Eye lens membrane junctional microdomains: a comparison between healthy and pathological cases

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The eye lens is a transparent tissue constituted of tightly packed fiber cells. To maintain homeostasis and transparency of the lens, the circulation of water, ions and metabolites is required. Junctional microdomains connect the lens cells and ensure both tight cell-to-cell adhesion and intercellular flow of fluids through a microcirculation system. Here, we overview membrane morphology and tissue functional requirements of the mammalian lens. Atomic force microscopy (AFM) has opened up the possibility of visualizing the junctional microdomains at unprecedented submolecular resolution, revealing the supramolecular assembly of lens-specific aquaporin-0 (AQP0) and connexins (Cx). We compare the membrane protein assembly in healthy lenses with senile and diabetes-II cataract cases and novel data of the lens membranes from a congenital cataract. In the healthy case, AQP0s form characteristic square arrays confined by connexons. In the cases of senile and diabetes-II cataract patients, connexons were degraded, leading to malformation of AQP0 arrays and breakdown of the microcirculation system. In the congenital cataract, connexons are present, indicating probable non-membranous grounds for lens opacification. Further, we discuss the energetic aspects of the membrane organization in junctional microdomains. The AFM hence becomes a biomedical nano-imaging tool for the analysis of single-membrane protein supramolecular association in healthy and pathological membranes.

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The eye lens is located in the anterior segment of the eye, behind the iris, which controls the quantity of light entering the eye via the pupil. The quantity of melanin, a pigment molecule, included in the iris determines the eye’s color (figure 1(A)) but the eye lens is crystal-clear transparent. This property makes the eye lens unique, as it is the only transparent organ in the body. On the macroscopic level, the crystalline transparency is ensured by a number of mechanisms, such as degradation of organelles and tight cell packing (Donaldson et al 2001). The lens epithelium (figure 1(B), top) consists of a monolayer of non-specialized cells, surfacing the anterior pole of the lens up to the equator. Epithelial differentiated cells, called lens fibers, are found in the lens from the equator to the bulk of the lens.

Homeostasis of lens fiber cells is maintained by an internal flow circulation within the tissue (figure 1(B), bottom) (Mathias et al 1997). This current brings water, metabolites and ions to the cells and evacuates waste products. Donaldson et al have characterized the microcirculation system of the eye lens (Donaldson et al 2001). The current is outward at the equator of the lens where a large number of young differentiating fiber cells featuring active transport proteins and energy-driven pumps create transmembrane gradients. These gradients induce an osmotically driven flow. The current directs inwards at the anterior and posterior poles of the lens, where the maturated fiber cells form the majority. The microcirculation model proposes that the flow of solutes arrives at the lens via the intercellular space, cross-fiber cell membranes, and then is redirected to the lens surface via an intracellular pathway through junctional microdomains (figure 1(C)).

The fiber cells have a particular shape about 5 µm across and up to 100 µm long and they organize in a typical ‘onion-like’ structure with a flattened hexagonal profile (figures 1(D) and (E)) (Pernget et al 2004, Song et al 2009). This facilitates tissue packing with intercellular distances smaller than the wavelength of visible light to avoid light scattering. Another adaptation to maintain lens transparency is degradation of organelles, such as the nucleus, mitochondria or endoplasmic reticulum in the mature fiber lens. Around 90% of the fiber cell proteins are soluble proteins called crystallins. Their main role is to create a high refractive index in the eye lens (Donaldson et al 2001).
Figure 1. The eye lens: architecture and function. (A) The iris is the pigmented part of the eye; the eye lens located behind the iris is completely transparent. (B) Top: onion-like structure of the eye lens. Anterior epithelial monolayer (red), differentiating fiber cells (DF) of the cortex (sky blue) and mature fiber cells (MF) in the core (blue) are shown. Bottom: schematic representation of the microcirculation in the eye lens: inward flow is located at the pole regions and outward flow at the equator. (C) Cross-section through the lens equator. Inward flow goes via the intercellular space, whereas outward flow is maintained by gap junctions between the cells (adapted from Donaldson et al. (2001), used with permission). (D) Ordered arrays of fiber cells (adapted from Song et al. (2009)). (E) Hexagonal profile of fiber cells ensuring optimal tissue packing (adapted from Perng et al. (2004), copyright 2004, with permission from Elsevier). Scale bars: (D) 25 μm; (E) 5 μm.

The microcirculation system of the lens could not exist without specialized structures linking the neighboring cells and connecting them in a unified continuum. In the lens, these requirements are fulfilled by protein microdomains that combine thin junctions and gap junctions and provide both adhesion between neighboring cells and microcirculation within the tissue. The organization of junctional microdomains that ensure those tasks is described here in membranes from healthy and cataract-affected eye lenses at the molecular level using atomic force microscopy (AFM).

2. The eye lens membrane protein channels

The eye lens membrane contains only a few membrane protein species. Among them, aquaporin-0 (AQP0) (Alcala et al. 1975) and the connexins (Cx) (Fleschner and Cenedella 1991) are the two major components. Another important class of proteins is represented by the tetraspanins (Gonen et al. 2008).
2.1. Aquaporin-0 (AQP0)

The lens-specific AQP0 is the major fraction of the membrane proteins, formerly known as the lens major intrinsic protein (MIP). It represents 50% or more of the total protein in an eye lens membrane extraction (Alcala et al 1975, Gonen et al 2004a, Mangenot et al 2009). AQP0 is coded by one of the 13 aquaporin family sequences in the human genome and is homologous to the founding member of the aquaporin water channel AQP1 (Agre 2004). In the lens core membrane, AQP0 exists in a full-length version AQP0-L263, and in a C-terminally truncated version AQP0-S245, which is more abundant (Buzhynskyy et al 2007b, Gonen et al 2004a, Mangenot et al 2009). It has been shown that AQP0 has a double function: it is a water channel and an adhesion protein (Costello et al 1989, Gonen et al 2004a, Varadaraj et al 1999).

Single- and double-layered 2D crystals of full-length and a mixture of full-length and truncated AQP0 were reconstituted, respectively. Both crystal forms displayed the same unit cell parameters (Gonen et al 2004a) as the native packing (Buzhynskyy et al 2007a, Scheuring et al 2007). In double-layered 2D crystals (Fotiadis et al 2000, Gonen et al 2004b, 2005) AQP0 tetramers pair with tetramers in the opposing membrane in a head-to-head manner, as they do in the junctional microdomains between cells (figure 2(A)). Such a type of adhesion is similar to that observed for gap junction connexons (Unger et al 1999) and cadherins with large extracellular domains (Al-Amoudi et al 2007). In the double-layered AQP0 crystal, the adhesion is mediated by proline-rich domains in the extracellular loops A and C (Gonen et al 2004a).

Water channeling is mediated in the middle of each AQP monomer through a channel that is surrounded by six transmembrane helices and lined by two half-helices, making the channel constriction and filter sites (Gonen et al 2004b, Harries et al 2004). The AQP0 structures derived from the double-layered two-dimensional (2D) crystals by electron crystallography (figure 2(A)) revealed narrow restrictions in the pore that were interpreted to be ‘closed’ for water flux (Gonen et al 2004b, 2005). The atomic structure derived from 3D crystals by x-ray crystallography of non-junctional full-length AQP0 tetramers revealed water molecules in the channel (Harries et al 2004) in agreement with water channel activity (Ball et al 2003). Since truncated APQ0 in oocytes also channels water (Ball et al 2003), it was concluded that the junction formation triggers the conformation, i.e. the closure of the channel (Gonen et al 2004b, 2005). Molecular dynamics studies of water flux across two head-to-head paired AQP0 tetramers in the junctional conformation indicated permeation (Han et al 2006). While a direct functional measurement of water flux across the AQP0 thin junction is lacking, the issue remains controversial.

2.2. Connexins

Connexins are the second most abundant proteins in lens fiber cells, constituting about 10% or more of the total membrane protein content (Fleschner and Cenedella 1991, Mangenot et al 2009). In humans, the most abundant connexins in the lens are Cx43, Cx45 and Cx50, homologous to Cx43, Cx44 and Cx49 in sheep (Yang and Louis 1996). Six connexins form a connexon or half-channel, and head-to-head docking of two connexons from adjacent cells creates a cell-to-cell gap junction channel (figure 2(B)). Electron crystallography has been used to produce a first density map of the recombinant cardiac gap junction (Unger et al 1999). More recently, x-ray crystallography has been used to produce a 3D structure of human Cx26 gap junctions (figure 2(B)) (Maeda et al 2009). In addition to cell–cell adhesion, gap junctions mediate transport functions (Kumar and Gilula 1996). They form
Figure 2. Eye lens membrane proteins AQP0 and connexin form head-to-head junctions. (A) The two adjacent tetramers of AQP0 form thin junctions connecting plasma membranes of neighboring fiber cells. Here, the junctional AQP0 structure is derived from electron crystallography of highly ordered double-layered 2D crystals (2B6O (Gonen et al. 2005)). (B) Connexins assemble in hexameric connexon channels that form gap junctions with hexameric connexon in the neighboring cell membrane. This gap junction structure was obtained by x-ray crystallography of 3D crystals (2ZW3 (Maeda et al. 2009)).

Intercellular communication channels that allow the passage of a variety of molecules with a size of up to 1 kDa from the cytoplasm of one cell to its neighboring cell, including ions, metabolites, nucleotides, peptides and secondary messengers. Gap junctions have been proposed to play an essential role in the microcirculation model through the stacked eye core lens cells (Donaldson et al. 2001). Since eye core lens cells contain no mitochondria and are therefore unable to perform complex bioenergetical and biochemical processes, gap junctions are likely to be crucial for keeping the intrinsic lens cells alive.

2.3. Other proteins

Another relatively abundant membrane protein is tetraspanin MP20, a scaffolding protein that is evidenced to assemble in a variety of oligomeric arrangements (Gonen et al. 2008). Members of the crystallin protein family are also repeatedly found in biochemical analysis of lens membrane preparations (Buzhynskyy et al. 2007a, Mangenot et al. 2009). These crystallins are supposed to be quite strongly associated with the cytoplasmic surface of AQP0 (Fan et al. 2004).
3. The supramolecular architecture of native lens junctional microdomains

The membrane proteins in the eye lens membranes are closely packed in junctional microdomains. Pioneering studies by thin-section transmission electron microscopy of samples labeled with gold-antibodies (figure 3(A)) showed the assembly of membrane proteins in microdomains (Zampighi et al 1982, 2002); however, the fine details of these protein assemblies remained unclear. Interestingly, freeze-fracture-labeling electron microscopy has revealed lines of connexons, although their significance was underestimated due to the necessity and imperfection of labeling techniques (Zampighi et al 2002). Similarly, sections normal to the cell membrane plane revealed ‘thin’ and ‘thick’ junctions, as judged from the membrane interspaces of neighboring lens cells (Zampighi et al 1982).

In recent years, AFM (Binnig et al 1986) has become a powerful tool for membrane studies (Engel et al 1997). AFM has an exceptionally high signal-to-noise ratio that allows direct observation of individual molecules; the sample is kept in physiological buffer at room temperature and normal pressure (Müller et al 1999, Scheuring and Sturgis 2005). The junctional microdomains in membranes of fiber cells were readily visible in overview deflection AFM images (figure 3(B), left inset). The membrane proteins occupied a total of about 15% of the cell membrane surface in the preparations (Buzhynskyy et al 2007b, Mangenot et al 2009). At medium resolution, the size, shape and distribution of junctional microdomains could be observed.

High-resolution AFM topographs show that the junctional microdomains consist of AQP0 and connexons. Remarkably, AQP0 tetramers form highly regular square arrays that are separated and confined by connexons (figure 3(B), right inset). The AQP0 lattice parameters were $a = b = 65.5\,\text{Å}$, $\gamma = 90^\circ$ in calf (Mangenot et al 2009), sheep (Buzhynskyy et al 2007b) and human (Buzhynskyy et al 2007a, Mangenot et al 2009) (figure 4(A)). The high-resolution AFM topographs were sufficiently well resolved ($\sim 9\,\text{Å}$ resolution (Fechner et al 2009)) to display individual surface protruding helix-connecting loops (figure 4(A), arrowheads). The features visible in the raw data topographs were enhanced through averaging (figure 4(B)). Comparison with the high-resolution structure indicated that the surface protruding features precisely match the three proline-rich regions of loops A and C on each AQP0 monomer that make the thin junctions linking the extracellular surfaces of head-to-head-oriented AQP0 (Buzhynskyy et al 2007b). Concomitantly calculated SD maps (figure 4(C)) highlight flexible domains on protein surfaces in AFM topographs (Müller et al 1998, Scheuring et al 2003), and revealed, for AQP0, that loop A close to the fourfold symmetry axis was highly flexible, although it protruded less than the surrounding loops (Buzhynskyy et al 2007a, Mangenot et al 2009). This was in agreement with the fact that the conformation of loop A differs between the high-resolution AQP0 structures derived from electron crystallography (Gonen et al 2005) and those from x-ray diffraction (Harries et al 2004). By docking the high-resolution structure of junctional AQP0 (Gonen et al 2005) into the averaged lattice of the native junctional microdomains, a structural model of the native AQP0 assembly was built (figure 4(D)) (Mangenot et al 2009, Scheuring et al 2007). This assembly is identical to the arrangement of AQP0 tetramers in double-layered 2D crystals; therefore, the high-resolution structure derived from junctional 2D crystals (Gonen et al 2005) is representative of the native state of AQP0 in eye lens junctional microdomains (Scheuring et al 2007).

The hexameric oligomerization state of connexons was only visible in some of the raw data connexon topographs (figures 4(E) and (F)), but averaging (figure 4(G), left) and symmetrization...
Figure 3. Junctional microdomains in native lens core membranes. (A) Immunolabel-freeze-fracture electron microscopy of eye lens membranes showing AQP0 microdomains visualized with gold-antibody labels (adapted from Zampighi et al (1989), © Rockefeller University Press, 1989). (B) Junctional microdomains in the lipid bilayer. Left inset: AFM deflection overview images of the lens membrane fragment on mica. Lipid bilayer areas appear as large smooth areas containing corrugated junctional microdomains. The junctional microdomains on the top left are still covered by the second cell membrane. After the removal of the covering material, the AFM has access to imaging the extracellular surface of the junctional microdomains, revealing the typical square pattern (arrowheads). Right inset: the AQP0 square arrays are often delineated by connexons, forming together stable junctional microdomains in the lipid membrane.

(figure 4(G), right) allowed us to gain more detailed insights into the connexon structure at the edges of junctional microdomains (Mangenot et al 2009, Scheuring et al 2007). Symmetry analysis confirmed sixfold symmetry of connexons (figure 4(H)), in agreement with structural studies of purified connexons (Maeda et al 2009, Oshima et al 2007, Unger et al 1999).
Figure 4. High-resolution AFM analysis of AQP0 and connexons in junctional microdomains. (A) In high-resolution AFM topographs, individual surface protruding loops are visible. The proline-rich domains that make the thin junction contacts of loop A (R33-W34-A35-P36) close to the fourfold symmetry axis and at the beginning (P109-P110-A111-V112) and the end (H122-P123-A124-V125) of loop C (arrowheads) are resolved. AQP0 is assembled in a square lattice with dimensions $a = b = 65.5$ Å, $\gamma = 90^\circ$. (B) Average topography (full color scale: $4 \text{ Å} < \text{height} < 14$ Å). (C) Standard deviation (SD) map (full color scale: $0.2 \text{ Å} < \text{SD} < 1.1$ Å). The central loop A is particularly flexible: its average topography is lower than loop C (see B), but its SD values are similar. (D) Surface representation of an atomic model of the average AQP0 assembly. (E) High-resolution AFM topographs of native lens membranes of sheep and (F) of calf. The individual connexin subunits are resolved. (G) Non-symmetrized (left) and symmetrized average topography (full color scale: $14 \text{ Å} < \text{height} < 26$ Å). (H) Symmetry analysis of the non-symmetrized connexon average (C, left) peaks at sixfold symmetry. (I) Radial profile plot analysis of connexon above the AQP0 array surface: central pore diameter, $\sim 20$ Å; outer diameter, $\sim 70$ Å, and top diameter, $\sim 50$ Å.

Connexons protruded 12 Å further out of the membrane than AQP0 (figure 4(I)), which resulted in a total height of the extracellular connexon surface of $\sim 26$ Å above the bilayer (Mangenot et al 2009). From the difficulties encountered in imaging the intercellular face of connexons, we anticipate that the extracellular domains of connexons are stabilized by the gap junction formation itself, and are flexible when connexons are separated.

Summing up data from high-resolution AFM analysis of healthy lens junctional microdomains in statistical terms (Mangenot et al 2009), individual junctional microdomains consist on average of $\sim 200$ AQP0 tetramers (figure 5(A)) on a membrane surface of $\sim 8500 \text{ nm}^2$. They are stochastically oriented within the membrane (figure 5(B)). The majority of native junctional microdomains have an aspect ratio of between 1 and 2 (figure 5(C)), considering the above number of AQP0 tetramers involved, the average microdomain diameter
Figure 5. (Caption on next page)
Figure 5. Statistical analysis of junctional microdomains. (A) Junctional microdomain size: The junctional microdomains contain ∼ 200 AQP0 tetramers. (B) Junctional microdomain orientation: all orientations appear equiprobable. (C) The average of the junctional microdomain aspect ratio is 1.8 ± 0.6. (D) Connexon repartition: surface occupancy maximum ∼ 60% at 75 Å outside the AQP0 array. Dashed line at 0-position represents the outermost AQP0 position. (E) PCF analyses of AQP0–AQP0 interactions: hard-body repulsion is 59 Å (ρ = 1.0) and the most frequent intermolecular distance is 65.5 Å (ρ = 7.7). (F) PCF analyses of connexon–connexon interactions: hard-body repulsion is 75 Å (ρ = 1.0) and the most frequent intermolecular distance is 92 Å (ρ = 5.3). (G) PCF analyses of AQP0–connexon interactions: hard-body repulsion is about 66 Å with progressively increasing probability until ρ = 1.0 at a distance of an AQP0 radius (around 400 Å).

is ∼ 100 nm. Connexons confine the AQP0 arrays occupying ∼ 60% of the AQP0 array circumference with highest location probability of a connexon at a distance of 75 Å from the AQP0 array edge (figure 5(D)). Packing analysis showed that AQP0 was arranged in 2D arrays with lattice parameters of a = b = 65.5 Å, γ = 90° (see figure 5, Buzhynskyy et al 2007b, Scheuring et al 2007). Pair correlation function (PCF) analysis provided additional information (figure 5(E)): the hard-body repulsion of AQP0 was 59 Å (normalized probability ρ = 1.0). In agreement with the lattice analysis, the most frequent intermolecular distance is 65.5 Å (ρ = 7.7). Several longer-range peaks appeared in the PCF analysis that documented high order of the AQP0 arrangement, i.e. multiples of the lattice dimension, the broadening of which documented small distortions over long distances. The distance at which ρ < 1.0 at ∼ 400 Å reflects the radius of the AQP0 array. The PCF analysis of connexons (figure 5(F)) demonstrated a hard-body repulsion of 75 Å (ρ = 1.0), in agreement with the lattice dimensions of connexon 2D crystals (Unger et al 1999). The most frequent intermolecular distance of 92 Å (ρ = 5.3) illustrated that eye lens connexons are not ordered in the membrane and do not make contact with each other but have considerable (∼ 20 Å) spacing between proteins. The PCF analysis between AQP0 and connexons (figure 5(G)) revealed a probability graph with a hard-body repulsion of ∼ 66 Å and progressively increasing probability until ρ ∼ 1 at ∼ 400 Å that corresponds to about half the diameter of an AQP0 array. Since connexons were located at and confined to most of the borders of AQP0 arrays, the probability is high for finding a connexon at such large distances from any AQP0. From a functional point of view, the specific co-assembly of AQP0 and connexons in an organized way is of particular interest as these protein domains ensure intercellular junctions and may favor an ensemble fluid flux.

4. Comparison of healthy and cataract lens junctional microdomains

Cataracts are opacifications of eye lenses and the leading cause of blindness in the world. They affect about two-thirds of the population over 65 years. The importance of cataract care and knowledge is increasing with the rise in population and age worldwide. Besides age, other recognized risk factors include long-term ultraviolet ray and radiation exposure. Cataracts can develop as secondary effects of diseases such as diabetes and hypertension, among others, or they can be genetically determined. Using AFM as a medical nano-imaging tool, the molecular
bases of cataracts of individuals were studied, i.e. from a senile cataract patient (Buzhynskyy et al 2007a) and from a type-II diabetes cataract patient (Mangenot et al 2009), and compared with healthy lens membranes from ovine (figure 6(A)) and bovine (figure 6(B)) lenses. At low resolution, the junctional microdomains in the senile (figure 6(C)) and diabetes-II (figure 6(D)) cataract cell membranes appeared to be enlarged compared with the junctional microdomains from healthy tissues. In the senile cataract tissue membranes as in the type-II diabetes patient, connexons were lacking at the borders of the junctional microdomains (figures 6(C) and (D)). The absence of connexons was corroborated using gel electrophoresis and mass spectrometry techniques (Buzhynskyy et al 2007a, Mangenot et al 2009). Individual AQP0 transmembrane channels could be imaged in the human pathological tissue at sub-nanometer resolution (figure 6(D)). Hence, it appeared that AQP0 remained unaffected, while connexons were degraded during cataract formation (Buzhynskyy et al 2007a). As a consequence of the absence of connexons, AQP0 arrays are malformed and fuzzy; indeed, they branched out into rows of a few AQP0 molecules.

A novel analysis of the cataract eye lens membranes originating from a 2-year-old patient suffering from congenital cataract showed the characteristic domains of 2D arrays of AQP0 tetramers embedded in lipid membrane (figure 6(E)). Interestingly, a group of connexons was observed in the boundary region of the AQP0 domain (arrowheads in figure 6(E)). These data confirm, on the one hand, that the protocol of surgery and sample preparation does not affect the presence of the connexons in the membranes and that our analysis of the senile and diabetes-II membranes reflects the pathological state. On the other hand, the data testify the polyvalent origins of cataract disease at the molecular level. Obviously, although we do not dispose of a genetic profile of this patient, the cataract cause was not membrane related.

5. Discussion and conclusions

Junctional microdomains represent well-defined functional platforms within the cellular membrane. Membrane-mediated interactions of several types may strongly influence the organization of junction-forming proteins (figure 7) and thereby play an important role in the functionality of intercellular junctions. Both AQP0 and connexin are transmembrane proteins and, as such, they locally modify the lipid organization in their vicinity. One common type of such deformation, the so-called hydrophobic mismatch (figure 7(A)), occurs when the transmembrane domain of a protein does not precisely match the thickness of the pure lipid bilayer in which it is embedded (Piknova et al 1993). The bilayer thickness of the native lens membranes was 46 Å (Buzhynskyy et al 2007b), significantly thicker than the 35 Å found for the bilayer thickness in junctional 2D crystals (Gonen et al 2005) and the length of the hydrophobic region of the transmembrane helices of AQP0. This is a strong hydrophobic mismatch, which induces either squeezing and bending of the bilayer to match the protein’s hydrophobic core, or recruitment of those lipids from the bilayer that fit best their properties, resulting, in both cases, in an interaction between proteins (Phillips et al 2009). The interaction is often attractive between identical proteins, and is characterized by a range \( \lambda \) of the order of a few nm and a strength \( \kappa (\delta u / \lambda)^2 \) possibly reaching several times the thermal energy \( kT \) (here, \( \kappa \approx 10 kT \) is the bending rigidity of a monolayer, and \( \delta u \approx 1/2 \) nm is the monolayer mismatch). Such a strong interaction is liable to drive protein aggregation into clusters. In the case of the purified AQP0 tetramer, these clusters are 2D crystals with a tetragonal lattice, which may reach a size of
Figure 6. Comparison of supramolecular architecture of lens junctional microdomains of various origins in native and cataract-affected states. Top: AFM images. Bottom: schematic representations. (A) Organization of a native junctional microdomain of sheep. The AQP0 square arrays are confined by connexons, forming together stable platforms in the lipid membrane. Inset: high-resolution view of junctional microdomain edge regions. Connexons confine AQP0 arrays either as densely packed assembly to a certain extent mixed with AQP0. (B) High-resolution topograph of native calf junctional microdomains showing five individual and differently oriented AQP0 arrays separated and edged by stronger protruding molecules, identified as connexons (arrowheads). (C) Human senile cataract lens membranes consist of large smooth lipid domains containing corrugated regions. Top: corrugated protein layers protrude strongly from the cell membrane. Using the AFM tip as a nano-dissector, the membranes of the junctional microdomain were separated, giving access to the extracellular surface. The edge region of a membrane adsorbed on mica shows the smooth lipid bilayer surface containing the square-patterned protein patches. Both the intracellular and extracellular surfaces of cell junctions are present in the image. Bottom: medium-resolution topograph of AQP0 junction arrays. No connexons were found at the AQP0 array edges (arrowheads). (D) Human type-II diabetes patient cataract membrane containing large smooth lipid membranes occupied by junctional microdomains that appear as square-patterned domains. The AQP0 arrays are not surrounded by connexons, and their supramolecular structure is fuzzier than in healthy membranes. AQP0 tetramers are found branching into single rows (outline 1), rows of 2 (outline 2) and 3 (outline 3). (E) Membranes from the eye lens of a 2-year-old patient suffering from congenital cataract. AQP0 tetramers form typical square lattices. A group of barrel-shaped connexons is present at the peripheral zone of the AQP0 array (arrowheads).

several micrometers. Gonen et al were able to grow 2D crystals of AQP0 comprising ~ 200 000 AQP0 tetramers (Gonen et al 2004b).

Another type of membrane-mediated interaction exists between proteins forming junctions between the membranes of neighbouring cells (Bruinsma et al 1994) (figure 7(B)). These
Interactions between membrane inclusions arise when the inclusion locally perturbs the optimal membrane organization (schematized here by the dashed straight lines), leading to the formation of clusters of inclusions. These include (A) interactions within one bilayer membrane, e.g. due to local variation of the bilayer thickness (hydrophobic mismatch), or (B) interactions between inclusions forming junctions between two membranes. In the case of a mixture of junctions of two different sizes, (C) the membrane-mediated interactions can induce size sorting within the inclusion clusters.

Interactions also originate from the fact that junctions perturb the intercellular spacing, by forcing the two cells to be in close contact, and can be responsible for the formation or large junction clusters. Interestingly, the clusters can contain junctions of different sizes, which will then end up being sorted according to their size within the clusters (figure 7(C)) (Weikl and Lipowsky 2004). Such a situation seems to correspond with our observations that the tight junction forming AQP0 are located in the core of junctional domains and surrounded by lines of the thicker gap junctions (figure 3). One question that remains to be answered is what mechanism dictates the (rather small, ~100 nm) size of the junction clusters observed in eye lens membranes. Since AQP0 alone can form micrometer-sized lattices, and since connexons are located at the edges and at the interfaces between AQP0 arrays in the native membrane, connexons may act as lineactants (Mangenot et al 2009), favoring the formation of many small AQP0 clusters with high translational entropy rather than micron-size assemblies. Lineactants, namely molecules that reduce the energetic cost by creating a (1D) interface between (2D) membrane domains, are thought to play a crucial role in many aspects of cell membrane organization, such as in protein–lipid raft formation. A lineactant function has readily been proposed for the lipid molecule cholesterol (Sparr et al 2002). Phase separation of constituents in a membrane is at the origin of domain formation; however, the formation of well-defined separated areas demands a lineactant without which phase separation would be global and result in a complete segregation of components. An additional complexity of the present situation is
that tight junctions form crystals, and that the AQP0 arrays that are separated by connexons tend to have different orientations. It is thus possible that the insertion of connexons actually reduces the energy of grain boundaries within AQP0 polycrystals (Phillips 2001).

To summarize, we propose two hypotheses that may energetically account for the supramolecular assembly of AQP0 and connexons found in the junctional microdomains. Firstly, the hydrophobic mismatch driving force of the AQP0 self-assembly is sufficiently dominant to push all connexons to the edges, i.e. AQP0 crystallize the connexons out. Secondly, AQP0 thin junctions create an intermembrane space of $\sim 20\,\text{Å}$, unambiguously shown by the junctional AQP0 structure (figure 2(A); Gonen et al 2005), and by the surface protrusion of 10 Å of AQP0 in the junctional microdomains (figure 5(A); Buzhynskyy(98,808),(710,870) et al 2007b, Mangenot et al 2009). In contrast, connexins form gap junctions that imply an intermembrane space of $\sim 40\,\text{Å}$, also clearly documented by the 3D structure (Maeda et al 2009) and by the surface protrusion of connexons of 22 Å in the junctional microdomains (figure 6(C)) (Buzhynskyy et al 2007b, Mangenot et al 2009). Therefore, the two cannot co-exist mixed in the junctional microdomains. Thus, it is probably energetically favorable to separate AQP0 and connexons, allowing a majority of both to form junctions. Most probably, the two effects are superposed and drive together the supramolecular assembly of junctional microdomains in the native membranes.

AFM proves to be a potential nano-imaging tool for the study of pathological material from individual patients in comparison with native morphology at the single molecule level. Eventual clinical applications are yet to be developed for this technique, which will be able to contribute to ‘medical proteomics’ of a patient. Such information, especially in combination with potential individual ‘genomics’, will enable the development of unique individual medical treatments.

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Appendix. Materials and methods

A.1. Sample preparation

The analyzed congenital cataract material originated from the right eye of a 2-year-old patient. The pathological eye lens was extracted by faceomulcification and the lens material was collected in a disposable recipient (connected to the fluid-control generator), which was transported on ice to the laboratory. The cataract debris, visible pieces of the lens tissue of millimeter size, was pelleted by high-speed centrifugation. Subsequently, the cells of the surgery debris were separated and broken using a homogenizer, followed by membrane washing and pelleting steps (Buzhynskyy et al 2007a, 2007b, Gonen et al 2004a).
A.2. Atomic force microscopy (AFM)

Mica supports were immersed in 40 µl adsorption buffer (10 mM Tris-HCl, pH 7.4, 150 mM KCl and 25 mM MgCl₂). Subsequently, 3–5 µl of membrane solution were injected into the buffer drop. After ~ 30 min, the sample was rinsed with recording buffer (10 mM Tris-HCl, pH 7.4, and 150 mM KCl). The AFM (Binnig et al. 1986) was operated in contact mode at ambient temperature and pressure. Imaging was performed with a commercial Nanoscope-E AFM (Veeco, Santa Barbara, CA, USA) equipped with a 160 µm scanner (J-scanner) and oxide-sharpened Si₃N₄ cantilevers (length 100 µm; k = 0.09 N m⁻¹; Olympus, Tokyo, Japan). For imaging, minimal loading forces of ~ 100 pN were applied, at scan frequencies of 4–7 Hz using optimized feedback parameters and manually accounting for force drift.

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