Aquaporin-0 (AQP0) is the most prevalent intrinsic protein in the plasma membrane of lens fiber cells where it functions as a water selective channel and also participates in fiber–fiber adhesion. We report the 3D envelope of purified AQP0 reconstituted with random orientation in phospholipid bilayers as single particles. The envelope was obtained by combining freeze-fracture, shadowing and random conical tilt electron microscopy followed by single particle image processing. Two-dimensional analysis of 2547 untilted images produced eight class averages exhibiting “square” and “octagonal” shapes with a continuum of variation. We reconstructed in 3D five class averages that best described the data set. The reconstructions (“molds”) appeared as metal cups exhibiting external and internal surfaces. We used the internal surface of the mold to calculate the “imprints” that represent the AQP0 particles protruding from the hydrophobic core of the phospholipid bilayer. The complete envelope of the channel, formed by joining the square and octagonal imprints, described accurately the size, shape, oligomeric state, orientation, and molecular weight of the AQP0 channel inserted in the phospholipid bilayer. Rigid body docking of the atomic model of the aquaporin-1 (AQP1) tetramer showed that the freeze-fracture envelope accounted for the conserved transmembrane domain (~73% similarity between AQP0 and AQP1) but not for the amino and carboxyl termini. We suggest that the discrepancy might reflect differences in the location of the amino and carboxyl termini in the crystal and in the phospholipid bilayer.

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Keywords: aquaporin-0; aquaporin-1; freeze-fracture; electron microscopy; envelopes, surface topology

Introduction

The ancient aquaporin family is comprised of channel proteins that select for small, non-charged but polar molecules, such as water and glycerol. The atomic structures of aquaporin-1 (AQP1) and the glycerol facilitator (GlpF) have been determined by diffraction methods. These studies demonstrate that the functional unit of both aquaporins is a bundle of six transmembrane alpha helices surrounding a conducting pore comprised of vestibules connected by a narrow 2-nm long selectivity filter. The particular arrangement of charged amino acid residues at the vestibule of the pore and the arrangement of non-polar amino acid residues along the selectivity filter can explain in a simple and elegant manner how AQP1 and GlpF select for water and glycerol.

The availability of the atomic structures opens the possibility of applying freeze-fracture electron microscopy to study aquaporins in membranes, an environment where their functional state, location of flexible domains and interactions with other proteins of the cell can be determined. Freeze-fracture is based on a conspicuous property of frozen membranes: mechanical stress propagates down the middle of the lipid bilayer producing complementary protoplasmic (P) and external (E) fracture faces. The hydrophobic center appears as a smooth surface and the proteins spanning the lipid bilayer (“integral” proteins) as distinct

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particles. A thin layer of heavy metals (the "replica"), deposited by evaporation, provides the relief (the "outer shell" or "envelope") of the domain protruding from the hydrophobic core (i.e. one-half of the protein molecule). In contrast to negative staining and cryo-electron microscopy methods, freeze-fracture and metal replication can be easily extended to study functionally characterized channels in cells.\textsuperscript{8–12} Attempts in determining the molecular envelope of “single” particles by shadowing methods have been limited,\textsuperscript{21,22} with the exception of envelopes calculated from soluble macromolecules, whose surfaces have been exposed by evaporation ("etching") of frozen water.\textsuperscript{23} Conventional thinking has long hypothesized that problems in specimen preparation as well as the intuitive interpretation of images are mostly responsible for the failure in calculating the envelope of functional single freeze-fracture particles in phospholipid bilayers.

Here, we applied freeze-fracture, random conical tilt (RCT) electron microscopy\textsuperscript{24} and computer image processing to calculate the envelope of aquaporin-0 (AQP0) reconstituted in phospholipid bilayers. The untilted images show class averages exhibiting two principal views ("square" and "octagon") with a continuous variation. The 3D imprints calculated from these class averages described the halves of the channel protruding from the hydrophobic core of the phospholipid bilayer. The complete envelope of the channel, formed by joining the two halves, predicted the size, shape, oligomeric state, and molecular weight of the AQP0 channel. The envelope fitted the conserved transmembrane domain but not the amino and carboxyl termini of the atomic model of the AQP1 tetramer.\textsuperscript{4} This discrepancy might reflect differences in the position that the more flexible regions of the channel adopt in crystal and in the lipid bilayer.

Results and Discussion

Freeze-fracture and shadowing

To prepare replicas describing the molecular envelope of AQP0 inserted in the lipid bilayer, it was necessary to resolve problems arising from the local curvature of the liposomes, the angle of deposition, as well as the preferential nucleation ("decoration") that results from metal interaction with the sample.\textsuperscript{25,26} The local curvature of liposomes made the exposed surface of the channel difficult to shadow completely and also provided images representing a range of orientations rather than the 0\degree views. We solved these problems by fracturing larger (~2 \mu m diameter) liposomes attached to a glass surface (Figure 1).\textsuperscript{27} This simple maneuver produced flat lipid monolayers that were easier to shadow and contained only freeze-fracture particles corresponding to the domains of the channel protruding from the hydrophobic core of the bilayer. The complementary “pits”, representing the impression that these domains made in the ice, partitioned in the monolayers were discarded during fracturing.

The preparation of the replicas also depended on the angle at which the metal was evaporated on the fractured surfaces. When shadowed at a fixed angle (unidirectional), the AQP0 channels
appeared as particles composed of a black region measuring 8.5±0.6 nm (n = 62) in diameter and a conical-shaped light region (the "shadows") extending from it (Figure 2(A)). Low-angle rotary shadowing, a method that rotates the specimen in the x–y plane during shadowing, eliminated shadows only partially because parts of the channel screened others ("self-shadowing"), which led to regions of the molecule without or with considerably less metal. We minimized self-shadowing by rotating the specimen along the x–y–z axes during evaporation ("multi-axis shadowing"). Using this method, the particles measured 9.0(±0.4) nm (n = 35) in size and, more importantly, many of them exhibited square shapes (Figure 2(B)) rather than the cone-shaped images resulting from the unidirectional shadowing method. The AQP0 measures 6.4 nm in size.19 Therefore, the increase in the dimension of the freeze-fracture particle was consistent with a platinum–carbon replica of 1.3(±0.2) nm in thickness, or composed of three to four metal grains only.11,12

The replica was also influenced by intrinsic properties of the substrate, such as affinity of the metal to specific sites as well as the presence of ions in the vapor, their energies, the rate of incidence, the temperature of the specimen and the vacuum conditions.16 The interplay of this complex set of conditions influenced image formation by a combination of pure shadowing and decoration phenomena.25,26 Pure shadowing represents the relief or molecular envelope of the particle. In contrast, decoration reflects physico-chemical properties of the fractured surfaces, such as, for example, the presence of bulky side-chains and charged groups. Here, we eliminated decoration by depositing a thin layer of carbon prior to metal shadowing.28 We determined experimentally that a thin carbon pre-coat layer (two seconds) produced the most reliable molecular envelopes of AQP0.

In summary, we prepared replicas that described the envelope of AQP0 by freezing and fracturing liposomes attached to glass surfaces, by using multi-axis shadowing and by depositing a thin pre-coat carbon layer prior to shadowing.

**Imaging conditions**

Reconstitution of AQP0 in liposomes imposes several constraints on the images of single particles gathered by freeze-fracture methods. First, AQP0 is oriented randomly in the lipid bilayer of the liposome ("molecular flipping") and thus, it produces images of particles viewed from the external or cytoplasmic domains of the channel. Second, the fracture process exposes the domain of the channel protruding from the hydrophobic core of the bilayer. Third, the fact that liposomes were fractured attached to the glass produced 0° views of the cytoplasmic or external domains of the AQP0 channel ("preferential orientation").

We used "RCT" to collect images sampling a suitable set of viewing directions for 3D reconstructions.24,29 RCT requires untilted (Figure 2(B)) and high-angle tilted (Figure 2(C)) views of the same particle. When the replica is tilted, the viewing directions of the particles lie on the surface of a cone, whose angle is the experimentally chosen tilt angle (50°). The azimuth angles, representing the random distribution of the particles in the plane of the membrane, were obtained a posteriori by alignment and classification of the images of the untilted particles (see below and Figure 3).24,29 Classification of the untilted images allowed the determination of the azimuth angles as well as independent RCT reconstruction of the subsets of particles exhibiting different preferential orientation in the untilted data set.
The goal was the computation of the 3D envelope of the AQP0 channel, a process that involved the determination of the symmetry, the alignment and classification of the untilted images, the 3D reconstruction of the replica (the mold), the calculation of the “imprints” of the E and cytoplasmic domains, and the computational annealing of the two imprints to reconstruct the complete channel (Figure 3). The analysis also included the docking of the atomic structure of the AQP1 tetramer in the freeze-fracture envelope.

**Symmetry**

The minimal functional unit of all known aquaporins is a bundle of six alpha helices surrounding a narrow conducting pore. In the lens fiber plasma membranes, however, AQP0 is a tetramer. Therefore, we first analyzed the untilted images to determine whether the tetramer arrangement was maintained after purification and reconstitution in liposomes.

Visual inspection of unprocessed images showed many particles with square shape, which is characteristic of the presence of fourfold symmetry (Figure 2B). An important task was to determine whether the fourfold symmetry seen in a few particles was representative of the entire data set. To address this, we applied computer image processing methods. First, the entire data set was aligned by translation only and processed by multi-variate statistical analysis (MSA) to calculate eigen (own) images. This procedure finds the main sources of interimage variations and, thus, avoids the danger of introducing symmetry that is not present in the data set. If the fourfold symmetry detected by visual inspection was a significant feature of the untilted images, the principal eigen images should appear as squares rotated at angles in the plane or fourfold rotationally symmetric structures. The result of this analysis was consistent with this prediction (Figure 4). Second, cycles of rotational alignment and classification of the untilted data set produced class averages that exhibited square shapes. Similar results were obtained by processing the data sets obtained by using carbon pre-coat films of different thickness (four to six seconds). Therefore, we imposed fourfold symmetry on the data set to reduce metal coat inhomogeneity, the principal source of noise in freeze-fractured and shadowed specimens.

**Classification**

The purpose was to partition the particles into subsets composed of images exhibiting defined geometric relationships. Heterogeneity arises from “molecule-related” variations, such as molecular flipping as well as the true noise introduced during freeze-fracture and shadowing. Ideally, classification of the untilted data set from fractured liposomes should produce two class averages corresponding to the external and cytoplasmic views of the channel (see Figure 1). In practice, the classification process of the 2547 particles in the data set produced eight stable class averages displaying a square and an octagon shape, with a continuum of variation (Figure 5, top panel). Yet, the
number of particles in the most distinctive square and octagon-shaped classes were approximately equal, suggesting that the square and octagon views represented the two sides of the channel. Similar class averages displaying square and octagonal shapes were also obtained by alignment and classification of the data sets obtained with heavier (four to six seconds) carbon pre-coating conditions (not shown).

The square and octagon class averages appeared as white outer frames surrounding a darker center. We interpreted the outer frame as the replica seen edge-on, thus its dimensions (1.2–1.4 nm in width) corresponded to the thickness of the replica. This dimension agreed with that estimated from the particle dimension. The darker center representing the channel measured 6.2–6.5 nm in size, which is in good agreement with that obtained from processing 2D crystals of AQP0 by cryo-electron microscopy. Therefore, classification of the untilted images produced eight stable classes displaying distinct square and octagonal views. This result was consistent with the expected random in-plane orientation of AQP0 reconstituted in liposomes.

Further confirmation that the square and octagonal views were of the entire data set was obtained by applying the parameters obtained after imposing fourfold symmetry (xy-shift, rotation, and class assignment) to the same images without imposing symmetry. This test also shows square and octagonal shapes that were in complete agreement with those obtained after imposing fourfold symmetry. Another test was the representation of the untilted images in non-linear maps, an analysis that has the advantage of showing less frequently represented images in the set that may have been lumped in the final class averages. Non-linear mapping showed that all class averages fit the square or octagonal views resulting from this classification (not shown). Similar results were obtained by processing data sets obtained with thicker carbon pre-coats (not shown). Therefore, we used the class-averages that represented best the square and octagonal views as references and performed iterative multi-reference alignment.

**Figure 4.** The six principal eigen images were used to determine the presence of fourfold symmetry in the data set. To calculate the eigen images, the complete untilted data set (2547 particles) was shift-aligned only and processed by MSA. The images are presented as pairs because they were computed on both positive (+, top images) and negative (−, bottom images) sides of the principal directions of the cloud formed by the images in multi-dimensional space. To aid the presentation, the images were computed by mixing the pure eigen images with the average image of the sector.

**Figure 5.** Results of the classification of the untilted data set after rotational alignment and imposition of fourfold symmetry. Top panel shows eight class averages resulting from classification of the entire data set (2547 particles). The classes were arranged showing the most distinctive square view at the left corner and the most distinctive octagonal view at the right corner. The number of particles contained in classes exhibiting square and octagonal shapes was approximately equal. Bottom panel shows five class averages resulting from classification of the untilted images after MRA using square and octagonal class averages as references. During this process, the number of particles decreased to 1108. The class averages contained 230, 179, 220, 237, and 242 particles, respectively. The class averages were used to partition the tilted data set and to perform the 3D reconstructions. The stars label the classes from which 3D reconstructions are displayed in Figure 6.
The mold and the “imprint”

The five stable class-averages resulting from the analysis of untilted images were used to partition the data set containing the tilted images. Using the weighted back projection algorithm\(^{35}\) and a direct Fourier method based on the discrete Radon transform,\(^{36}\) we obtained the 3D models (the molds) of the five class averages resulting from the analysis of the untilted images. The mold of the square and octagonal views (stars, Figure 5, bottom panel) is shown in Figure 6(A) and (B), and (D) and (E). The estimation, resolved by Fourier shell correlation\(^{25}\) using a criterion on the basis of 0.5 thresholds, varied from 1.6 nm to 1.9 nm depending on the class average and the thickness of carbon pre-coating film used to prepare the replicas (thickest pre-coat films exhibited the lowest resolution).

Both views produced 3D molds that appeared as cups exhibiting external convex (Figure 6(A) and (D)) and internal concave surfaces (Figure 6(B) and (E)). The internal surface of the mold was in direct contact with the channel and thus closely represented its molecular envelope. Because of the nature of the replication process, the internal concave surface was the negative image of the envelope, a fact that hampered its interpretation. To resolve this problem, we computed the imprints of the molds calculated from the five classes seen in Figure 5 (bottom panel), a procedure that consists of computing the shape of the volume contained inside the mold.\(^{23}\)

The imprints of the square and octagonal views resulted in particles measuring 6.2–6.5 nm in side and ~3 nm in height. The particles rose from flat platforms representing the middle of the bilayer, which were removed from the final images (Figure 6(C) and (F)). The particles representing the imprints of the class averages seen in Figure 5 (bottom panel) were similar in size and shape but they also exhibited some significant differences. The square imprints revealed a small protrusion at the fourfold axis of the particle (Figure 6(C)). In contrast, the octagonal imprints exhibited small peaks at the border of the particle that encircled a shallow depression (Figure 6(F)).

The complete envelope of the channel (Figure 7) was computed by joining the imprints of the square (Figure 6(C)) and the octagonal views (Figure 6(F)). This process produced four combinations (we did not use the imprint of one class average) with two possible hands; a total of eight models. While the envelopes exhibited small differences, they all predicted the correct size and shape of the channel. Moreover, the volume of the envelope predicted a molecular mass of 124–134 kDa (6000 voxels or 1.74×10⁵ Å³ and a partial (MRA) of the particles in the entire set.\(^{34}\) This process reduced the number of stable class averages from eight to five and the number of particles from 2547 to 1108 (Figure 5, bottom panel).

![Figure 6](image-url) Molds and imprints calculated after partition of the tilted data set using the parameters resulting from alignment and classification of the untilted images. (A)–(C) The mold and imprint from the square view. (D)–(F) The mold and imprint from the octagonal view (see Figure 5). The molds appear as cups with external convex (A) and (D)) and internal concave ((B) and (E)) surfaces. They represent the 3D distribution of metal in the replica. The concave internal surface was in direct contact with the surface of the protein exposed by fracturing. Therefore, its topology more closely represents the envelope of the protein. The concave internal surface of the cup was used to calculate the volume contained under the replica (the imprint). Because the freeze-fracture particle extends from the hydrophobic center of the phospholipid bilayer, the imprints represent either the external or cytoplasmic domain of the AQP0 channel. The imprints from the square and octagonal views differed from each other in the overall shape and in smaller details, such as the presence of a small protrusion at the fourfold axis in the square view (C) and a small depression in the same region in the octagonal view (E).

![Figure 7](image-url) Small lateral protrusions at the center of the side view (A) point to the line where both imprints were joined together. The images represent orthogonal views of the envelope. The side view (A), with the octagonal view upward, also exhibits the location of the lipid bilayer to indicate that only small domains protrude from the phospholipid bilayer into the external and cytoplasmic compartments. (B) and (C) The top and bottom views, respectively, of the structure shown in (A). The octagonal and square views differ in their overall shape and in the location of smaller protrusions. In the octagonal view (B), the center of the particle contains a small depression that is also evident in the upper part of the side view (A). The square view (C), on the other hand, exhibits a small elevation in the equivalent region.
joined together (Figure 7(a)) was removed from the envelope shown here. The external surface of the tetramer (with loops) is shown fitting the square imprint and the cytoplasmic domain the octagonal imprint. However, this fitting was not unique and the inverse orientation also docked in the envelope with a similar correlation coefficient. (A) A side view, indicating that the envelope fitted the transmembrane domain of the AQP1 channel but not the external loops and the alpha helices at the amino and carboxyl termini. The fact that these domains are outside the envelope explains why the transmembrane domains equally well in both orientations across the bilayer. (B) A top view from the external domain to represent the tight fitting of the protein inside the envelope. (C) A tilted view with the cytoplasmic surface oriented upward to demonstrate that both amino and carboxyl termini of the atomic structure of AQP1 are outside the envelope. At the top and bottom of the envelope, there are regions with sufficient volume to account for the external loops and the alpha helices protruding out of the cytoplasmic surface, indicating a possible location for these domains.

Docking of the X-ray structure

We docked the structure of AQP1 into one of the complete envelope of the AQP0 channel (Figure 8). Selection of this particular model was on the basis of coincidence of the hand of the two imprints with the X-ray model using rigid body docking (SITUS 2.0). First, we docked the imprints from the square and octagonal views into the external and cytoplasmic halves of the atomic model in an effort to find out which view corresponded to the different domains of the channel (not shown). All imprints fitted the external domain better than the cytoplasmic domain of the channel. This preference for the external domain made the assignment of one of the imprints to either the external or cytoplasmic domains impossible. Second, we docked the tetramer in the envelope of the entire channel calculated by joining the square and with the octagonal imprints (Figure 7). The envelope fitted well the dimensions and shape of the atomic model of the tetramer (correlation coefficient 0.755 and fitting parameters 1.5 Å and 6.3°). It differed from the X-ray model in the position of the external loops and the amino and carboxyl ends in the cytoplasmic domain of the channel (Figure 8). In the X-ray model, the amino and carboxyl termini (~20% of the molecular mass) were protruding from the outside border of the channel, a region that exhibited no density in the freeze-fracture envelope (Figure 8(A) and (C)). Therefore, the small alpha helices in the termini were located completely outside of the envelope (Figure 8).

A possible explanation is the limited resolution of the envelope obtained from the freeze-fracture replicas (~1.6 nm) and the presence of plastic deformation that might occur during fracturing and shadowing. Yet the difference in mass is substantial (~20 kDa for the tetramer) and predicts an equivalent particle of ~1.8 nm in radius. We believe that such a sizable volume should have been detected even at the resolution accomplished here. Alternatively, this discrepancy can also be explained by comparing the amino acid sequence of AQP0 and AQP1. The transmembrane domains of both channels exhibit ~73% sequence similarities, but the amino and carboxyl termini exhibit no significant similarity (Pairwise Blast). Therefore, it appears that the freeze-fracture envelope accounted for the regions of the transmembrane domain exhibiting high sequence homology but not for the more flexible domains exhibiting no significant sequence homology. Perhaps the placement of the carboxyl terminus extending out of the tetramer in the crystal form is only one of several possible locations that this region of the channel might adopt when inserted in a lipid bilayer.

Conclusions

We determined the 3D envelope of lens AQP0 water channels inserted in the lipid bilayer from images of single freeze-fracture particles. The envelope accurately predicted the size, shape, oligomeric structure, and molecular mass of the channel. Docking the atomic structure of AQP1 showed that the freeze-fracture envelope accounted for the transmembrane domains of the channel but not for the less conserved regions in the amino and carboxyl termini. Analysis of the freeze-fracture envelope of functional integral proteins might contribute to our understanding of the contribution of accessory subunits to channel structure as well as the visualization of genetically engineered flags,
such as the green fluorescent protein, coded in their amino acid sequences. Genetic manipulations that produce changes in the molecular mass of the protein should translate in changes in the volume of the envelopes, thus allowing a precise determination of the orientation at the present resolution.

Materials and Methods

Purification of AQP0

AQP0 was purified from plasma membrane fractions isolated from lens fiber cells of calf lenses following procedures already published.39 In summary, isolated lens fiber plasma membranes were solubilized in 10 mM Hepes (pH 7), 50 mM NaCl, 5 mM EDTA, 3 mM NaN3 at a final β-octylglucopyranoside (OG) concentration of 3.5% (w/v) and centrifuged at 120,000g for one hour. Essentially complete purification of AQP0 was achieved by passing the OG-solubilized plasma membranes through a cation exchange column (MonoS column), to exploit the fact that AQP0 is the major positively charged protein (pI = 9.4) in the solution. AQP0 eluted reproducibly in a single peak at 109(± 1) mM NaCl. The procedure also requires gel filtration column chromatography using a Superdex 200 to exchange OG for decylmaltopyranoside. The detergent exchange provides stability to the purified protein. The procedure yields ∼3 mg of highly purified AQP0 per 10 mg of isolated plasma membrane protein. The purified AQP0 has been extensively characterized by column chromatography, hydrodynamic measurements, partial proteolysis, and single particle negative staining electron microscopy.39

Reconstitution of AQP0

Liposomes were prepared by the β-cyclodextrin method described.40 Asolectin soy lecithin and cholesterol were dissolved in diethyl ether, dried under N2 and then under vacuum. The lipids were suspended in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1% solution of Alcian Blue and dried in a 0.1%

Freezing

The procedure used to freeze and fracture membranes has been described.27 Cover slips were polished using a mixture of alumina and detergent and washed extensively with distilled water. The cover slips were immersed in a 0.1% solution of Alcian Blue and dried in an oven. After drying, the cover slips were scored with a diamond and broken into smaller pieces. Aliquots (1–5 µl) of the liposome solution were spread on the treated glass surface and the excess drained with a piece of filter paper. A regular copper specimen carrier (BalTec), cleaned prior to use in nitric acid, was placed on top. The solution of liposomes sandwiched between glass and copper substrates was frozen by immersion in liquid nitrogen. The sandwiches were stored in a liquid nitrogen refrigerator before their transfer into the freeze-fracture apparatus. Fractures and shadowing

Fracturing was performed by knocking the copper hat on top of the glass surface with a liquid nitrogen-cooled knife at −145 °C and at 1 × 10−3 mbar in a JEOL RFD 9010CR freeze-fracture apparatus (Figure 1(B)). The cleaved surface was shadowed first with a thin layer of pure carbon to eliminate decoration.28 We deposited carbon layers of different thickness by varying the time of evaporation (two, four, six seconds). After pre-coating, the surface was shadowed with platinum and carbon. Instead of shadowing with the electrodes at a fixed angle with respect to the specimen (unidirectional shadowing), we rotated the specimen simultaneously in the x, y, and z planes during evaporation (multi-axis shadowing). Our protocol used two four-second pulses separated by an interval of two to three seconds. We found empirically that “pulsing” during evaporation greatly improved the mechanically stability of the replicas. The replica was a homogeneous layer of metal 1.2–1.3 nm in thickness that covered the domains of the channel exposed by fracturing. The replicas were separated from the glass surface by floating them on a 10% hydrofluoric acid solution and immediately transferred to distilled water.

Mechanical support of the replica during imaging

In the standard freeze-fracture method, the replica is deposited on copper grids coated with a layer of plastic (parlodion or formvar) coated with carbon to provide mechanical stability. The plastic/carbon support film is thinned (∼50 nm) and imaged with improved resolution, particularly during tilting. We eliminated the interference of the plastic film by depositing the replica on substrates with holes of 1–2 µm diameter (“holey” grids), and imaging the region of the replica spanning the holes only.

Electron microscopy

To apply RCT strategy,24,29 the fractured liposomes were imaged tilted at 50° and untilted. Both untilted and tilted images were collected at 60,000 magnification in a Philips CM120 equipped with a CCD camera (1024 × 1024 pixels). The digital micrographs had a pixel size of 0.31 nm on the molecular scale. The untilted images were recorded at ∼600 nm under-focus. The tilted images exhibited a gradient of focus that extended from perfect focus to 200–300 nm under-focus. Therefore, the images did not require correction for the contrast transfer function (CTF). The images were recorded under low electron dose conditions by focusing in regions away from the area of interest. While metal replicas are essentially insensitive to radiation,30 organic material left attached during cleaning was a source of contamination during imaging. The location of the tilt axis was determined by the deformation of holes in the substrate and by analysis of the tilted data set. Astigmatism, specimen drift, and level of under-focus were assessed from Fourier transforms calculated during imaging.

Image processing

Figure 3 shows the sequence of steps involved in image processing. Particle selection was done interactively by displaying simultaneously the image pair in
a dual monitor LINUX workstation. We used “boxer”, a program included in that EMAN software package to produce galleries of untitled and tilted images centered inside 64-pixel side boxes. The particles from the three carbon pre-coating conditions were processed separately. The two-seconds carbon pre-coat data set contained 2547 particles, the four-seconds pre-coat data set contained 3669 particles, and the six-seconds pre-coat data set contained 2889 particles. The three data sets were processed independently at Milan, using software packages developed in the laboratory, and at UCLA using Imagic-5 software package.

The untitled particles were processed first to identify homogeneous subsets and to determine the viewing directions of the corresponding tilted image of the pair (left side, Figure 3). Shift alignment and search for symmetry was followed by MSA, reference-free alignment of class averages, and MRA. The alignment/classification process was repeated until stable and homogeneous subsets were extracted from the data set. The parameters obtained from these subsets were then used to partition the tilted set (right side, Figure 3).

To perform the 3D reconstruction, we determined the tilt and the azimuth angles for each image of the class average. The tilt angle (50°) was known from the experiment. The azimuth angles were found from the relative rotation of the untitled images with respect to class average. After determining the angles, the tilted images were rotated to bring the direction of the tilt axes along the Y axis, and the 3D reconstruction was calculated using the weighted back projection algorithm, and a direct Fourier method on the basis of the discrete Radon transform.

The tilt and azimuth angles were refined by comparing each tilt image with a number of projections obtained from the 3D-reconstruction and computed at various viewing directions close to the one previously assigned to the image (Figure 3). The comparison assigned a pair of new tilt and azimuth angles to the images, on the basis of the best match with the references (Figure 3). This refinement of the viewing directions, on the basis of the projection matching strategy, was iterated several times until reaching convergence.

RCT geometry did not provide all the information necessary for an ideal 3D reconstruction because of the limited tilt angle employed (the “missing cone” problem). We partially solved the problem by using the projection onto convex set theory (POCS), and the POCS algorithm on the basis of the internal consistency of the Radon transform. These corrections should be applied with caution because they could induce a tapering of the particle. The resulting 3D reconstructions (the molds) were used to calculate imprints, which represented the positive image of the inner surface of the mold. Selection of the threshold level and the plane at which the molecule begins (the “zero” plane) influenced the final map.

The 3D imprint of the AQP0 channel was docked into the atomic resolution model of the AQP1 tetramer. We used SITUS 2.0 to perform automatic rigid body fitting and VMD to visualize the docked model. Because the fracturing process revealed only one-half of the channel, we split the atomic models and docked each half to the imprints calculated from the freeze-fracture particles. The number of vectors was varied from three to nine, and the best solutions were selected on the basis of the values of rmsd, the correlation coefficients, and the fitting parameters.

Acknowledgements

We thank P. L. Bellon for help and encouragement during the analysis of the data. NIH grants EY-04110 (G.A.Z.), DK-60846 (G.A.Z.), DK-44602, DK-44582 (E.M.W.), and GM-53933 (S.E.) supported this work. We also thank the COFIN2000 grant of the Italian MIUR and the FIRST2000 grant of the University of Milano (S.L.).

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*Edited by Sir A. Klug*

(Received 19 July 2002; received in revised form 17 October 2002; accepted 18 October 2002)