with Ras before cDNA subtraction. cDNA prepared from RNA isolated on day 3 of induction was subtracted using cDNA prepared from undifferenitized cell RNA using a polymerase chain reaction (PCR)-select cDNA subtraction kit (Clonetics). The remaining cDNAs were randomly subcloned into a T-vector (Promega). Sixty-six clones were sequenced, and their sequences were compared with those in the GenBank/EMBL/DDBJ database. One clone (5m-1) was found to encode the 5′-UTR of a previously isolated seven-span orphan receptor10. The cDNA corresponding to the ORF of the orphan receptor was amplified by PCR from 1 μg of human genomic DNA. The primers used for PCR were 5′-GGGATCCCGCGAGCGTCAGGAACCGTTG-3′ (sense) and 5′-GGATTCCTTGTTCGTTTAACTTG-3′ (antisense). The PCR conditions were as follows: denaturation at 96°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 3 min; 30 cycles. The amplified fragment was then randomly labelled with 32PdCTP, and was used to screen an HL-60 cDNA library, which was constructed in λ Zap-II (Stratagene) from 5 μg poly(A)+ RNA of HL-60 cells differentiated by exposure to 1 μM retinoic acid for 3 days. 5 × 106 independent clones were screened and five clones (HL-1 to HL-5) were isolated by high-stringency washing. DNA sequencing revealed that HL-1 and HL-5 contain identical full-length ORFs. The ORF of HL-5 was subcloned in the mammalian expression vector pcDNA3 (Invitrogen), and the resulting HL-5 contain identical full-length ORFs. The ORF of HL-5 was subcloned in the mammalian expression vector pcDNA3 (Invitrogen), and the resulting HL-60 cells differentiated by exposure to 1 μM retinoic acid for 3 days.

The three-dimensional structure of aquaporin-1

Thomas Walz†, Teruhisa Hirai, Kazuyoshi Murata, J. Bernard Heymann*, Kaoru Mitsuoka, Yoshinori Fujiyoshi, Barbara L. Smith‡, Peter Agrup* & Andreas Engel†

of an erythrocyte integral membrane protein of relative molecular mass 28,000, identified it as the mercury-sensitive water channel, aquaporin-1 (AQP1). Many related proteins, all belonging to the major intrinsic protein (MIP) family, are found throughout nature. AQP1 is a homotetramer containing four independent aqueous channels. When reconstituted into lipid bilayers, the protein forms two-dimensional lattices with a unit cell containing two tetramers in opposite orientation. Here we present the three-dimensional structure of AQP1 determined at 6 Å resolution by cryo-electron microscopy. Each AQP1 monomer has six tilted, bilayer-spanning α-helices which form a right-handed bundle surrounding a central density. These results, together with functional studies, provide a model that identifies the aqueous pore in the AQP1 molecule and indicates the organization of the tetrameric complex in the membrane.

MIP-related proteins all have six stretches of hydrophobic sequence that are suggestive of six transmembrane helices. In addition, these proteins share an NPA (Asn-Pro-Ala) motif in each of the two most prominent loops. The membrane topology has been confirmed by epitope insertion studies of AQP1 which showed the amino- and carboxy-termi to be on the cytoplasmic side of the membrane. Site-directed mutagenesis of the loops containing the NPA motifs further indicated that these segments probably line the path of water permeation. These results led to the hourglass model, in which the NPA loops project from the outer and inner leaflets towards the centre of the membrane.

Figure 1a shows the unit cell calculated from 11,054 diffraction intensities and 13,734 phases measured over a tilt angle range of 60° (Table 1). The AQP1 tetramer in the centre is viewed from the extracellular side, whereas adjacent tetramers, which have the opposite orientation, are seen from the cytosolic side. The sidedness has been determined by surface relief reconstruction from metal-shadowed samples and atomic force microscopy of native membranes. Figure 1b shows a vertical slice containing four AQP1 monomers overlaid by the relief reconstruction. The cytoplasmic side of the tetramer protrudes further from the membrane than the extracellular side, and the surface relief between tetramers defines the boundaries of the bilayer (marked by a two-headed arrow in Fig. 1b). Narrow vertical clefs between monomers span the membrane (arrows in Fig. 1b). These gaps define the molecular boundaries within the tetramer. A loop (marked by an asterisk in Fig. 1a) links adjacent monomers on the cytoplasmic side, but helix–helix contacts in the narrow cleft may also contribute to the unusual stability of AQP1 tetramers. The square-shaped tetramers are rotated slightly about their fourfold axes, probably to provide the contacts between adjacent tetramers required for crystallization.

Different views of an AQP1 monomer (Fig. 2a–d) reveal six distinct tilted rods of variable lengths that form a right-handed bundle. Their shapes and dimensions suggest that these rods are membrane-spanning α-helices, in agreement with the sequence-based structure prediction (Fig. 2e). The six helices surround a complex central density X, as illustrated by the molecule viewed from the extracellular side in Fig. 2a.

The helix assignment in Fig. 2 is based on several features in the three-dimensional map. First, the C terminus has been identified as the most prominent cytosolic protrusion by comparing surface reliefs of metal-shadowed crystals before and after digestion with carboxypeptidase Y. As this protrusion is close to the fourfold axis, helix 6 should be in this region (Fig. 2a). Rotating the monomer by 45° clockwise around the x-axis (Fig. 2b) unveils the end of helix 6 which is seen to protrude away from the AQP1 molecule, thus supporting its assignment as the C terminus. The loop of the next helix to the right in Fig. 2b extends to the adjacent monomer (asterisk in Fig. 1a) and is likely to be the N terminus of AQP1. At the other end, helix 1 is connected to helix 2 by way of the density indicated by an arrow in Fig. 2b, consistent with the short loop A (∼12 residues). This connection becomes clearer when the molecule is contoured to include a larger volume. Helix 2 continues on the cytoplasmic side into a loop projecting back into the centre of the monomer, most probably loop B. The predicted loop D is very short (∼6 residues), and is represented by a bulge on the cytoplasmic side (Fig. 2c, arrow). No contiguous density could be assigned to the long loop C between helices 3 and 4. This region coincides, however, with a prominent protrusion of about 5 Å on the extracellular side observed by AFM. In addition, the surface topography measured with the AFM supports the right-handed α-helical bundle presented here.
In Fig. 2d, helices 5 and 6 were cut away to show density X. This unusual structure is wide on the extracellular side, has a side arm and seems to end above the prominent loop connected to helix 2. Although loops connected to density X are not clear for helices 5 and 6 on the extracellular side, the widening of the density towards this end suggests connections on that side. Therefore we propose that density X contains loop E with one of the NPA motifs. Loop B with the other NPA motif is seen to extend from helix 2 on the cytoplasmic side of the molecule. The hourglass model positions these densities in close proximity to the water channel.

An extensive spectroscopic comparison of AQP1 with bacterio-rhodopsin has suggested that AQP1 is an all-helical protein with an α-helix content that is consistent with six membrane-spanning segments of 17–22 residues[1]. Furthermore, the measured dichroic ratio has indicated that the helices are tilted with respect to the normal of the bilayer by an average of 21°.

We conclude that the present helix assignment is a reasonable working hypothesis. The right-handed twist in the helix bundle is also a feature commonly observed in soluble proteins[1]. We propose that the central mass is formed by the extended loops B and E which carry the highly conserved residues involved in water permeation. Much-higher-resolution information is required to gain insight into the tantalizing specificity of AQP1 for water.

**Methods**

Two-dimensional crystals of AQP1 were produced as described[15] and prepared for cryo-electron microscopy by addition of trehalose to a final concentration of 3–15%. The crystal suspension was applied to a molybdenum electron microscope grid coated with a thin carbon film. The grid was blotted with filter paper, immediately plunged into liquid ethane and transferred into a JEOL JEM-3000SF electron microscope. The JEM-3000SF, which is equipped with a liquid helium-cooled stage[16] and a field emission electron source, was operated at an acceleration voltage of 300 kV. Electron micrographs were recorded under low dose conditions (<20 electrons Å-2) at nominal magnifications of ×50,000 on Kodak SO163 film and developed with Kodak D19 developer. The images and electron diffraction patterns were taken at a stage temperature of 4.2K. Micrographs displaying well-ordered two-dimensional arrays were selected by optical diffraction and areas of 6,000 × 8,000 pixels were digitized with a Scitex LeafScan 45 (ref. 17) using a step size of 5 μm.

Images were processed on DEC/Alpha workstations with a modified version of the MRC image processing suite[18]. After two cycles unbending and correction for the contrast transfer function, 63 images were averaged to generate a merged phase data set. Electron diffraction data were recorded with a GATAN 2K × 2K slow-scan CCD camera connected to an Apple Macintosh computer. Forty-seven electron diffraction patterns were evaluated to calculate the amplitudes, which were combined with the image phases to produce the final three-dimensional electron-density map. Density maps were ison-

![Figure 2](image_url)

**Figure 2** Top, AQP1 monomer as seen a, from the extracellular side, and b, after a clockwise rotation around the x-axis by 45°. Bottom (from right to left), the monomer after another 45° clockwise rotation around the x-axis (c), and cut open to expose the central density X (d). The slice shown in d is outlined in a, and it is 16 Å thick. The red diamond marks the fourfold axis in a, and the horizontal line in b indicates the x-axis. The sequence-derived prediction of the AQP1 structure is displayed in e, and specifies the helix and loop labels.

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**Table 1 Crystallographic data**

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*High because of poor electron statistics.
*Phase residuals were calculated from 6,854 reflections with an IQ ≤ 7 (ref. 20).

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Aquaporins (AQP) are members of the major intrinsic protein (MIP) superfamily of integral membrane proteins and facilitate water transport in various eukaryotes and prokaryotes. The archetypal aquaporin AQP1 is a partly glycosylated water-selective channel that is widely expressed in the plasma membranes of several water-permeable epithelial and endothelial cells. Here we report the three-dimensional structure of deglycosylated, human erythrocyte AQP1, determined at 7 Å resolution in the membrane plane by electron crystallography of frozen-hydrated two-dimensional crystals. The structure has an in-plane, intramolecular 2-fold axis of symmetry located in the hydrophobic core of the bilayer. The AQP1 monomer is composed of six membrane-spanning, tilted α-helices. These helices form a barrel that encloses a vestibular region leading to the water-permeable epitope, the level of glycosylation, the source of the protein, and the architecture differed in the medium used for specimen cryopreservation. The overall features observed in all three projection maps were similar, but the interpretation in terms of the possible secondary structure varied. To delineate further the secondary and tertiary structure in detail, we have determined the three-dimensional density map of deglycosylated, human erythrocyte AQP1 at 7 Å resolution by electron cryocystallography (Fig. 1a).

The unselected structure in the bilayer was visualized by analysing minimal-dose images and diffraction patterns recorded from frozen-hydrated two-dimensional crystals tilted by up to 45° in the electron microscope. To our knowledge, this is the highest-resolution structure of a membrane protein determined by electron crystallography for a specimen preserved in vitrified buffer.

AQP1 monomers pack as tetramers (~60 × 60 Å2 in the bilayer plane) in a tetragonal crystalline lattice (two-sided plane group symmetry, p42; a/b = 99.6 ± 0.5 Å). Each monomer is approximately cylindrical with a diameter of ~30 Å and a height of ~60 Å. The three-dimensional density map shows that the prominent feature in each monomer is a group of six identifiable, tilted (18–30°), approximately cylindrical rods (A–F) which form a barrel surrounding a vestibular region (Fig. 1). The dimensions of these rods (36–44 Å long and ~7 Å in diameter) are similar to those for transmembrane α-helical segments seen in three-dimensional density maps of bacteriorhodopsin and LHC II (ref. 13) calculated at comparable resolution. We therefore interpret these rods of density as α-helices that form a six-helix barrel (Fig. 1). Such a six-span transmembrane arrangement is consistent with the general topology proposed for the MIP family, as well as that for AQP1 (ref. 15), which has the N and C termini on the cytoplasmic side. The α-helices tend to pack with a left-handed twist, the six-helix barrel displays a right-handed twist (Fig. 1). As a consequence of the significant tilt of the helices, the densities observed in the previous projection maps do not correspond to single helices, but rather to regions where adjacent helices overlap. The six tilted α-helices within each monomer appear to pack in three two-helix pairs (Fig. 1b, c), which is common for α-helices. Neighbouring monomers in a tetramer interact through helices that are packed as tightly as helices within individual monomers (Figs 1a and 2a). Further, densities for adjacent monomers overlap on one side of the bilayer (Fig. 2b), suggesting interactions between monomers that may stabilize the tetramer. This density implies an asymmetric disposition of the monomer with respect to the bilayer, and is interpreted as mass residing on the cytoplasmic face. We therefore attribute this density to the C and N termini, as well as the interhelical loops from adjacent monomers.

Non-crystallographic pseudo-2-fold symmetry is observed in the AQP1 monomer (Figs 1b, 2a and 3), which is consistent with the well-known tandemly repeating motif in AQP1 and the MIP family. This 2-fold rotation axis is located ~3 Å away from the putative centre of the bilayer (Fig. 1b), is inclined by 8° to the (a or b) unit cell axis, and passes through the crystallographic 4-fold rotation axis (Fig. 2a). This symmetry was suggested by visual inspection of the three-dimensional density map, and was confirmed by calculation of the rotation function (see Methods). Because the N and C termini are both located on the cytoplasmic side of the bilayer, this non-crystallographic symmetry would apply only to the transmembrane domains. Counting the six major hydrophobic segments sequentially from the N terminus, the homologous segments 1 and 4, 2 and 5, and 3 and 6 are oriented in opposite directions. This is a consequence of the six-span arrangement of the AQP1 polypeptide chain and is consistent with the observed in-plane pseudo-2-fold symmetry. The pseudo-2-fold symmetry also imposes strong constraints on models for the transmembrane topology of AQP1. Taking into account the two possible vectorial orientations of the AQP1 molecule in the synthetic lipid bilayer, the number of arrangements of the six transmembrane segments in the three-dimensional density map is reduced drastically from 1,440 (2 × 61) to 96 (2 × 2 × 3 × 3).

Within the six-helix barrel, the pseudo-2-fold axis passes through a central block of density, which appears to be connected to the

Three-dimensional organization of a human water channel
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Aquaporins (AQP) are members of the major intrinsic protein (MIP) superfAMILY of integral membrane proteins and facilitate water transport in various eukaryotes and prokaryotes. The archetypal aquaporin AQP1 is a partly glycosylated water-selective channel that is widely expressed in the plasma membranes of several water-permeable epithelial and endothelial cells. Here we report the three-dimensional structure of deglycosylated, human erythrocyte AQP1, determined at 7 Å resolution in the membrane plane by electron crystallography of frozen-hydrated two-dimensional crystals. The structure has an in-plane, intramolecular 2-fold axis of symmetry located in the hydrophobic core of the bilayer. The AQP1 monomer is composed of six membrane-spanning, tilted α-helices. These helices form a barrel that encloses a vestibular region leading to the water-selective channel, which is outlined by densities attributed to the functionally important NPA boxes and their bridges to the surrounding helices. The intramolecular symmetry within the AQP1 molecule represents a new motif for the topology and design of membrane protein channels, and is a simple and elegant solution to the problem of bidirectional transport across the bilayer.

Projection density maps of AQP1 (originally called CHIP28, for channel-forming integral protein, relative molecular mass 28K) have been determined by electron cryocystallography. These analyses differed in the medium used for specimen cryopreservation, the level of glycosylation, the source of the protein, and the nominal resolution of the maps. The overall features observed in all three projection maps were similar, but the interpretation in terms of the possible secondary structure varied. To delineate further the secondary and tertiary structure in detail, we have determined the