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Co-axial Association of Recombinant Eye Lens Aquaporin-0 Observed in Loosely Packed 3D Crystals

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Aquaporin-0 (AQP0) is the major membrane protein in vertebrate eye lenses. It has been proposed that AQP0 tetramers mediate contact between membranes of adjacent lens fiber cells, which would be consistent with the extraordinarily narrow inter-cellular spacing. We have obtained 3D crystals of recombinant bovine AQP0 that diffract to 7.0 Å resolution. The crystal packing was determined by molecular replacement and shows that, within the cubic lattice, AQP0 tetramers are associated head-to-head along their 4-fold axes. Oligomeric states larger than the tetramer were also observed in solution by native gel electrophoresis and analytical ultracentrifugation methods. In the crystals, there are no direct contacts between octamers, and it can thus be inferred that crystalline order is mediated solely by the detergent belts surrounding the membrane protein. Across the tetramer–tetramer interface, extracellular loops A and C interdigitate at the center and the perimeter of the octamer, respectively. The octamer structure is compared with that of the recently determined structure of truncated ovine AQP0 derived from electron diffraction of 2D crystals. Intriguingly, also in these crystals, octamers are observed, but with significantly different relative tetramer–tetramer orientations. The interactions observed in the loosely packed 3D crystals reported here may in fact represent an *in vivo* association mode between AQP0 tetramers from juxtaposed membranes in the eye lens.

Keywords: eye lens; MIP26; X-ray crystallography; membrane protein; mixed micelle

The major intrinsic protein (MIP26, AQP0), an integral membrane protein present in vertebrate eye lenses,[1] is the founding member of the aquaporin superfamily.[2,3] AQP0 is the most abundant protein in the plasma membrane of lens fiber cells constituting almost 50% of the total protein.[4] There is extensive intercellular coupling in this tissue, which renders AQP0 a possible candidate to mediate cell–cell interactions.[5] Knock-out of this protein in Cat^{Fr} mice[6] causes cataract formation. Similarly, mutations in AQP0 that prevent proper folding or targeting cause lens opacification supporting a crucial role of AQP0 in the development of the transparent lens.[7] Water channel function of AQP0 (though about 40 times less efficient compared to AQP1[8]) and facilitation of glycerol translocation[9] have been demonstrated for AQP0, but its putative role in mediating cell–cell interactions appears unique amongst aquaporins.

The arrangement of AQP0 in native membranes has been examined in freeze and label-fractured preparations by electron microscopy using immunolabeling.[10,11] AQP0 was found only in tightly abutting membranes (thin and wavy junctions) but not in thick junctions.[10] Bok *et al*. were able to show close interaction between membrane patches that contained AQP0 on both sides.[11] This was confirmed later by analogous studies on AQP0 containing proteoliposomes.[12] In another study, however, no interaction between proteoliposomes, but only between proteoliposomes and phosphatidylserine liposomes was demonstrated employing energy

Abbreviations used: AQP, Aquaporin; CMC, critical micellar concentration; OG, *n*-octyl-β-D-glucoside; DM, *n*-decyl-β-D-maltoside; DDM, *n*-dodecyl-β-D-maltoside; NG, *n*-nonyl-β-D-glucoside; EM, electron microscope; TLS, translation-libration-screw.

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transfer measurements.[13] This might indicate that *in vivo* AQP0 also interacts directly with the lipids of the juxtaposed cell membrane. Later, studies on 2-layered crystalline sheets of ovine AQP0[14] analyzed by atomic force microscopy (AFM) showed a precise ("tongue-and-groove") interaction of the extracellular AQP0 surfaces. AQP0 (monomer mass 26 kDa) is a homolog of AQP1 from red blood cells (44% sequence identity). The structure of AQP0 has been determined by X-ray crystallography[15] and is very similar to AQP1.[16,17] Both proteins are composed of six transmembrane helices and five connecting loops obeying an internal 2-fold symmetry. Both loops B and E contain the consensus motif Asn-Pro-Ala and dip into the membrane from opposite sides contributing to form the water channel. The structural unit of AQP0 is a tetramer with four independent hour glass-shaped channels. Recently, the structure of AQP0 from sheep eye lens was determined by electron crystallography to 3.0 Å resolution.[18] Within the double-layered 2D crystal tight head-to-head association of tetramers is observed. This has been proposed[14–18] to resemble the situation in membrane junctions. Here, we report a similar, though not identical, association of AQP0 tetramers in loosely packed 3D crystals.

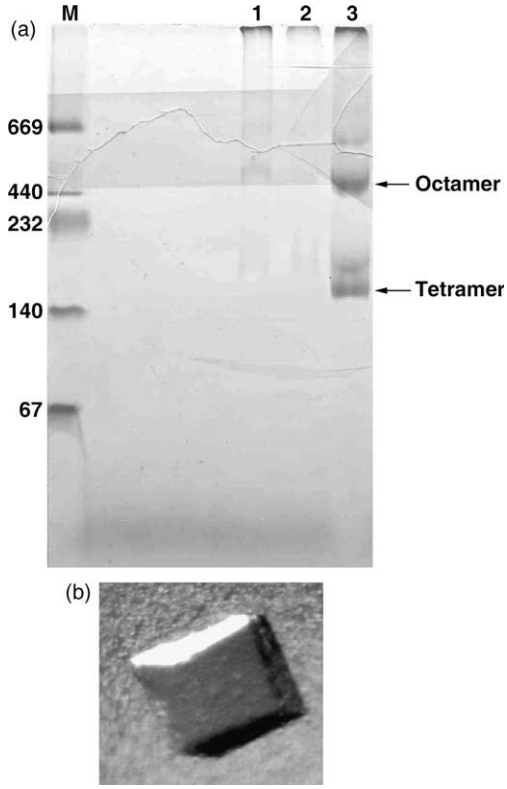

Figure 1. (a) Blue native gel electrophoresis (5–13%) of purified AQP0 samples (lanes 1, 2 and 3). The predicted position of AQP0 tetramers and octamers based on molecular markers (M; HMW-native protein marker kit, Amersham Biosciences) is shown on the right. (b) Cubic crystal of recombinant bovine AQP0, diameter = 150 μm. Bovine AQP0 (native sequence with a N-terminal $(His)_{10}$-tag and a Factor Xa cleavage site) was cloned in yeast vector pYES2.0 with a GAL6 promoter and expressed in yeast strain Sc334. Yeast cells were grown in SD–URA media (6×100 ml) and expressed in YPGal media (6× 1.25 l). This yielded 70 g of cells that were broken by a Dyanomil using glass beads. Cell debris was removed by centrifugation at 9000 rpm (Sorval GSA) and the membrane fraction was obtained by ultracentrifugation at 40,000 rpm (TFT45.94). AQP0 was extracted from membrane fractions using 10% (w/v) *n*-octyl-β-D-glucoside (OG). Detergent exchange was performed on Ni-NTA Wizard Midi Columns (Catalys AG, Switzerland) against *n*-decyl-β-D-maltoside (DM; 0.3% (w/v)), *n*-dodecyl-β-D-maltoside (DDM; 0.15% (w/v)), *n*-nonyl-β-D-glucoside (NG; 1% (w/v)), *n*-nonyl-β-D-maltoside (1% (w/v)) or *n*-decyl-β-D-glucoside (1% (w/v)). In DM (0.4% (w/v)) or DDM (0.15% (w/v)) AQP0 was found only partially aggregated as judged by analytical ultracentrifugation, whereas severe precipitation was observed with the other detergents. Further improvement, i.e. monodispersity, was achieved by employing DM or DDM at the extraction (at a concentration of 2% (w/v)) as well as purification step (0.4% (w/v) or 0.15% (w/v), respectively). During protein concentration also enrichment of the detergent (from 0.4% to about 6–7% (w/v) for DM) was observed using a drop-shape based method (? Kaufmann *et al.*, unpublished data), although a large cut-off membrane (100 kDa cut-off, Ultrafree or Amicon Ultra centricon, Millipore) was used. This was overcome by resuspending the solution (starting volume 3 ml) four times every 3 min with 1 ml of buffer solution resulting in a final detergent concentration below 1% (w/v) DM. Sample monodispersity was checked by sedimentation velocity runs on a Beckman Optima XL-A analytical ultracentrifuge at 54,000 rpm. Sedimentation equilibrium runs were carried out at different angular velocities to investigate the self-association behavior of the sample. Fitting to various self-association models[30] was performed by non-linear regression using program PROFIT (version 6.0). Crystals were grown from sitting drops at 20 °C under similar conditions as found earlier for native AQP0[25] and had similar shape. Equal volumes (3+3 μl) of protein solution (8.5 mg/ml in 10 mM Tris (pH 8.0), 300 mM NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, 0.4% (w/v) DM) and reservoir solution (100 mM bicine NaOH (pH 8.9), 350 mM NaCl, 34% (v/v) PEG400) were mixed. Using 1% (w/v) NG crystals of similar quality were obtained.

Biophysical characterization

His-tagged bovine AQP0 was heterologously expressed in *Saccharomyces cerevisiae* and purified by nickel affinity chromatography. This had several advantages including straightforward expression and purification of milligram quantities of protein. Also, heterologous expression lessened the likelihood that protein interactions are mediated by the co-factors found in the native environment, rather than driven by intrinsic structural properties. To assess for its homogeneity and oligomeric state, solubilized AQP0 was subjected to several tests. Electron micrographs indicated monodispersed tetramers with the occasional occurrence of coaxial octamers.[19] Native gel electrophoresis[20] revealed a major band with an apparent mass between