The three-dimensional structure of human erythrocyte aquaporin CHIP

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Water-permeable membranes of several plant and mammalian tissues contain specific water channel proteins, the 'aquaporins'. The best characterized aquaporin is CHIP, a 28 kDa red blood cell channel-forming integral protein. Isolated CHIP and *Escherichia coli* lipids may be assembled into 2-D crystals for structural analyses. Here we present (i) a structural characterization of the solubilized CHIP oligomers, (ii) projections of CHIP arrays after negative staining or metal-shadowing, and (iii) the 3-D structure at 1.6 nm resolution. Negatively stained CHIP oligomers exhibited a side length of 6.9 nm with four-fold symmetry, and a mass of 202 ± 3 kDa determined by scanning transmission electron microscopy. Reconstituted into lipid bilayers, CHIP formed 2-D square lattices with unit cell dimensions $a = b = 9.6$ nm and a $p422_1$ symmetry. The 3-D map revealed that CHIP tetramers contain central stain-filled depressions about the four-fold axis. These cavities extend from both sides into the transbilayer domain of the molecule leaving only a thin barrier to be penetrated by the water pores. Although CHIP monomers behave as independent pores, we propose that their particular structure requires tetramerization for stable integration into the bilayer.

Key words: aquaporin/CHIP/erythrocyte/two-dimensional crystal/water channel

Introduction

Discovered in 1988 (Denker *et al.*, 1988), channel-forming integral protein (CHIP) is the archetypal member of the aquaporins, a growing family of membrane water channels from diverse plant and animal species (Agre *et al.*, 1993). CHIP is a major protein of the red cell membrane (2×10^5 copies per cell; Smith and Agre, 1991), allowing rapid swelling or shrinkage to occur in response to small changes in extracellular osmolality. CHIP comprises 4% of the total brush border membrane protein in renal proximal tubules where it promotes reabsorption of water from the glomerular filtrate (Sabolic *et al.*, 1992; Nielsen *et al.*, 1993a). CHIP is also expressed in several other mammalian tissues including choroid plexus, ocular ciliary epithelium, hepatobiliary ductules and capillary endothelium, where

the protein participates in various secretory or reabsorptive processes (Nielsen *et al.*, 1993b). The developmental expression patterns in these tissues are complex (Bondy *et al.*, 1993; Smith *et al.*, 1993).

The CHIP cDNA (Preston and Agre, 1991) encodes a 28 kDa protein similar to MIP, the major intrinsic protein of lens fiber membranes (Gorin *et al.*, 1984). While the function of MIP is unresolved, the water channel activity of CHIP was shown by expression in *Xenopus laevis* oocytes (Preston *et al.*, 1992). Purified CHIP reconstituted in proteoliposomes (Zeidel *et al.*, 1992, 1994), and in 2-D lipid-protein crystals (Walz *et al.*, 1994) exhibited an osmotic water permeability indistinguishable from that of native water channels in erythrocyte membranes, i.e. $\sim 2 \times 10^9$ water molecules per CHIP subunit per second. The low Arrhenius activation energy (<4 kcal/mol; Zeidel *et al.*, 1992; Walz *et al.*, 1994) indicates that CHIP-mediated transmembrane water movement occurs as a continuous, unbroken stream with the direction of flow determined by the osmotic gradient. Nevertheless, CHIP will not permit passage of ions or urea, supporting the idea that a highly specific water channel structure must exist.

Based on its cDNA-derived primary sequence, the structure of the CHIP monomer was predicted to contain six bilayer-spanning helices, five connecting loops (A–E), and intracellular NH₂- and COOH-termini (Preston and Agre, 1991). Spectroscopic analyses established that about half of the secondary structure of MIP (Horwitz and Bok, 1987) and of CHIP (van Hoek *et al.*, 1993) is α -helical. Like MIP and all other homologous proteins, the primary sequence of CHIP exhibits a striking symmetry with two internal tandem repeats corresponding to the NH₂- and COOH-terminal halves of the protein, each comprising three transmembrane helices. Accordingly, homologous helices are believed to be of opposite orientation in the bilayer (Reizer *et al.*, 1993). This membrane topology was confirmed with biologically active CHIP recombinants (Preston *et al.*, 1994).

The osmotic water permeability of water channels in native erythrocyte membranes is known to be reversibly inhibited by HgCl₂ (Macey and Farmer, 1970). The most highly conserved domains of the MIP family are the connecting loops B and E which contain the motif Asn-Pro-Ala (NPA) which is predicted to lie near the inner (B) or outer (E) leaflets of the bilayer (Preston and Agre, 1991). Preceding the second NPA motif is Cys189, the site of mercury-sensitivity. Replacing this Cys with a Ser eliminated the mercury-sensitivity, whereas replacing Cys189 with larger amino acids abolished the water permeability of the molecule (Preston *et al.*, 1993). The position of Ala73 in the first repeat corresponds to that of Cys189 in the second repeat, as demonstrated by the double-mutant Ala73→Cys/Cys189→Ser which exhibited

the same mercury-sensitivity as the wild-type CHIP (Jung *et al.*, 1994). Although solubilized red cell CHIP behaves as a multi-subunit complex (Smith and Agre, 1991), coexpression of mercury-sensitive and mercury-insensitive CHIP recombinants suggests that the monomers are individually active water pores (Preston *et al.*, 1993). The apparent discrepancy between the function of CHIP monomers and the existence of native CHIP oligomers remains unexplained.

To elucidate the 3-D structure of CHIP, we reconstituted octyl- β -D-glucopyranoside (OBG)-solubilized CHIP in the presence of phospholipids under conditions that are known to promote the 2-D crystallization of other membrane proteins (Jap *et al.*, 1992). Our protocol has produced 2-D square arrays of biologically active CHIP (Walz *et al.*, 1994). These arrays fold into vesicles in the absence of divalent cations, whereas the presence of Mg^{2+} or Ca^{2+} induces planar single layered sheets. Here we characterize the OBG-solubilized CHIP oligomer, and describe the 3-D model of the aquaporin CHIP derived from data collected to a resolution of 1.6 nm with fixed beam and scanning transmission electron microscopy.

Results

Solubilized CHIP particles

Micrographs of negatively stained, OBG-solubilized CHIP recorded at a magnification of 100 000 $\times$ show a rather homogeneous size distribution (Figure 1a, left panel). Many particles exhibit a square shape and often a central, stain-filled pit. To enhance these features, ~350 particles were selected by eye, aligned with respect to a reference, and averaged. Besides a central depression, aligned particles reveal a vertical or horizontal groove, in some cases both of them, dissecting the particle into four quadrants (Figure 1a, right panel). An average of those 125 particles

yielding the highest correlation coefficient with the final reference is shown in Figure 1b: even without symmetrization the tetrameric structure of the particle is distinct. A possible asymmetry resulting from the glycosylation of one CHIP per tetramer (Smith and Agre, 1991) could not be detected after uranyl formate staining. Therefore, the average displayed in Figure 1b was four-fold symmetrized and low-pass filtered to a resolution of 1.6 nm (Figure 1c). The tetramer has a side length of 6.9 nm and displays a square-shaped central depression with a side length of 2.5 nm. The four major stain-excluding units arranged about the central depression possess an elongated shape of 3 nm length with one protrusion extending towards the four-fold axis. The morphology and dimensions of these OBG-solubilized CHIP particles are similar to the intramembrane particles observed by freeze-fracture electron microscopy of liposomes reconstituted with CHIP, CHO cells transfected with CHIP cDNA, or native kidney tubule plasma membranes (Verbavatz *et al.*, 1993).

Freeze-dried, OBG-solubilized CHIP particles adsorbed to a thin carbon film and imaged in the scanning transmission electron microscope (STEM) at 80 kV acceleration voltage exhibit uniform size and brightness (Figure 2). The mass of 3309 particles was evaluated from such dark-field images recorded at doses between 200 and 1100 electrons/nm². The mass values plotted in a histogram (Figure 2, inset) exhibit a peak at 130 kDa. To compensate for the beam-induced mass loss, the measured mass values were extrapolated to zero recording dose by linear regression to yield an average mass of 202 kDa with 99% confidence limits of ± 3 kDa.

Reconstitution

CHIP has been reconstituted at a lipid:protein ratio (LPR; w/w) of ~100 into proteoliposomes (Zeidel *et al.*, 1992), thus providing a basis for 2-D crystallization experiments.

Fig. 1. Electron microscopy and digital image processing of solubilized CHIP oligomers. (a) The left frame shows a typical electron micrograph of negatively stained CHIP particles solubilized in OBG and recorded at a magnification of 100 000 $\times$. The particles are rather homogeneous in size, and many particles exhibit a square shape (arrowheads). The right side displays a gallery of particles after translational and angular alignment, revealing fine grooves running horizontally and vertically, and a central stain-filled pit. The final average of 125 particles is shown before (b) and after (c) four-fold symmetrization and low-pass filtering to 1.6 nm resolution. The subframes of the gallery have a side length of 16.8 nm and the scale bars correspond to 50 nm in (a) and 3 nm in (c).

A variety of crystallization conditions were explored using *E. coli* lipids for the reconstitutions. 2-D crystals formed in 20 mM Tris-HCl buffer containing 0.25 M sodium chloride for LPRs between 0.25 and 2. These arrays folded into vesicles with an average diameter of 3 μm (Figure 3a-c; Walz *et al.*, 1994). The highest yield of well-ordered 2-D crystals was found at an LPR of 0.5. Higher values led to a decrease of the crystallinity, while lower LPRs promoted aggregation of the protein. The pH value was tested in the range 6–9, but did not significantly influence crystal formation, since 2-D arrays were found over the full pH range. Nevertheless, as observed with cytochrome reductase (Hovmoeller *et al.*, 1983), slightly acidic pH values seemed to produce better ordered crystals. Vesicles frequently spread-flattened during adsorption to the hydrophilic carbon film and allowed the thickness of the double layer to be estimated from the shadow length as 12 ± 1.7 nm ($n = 75$) after freeze-drying and unidirectional metal-shadowing.

Addition of divalent cations such as Mg^{2+} or Ca^{2+} to the reconstitution buffer prevented the formation of vesicles but promoted the growth of sheet-like structures. The borders of these sheets were sometimes decorated with aggregated protein. As illustrated in Figure 3d, sheets were found clustered, arranged side-by-side, and stacked as multi-layers. In an attempt to improve the crystallinity of CHIP arrays, phospholipase A_2 digestions were performed (Mannella, 1986). However, phospholipase treatment reduced the crystal order (data not shown), suggesting that the presence of ~20 lipid molecules per CHIP monomer corresponding to an LPR of 0.5 is essential for the crystalline packing arrangement. This finding is in excel-

lent agreement with our previous finding that ~42% of the unit cell area is covered by the lipid bilayer (Walz *et al.*, 1994), thus accommodating ~20 lipid molecules of 0.5 nm^2 cross-section per CHIP monomer.

2-D structural analysis of the crystals

Under all reconstitution conditions examined the same crystal type occurred, with CHIP tetramers packed in a square lattice of unit cell dimensions $a = b = 9.6$ nm. As demonstrated by STEM mass measurements, one unit cell houses eight CHIP monomers, i.e. two tetramers (Walz *et al.*, 1994). A spread-flattened vesicle-type array (Figure 4a) exhibited the best crystal order, with diffraction spots extending to order (6,0), corresponding to a resolution of 1.6 nm (Figure 4b). A set of faint first order spots rotated by 20° anticlockwise indicated the presence of a second, weakly stained layer. The unsymmetrized correlation average from the well stained layer (Figure 4c) displays tetrameric units that are similar in size and shape to the single particle average shown in Figure 1c. Upon closer inspection, two types of tetramers can be discerned, one with distinct four-fold symmetry and the other with one of the four domains prominent. In agreement with this finding the correlation average of freeze-dried, metal-shadowed vesicles (Figure 4d–f) showed only one prominent protrusion per unit cell. Therefore, adjacent CHIP tetramers are likely to be incorporated in the membrane in opposite orientation, which is compatible with space group p422_1 . Single-layered sheets reconstituted at an LPR of 0.5 (Figure 4g) were less well ordered than spread-flattened vesicles. This is documented by the diffraction pattern in Figure 4h, which shows prominent spots up to order (2,0). The correlation average of negatively stained sheets revealed two types of tetramers as well, one with strong contrast and the other with weak contrast (Figure 4i). This may be the result of asymmetric staining and interaction with the supporting film.

3-D structural analysis of the vesicle-type crystals

Reciprocal lattice line data were extracted from 58 projections collected in five tilt series. The data were four-fold symmetrized, and smooth curves were fitted up to lattice line (6,0) using a $\sin(x)/x$ interpolation algorithm (Smith, 1981) to calculate the 3-D density map. As documented by the lattice line plots displayed in Figure 5, low order lattice line data are truncated as result of the missing cone, while higher order lattice lines comprise data to a vertical resolution beyond $(2 \text{ nm})^{-1}$. In addition, orders (1,0) and (3,0) vanish for $z^* = 0$, confirming space group p422_1 . Plots of either the standard deviation or the dynamic range of horizontal sections against section number indicated that the reconstructed layer exhibited a nominal thickness of 4.5 nm (data not shown). As flattening during dehydration and shrinkage resulting from the electron irradiation (Berriman and Leonard, 1986) need to be taken into account, the effective thickness of a single layer was adjusted to the value calculated from unidirectionally shadowed freeze-dried membranes and from mass measurements by STEM (Walz *et al.*, 1994). The resulting horizontal sections of 0.2 nm thickness (Figure 6, left panel) show two tetramers of membrane-spanning CHIP monomers per unit cell that are incorporated in the bilayer with opposite orientation. This results in adjacent tetramers

Fig. 2. Mass measurement of single particles by STEM. The elastic dark-field image reveals particles of rather homogeneous size and brightness. To visualize CHIP oligomers distinctly, the image displayed was recorded at a dose of 1100 electrons/ nm^2 . 3309 particles were evaluated and the mass values plotted without mass loss correction in the histogram shown in the inset. Extrapolation of the data set recorded over a dose range of 200–1100 electrons/ nm^2 to zero dose by linear regression yields a mass of 202 ± 3 kDa (99% confidence band). Scale bar represents 100 nm.

Fig. 3. Electron micrographs of negatively stained reconstituted CHIP membranes. The upper panel (a–c) shows typical vesicles assembled in buffer devoid of divalent cations. Spread-flattened vesicles often expose flat areas suitable for digital image processing. Sometimes aggregated protein sticks to the collapsed vesicles. As illustrated in the lower panel (d), addition of divalent cations to the reconstitution buffer causes the formation of sheet-like structures. These sheets have a mosaic appearance, are often multi-layered, and often have their borders decorated with aggregated protein. Scale bar represents 100 nm.

having different surface topographies as displayed by the perspective view in the right panel of Figure 6. Each tetramer exhibits stain penetrated indentations about the four-fold axis which extend from both membrane surfaces towards the inside. Vertical sections (Figure 7c–e) further illustrate the pronounced asymmetry of the CHIP tetramer with respect to the plane of the membrane: one face of the tetramer possesses a wide cavity that is surrounded by four small domains and extends far into the bilayer, whereas the other face consists of four larger domains that form a narrow central cavity. This is more clearly documented by Figure 7b which represents the horizontal projection of all vertical sections comprising a row of three tetramers (i.e. all sections between lines 1 and 4 in Figure 7a). In addition, the difference in structural

preservation of the two sides is evident: the smaller domains are seen to protrude from the membrane surface of the top side in Figure 7b, whereas they are not resolved at the bottom side.

Discussion

The challenge is to merge the structural data presented here with the known primary sequence of CHIP and the observed consequences of site-directed mutagenesis on CHIP-mediated water permeability. Although only a subset of proteins homologous to MIP has been demonstrated to function as aquaporins, the functions of the other homologs are not resolved; it is possible that most are also water channels. Therefore solving the structure of CHIP to

Fig. 4. Electron microscopy and digital image processing of reconstituted 2-D CHIP crystals. **(a)** The micrograph shows a typical area of a negatively stained, spread-flattened vesicle used for digital image processing. **(b)** The distinct square lattice ($a = b = 9.6$ nm) diffracts up to order (6,0), indicating a resolution of 1.6 nm. **(c)** The correlation averaged patch exhibits unit cells containing eight monomers organized as two tetramers (protein in bright shades and negative stain in dark shades). As the average is not symmetrized, systematic differences between the subunits are distinct, such as the bright subunit marked by the arrowhead. **(d)** The crystal lattice is hardly visible on micrographs of freeze-dried/unidirectionally metal-shadowed CHIP arrays indicating very small surface corrugations. **(e)** The diffraction pattern exhibits only a small number of spots. **(f)** Nevertheless, the correlation average reveals a complex morphology with one prominent protrusion per unit cell, suggesting an opposite orientation of the two CHIP tetramers comprising one unit cell. **(g)** Sheets assembled from CHIP tetramers and lipids in the presence of divalent cations. As demonstrated by the diffraction pattern shown in **(h)**, these lattices always diffracted less well than vesicular 2-D crystals. Both, vesicle-type and sheet-type crystals have the same unit cell dimensions and space group $p422_1$. However, the difference in the contrast between the two oppositely incorporated tetramers in the correlation average **(i)** is more distinct than in the average **(c)**. The lattice vectors are marked by white arrows. Scale bars represent 100 nm in panels a, d and g, $(2.5 \text{ nm})^{-1}$ in panels b, e and h, and 10 nm in panels c, f and i; the arrow in (d) indicates the shadowing direction.

Fig. 5. Amplitudes and phases of eight prominent lattice lines. Data from 58 projections were merged, four-fold symmetrized and interpolated by a $\sin(x)/x$ algorithm (Smith, 1981). Amplitude values in the top box and phase values in the bottom box are plotted as function of the reciprocal coordinate z^* measured in $1/\text{nm}$.

atomic resolution will probably provide insight applicable to all members of this growing family of proteins.

The major revelation of the primary sequence was the identification of six probable transmembrane helices and five connecting loops (A–E). The first three and second three bilayer spans form two tandem repeats, and connecting loops B and E have the most closely related sequences (Preston and Agre, 1991). Membrane topology studies have established that the two repeats span the bilayer in opposite orientation and localized loop B at the cytoplasmic face and loop E at the extracellular face of the membrane (Preston *et al.*, 1994).

Previous experiments revealed that Triton X-100-solubilized CHIP immobilized on a wheat germ agglutinin column eluted as tetramers containing three unmodified polypeptides of 28 kDa each and one glycosylated polypeptide of 50 kDa (total mol. wt = 134 kDa; Smith and Agre, 1991). OBG-solubilized CHIP tetramers have a

mass of 202 kDa as measured by STEM. The difference of 68 kDa corresponds to 225 OBG molecules, or approximately two OBG micelles, close to what is expected for the solubilization of a tetrameric integral membrane protein (Zulauf, 1991). Therefore, our STEM mass data corroborate the previous observation that only one site per CHIP tetramer is glycosylated. Further evidence is provided by the correlation average displaying adjacent tetramers in alternating orientation with respect to the membrane (Figure 4c). The sugar chain of the tetramer that had adsorbed with its glycosylated side towards the carbon film is likely to be deformed and thus not contoured by the stain, provoking a distinct four-fold symmetry. The sugar moiety of the oppositely oriented tetramer, however, may collapse onto the protein and thus increase the stain exclusion volume of the glycosylated monomer (arrow in Figure 4c).

The perspective view of CHIP tetramers integrated in

Fig. 6. Horizontal sections and surface-rendered view of the 3-D density map obtained from spread-flattened, vesicle-type crystals. The left panel shows 20 horizontal sections of 0.4 nm thickness showing two tetramers of transmembrane CHIP monomers per unit cell that are incorporated in opposite orientation. This results in the different surface topographies of adjacent tetramers revealed by the perspective view obtained by surface rendering the 3-D map at a contour comprising 75% of the total mass (right panel). CHIP monomers protrude by ~ 0.7 nm from the bilayer on one side and surround a cavity of 3 nm diameter (arrow). The CHIP tetramer extends by ~ 1 nm on the other side, and the narrow pore is just indicated (arrowhead).

Fig. 7. Vertical sections through the 3-D density map. (a) Projection through the stack of 0.2 nm thick horizontal sections of the 3-D map. One unit is outlined in white. (b) Horizontal projection of all vertical sections between lines 1 and 4 marked in panel a. Panels c–e correspond to vertical sections through the 3-D map at the positions of lines 1, 2 and 3, respectively indicated in panel a. The horizontal lines in panels b–e represent the bilayer boundaries. All panels have a side length of 19.2 nm.

opposite orientation in the bilayer displays their two faces (Figure 6). One face probably corresponds to the extracellular side of the tetramer and has subunits which project ~0.7 nm from the bilayer and surround a central cavity of ~3 nm in diameter. The second face probably corresponds to the cytoplasmic side of the tetramer and has subunits which project ~1 nm from the bilayer and surround a narrower central cavity. The primary sequence of CHIP predicts that the extracellular and cytoplasmic faces of the protein will have different structures with both the NH₂- and COOH-termini projecting into the cytoplasm, while the wide and narrow cavities must be separated by an unresolved, thin barrier about the four-fold axis. Consistent with structure prediction and the measured α -helical structure content of several members belonging to the MIP family, we expect that the six transmembrane helices represent the essential part of the CHIP monomer that exists in α -helical conformation. The compelling homology of connecting loops B and E throughout the MIP family (Reizer *et al.*, 1993) indicates that these loops are directly involved in the molecular architecture of the CHIP oligomer. Their internal homology and their localization at both surfaces of the membrane suggest that loops B and E form a water channel with a distinct symmetry. This is supported by systematic point mutations close to the NPA motifs in loops B and E: the double mutant Ala73→Cys, Cys189→Ser exhibits a wild-type, mercury-sensitive osmotic water permeability (Jung *et al.*, 1994). Taken together, these data support our hypothesis that loops B and E form the barrier about the four-fold axis containing four independent water pores, while the α -helices assemble into the peripheral wall surrounding the central cavity. Such an arrangement would explain the necessity for oligomerization: similar to bacterial porin monomers (Weiss *et al.*, 1991; Cowan *et al.*, 1992), a CHIP monomer may not have a hydrophobic belt along the entire circumference that would span the bilayer.

Although radiation target analysis (van Hoek *et al.*, 1991) and site-directed mutagenesis (Preston *et al.*, 1993) indicate that the individual monomeric subunits are functional water pores, the data presented here support the hypothesis that the tetramer is the native, biologically active unit. Analysis of the 2-D crystals together with information from site-directed mutagenesis of the primary sequence has provided significant insight into the structure and function of aquaporin CHIP, a tetrameric complex with individually functional subunits. Improved definition of these features is being attempted by further crystallization and mutagenesis studies.

Materials and methods

Isolation of CHIP

CHIP was purified following a protocol described previously (Smith and Agre, 1991) with a few modifications. Briefly, KI-stripped red cell membrane vesicles from 900 ml of human blood were extracted with 1% (w/v) *N*-lauroylsarcosine and solubilized in 4% (v/v) Triton X-100 (Nielsen *et al.*, 1993a). The solubilized material was filtered through a 0.22 μ m Millipore Steril D-GV apparatus. A 600 ml aliquot of the filtrate (corresponding to 200 ml of packed membranes) was loaded onto a 10 mm $\times$ 100 mm Poros Q/F column (PerSeptive Biosystems, Cambridge, MA) equilibrated with 0.1% Triton X-100, 20 mM Tris-HCl (pH 7.8), 1 mM Na₂S₂O₃, 1 mM dithiothreitol (DTT) driven at 3 ml/min by a Pharmacia FPLC apparatus. The column was washed with 40 ml of equilibration buffer and eluted with a 120 ml 0.2–0.6 M NaCl

gradient at 4 ml/min while the A₂₈₀ was monitored. Peaks eluted at 0.35 M NaCl, and fractions from two runs were combined, diluted 6-fold with 1.2% OBG (Calbiochem), 20 mM Tris-HCl (pH 7.8), 1 mM Na₂S₂O₃, 1 mM DTT, and loaded onto the same Poros column equilibrated with the same buffer while running at 2 ml/min. The column was washed until the A₂₈₀ baseline was recovered and then eluted at 1 ml/min with a 40 ml gradient of 0–0.6 M NaCl in the same buffer. The combined peak fractions eluted at 0.25 M NaCl, contained pure CHIP at 630 μ g/ml (BCA protein method, Pierce, Rockford, IL), and were snap frozen and stored at –80°C until reconstitution.

Reconstitution

Isolated CHIP in 1.2% OBG, 20 mM Tris-HCl, 0.25 M NaCl, 1 mM D,L-DTT, 1 mM sodium azide was mixed with *E. coli* lipids solubilized in 2% OBG in the same buffer and reconstituted into membranes by detergent removal in a continuous flow dialysis system (Jap *et al.*, 1992). The protein concentration in the final reconstitution mixture was adjusted to 0.5 mg/ml and the lipid concentration was varied to yield lipid:protein ratios (LPRs, w/w) between 0.1 and 2. The samples were preincubated for 2 h at room temperature and subsequently dialyzed for 12 h at 25°C, for 12 h with a linear ramp from 25°C to 35°C, for 24 h at 35°C, and finally for 6 h with a linear ramp from 35°C to 25°C. The reconstitution buffers always contained 20 mM Tris-HCl, 0.25 M NaCl, 1 mM DTT and 1 mM sodium azide. Depending on the experiment, the pH was adjusted to between 5.8 and 8.8. In some reconstitutions, divalent cations (Mg²⁺ or Ca²⁺) were added to the buffer to a concentration of 10 or 100 mM.

Electron microscopy

Solubilized CHIP or reconstituted membranes were adsorbed to glow-discharged, carbon-coated Parlodion films that were mounted on electron microscope grids. After washing in double-distilled water, the membranes were negatively stained with 0.75% uranyl formate. Alternatively, adsorbed membranes were freeze-dried in a Balzers BAF 300 unit at –80°C and unidirectionally shadowed with platinum-iridium-carbon at an elevation angle of 30° at the same temperature. Images were recorded on Kodak SO-163 film at a nominal magnification of 50 000 $\times$ and a recording dose of typically 2000 e[–]/nm² using a Hitachi H-7000 transmission electron microscope operated at 100 kV. For 3-D reconstructions, tilt series were recorded with tilt angles ranging between –60° and +60° comprised of either nine or 15 projections, yielding a total dose of 1.8 $\times$ 10⁴ or 3 $\times$ 10⁴ electrons/nm². The magnification was calibrated with negatively stained catalase crystals as described by Wrigley (1968).

Image processing

For single particle averaging a reference was first generated by selecting 10 particles with a distinct square shape by eye, aligning them translationally as well as angularly, and calculating the average. Approximately 350 particles were then selected, aligned angularly either directly or by their autocorrelation function and translationally with respect to the reference for subsequent averaging (Frank, 1979). To test the bias of the initial reference, arbitrary sets of 10 particles were averaged and used as alternative references. Alignment of the 350 particles remained unchanged within the resolution (1.6 nm, radial amplitude correlation function; Saxton and Baumeister, 1982), thus leading to similar averages.

Micrographs of 2-D crystals were selected with an optical diffractometer (Aebi *et al.*, 1973) based on the alignment of the microscope and the crystal order. Suitable areas were digitized as described (Ford *et al.*, 1990) using an Eikonix 850 CCD camera at a pixel size corresponding to 0.64 nm on the sample. The averaged CHIP projection was calculated using the SEMPER image processing system (Saxton *et al.*, 1979). A rapid evaluation of crystal quality and unit cell morphology was achieved by Fourier peak filtration (Aebi *et al.*, 1973). Residual lattice disorder was eliminated by correlation averaging (Saxton and Baumeister, 1982). Correlation averaged unit cells were finally four-fold symmetrized. Five out of ten tilt series of negatively stained CHIP crystals were selected by optical diffraction and individually processed, and independent 3-D reconstructions were calculated using the SEMPER image processing package. The best 3-D reconstruction was then used to align and normalize the projections from the other tilt series, eliminating five out of 63 projections based on their cross-correlation coefficient with the corresponding projection calculated from the reference data. Lattice line data from 58 projections were fitted by linear interpolation and backtransformed. A plot of the dynamic range or the standard deviation of horizontal sections versus section number was produced to estimate

the layer thickness. A refined interpolation was then achieved by the use of a $\sin(x)/x$ algorithm (Smith, 1981). Stacks of horizontal sections separated by a nominal distance of 0.15 nm were computed from the interpolated lattice lines. The vertical scale was subsequently adapted to accommodate the mass/area of 4.1 kDa/nm² (Walz *et al.*, 1994) taking into account a density of 0.756 kDa/nm³ for the lipid-protein layer, yielding a section thickness of 0.2 nm. This thickness was cross-checked with the shadow length measured on five individual spread-flattened, freeze-dried and unidirectionally shadowed vesicles. For visualization, the 3-D reconstruction stacks were surface-rendered at a contour level including 75% of the total protein and lipid mass by a marching cube algorithm (Lorensen and Cline, 1987) implemented in our laboratory by Mr C.Henn.

STEM mass determinations

Reconstituted membranes or solubilized CHIP oligomers were adsorbed onto glow discharged grids covered with a 3 nm thick carbon film mounted on a fenestrated plastic film, and washed extensively in double-distilled water. Grids were plunged into liquid nitrogen and freeze-dried within the pretreatment chamber attached to a Vacuum Generators HB5 STEM at -80°C. Elastic annular dark-field images containing 512 × 512 pixels were acquired in digital format at 80 kV, at either 100 000× (pixel size 1.87 nm) or 200 000× (pixel size 0.93 nm) magnification and at doses between 200 and 1100 electrons/nm². The processing of digital STEM images for mass determination was carried out as described (Müller *et al.*, 1992), using for linear regression and statistical analysis the program StatView implemented on a Macintosh computer.

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References

- Aebi,U., Smith,P.R., Dubochet,J., Henry,C. and Kellenberger,E. (1973) *J. Supramol. Struct.*, **1**, 498–522.
- Agre,P., Preston,G.M., Smith,B.L., Jung,J.S., Raina,S., Moon,C., Guggino,W.B. and Nielsen,S. (1993) *Am. J. Physiol.*, **265**, F463–F476.
- Berriman,J. and Leonard,K.R. (1986) *Ultramicroscopy*, **19**, 349–366.
- Bondy,C., Chin,E., Smith,B.L., Preston,G.M. and Agre,P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 4500–4504.
- Cowan,S.W., Schirmer,T., Rummel,G., Steiert,M., Ghosh,R., Paupit,R.A., Jansonius,J.N. and Rosenbusch,J.P. (1992) *Nature*, **358**, 727–733.
- Denker,B.M., Smith,B.L., Kuhajda,F.P. and Agre,P. (1988) *J. Biol. Chem.*, **263**, 15634–15642.
- Ford,R.C., Hefti,A. and Engel,A. (1990) *EMBO J.*, **9**, 3067–3075.
- Frank,J. (1979) *J. Microsc.*, **117**, 25–38.
- Gorin,M.B., Yancey,S.B., Cline,J., Revel,J.-P. and Horwitz,J. (1984) *Cell*, **39**, 49–59.
- Horwitz,J. and Bok,D. (1987) *Biochemistry*, **26**, 8092–8098.
- Hovmoeller,S., Slaughter,M., Berriman,J., Karlsson,B., Weiss,H. and Leonard,K. (1983) *J. Mol. Biol.*, **165**, 401–406.
- Jap,B.K., Zulauf,M., Scheybani,T., Hefti,A., Baumeister,W., Aebi,U. and Engel,A. (1992) *Ultramicroscopy*, **46**, 45–84.
- Jung,J.S., Preston,G.M., Smith,B.L., Guggino,W.B. and Agre,P. (1994) *J. Biol. Chem.*, **269**, 14648–14654.
- Lorensen,W. and Cline,H.E. (1987) *Comp. Graph.*, **21**, 163–169.
- Macey,R.I. and Farmer,R.E.I. (1970) *Biochim. Biophys. Acta*, **211**, 104–106.
- Mannella,A.C. (1986) *Methods Enzymol.*, **125**, 595–609.
- Müller,S., Goldie,K.N., Bürki,R., Häring,R., Engel,A. and Reichelt,R. (1992) *Ultramicroscopy*, **46**, 317–334.
- Nielsen,S., Smith,B.L., Christensen,E.I. and Agre,P. (1993a) *Proc. Natl Acad. Sci. USA*, **90**, 7275–7279.
- Nielsen,S., Smith,B.L., Christensen,E.I., Knepper,M.A. and Agre,P. (1993b) *J. Cell Biol.*, **120**, 371–383.
- Preston,G.M. and Agre,P. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 11110–11114.
- Preston,G.M., Carroll,T.P., Guggino,W.B. and Agre,P. (1992) *Science*, **256**, 385–387.
- Preston,G.M., Jung,J.S., Guggino,W.B. and Agre,P. (1993) *J. Biol. Chem.*, **268**, 17–20.
- Preston,G.M., Jung,J.S., Guggino,W.B. and Agre,P. (1994) *J. Biol. Chem.*, **269**, 1668–1673.
- Reizer,J., Reizer,A. and Saier,M.H., Jr (1993) *Crit. Rev. Biochem. Mol. Biol.*, **28**, 235–257.
- Sabolic,I., Valenti,G., Verbavatz,J.M., van Hoek,A.N., Verkman,A.S., Ausiello,D.A. and Brown,D. (1992) *Am. J. Physiol.*, **263**, C1225–C1233.
- Saxton,W.O. and Baumeister,W. (1982) *J. Microsc.*, **127**, 127–138.
- Saxton,W.O., Pitt,T.J. and Horner,M. (1979) *Ultramicroscopy*, **4**, 343–354.
- Smith,B.L. and Agre,P. (1991) *J. Biol. Chem.*, **266**, 6407–6415.
- Smith,B.L., Baumgarten,R., Nielsen,S., Raben,D., Zeidel,M.L. and Agre,P. (1993) *J. Clin. Invest.*, **92**, 2035–2041.
- Smith,P.R.S. (1981) *Ultramicroscopy*, **7**, 155–160.
- van Hoek,A.N., Hom,M.L., Luthjens,L.H., deJong,M.D., Dempster,J.A. and van Os,C.H. (1991) *J. Biol. Chem.*, **266**, 16633–16635.
- van Hoek,A.N., Wiener,M., Bicknese,S., Miercke,L., Biwersi,J. and Verkman,A.S. (1993) *Biochemistry*, **32**, 11847–11856.
- Verbavatz,J.-M., Brown,D., Sabolic,I., Valenti,G., Ausiello,D.A., Van Hoek,A.N., Ma,T. and Verkman,A.S. (1993) *J. Cell Biol.*, **123**, 605–618.
- Walz,T., Smith,B.L., Zeidel,M.L., Engel,A. and Agre,P. (1994) *J. Biol. Chem.*, **269**, 1583–1586.
- Weiss,M.S., Abele,U., Weckesser,J., Welte,W., Schiltz,E. and Schulz,G.E. (1991) *Science*, **254**, 1627–1630.
- Wrigley,N.G. (1968) *J. Ultrastruct. Res.*, **24**, 454–464.
- Zeidel,M.L., Ambudkar,S.V., Smith,B.L. and Agre,P. (1992) *Biochemistry*, **31**, 7436–7440.
- Zeidel,M.L., Nielsen,S., Smith,B.L., Ambudkar,S.V., Maunsbach,A.B. and Agre,P. (1994) *Biochemistry*, **33**, 1606–1615.
- Zulauf,M. (1991) In Michel,H. (ed.), *Crystallization of Membrane Proteins*. CRC Press, Boca Raton, FL, pp. 53–72.

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