THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 3, Issue of January 21, pp. 1583-1586, 1994
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Printed in U.S.A.

Communication

Biologically Active Two-dimensional Crystals of Aquaporin CHIP*

(Received for publication, October 25, 1993, and in revised form, November 22, 1993)

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Plasma membranes of several mammalian tissues are highly permeable to water due to the presence of CHIP, the 28-kDa channel-forming integral protein which is the archetypal member of the aquaporin family of water channel proteins. To define its native structure, purified red cell CHIP protein was reconstituted into lipid bilayers at a high protein-to-lipid ratio, and the resulting 3-µm diameter membrane vesicles were examined by high resolution electron microscopy. The reconstituted membranes contained highly ordered two-dimensional crystalline lattices of $p422_1$ symmetry in which each CHIP tetramer contained a central depression extending from the outer and inner surfaces of the membrane into the transbilayer domain of the molecule. The reconstituted membranes also exhibited extremely high osmotic water permeability, P_f = 0.472 cm/s, corresponding to the sum of activities of all incorporated CHIP molecules. These studies report the first two-dimensional crystallization of a biologically active water channel and provide direct evidence of the structure responsible for its pore-like behavior.

The existence of constitutively activated water-selective channels has been postulated to explain the known water permeability of the plasma membranes of several mammalian and amphibian tissues (reviewed by Finkelstein (1987), Verkman (1989), and Harris *et al.* (1991)); however, the molecular water channel structure eluded identification until recently. CHIP is the 28-kDa channel-forming integral protein of red cells and renal proximal tubules (Denker *et al.*, 1988; Smith and Agre, 1991; Preston and Agre, 1991). CHIP was first shown to function as a water pore by expression in *Xenopus laevis* oocytes (Preston *et al.*, 1992) and then by reconstitution of highly purified CHIP protein into proteoliposomes (Zeidel *et al.*, 1992). CHIP protein is also distributed within renal proximal tubules and water-permeable epithelia of several other tissues (Nielsen, 1993a, 1993b). These studies were confirmed and further defined in several laboratories (reviewed by Agre *et al.* (1993b)), and CHIP is now recognized as the archetypal member of a family of plant and mammalian water channels known as the "aquaporins" (Agre *et al.*, 1993a).

Although the current understanding is incomplete, the structure of CHIP is known to contain several unique features. Near and far ultraviolet circular dichroism analyses of MIP26, a homologous protein from lens, predicted that ~50% of the protein is α-helical and ~20% is β structure (Horwitz and Bok, 1987). Hydropathy analysis of the deduced amino acid sequence predicted that each CHIP subunit has six bilayer-spanning domains (Preston and Agre, 1991); the N- and C-terminal halves of the CHIP molecule are sequence-related and are oriented at 180° to each other within the membrane bilayer (Preston *et al.*, 1992). This topology was confirmed by vectorial proteolysis of biologically active CHIP molecules in which cleavage sites had been introduced (Preston *et al.*, 1994). Hydrodynamic studies revealed that CHIP is a multisubunit oligomer comprised of approximately four 28-kDa subunits, which are identical except that only one bears a large *N*-linked glycan (Smith and Agre, 1991). Radiation inactivation studies of water channels in native membranes indicated a functional size of 30 kDa (van Hoek *et al.*, 1991). Moreover, site-directed mutagenesis studies suggested that the CHIP oligomer does not function as a single channel, analogous to ion channels, but that each subunit contains an individually functional water-conducting pore, analogous to the bacterial porins (Preston *et al.*, 1993). In order to examine the native ultrastructure with high resolution, pure CHIP was reconstituted into artificial membranes at a high protein-to-lipid ratio, and two-dimensional crystallographic analyses and biophysical studies of water permeability were performed.

EXPERIMENTAL PROCEDURES

CHIP was purified to homogeneity using methods adapted from Smith and Agre (1991). Red cell membrane vesicles from 900 ml of human blood were stripped with 1 M KI, extracted with 1% (w/v) *N*-lauroylsarcosine, solubilized in 4% Triton X-100 (v/v) (Nielsen, 1993a), and filtered through a 0.22-µm Millipore Sterifil D-GV apparatus. A 600-ml aliquot (equivalent to 200 ml of packed membranes) was loaded onto a 10 mm × 100-mm POROS Q/F column (PerSeptive Biosystems, Cambridge, MA) driven by a Pharmacia FPLC apparatus. The column was then washed with 40 ml of 0.1% Triton X-100, 20 mM Tris-HCl (pH 7.8), 1 mM NaN_3, 1 mM dithiothreitol and eluted with a 120-ml 0.2–0.6 M NaCl gradient at 4 ml/min while monitoring the A_{280}. Peak fractions eluted at 0.35 M NaCl and were combined and diluted to six volumes with 1.2% octyl glucopyranoside (Calbiochem), 20 mM Tris-HCl (pH 7.8), 1 mM NaN_3, 1 mM dithiothreitol. Peaks from two runs were reloaded onto the POROS column and washed until the A_{280} base line was stabilized. The column was then eluted at 1 ml/min with a 40-ml gradient of 0–0.6 M NaCl in the same buffer. Peak fractions eluted at 0.25 M NaCl and contained 630 µg/ml pure CHIP (bicinchoninic acid protein method, Pierce Chemical Co.). These were divided into aliquots, which were snap-frozen and stored at −80 °C.

A 100-µl aliquot of purified CHIP was mixed with purified *Escherichia coli* lipids (70% phosphatidylethanolamine, 15% phosphatidylglycerol, 15% cardiolipin (w/w); Ambudkar and Maloney (1986)) solubilized in the same buffer containing 2% octyl glucopyranoside at a

* This work was supported by Grants HL33991 and HL48268 (to P. A) and DK43955 (to M. L. Z.) from the National Institutes of Health, a Department of Veterans Affairs Career Development Award (to M. L. Z.), Swiss National Foundation for Scientific Research grant 31-32536.91 (to A. E.), and a grant from the M. E. Müller-Foundation of Switzerland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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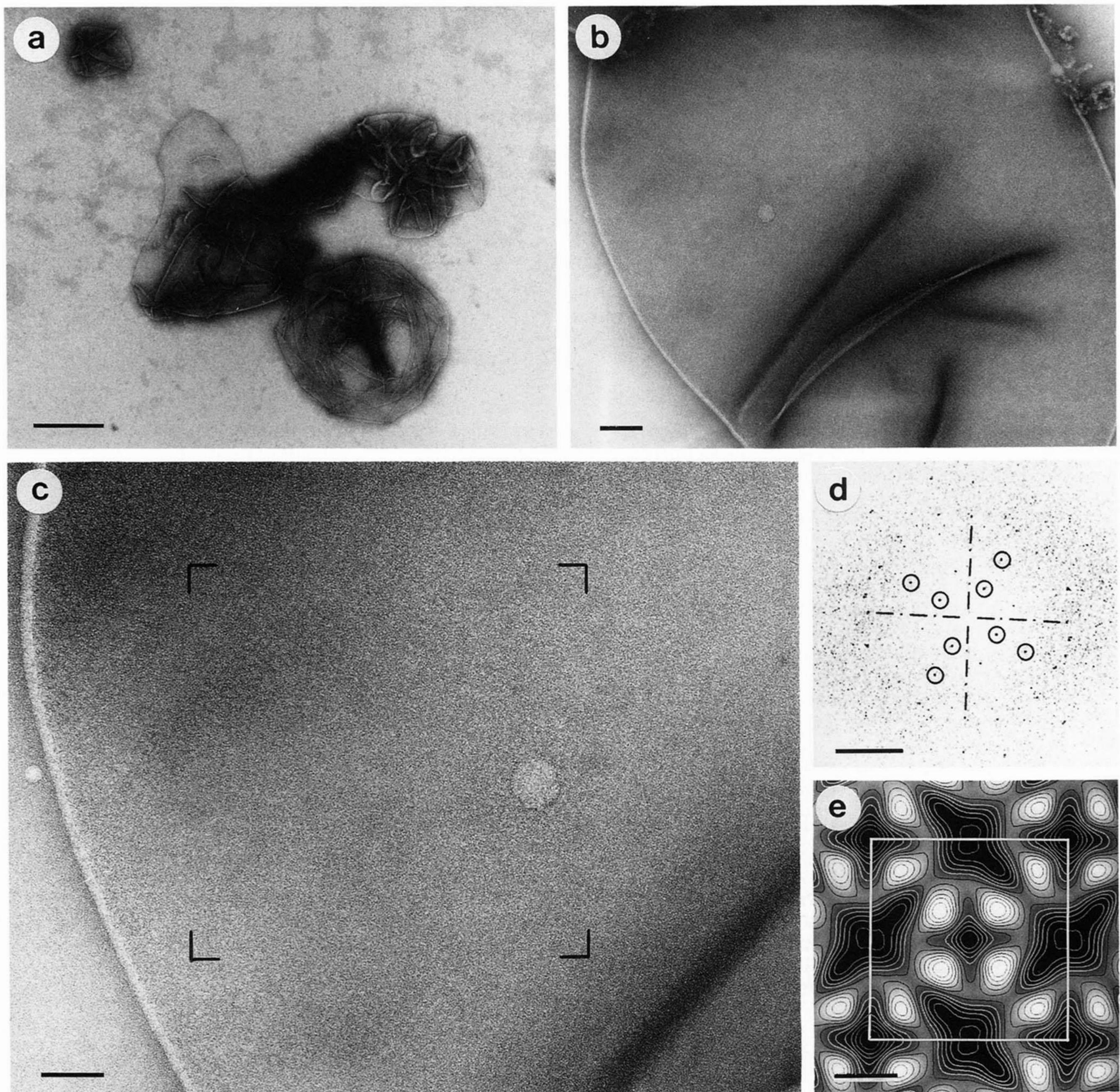

FIG. 1. **Electron microscopy and image analysis of two-dimensional CHIP crystals.** *a*, the two-dimensional CHIP crystals folded into closed unilamellar vesicles that collapsed into convoluted structures during negative staining. *b*, spread-flattened vesicles occasionally exhibited only a few folds. *c*, at higher magnification spread-flattened vesicles revealed square lattices formed by the CHIP tetramers. *d*, the power spectrum calculated from the area marked in *c* shows two sets of diffraction spots corresponding to the crystalline top and bottom layers of the collapsed vesicle. *e*, averaged and 4-fold symmetrized unit cell calculated from six independent crystals is shown. Protein is displayed in *bright shades*, the surrounding stain in *dark shades*. The unit cell outlined houses one tetramer centered on the 4-fold axis perpendicular to the membrane and four monomers belonging to adjacent tetramers in the four corners. *Rhomboid dark areas* between CHIP tetramers represent the lipid bilayer. *Scale bars* represent 1 μm in *a*, 100 nm in *b*, 50 nm in *c*, $(5\ nm)^{-1}$ in *d*, and 3 nm in *e*.

protein-to-lipid ratio (w/w) of 2.0. After 2 h of preincubation, the sample was dialyzed in a continuous flow dialysis system (Jap *et al.*, 1992) using the following temperature profile: 25 °C for 12 h, a linear increase to 35 °C over the next 12 h, 35 °C for 24 h, and a linear decrease to 25 °C over 6 h. Reconstituted vesicles were adsorbed to carbon coated copper grids and stained with 0.75% uranyl formate. Micrographs were recorded at 2,000 electrons/nm² in a Hitachi H7000 electron microscope operated at 100 kV. Averaged projections of CHIP were calculated by correlation methods using the SEMPER image processing system (Ford *et al.*, 1990).

Membrane vesicles in 250 mM NaCl, 20 mM Tris-HCl (pH 7.55), 0.01% NaN_3 were incubated overnight at 4 °C in 20 mM carboxyfluorescein and pelleted by centrifugation for 15 min at 11,000 × *g*, and the entire pellet was resuspended in 100 μl of buffer. Measurements of the osmotic water permeability were performed as previously described (Zeidel *et al.*, 1992) using a stopped-flow fluorometer (SF.17MV, Applied Photophysics, Leatherhead, United Kingdom). The vesicles were rapidly mixed with the same buffer containing sufficient sucrose to raise the osmolality of the mixture to 150%. Excitation wavelength was set at 490 ± 1.5 nm, and emissions at wavelengths >515 nm were collected through a cut-on filter (Oriel Corp., Stratford, CT). Relative decreases in proteoliposome volumes were linearly related to relative fluorescence. Data from five to eight individual recordings were averaged and fitted to single exponential equations, and the resulting coefficients were used to solve the water permeability equation (MathCAD software, MathSoft, Cambridge, MA) shown in Equation 1,

$$dV(t)/dt = (P_f)(SAV)(MVW)[(C_{in}/V(t)) - C_{out}] \quad \text{(Eq. 1)}$$

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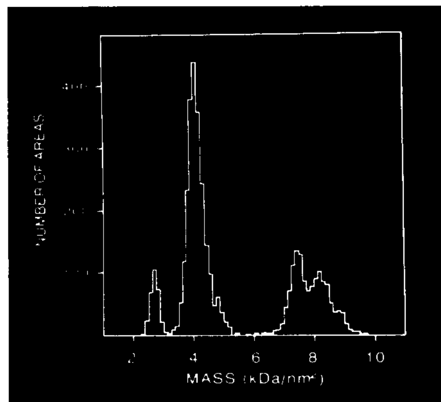

FIG. 2. **Mass analysis of two-dimensional CHIP crystals by scanning transmission electron microscopy (STEM).** Membranes were adsorbed to thin carbon films supported by fenestrated films, washed in double-distilled water, and air-dried. Elastic dark-field images were recorded in STEM at doses of 260 ± 38 electrons/nm^2 and 80 kV acceleration voltage. The mass/area values of 2700 circular areas of 580 nm^2 were evaluated as described (Müller *et al.*, 1992) and plotted in the histogram displayed.

in which $V(t)$ is relative vesicle volume with time, P_f is osmotic water permeability (cm/s), *SAV* is the ratio of proteoliposome surface to volume, *MVW* is the molar volume of water (18 cm^3/mol), and C_{in} and C_{out} are the osmolalities inside and outside the vesicle at outset of the experiment.

RESULTS

Purified CHIP protein exhibited a marked propensity to assemble into square arrays when reconstituted with pure phospholipids at high protein-to-lipid ratio. In the absence of divalent cations, closed unilamellar vesicles formed with an average diameter of 3 µm (Fig. 1*a*). Some vesicles flattened to planar, double-layered structures when adsorbed to carbon-coated Formvar films rendered hydrophilic by glow discharge at low air pressure (Fig. 1*b*). Homogeneously stained flat areas (Fig. 1*c*) revealed a faint regular pattern of lattice lines that gave rise to diffraction spots belonging to two square arrays with unit cell dimensions $a = b = 9.6 \pm 0.1$ nm ($n = 50$, Fig. 1*d*). The presence of two distinct sets of spots rotated with respect to one another by an arbitrary angle provided evidence that both layers of the spread-flattened vesicles were crystalline and suggested that the entire vesicle surface was covered by the square array of CHIP oligomers. Correlation averages typically exhibited a resolution of 2 nm (best value: 1.6 nm) and a 4-fold rotational symmetry (root-mean-square deviation = 5.9 ± 1.8%, $n = 12$) with a unit cell housing two tetrameric units (Fig. 1*e*). Each CHIP tetramer had four stain-excluding elongated domains (*bright shades* in Fig. 1*e*) of approximately 2.7 nm length and 1.6 nm width surrounding a central stain-filled depression (*dark shades*). The tetramers were separated by rhomboid stained areas of approximately 7.3 nm length and 4.7 nm width that represent the lipid bilayer. On closer inspection of the computer-averaged projection, a distinct difference in the stain-filled central depression was evident in the two types of CHIP tetramers (Fig. 1*e*, compare central tetramer to tetramers at four corners).

Mass measurements performed with the STEM[1] (Müller *et al.*, 1992) yielded a mass/area histogram with distinct peaks (Fig. 2). The first peak at 2.7 ± 0.14 kDa/nm^2 ($n = 294$) represents lipid bilayers, whereas the peak at 4.1 ± 0.27 kDa/nm^2 ($n = 2160$) results from CHIP square arrays. Therefore each unit cell comprising an area of 92.2 nm^2 has a mass of 378 kDa and contains two CHIP tetramers and surrounding phospholipids.

[1] The abbreviation used is: STEM, scanning transmission electron microscopy.

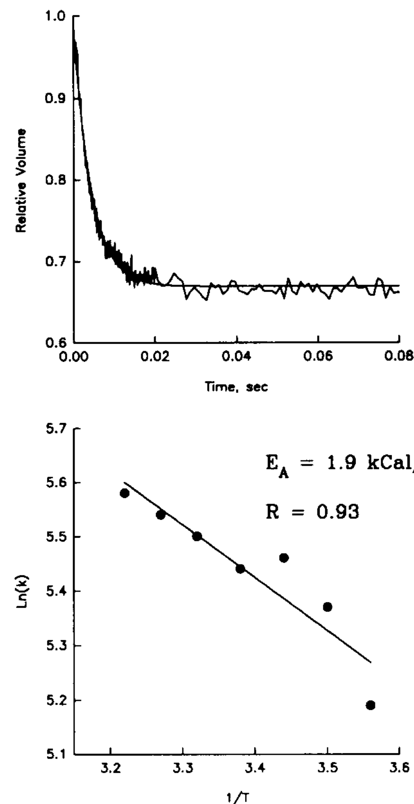

FIG. 3. **Osmotic water permeability of reconstituted CHIP vesicles at 22 °C (*top*) and Arrhenius plot of determinations at 8–39 °C (1/K × 10^{-3}, *bottom*).** CHIP vesicles were incubated overnight at 4 °C in a buffer (250 mM NaCl, 20 mM Tris-HCl (pH 7.55), 0.01% NaN_3) containing 20 mM carboxyfluorescein. The vesicles were pelleted and resuspended in the same buffer, and fluorescence at 490 nm was measured after exposure to a 50% increase in osmotic strength by stopped-flow analysis as described (Zeidel *et al.*, 1992). Relative decreases in vesicle volumes were directly related to reduced fluorescence. Data from five to eight individual recordings served to determine the P_f.

Only one subunit of the CHIP tetramer is likely to be glycosylated, yielding an estimated tetramer mass of 134 kDa, allowing for 110 kDa of interspersed lipid bilayer. Packed at 4 lipids/nm^2 (2.7 kDa/nm^2), the bilayer would cover 44% of the unit cell area, consistent with the area of the rhomboid structures that contribute 40% of the unit cell area (Fig. 1*e*).

The reconstituted protein-lipid vesicles containing CHIP tightly packed in two-dimensional crystals were evaluated for water channel activity. After the vesicles were loaded with carboxyfluorescein, the osmotic water permeability was measured by abruptly increasing the osmolality by 50% with a stopped-flow device while monitoring the quenching of fluorescence. Although large, the 3-µm diameter vesicles exhibited a high degree of water permeability and were fully shrunken in <20 ms (Fig. 3). The coefficient of osmotic water permeability was calculated from the averages of multiple recordings, $P_f = 0.472$ cm/s. The density of CHIP within the two-dimensional crystals in Fig. 1, 8 subunits/(9.6 nm)^2, permitted calculation of the unit water permeability, $p_f = P_f \times$ area/subunit = 5.43 × 10^{-14} cm^3/s/subunit. This value agrees with previous determinations of osmotic water permeability, p_f = 4.6 × 10^{-14} cm^3/s/subunit, measured from smaller vesicles (approximately 0.15 µm in diameter), which were made by reconstitution of pure CHIP at low protein-to-lipid ratios (<0.01, w/w), and is similar to the known magnitude of water permeability of the intact red cell (Zeidel *et al.*, 1992, 1994). Other features of native water channels were also exhibited by the two-dimensional crystals. Incubation with submillimolar $HgCl_2$ reduced the osmotic water

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permeability to <10% of the original level (data not shown). Water permeability measurements over a range of temperatures revealed a remarkably low Arrhenius activation energy, E_a = 1.9 kcal/mol (Fig. 2, bottom), which is similar to diffusion of water in bulk solution and indicates that CHIP provides a pathway by which water may permeate the bilayer as a continuous unbroken stream.

DISCUSSION

This report describes the first two-dimensional crystallization of a water channel protein. Computer-averaged projections of negatively stained square arrays revealed two tetrameric structures/unit cell. Adjacent tetramers were stained differently, subtle distinctions even remaining in the best averages such as shown in Fig. 1*e*. This most likely corresponds to the outer and inner surfaces of CHIP tetramers, since at low protein-to-lipid ratios CHIP is known to insert into proteoliposomes in both native and inverted orientations (Zeidel *et al.*, 1994). Disregarding the faint differences in staining, the unit cell exhibited 2-fold axes as well as 2-fold screw axes parallel to the *a* and *b* axes, and a 4-fold axis perpendicular to the membrane. The space group of the CHIP crystal is therefore $p422_1$. Surprisingly, the CHIP oligomer fully retained the ability to conduct water despite the physical constraints of the highly ordered lattice, which contains no other components and which will tolerate negligible lateral or rotational mobility.

These features are highly consistent with the prevailing view that CHIP is a structure comprised of four pore-like subunits, which are embedded in the lipid bilayer and selectively permit passive movements of water in the direction of an osmotic gradient (reviewed by Agre *et al.* (1993b)). The structural information at better than 2-nm resolution provides predictions that will most likely be valid for other plant and mammalian aquaporins (Maurel *et al.*, 1993; Fushimi *et al.*, 1993) and related proteins including MIP26 for which functions remain undefined (Gorin *et al.*, 1984; Pao *et al.*, 1991). Although negative-stain electron microscopy did not unveil the structure of the major body of the CHIP tetramer that is embedded in the membrane, the bilayer-spanning domains of CHIP may eventually be visualized by cryoelectronmicroscopic analysis of the crystals. As further suggested by these studies, two-dimensional crystallization in the presence of lipids may also reveal the structures of other types of membrane channels, transporters, and receptors in functionally active form.

The salient feature of the CHIP tetramer is the stain-penetrated depression about the 4-fold axis, which requires additional consideration. Both the STEM mass determination and the analysis of the rhomboid stained areas suggest that the lipid bilayer covers 40–44% of the unit cell allowing 52–55 nm^2 for the cross-section of two CHIP tetramers. Assuming a protein of density 0.822 kDa/nm^3 that spans the full lipid bilayer of 5-nm thickness and possesses a cross-section of 6.7 nm^2, the subunit mass would be 27.5 kDa. This is larger than the water-conducting 24-kDa transbilayer domain of a CHIP subunit (Smith and Agre, 1991; Zeidel *et al.*, 1994), so the stain-filled central depression must penetrate far into the bilayer from above and below, possibly leaving only a thin barrier that is crossed by individual water pores. Although the 28-kDa CHIP monomers may be independently active units (van Hoek *et al.*, 1991; Preston *et al.*, 1993), their stability may require assembly into tetramers, since sedimentation studies under nondenaturing conditions revealed only the oligomeric form of the protein (Smith and Agre, 1991). In any case, these first structural data suggest that CHIP subunits have a novel structure unlike known membrane channels that contain multiple bilayer-spanning helices connected by extracellular or intracellular connecting loops.

Acknowledgments—We thank Suresh V. Ambudkar for purified phospholipid and helpful suggestions and Ueli Aebi for continuous encouragement.

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