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Improvisations in phototrophy. Protein engineering and functional investigation of rhodopsin proton-pumps

Ganapathy, S.

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Chapter 1

Introduction to this thesis

Our planet faces a challenging situation driven by the depletion of fossil fuels and the imminent threat of global warming. In the near future, we anticipate a time when the available fuel supplies will no longer meet the rising demands of the population and industry. Thus, we urgently require alternative means to generate and harness energy, to reduce our over-reliance on fossil fuels. The solution clearly lies in renewable sources of energy, of which solar energy is the most prevalent form available to us. Photosynthesis and phototrophy are among the most important biological processes on the planet, and these already make efficient use of the incident solar flux to generate chemical energy for the growth and survival of organisms [1]. Photosynthetic microorganisms represent promising systems for the sustainable production of biofuels or important biochemicals. However, photosynthesis is expensive with respect to the resources required. Organisms have only evolved the photosynthetic capabilities to meet their energy requirements for growth and survival, within the environmental constraints imposed on them. Furthermore, non-photochemical quenching limits the amount of light that can be converted to product. From a bioengineering perspective, the complexity of photosynthetic light harvesting processes imposes another challenge.

Retinal-based microbial phototrophy may present an attractive alternative to harness solar energy. Microbial rhodopsins are relatively simple tunable photosystems, which are highly amenable for a host of bioengineering strategies targeted towards solar-fuel conversion [2, 3].

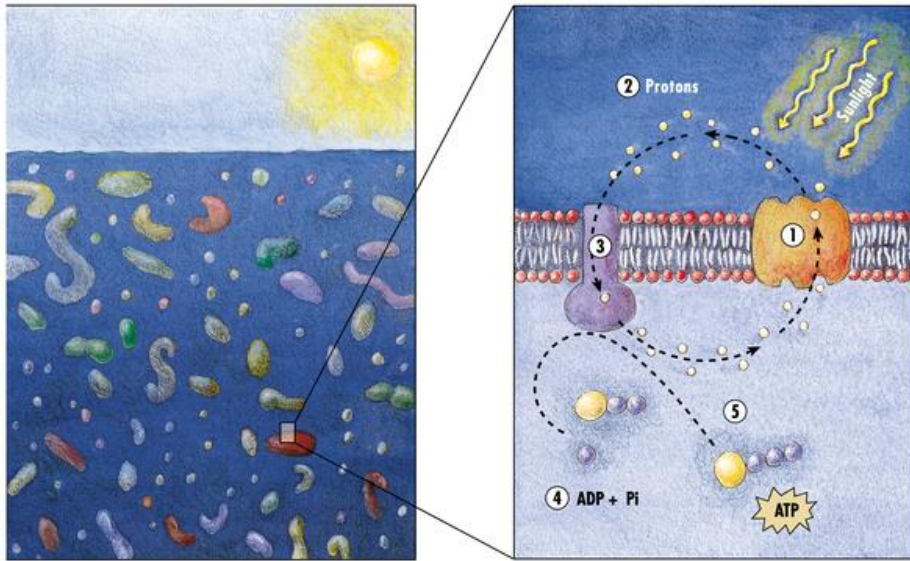


Figure 1.1 An artist's illustration of the distribution and function of microbial proton-pumping rhodopsins in aquatic microbes [4]. Illumination induces proton transport across the cell membrane, which is coupled to ATP synthesis.

1.1 Microbial rhodopsins

Microbial or type-I rhodopsins are light-driven seven-helical transmembrane proteins found in a broad phylogenetic range of microbial life (Figure 1.1) [5]. These proteins bind a molecule of retinal as a chromophore (Figure 1.2) and use visible light, in the range of 400-700 nm, to facilitate various functions in their hosts. These functions include the active transport of ions via proton, sodium, potassium or chloride pumps as well as sensory signaling pathways. The ion transporting rhodopsins provide energy to their hosts by hyperpolarizing the cell membrane. This energy is used during conditions of stress or nutrient limitation, when the respiratory electron transport activity is low. Sensory rhodopsins allow cells to sense and respond to their immediate environment using phototactic signaling mechanisms. Consequently, these proteins are physiologically important for the survival and adaptation of their hosts.

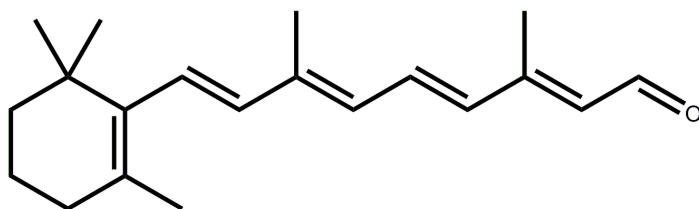


Figure 1.2 The all-*trans* retinal chromophore, common to all microbial rhodopsins

The light-driven outward proton pump bacteriorhodopsin (bR) was the first microbial rhodopsin to be discovered. It was detected in 1971 in the purple membrane of the halophilic marine archaeon *Halobium salinarum* [6]. This archaeon was later shown to also contain an inward chloride pump halorhodopsin (HR) [7] and the positive and negative phototaxis sensors sensory rhodopsin-I (SRI) and II (SRII) respectively [8]. These four archaeal rhodopsins have served as functionally distinct prototypes of the microbial rhodopsin family, and have been investigated by various crystallographic and spectroscopic techniques over the past four decades [9].

At the turn of the 21st century, scientists discovered that microbial rhodopsin genes are far more widespread and functionally diverse than previously imagined. No longer confined to Archaea, variants of these proteins were now also found in Eubacteria, such as proteobacteria [10], cyanobacteria [11] and Eukarya, such as fungi [12], algae [13], as illustrated by the various classes presented in figure 1.3. Metagenomic analyses have shown that lateral gene transfer may have facilitated this distribution across divergent microbial populations [14]. Thus, microbial rhodopsin-based phototrophy is ubiquitous in all three domains of life, making it the most abundant light-driven process on the planet.

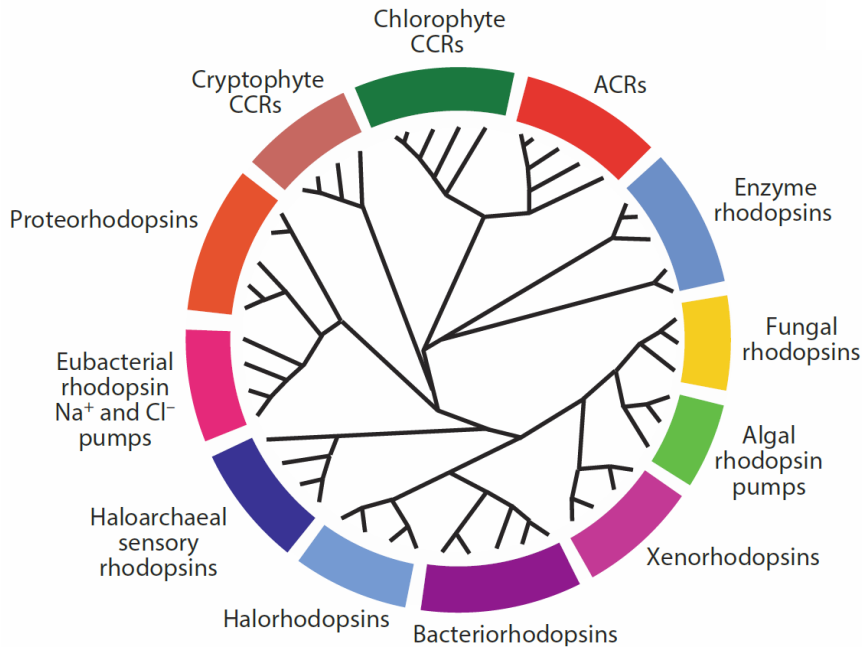


Figure 1.3 Cladogram showing the relatedness (black lines) between the major classes of the microbial rhodopsin superfamily [3]. The proteorhodopsin family of proton-pumps discussed below is represented by the orange arc.

In this thesis, we focus on two eubacterial rhodopsin proton-pumps, which have stimulated considerable interest recently, namely proteorhodopsin and *Gloeobacter* rhodopsin.

1.2 Proteorhodopsin

Proteorhodopsins are the first rhodopsins to be isolated from eubacteria. They are an archetype of the class of rhodopsin proton-pumps, showing ~22% homology to the well-studied bR. The first proteorhodopsin (PR; $\lambda_{\max}$ 520 nm, Figure 1.4a) was discovered in 2000 during a metagenomic screen of marine uncultured gamma-proteobacteria from Monterey Bay in California [10]. Since then, PR-like variants have been found widely distributed in various organisms across the photic zone, making them a hot topic of current research [15].

Since its discovery in bacteria, homologs of PR have been found in archaeal and eukaryotic hosts [16-20], and even in some giant viruses [21]. These hosts inhabit diverse ecosystems, ranging from marine [22, 23] and freshwater habitats [24, 25], to Antarctic sea ice [26], permafrost [27], hot-springs [28], and even samples from terrestrial leaf surfaces [29]. Furthermore, they are found stratified in deep or shallow environs, and are spectrally tuned to the light availability at that depth [30]. Though their exact physiological role is unclear, various studies have shown that PRs generate a light-driven proton-motive force *in vivo*, which can be coupled to ATP synthesis [31]. This contributes towards the growth and survival of host organisms under energy limiting conditions. For instance, upon illumination, PR was shown to stimulate the growth of a marine flavobacterium *Dokdonia* sp. MED134 under carbon limitation [32], and of *Vibrio* sp. during starvation [33]. Due to these light driven functions and their prolific distribution, PRs are thought to be key players in maintaining the phototrophic energy balance in various biospheres.

PR can also be recombinantly expressed in heterologous hosts such as *Escherichia coli* in good quantities [10], unlike bR which shows poor bacterial expression [34]. Its phototrophic potential can be exploited to drive physiological activities in *E. coli*, like flagellar motility [35] or ATP synthesis [36]. One study showed that upon illumination, PR could contribute towards the growth of *E. coli* under anaerobic conditions [37]. Furthermore, heterologous co-expression of PR with a hydrogenase could drive the production of bio-hydrogen [38]. These studies, taken in concert, demonstrate that PR has broad potential for a variety of light-driven biotechnology applications.

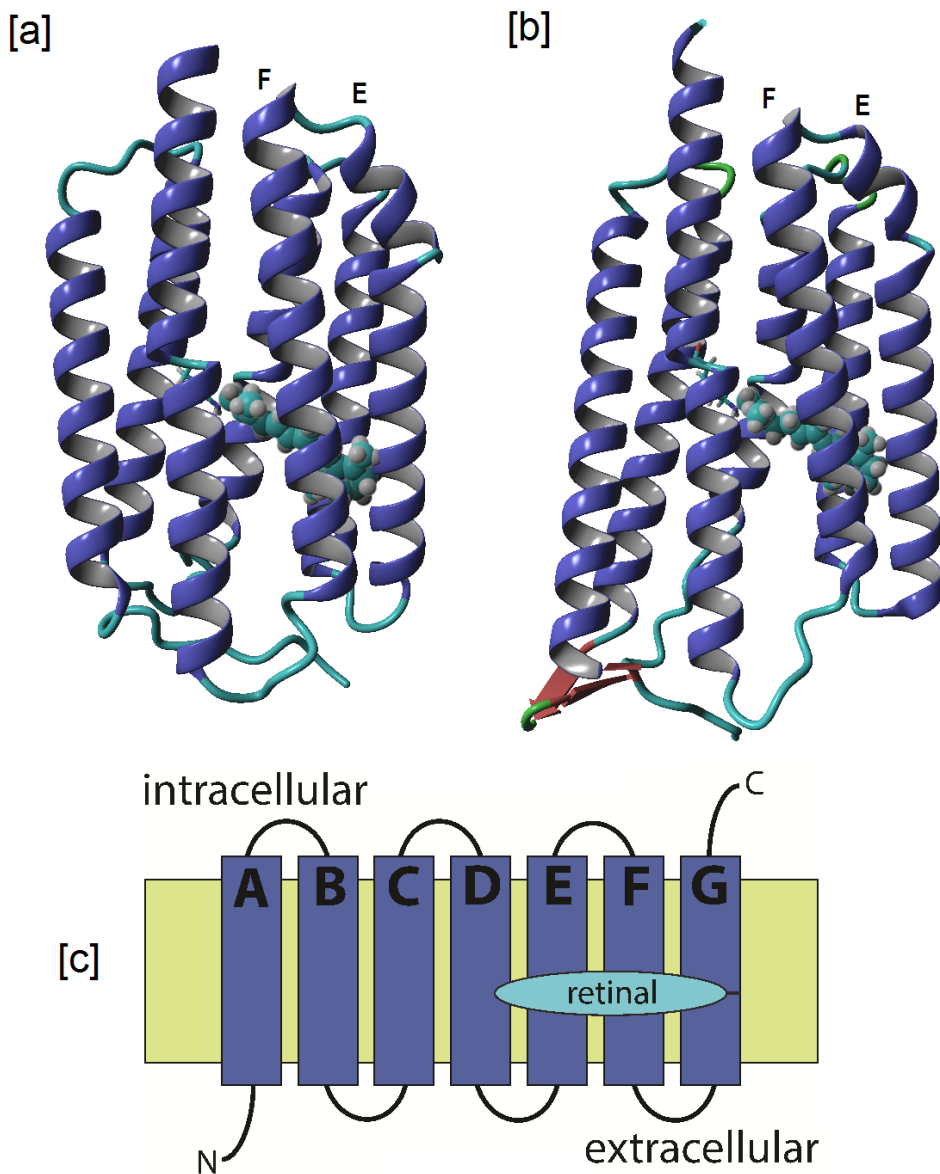


Figure 1.4 Homology model of [a] proteorhodopsin (PR) and [b] *Gloeobacter* rhodopsin (GR), generated using YASARA (www.yasara.org). See chapter 2 for further information. The labeling of helices E and F is indicated. The homology model of GR indicates the presence of a small β -sheet shown in red. The retinylidene chromophore is displayed in cyan as a space filled residue. [c] 2-D snake plot displaying the labeling of the seven helices of PR and GR.

1.3 *Gloeobacter* rhodopsin

Gloeobacter rhodopsin (GR; $\lambda_{\text{max}} = 540$ nm, Figure 1.4b), a distant relative of PR, has been studied less extensively. It was discovered in the thylakoid-less cyanobacterium *Gloeobacter violaceus* PCC 7421, isolated from a calcareous rock in Switzerland [39]. GR also functions as a light driven proton-pump in its native host [40] and shares ~30% sequence identity with PR, while conserving several key residues involved in its proton pumping function [40, 41]. GR shows excellent heterologous expression in *E. coli* as well [42] and can contribute towards light-driven ATP production *in vivo* [43].

Phylogenetically, GR is closely related to the eubacterial proton pump xanthorhodopsin (XR), which binds the carotenoid salinixanthin, in addition to the retinylidene chromophore [44, 45]. *In vitro* reconstitution experiments have shown that GR binds the carotenoids salinixanthin [42], and echinenone [46]. The carotenoid is speculated to behave as an antenna, by facilitating energy transfer to the retinal [47], in the order of 30% [48]. The enhanced spectral sensitivity of this phototrophic system, in addition to its rapid photocycle [49] and ease of bacterial expression make GR another highly amenable candidate for bioengineering applications.

1.4 Mechanism and photocycle of PR and GR

PR and GR contain the typical seven trans-membrane α -helical protein motif common to all microbial rhodopsins, called the “opsin”. Residues from all helices, which are labeled from A to G (Figure 1.4), contribute towards the formation of the retinal-binding pocket. The holo-protein is formed upon binding a molecule of all-*trans* retinal, via a covalent linkage with the ϵ -amino group of a lysine residue in helix G (Figures 1.4 and 1.5; K231 in PR, K257 in GR). This generates the retinylidene Schiff-base (SB), which is protonated (PSB) in the resting state. In the dark-adapted form of

PR, the retinylidene moiety predominantly remains in an all-*trans*-15-*anti* conformation [50, 51]. This is in contrast to bR, which contains a mixture of the all-*trans*-15-*anti*, and 13-*cis*-15-*syn* retinal isomers in its dark state [52]. Light excitation drives the isomerization of retinylidene, followed by thermal relaxation of the chromophore and the opsin. A series of conformational changes ultimately result in the transfer of one proton across the cell membrane per photon absorbed. Using ultra-fast spectroscopy, this entire photocycle can be spectrally and kinetically resolved into distinct intermediates[53].

The photocycle is initiated by electronic excitation of the PSB from the ground state (S_0) to its first excited state (S_1), upon the absorption of a single photon of light. The transfer of an electron from the lower energy π -bonding orbital to the higher energy π^* anti-bonding orbital induces rotation about the C13-C14 bond, leading to the isomerization of the PSB from an all-*trans* to a 13-*cis* configuration (Figure 1.5). This isomerization takes place in less than 200 femtoseconds, forming the red-shifted J and K-intermediates [54, 55]. The J intermediate is a vibrationally hot form of the subsequent K state, which contains a highly strained 13-*cis* chromophore.

Relaxation of the 13-*cis* chromophore leads to structural rearrangement within the protein, causing a proton to be transferred from the PSB to a nearby Asp residue (D97 in PR, D121 in GR). The deprotonated SB accumulates over a time scale of ms, forming the blue-shifted M-intermediate, which often exists in equilibrium with an earlier L-state [56, 57]. The deprotonated SB is re-protonated by a Glu residue (E108 in PR, E132 in GR) situated midway between the retinylidene and the cytoplasmic side, forming the red-shifted N-intermediate [41]. This Glu picks up a proton from the cytoplasm, while the retinal thermally re-isomerizes into a twisted all-*trans* form, characteristic of the O-intermediate [58]. In the last step of the photocycle, D97/D121 undergoes deprotonation leading to

extracellular proton release, accompanied by relaxation of retinal back to the ground state. The entire photocycle is completed within a time scale of milliseconds.

The photocycle can thus be simplified into the following main stages: isomerization of the PSB, deprotonation to form the SB, reprotonation of the SB, proton-uptake from the cytoplasm and extracellular proton-release. The photocycle of PR resembles that of bR. However, there are some differences, the most notable being the lack of ionizable proton release groups. In bR, Asp residues are involved in proton release to the extracellular surface [59, 60], which are absent in PR and GR. The strongly hydrogen bonded network in the retinal binding pocket could play an important role in proton transport via a bucket brigade of water molecules [61], and proton uptake from the cytoplasm is shown to be influenced by the mobile loop between helices E and F (Figure 1.4) [62]. However, the precise mechanism of extracellular proton release and proton uptake for PR and GR is still unclear.

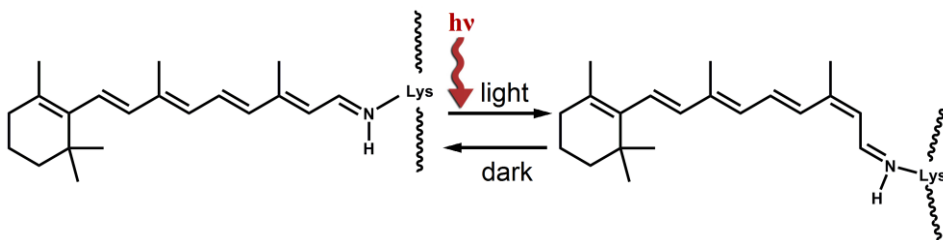


Figure 1.5 Light induced isomerization of the all-*trans* to the 13-*cis* retinylidene group bound to the opsin, eliciting reorganization of the protein environment.

Despite the ambiguity in atomic details of its mechanism, the overall proton-pumping function of PR has been well investigated under a range of conditions. Its function is strongly pH dependent. It shows outward directed proton-pumping in alkaline pH [58], and it has been claimed that the direction of proton transport can be reversed under acidic conditions

[56, 63], though this is the subject of some debate [64]. Nevertheless, there is a clear consensus that the primary proton acceptor D97 has to be deprotonated [65] for a normal photocycle to proceed. The pKa of D97 is reported to be ~ 7.1 [58], which is much higher than the pKa of the analogous D85 in bR, which is ~ 2.9 [66]. A nearby His residue, namely H75 in helix B, is thought to have an influence on this high pKa of D97. Solid-state nuclear magnetic resonance (NMR) analyses in combination with mutagenesis have revealed the importance of a specific hydrogen bonding interaction between H75 and D97 [67], which also has an effect on the photocycle of PR. The acidity of D97 is further influenced by the formation of a complex counterion with the residues D227 and R94, as shown in figure 1.5. At alkaline pH, a water molecule forms a complex with the two Asp residues, and the proton from the PSB [56, 68]. R94 further contributes a weak coupling interaction with D97, and possibly also to H75 [69].

Figure 1.6 highlights the important residues discussed above, which influence proton transfer and stability of the counterion complex. It also highlights residues which affect the spectral tuning of proteorhodopsins in general. In the next section, we discuss some of these tuning processes.

1.5 Colour tuning mechanisms

The colour of microbial rhodopsins originates from the electronic transitions occurring within the all-*trans* retinylidene chromophore. Retinal consists of a β -ionone ring attached to an elongated polyene chain, which has the characteristic chemical structure of alternating single and double bonds, as shown in figure 1.2. A delocalization of π electrons over the polyene chain results in a main absorbance band peaking at 360-380 nm for free retinal, which is visually seen as a yellow compound. Studies on SB model compounds have shown that the unprotonated retinylidene SB is relatively insensitive to the solvent environment, with a slightly blue-shifted absorbance band (relative to free retinal) in the UV region [70].

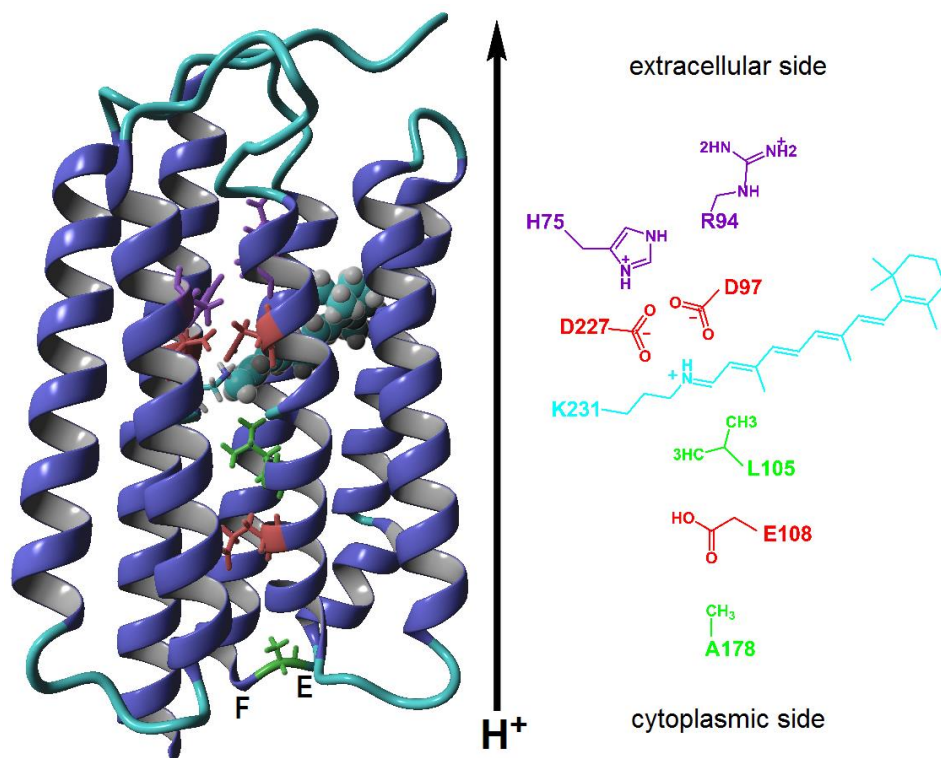


Figure 1.6 Homology model of PR highlighting important residues for the proton pumping function (red), for stability of the counter-ion complex (purple) or residues affecting colour tuning (green). The retinal bound via a protonated SB to a lysine residue is displayed in cyan. The labeling of helices E and F is indicated.

The PSB, however is significantly red-shifted to ~ 440 nm in methanol, and is much more sensitive to its microenvironment [70, 71]. Upon binding to the opsin, the interaction with the protein environment further shifts this absorbance band towards the much longer wavelengths of $\lambda_{\text{max}} = 520$ and $\lambda_{\text{max}} = 540$ nm for PR and GR respectively, which is called the “opsin-shift”. The distance of the PSB from the counterion and specific interactions with residues lining the binding pocket influence the energy gap between the ground and excited state of the retinylidene chromophore, and therefore the resulting colour of the pigments. A smaller energy gap represents a bathochromic shift and a larger gap corresponds with a hypsochromic shift

in the absorbance band of the pigment (Figure 1.7d). These effectors are examined in further detail below.

The interaction with the negatively charged counterion is an important factor affecting the colour of the retinylidene SB in the binding pocket (Figure 1.7). In the ground state, the PSB is positively charged. The excited state has a tendency to displace this positive charge in the direction of the β -ionone ring, thereby neutralizing the PSB. The counterion however stabilizes the positive charge on the ground state, increasing the pKa of the Schiff base, and leading to a blue shift (Figure 1.7a). The overall effect of the opsin environment, therefore, is to modulate this blue shift induced by the counterion. To further understand this phenomenon, mutational studies have been used to identify specific amino acid residues, which impact the absorbance band of the pigment. Several mutations of PR have thus been described by site-specific mutagenesis [72] or random mutagenesis screens [73], which exert an effect on the PSB via direct or long-range interactions.

A detailed solid-state NMR study in combination with site-directed mutagenesis probed the effect of the EF loop on the photocycle and spectral properties of PR [62]. A specific Ala residue on this loop (Figure 1.6) was shown to exert a long-range effect on the retinal binding pocket, with the A178R mutation leading to a 20 nm red-shift in the absorbance band. This was accompanied by a longer photocycle, due to an elongated life-time of the N/O intermediate, the decay of which is correlated with the re-protonation pathway. One can thus conclude that a complex interaction pathway exists between the EF loop and the retinal-binding pocket, which influences both the colour and photocycle of PR.

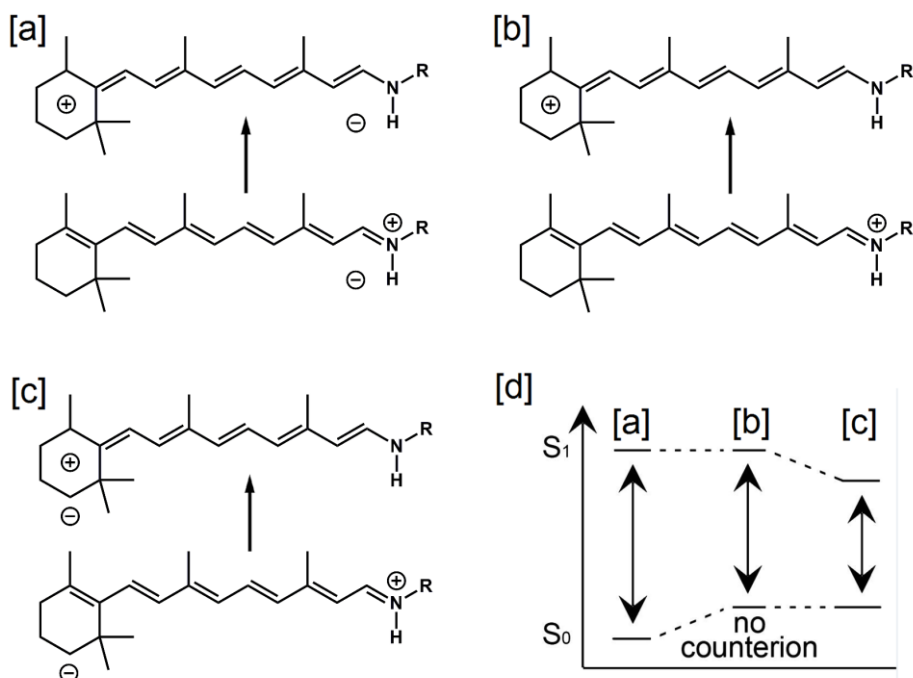


Figure 1.7 Photoexcitation causes switching of the bond alteration and a displacement of positive charge towards the β -ionone ring. Scenarios when the counterion is located: [a] close to the protonated SB, [b] absent, [c] near the β -ionone ring. [d] Electrostatic interactions with the counterion or other residues will increase or decrease the energy gap between the ground and excited states of the retinylidene chromophore.

Two main spectral variations of PR have been described, namely a blue absorbing PR (BPR, $\lambda_{\max}$ 490 nm) [74, 75] and the green absorbing PR (PR, $\lambda_{\max}$ 520 nm), included in this study. From structural modeling and mutagenesis, it was seen that a single amino acid substitution at position L105 in the retinal binding pocket of PR, (which is a Q in BPR) functions as a colour tuning switch (Figure 1.6). The mutation PR L $\rightarrow$ Q shifts the absorbance maxima into the blue, while the reverse mutation shifts it back to the green form [30, 75]. This mutation is thought to represent an ecological adaptation strategy, to spectrally tune PR to the light-availability at the depth where its respective host is located.

The conformational properties of the ligand also have an influence on the spectral properties and function of these proteins. The electronic distribution in the chromophore will affect the pKa of the SB [76-78], and its structure and fit into the binding pocket will determine the distance to the counterion and proton acceptor, and may also have long-range effects on the protein structure and thereby the interaction with the protein. From a bioengineering perspective, the influence of the PSB and its binding pocket on the colour and function of these proteins is very convenient. Spectrally tuned variants of these proton pumps can be generated by modifying the electronic properties of retinal, or with specific opsin mutations. These proteins can thus be engineered to exploit desired wavelengths of light for specific applications, especially the near-infrared part of the solar spectrum, which is discussed further below.

1.6 Potential of near-infrared active proteorhodopsins

PR and GR are attractive model systems for various synthetic biology applications. However, a great challenge in many of these applications is to extend the action spectrum into the far-red and near-infrared (NIR) range of the electromagnetic spectrum (≥ 700 nm), a range which is relatively unexplored. It has been suggested that PRs can complement oxophototrophy when their spectral band is shifted bathochromically to utilize photons outside the range of photosynthetically active radiation (PAR; 400-700 nm) [79], which is hardly exploited by oxygenic photosynthesis [80, 81]. A step has been made in this direction, by expressing PR in the cyanobacterium *Synechocystis* sp. PCC 6803, where it was shown to stimulate growth upon illumination, when compared to a non-functional PR mutant [82].

Red-light activation is also extremely desired in the field of optogenetics, where microbial rhodopsins like Channelrhodopsins are used to modulate the activity of neurons or other mammalian cells by light [83]. Light ≥ 700

nm penetrates much further into biological tissue, which for instance would be very useful for the optogenetic stimulation of deeper brain regions. Recently, a red-shifted variant of the widely used Channelrhodopsin2 (ChR2) termed ReaChR was engineered by opsin modification [84]. ReaChR can be activated by 630 nm illumination, and is thus far the most shifted red-light activable optogenetic tool available for the stimulation of neurons.

Chromophore modification strategies have been the most successful in the spectral modulation of microbial rhodopsins. The largest spectral red-shift (~225 nm) reported thus far was obtained with the azulenic pigment of bR, displaying a λ_{max} at 795 nm [85]. However subsequent studies showed that the resulting pigments could not undergo a complete photocycle [86], thereby lacking pump activity which represents a major drawback. Altering the retinylidene environment can significantly affect the isomerization and proton transfer reactions within the retinal-binding pocket, thereby influencing the function of these proteins. Thus, generating a NIR active microbial rhodopsin is still a significant challenge, and the spectral window >700 nm remains largely elusive.

1.7 Structural characterization of proteorhodopsins

Protein and chromophore engineering require an understanding of the structure-function relationship of the system at hand. This is greatly facilitated by detailed and precise structural information available via crystallographic or NMR analyses [87, 88]. These techniques typically require a highly purified intact protein sample, which in the case of membrane proteins is also stabilized in a micellar or lipid bilayer microenvironment. Recombinant protein expression in heterologous hosts is the most convenient and preferred method towards this end. The gram-negative bacterium *E. coli* is widely used as a model organism for the

inducible expression, purification and characterization of many membrane proteins, due to its high turnover, transformation efficiency and well-characterized genetic toolbox. Both PR and GR can be recombinantly expressed in *E. coli* with high yields in the order of 10^5 - 10^6 molecules/cell [10, 41]. Due to their insoluble nature, PR and GR require prior solubilization and purification with a detergent such as n-dodecyl- β -D-maltopyranoside (DDM), and if required, can subsequently be embedded into an artificial membrane environment in the form of liposomes or nanodiscs [89]. The above-described photophysical and spectral properties of PR have been investigated both in detergent micelles, as well as in a more native-like liposome environment.

The quarternary structure of membrane proteins has an important impact on their function. Cryo-electron and atomic force microscopy have shown that PR exists predominantly in a pentameric or hexameric assembly, both in lipid bilayers and in DDM micelles, which is likely to represent its native oligomeric state [90, 91]. This is in contrast to bR, which is embedded in the purple membrane as a hexagonal lattice composed of trimeric units. Trimers of GR have also been reported, due to an involvement of the His-Asp cluster [92]. Till date, no crystal structure of PR has been reported. However, solid and liquid-state NMR have been used to investigate the structure of PR in liposomes and detergent micelles respectively [93, 94], though these NMR structures deviate from the crystal structure of BPR [95]. Therefore, one can conclude that reliable structural information for these proteins in a native-like membrane environment is still lacking, and a more detailed characterization under such conditions is imperative.

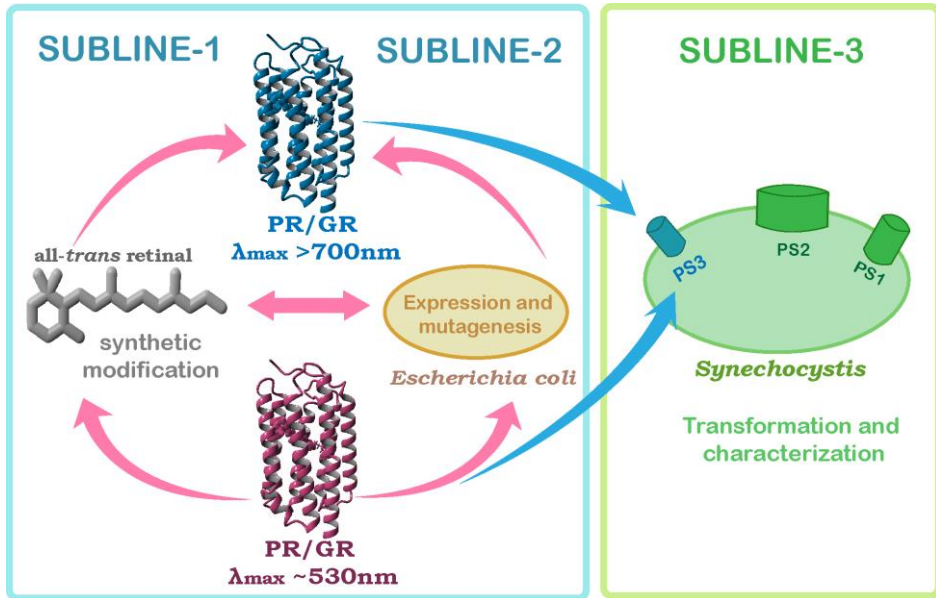


Figure 1.8 An overview of the research goals of this project. Sublines-1 and 2 refer to the spectral modulation of WT PR and GR, by modification of the retinal chromophore and the opsin, to generate a NIR active proton pump with a $\lambda_{\max} > 700\text{nm}$. Both the WT and NIR active proto-pumps (PS3) are incorporated into the cyanobacterium *Synechocystis*, to assess their contribution towards phototrophic growth of the organism.

1.8 Research goals

The major goal of this research is to generate and characterize a NIR active rhodopsin proton pump, towards the ultimate intention of complementing natural photosynthesis. Figure 1.8 shows a broad overview of this project, which can be defined by the following sublines. Sublines-1 and 2 refer to the engineering of NIR active PR and GR, and the characterization of their spectral properties and proton-pump activity using *E. coli* as a model organism. **Subline-1** involves the use of synthetically engineered analogs of all-*trans* retinal to regenerate the wild type (WT) proteo-opsins. In **subline-2**, site-directed mutagenesis and/or directed evolution are used to generate red-shifted opsin mutants. The mutants are first tested with the native

ligand to characterize their properties, and then further combined with suitable retinal analogs obtained from subline-1.

From this combinatorial strategy, we aim to generate an active variant of PR/GR displaying an absorbance maximum >700 nm. This NIR active proton-pump, nicknamed photosystem-3 (PS3) will be incorporated in the cyanobacterium *Synechocystis* sp. PCC6803 in **subline-3**. This subline is undertaken by our collaborators in the University of Amsterdam, towards the aim of utilizing PS3 as a complement to photosystems-1 and 2 (PS1, PS2). For this subline, a suitable expression system for the proteorhodopsins in *Synechocystis* had to be generated, which was optimized using WT PR and GR. The ultimate goal is to express PS3 in *Synechocystis*, and assess its contribution towards phototrophic growth of the organism, using complementary or sole illumination >700 nm.

1.9 Scope of this thesis

The central aim of this thesis project is to investigate the spectral modulation and characterization of PR and GR in various micro-environments. In **Chapter 2**, we tested retinal analogs containing specific ring modifications, and were able to generate active red and blue shifted variants of PR and GR. The red-shifted A2 analog studied in this chapter was further modified in **Chapter 3**, by elongating the conjugation on the retinal β -ionone ring. One promising retinal analog, in combination with a specific PR mutant, generated a novel near-infrared absorbing proton-pump. In **chapter 4**, we describe the construction of a novel concept for a directed evolution assay, which enables us to perform random mutagenesis as well as select for active red-shifted variants of PR and GR simultaneously *in vivo*. In **Chapter 5**, we investigate the influence of the membrane or detergent microenvironment on the stability and spectral properties of PR/GR and some of their red-shifted variants. The biotechnological

potential and future scope of the novel proteorhodopsin variants generated in this project is discussed in **Chapter 6**.

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