Comparative electron microscopy and image analysis of oxy- and deoxy-hemocyanin from the spiny lobster *Panulirus interruptus*

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Structural differences between oxy-hemocyanin and deoxy-hemocyanin from the spiny lobster *Panulirus interruptus* were studied by electron microscopy and image analysis of negatively stained preparations. Projections of the hexameric *Panulirus interruptus* hemocyanin from electron microscopy were compared with simulated projections of the high-resolution structure determined by X-ray crystallography previously. Image analysis was focused on the hexagonal views. Four independent data sets of hexagons (two of oxy- and two of deoxy-preparations) were analyzed and compared. Despite the small size of the projections, a resolution in the average images of better than 1.5 nm was obtained. A significant difference in stain modulation could be noticed between the oxy- and deoxy-preparations in two areas of the projections. The first difference in stain exclusion occurred in the center of the hexagonal views. The second is the shape of the domain 3 region. In the deoxy-images this region has a triangular shape, in the oxy-images it appears to be more rounded. The overall differences suggest a "breathing" of the hexamer upon oxygenation/deoxygenation.

1. Introduction

Hemocyanin (Hc) serves as oxygen transporter in the hemolymph of certain invertebrates. It is capable of binding oxygen reversibly and co-operatively. The co-operativity can be influenced by addition of certain allosteric effectors like H^{+}, Ca^{2+}, Mg^{2+}, Cl^{−}, lactate. A great difference in assembly exists between Hc from mollusca and arthropoda. Molluscan Hc is assembled into drum-like structures while arthropodan Hc occurs as hexamers which further associate into multi-hexamers [1–3]. The Hc from the spiny lobster *Panulirus interruptus* is of the arthropodan type and exists as single hexamers in its hemolymph. This Hc can bind oxygen co-operatively and consists of 3 subunits termed a, b and c, which differ in their amino acid sequence [4,5]. Subunits a and b are almost identical with 97% sequence identity, whereas subunit c has 59% sequence identity with a or b. The X-ray structure was determined from crystals that consisted exclusively of subunits a and b [6].

The Monod–Wyman–Changeux model [7] describes co-operative substrate binding theoretically by assuming that a multimeric molecule has at least two states in which the affinity for a substrate is high and low and that the equilibria are linked. Between these states a conformational change in one of the subunits induces a similar structural alteration in neighbouring subunits. This change occurs in a concerted mechanism. In the transition between them, the symmetry of the molecule would be conserved [8].

The conformational alterations upon oxygen binding for the four-hexamer Hc of the tarantula *Eurypelma californicum* were determined by Decker et al. [9] to be approximately 2 nm, using small-angle X-ray scattering. In principle, such large structural differences are observable by electron microscopy and image analysis. In order to test this hypothesis the hexameric Hc of *P. interruptus*...
interruptus was taken as object of this study. Some clear differences between the oxy- and deoxy-preparations could be detected. These were correlated with the X-ray structure.

2. Material and methods

2.1. Isolation and purification

The spiny lobster Panulirus interruptus was purchased from Pacific Bio-Marine Laboratories Inc. (Venice, CA, USA). Hc was isolated and purified as described previously [5,10].

2.2. Preparation of specimens for electron microscopy

An atmos-bag (SIGMA) filled with pure nitrogen was used for making the specimens in an oxygen-free environment [11]. All solutions used (buffer, negative stain, Hc solution) were made oxygen-free by flushing with pure nitrogen for 3 h. Before preparation the Hc solution was checked spectrophotometrically for absence of the copper–oxygen peak at 340 nm. The optimal Hc concentration for electron microscopy was achieved by dilution of the Hc solution to approximately 0.1 mg/ml with 50mM Tris-HCl buffer pH 8.5 containing 10mM CaCl₂, 10mM MgCl₂. Specimens were prepared by the spray-droplet technique with 2% ammonium molybdate pH 8.5 as negative stain. The grids were left drying in the glove box overnight until use.

The oxygen-containing Hc was prepared in air (usual preparation conditions). The presence of the copper–oxygen complex in the Hc was confirmed spectrophotometrically (broad peak at 340 nm).

Micrographs were taken at 75 000 × with minimal exposure procedures using a JEOL 1200 EX microscope with an acceleration voltage of 80 kV.

2.3. Image processing and image analysis

Electron micrographs were taken from 2 grids originating from a deoxy-preparation and 3 grids from an oxy-preparation. Different grids were used in order to minimize variations caused by the preparation procedure. Micrographs were checked for optimal defocus, minimal astigmatism and lack of drift. The micrographs were digitized with an Kodak EIKONIX CCD-camera with 25 μm step size corresponding to a pixel size of approximately 0.3 nm at the specimen level.

About 4000 molecular projections were selected from the oxy/deoxy-micrographs and grouped arbitrarily into two sets of oxy- and two sets of deoxy-images. The image format of the selected projections was 48 × 48 pixels. All the images were aligned rotationally and translationally by cross-correlation methods to a single common reference by multiple-alignment steps [12,13] by using the IMAGIC program package [14]. Afterwards, the original images were oriented in just one interpolation step by the so-called equivalent rotation and translation procedure. From each of these four image sets the first 183 images with the highest cross-correlation value were used to create a single set of 732 images in which the first 366 were from oxy-preparations and the last 366 from deoxy-preparations. To investigate structural differences in the 2 nm range the images were treated by a bandpass filter which allowed image details to pass from 30 nm to 1 nm. In this way the influence of large stain modulations and small defocus differences on the statistical analysis and classification was suppressed. Each image was provided with a narrow mask around the hexagons and three-fold averaged.

Multivariate statistical analysis (MSA) [15] and hierarchical ascendent classification (HAC) [16] was then performed on this set of 732 oxy/deoxy-images.

2.4. Simulation of average images

The X-ray structure from P. interruptus hexameric Hc [6] was used to calculate hexagonal projections of the hexamer. Only Cₐ-atom x,y,z positions were used in this calculation with a radius of 0.7 nm. The density outside the Cₐ-atom spheres was 0; inside it linearly decreased from 100 to 0 along their radius. By alignment the calculated projections were rotated in the same orientation as one of the average images from fig.
Fig. 1. Electron micrographs of *P. interruptus* Hc: (a) From a preparation in air; (b) from a preparation in an oxygen-free environment. Specimens were stained with a 2% ammonium molybdate solution at pH 8.5. The bar indicates 50 nm.
4. Stain height was simulated by taking into account only those atom positions below a certain distance from the support film. It is assumed that protein parts which are not embedded in the stain do not contribute to the projection seen in the electron microscope. The calculated images were treated with a bandpass filter which suppressed image details below 1 nm and above 30 nm in accord with the average images.

3. Results and discussion

We investigated structural differences between oxy-Hc and deoxy-Hc from *P. interruptus* by image analysis of negatively stained preparations. This study was stimulated by the work of Decker et al. [9]. They detected structural differences equivalent to 2 nm rearrangements with small-angle X-ray scattering for *E. californicum* four-hexameric Hc under oxy- and deoxygenated conditions. In principle such differences can become visible also by image analysis of negatively stained molecules. The hexameric *P. interruptus* Hc was chosen by us because the projections seen in the electron microscope are easy to interpret. The differences, if found, would not be obscured by orientation problems of the molecules on the carbon support, such as “flip/flop” and “rocking”, which are characteristic of four-hexamer Hc [17,18]. Another advantage is the availability of its X-ray structure [6].

The micrographs of *P. interruptus* Hc in both oxy- (fig. 1a) and deoxy-form (fig. 1b) show the hexamer in hexagonal and rectangular views. The hexagonal views have a diameter of 12 nm, whereas the rectangular views show a short edge of 9 nm and a long edge of 12 nm.

Image analysis was focused on the hexagonal views. The rectangular views were not analysed because of considerable variations in orientation of this view. The hexagonal view is much more stable because the molecule rests on its flat side. The structure of the *P. interruptus* Hc subunit is organized into three domains, i.e. 1, 2 and 3 [6]. This terminology is used by us also for the hexagonal projections of this Hc. In the hexagonal projections regions can be discriminated in which the same domains are superposed. Fig. 2b shows the position of these superposed domains indicated by the domain number in Roman style.

One of the most crucial steps in analysis of single-particle projections is the choice of the reference. In some cases a strong influence of the reference on the result of the alignment was found [12,13]. However, in the case of Hc from *P. interruptus*, the alignment parameters were not very dependent on the reference choice. Thus one reference was used for iterative alignment of the data set. The resolution in the average images of both the oxy- and deoxy-set was determined by Fourier ring correlation [19,20] to be better than 1.5 nm as shown in fig. 3. This resolution is one of the best ever obtained for single particle averaging and to our knowledge the best obtained so far for a molecule of the size of *P. interruptus* Hc. Possibly, the preservation of the conformational integrity in the relatively thick stain layer, the very even stain distribution in a broad zone around the molecules and the molecule attached to the carbon support on its flat side are factors that positively influence the resolution.

Due to overlap in the hexagonal projection it is difficult to interpret visible details in terms of structural features which are known from the high-resolution X-ray structure. But on the periphery there is less overlap and the X-ray structure shows a protrusion consisting of amino acids 536–559. This protrusion contains helix $\alpha3.3$ and a loop of about 10 residues. In the hexagonal projection of the X-ray structure (figs. 2a and 2b), this protrusion was taken into account at four positions (straight arrows) and omitted at two positions (bent arrows). To our surprise, the protrusions are also somewhat visible at the corresponding positions in the hexagonal average images from electron microscopy (fig. 2c). The local density at the position where the protrusions can be expected is just somewhat higher than for the average background. It should be noted that no symmetry was imposed and that nevertheless the protrusions are seen in all positions where it could be expected. The three-fold average shown in fig. 2d still shows the same features. This demonstrates that small details (mass 2 kDa) can be visualized under optimal
negative staining conditions. This finding also gave us the confidence that differences between oxy- and deoxy-sets at the 1.5 nm level, if present, can be detected in principle and that it might be possible to relate them to local differences in the Hc structure caused by oxygenation.

Fig. 2. The *P. interruptus* Hc X-ray structure supports the interpretation of details in the EM-projections into structural features. (a) The main chain of the polypeptide represented by bonds between $C_{\alpha}$ atoms only. The straight arrows point at the protruding $\alpha_{3.3}$ helix. The bent arrows indicate the two $\alpha_{3.3}$ helices which are removed to check their absence in the simulation. (b) A calculated projection of the X-ray structure at low resolution. The $\alpha_{3.3}$ protrusions are visualized by the contour lines around the hexagonal projection. The removed $\alpha_{3.3}$ helices are indeed not observable in this simulation. The Roman capitals indicate the regions where corresponding domains superpose in this projection. (c) An average projection of the first 50 best images from the reduced image set. Contour lines show more clearly the $\alpha_{3.3}$ protrusions. (d) A three-fold average of the projection in c.
To detect possible differences between oxy- and deoxy-states, a further comparison of aligned images of the hexagonal view was performed by multivariate statistics (MSA) and classification (HAC).

Prior to MSA the aligned images were three-fold averaged. The mechanism of co-operativity is operating in a concerted way [7,8] on a time scale much faster compared to the time scale on which specimens were prepared. Therefore, we do not expect to find molecular images in which the hexamer is caught in between the low- and high-affinity states and thus three-fold averaging is allowed.

Inspection of the MSA maps and the importance images [17,21] showed us that the first two factorial axes 1 and 2 did discriminate the set at best. A weighting of the first 2 factorial axes by 1 and the others by 0.5 was performed and this led to the classification map shown in fig. 4. After MSA the combined oxy/deoxy-set of 732 images was grouped into 6 classes in order to discover the oxy/deoxy-features in the images. The number of six classes is a compromise between maximal number of classes and a minimal number of 100 members per class. The MSA map shows an arrangement along the diagonal into classes A to F. Analysis of the oxy-/deoxy-image content of the classes (table 1) shows a decrease of oxy-images from 75% (class A) to 19% (class F). The resolution determined by Fourier ring correlation for the 6 classes separately remained better than 1.5 nm. Figs. 4a–4f show the class-average images in the decreasing order of their oxy-image content. We will focus on the extremes of this arrangement. Both extremes show images of equal size, which indicates that the differences between the particles of both sets cannot be related to deformations such as a flattening which can be caused by surface tension effects during the specimen preparation. Both images show an overall difference in modulation of image contrast. Clearly different are the centers of these images. In fig. 4a the central stain spot is not as dark as in fig. 4f. From the X-ray structure we know that a channel is present in the hexamer running along the three-fold axis from one flat side to the other.

**Table 1**
The six classes with the corresponding figure, the number of images per class and the fraction of images originating from a preparation in air

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Members/class</th>
<th>Oxy-images (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>127</td>
<td>75</td>
</tr>
<tr>
<td>4b</td>
<td>116</td>
<td>70</td>
</tr>
<tr>
<td>4c</td>
<td>124</td>
<td>60</td>
</tr>
<tr>
<td>4d</td>
<td>119</td>
<td>50</td>
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<tr>
<td>4e</td>
<td>130</td>
<td>27</td>
</tr>
<tr>
<td>4f</td>
<td>116</td>
<td>19</td>
</tr>
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</table>

Fig. 3. The Fourier ring correlation curve shown with the images from which it was obtained. The diagram shows that the resolution is better than 1.5 nm by the crossover point between the Fourier-ring correlation curve (continuous line) and the threshold curve (dotted line) as indicated by the arrow.
Fig. 4. Image analysis of the best 732 images of the combined oxy- and deoxy-Hc data set. In the classification step, the data set was grouped into 6 classes. Weighting of the factors 1 and 2 by 1.0 and 3 through 8 by 0.5, resulted in this map. Images, represented by letters, indicate to which class they belong. The asterix ( * ) roughly marks the center of a class cluster. Below this map the six class-average images are presented in order of their oxy-image-fraction.
The wall of this channel is mainly formed by six helices which are termed $\alpha 2.3$. This suggests local rearrangements in the central part of the hexamer, but with rather fixed outer areas. This reminds us of the iris aperture in a camera, which opens and closes, not altering the outer dimensions. Another distinction between these extremes is the shape of the region indicated as III in fig. 2b. In fig. 4a it looks rounded whereas in fig. 4f region III is of triangular shape. In fig. 4f

![Fig. 5. A simulation of the stain exclusion profile of the hexagonal projection from *P. interruptus* hexamer Hc. The X-ray structure [6] was used to calculate projections of the hexamer in the same orientation as in figs. 4a–f. An idea of the influence of stain height on the projection is obtained from image (a) to (i), where the stain level decreases from 100% to 50% of the height of the hexamer. In this simulation it is assumed that protein parts that are not embedded in the stain do not contribute to the projection.](image-url)
region III seems more loosely connected to the rest of the protein than in fig. 4a, judging by the amount of stain in between region II and III.

In order to obtain better insight into the way the amount of negative stain in and around the particles influences the appearance of the projected hexamer, a set of hexagonal projections with a decreasing height of stain embedding was calculated from the X-ray structure. Fig. 5 shows how the hexagonal stain exclusion profile alters in projection, starting from the fully embedded hexamer (fig. 5a) towards a “stain height” of 50% (fig. 5i) in steps decreasing by 7%. Lowering the stain height causes gradual disappearance of the mirror planes running through the projection, as indicated by the white arrows in fig. 5a. After checking for the presence or absence of a mirror plane in the average images of figs. 4a–4f it can be concluded that the hexamers were embedded close to 100%. This means that the differences present in the average images of fig. 4 might, only to a small extent, be the effect of differences in stain height. The difference in shape of region III and the central hole of the hexamer (which is present in all the simulated images of fig. 5) are hardly influenced by variations in the level of negative stain and may therefore correspond with structural changes caused by oxygen binding.

Comparison of the average images of fig. 4a (oxy-state) and fig. 4f (deoxy-state) with the calculated projections of fig. 5 shows a large correspondence between fig. 4f and fig. 5b. From this we conclude that the X-ray structure of the P. interruptus Hc represents the hexamer in the deoxy-state. The difference in stain modulation between fig. 4a and 4f can be explained by slight changes in the orientation of the subunits and the domains with respect to each other. The overall movement in the hexamer during oxygenation/deoxygenation reminds us of the “breathing” as observed earlier for a mollusc Hc by Van Breemen et al. [22].

4. Conclusions

Image processing with multivariate statistical analysis of molecular projections of native P. interruptus Hc prepared under oxygen-containing and oxygen-free conditions for electron microscopy showed that it is possible to discriminate between oxygenated and deoxygenated Hc projections.

Comparison with the X-ray structure shows that in the average images a very small feature, the helix α3.3, can be observed as a slight protrusion at the periphery of the hexagonal projection.

The large correspondence of the calculated projection to the average image obtained from the oxygen-free preparation led us to the conclusion that the oxygenation state of the published P. interruptus Hc X-ray structure might be deoxy.

It was shown, by comparison of average images to molecular projections calculated at different stain heights from the X-ray structure of P. interruptus Hc, that the hexameric molecule was embedded in the stain layer close to 100%. This seems to be advantageous for obtaining the highest resolution for negative staining.

In analogy to the oxygenation studies performed on molluscan Hc we observed also a “breathing”-like movement in arthropodan Hc.

Acknowledgements

We dedicate this article to Professor E. Zeitler on the occasion of his 65th birthday and are grateful for his interest in our studies during the many years of his scientific leadership. We thank Drs. F. Perton and J.J. Beintema for their kind donation of lobster hemocyanin and Drs. B. Hazes and W.G.J. Hol for making the X-ray structure coordinates available to us. This study was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

References