6 Sodium and Engineered Potassium Light-Driven Pumps

Vitaly Shevchenko, Ivan Gushchin, Vitaly Polovinkin, Kirill Kovalev, Taras Balandin, Valentin Borshchevskiy, and Valentin Gordeliy

6.1 Introduction

Optogenetics is one of the most important recent technological advances in biology. It revolutionized our ability to study the processes in neuronal circuits, promising new approaches to the treatment of different diseases and disabilities. The core tools of optogenetics are light-driven retinal membrane proteins. Retinal-containing membrane proteins are present in all domains of life. They employ light energy for a wide range of different functions such as ion transport, photosensing and channel activity (Gushchin *et al.*, 2013). All of these proteins contain seven transmembrane α-helices and a chromophore-retinal molecule, which is covalently bound via the Schiff base to the side-chain of a lysine amino acid (Oesterhelt and Stoeckenius, 1971; Stoeckenius *et al.*, 1979).

Microbial rhodopsins (MRs) were first discovered in the early 1970s in the archaeal halophiles (salt-loving microbes that live in saturated brines). These extremophiles have a remarkable variety of rhodopsins. The first known was called bacteriorhodopsin (BR) because of its structural similarities with vertebrate eye rhodopsins (Oesterhelt and Stoeckenius, 1971; Stoeckenius *et al.*, 1979). However, BRs that are widely distributed in different genera of archaeal halophiles are proton pumps used to generate light-driven proton gradients as sources of energy for the cell. Then, in 1979, again in the same microbe, a second pump was found; however, it was erroneously concluded that this pump functions as an outward sodium pump (Greene and Lanyi, 1979; Lindley and MacDonald, 1979; MacDonald *et al.*, 1979). Only later was it recognized that, in fact, this rhodopsin is an inward-directed chloride pump that helps maintain the proper electrochemical balance (Schobert and Lanyi, 1982). Soon after halorhodopsin, two sensory rhodopsins (also known as slow rhodopsins) were found in the same microbe (*Halobacterium salinarum*) (Bogomolni and Spudich, 1982). These are light sensors (each tuned to a different wavelength) that produce complex photactic behavior in these microbes. All of these rhodopsins were found in different representatives of this archaeal clade.

It was only after the 2000s that, thanks to metagenomics, rhodopsins were also found in bacteria. An uncultivated marine gammaproteobacterium of the 16S rRNA-defined clade SAR86 was confirmed to have a functional proton pump that was named proteorhodopsin (Béjà *et al.*, 2000). The rapid increase in sequenced bacterial genomes of the first decade of the twenty-first century enabled the discovery of several examples of rhodopsin genes in multiple branches of bacteria and archaea (de la Torre *et al.*, 2003; DeLong and Karl, 2005; O'Malley, 2007). A clear illustration of the widespread transmission of these genes in the prokaryotic domain was found in the extremely halophilic bacterium *Salinibacter*, the genomes of which produced four rhodopsins, one proton pump, one chloride pump and two sensory rhodopsins, representing exactly the same combination as the environment-sharing archaeon *Halobacterium* (Mongodin *et al.*, 2005). But nature plays tricks on us. *Salinibacter*'s proton pump, later called xanthorhodopsin, is different from the *Halobacterium* one in both structure and function, being the first discovered rhodopsin to use a carotenoid pigment antenna in the same manner as chlorophylls to increase the wavelength functional range (Balashov *et al.*, 2005). Since then, rhodopsins have been found in most clades of microbes exposed to light, including firmicutes, actinobacteria, bacteroidetes, most proteobacteria and marine archaea.

The most studied protein of this family is BR from a halophilic archaea *H. salinarum*, which is a proton pump that provides the first key universal step of transformation of energy in cells: the generation of a proton electrochemical gradient across the cell membrane. Beginning with electron microscopy (Henderson *et al.*, 1990) and then X-ray high-resolution studies, the analysis of halorhodopsin and sensory rhodopsins provided a basis for the fundamental hypothesis of transport mechanisms (Luecke *et al.*, 1999; Kolbe *et al.*, 2000; Gordeliy *et al.*, 2002; Moukhametzianov *et al.*, 2006) and motivated the development of new experimental technologies (Gushchin *et al.*, 2013).

Among the most important applications of rhodopsins is optogenetics. Unfortunately, few retinal proteins are available for optogenetics. Specifically, the most used are the non-selective channel rhodopsin, ChR2, which is used to depolarize and thus activate neurons (Nagel *et al.*, 2003), the Cl^- pump halorhodopsin, NpHR (Zhao *et al.*, 2008), and the H^+ pump archaerhodopsin, Arch3 (Han *et al.*, 2011). The latter two are used to hyperpolarize and thus silence neurons. A crucial aspect for future progress in optogenetics is to identify and/or engineer new light-driven proteins with novel properties, such as channels and transporters with high selectivity and conductivity for a particular ion. The ions Na^+, K^+ and Ca^{2+}, which are relevant for neuronal function, have not been addressable by MR pumps so far (the first experiments with Na^+ were conducted very recently). In particular, an outward K^+ pump would be highly desirable, as K^+ is the main ion used for neuronal re- or hyper-polarization. In our review, we describe very recent work on the structure–function characterization and engineering of sodium and potassium light-driven pumps.

Interestingly, the known structures led to a widely accepted belief that sodium pumps cannot exist. This erroneous conclusion was based on the assumption that

the known rhodopsins had to have quite the same structures; for instance, similar to that of BR. If this were true, it would not be clear how a cation can be transferred from extracellular to cytoplasmic parts of the protein by the positively charged Schiff base of the chromophore upon its isomerization. However, the recently elucidated structure of the KR2 sodium pump, as is discussed in this chapter, is remarkably different from that of BR, and this difference explains why a cation pumps exist. Indeed, while investigating the marine bacterium *Dokdonia eikasta*, Inoue and co-workers discovered that *D. eikasta* cells are able to pump out sodium ions during early periods of growth under illumination (Inoue *et al.*, 2013). It turned out that the corresponding ion transporter, dubbed KR2, belongs to a new type of MR in which some of the most important ionizable amino acids are replaced with polar ones, and vice versa. Even when expressed in another commonly employed bacterium, *Escherichia coli*, KR2 could still transport sodium ions. Yet it appeared that potassium ions, despite being very similar to sodium ions, were not transported by KR2.

This and other curiosities displayed by KR2 attracted our interest because we had already carried out several structural studies of other MRs in order to investigate the molecular mechanisms behind their actions. However, membrane proteins are notoriously difficult to handle, and producing them is usually expensive and time consuming, with KR2 being no exception. Since the protein could be produced only in batches of several milligrams despite our new efficient approaches to membrane protein crystallization, while thousands of crystallization conditions should have been tested, we had to miniaturize and automate the process using robotic and imaging systems. The diffraction signal from the first crystals that were obtained, which measured less than 20 μm across, was too weak to be measured using in-house X-ray sources. Recent advances at the European Synchrotron Radiation Facility's macromolecular crystallography beamlines – such as microfocus X-ray beams, automatic sample changers and convenient software protocols – greatly helped with the search for the best crystals. After several months of trials at beamlines ID23-1 and ID29, we were able to improve the resolution of the diffraction data collected from around 20 Å to better than 1.5 Å. Meanwhile, we were able to extract information about KR2's spectroscopic characteristics using optical absorption spectra at the cryobench laboratory at beamline ID29S. Before we describe Gordeliy and Kandori's groups' structures (Gushchin *et al.*, 2015b; Kato H.E. *et al.*, 2015), we shall describe the family of sodium pumps and illustrate their general functional characterization.

6.2 The Family of Sodium-pumping Rhodopsins

The first light-driven sodium pump KR2, from *D. eikasta*, was discovered and characterized in 2013 (Inoue *et al.*, 2013). Other representatives rapidly populated this class of rhodopsins. The gene sequences had already been acquired during whole-genome sequencing of various marine, sedimentation and more exotic species (Figure 6.1) (Ivanova *et al.*, 2011; Riedel *et al.*, 2012; Singh *et al.*, 2013;

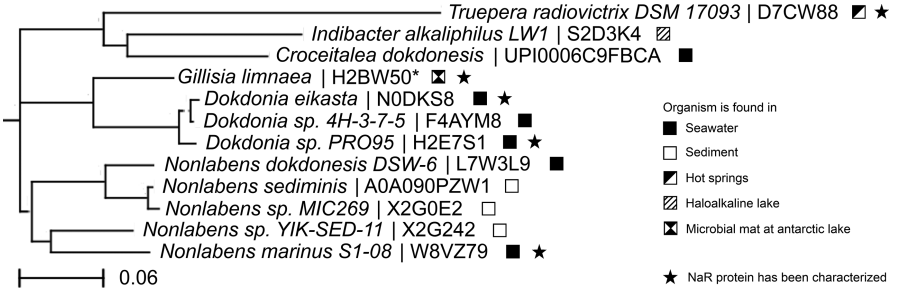

Figure 6.1 Phylogenetic tree of rhodopsins with the NDQ motif, and putative sodium-pumping rhodopsins. The name of the organism and corresponding UniProtID of the protein is shown. The media in which the species have been found are indicated by squares with different fills. The star indicates that the NaR protein has been demonstrated to pump sodium ions. * *Gillisia limnaea* GLR protein is represented in its truncated version (61 amino acids are truncated from the N-terminus).

Nakanishi *et al.*, 2014; Kwon *et al.*, 2016). The proteins are distinguished by their characteristic "NDQ motif" of the active site residues (N112, D116 and Q123 in KR2), replacing the "DTD motif" (D85, T89 and D96) in BR or DTE/DTK in other proton pumps (Balashov *et al.*, 2013; Ernst *et al.*, 2014) and NTQ/TSA in chloride pumps.

Sequence alignment of all available members of putative sodium-pumping rhodopsins shows high sequence similarity among them (Figure 6.2), with the transmembrane regions being more conserved than extra- and intra-cellular loops. In order to analyze the conservation of the functional units in the protein, we provide a brief description of the structure of KR2 in the following section.

6.3 Structures of the Wild-type Sodium Pump KR2

Several crystallographic structures of the light-driven sodium pump KR2 have been determined (Gushchin *et al.*, 2015b; Kato H.E. *et al.*, 2015). The structures have recently been reviewed by us (Gushchin *et al.*, 2015a) and here we will provide a brief synopsis.

Overall, similarly to other MRs, KR2 consists of a seven-transmembrane helical framework, with the light-absorbing cofactor retinal bound in the middle. Somewhat unexpectedly, the extracellular side of the protein is capped with the N-terminal α-helix, which is not observed in other MRs. The elongated loop connecting helices B and C is similar to xanthorhodopsin's B–C loop in structure (Luecke *et al.*, 2008) and makes β-sheet interactions with the linker joining the N-terminal α-helix and helix A.

Inside the protein, the crystallographic structures reveal the ion translocation pathway. Structural and mutational analysis enabled functional assignment of three major segments: the ion uptake cavity, the Schiff base cavity and the putative ion release cavity (Figure 6.3) (Gushchin *et al.*, 2015b; Kato H.E. *et al.*, 2015).

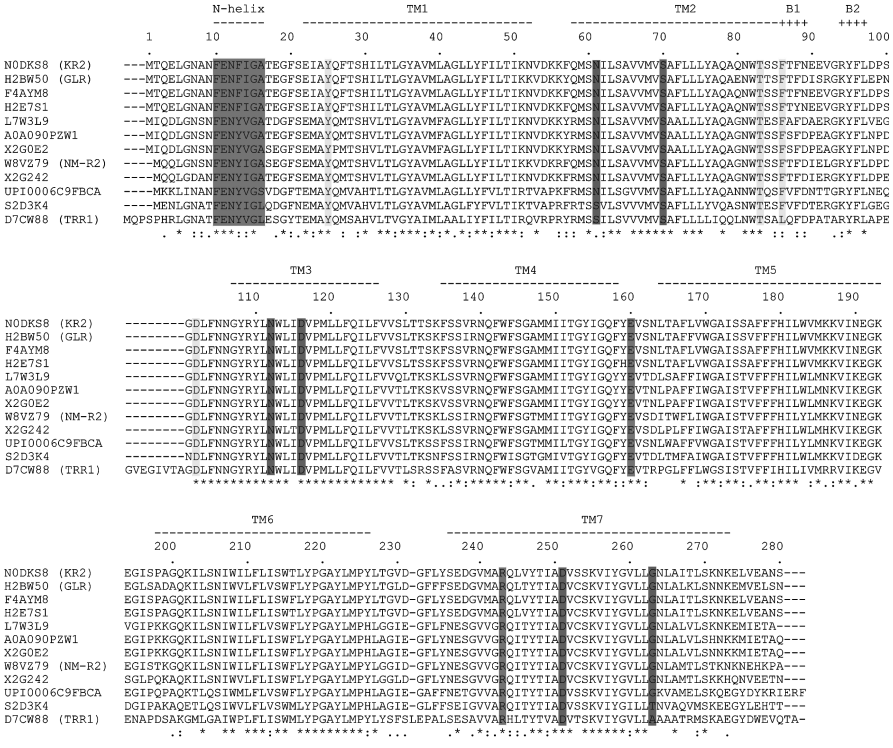

Figure 6.2 Structure-based sequence alignment of putative sodium-pumping rhodopsins. In order to precisely specify the proteins, we indicate the UniProtIDs. However, some of the proteins have "established" common names in the literature, so we cite them as well. TM indicates transmembrane regions, B shows the location on beta-sheets. The ion uptake cavity (cyan) is represented by an N61–G263 pair, the two latter members have an S61–T/A263 pair. The cavity in proximity to retinal (magenta) is completely conserved in the class (i.e. S70, N112, D116 and D251). The ion release cavity contains a cluster of three conserved ionizable residues: E11, E160 and R243 (green). All members possess an N-terminal α-helix that caps the putative ion release cavity. The oligomerization interface is also well conserved (yellow) among the members of sodium-pumping rhodopsins (Y25, T83, F/L86 and D102). (A black-and-white version of this figure will appear in some formats. For the color version, please refer to the plate section.)

The ion uptake cavity has a large opening into the cytoplasm. Obstructing this cavity through various mutations either inhibits ion pumping or imparts the protein with novel selectivities toward monovalent cations (Gushchin *et al.*, 2015b; Kato H. E. *et al.*, 2015; Konno *et al.*, 2016). Indeed, while the wild-type KR2 can only pump H^+, Li^+ and Na^+, the mutations G263F and G263W result in potassium-pumping variants, whereas the double-mutant N61L/G263F can pump ions as large as Cs^+. Overall, it has been found that raising the side-chain volumes at positions 61 and 263 leads to stronger conductivity of larger cations (Konno *et al.*, 2015). At the bottom of the KR2's ion uptake cavity is a polar residue Glu-123, the analog of the ionizable proton donor residues of the proton pumps. Mutations of Glu-123 to either alanine or valine result in decreased efficiency of the pump (Inoue *et al.*, 2015).

The retinal-binding pocket contains all-*trans* retinal, with its Schiff base embedded in a polar cavity, lined with Ser-70, Asn-112, Asp-116 and Asp-251.

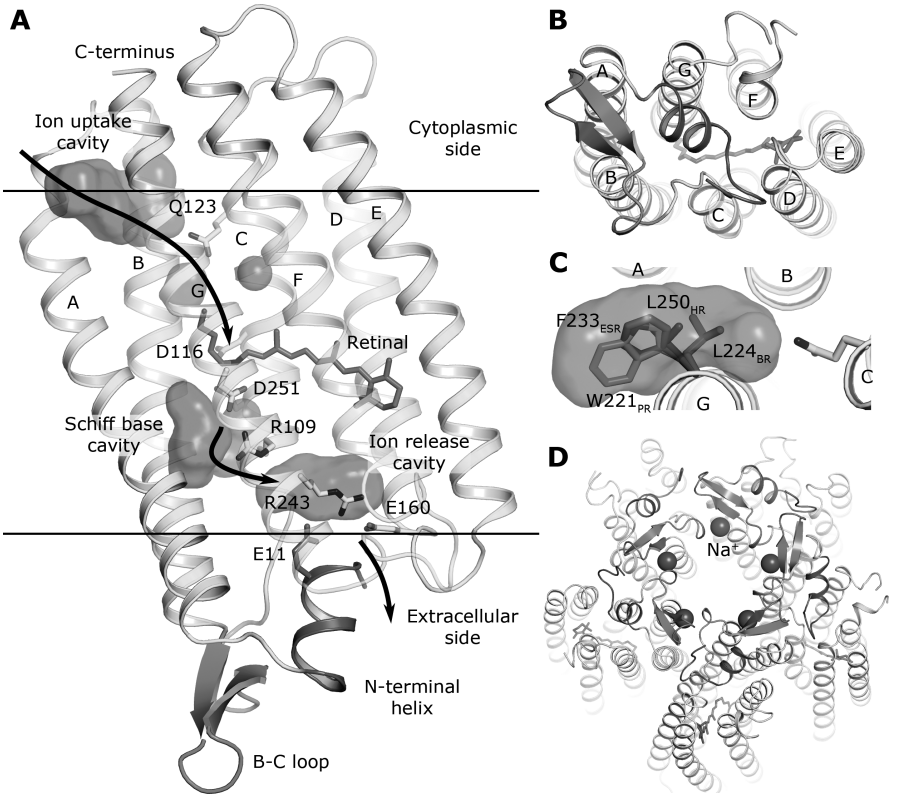

Figure 6.3 Structure of KR2. The N-terminal helix is shown in blue and the B–C loop is shown in orange. Water-accessible cavities are shown as red surfaces. (A) Side view. The ion path is shown with arrows. (B) View from the extracellular side. (C) KR2 ion uptake cavity. Hypothetical effects of the G263L, G263F and G263W mutations are shown using the conformations of the homologous residues L224 of bacteriorhodopsin (BR), L250 of [20], F233 of *Exiguobacterium sibiricum* rhodopsin (ESR) and W221 of blue proteorhodopsin (PR). (D) Pentameric assembly of KR2. Sodium ions are shown in violet. (A black-and-white version of this figure will appear in some formats. For the color version, please refer to the plate section.)

The Schiff base cavity is separated from the extracellular side of the protein by an Arg-109 side-chain. Asn-112 and Asp-116 are among the residues defining the sodium pumping ability of KR2, and all of the polar and ionizable residues of the Schiff base cavity are crucial for the pump's function (Inoue *et al.*, 2013).

There are several conformations of the Schiff base cavity that have been observed in the crystallographic structures (reviewed in Gushchin *et al.*, [2015a]), most notably a compact one and an expanded one. This is similar to the case of the ion-free and ion-bound chloride pump halorhodopsin (Kouyama *et al.*, 2010; Kanada *et al.*, 2011). Consequently, we hypothesized this ability of the Schiff base cavity to expand to be a requisite for a MR to be able to pump an ion that is not a proton (Gushchin *et al.*, 2015a).

Finally, the ion release cavity of KR2 is centered on three ion-bridged residues Glu-11, Glu-160 and Arg-243. Since mutations of any of these residues do not result in qualitative changes in the pump's function, it must be concluded that these

residues have a mostly structural function (Inoue *et al.*, 2013; Gushchin *et al.*, 2015b; Kato H.E. *et al.*, 2015).

While KR2 crystallizes both as a monomer and a pentamer, we believe that the physiological state is pentameric (Gushchin *et al.*, 2015a, 2015b), similar to pentameric proteorhodopsins (Ran *et al.*, 2013). The sodium ion that binds to KR2 in the ground state is found both in monomer and at the pentamerization interface of a pentamer (Gushchin *et al.*, 2015a) and most probably plays a structural role and is not the ion that is being transported.

6.4 The Mechanism of Sodium Translocation

A number of representatives of putative sodium-pumping rhodopsins have been functionally characterized (Figure 6.1), and it has been demonstrated that they can pump sodium ions (Inoue *et al.*, 2013; Balashov *et al.*, 2014; Yoshizawa *et al.*, 2014; Bertsova *et al.*, 2015). Many of the studied proteins were found to be hybrid Na^+/H^+ pumps. The proteins transport sodium ions if they are present in sufficiently large amounts, but transport protons if the opposite is the case. KR2 from *D. eikasta*, GLR from *G. limnaea* and TRR1 from *Truepera radiovictrix* (Figure 6.4) are hybrid Na^+/H^+ pumps. NaR from *Dokdonia* sp. PRO95 and NM-R2 from *Nonlabens marinus* S1-08 display less pronounced H^+ transport in the absence of sodium ions (Yoshizawa *et al.*, 2014; Bertsova *et al.*, 2015).

All rhodopsins that have been characterized in this class have a common absorption maximum in the ground state of 523 nm. The presence or absence of sodium ions barely affected this value.

Time-resolved visible spectroscopy has been done for KR2 and GLR, sharing the primary result that photocycle turnover is faster in the presence of sodium ions. During the photocycle, a red-shifted K intermediate is rapidly formed; blue-shifted L/M states then appear, and then a red-shifted O state appears. The rate of formation of the O state is affected by the sodium concentration and it is increased with an increase of sodium concentration, implying that Na^+ uptake

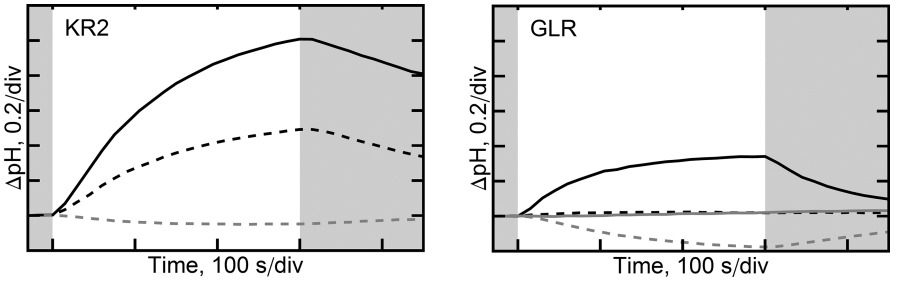

Figure 6.4 *Escherichia coli* activity tests of KR2 and GLR. The pH changes upon illumination in the media containing KR2 or GLR-expressing *E. coli* cells. The solutions contain 100 mM NaCl (black, dashed), 100 mM NaCl and 30 μM carbonylcyanide m-chlorophenylhydrazone (CCCP) (black, solid), 100 mM KCl (gray, dashed) and 100 mM KCl and 30 μM CCCP (gray, solid). The cells were illuminated for 300 s (light areas on the plots).

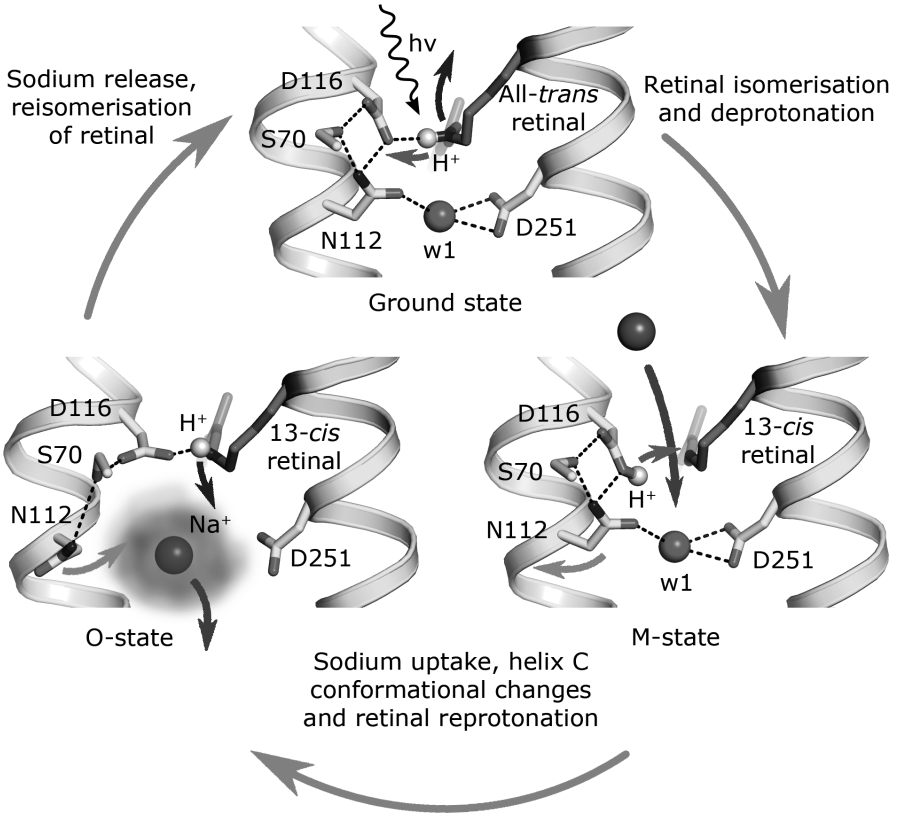

Figure 6.5 Proposed model of the structural changes during sodium translocation. (A black-and-white version of this figure will appear in some formats. For the color version, please refer to the plate section.)

occurs in the M-to-O transition. A natural competitive model was recently proposed for the Na^+/H^+ uptake for KR2. According to the presented kinetic analysis, the H^+/Na^+ selectivity is determined by the ion uptake rate constants k_H/K_{Na} and is approximately 7000–9000 at a neutral pH. This means that under physiological conditions KR2 will pump sodium much more efficiently than protons (Kato Y. *et al.*, 2015).

Crystallographic structures of KR2 together with spectroscopic data allowed us to formulate a hypothesis on the mechanism of the sodium translocation in the family of sodium-pumping rhodopsins (Figure 6.5) (Gushchin *et al.*, 2015a). Briefly, absorption of a photon by the retinal leads to its isomerization and consequent deprotonation. Then, in the M state to O state transition, the Schiff base cavity expands and the sodium ion enters the pump. During the relaxation of the O state to the ground state, the ion is released into the extracellular space and the Schiff base contracts to the resting conformation. The directionality of the sodium translocation is achieved via the proton residing at the Asp-116 side-chain that prevents the sodium from exiting into the cytoplasm.

The proposed mechanism is still under debate, and the experience obtained with BR may be valuable in this respect. BR is the most heavily studied rhodopsin

protein and is regarded as a reference model for the family. The enormous scientific efforts and critical analysis applied to BR resulted in a unified view on the mechanism of unidirectional proton pumping (Wickstrand *et al.*, 2015), which is not consistent with all of the experimental data. The unified view gave answers to two major questions: (1) what forces a proton to move uphill against a transmembrane potential? And (2) what ensures the unidirectionality of the proton transfer? The state-of-the-art answer to these questions is that structural changes caused by retinal isomerization affect the local molecular surrounding of the proton translocation pathway groups (mostly the Schiff base and Asp-85), which changes their pKa, and the change of proton affinity drives proton translocation. At the same time, proton accessibility to the Schiff base switches from the extracellular to the cytoplasmic side and back during the photocycle, which ensures the unidirectionality of the proton transfer.

The KR2 crystal structure shows a putative ion translocation pathway in fine detail. However, the exact force that drives the ions and the mechanism for preventing backward ion leakage are still to be elucidated. Clear answers, just like those in the BR case, are important for rational manipulations of KR2 optogenetic properties.

To completely characterize KR2 and understand the possible mechanism of its function, we discuss in the next section an interesting phenomenon – oligomerization of KR2, which seems to be coupled with the switch from H^+ to Na^+ pumping.

6.5 Oligomeric States of KR2

KR2 can form both a monomeric state at low pH (around 4.3) and a pentameric state at higher pH while crystallizing *in meso*. It also appears as a pentamer in detergent when solubilized and purified by size-exclusion chromatography (SEC) at higher pH values, but comes in a variety of forms at low pH values (Figure 6.6). Because no sodium pumping activity has been observed at pH values under 5.0, we tried to determine whether the oligomeric state directly affects ion transport.

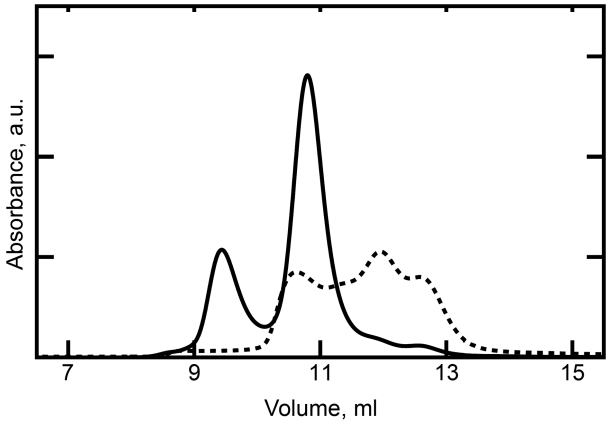

Figure 6.6 Size-exclusion chromatography of wild-type KR2 at pH 7.5 (solid) and pH 4.6 (dashed).

Crystal structures of KR2 show that in the red pentameric state, the His-30 residue of helix A can form hydrogen bonds with the Tyr-154 residue of helix D of a nearby protomer. To break this bond, H30A, H30L and Y154F mutants were expressed in *E. coli*. The H30L mutant showed no sodium pumping activity in cell suspensions at neutral pH values, nor did it form pentamers when purified. Pentamer formation is completely abolished by introducing amino acids with large side-chains and removing charge. The Y154F and H30A mutants displayed observable sodium pumping activity, although this was reduced in comparison to that of the wild-type protein (Figure 6.7). In SEC, these mutants partially appear as pentamers, thereby explaining the lower transport activity.

As histidine residues have relatively low pKa values and can undergo changes in their biochemical properties even if pH changes are small, we prepared a H30K mutant in order to make the protein less sensitive to proton concentrations at pH values of approximately 7. The H30K mutant showed the same sodium pumping activity as the Y154F mutant (Figure 6.7). SEC at physiological pH showed that the pentameric state fraction is even lower. The protein is found only in a monomeric state when crystallized *in meso* at pH 8.0 or lower (crystal packing is similar to one of the wild type in the blue monomeric form (PDB ID 4XTL). Its structure is almost the similar as that of wild-type KR2 in the blue state. This indicates that the "switch" between oligomeric states happens at higher pH values as compared to the wild-type protein, as was expected.

6.6 Optogenetic Applications of the Sodium Pump and Structure-based Engineering of the Light-driven Potassium Pump

Gordeliy and Kandori's groups suggested that KR2 could work as an inhibitory optogenetics tool in heterologous systems. Indeed, Kato *et al*. have demonstrated successful application of a eubacterial rhodopsin (KR2) in optogenetics (Kato, H.E. *et al.*, 2015). In contrast to halorhodopsin and archaearhodopsin-3, KR2 is not expected to induce unintentional side effects, such as the activation of pH-sensitive channels. It has been argued that the flow of Na^+ would be more physiological than that of proton or chloride ions. It is also natural that the voltage-independent photocurrent of KR2 would enable efficient sodium flux, even under highly negative membrane potential conditions. KR2 may have the potential to become an ideal tool in many optogenetics experiments.

As we mentioned previously, an interesting feature of KR2 is the unusual structure of the inward-facing ion uptake cavity, which is unexpectedly large and protrudes from the protein surface, suggesting that this structure could act as a filter that allows KR2 to be selective for sodium ions. We swapped specific amino acids at the relevant site using targeted mutations and found that KR2 indeed loses its sodium-pumping ability under such conditions. However, we also found that one of the mutations (G263F) transforms KR2 into a light-driven potassium pump – the first of its kind. This result is especially interesting in terms of potential optogenetic applications because transporting potassium ions from the cell is a natural neuron deactivation

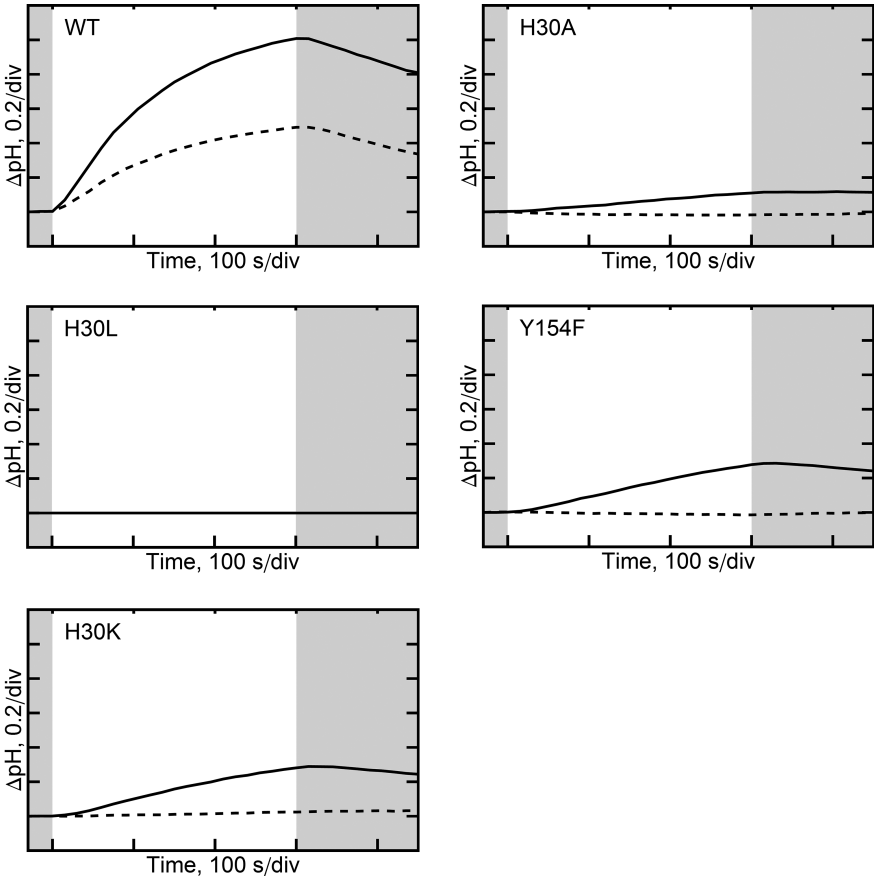

Figure 6.7 *Escherichia coli* activity tests of KR2 mutants related to pentamerization. pH changes upon illumination in the media containing KR2 or GLR-expressing *E. coli* cells. The solutions contain 100 mM NaCl (black, dashed) and 100 mM NaCl and 30 μM CCCP (black, solid). The cells were illuminated for 300 s (light areas on the plots).

mechanism. Normally, activated neurons release the ions through passive potassium channels in the membrane, but a light-activated potassium pump would enable this process to be precisely controlled; therefore, KR2 provides a highly effective off switch for neurons (Kato, H.E. *et al.*, 2015). The next step is to find ways of integrating the pump into different types of cells. The light-activated channelrhodopsin 2, which is a well-known molecular off switch, in combination with the KR2 potassium pump would form a perfect pair of tools for the precise control of nerve cell activity.

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