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The Aquaporin family of membrane water channels

Andreas Engel, Thomas Walz and Peter Agre

M E Müller-Institut for Microscopic Structural Biology, Basel, Switzerland
and Johns Hopkins University School of Medicine, Baltimore, USA

The rapid movement of water across the plasma membranes of certain cells has been a long-standing puzzle to membrane biophysicists and physiologists; the discovery of the red cell channel-forming integral protein has provided a molecular solution to this puzzle. The identification of this protein has led to the recognition of a family of related water-selective channels, the aquaporins, that are found in animals, plants and microbial organisms. In order to provide insight into the remarkable but simple function of these membrane proteins, their structures are being elucidated.

Introduction

All biological membranes exhibit some water permeability as a result of diffusion across the lipid bilayer; however, the degree of water permeability differs considerably between tissues and cell types. Mammalian red blood cells and renal proximal tubules are extremely permeable to water molecules. This remarkable feature led biophysicists to propose the hypothesis that water-selective channels must exist. The physiological importance of red cell water channels is still unclear; however, the kidney has a major role in body water balance, and permits survival despite severe water deprivation. The plasma membranes of certain other tissues are also highly permeable to water molecules, and transmembrane water movements are involved in diverse physiological processes including the secretion of cerebrospinal fluid, aqueous humor, sweat, tears, saliva, bile and amniotic fluid.

Several observations have provided clues about how water channels function (reviewed in [1]). For example, it is thought that water traverses water channels as a single-file column of molecules, because the ratio of osmotically-driven water permeability ($P_f$) to diffusional water permeability ($P_d$) is much greater than one. The selectivity for water is apparently explained by a narrow aqueous pore since such a channel would be permeable to water but not to protons, other ions, or small molecules. The Arrhenius activation energy measured for red cell $P_f$ is similar to the diffusion of water in bulk solution ($<5$ kcal mol$^{-1}$), which is considerably below the activation energy of the diffusional water permeability of simple phospholipid bilayers. Red cell and renal tubule water channels are selectively inhibited by HgCl$_2$ and certain organomercurials, indicating the presence of a critical sulphydryl group in the pore [2]. Also, the water channels of red cells and renal proximal tubules seem to be constitutively activated, whereas the renal collecting duct is only permeable to water in the presence of vasopressin, which leads to the redistribution of collecting duct water channels from intracellular vesicles to the apical surface (reviewed in [3]). Radiation target analysis initially showed that the size of the molecular water channel is 30 kDa [4], but attempts to determine its structure by biochemical approaches [5] and expression cloning [6] were unsuccessful. This was in part due to the inability to label the water channel with its substrate (as water is ubiquitous), the lack of a highly specific inhibitor (HgCl$_2$ labels all free sulphydryls), and the diffusional permeability of cell membranes. Recently, however, significant progress has been made in identifying and characterizing the proteins responsible for water permeability, and the first steps towards a determination of their structure have been made.

CHIP, the archetypical Aquaporin

The first molecular explanation for transmembrane water movements emerged from the study of a novel red cell membrane protein identified by the serendipitous development of an antibody. This 28 kDa channel-forming integral protein (CHIP) has not been noticed by traditional SDS–PAGE as it fails to bind Coomassie stain [7]. CHIP resides within the lipid bilayer, as it is quantitatively included in the membrane vesicle fraction even after extraction with 1 M KI. CHIP is very abundant in red blood cells, ~200 000 copies per cell [8], and in apical brush border cells of renal tubules where it constitutes 4% of the total protein [9].

Abbreviations

CHIP—channel-forming integral protein; MIP—major intrinsic protein; STEM—scanning transmission electron microscope.
Analysis of CHIP was facilitated by its unusual insolubility in certain detergents such as N-lauroylsarcosine which made purification easy (Fig. 1a) [7,8]. Determination of the amino-terminal amino acid sequence revealed a homology with major intrinsic protein of lens (MIP), a putative channel of unknown function [10]. The structural composition of MIP was previously established by near- and far-ultraviolet circular dichroism to be ~50% α-helix and ~20% β-structure [11]. When CHIP was similarly investigated, the structure was also predicted to be 40–50% α-helix and 15–40% β-structure [12], and, as expected for a membrane channel protein, fluorescence-quenching studies revealed a nonpolar environment for the tryptophans [13]. The amino- and carboxy-terminal domains of CHIP were demonstrated to be cytoplasmic by vectorial proteolysis and the use of terminus-specific antibodies [8].

Several biochemical and electron microscopic studies provided evidence that CHIP exists as a tetramer. There are two forms of CHIP, a 28 kDa nonglycosylated form (CHIP28) and a glycosylated form (glyCHIP) of 40–60 kDa which has a large N-linked glycan. Unlike other oligomeric integral membrane proteins, CHIP28 and glyCHIP occur in a ratio of about three to one [7]. Both CHIP28 and glyCHIP adsorbed to wheat germ agglutinin columns when the protein was purified under non-denaturing conditions, but only glyCHIP adsorbed after the protein was denatured, indicating that one polypeptide per tetramer is glycosylated. Glutaraldehyde crosslinking generated a ladder of bands on SDS-PAGE with the largest being approximately 120 kDa. Gel filtration in Triton X-100 revealed that the protein has a Stokes radius of 6.1 nm and a sedimentation coefficient of 5.7 S, providing a calculated molecular weight of 135 kDa when corrected for detergent binding [8]. Mass measurements performed with the scanning transmission electron microscope (STEM) yielded a value of 202 kDa for the solubilized CHIP oligomer including bound detergent. Negative stain electron microscopy and digital image analysis of purified CHIP particles showed a square shaped oligomer with sides 7 nm long, in which four domains surround a central stain-filled pit (Fig. 1b) [14*].

Freeze-fracture electron micrographs of renal tubules and CHIP proteoliposomes [15*,16*] suggest that the native structural organization of purified CHIP is retained when it is solubilized in non-ionic detergents or reconstituted into proteoliposomes. Distinct intramembranous particles were seen in these studies, and morphometric analyses were consistent with a tetrameric organization, although octameric complexes may also exist when the protein is reconstituted at higher concentrations [16*]. Detailed freeze-fracture studies of CHIP proteoliposomes and Chinese hamster ovary cells transfected with CHIP cDNAs revealed the presence of intramembranous particles that were 9 nm in diameter, consistent with a tetrameric organization (Fig. 1c) and that they were similar to intramembranous particles found in the plasma membranes of the S3 segment of renal proximal tubules [15*].

The cDNA for CHIP was cloned from a human fetal erythroid cDNA library using oligonucleotides designed from CHIP protein sequences and used to isolate a nearly full-length recombinant clone from a human bone marrow cDNA library [17]. The 5’ end of the human CHIP cDNA was obtained by polymerase chain amplification methods, as the transcription initiation site is located very close to the initiating ATG, and this sequence was in complete agreement with the amino-terminal protein sequence. The full length cDNA was used to probe Northern blots, demonstrating equivalent strong hybridizations with transcripts of ~3 kbases from bone marrow, anemic murine spleen, and kidney. When their amino-termini were aligned, the overall sequences of CHIP and MIP were found to be 40% identical, with several specific domains being identical. The sequences of the mouse and rat CHIP homologs were subsequently established to be 93% identical to human [18,19]. The sequence of the CHIP from a human kidney cDNA was demonstrated to be identical to red cell CHIP, and the intron-exon organization of the single CHIP gene located at human chromosome 7p14 was established [20]. CHIP protein has been demonstrated histochemically in
Apical and basolateral domains of renal proximal tubules and descending thin limbs of Henle's loop [7,9,21], male reproductive tract [22], as well as choroid plexus of brain, ciliary epithelium of eye, biliary epithelium, and endothelium of capillaries and post-capillary venules [23]. In situ hybridizations in fetal rat demonstrated that distinct expression patterns exist in different tissues [24], with expression in red cells and renal tubules first occurring at the time of birth [25].

Physiological studies of Aquaporin CHIP

The identification of CHIP in known water-permeable membranes and the subsequent recognition of homologous proteins in plants indicated that CHIP might be involved in membrane water transport. This led to CHIP and related proteins being termed the 'Aquaporins' (reviewed in [26]). The first direct demonstration that CHIP can mediate water transport across membranes involved the injection of defolliculated Xenopus oocytes with in vitro transcribed CHIP RNA. After three days of incubation, the oocytes were exposed to hypotonic buffer. Oocytes containing CHIP swelled and ruptured, whereas control oocytes exhibited only a marginal increase in osmotic water permeability (Pf) [27]. The behavior of CHIP in oocytes resembled that of water channels in native membranes; the channels were reversibly inhibited by HgCl₂ and had a low Arrhenius activation energy. The behavior was specific for water, as no increase in permeability to glucose, urea or other small molecules was noted, nor was the swelling accompanied by an increase in membrane conductance. The increased Pf of oocytes injected with mRNA from kidney and cornea could be inhibited with antisense probes to CHIP, demonstrating that CHIP makes a major contribution to water movements in those tissues [28-30].

CHIP was demonstrated to be both necessary and sufficient for transmembrane water movement by analysis of highly purified CHIP reconstituted into proteoliposomes [31]. When CHIP proteoliposomes containing carboxyfluorescein were abruptly exposed to an increase in extraliposomal osmolality by stopped-flow techniques, the membranes shrunk rapidly, reaching equilibrium in ~20 milliseconds (Fig. 2b), whereas control liposomes lacking CHIP required ~0.4 seconds to reach equilibrium (Fig. 2a). The Pf of these membranes was sufficient to account for most of red cell membrane water permeability, and the Arrhenius activation energy was ~3 kcal mol⁻¹. These conclusions were confirmed by analysis of red cell membranes that had been partially depleted of other proteins with N-lauroylsarcosine [32]. Detailed studies of CHIP proteoliposomes revealed that water transport was not dependent on the specific lipid compositions and closely reflected that of water channels in native membranes [16*]. CHIP proteoliposomes thus displayed a large unit permeability of water (Pf=2×10⁹ water molecules sec⁻¹ subunit⁻¹) were impermeable to urea or protons and were sensitive to known inhibitors of water channels.

Crystalline arrays of Aquaporin CHIP

CHIP has been reconstituted into crystalline arrays in the presence of Escherichia coli lipids using a temperature controlled dialysis apparatus [33], and crystallization has been optimized by variation of the lipid-to-protein ratio, the ionic strength and pH. Under all reconstitution conditions examined, the same crystal type occurred, with CHIP tetramers packed in a square lattice of unit cells with dimensions a= b= 9.6 nm that house two CHIP tetramers [14*,34**]. Either vesicles (Fig. 3a) or single layered sheets [14*] assembled depending on the pres-

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**Fig. 2.** Osmotic water permeability of (a) liposomes, (b) CHIP proteoliposomes, and (c) vesicles folded from two-dimensional CHIP-lipid crystals. The osmotic water permeability of E. coli lipid vesicles (a) is increased by an order of magnitude as a result of the incorporation of approximately 50 CHIP tetramers per vesicle (b) [31]. A further increase of the water flux is observed when CHIP tetramers are densely packed in a crystalline array (c) [34**].
ence of Mg\(^{2+}\) ions. STEM mass measurements (Fig. 3b) yielded a mass-per-area of single-layered CHIP square arrays of 4.1±0.27 kDa nm\(^{-2}\) (n=2160). The mass of one unit cell is therefore 378 kDa, accommodating two CHIP tetramers of 268 kDa and phospholipids. Correlation averages calculated from electron micrographs of negatively stained CHIP lattices exhibited a prominent four-fold rotational symmetry \([34**]\). As shown in Fig. 3d each CHIP tetramer has four stain-excluding elongated domains of approximately 2.7 nm length and 1.6 nm width (bright shades) surrounding a central stain filled depression (dark shades). The tetramers are separated by rhomboid stained areas of approximately 7.3 nm length and 4.7 nm width that represent the lipid bilayer.

Vesicles folded from CHIP lattices have been shown to retain full biological activity \([34**]\). The 3 \(\mu\)m diameter vesicles exhibited a high degree of water permeability and were shrunken within 10 msec. The osmotic water permeability, \(P_f\), was calculated as \(0.472 \text{ cm sec}^{-1}\), allowing the unit water permeability, \(p_f/P_f\times \text{area per subunit} = 5.43 \times 10^{-14} \text{ cm}^3 \text{sec}^{-1} \text{subunit}^{-1}\) to be calculated from the packing density of CHIP within the two-dimensional crystals. This value agrees with that measured from smaller proteoliposomes, \(p_f=4.6 \times 10^{-14} \text{ cm}^3 \text{sec}^{-1} \text{subunit}^{-1}\) \([16*]\).

Projections of tilted negatively-stained two-dimensional CHIP crystals allowed the three-dimensional density distribution of the CHIP tetramer to be determined at a resolution of better than 2 nm \([14*]\). The three-dimensional map showed two tetramers of membrane-spanning CHIP monomers per unit cell that are incorporated in the bilayer with opposite orientation. This results in different surface topographies of adjacent tetramers as shown by the perspective view in Fig. 4. Each tetramer exhibits two stain penetrated indentations about the four-fold axis. One face corresponds to the extracellular side of the tetramer and has subunits that project approximately 0.7 nm from the bilayer and surround a deep central cavity of \(~3\) nm in diameter. The second face corresponds to the cytoplasmic side of the tetramer and has subunits that project approximately 1 nm from the bilayer and surround a narrower central cavity. The wide and narrow cavities must be separated by an unresolved, thin barrier about the four-fold axis.

**Primary sequence of Aquaporin CHIP**

The initial structural predictions for CHIP were derived from the primary sequence by the Kyte and Doolittle hydropathy analysis (Fig. 5a) \([17]\). This plot strongly resembles that first deduced for MIP \([10]\) and is shared by the aquaporins and all members of the MIP family, whose function is still unknown (reviewed in \([35]\)). The predicted topology features a small cytoplasmic amino terminus, a 35 residue cytoplasmic carboxyl terminus, six bilayer-spanning domains of 20–25 residues (which may be helical), and five connecting loops of which A, C, and E are extracellular and B and D are cytoplasmic (Fig. 5b).
loops B, C, D, and E. The water permeabilities of oocytes expressing each of the recombinant proteins were determined; only recombinants with inserts in loops B and E were not active, indicating that these loops are important for function. Use of antiserum specific for the E1 and epitope selective proteolytic degradation of intact oocytes expressing recombinants established that loop C is extracellular. Similar analyses of microsomes isolated from oocytes established that the amino- and carboxyl-termini and loops B and D are cytoplasmic. These findings provide experimental evidence that the tandem repeats span the bilayer in opposite orientation.

Red cell membrane water channels are known to be reversibly inhibitable by HgCl₂, presumably because the HgCl₂ modifies sulphydryl group near the outer face of the water channel aperture [2]. When the four cysteines in CHIP were individually replaced by serine and expressed in oocytes, each recombinant exhibited wild type biological activity, but the Cys189→Ser mutation in loop E was no longer inhibitable by HgCl₂, indicating that Cys189 is the mercury-sensitive site [39]. When residue 189 was replaced by a smaller residue, alanine, similar behavior was noted. When larger residues such as methionine were introduced, the P₁ was greatly reduced, suggesting that residue 189 is critical for the function of the CHIP water pore. The glycosylation pattern was high mannose, indicating that the non-functional mutants at position 189 are not properly targeted to the Golgi, presumably as result of aberrant folding. These findings were subsequently confirmed by others [40].

The identification of the mercury-sensitive residue at position 189 in loop E raised the question of whether residue Ala73, which is found at the corresponding site in loop B with respect to the Asn→Pro→Ala motif, might behave similarly. The double mutant Ala73→Cys/Cys189→Ser was constructed, and the P₁ indicated full biological activity. When treated with HgCl₂, this double mutant was reversibly inhibited, although the sensitivity to the reagent was approximately one-third that of the wild-type CHIP [41]. Although located in opposite halves of the molecule, both residues 73 and 189 appear to reside near critical narrowings of the water channel through CHIP. Other studies in which similar conservative mutations were introduced at corresponding sites in loop B or loop E confirmed that these locations were structurally similar. A possible explanation of this behavior is that loop B may fold into and out of the cytoplasmic leaflet of the bilayer, and loop E may do the same at the extracellular side (similar to the H5 loop of potassium channels). If the two halves of the molecule are juxtaposed, loops B and E could form a single central sleeve surrounded by the six bilayer spanning domains (Fig. 5b). This is referred to as the 'hourglass' model.

Although CHIP exists exclusively as a tetramer, numerous experiments suggest that individual subunits have independently functioning water pores. Coex-
Fig. 5. Primary sequence analysis of CHIP and a proposed model of the essential structural features of the aquaporins. (a) The primary sequence of CHIP comprises two repeats of three membrane spanning domains and exhibits a remarkable homology to the MIP26 gene (indicated by residues in black). Loops B and E contain the highly-conserved Asn-Pro-Ala motifs, while loop A possesses the only glycosylation site, which is marked by an asterix (from [17]). (b) Insertional and site-directed mutagenesis have identified loops B and E as the most critical segments of aquaporin CHIP. Cys189 confers the mercurial sensitivity which can be suppressed by the mutation Cys189→Ser. The double mutant Cys189→Ser/Ala73→Cys exhibits wild-type mercurial sensitivity, documenting the functional symmetry of loops B and E (from [41*]). (c) The cross-section obtained by negative stain electron microscopy and digital image processing suggests a thin barrier about the four-fold axis of the tetramer. (d) Provided that the putative α-helical membrane spans form the peripheral wall of the CHIP tetramer, loops B and E must assemble into a thin barrier that carries four water pores and joins the CHIP monomers into a tetrameric complex. Reproduced with permission from [17] and [41*].

Expression of certain in vitro transcribed RNAs encoding mercury-insensitive CHIP mutants and wild-type CHIP resulted in membranes with Ps that were partially inhibitable by HgCl2 [39] and expression of dimeric CHIP constructs of varying compositions produced similar results [41**]. Expression of certain CHIP mutants (such as Cys189→Met and a truncation mutant, Asp237→STOP) in oocytes did not result in increased water permeability; however, when these two mutants were coinjected, the subunits complemented each other, and the oocytes exhibited a Pcf characteristic of wild-type CHIP [41**]. These studies confirm that multiple subunits are needed to form active CHIP water channels.

The tetrameric hourglass model

CHIP has been studied by biochemical, biophysical, cell biological, physiological, ultrastructural, crystallographic, and molecular biological techniques. These studies combined have demonstrated that CHIP is an oligomer which resides primarily between the leaflets of the lipid bilayer. It is distributed in many but not all water-permeable epithelia and has a remarkably high permeability for water, yet it is not permeable to ions or other small molecules. The protein assembles as a tetramer with a central cavity, which extends from the outer surface deep into the molecule, and with a smaller central cav-
Aquaporins are involved in the transport of water across cell membranes, primarily in the collecting duct and intestine. They are single-subunit proteins that form channels in the plasma membrane, allowing water to pass through. The structural model presented here is based on a low-resolution three-dimensional map obtained by negative stain electron microscopy, molecular biological analyses, and on the putative presence of six helical membrane-spanning regions suggested by structural prediction. The first aim of high-resolution electron microscopy will be the identification of these membrane-spanning domains, and this goal may be reached by cryo-microscopy of the two-dimensional CHIP crystals that are currently available. The particular properties of loops B and E indicate that a full understanding of the water permeability requires the elucidation of the atomic structure.

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Conclusions

Since our efforts began, cDNAs encoding several homologous proteins have been isolated from various mammalian and plant tissues. Some of these proteins cause membranes to become permeable to water as assessed by the oocyte swelling assay and are termed aquaporins. The first aquaporin identified was CHIP (gene symbol AQP1). Newly identified mammalian homologs include: AQP-CD (gene symbol AQP2); the probable vasopressin-regulated water channel of collecting duct [42], AQP3, the product of which forms the basolateral water channel of collecting duct and intestine [43*], and a mercury-insensitive aquaporin from neural tissues [44*]. Plant homologs include: tonoplast intrinsic protein AQP-TIP, which has been identified in seeds [43°], and a mercury-insensitive aquaporin from neural tissues [44°]. Plant homologs include, tonoplast intrinsic protein AQP-TIP, which has been identified in seeds [43°], and a mercury-insensitive aquaporin from neural tissues [44°].

Aquaporins are also likely to be involved in certain diseases of animals and plants. The presence of CHIP in alveolar capillary endothelium suggests that it may be important in the pathogenesis of fresh water drownings, and its presence in ciliary epithelium indicates a possible involvement in glaucoma. A patient with nephrogenic diabetes insipidus was found to be a compound heterozygote for two mutations in AQP2 [47*]. In addition a plant homolog is known to be pathologically induced in roots during nematode infestations, a major scourge in agriculture [46*].

The structural model presented here is based on a low-resolution three-dimensional map obtained by negative stain electron microscopy, molecular biological analyses, and on the putative presence of six helical membrane-spanning regions suggested by structural prediction. The first aim of high-resolution electron microscopy will be the identification of these membrane-spanning domains, and this goal may be reached by cryo-microscopy of the two-dimensional CHIP crystals that are currently available. The particular properties of loops B and E indicate that a full understanding of the water permeability requires the elucidation of the atomic structure.

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