Van Heel M, Keegstra W, Schutter W, van Bruggen EFJ:

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In: *Life Chemistry Reports, Suppl.* 1, "The Structure and Function of Invertebrate Respiratory Proteins", EMBO workshop, Leeds 1982, Ed. E.J. Wood, pag. 69-73

ARTHROPOD HEMOCYANIN STRUCTURES STUDIED BY IMAGE ANALYSIS

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The various Arthropod hemocyanins (large, copper containing oxygen-binding proteins, Mw 450 000 to $\sim 4.10^6$) form a 'building block' family of oligomeric proteins with the hexameric unit as elementary building block.[4] The structure of the Panulirus interruptus hemocyanin, consisting of just one hexameric unit, was solved by X-ray diffraction methods to a resolution of $\sim$4Å.[2] The structure consists of six bean-shaped monomers in P_{32} arrangement. Fig 1a shows a projection through the low-pass filtered (7Å) X-ray electron density data along the 3-fold axis. The diameter of the molecule is $\sim$110Å. Fig 1b shows a projection through only the top half of the molecule with projections of three individual 'beans' visible. Fig 1c is the result of an image analysis pilot study of EM images of the same protein negatively stained with uranyl acetate. All image analysis was done with the IMAGIC software package.[5] Of 33 computer-aligned[3] single molecular images, the 18 best were selected based on correspondence analysis[1] results. These formed a compact class,[10] ie they look 'more alike' than the remaining molecules. The 18 images of the 'purified' subpopulation were summed, and the sum was 3-fold rotationally averaged. The resulting image was filtered to enhance the relative contributions of the higher frequency components, emphasising finer details (fig 1c). Fig 1d resulted from averaging a 2D crystal of the same hemocyanin ($\sim$200 unit cells). This image was not rotationally averaged. A rough comparison of projections indicates very many details in common. But there are minor differences, which tend to disappear if a weight function is applied during projection through the X-ray data, which gives a higher weight to peripheral areas of the molecule, thus stimulating the accessibility of such areas to the stain during EM preparation. Detailed comparative study of X-ray and EM data with different stain models is underway.

A complication in comparing images is that visual comparison provides no objective measure of similarity. At the same time the (objective) correlation coefficient is not a reliable measure, tending to be dominated by the lower frequency components. Hence we designed the so-called Fourier-ring correlation function (FRC) which calculates the correlation coefficients per radial frequency component (R) separately. Let F_1 and F_2 be the Fourier transforms of the aligned images f_1 and f_2. Then:

$$FRC(R) = \sum_{RingR} F_1 \cdot F_2^* \Big/ \sqrt{\sum_{RingR} |F_1|^2 \cdot \sum_{RingR} |F_2|^2}$$

in which the summations are over rings in Fourier space at a distance R from the origin. It can be shown directly that the FRC function is a real function as f_1 and f_2 are real. This measure was inspired by the 'phase residual' function[3] which is also measured along rings in Fourier space. A genereralization of the FRC function to the 3D case, to compare two 3D reconstructions, is obvious. But the FRC as defined here will only measure isotropical 'similarity' and might thus need redefinition for the 3D case, in which the resolution in the third dimension is typical lower.

A need for better understanding of the negative strain is apparent in our very first comparison. If we assume the carbon-foil side of the molecule to be preferentially stained, the average image (fig 1c) would mainly show the lower half, where it really looks more like the top half of the X-ray reconstruction, fig 1b. Thus we have an enantiomeric conflict! The single hexameric images agree well with the averaged double-hexameric structures (fig 2,3). These 2x6-mers are a dissociation product of the Limulus polyphemus hemocyanin.[4]

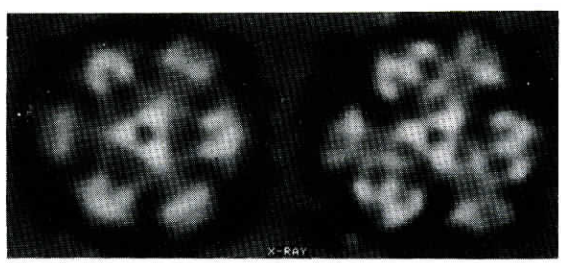

Figure 1a

Figure 1b

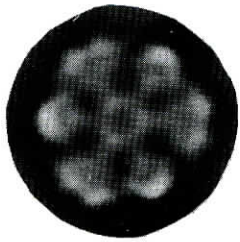

Figure 1c

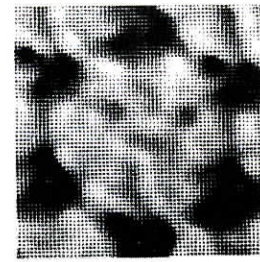

Figure 1d

Computer alignment, correspondence analysis[1] and subsequent classification[10] over 170 molecular images led to the map of factor 1 vs factor 3 in fig 2. There are only two main images classes (types A and B in fig 2). The long shape of the molecule did not, as expected, lead to a continuous 'rotation' of the molecule on the support film.[9] The interpretation of the A and B type views (face-up and face-down positions of the same structure!) is shown in fig 3, with 90° rotations between two frames. The 4x6-meric structure was already studied extensively[1,7] but its 2x6-meric components are in 45° rotated position relative to the single 2x6 molecules (fig 2,3), making it difficult to determine the exact position of the 2x6-mers in the 4x6 projections. We have, however, found another stable position of the 4x6-meric structures which we call the '45° position' (fig 4). This position is interesting because: (1) it gives direct evidence that the four hexamers in the 4x6 structure are not co-planar such that the 'rocking' behaviour found earlier[1,7] is indeed a very real phenomenon;(2) the 2x6-mers are seen 'face-on' and can thus be correlated to our knowledge of the 2x6 structure;(3) this 4x6-meric projection can immediately be identified in the projections of the 8x6-meric native Limulus hemocyanins ('pentagonal' views[8]), an important clue in determining the 8x6 structure;(4) the molecular images can be used to determine the absolute hand of the 4x6 structure.

After alignment, correspondence analysis and classification, the map of fig 5 was obtained. Four classes are seen: class 1 is thought to have a 'flip-flop'[1] relation to class 3; class 4, is thought to be the same as class 1, however 'rocked' slightly out of its most extreme position (fig 4); the relation between classes 2 and 3 is thought to be equivalent. From determination of the absolute hand it followed that the earlier models[1,6] showed a wrong enantiometric form. This has serious consequences[6] as the antibody labelling experiments interpretation cannot be simply 'transposed' t' another, mirror-related, enantiomer! But

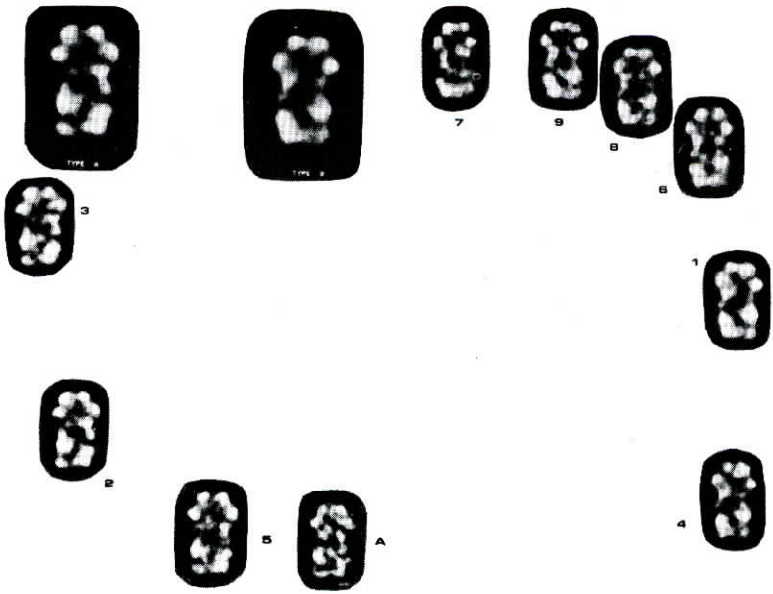

Figure 2.

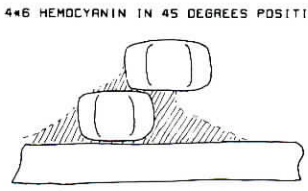

Figure 4.

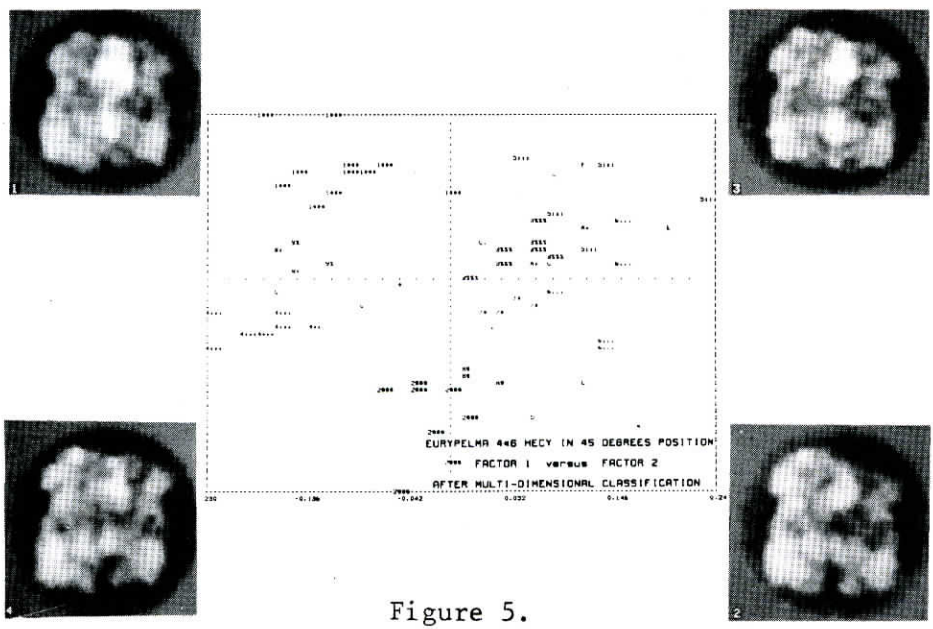

Figure 5.

even with the correct 'rocking' direction, enantiomeric forms remain possible and we hope to solve this problem in the near future.

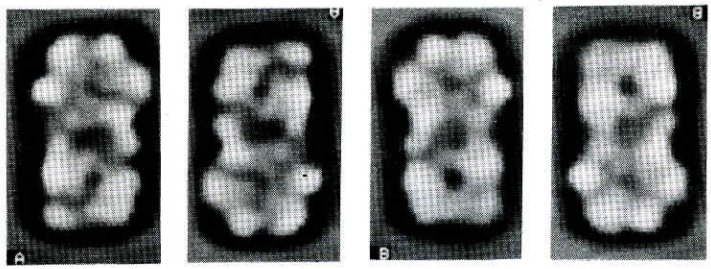

Figure 3

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