing that subunit 6 is effectively present at the four corners of the molecule. The agreement in the other three cases is not so good as for subunit 6. Two and sometimes three copies of subunits 2 and 5A and one copy of subunit 4 are seen in the native molecule instead of the expected four copies. This discrepancy can be explained in several ways. First, the Fab fragments are bound but not visible because they do not project out of the outline of the molecule. This phenomenon may be amplified by "rocking" of the four noncoplanar hexamers. For subunit 4, this provides an acceptable explanation for the fact that only one Fab is seen on the molecule. Second, the number of accessible antigenic determinants in the whole molecule causes limitation to the binding of Fab fragments. Such a reduction of the number of antigenic sites is probable for subunit 4, because the ratio of the native hemocyanin line to the free subunit line is highest with this subunit (Figure 4). This implies that each copy of subunit 4 can only bind a few Fab fragments. The same explanation holds to a lower degree for subunits 5A and 2. A third explanation is that no Fab is bound to several antigenic sites, although they are accessible, due to the fact that these kind of Fab molecules have been removed during the purification of the specific antiserum.

Integration of Reassembly Data in the Model. It has been previously shown that in a reassembly experiment in the presence of calcium subunits 1, 2, 3B, and 5A are essential for the reconstruction of (4 × 6)-mers (Lamy et al., 1977a, 1980b). The requirement for these subunits suggests that they play a specific structural role and that they can replace subunits 3A, 4, and 6 at least from a structural point of view. The proposed model shows that subunits 1, 3A, 3B, and 5A are engaged in the interhexamer contacts either between two half molecules (subunit 1) or between the two hexamers of each half-molecule (subunits 3A, 3B, and 5A). Reassembly experiments from an unfractionated mixture of subunits show that 3A is not incorporated into the (4 × 6)-mers while 3B is incorporated in a double amount (Lamy et al., 1980c). Since 3B is able to replace 3A, we conclude that three of the four essential subunits (1, 3B, and 5A) occupy important positions in the interaction site between the four hexamers. That the fourth essential subunit, 2, can fill the position of subunits 4 and 6 in addition to its own is supported by the following data. Hexamers can be formed from mixtures of the subunits 2 + 4, 2 + 6, 4 + 6, and 2 + 4 + 6. The hexamers from this last mixture are more stable than the others and show a tendency to adhere to each other in twos and threes (Lamy et al., 1977b). This is a strong argument in favor of the existence of a trimer at the corners of the molecule. However, it remains unclear why subunit 2 can replace 4 and 6 while subunits 4 and 6 are unable to replace subunit 2 in the reassembled (4 × 6)-mers.

Conclusion

In this model, we have defined some molecular areas and localized all the 24 subunits within them. To reach a higher degree of precision in the localization, we need to know with accuracy a center of asymmetry. This procedure is analogous to the preparation of heavy metal derivatives for X-ray

crystallography. The four so-called internal subunits (3A, 3B, 3C, and 5B) seem all good candidates for this role.

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