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REVIEW

Biophysics of rhodopsins and optogenetics

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Abstract

Rhodopsins are photoreceptive proteins and key tools in optogenetics. Although rhodopsin was originally named as a red-colored pigment for vision, the modern meaning of rhodopsin encompasses photoactive proteins containing a retinal chromophore in animals and microbes. Animal and microbial rhodopsins respectively possess 11-*cis* and all-*trans* retinal, respectively. As cofactors bound with their animal and microbial rhodopsin (seven transmembrane α-helices) environments, 11-*cis* and all-*trans* retinal undergo photoisomerization into all-*trans* and 13-*cis* retinal forms as part of their functional cycle. While animal rhodopsins are G protein coupled receptors, the function of microbial rhodopsins is highly divergent. Many of the microbial rhodopsins are able to transport ions in a passive or an active manner. These light-gated channels or light-driven pumps represent the main tools for respectively effecting neural excitation and silencing in the emerging field of optogenetics. In this article, the biophysics of rhodopsins and their relationship to optogenetics are reviewed. As history has proven, understanding the molecular mechanism of microbial rhodopsins is a prerequisite for their rational exploitation as the optogenetics tools of the future.

Keywords Microbial rhodopsin · Retinal · Photoisomerization · Pump · Channel · Photo-cycle

Light absorption in animal and microbial rhodopsin

The word "rhodopsin" comes from a conjunction of the Greek words "rhodo" and "opsis," which indicate rose and sight, respectively (Kandori 2015). Therefore, the classical meaning of rhodopsin is the red-colored pigment for vision, which is localized in the retinal rods of eyes. The chromophore molecule that absorbs light is retinal, which is the origin of red color. Then, different colored retinal-binding proteins were found in microbes, largely expanding the definition of the word rhodopsin. The modern meaning of rhodopsin encompasses photoactive proteins containing a retinal chromophore in animals and microbes (Ernst et al. 2014). Animal and microbial rhodopsins share a common architecture of seven transmembrane α-helices, but they have no sequence homology with each other. Figure 1a shows the structures of bovine rhodopsin (top panel) and bacteriorhodopsin (BR) (bottom panel), which act as good archetypes of the typical animal and microbial rhodopsins, respectively.

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Retinal, the aldehyde of vitamin A, is bound to the protein by a protonated Schiff base linkage to the ε-amino group of a lysine side chain in the shape of 11-*cis* and all-*trans* forms in animal and microbial rhodopsins, respectively (Fig. 1b). The thermal stability of the 11-*cis* and all-*trans* forms of retinal is highly dependent upon its surroundings. The 11-*cis* retinal is stable in animal rhodopsins, and thermal isomerization to the all-*trans* does not readily occur when co-factored to the protein. However, 11-*cis* retinal is not thermally stable in other environments and easily isomerizes into the all-*trans* form when not co-factored to the protein. Consequently, the 11-*cis* form of retinal is not abundantly populated in animal cells whereas the all-*trans* isomeric form of retinal is highly populated in animal cells. This is one of the important factors in optogenetics. Upon light absorption, photoisomerization takes place from the 11-*cis* to all-*trans* form in animal rhodopsins, and from the all-*trans* to the 13-*cis* form in microbial rhodopsins (Fig. 1b) (Ernst et al. 2014). While the isomerization reaction is common between animal and microbial rhodopsins, the end stage of the photoreaction differs. The isomerized all-*trans* retinal is released in our visual pigments and does not return to the 11-*cis* form, which is thus called "photobleaching" (Fig. 1b). This is not a problem in human visual cells because enzymatically isomerized 11-*cis* retinal is newly supplied, but this is not the case in other animal cells. In contrast, the 13-*cis* form is thermally isomerized into the all-

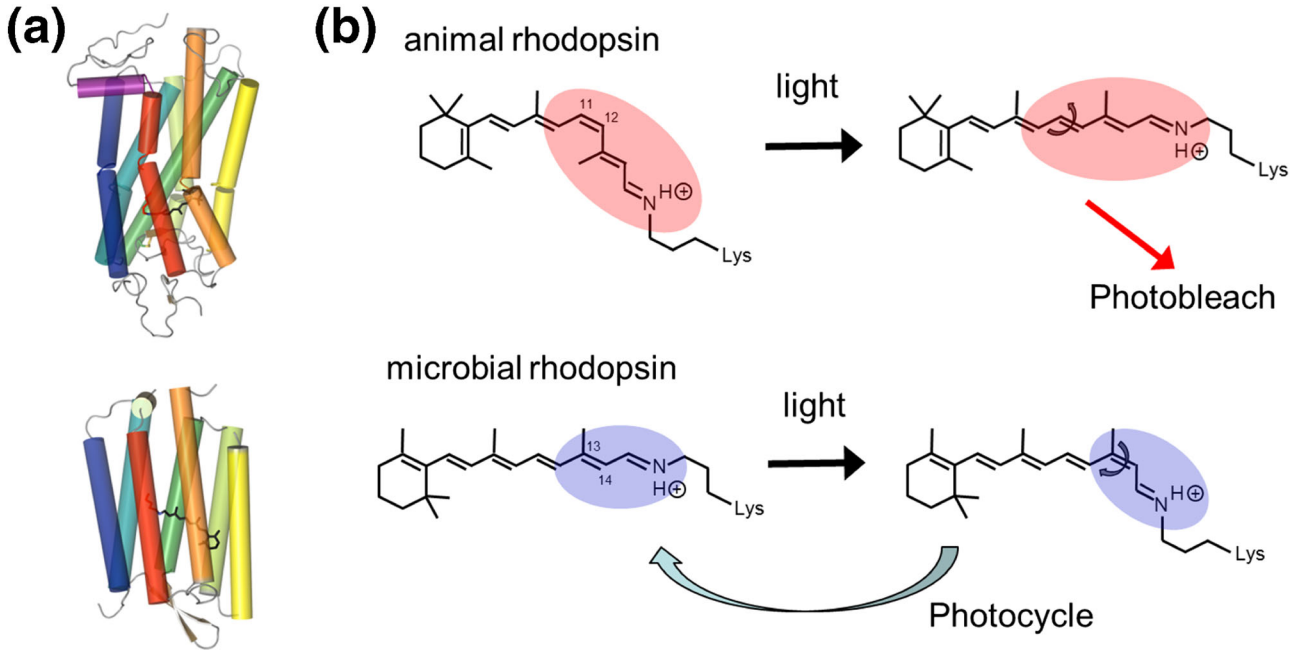

Fig. 1 **a** Structures of bovine rhodopsin (upper panel) and bacteriorhodopsin (lower panel), typical of animal and microbial rhodopsins, respectively. Top and bottom sides correspond to cytoplasmic and extracellular region, respectively. **b** Chromophore of animal (upper panel) and microbial (lower panel) rhodopsins. Animal and microbial rhodopsin possesses 11-*cis* and all-*trans* retinal as its chromophore, respectively, bound to a Lys residue via a Schiff base, which is normally protonated and exists in the 15-anti configuration. Upon light absorption, photoisomerizations take place from the 11-*cis* to the all-*trans* form and from the all-*trans* to the 13-*cis* form in animal and microbial rhodopsins, respectively. In our visual pigments, the all-*trans* retinal is released from protein, which is called "photobleach" (upper panel). In contrast, the 13-*cis* form is thermally returned to the original state in microbial rhodopsins, which is called "photocycle" (lower panel)

trans form, and the spontaneous return is termed the "photocycle" in microbial rhodopsins (Fig. 1b). The fact that smaller structural alteration occurs between the all-*trans* and 13-*cis* forms than between the 11-*cis* and all-*trans* forms is one of the reasons why photocycling occurs in microbial systems, with this phenomena significantly influencing the course of optogenetics.

Ion-transporting microbial rhodopsins and optogenetics

The architecture of animal and microbial rhodopsins is illustrated in Fig. 2a. The retinal chromophore is bound to a lysine side chain in the 7th helix in both animal and microbial rhodopsins. In the rhodopsin field, by convention the cytoplasmic and extracellular sides are drawn at the upper and lower sides of figure, respectively, which is based on the first paper of the structural determination of BR by electron microscopy (Henderson and Unwin 1975). This style of drawing is opposite to the conventional drawing of membrane proteins such as G protein coupled receptors and transporters. This convention sometimes causes inconvenience for researchers of rhodopsin and non-rhodopsin in international conferences.

Animal rhodopsins are a specialized subset of G protein coupled receptors (GPCRs). Therefore, they can be classified as a photosensor that activates the soluble transducer protein (Fig. 2b). In contrast, the function of microbial rhodopsins is highly divergent. The first discovered microbial rhodopsin was BR, a light-driven H^+ pump from halophilic archaea (*Halobacterium salinarum*) (Oesterhelt and Stoeckenius 1971). It was subsequently found that *H. salinarum* also contains both a light-driven Cl^- pump halorhodopsin (HR) and the sensory rhodopsins I and II that activate transmembrane transducer protein (Fig. 2b) (Govorunova et al. 2017). As ion pumps, BR and HR perform uni-directional transport of H^+ and Cl^-, respectively, by which light energy is converted into chemical energy that can be used by *H. salinarum*. In pumps, the transport pathways between the two sides of the membrane cannot be fully connected because otherwise the gradient formed by active transport will collapse. This view is an important aspect when distinguishing pumps from channels. The former needs energy input, which ensures the unidirectionality of transport across the membrane. The active transport mechanism is generally explained by the alternating access model, or the Panama Canal model (Kandori et al. 2018), where two gates are required for pump action (Fig. 2c). In contrast, a channel needs a fully connected ion pathway

for passive transport of ions upon opening, for which only one gate is sufficient to explain the mechanism (Fig. 2c). Architectures of natural pumps and channels are very different from each other, and initial thoughts within the field were that channel function was impossible for microbial rhodopsins.

In 2002–2003, three groups independently identified novel DNA sequences that encode microbial-type rhodopsins in *Chlamydomonas* (Nagel et al. 2002; Sineshchekov et al. 2002; Suzuki et al. 2003). Furthermore, a light-gated ion channel function was proved for two proteins, channelrhodopsin-1 (ChR1) and channelrhodopsin-2 (ChR2), in *Xenopus* oocytes using two electrode voltage clamp measurements (Nagel et al. 2002; Nagel et al. 2003). ChR2 was also shown to be expressed and used to depolarize mammalian cells in response to light (Boyden et al. 2005; Ishizuka et al. 2006). Following these initial discoveries, several groups began to work with ChRs, primarily with a truncated version of ChR2 that expressed better than the full-length ChR2 protein and much better than ChR1 (Ernst et al. 2014; Deisseroth 2011). This was the dawn of optogenetics. The mammalian brain contains sufficient retinoid levels to allow wild-type ChR2 to function without the addition of exogenous retinoid cofactors. In ChRs, the channel is closed in the dark, and opens upon light absorption. Although it was suspected initially, we now know that the ion conduction pathway lay inside the 7 transmembrane helices (Deisseroth and Hegemann 2017). Interestingly, the crystal structure of ChR showed that the protein architecture is common to all microbial rhodopsins (Kato et al. 2012; Volkov et al. 2017), suggesting that structural changes are linked to their unique function.

While ChR2 was used for neural excitation by light-induced depolarization of cells, neural silencing tools were in high demand from the very beginning. Light-driven ion pumps such as the H^+ pump BR and Cl^- pump HR were the two most promising candidates as they can hyperpolarize cells upon exposure to light. At that time, many were skeptical (including the author) about the practical usage of these pumps because of reasons related to efficiency. In light-driven pumps, a single ion, which is initially bound, is translocated by a single photocycle, while multiple ions can be transported when light-gated ion channel is activated by a single photocycle (Fig. 2c). Nevertheless, the light-driven Cl^- pump HR and a BR-like H^+ pump were later successfully used for neural silencing by the light-induced hyperpolarization of cells (Zhang et al. 2007; Chow et al. 2010).

Recently, new microbial rhodopsins have been reported, for use in optogenetics. In 2013, we reported on the existence of a light-driven Na^+ pump rhodopsin (Fig. 2b) (Inoue et al. 2013). A light-driven Na^+ pump KR2 can also pump Li^+, but pumps H^+ in K^+, Rb^+, and Cs^+. An ion selectivity filter is located at the intracellular surface and modification of this filter via designed mutation led to the creation of light-driven pumps for K^+, Rb^+, and Cs^+ (Kato et al. 2015; Konno et al. 2016). In active transporters, substrate is bound to the resting state, which is very important for energy coupling purposes. While this is also the case for light-driven H^+ and Cl^- pumps (H^+ and Cl^- are bound), the light-driven Na^+ pump does not need Na^+ to be bound to the resting state (Kandori et al. 2018). Thus the light-driven Na^+ pump is unique among active transporters. In 2015, anion channelrhodopsin (ACR) was discovered from a cryptophyte *Guillardia theta* (Fig. 2b) (Govorunova et al. 2015). The cellular interior is negatively charged (− 70 mV), but Cl^- ions are still taken up upon opening of the anion channel, because the concentration of Cl^- is much higher in the extracellular side than within the cytoplasm (Fig. 3a). Light-gated anion channels are particularly desirable for neural silencing, because of multiple ion permeation in channels. While ACRs have been engineered from cation channels (Wietek et al. 2014; Berndt

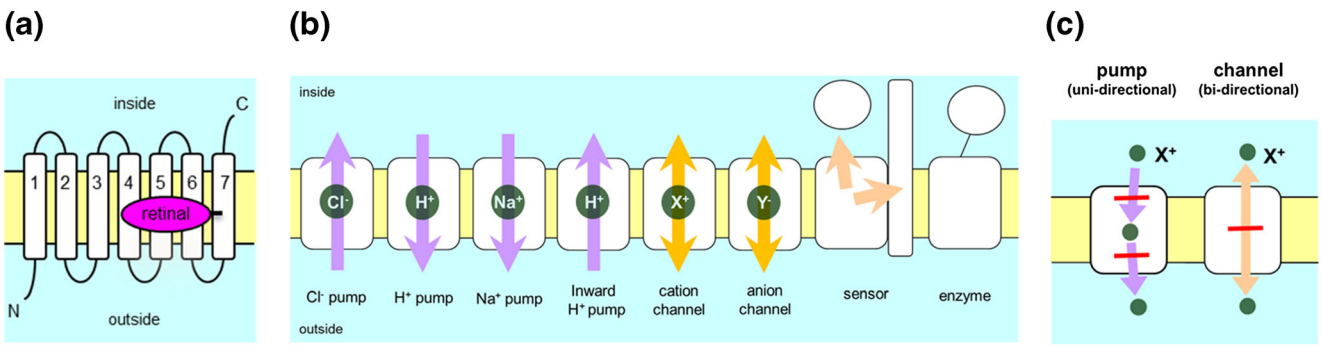

Fig. 2 **a** Architecture of rhodopsins. N- and C-terminus face the extracellular and cytoplasmic region, respectively. Seven α-helices span the lipid bilayer, and the chromophore retinal is covalently attached to a lysine side chain on the 7th helix. **b** Function of rhodopsins; light-driven Cl^- pump, light-driven H^+ pump, light-driven Na^+ pump, light-driven inward H^+ pump, light-gated cation channel, light-gated anion channel, light sensor with transmembrane transducer and soluble transducer, and light-activated enzyme. Purple or orange arrows indicate uni-directional or bi-directional transport of ions in pumps or channels, respectively. On the other hand, pale orange arrows show the signal transduction from rhodopsins to either soluble or transmembrane transducer proteins. **c** Ion transports of ion pump and channel are uni-directional and bi-directional, respectively

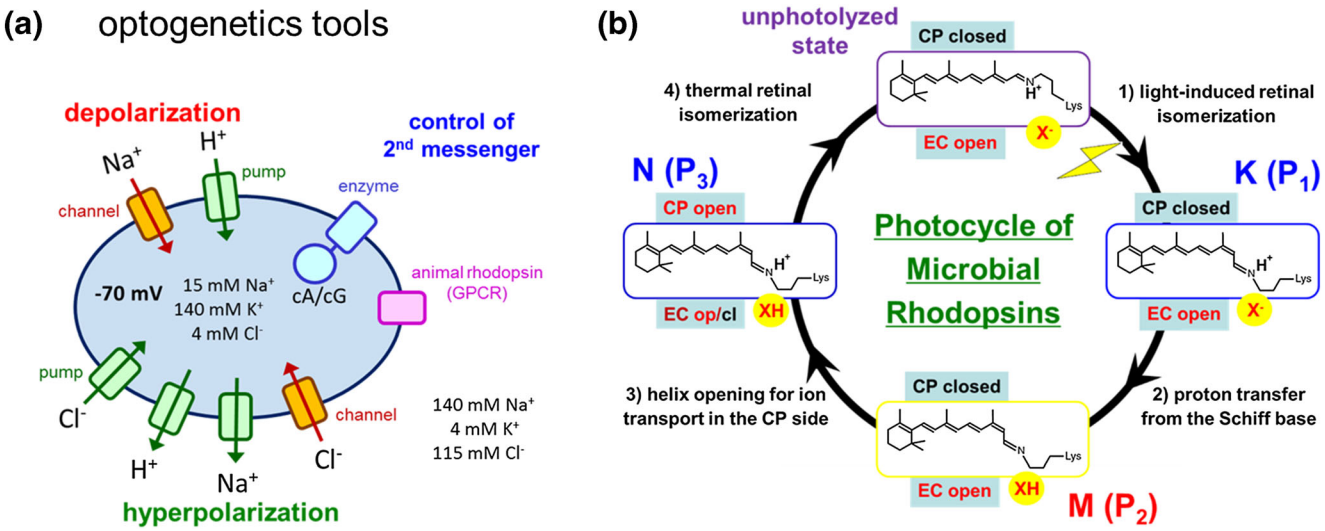

Fig. 3 **a** Role of photoreceptive proteins in optogenetics. Channels and pumps perform passive and active transports of ions, respectively. At the beginning of optogenetics (in 2007), light-gated cation channel (ChR2) depolarizes neural cells by influx of Na^+, leading to generation of action potential (neural excitation), while light-driven Cl^- pump (HR) hyperpolarizes neural cells by influx of Cl^-, leading to inhibition of action potential (neural silencing). More optogenetics tools are now at hand. Light-driven inward H^+ pumps depolarize cells, while light-driven (outward) H^+ and Na^+ pumps and light-gated anion channels hyperpolarize cells. Recently discovered enzyme rhodopsins, one family of microbial rhodopsins, are used to control concentrations of cAMP and cGMP. In addition, animal rhodopsins are also used for optogenetics. **b** Typical photocycle of microbial rhodopsins showing isomeric and protonation state of the retinal chromophore. Names of the photocycle intermediates are for BR, while those in parenthesis are for ChR2. X^- represents the Schiff base counterion, which corresponds to Asp85 in BR and Glu123 in ChR2. Asp85 in BR also acts as the proton acceptor from the Schiff base. CP and EC indicate cytoplasmic and extracellular domains, respectively. In the unphotolyzed state of microbial rhodopsins, the EC side is open through a hydrogen-bonding network, but the CP side is closed. While this is persistent in the K (P1) and M (P2) states, the CP side is open in the N (P3) state. When the EC side is closed (black), the CP side is open, as is the case for an ion pump, the N intermediate of BR. When the EC side is open (red), the CP side is open, as is the case for an ion channel, the P3 intermediate of ChR2

et al. 2014), natural ACR is still considered the best neural silencer. Structures of ACR and other engineered anion channelrhodopsins were determined (Kim et al. 2018; Kato et al. 2018), which will be used for the improvement of these tools. In 2016, we reported the existence of a light-driven inward H^+ pump (PoXeR) (Inoue et al. 2016). As inward H^+ pumping competes with ATP synthase function, its existence provided a surprise and its physiological role is still unknown. We previously succeeded in creating an inward H^+ transport using a mutant of the photochromic sensor, Anabaena sensory rhodopsin (D217E ASR) (Kawanabe et al. 2009), and PoXeR is in the same family of ASR. Inward H^+ pumps depolarize cells (Fig. 3a), but cation channels are far superior in the depolarization of neural cells. However, inward H^+ pumps are more beneficial for intracellular organelle optogenetics. While intracellular organelle can be acidified by action of light-driven H^+ pumps, a reverse alkalization tool for use in intracellular organelles has been much sought after. In this role, a mutant D217E ASR was used to alkalize AMPA-type glutamate receptor endocytosis in the study of long-term depression of AMPA-type glutamate receptor (AMPA receptor)-mediated synaptic transmission for learning and memory (Kakegawa et al. 2018).

Enzyme rhodopsins do not transport ions, but are promising optogenetic tools, as they can alter the concentration of cyclic nucleotides. In 2014, a fungal light-activated guanylyl cyclase (Rh-GC) was reported (Avelar et al. 2014), which increases the concentration of cGMP upon light absorption. In 2017, we reported Rh-PDE in a unicellular and colonial single flagellate eukaryote *Salpingoeca rosetta* (Yoshida et al. 2017), which decreases the concentrations of cGMP and cAMP in a light-dependent manner. These proteins are composed of a membrane-embedded rhodopsin domain and a C-terminal cytoplasmic enzyme domain that are activated when light is absorbed by the all-*trans*-retinal chromophore (Fig. 2b; Fig. 3a).

Photocycle dynamics of pump and channelrhodopsins

As described, microbial rhodopsins including pumps and channels undergo the "photocycle." A summary of the photocycle in BR is shown in Fig. 3b, which illustrates key intermediate states for most microbial rhodopsins. Although the photocycle of BR contains six intermediates, namely J, K, L, M, N, and O states that are named alphabetically, only three states (K, M, and N) are shown in Fig. 3b to show the mechanism clearly. After light absorption, photoisomerization occurs from the all-*trans* to 13-*cis* form in 10^{-13} s (Gozem et al.

2017). This ultrafast retinal isomerization yields the formation of red-shifted J and K intermediates in which J is the precursor of the K state. As the protein cavity, which accommodates retinal, cannot change its shape promptly, the K intermediate contains twisted 13-*cis* retinal and an altered hydrogen-bonding network in the Schiff base region, which yield higher free energy in K than in the original state. These structural changes observed by spectroscopy have been recently visualized by time-resolved X-ray crystallographic studies (Nogly et al. 2018; Nass Kovacs et al. 2019). This highly distorted chromophore leads to subsequent protein structural changes that accompany relaxation.

In the case of BR, relaxation of the K intermediate leads to the formation of the blue-shifted L intermediate. For the H^+ pumping (and some of the photosensory) rhodopsins, the L intermediate serves as the precursor of the proton transfer reaction from the Schiff base to its primary carboxylic proton acceptor, by which the M intermediate is formed. This is a key step in proton transport. Since the M intermediate has a deprotonated 13-*cis* chromophore, it exhibits a characteristically strong blue-shifted absorption (λ_{max} at 360–410 nm), and is well isolated from other intermediates. In BR, the proton acceptor (X^- in Fig. 3b) is Asp85, so that the primary proton transfer takes place from the Schiff base to Asp85. In the case of Cl^- pump HR, the Schiff base does not deprotonate during the photocycle, because Asp85 in BR is replaced by Thr. In HR, X^- in Fig. 3b is Cl^-, which is directly translocated upon decay of the L intermediate.

If the Schiff base of M is reprotonated from Asp85 in BR, no H^+ transport occurs. In reality, the Schiff base is reprotonated from Asp96 in the cytoplasmic region upon which the N intermediate is formed. The molecular mechanism of uni-directional transport of protons in BR has attracted the attention of many researchers, and it is believed that the primary proton transfer from the Schiff base to Asp85, and the subsequent proton transfer from Asp96 to the Schiff base determine the uni-directionality from the cytoplasmic to the extracellular region (Ernst et al. 2014). The crystal structure of BR exhibits an asymmetric pattern of hydration, while seven internal water molecules are found in the extracellular half, only two are observed in the cytoplasmic half. Such asymmetry makes sense in view of BR's function, as the water molecules build a hydrogen-bonding network on the extracellular side for fast proton release, while the cytoplasmic side is likely inaccessible in the dark and allows proton uptake only after the light-induced accessibility switch. Such asymmetric access (EC open and CP closed) is not only the case for the unphotolyzed state, but is also the case for the K and M intermediates, as shown in Fig. 3. The important roles of protein-bound water molecules has been studied by using FTIR spectroscopy (Kandori 2000; Gerwert et al. 2014), and has also been monitored by using time-resolved X-ray crystallography (Nango et al. 2016).

To make proton conduction in the cytoplasmic region possible (CP open), an additional conformational change allowing the entrance of water into the vicinity of Asp96 should take place. Such a conformational alteration is realized mainly by changes in helical tilts (especially of the cytoplasmic half of helix F), and the N intermediate is often characterized by the largest changes in the protein backbone conformation, most notably, outward tilts of the cytoplasmic end of helix F (Kamikubo et al. 1996; Shibata et al. 2010). Such helical motions are functionally significant both for ion transport and interactions with transducers of sensory rhodopsins. The photocycle usually ends with another red-shifted intermediate, known as the O intermediate, serving as a last step in resetting the original unphotolyzed conformation.

Like BR, the ChR photocycle has been studied by various methods, and it is now established that ChR has a photocycle similar to other microbial rhodopsins (Ernst et al. 2014; Lórenz-Fonfría and Heberle 2014; Schneider et al. 2015). After the absorption of light, photoisomerization occurs from the all-*trans* to the 13-*cis* form very rapidly, and forms the red-shifted K-like intermediate (P1 in Fig. 3b). In a similar manner to the mechanism of BR, the protein cavity accommodating retinal equilibrates on a slower time scale and therefore the P1 intermediate contains twisted 13-*cis* retinal and an altered hydrogen-bonding network in the Schiff base region. This structure is unstable in comparison with the original state and hence protein structural change ensues as for the case in BR. Then, the proton is transferred from the Schiff base, forming the M-like P2 intermediate. The P2 intermediate has a deprotonated 13-*cis* chromophore whose absorption is strongly blue-shifted (λ_{max} at 380 nm). The N-like P3 intermediate is formed by reprotonation of the Schiff base, and is believed to be the ion-conducting state. It is reasonable to assume that the N-like state exhibits the largest conformational changes in microbial rhodopsins. When the CP side is open, the EC side must also be open in ChR, so that a transient ion conduction pathway is created. Therefore, in Fig. 3b, pumps and channels show the N-like intermediate in CP-open/EC-closed and CP-open/EC-open conformations, respectively.

The channel property of ChR is important for optogenetic applications. In particular, absorption color, ion selectivity, and open/close dynamics should be taken into account. Thus far, various mutants have been shown to improve the properties of ChR (Ernst et al. 2014; Schneider et al. 2015; Govorunova et al. 2017; Deisseroth and Hegemann 2017). Before ChR structural determination, knowledge of BR contributed significantly to the design of ChR2 mutants. Despite the creation of useful ChR2 variants, there are still many additional needed improvements for optimized channel function. The limited conductance of ChR2 needs to be improved. However, unlike other channel proteins, ion pathway is not straight, as ions are conducted inside 7 transmembrane helices in ChR2. This is a substantial limitation causing the low

conductance of ChR2. The creation of wider pores through molecular engineering may be a solution to this challenge. Ion selectivity of ChR2 is much higher for H^+ than for Na^+ (the permeability ratio, P_H/P_{Na}, is about 10^6) (Nagel et al. 2003), indicating that ChR2 should be recognized as a light-gated H^+ channel rather than a Na^+ channel, and if pH differs between inside and outside of cells, ChR2 acts as a proton channel. ChR2 also displays some permeability to Ca^{2+}. Either the elimination or enhancement of Ca^{2+} conduction is highly demanded, and Ca^{2+}-permeable channelrhodopsin CatCh (L132C ChR2) was reported, though it does not remarkably increase Ca^{2+} permeability (Kleinlogel et al. 2011). The absorbing color is another problem of ChR2, which absorbs blue light maximally (470–480 nm). Blue light is harmful to organisms and also penetrates through tissue less than red light. Therefore, red-absorbing ChR has been reported such as ReaChR and Chrimson (Zhang et al. 2008; Lin et al. 2013; Klapoetke et al. 2014).

Conclusion

The original meaning of rhodopsin is the red-colored pigment for vision, but colored retinal proteins were also found in microbes. Consequently the definition of the word rhodopsin has been largely expanded, and the modern meaning of rhodopsin encompasses photoactive proteins containing a retinal chromophore in animals and microbes. While the meaning of rhodopsin has been altered in history, a similar situation has also arisen with the word "optogenetics." Optogenetics is originally derived from the combination of "optics" and "genetics," which describes optical control of neural activity by use of ion-transporting microbial rhodopsins such as ChR and the light-driven Cl^- pump. This allows for rapid temporal response, which has greatly enhanced experimental capabilities in brain function research. Numerous studies have led to an expanded definition of the word optogenetics, with the current meaning encompassing light control in biology. The molecular mechanism of rhodopsin has attracted many researchers in biophysics. In these studies, one major goal is to understand how light is converted into each function. Interest in these proteins will be further enhanced by advancements in the field of optogenetics, as fundamental and application-based research are inseparable and proceed in unison.

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