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Functional Reconstitution of Lens Gap Junction Proteins into Proteoliposomes

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Summary. Membranes rich in junction complexes were prepared from bovine lens, and the fragments of the membranes were reconstituted into proteoliposomes with a large excess of phosphatidylcholine and dicetylphosphate. The osmotic swelling behavior of these liposomes showed that the lens junction membranes contributed protein components that produced channels with a nominal diameter of 1.4 nm. Most preparations of lens junctions produced rates of osmotic swelling much slower than those found in proteoliposomes containing equivalent amounts of *Escherichia coli* porin, and we discuss several possible explanations for this observation.

Key Words proteoliposome reconstitution · bovine lens · gap junctions

Introduction

Many cells in animal tissues are connected together through "communicative junctions," such as gap junctions [14, 21]. Gap junctions are characterized by the apposition of two plasma membranes with an apparent "gap" of 2–4 nm, and protein oligomers with an apparent central channel have been isolated from the regions of liver plasma membrane enriched in such junctions [4, 10, 13, 30]. Although ingenious approaches such as dye injection [14] have been used to characterize the properties of the channel, to our knowledge there has been no report of direct reconstitution studies of the type widely used with the bacterial channel-forming protein, porin [18, 19, 26], or with its analog from the mitochondrial outer membrane, mitochondrial porin [6, 32].

More recently membranes from lens fiber have been studied intensively, because they contain essentially only one protein, which appears to be the subunit of intercellular junctions [1, 3, 11, 12, 28, 33]. However, the subunit of the lens junction is different from that of liver gap junctions in its molecular weight, peptide map, and immunological specificity [12]. Furthermore, proteins in rat and bovine lens membranes were reported to occur frequently in square arrays in contrast to the hexagonal arrangement found in liver junctions [28, 33], although hexagonal and rhombic arrays have been observed in lens fiber membrane preparations in other laboratories [1, 13]. Because of these differences, some workers expressed doubts on whether the lens junctions were indeed communicative junctions like the liver gap junctions [12, 28, 33].

In this study we incorporated proteins from bovine lens fiber membranes into phospholipid bilayers of liposomes and showed, by the osmotic swelling of these proteoliposomes, the existence in these membranes of protein complexes that produced large, water-filled channels that allowed the rapid diffusion of molecules as large as maltoheptaose (1,152 daltons). These results are evaluated in relation to the probable function of lens junctions.

Materials and Methods

In most experiments, membranes containing predominantly junction proteins were isolated from bovine lens (obtained frozen from Pel-Freeze) by the procedure of Brockhuyse and Kuhlman [2] as modified by Zampighi et al. [33], i.e., by removing crystallin and other cytoplasmic proteins by using 4 and 7 M urea in the presence of 1.6×10^{-8} M Ca^{2+}. Where specified, the procedure was carried out in the total absence of Ca^{2+}, by substituting the ethylenediaminotetraacetic acid (EDTA)-Ca^{2+} pair in all solutions by 4 mM EDTA or ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). For brevity, these junction-enriched membrane preparations will be called "lens junctions."

The proteoliposome reconstitution was performed essentially as described in ref. 19, except that the intravesicular solution contained 17% (wt/vol) Dextran T-20 (Pharmacia, Uppsala, Sweden) instead of stachyose or nicotinamide adenine dinucleotide. Briefly, acetone-washed preparation of egg phosphatidylcholine (Type IX-E, Sigma, St. Louis, Missouri), 2.4 μmol, and dicetylphosphate (Sigma), 0.1 μmol, were dried at the bottom of a tube, and the lipid was resuspended in an aqueous dispersion (usually 0.1–0.2 ml) containing up to 10 μg (protein) of the lens junction. After sonication, the translucent mixture was rapidly dried under reduced pressure, and the dry film was resus-

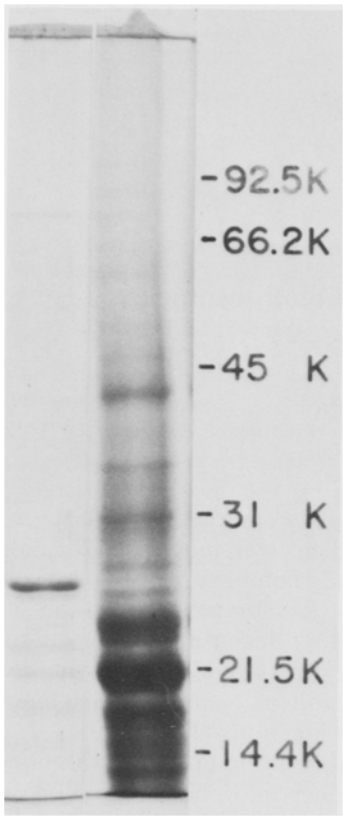

Fig. 1. SDS-polyacrylamide electrophoresis of preparations from bovine lens. *Left lane:* Purified lens junction membranes. *Right lane:* Crude homogenate of the bovine lens, used as the starting material of purification. Molecular weight markers were: phosphorylase B (92.5 kilodaltons, shown as 92.5K), serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), and lysozyme (14.4K)

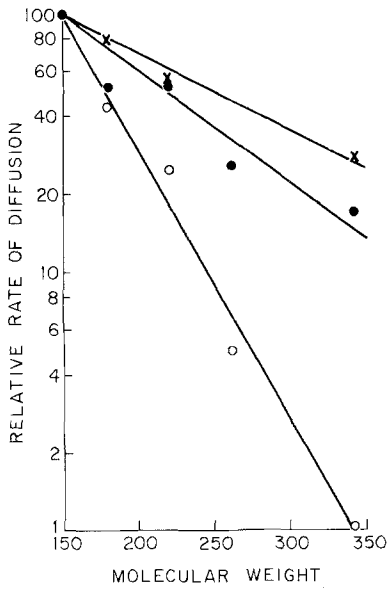

Fig. 2. Initial rates of swelling of proteoliposomes in various sugars. Proteoliposomes containing *P. aeruginosa* porin (×), bovine lens junctions (●), or *E. coli* OmpF porin (○), were diluted into isotonic solutions of L-arabinose, D-glucose, N-acetyl-D-glucosamine, 2,3-diacetamido-2,3-dideoxy-D-glucose, and sucrose, and their initial rates of swelling were plotted after normalization to the rate in L-arabinose. The data with the *P. aeruginosa* and *E. coli* porins were taken from references 31 and 19, respectively

pended in 0.6 ml of 17% Dextran T-20 containing 5 mM Tris-Cl buffer, pH 7.5. The liposomes (usually 20 μl) were then diluted into 0.6 ml of isotonic solutions of various sugars, all made up in 5 mM Tris-Cl buffer, pH 7.5, and the initial rates of osmotic swelling due to the influx of the sugars through the junction channel were measured by following the turbidity of the suspension at 400 nm. This method of preparation is expected to produce multilayered liposomes, and therefore only the initial rates of swelling, presumably representing the swelling of the outermost layer, were used in calculation [19].

The results of the swelling assay above were confirmed by carrying out an isotope efflux assay, outlined in ref. 19. [^{3}H]-Maltoheptaitol was made by reducing maltoheptaose (obtained by the limited acid hydrolysis of β-cyclodextrin [9]) with [^{3}H]-$NaBH_4$.

Protein was measured by the Lowry method [15], using bovine serum albumin as the standard. Polyacrylamide gel electrophoresis was performed with the slab method [16] in the presence of sodium dodecylsulfate; 15% acrylamide was used.

Results

The procedure of Zampighi et al. [33] produced a membrane fraction that contained predominantly the polypeptide with an apparent molecular weight of 27,000. In the particular preparation shown in Fig. 1, the 21,000-dalton and 14,000-dalton bands, which are presumed to be the proteolytic degradation products of the 27,000-dalton protein [33], were almost completely absent. When this preparation was reconstituted with a large excess of phospholipids as described in Materials and Methods, the proteoliposomes showed significant permeability toward sugars, as seen from the rapid decrease of turbidity upon their dilution into isotonic sugar solutions. The relative rates of permeation of sugars through the bilayer showed clear dependence on the size of the solute sugar molecule, as shown in Fig. 2. This is a characteristic behavior expected for diffusion through water-filled pores. In fact, the equation proposed by Renkin [24] allows us to predict the behavior of cylindrical pores of various diameters toward a series of solutes, and the calculation showed that the experimental results fitted best with those predicted for a pore of 1.4 nm diameter (Fig. 2). However, we note that this number is a very rough estimate. Firstly, the Renkin equation postulates a cylindrical pore, whereas the real channel may deviate significantly from this shape. In addition, the equation postulates that small molecules are affected by various forces in such a manner as are large macroscopic bodies, an assumption that cannot be correct in a rigorous way. Secondly,

as one sees from the comparison of lens junction pores with the *Pseudomonas aeruginosa* porin channel with an estimated diameter of 2.0 nm (Fig. 2), the diffusion rates of the tested solutes are not very sensitive to the small variations in pore diameter in this range. In contrast, the size-dependence curve for *E. coli* porin (Fig. 2), which has a smaller diameter (around 1.15 nm; ref. 19), shows that a further reduction in pore diameter produces a much stronger dependence of permeability on the size of the solutes.

When 5 μg of the junction protein was used in reconstitution, dilution of 20 μl of the proteoliposome suspension into 0.6 ml of L-arabinose solution usually produced the initial rate of optical density decrease in the range of 0.2 to 0.35 per min. With *E. coli* porin, similar rates were observed when less than 0.5 μg was used in reconstitution. Thus, although the pores produced by the junction complex are large, the absolute degree of permeability or the rate of solute flux in the presence of a given amount of driving force was somewhat lower in comparison with the proteoliposomes reconstituted with *E. coli* porin. These observations will be considered in the Discussion.

The result of the swelling assay was confirmed by performing isotope efflux assay (Table). When proteoliposomes were reconstituted with the lens junction protein in a way in which they contained isotopically labeled oligosaccharides initially in the intravesicular space, there was nearly complete efflux of a trisaccharide, raffinose, as well as a tetrasaccharide, stachyose, during the gel filtration of the vesicles. Even a heptasaccharide, maltoheptaitol, was seen to diffuse out of the proteoliposome vesicles to a significant extent. These results form a striking contrast to the proteoliposomes containing *E. coli* porins with much narrower channels; these vesicles allowed no measurable diffusion of stachyose [19].

Channels of gap junctions from several tissues apparently become "closed," as judged from the electrical uncoupling, when the cytoplasmic concentration of Ca^{2+} increases beyond a certain level [14, 25]. The clearest explanation, at the molecular level, of this phenomenon was presented by Unwin and Ennis [29], who showed that in the presence of Ca^{2+} the subunits of liver gap junctions take an orientation more perpendicular to the membrane surface, and that this rearrangement could result in the closing of the channel [30]. In addition, however, other alterations may be induced. For example, it has been reported that the size and the two-dimensional arrangement of the junctions become altered under uncoupling conditions [17, 20], and that such changes were reproduced by treating the lens junctions, isolated in the absence of Ca^{2+}, with Ca^{2+} or higher concentrations of Mg^{2+} [23]. In view of these observations, we tried to see whether lens junction preparations isolated in the absence of Ca^{2+} showed permeability properties different from those isolated in the presence of Ca^{2+} according to Zampighi et al. [33]. Lens junctions were isolated as described in Materials and Methods, with EDTA or EGTA present throughout the procedure. When one such preparation made in EGTA was reconstituted into proteoliposomes, the size of the channel was identical to that seen with preparations prepared in the presence of Ca^{2+} in parallel and reconstituted in the presence of 5×10^{-6} M Ca^{2+} (Fig. 3). Furthermore, the initial swelling rates of proteoliposomes containing junction preparations made in the total absence of Ca^{2+} were similar, in most cases, to the rates of proteoliposome containing the preparations made with 1.6×10^{-8} M Ca^{2+}. Thus we could not observe the expected massive opening or closing of the junction channels through the removal or addition of Ca^{2+}.

Table. Oligosaccharide retention assay of proteoliposome permeability

Oligosaccharide used	Mol wt	Fraction diffused out during gel filtration (%)
Sucrose	342	94
Raffinose	505	76
Stachyose	666	74
Maltoheptaitol	1154	34

Lens junction membranes (prepared in the presence of EDTA throughout) containing 30 μg protein were reconstituted with 1.35 μmol egg phosphatidylcholine and 50 nmol dicetylphosphate in 300 μl aqueous solution containing 1 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH buffer, pH 7.4, [^{14}C]-dextran, and ^{3}H-labeled oligosaccharide listed above. The vesicles were slowly (approx. 20 min) passed through a 1.27 × 16 cm column of Sepharose 4B (Pharmacia, Uppsala, Sweden), which was eluted with 1 mM HEPES-NaOH buffer, pH 7.4. The vesicles, eluted at the void volume, were collected, and the fraction of the ^{3}H-labeled oligosaccharides still present in intravesicular space was calculated from the ^{3}H and ^{14}C contents of the vesicles and the reconstitution mixture.

Discussion

In this study we showed that preparation of junctional membranes obtained from bovine lens could be successfully reconstituted into proteoliposomes. These vesicles showed rapid swelling when diluted into isotonic solutions of various sugars, and the dependence of the influx rates on the sizes of the solutes suggested the pore diameter of 1.4 nm (Fig. 2), in good agreement to previous suggestions from the fluorescent marker diffusion experiments performed with cultured mammalian cells [27]. Be-

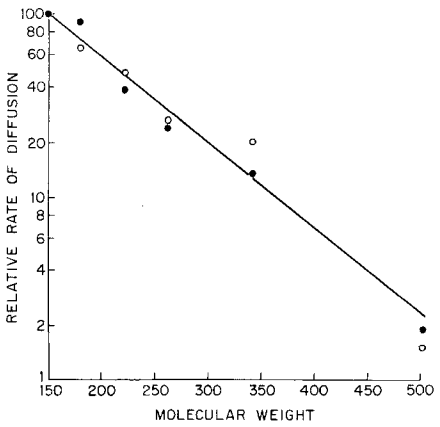

Fig. 3. Initial rates of swelling of proteoliposomes containing lens junctions prepared in the presence and absence of Ca^{2+}. Lens junctions prepared in the presence of Ca^{2+} (1.6×10^{-8} M) according to Zampighi et al. [33] and reconstituted in the presence of 5×10^{-6} M Ca^{2+} (○). Lens junctions prepared in the absence of Ca^{2+}, i.e., in the presence of EGTA, and reconstituted in the absence of Ca^{2+} (●). Liposome preparations were diluted into isotonic solutions of various sugars listed in the legend to Fig. 2, and their *relative* initial rates of swelling were plotted against the mol wt of the sugar

cause the permeability conferred on the vesicles was rather low in comparison with the activity of *E. coli* porin (*see* Results), and because junctional membrane rather than purified protein was used as the source of the channel-forming protein, we cannot completely exclude the possibility that minor protein(s) other than the lens junction complex was responsible for the generation of channels in the liposomes. However, this appears rather unlikely because of the following reasons. (i) One source of protein forming large channels is the mitochondrial outer membrane [6, 32]. The fiber cells of lens cortex and nucleus, however, are known to contain few mitochondria [5]. Furthermore, the mitochondrial porin appears to produce pores much larger than those seen in this study, with a size similar to those of *P. aeruginosa* porin channel [32], which was estimated to have a diameter of 2.0 nm [31]. (ii) Another potential source could be the nuclear membrane, but nuclei are not found in these cells [5]. (iii) Finally, the preparation contained very little contaminants other than the 27,000-dalton protein (Fig. 1).

The junction proteins are rather inefficient in producing the osmotic swelling of proteoliposomes. There are some trivial explanations of this observation. (i) Since our reconstitution technique includes a rather drastic step in which the protein-lipid complex is dried up from an aqueous suspension, a fraction of the junctional proteins may have become denatured during the reconstitution process. (ii) Because one junctional complex has two regions that would normally interact with the hydrophobic interior of membranes, many of these units could connect two liposome vesicles together, making them isolated from the external medium. (iii) Because the junction proteins occur in patches or aggregates, the reconstituted vesicle population may be heterogeneous, and may contain only small numbers of vesicles with large amounts of junction proteins. Possibly some of these mechanisms do make partial contributions in lowering the permeability of proteoliposomes under certain conditions. However, the nearly complete efflux of labeled sucrose from the reconstituted proteoliposomes (Table) suggests that there was at least one open pore per vesicle made under these conditions, and this result could therefore be taken as being inconsistent with suggestions ii and iii above.

In addition to these trivial explanations, however, there is a more interesting possibility that only a portion of the population of the 27,000-dalton protein is involved in the formation of communicating junctions. Thus FitzGerald and coworkers [8] recently found that antibody specific for the 27,000-dalton protein stains all areas of the bovine lens fiber cell membrane evenly, in spite of the concentration of the junctions to small patches in such a membrane. Costello and coworkers [7] found that a large fraction of the 27,000-dalton protein is involved in the formation of square arrays, which appeared to produce waviness in the contour of the membrane but no junctions traversing the thickness of the two membranes. In view of these results, it seems quite possible that not all of the 27,000-dalton proteins could be assembled to form channels in our proteoliposomes. Lens junction membranes made in the presence of Ca^{2+} tend to contain crystalline arrays of junction proteins [33]. Furthermore, *in vivo* such crystalline arrays are usually found in situations in which the cells are electrically uncoupled [20, 23]. This provides another explanation of why the permeability was rather low in reconstituted proteoliposomes: our membranes have been prepared usually in the presence of Ca^{2+}, and therefore most of the junctions could have been in crystalline array in which the channels are in the uncoupled or closed state. If this is the case then the preparations with noncrystalline distributions of "open" junctions should produce a much higher permeability. As described in Results, however, most preparations made in the absence of Ca^{2+} did not produce significantly more permeable proteoliposomes. But our starting material was the frozen preparations of bovine lens, and freezing and thawing under certain conditions were found to produce crystalline arrays of junctions (and therefore presumably the closing of the channel) [1]. Thus most of the junctions in

our starting material could have been in a crystalline state from the beginning. In this connection, one sample prepared with EDTA throughout did produce at least an order of magnitude higher permeability in proteoliposomes (not shown), a result consistent with the interpretation presented above. It is also interesting that, after this work had been completed, Peracchia and Girsh [22] reported a similar reconstitution of lens junctions in proteoliposomes and found that the opening and closing of the channel requires calmodulin in addition to Ca^{2+}. This work utilized 27,000-dalton protein recovered from SDS-polyacrylamide gels, a preparation probably largely denatured, and consequently its pore-forming activity appeared to be about three orders of magnitude lower than ours. Nevertheless, these results strongly suggest the need to repeat our experiment by using calmodulin.

In conclusion, we have shown by reconstitution approach that membranes from lens fiber, enriched in lens junctions, do indeed produce transmembrane channels of approximately 1.4 nm in diameter. Because the preparations contained essentially only the 27,000-dalton lens junction protein, it appears most likely that the permeability of the proteoliposomes is due to the major lens junction protein. We believe that this reconstitution approach will be useful in the study of the assembly and disassembly of junctions as well as of the opening and closing of the preassembled channel by physiological stimuli.

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References

1. Bernardini, G., Peracchia, C. 1981. Gap junction crystallization in lens fibers after an increase in cell calcium. *Invest. Ophthalmol.* **21**:291–299
2. Brockhuyse, R.M., Kuhlman, E.D. 1978. Lens membrane: IV. Preparative isolation and characterization of the membranes and various membrane proteins from calf lens. *Exp. Eye Res.* **26**:305–320
3. Brockhuyse, R.M., Kuhlman, E.D., Bijvelt, J., Verkleij, A.J., Ververgaert, P.H.T. 1978. Lens membranes: III. Freeze fracture morphology and composition of bovine lens fibre membranes in relation to ageing. *Exp. Eye Res.* **26**:147–156
4. Caspar, D.L.D., Goodenough, D.A., Makowski, L., Phillips, W.C. 1977. Gap junction structures: I. Correlated electron microscopy and X-ray diffraction. *J. Cell Biol.* **74**:605–628
5. Cohen, A.I. 1965. The electron microscopy of the normal human lens. *Invest. Ophthalmol.* **4**:443–446
6. Colombini, M. 1979. A candidate for the permeability pathway of the outer mitochondrial membrane. *Nature (London)* **279**:643–645
7. Costello, M.J., McIntosh, T.-J., Robertson, J.D. 1984. Square array fiber cell membrane in mammalian lens. *In*: Proceedings 42nd Meeting of Electron Microscopy Society of America. D.W. Dailey, editor. pp. 126–129. San Francisco Press, San Francisco
8. FitzGerald, P.G., Bok, D., Horwitz, J. 1983. Immunocytochemical localization of the main intrinsic polypeptide (MIP) in ultrathin frozen sections of rat lens. *J. Cell Biol.* **97**:1491–1499
9. French, D., Levine, M., Pazur, J.H. 1949. Studies on the Schardinger dextrins: II. Preparation and properties of amyloheptaose. *J. Am. Chem. Soc.* **71**:356–358
10. Goodenough, D.A. 1974. Bulk isolation of mouse hepatocyte gap junctions. *J. Cell Biol.* **61**:557–563
11. Goodenough, D.A. 1979. Lens gap junctions: A structural hypothesis for nonregulated low-resistance intercellular pathways. *Invest. Ophthalmol.* **18**:1104–1122
12. Hertzberg, E.L., Anderson, D.J., Friedlander, M., Gilula, N.B. 1982. Comparative analysis of the major polypeptides from liver gap junctions and lens fiber junctions. *J. Cell. Biol.* **92**:53–59
13. Kuszak, J.R., Rae, J.L., Pauli, B.U., Weinstein, R.S. 1982. Rotary replication of lens gap junctions. *J. Ultrastruct. Res.* **81**:249–256
14. Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* **61**:829–913
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275
16. Lugtenberg, B., Meijers, J., Peters, R., Hoek, P. van der, Alphen, L. van 1975. Electrophotretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. *FEBS Lett.* **58**:254–258
17. Makowski, L., Caspar, D.L.D., Phillips, W.C., Goodenough, D.A. 1977. Gap junction structures: II. Analysis of the X-ray diffraction data. *J. Cell Biol.* **74**:629–645
18. Nakae, T. 1976. Outer membrane of *Salmonella*. Isolation of protein complex that produces transmembrane channels. *J. Biol. Chem.* **251**:2176–2178
19. Nikaido, H., Rosengerg, E.Y. 1983. Porin channels in *Escherichia coli:* Studies with liposomes reconstituted from purified proteins. *J. Bacteriol.* **153**:241–252
20. Peracchia, C. 1977. Gap junctions: Structural changes after uncoupling procedures. *J. Cell Biol.* **72**:628–641
21. Peracchia, C. 1978. Structural correlates of gap junction permeation. *Int. Rev. Cytol.* **66**:81–145
22. Peracchia, C., Girsh, S.J. 1984. Calmodulin-mediated gating of lens gap junction channels in vesicles. *In*: Proceedings 42nd Meeting of Electron Microscopy Society of America. G.W. Dailey, editor. pp. 134–137. San Francisco Press, San Francisco
23. Peracchia, C., Peracchia, L.L. 1980. Gap junction dynamics: Reversible effects of divalent cations. *J. Cell Biol.* **87**:708–718
24. Renkin, E.M. 1954. Filtration, diffusion, and molecular sieving through porous cellulose membranes. *J. Gen. Physiol.* **38**:225–243
25. Rose, B., Loewenstein, W.R. 1975. Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature (London)* **254**:250–252
26. Schindler, H., Rosenbusch, J.P. 1978. Matrix protein from *E. coli* outer membrane forms voltage-controlled channels in lipid bilayers. *Proc. Natl. Acad. Sci. USA* **75**:3751–3755
27. Schwarzmann, G., Wiegandt, H., Rose, B., Zimmerman, A., Ben-haim, D., Loewenstein, W.R. 1981. Diameter of the

cell-to-cell junctional membrane channels as probed with neutral molecules. *Science* **213:**551–553
28. Simon, S.A., Zampighi, G., McIntosh, T.J., Costello, J., Ting-Beall, H.P., Robertson, J.D. 1982. The structure of junctions between lens fiber cells. *Biosci. Rep.* **2:**333–341
29. Unwin, P.N.T., Ennis, P.D. 1983. Calcium-mediated changes in gap junction structure: Evidence from the low angle X-ray pattern. *J. Cell Biol.* **97:**1459–1465
30. Unwin, P.N.T., Zampighi, G. 1980. Structure of the junction between communicating cells. *Nature (London)* **283:**545–549
31. Yoshimura, F., Zalman, L.S., Nikaido, H. 1983. Purification and properties of *Pseudomonas aeruginosa* porin. *J. Biol. Chem.* **258:**2308–2314
32. Zalman, L.S., Nikaido, H., Kagawa, Y. 1980. Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels. *J. Biol. Chem.* **255:**1771–1774
33. Zampighi, G., Simon, S.A., Robertson, J.D., McIntosh, T.J., Costello, M.J. 1982. On the structural organization of isolated bovine lens fiber junctions. *J. Cell Biol.* **93:**175–189

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