

The milestone of membrane protein research: Nobel Prize in Chemistry for 2003

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The Nobel Prize in Chemistry for 2003 was awarded to two biologists, Peter Agre in Johns Hopkins School of Medicine and Roderick MacKinnon from Howard Hughes Medical Institute in Rockefeller University, who have made fundamental discoveries concerning channels in cell membranes. Peter Agre discovered and characterized the first water channel protein and Roderick MacKinnon mainly elucidated the structural and mechanistic basis for potassium channels.

1 The discovery of aquaporins

Because water is the major component of all living cells, the ability to absorb and release water must be considered a fundamental property of life. For rather a long time, how water passes through the cell membrane remained controversial. At first, it is agreed by most scientists that water passes through biological membranes by simply diffusing through the lipid bilayer, however, more and more contradicting experimental observations were made. The basal water permeability of red cell membranes is much higher than that observed for other cell types and artificial lipid bilayers. The activation energy of this process (E_a 21 kJ/mol) is equivalent to the diffusion of water in solution, indicating that water moves through aqueous pathways across these membranes. Reversible inhibition by $HgCl_2$ and other organomercurials suggested the protein nature of the pathway. Further evidence was provided by the observation that some epithelial tissues exhibit changes in water permeability on a timescale that is not compatible with changes in lipid composition. These facts made people realize that there should be special and high efficient water channels in the cell membranes. However, the failures of all tempts to isolate water channel proteins and high background permeability of membrane bilayer to water leave the existence of water channels in doubt^[1].

It was not until the late 1980s that Agre discovered the water channels by chance. In the process of isolating a 32-kD membrane-spanning polypeptide component of the red cell Rh blood antigen group, a 28-kD polypeptide was obtained, which was initially assumed to represent a proteolytic fragment of Rh, later it was proved to be a new

protein. Further studies demonstrated that the protein was abundant in red cells and renal proximal tubules, and existed in two forms: a non-glycosylated 28-kD polypeptide and a 40- to 60-kD N-glycosylated polypeptide^[2]. Sequence alignment analysis suggested that it showed a strong homology with a group of putative membrane channel proteins^[3]. At the same time, radiation inactivation studies of water permeability by renal vesicles yielded a target size of 30 kD^[4]. Because of the characteristic tissue distribution, Agre began to realize that this protein might be the sought-after water channel.

Now the protein was known as aquaporin1 (AQP1). The possibility that the 28-kD protein may confer water permeability was tested by expression in *Xenopus laevis* oocytes which normally have low water permeability. When transferred to relatively low-concentration buffer, the oocytes microinjected with *in vitro*-transcribed AQP1 RNA were found to have markedly increased water permeability, causing them to swell and burst. The process was reversibly inhibited by Hg^{2+} ^[5]. In addition, the purified AQP1 was reconstituted into proteoliposome which exhibited up to 50-fold above that of control liposomes. The unit water permeability was found to be extremely high, about 3×10^9 water molecules per subunit per second, while transport of urea and protons was not increased and negligible^[6]. More direct evidences came from von Hoek. The water permeability in the red cell membrane depleted of non-aquaporin protein was similar to that of the original membrane^[7]. Thus, the debates on the existence of water channels should be over. The recent viewpoint on water transport through cell membranes was that the lipid bilayer and water channel both contributed to the water permeability and the latter was related to regulated and efficient water transport.

The functional unit of AQP1 is a tetramer with each six-transmembrane monomer providing an independent water pore^[8]. Sequence analysis indicates that it contains two tandem repeats, which the N- and C-halves are sequence-related and each contains the signature motif Asn-Pro-Ala (NPA)^[3] (Fig. 1). There are four cysteines in AQP1, but only one (Cys-189, near the NPA motif) confers mercury sensitivity, suggesting that it may reside within the aqueous pore^[8]. By inserting the epitope from the E1 coronavirus glycoprotein and analysis with specific protease, Agre revealed the topology of AQP1 and put forward the “hourglass model”—a six-bilayer-spanning α -helices surrounding the aqueous pore formed from the two NPA-containing loops that enter the bilayer from the opposite surface and overlap at the junction of the two NPA motifs^[10,11]. The model was biochemical summarization of the AQP1 structure.

Since kidney is highly related with the water conservation and excretion, most attempts to seek for new water channels pointed to this organ. Soon AQP2, AQP3, AQP4 and so on were isolated one after another. Until now at

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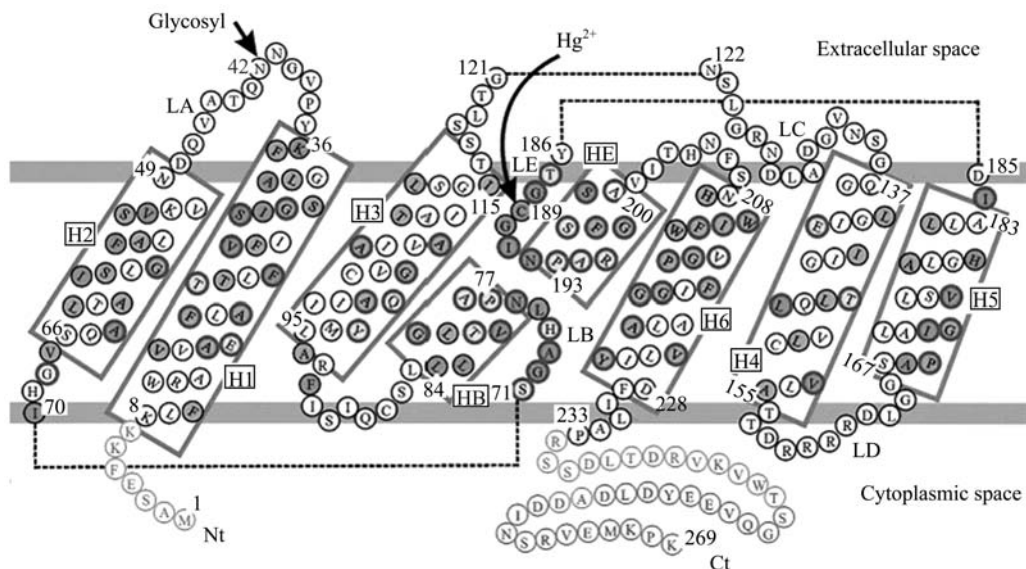


Fig. 1. Schematic representation of AQP1 topology. It can be seen that each monomer of AQP1 contains six-transmembrane helices (H1, H2, H3, H4, H5 and H6) and two short helices (HB and HE). The signature NPA motifs are located at the end of the short helices. Those gray circles stand for structurally important amino acids^[9].

least eleven human aquaporins have been identified, and their distributions were no longer confined to kidney, still found in brain, eye, liver, salivary gland, etc.^[12]. These aquaporins conform to two subsets: those selectively permeated by water (classic aquaporins) and those permeated by water plus glycerol (aquaglyceroporins). They participate in a range of pathophysiological events: Inherited defects in the gene encoding AQP0 can cause congenital cataracts and individuals with mutation in AQP2 gene suffer from a severe form of nephrogenic diabetes insipidus (NDI)^[13,14].

Aquaporins present to be highly specific for water and to prevent other solutes and ions (including anions and cations) especially protons (H_3O^+) from crossing the membrane. The problems are rather puzzling, for columns of water molecules joined by hydrogen bonds are known to permit exceedingly rapid conduction of protons, analogous to the conduction of electron through a copper wire. How can aquaporins rapidly transport water but not proton? Recently, through the high resolution three-dimensional structure provided by electron crystallography and X-ray crystallography plus molecular dynamics simulation, the selective transport mechanisms for aquaporins are being elucidated^[9,15–17].

Each monomer of aquaporins provides an hour-glass-like pore with 20 Å in length, which is mainly surrounded by hydrophobic amino acids, yet there are still four hydrophilic sites for water binding and lowering the energy barrier. These two opposing facts balance with each other to transport water selectivity while optimizing permeability^[15]. The narrowest segment of the channel occurs about 8 Å above the channel center where the entry

of molecules is controlled by size restriction and electrostatic repulsion. At this level, the wall of the pore is mainly formed by the side chains of arginine (Arg-195) and histidine (His-180), which provide a pore with diameter of 2.8 Å, the same size of van der Waals diameter of water, to filter the molecular size larger than water, while the positive charge of arginine and histidine at neutral pH would repel cations and tightly bind with anions to prevent from passing through the channel. The most remarkable character of aquaporins is the two-signature NPA motifs, which are juxtaposed by Van der Waals force and associated with each other at the center of the pore. The dipoles caused by two short α -helices whose N-termini are facing the pore result in partial positive charges surrounding the nearby conserved asparagines. An isolated water molecule is now believed to transiently form partial hydrogen bonds with both asparagines, thus undergoing a temporary dipole reorientation of the water molecule—the oxygen faces down when the water molecule enters from the extracellular side, and then flips, moves further down the channel with the oxygen facing upward. This results in the broken of the column of the hydrogen bonds, thus prevents proton transport through the membrane (Fig. 2).

2 Understanding the mechanism of potassium channels

The research history of potassium channels is quite long. About fifty years ago, Hodgkin and Huxley (Nobel laureates in physiology or medicine for 1963) had clarified the mechanism of action potential, which attributed to the sequential rise of permeability to sodium and potassium ions in nerve cell membrane. It in fact hints the exis-

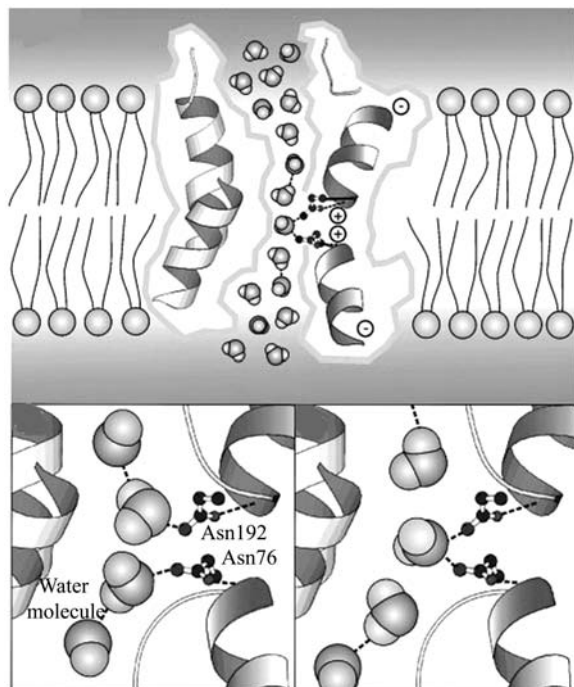


Fig. 2. Schematic representation of the mechanism for selectivity of water channels. Diagram shows that the hydrogen bonds are formed with both asparagines, thus undergoing a temporary dipole reorientation of the water molecule to break the continuous hydrogen bonds^[9].

tence of selective ion channels. In the 1980s, cloning of the pore-forming α subunits of voltage-gated Na^+ , K^+ and Ca^{2+} channels was reported^[18]. The sequences of Na^+ and Ca^{2+} channels indicate four homologous repeats, each with six putative transmembrane segments (S1—S6), while the subsequently cloned K^+ channels are only about one-fourth as large as Na^+ channels and contain one copy of the S1—S6 motif. Thus, K^+ channels may be implied to be homotetramer, which was then proved by MacKinnon^[19] by stoichiometry.

Among the six-transmembrane voltage-gated K^+ channels, S1—S4 is considered to form the voltage-sensors (Fig. 3(a)). Certain charged amino acids are within the voltage sensors, particularly the first four arginines in S4, accounting for most of the gating charge, which may move across the membrane in the electric field to control the pore open or closed^[21,22]. The pore helix and conserved signature sequence between S5 and S6 which is taken as the site for selective transport are quite important for its specific transport^[23]. Besides six-transmembrane K^+ channels, there are still two-transmembrane K^+ channels, whose sequences show homology with S5 and S6 in the voltage-gated K^+ channels and serve for the same function.

For rather a long time, a lot of studies on molecular mechanism had been done by electrophysiology, biophysics, mutation, etc., and big progress had been made in

mapping different functional regions of K^+ channels. In the middle of the 1990s, the function for selectivity of K^+ channels was mapped to a quite conserved region of their outward. However, the detailed molecular mechanism for the selective transport, gating and voltage sensor would be unknown unless high-resolution structural data could be obtained. Since it was failed to determine atom resolution 3D structure, people knew little on these fundamental problems.

The breakthrough came in 1998, when Roderick MacKinnon succeeded in determining the first high-resolution structure of an ion channel, the KcsA K^+ channel from *Streptomyces lividans*, which provides some explanations for selective transport of K^+ (Fig. 3(a))^[23]. Each subunit has two transmembrane α -helices connected by short pore region that consists of pore helices, selectivity filter, etc. A subunit is inserted into tetramer such that one transmembrane helix (inner helix) faces the central pore while the other faces the lipid membrane. The inner helices are tilted with respect to the membrane normal by about 25° and are slightly kinked. The four subunits of K^+ channels enclosed a pore for K^+ transport, whose narrow outer part consists of a 12 Å selectivity filter formed by conserved signature sequence, while the inner part is quite wide and contains a central cavity with a diameter of 10 Å at the center. Near the intracellular aspect of membrane, the four inner helices are packed against each other as a bundle. Besides the structure of KcsA, MacKinnon further determined Ca^{2+} -gated potassium channels, voltage-gated potassium channels, etc., and combined with a series of functional experiments, he clarified the fundamental mechanisms of many ion channels (Table 1).

The mechanism for selective conduction is elucidated firstly. K^+ selectivity occurs in the selectivity filter, which is formed by signature sequence amino acids as threonine-valine-glycine-tyrosine-glycine. These amino acids provide carbonyl oxygen atom, and form four cages along the pore, each offering a binding site for K^+ ions (Fig. 3(b)). At these sites K^+ ions bind in an essentially dehydrated state, surrounded by eight oxygen atoms from the protein, four above and four below each ion, which is the same case as the K^+ ions in the water surrounded by eight oxygen atoms came from water molecules. The binding sites mimic the hydrated K^+ in the water, thus the energy cost of K^+ diffusing from water into selectivity filter could be largely compensated. The dehydrated atom radius of Na^+ (0.95 Å) is smaller than K^+ (1.33 Å), failing to be fit for the selectivity filter, thus the energy barrier is rather high.

The throughput of K^+ channel could be up to 10^8 ions per second. There must be some mechanisms for the high efficiency. The central cavity of the K^+ channel is wide and contains a large amount of water to hydrate and stable K^+ ions, so that when the channel is open, K^+ ions can diffuse between cytoplasm and central cavity freely. The

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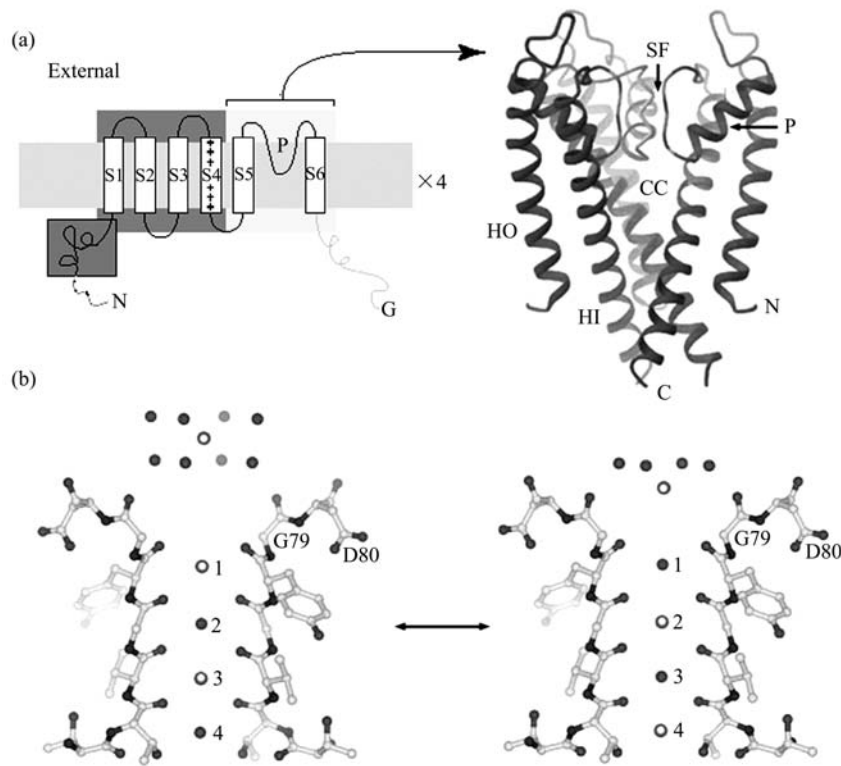


Fig. 3. The structure of potassium channel. (a) Three-dimensional structure of KcsA. The pore for K⁺ transport is surrounded by four subunits and the subunit closest to the viewer is removed for the sake of easy observation. The KcsA contains two-transmembrane helices, which are corresponding to S5 and S6 of six-transmembrane potassium channel. HO and HI stand for outer and inner helices respectively, while P for pore helix, CC for central cavity and SF for selectivity filter. (b) Electron density at selectivity filter indicate the location of K⁺——1, 3 configuration or 2, 4 configuration for K⁺. When blank circles stand for K⁺ and solid circles for water molecule, the diagram shows that the K⁺ diffuses through the pore in a row.^[29]

Table 1 MacKinnon's main contributions on the structure of ion channels

Name	Source
PDZ domain (from the synaptic protein PSD-95) ^[24]	Cell, 1996
KcsA (two-transmembrane K ⁺ channels from <i>Streptomyces Lividans</i>) ^[23]	Science, 1998
Eukaryotic PAS domain (from the N-terminal domain of HERG K ⁺ channels) ^[25]	Cell, 1998
β subunit of Kvβ2 ^[26]	Cell, 1999
The complex assembled by the T1 structure of Kv1.1 and the oxidoreductase β subunit ^[27]	Science, 2000
RCK domain (from the six-transmembrane K ⁺ channel of <i>Escherichia coli</i>) ^[28]	Neuron, 2001
KcsA-Fab complex ^[20]	Nature, 2001b
CIC chloride channel ^[29]	Nature, 2002
MthK (from Ca ²⁺ -gated K ⁺ channels of <i>Methanobacterium thermoautotrophicum</i>) ^[30,31]	Nature, 2002a, 2002b
Cytoplasmic domain of the G-protein-gated inward rectifier GIRK1 ^[32]	Cell, 2002
The complexes of KvAP (from <i>Aeropyrum pernix</i>)-Fab and voltage sensor paddle-Fab ^[33]	Nature, 2003
The complex of CIC-Fab (from <i>Escherichia coli</i>) ^[34]	Science, 2003

pore helices above central pore have their negative ends (C-termini) facing the central cavity so as to attract and stabilize cations. The concept is mirrored in quite different architecture of channels designed to conduct the negatively charged chloride ions—they have the positive ends of multiple helices pointed toward the central ion site^[29]. Besides the structural study, a comparison with the distribution of other ions (e.g. Rb⁺) in the filter^[35] showed

that the selectivity filter contains two K⁺ ions, separated by about 7.5 Å, roughly the average distance between K⁺ in a 4 mol/L KCl solution. The mutual repulsion between the two ions is quite large and prefers high speed conduction.

However, we have only seen a closed K⁺ channel-KcsA, and are not able to understand how K⁺ channel regulates itself between the open and closed states. In 2002, through the atom-resolution structure of MthK

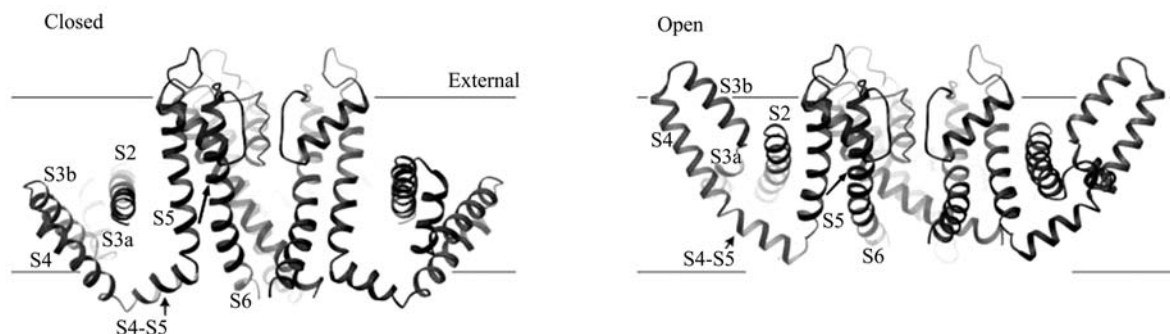


Fig. 4. The supposed opened and closed states of voltage-gated K^+ channels. The voltage-sensor paddles composed of S3b-S4 are located at the channels' out perimeters. The arrowed point on S6 represents gating hinge and the opened and closed conformation conform to the structures of KcsA and MthK. The figure indicates that at the change of the membrane electric field, voltage-sensor paddle moves to the outer part of the membrane to pull it open^[36].

determined and comparison with KcsA, MacKinnon succeeded in understanding the gating mechanism^[31]. KcsA was crystallized under conditions that favor its closed conformation in membranes, while MthK, a Ca^{2+} -gated K^+ channel from *Methanobacterium thermoautotrophicum*, was crystallized in the presence of high Ca^{2+} concentrations that open its pore. We can compare the two structures so as to infer the conformational change during gating. At the closed state, the inner helices of K^+ channels are straight and form a helix bundle near the intracellular membrane surface. At the bundle crossing, the pore narrows to 3.5 Å in diameter and is lined with hydrophobic amino acids, creating a barrier to the flow of K^+ ions. In contrast, when at the open state the inner helices of K^+ channels are bent (approximately 30°) at hinge point and splayed open to 12 Å in diameter so that the central cavity becomes confluent with the cytoplasm. The hinge point was named gating hinge, corresponding to glycine-83 in MthK and glycine-99 in KcsA (Fig. 4). Since glycine is unique in its ability to adopt a wide range of main-chain dihedral angles and confer flexibility, it is adapted to be gating hinge.

Along with the elucidation of mechanisms for selectivity and gating, the most difficult and important problem for K^+ channels, the mechanism for voltage sensing, is in special need to understand. After a six-year effort, MacKinnon published the first structure of six-transmembrane voltage-gated K^+ channel in 2003^[33]. Although the structure (KvAP) from *Aeropyrum pernix* may be in a non-native state, it still provides a simple possibility for us to understand how the voltage sensors might transfer the gating charges through the membrane electric field.

The KvAP structure shows that the traditional definition of S3 helix is in fact separated into two individual helices (S3a and S3b) by a loop (S3 loop). The second helix in S3 (S3b) and the N-terminal half of the defined S4 are packed tightly against each other, forming a helix-turn-helix structure that is mainly hydrophobic, with the notable exception of distributed arginine residues. We call

this S3b-S4 unit the voltage-sensor paddle. It is shown that the first four arginine residues in S4 account for most of the gating charges movement that open and close the pore by pulling S4-S5 linker (Fig 4). Through the method of molecular ruler, MacKinnon got to know that the total displacement of the voltage-sensor paddles is approximately 20 Å perpendicular to the membrane^[36].

3 Comments

The achievements of Agre and MacKinnon have solved the key problems in the area of water and potassium channels. The most important work on water channels was over in 1988 and the later work on high-resolution structure of water channels was not only attributed to Agre. Since he is the first one to discover and isolate water channels, this contribution has made the out-pouring of research on aquaporins world-wide and him the Nobel laureate. In contrast to water channels, the difficulty in the investigation of K^+ channels is quite different—its existence has been inferred early, but in lack of atom-resolution structure. Under this background MacKinnon turned to structural biology to study K^+ channels and since 1998, he determined a series of the atomic structure on ion channels, succeeding in setting up the model of K^+ channels (Cl^- channels also). He is worthy of Nobel Prize.

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