A new group of eubacterial light-driven retinal-binding proton pumps with an unusual cytoplasmic proton donor

by

Andrew Harris

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ABSTRACT

A NEW GROUP OF EUBACTERIAL LIGHT-DRIVEN RETINAL-BINDING PROTON PUMPS WITH AN UNUSUAL CYTOPLASMIC PROTON DONOR

Andrew Harris

University of Guelph, 2016

Advisor:

Professor Leonid Brown

Microbial rhodopsins are a versatile family of photoactive retinal-binding membrane proteins which are widespread geographically and taxonomically. One of the main functions of microbial rhodopsins is outward-directed light-driven proton transport across the plasma membrane, which can provide sources of energy alternative to respiration and chlorophyll photosynthesis. Proton-pumping rhodopsins are found in Archaea (Halobacteria), multiple groups of Bacteria, numerous fungi, and some microscopic algae. This work describes a new group of efficient proteobacterial retinal-binding light-driven proton pumps which lack the carboxylic proton donor on helix C (most often replaced by Gly) but possess a unique His residue on helix B. The typical representative of the group (from Pseudomonas putida) was characterized spectroscopically and through site-directed mutagenesis, which suggested that the unique histidine at position 37 likely forms a proton-donating complex involving water molecules compensating for the loss of the carboxylic proton donor.
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LIST OF ABBREVIATIONS

ApR ≡ *Asaia platycodi* rhodopsin
ASR ≡ *Anabaena* sensory rhodopsin
ATP ≡ Adenosine triphosphate
BR ≡ bacteriorhodopsin
CCCP ≡ Carbonyl cyanide m-chlorophenyl hydrazone
Cl⁻ ≡ chloride ion
D₂O ≡ deuterium oxide
DDM ≡ n-dodecyl β-D-maltoside
DMPA ≡ 1,2-dimyristoyl-sn-glycero-3-phosphate
DMPC ≡ 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DNA ≡ Deoxyribonucleic acid

*E. coli* ≡ *Escherichia coli*

ESR ≡ *Exiguobacterium sibiricum* rhodopsin

F-lids ≡ phenylalanine lids

FTIR ≡ Fourier transform infrared spectroscopy

GPR ≡ green-light absorbing proteorhodopsin from gamma-proteobacterium

GR ≡ *Gloeobacter* rhodopsin

H⁺ ≡ proton

H-bonding ≡ hydrogen-bonding

HR ≡ halorhodopsin

HVPS ≡ high voltage power supply

IPTG ≡ Isopropyl-β-D-thiogalactoside

KIE ≡ kinetic isotope effects

L-barrel ≡ leucine barrel

LED ≡ light emitting diode

LN₂ ≡ liquid nitrogen

MD ≡ molecular dynamics
MIR ≡ mid infrared

Na⁺ ≡ sodium ion

ND ≡ no data

NDQ ≡ sodium-pumping rhodopsins

NM-R2 ≡ Nonlabens marinus rhodopsin

OD600 ≡ optical density at 600 nm

PaR ≡ Pantoea ananatis rhodopsin

PPMRs ≡ proton pumping microbial rhodopsins

PR ≡ proteorhodopsin

PRC ≡ proton release complex

PspR ≡ Pseudomonas putida rhodopsin

PvR ≡ Pantoea vagans rhodopsin

SB ≡ Schiff base

SOPs ≡ Standard operating procedures

SpR ≡ Spingomonas rhodopsin

SR ≡ slow rhodopsin like pigment

SRI ≡ sensory rhodopsin I
SRII ≡ sensory rhodopsin II

TM ≡ transmembrane

VMD ≡ visual molecular dynamics

w/w ≡ weight per weight

WT ≡ wild-type

XR ≡ xanthorhodops
1) INTRODUCTION

1.1) Rhodopsins

1.1.1) General information

Rhodopsins are a family of versatile membrane bound photoreceptor proteins which are capable of absorbing and utilizing electromagnetic radiation in order to perform a diverse range of functions with high efficiency\(^1\)-\(^3\). The host organisms of rhodopsins can be found in a wide range of environments, both terrestrial and from polar to equatorial waters, and are responsible for a considerable fraction of the solar energy conversion in the biosphere\(^4\)-\(^9\). Rhodopsins in general are classified into two groups, type-I and type-II, representing microbial rhodopsins and animal rhodopsins respectively\(^1\). Microbial rhodopsins have been impressive in their evolutionary success as they are highly ubiquitous in nature; initially believed to be restricted to \textit{Archaea}, they have been discovered in all three kingdoms of life\(^2,10\). They are expressed in unicellular archaea, bacteria, algae, fungi, and viruses, while their non-microbial counterparts (animal rhodopsins) reside in complex eukaryotic organisms such as humans, who rely on photosensory rhodopsins for vision\(^11\)-\(^14\). At present there are six known light-induced microbial rhodopsin functions: ion-transporters (H\(^+\), Cl\(^-\) or Na\(^+\)), photosensors, channel rhodopsins, and enzyme rhodopsins\(^1,15\)-\(^24\). While the focus of this work is on proton pumping microbial rhodopsins, all rhodopsins possess the same characteristic structure\(^1\).
1.1.2) Structure of rhodopsins

Rhodopsins all share a common seven transmembrane (TM) alpha helical structure, where the helices are labelled A through G (typical of type-I rhodopsins; a 1-7 nomenclature is typical for type-II rhodopsins) and their N- and C- termini lie on the extracellular and cytoplasmic sides of the membrane, respectively\(^1,11\) (See Figure 1.1). The retinal chromophore, which is responsible for absorbing light, giving colour to the protein, and allowing it to perform its function, is covalently bound via a lysine side chain to the middle of helix G\(^1,25,26\) (See Figure 1.1 & 1.2). This bond forms the structure known as the retinal Schiff base (SB) which is

Figure 1.1: Microbial rhodopsin structure. A) Shows the seven TM \(\alpha\) helices labelled A through G, the retinal binding site in the middle of helix G and the N and C termini which lie on the extracellular and cytoplasmic sides of the membrane respectively. B) Representation of the helical structure of microbial rhodopsins (bacteriorhodopsin, PDB ID: 1C3W). Reproduced with permission from Ernst et al. *Chem. Rev.* **114**, 126–63 (2014).
Figure 1.2: Retinal in its all-trans and 13-cis conformations with the SB nitrogen present and protonated. Also present is the lysine sidechain from helix G (Lys-216 in the case of BR).

functionally important for proton pumps and some photosensors as it quickly changes protonation states in a systematic way to support the transport of protons in the extracellular direction. Rhodopsins are efficient light capture machines which use retinal to absorb and use the energy from a single photon as fast as in tens of milliseconds. One exception to this single chromophore system employed by most rhodopsins is the so called xanthorhodopsin (XR) from the bacterium *Salinibacter ruber*, which harvests light using retinal and a carotenoid, salinixanthin.
1.1.3) A brief history of microbial rhodopsin research

Oesterhelt and Stoeckenius were the first to discover a microbial rhodopsin, in 1971\textsuperscript{28}. They studied the protein from \textit{Halobacterium salinarum} (then \textit{halobium})\textsuperscript{15} of the domain \textit{Archaea}, which was later termed bacteriorhodopsin (BR) and found to pump protons in the extracellular direction to build an electrochemical gradient\textsuperscript{29}. In 1977, another protein was discovered in the same organism\textsuperscript{16}, later termed halorhodopsin (HR) and found to pump chloride ions in the cytoplasmic direction\textsuperscript{17}. By 1982, researchers were beginning to understand the complexity of the \textit{H. salinarum} rhodopsin machinery as light-induced absorption changes of the purple membrane revealed features that couldn’t be attributed to BR or HR. The part of the pigment which exhibited a slow photocycle was also shown to bind retinal and was named slow rhodopsin like pigment (SR)\textsuperscript{18}. This pigment, later called Sensory Rhodopsin I (SRI) allows \textit{H. salinarum} to seek favourable light (>500 nm) and avoid UV light\textsuperscript{18–20}. The second photophobic sensor, sensory rhodopsin II (SRII) was discovered in 1986 and is responsible for avoiding oxidative stress in response to blue light\textsuperscript{21–23}.

Originally believed to be confined to \textit{Archaea}, in 1999, the first eukaryotic rhodopsin of the microbial type was discovered in the filamentous fungus \textit{Neurospora crassa}\textsuperscript{30} and was later found to exhibit sensory function\textsuperscript{31}. The first eubacterial rhodopsin was discovered at the turn of the century\textsuperscript{26}. Through DNA sequencing of environmental samples, a rhodopsin was found in marine \textit{γ-Proteobacteria} of SAR86 group, and these rhodopsins became known as proteorhodopsins (PRs)\textsuperscript{26,32}. This spawned a great acceleration in the discovery of microbial rhodopsins. Researchers had the necessary data in genomic DNA and cDNA libraries to
investigate rhodopsins in a more systematic way\textsuperscript{32}. As gene sequencing technology improved, so too did the general understanding of the taxonomic diversity of microbial rhodopsins, especially proton pumping microbial rhodopsins\textsuperscript{11}. This established widespread presence of proton-pumping rhodopsins in marine and freshwater eubacteria. Related to but distinct from PRs, some of these proteins became known as xanthorhodopsins (XRs) and actinorhodopsins\textsuperscript{34–36}. In parallel, it was realized that various lower eukaryotes, including multiple fungi and some algae, possess retinal-binding light-driven proton pumps and other types of microbial rhodopsins as well\textsuperscript{25,36–38}.

Light-driven proton transport is one of the most basic functions of microbial rhodopsins and it is studied extensively. The interest in proton pumping rhodopsins is a result of their evolutionary success but also because they are so critical to the survival of the organisms in which they are expressed. The outward transport of protons (alternatively viewed as inward hydroxyl ion transport)\textsuperscript{39–41} is important bioenergetically, as it can supplement production of ATP under the conditions when respiration is inefficient due to the scarcity of carbon sources and/or oxygen\textsuperscript{10,42}. Nearly all hosts of proton pumps convert the proton gradient into chemical energy using an integral membrane protein, ATP synthase\textsuperscript{41}. These proteins are also interesting to study because the mechanisms of proton transport are incredibly complex and efficient, and as such, satisfactory explanations for some aspects of the transport mechanism remain elusive.
1.1.4) Photocycle of proton pumping microbial rhodopsins

Absorption of a photon by the retinal chromophore causes the molecule to isomerize from its all-trans to its 13-cis configuration and initiates the cascade of conformational changes which ultimately leads to protein function\(^1\text{-}^3,^35\). As the protein undergoes these structural changes, it transitions through a set of well-defined photo-intermediates which are detectable spectroscopically as they absorb different wavelengths of light to different extents\(^1,^3\). The transitioning of the protein through these observable intermediates is what is known as the photocycle (See Figure 1.3).

Briefly, the photocycle is initiated by the absorption of a photon by retinal which causes isomerization of the chromophore, and this corresponds to the K-intermediate\(^1\) (absorption of 620 nm monitored in this work). Isomerization of retinal causes residues to rearrange which changes electrostatics in the region and effectively reduces the pKa of the SB and increases the pKa of the proton acceptor (Asp-85 in BR); this is known as the L-intermediate\(^41\) (absorption of 460 nm monitored in this work). This is in preparation for the proton transfer from the SB to the acceptor, or deprotonation of the SB which is called the M-rise\(^1,^10\) (absorption of 420 nm monitored in this work). The M-decay (absorption of 420 nm monitored in this work) corresponds to the SB reprotonation from the cytoplasmic proton donor\(^1,^10\) (Asp-96 in BR). In the N-intermediate, the F-helix tilts to open the cytoplasmic proton channel and allow for the entry of water molecules resulting in reprotonation of the proton donor\(^1,^10\). Lastly, retinal isomerizes from 13-cis back to all-trans, and this corresponds to the O-intermediate\(^1,^10\) (absorption of 620 nm monitored in this work).
Figure 1.3: Typical photocycle of proton pumping microbial rhodopsins exemplified by BR. The wavelengths shown correspond to the wavelengths of light that were monitored for each intermediate in time-resolved spectroscopy experiments in this work. Modified with permission from Ernst et al. *Chem. Rev.* **114**, 126–63 (2014).

The primary motivation for studying a rhodopsin’s unique photocycle is that it provides the basis for the determination of its function. For example, the rate of photocycle turnover sheds light on the function of the protein of interest; namely sensors possess slow photocycles (on the order of seconds) and pumps possess fast photocycles (on the order of milliseconds)\(^2,3,5\). Further, the M-intermediate corresponds to the deprotonated state of the SB which is critical for the signalling or transport mechanism of rhodopsins. However, M can only form when there are
proton transfers involving the SB and as such the Cl⁻ pump, HR, produces no M-intermediate (under physiological conditions). If an M-intermediate is detected, the protein may be a proton pump, a sodium pump or a photosensor but it cannot be a chloride pump; sodium pumps do possess an M intermediate as the initial proton transfer is required for sodium transport. The proton transfers involving the SB are critical for proton translocation, but are only a small (even though the most important) portion of the complex mechanism by which microbial rhodopsins transport protons.

1.1.5) Mechanism of proton transport by microbial rhodopsins

Since the discovery of bacteriorhodopsin nearly a half century ago, researchers have been investigating the mechanism by which microbial rhodopsins transport protons. The main features of the mechanism of the light-driven proton transport have been elucidated for BR and its close relatives such as archaerhodopsins, cruxrhodopsins and deltarhodopsins from various species of Halobacteria. Biophysical studies have provided information on the essential (conserved) and optional (variable) elements in the molecular mechanism of such proteins. The consensus on the general mechanism emerging from these studies involves three important elements: the retinal Schiff base with lysine side chain on helix G (Lys-216 in BR), the primary proton acceptor on the extracellular side of helix C (Asp-85 in BR, usually in complex with His in PR and XR), and the proton donor (Asp-96 in BR) on the cytoplasmic side of helix C. The most characteristic scenario is the one in which the donor, Asp on helix C, is interacting with Thr on helix B (typical for archaeal and fungal rhodopsins), or Glu on helix C is interacting with Ser on helix B (typical for eubacterial rhodopsins). It was shown that, in
some cases, disruption of this Asp/Thr (Glu/Ser) pairing results in a strong perturbation of the proton transport function\textsuperscript{58,59}. On the other hand, one should note two exceptions from this general trend. First, in algal rhodopsin from Acetabularia the aspartic proton donor is conserved, but interacts with Cys on helix G\textsuperscript{60}. The second, more striking departure from “the carboxyl rule”\textsuperscript{61} is that found in Exiguobacterium (ESR), where the carboxylic proton donor is replaced by Lys\textsuperscript{62}. Unlike the typical carboxylic proton donors, this lysine residue resides in a hydrophilic cavity and may form a proton-donating complex involving water molecules\textsuperscript{61}.

While this polar environment for the cytoplasmic proton donor of ESR is atypical of proton pumping microbial rhodopsins (PPMRs), the use of water molecules to form polar networks is not. PPMRs would be rendered useless without the extensive, polar, hydrogen bonded networks provided by water\textsuperscript{32}. The polar chains that are created by hydrogen bonding (H-bonding) of water molecules bridge gaps between polar residues that would otherwise be too far apart for proton transport to occur. A correlation between strongly bound water molecules near the SB and the ability to pump protons has been observed in many retinal proteins\textsuperscript{32}. While all microbial rhodopsins contain protein-bound water molecules near the SB\textsuperscript{1}, it has been suggested that strongly hydrogen bonded water molecules are only found in proteins that exhibit proton pumping function\textsuperscript{63}. For example, there is a specific pentagonal arrangement of water molecules which is believed to be critical for PPMRs\textsuperscript{64–66}. This arrangement involves three water molecules (wat-402, wat-401 and wat-406), where the proton acceptor (Asp85) and the SB counterion (Asp212) are H-bonded to two water molecules each, and share one with the retinal SB (wat-402)\textsuperscript{64}. Additional water molecules can be found on the extracellular side of the proton acceptor, in the proton release complex (PRC). This complex facilitates proton release to the
extracellular surface and is comprised of three residues (Arg-82, Glu-204 and Glu-194) H-bonded to several water molecules and also H-bonded to Tyr-83 and Tyr-57\textsuperscript{51}. The first three residues which comprise the PRC (R82, E204 and E194) are essential for normal (early) proton release but not all of these glutamates are conserved in all proton pumps\textsuperscript{51}. However, the carboxylic SB counterion does appear to be critical for proton transport as all PPMRs conserve the homologue of this residue (Asp-212)\textsuperscript{32}. Mutation of this residue in BR causes drastic perturbations to the photocycle, including delayed proton release, delayed decay of M and delayed proton uptake\textsuperscript{67}. Mutation of the homologous residue in PR results in the loss of specificity of the retinal isomerization (all-\textit{trans} to 9-\textit{cis} as opposed to 13-\textit{cis})\textsuperscript{32}.

The situation in the cytoplasmic side of BR is somewhat different but water molecules are still present. There are two water molecules (Wat-501 and Wat-502) in the proton uptake path (the space between the carboxylic proton donor (Asp-96) and the retinal SB) which connect helix G to functionally important regions\textsuperscript{64,68}. These joined regions are the C\textsubscript{13}=C\textsubscript{14} double bond of retinal through Trp-182 and Asp-96 through H-bonding with Thr-46. Wat-501 is H-bonded to Ala-215 (of helix G) and Trp-182 (of helix F) while Wat-502 is H-bonded to Thr-46 (of helix B) and the SB (Lys-216 of helix G)\textsuperscript{68}. Interestingly, Wat-502 mediates the proton transfer from the donor to the SB and is located 5Å from Asp-96 and 7.8Å from the SB\textsuperscript{68}. It is clear that polar residues and water molecules are necessary for the transport of protons by microbial rhodopsins, but how can the mechanism be described in detail?

Below, the mechanism of proton transport employed by BR will be presented. In order to imagine the mechanism of proton transport, and the rearrangement of residues and water
molecules, it is helpful to track conformational changes of the protein through the five different proton transfers that occur during the photocycle. See Figure 1.4 for a visual representation of BR with critical amino acids and the five proton transfers identified.

*Step one* is a proton transfer from the SB to the proton acceptor, Asp-85. The SB is initially accessible to Asp-85 and upon photo-isomerization of the retinal chromophore (from all-trans- to 13-cis-), the SB proton is transferred to the primary proton acceptor, and this transfer is mediated by a strongly hydrogen-bonded water molecule, wat-402 (which is part of the pentagonal arrangement discussed earlier)\(^{64-66}\). Retinal, once isomerized, causes the binding pocket to become distorted. The 13-methyl group interacts sterically with the indole ring of Trp-182, which causes this residue to tilt upward\(^{69}\). This initiates local displacements, breaking the hydrogen bond between Trp-182 and wat-501 which disconnects helix F from Ala-215 on helix G and ultimately leads to the influx of water molecules from the cytoplasm which mediate the proton transfer in the third step\(^{69}\). Once Asp-85 becomes protonated, the pentagonal arrangement of water molecules dissociates, raising the pKa and effectively stabilizing the protonated state of the acceptor\(^{64}\).
Figure 1.4: Bacteriorhodopsin structure showing the five internal proton transfer steps, resulting in outward proton translocation. Notice the proton donor (Asp-96), the retinal chromophore bound to helix G via Lys-216 forming the SB, the proton acceptor (Asp-85), and key members of the PRC (Arg-82, Glu-204 and Glu-194). Reproduced with permission from Wickstrand et al. *Biochim. Biophys. Acta –Gen. Subj.* **1850**, 536–53 (2015).
**Step two** is the proton release to the extracellular side of the membrane. The protonation of Asp-85 is coupled to the release of the extracellular proton that is likely bound to two water molecules in the PRC. Here, the proton is not bound to a single amino acid but rather is delocalized by having multiple binding sites including the two water molecules and the members of the PRC (Arg-82, Glu-194 and Glu-204), which are also H-bonded to Tyr-57 and Tyr-83. Once Asp-85 is neutral, the electrostatic interaction between it and Arg-82 is weakened. This forces the positive side chain of Arg-82 to shift 1.7 Å toward the glutamates of the PRC, which shifts water molecule positions and causes the proton to become localized at the release site (either Glu-194 or Glu-204). In the ground state, Ser-193 and Glu-204 form a gate that seals the protonated water cluster from external water molecules (See Figure 1.5). The localization of the proton (at Glu-194 or Glu-204) weakens the H-bonding between Ser-193 and Glu-204 and disrupts “the gate”. This opens the cavity to the extracellular bulk, allowing for the influx of water molecules, finally resulting in proton release.

**Step three** is SB reprotonation from the carboxylic cytoplasmic proton donor, Asp-96, which does not interact with the Schiff base directly, but rather communicates with it via a number of water molecules entering the cytoplasmic half of the protein after a conformational change. In the resting dark state, the environment of the cytoplasmic proton donor is hydrophobic, which keeps it protonated and prohibits communication of the Schiff base with the cytoplasmic bulk, preventing back-flow of protons in the dark. This hydrophobic region is often called the leucine barrel with phenylalanine lids (L-barrel and F-lids) and is comprised of four leucine residues (Leu-95, Leu-97, Leu-100 and Leu-223) and two phenylalanine residues (Phe-42 on the cytoplasmic side and Phe-219 facing the SB). Tilting
of helix F and to a lesser extent helix G (mentioned earlier in step one) disrupts the L-barrel and F-lids to allow for three water molecules to enter the protein, form a H-bonded chain with two other water molecules (Wat-501 and Wat-502), and bridge the two groups which are the SB and the proton donor/donor partner pair (Asp-96/Thr-46)\textsuperscript{32,64,69}. Just prior to this, the deprotonated SB switches its accessibility toward the cytoplasmic side through relaxation of the twisted retinal chromophore, allowing for the reprotonation\textsuperscript{32,69}.

Figure 1.5: Image generated through MD simulations illustrating the proton release site of BR. Serine and glutamic acid gate is shown with water molecules entering the cavity after the movement of Arg-82. See text for more details. Reproduced with permission from Gerwert et al. *Biochim. Biophys. Acta* \textbf{1837}, 606–13 (2014).

*Step four* is reprotonation of Asp-96 from the cytoplasmic bulk but this is not yet well understood. However, it seems that this transfer relies on the rearrangement of water molecules after a conformational change, much like the earlier steps of this photocycle\textsuperscript{64}. In the ground
state of BR, the pKa of Asp-96 is high, above 11. Increased connectivity to the cytoplasmic surface decreases the pKa of Asp-96 to 7.2-7.5 and its pKa raises again after it is reprotonated. Also, it has been proposed that BR makes use of negatively charged carboxylic acids to form a proton collecting antenna on the cytoplasmic side of the protein. At the cytoplasmic surface there are four aspartic acids (Asp-36, Asp-38, Asp-102 and Asp-104), on the EF loop there are two glutamic acids (Glu-161 and Glu-166), and on the C-terminus there are three glutamic acids and one aspartic acid (Glue-232, Glu-234, Glu-237, and Asp-242). Mutations of Asp-36, Asp-38 and Glu-161 with cysteine each result in a delay of proton uptake by two fold. Mutations of Asp-38 with cysteine and asparagine slowed internal proton transfer from Asp-96 to the SB by five and three fold, respectively. While researchers understand the process by which BR collects protons from the cytoplasmic space to reprotonate Asp-96 more rapidly, the exact mechanism by which one of these protons is transferred from the antenna to Asp-96 remains to be seen. However, the reprotonation of Asp-96 is known to be coupled to the re-isomerization of retinal back to all-trans.

*Step five* is reprotonation of the PRC from the proton acceptor, Asp-85, and its explanation is the most elusive to researchers. It is a long range proton transfer and there is a positive charge supplied by Arg-82, that must be overcome. At present, the understanding of this process is as follows. Helices A, D and E tilt outward to allow for rearrangement of water molecules and polar residues in the PRC, and reprotonation from Asp-85 may be mediated by Asp-212, the SB counterion. Arg-82 only returns to its original position once the PRC is reprotonated.
As the mechanisms for ion transport and photosensory transduction are complex and involve many residues, it would be prohibitively time consuming to refer to a group of rhodopsins by all of their functionally important residues. Instead, researchers have identified what appear to be the three most critical amino acids for rhodopsins, and use these three residues to categorize rhodopsins.

1.1.6) Amino acid helix C motifs

While the process of light-activated proton transport by microbial rhodopsins is intricate and complex, one can notice trends for their amino acid sequences, especially on helix C. These trends are the so-called amino acid motifs which are a string of three letters representing (in the case of proton pumps) the proton acceptor, the proton acceptor partner and the proton donor which all reside on helix C.

Proton pumping microbial rhodopsins

The proton acceptor and acceptor partner lie on the extracellular side of the SB and are always an aspartic acid and a threonine, respectively (Asp-85 and Thr-89 in the case of BR)\textsuperscript{10,43}. Variations in the helix C motifs come solely from the proton donor which is most commonly a carboxylic acid (Asp-96 in the case of BR and Glu in the case of PR and XR)\textsuperscript{33,43}. Therefore, the amino acid motif for BR is DTD, and that of PR and XR are DTE. The most surprising departure from this trend comes from the rhodopsin of \textit{Exiguobacterium sibiricum} (ESR) which has a
lysine at the proton donor position creating the unique DTK motif (mentioned earlier, see 1.1.5). The finely tuned architecture of proton pumps appears to be altered in other microbial rhodopsins to allow for the translocation of other ions.

Other ion pumps and photosensors

The halobacterial chloride pump, HR discussed earlier, has a threonine and a serine at the acceptor and acceptor partner positions respectively and an alanine at the donor position, yielding a TSA helix C motif. Chloride is stabilized by H-bonding to hydroxyl groups of serine and threonine, sometimes in complexes involving water molecules. The chloride pump expressed in marine flavobacterium, Nonlabens marinus, named ClIR (representing Cl− rhodopsin) has a motif starkly different from HR - NTQ. Nonlabens marinus also expresses a PR-like proton pump and a sodium pump named NM-R2, becoming the first known organism to express three different types of pumps. The motif for the sodium pumping NM-R2 is NDQ, similar to its chloride pump counterpart. The chloride and sodium pumps exhibiting NTQ and NDQ motifs respectively, parallels the functional switch between BR and HR by D/T replacement, although at a different position. Bacteriorhodopsin can be forced to pump chloride by replacing Asp-85 with threonine creating a TTD motif.

Photosensory rhodopsins of halobacterial type are also closely related to PPMRs. They share the same proton acceptor and proton acceptor partner (Asp and Thr) but replace the proton donor with aromatic amino acids, Tyr or Phe, yielding a DTY/F motif. Archaeal sensory rhodopsins from Halobacterium salinarum, SRI and SRII have DTY motif. Interestingly,
without their soluble transducers bound, SRI and SRII efficiently pump protons in response to light\textsuperscript{77}.

Returning to proton pumps, exploring variations on the normal DTD/E motifs is important as they extend our understanding of the diversity of the mechanisms by which rhodopsins transport protons. This type of research is advantageous as microbial rhodopsins with atypical amino acid motifs are more likely to reveal new microbial rhodopsin functions.

1.2) Current work

1.2.1) Significance

Beyond the rhodopsin family being functionally, taxonomically and geographically diverse, they are also interesting to study because there are a great number of potential biomedical applications. For example, the emerging field of optogenetics allows researchers to suppress or activate neurons which have expressed rhodopsins\textsuperscript{78}. More specifically, proton and chloride pumping rhodopsins have been shown to induce neural silencing and channel rhodopsins can activate neurons\textsuperscript{78}. Having the ability to control the activity of neurons allows researchers to identify the roles of neural networks in vivo\textsuperscript{79,80}. This work may also be interesting to evolutionary scientists because it is believed that rhodopsins all have a common ancestor or that animal rhodopsins and microbial rhodopsins evolved from distinct ancestors and there is a missing ancestral link between these two types of rhodopsins\textsuperscript{81}. At any rate, there remain aspects of the evolutionary history of rhodopsins which are unknown and when the function of a new
group of rhodopsins is determined, it adds a piece to the evolutionary puzzle. This work is also exciting for researchers interested in molecular mechanisms, because most rhodopsins possess sophisticated mechanisms of action. There is a lot to learn with respect to rhodopsins with atypical amino acid motifs, and even more still if a new rhodopsin function is discovered. Finally, microbial ecology researchers would be attracted to this work since rhodopsins are membrane proteins which interact with the extracellular environment and whose efficacy depends on the concentrations of substrates in the surroundings, for example, the rate of photocycle turnover in proton pumps is accelerated at lower pH and reduced at higher pH\textsuperscript{10}. Rhodopsin research stands to benefit many different fields of research in these ways.

1.2.2) Objective

The main objective of this study is to determine the function of the new group of rhodopsins (See Figure 3.1, in section 3.1). A group is a collection of rhodopsins which are all highly related in their amino acid sequences and all share a common helix C motif. Typically, all members of a group have the same function and this is one of the reasons why this approach can be so powerful. Investigating groups of rhodopsins with unknown amino acid motifs is significant because it may result in the discovery of a new function and it broadens the current understanding of what is believed to be possible for rhodopsins of various taxonomic groups if it is found that the function is already known.

The representative member of this group is \textit{Pseudomonas putida} rhodopsin (PspR) which is expressed in the soil bound proteobacteria, \textit{Pseudomonas putida}. PspR was expressed in host
*Escherichia coli* (*E. coli*) cells. Time-resolved spectroscopy in the visible range was performed to investigate the photocycle; Raman spectroscopy was used to investigate the conformation, environment, and the light and dark adaptation of retinal; the proton pumping ability was assessed through proton transport assays; and finally, site-directed mutagenesis was used to identify the role of a unique histidine residue on helix B which is believed to participate in a polar, cytoplasmic proton donating complex involving water molecules. Collaborators from Japan investigated another member of this group, *Pantoea ananatis* rhodopsin (PaR) and the data is available in the supplementary information. Additionally, most of the results which will be discussed in detail in the following sections have been published in BBA – Bioenergetics: Harris, A. *et al.* A new group of eubacterial light-driven retinal-binding proton pumps with an unusual cytoplasmic proton donor. *Biochim. Biophys. Acta.* **1847**, 1518–29 (2015).

2) MATERIALS AND METHODS

2.1) Protein expression

Genes encoding wild-type (WT) (gi 558523492) and mutant PspR were cloned into pET21a(+) vector (EMD Millipore, Billerica, MA) by GenScript (Piscataway, NJ) using NdeI–XhoI restriction sites, which added C-terminal 6× His-tag after the LE insert. *Escherichia coli* (*E. coli*) C41 (DE3) OverExpress Chemically Competent Cells (Lucigen, Middleton, WI) were transformed with the plasmids using Lucigen's heat-shock transformation protocol. After thawing the *E. coli* C41 cells on ice for 20 min, 1 μL of plasmid solution (up to 200 ng DNA) was added to 50 μL of cells and incubated on ice for 30 min. The cells were heat-shocked in a 42
°C water bath for 45 s, and then incubated on ice for 2 min. The cells were added to 950 μL of Expression Recovery Media (Lucigen) and incubated at 37 °C and 250 rpm for 1 h. 100 μL of cell culture was spread onto 2×YT agar plates (1% yeast extract, 1.6% tryptone, 1% NaCl, 1.5% agar, 0.1 mg/mL ampicillin, pH = 7.0) and incubated overnight at 37 °C. For the small scale colony screening, eight isolated bacterial colonies were selected to inoculate 2 mL of 2×YT media for incubation at 37 °C and 240 rpm overnight and transferred to 25 mL 2×YT media.

When the 25 mL culture reached an optical density at 600 nm (OD600, measured with a Cary50, Varian) of ~0.4, it was induced by 1 mM isopropyl-β- D-thiogalactoside (IPTG) and 7.5 μM all-trans retinal. The induced cell culture was incubated for 3 h at 37 °C and 240 rpm. The cells were collected through low-speed centrifugation at 2400×g and 4 °C. The intensity of color between the cell pellets was compared, and the colony with the most intense pink color and largest pellet was selected as the best protein expressing colony. The best protein expressing colony was grown in 1 L of 2×YT media using shake flask cultures. The colony material was used to inoculate 2 mL media and was incubated overnight at 37 °C and 240 rpm. The 2 mL cell culture was used to inoculate 25 mL of media which was then incubated overnight at 37 °C and 240 rpm. The 25 mL cell culture was used to inoculate 1 L media and was incubated at 37 °C and 275 rpm. Once an OD600 of ~0.4 was reached, the 1 L cell culture was induced with a total concentration of 1 mM IPTG and 7.5 μM all-trans retinal and was incubated for 3 h. The cells were collected with low-speed centrifugation at 2400×g and 4 °C for 10 min. The cell pellet was re-suspended in 150 mM NaCl and was centrifuged again at 2400×g and 4 °C for 10 min. The cell pellet was re-suspended in 40 mL of lysis buffer (0.15 M NaCl, 0.05 M Tris base, 1 mM MgCl₂, 2 μg/mL DNase I, 0.2 mg/mL lysozyme, pH = 7.2). The suspended cells were left to shake (IKA VIBRAX) at 400 rpm at room temperature for 3 h and frozen for future use.
2.2) Time-resolved spectroscopy in the visible range

2.2.1) Sample preparation

The thawed cells were sonicated (Fisher Sonic Dismembrator Model 500) three times at 45% amplitude for 2:30 min in 30 second intervals. The sample was spun down to check for complete cell lysis at 2400×g and 4 °C for 15 min and the supernatant was collected. The pellet, containing unbroken cells with expressed protein, was re-suspended in 20 mL of lysis buffer, sonicated, and then centrifuged again as before. This process was repeated typically three times until the supernatant after low-speed centrifugation showed little to no pink color intensity. The broken membrane fragments were then collected with the ultracentrifuge at 150,000×g and 4 °C for 1 h. Flash-photolysis experiments were run on the crude membrane fragments encased within polyacrylamide gels. The membranes were resuspended in 0.4 mL of pH 8 buffer (50 mM KH$_2$PO$_4$ and 50 mM Tris) and the following was added: 0.3 mL of 33% acrylamide and 1% bis-acrylamide solutions, 2.4 μL of 10% ammonium persulfate, and 3 μL of N, N, N', N'-tetramethylethylenediamine. After solidification, the gels were washed with deionized water for a total of 4 h at room temperature, and stored at 4 °C in deionized water or buffer. The following buffers were used unless indicated otherwise: pH 1–5 (50 mM KH$_2$PO$_4$, 50 mM potassium acetate); pH 6 (50 mM KH$_2$PO$_4$, 50 mM MES); pH 7–8 (50 mM KH$_2$PO$_4$, 50 mM TRIS); and pH 9 (50 mM KH$_2$PO$_4$, 50 mM CHES).
2.2.2) Experimental setup

Investigating rhodopsins using time-resolved difference spectroscopy in the visible range (also called laser spectroscopy or flash-photolysis) provides evidence for the determination of the protein’s function. When applied with site-directed mutagenesis in parallel, critical amino acids can be determined by observing changes to the photocycle that originate from a given amino acid replacement. Our experimental set-up for this method is discussed briefly below and a simplified schematic is shown in Figure 2.1.

The single-wavelength laser spectrometer was built in house previously. The photocycle was initiated with 7 ns pulses of the second harmonic of an Nd-YAG laser at 532 nm (Continuum Minilite II). Time-resolved measurements were made possible through the short laser pulses generated by the Q-switch circuit. Absorption changes of the monochromatic light [provided by an Oriel quartz tungsten halogen (QTH) source and two monochromators] were followed using an Oriel photomultiplier with a 350 MHz bandwidth, an amplifier and a Gage analog-to-digital converter (CompuScope 12100-64M)\textsuperscript{25}. Kinetic traces were averaged and converted into a quasi-logarithmic time scale using in-house software (GageCon, written by Dr. Brown). Global multiexponential analysis was performed by FITEXP\textsuperscript{82} and exponential analysis.
of single kinetic traces was performed by SPSERV (C. Bagyinka, BRC Szeged, Hungary). The kinetic traces that are collected represent a difference in visible light absorption between one or more intermediates and the ground state (i.e. photo-intermediate state – dark state). If we let the applied offset voltage \( V_{\text{offset}} \) represent the voltage when no light reaches the photomultiplier and \( V_0 \) when a monochromatic light is selected then \( V_0 - V_{\text{offset}} \) is proportional to the static light intensity at the selected wavelength. Once the sample is excited, the voltage will change corresponding to \( \Delta V(t) \) as the photocycle progresses through detectable intermediates and \( V_0 - V_{\text{offset}} - \Delta V(t) \) is proportional to the transient light intensity at the corresponding time. In the GageCon program, the detected voltage is converted to transient absorption change via: \( \Delta A(t) = -\log\left(\frac{V_0 - V_{\text{offset}} - \Delta V(t)}{V_0 - V_{\text{offset}}}\right) \), as reported previously\(^{83}\). In this way, positive voltage signal corresponds
to the formation of an intermediate and negative signal represents disappearance of the ground state, which are recalculated into respective absorption changes.

2.3) Raman spectroscopy

2.3.1) Sample preparation

Vibrational spectroscopy was performed on His-tag-affinity purified lipid-reconstituted samples. The membrane pellet was re-suspended in a solubilization buffer (5 mM Tris base, 1% DDM, pH = 7.5) and was left to stir overnight at 4 °C in the dark. The insoluble membrane debris was removed at 150,000×g and 4 °C for 1 h. The supernatant was collected, and the protein yield was estimated spectrophotometrically (Cary50) using the extinction coefficient of BR, to determine the amount of nickel-nitrilotriacetic acid (Ni²⁺-NTA) resin (Qiagen) to be added. The resin was washed with 200 mL of 150 mM NaCl on a membrane filter, re-suspended in 10×binding buffer (3 M NaCl, 0.5 M Tris base, 0.05% DDM, pH = 8), and added to the solubilized protein sample to stir overnight at 4 °C. A membrane filter (Thermo Scientific Nalgene MF75 Filter, 0.8 μm pore size) was used to wash the protein bound to resin sample approximately eight times with 25 mL of washing buffer (0.3 M NaCl, 0.05 M Tris, 40 mM imidazole, 0.05% DDM, pH = 8) to remove any undesired proteins. The washed protein-resin sample was collected by low-speed centrifugation at 2400×g and 4 °C for 5 min. The supernatant was discarded and 5–10 mL of elution buffer (0.3 M NaCl, 0.05 M Tris, 0.5 M imidazole, 0.05% DDM, pH = 8) was added and stirred on ice for 10 min. Typically a total of 50 mL of eluted protein in elution buffer was collected, and this sample was syringe filtered (0.22 μm pore size)
to remove any large debris or accidentally collected resin. An Amicon Ultra 15 mL centrifugal filter tube was used to concentrate the sample down to a small volume through repeated centrifugation at 1500×g and 4 °C for 20 min. The buffer was exchanged to reconstitution buffer (5 mM NaCl, 10 mM Tris, 0.05% DDM, pH = 8) through washing it with a total volume of 50 mL through repeated centrifugation in the filter tube.

For Raman spectroscopy, the liposomes used for reconstitution were made of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA) in a 9 to 1 w/w ratio. The lipids were dissolved in chloroform and stirred for 90 min; the chloroform was then removed under vacuum inside a desiccator for 5 h. The lipid film was rehydrated with 1 mL of reconstitution buffer to form an 11.1 mg/mL suspension. The solubilized, concentrated pure protein was combined with the lipid suspension at a 2:1 protein to lipid ratio (w/w), and Triton X-100 stock of 0.2 mg/μL was added dropwise to the sample until the noticeable decrease in turbidity was observed. After 6 h of stirring at 4 °C, 0.6 g/mL of BioBeads SM-2 (Bio-Rad) was added to the sample to absorb the detergent and mixed on the Orbitron Rotator for 24 h. A 27 G needle (BD) was used to remove the reconstituted protein and the BioBeads were washed with a buffer (10 mM NaCl, 24 mM CHES, pH = 9) to collect all reconstituted protein. The sample was centrifuged at 150,000×g and 4 °C for 1 h to collect proteoliposomes.
2.3.2) Experimental setup

In Resonance Raman spectroscopy, a laser provides continuous monochromatic illumination, which changes direction at a prism, exciting a sample and the scattered light is collected at a detector. Photons incident on the sample may be absorbed, they may pass through the sample or they may be scattered inelastically\(^\text{84}\). Approximately 1 in \(10^6\) photons are scattered inelastically which is known as Raman scattering. The detector measures the frequency difference between the incoming and scattered photons which is proportional to the vibrational frequency of the bond the photon scattered from\(^\text{84,85}\). The frequency represents a vibrational fingerprint of the molecule of interest\(^\text{86}\). Since retinal is a photoactive molecule, Raman spectroscopy of rhodopsins mainly provides structural details of retinal and the surrounding environment, known as the retinal binding pocket\(^\text{85}\). There is a significant advantage to using resonance Raman spectroscopy to study chromophores which is the resonance enhancement effect\(^\text{85}\). There is a large increase in the cross section of Raman scattering as the incident laser frequency approaches the frequency separation between the chromophore’s ground and excited electronic states; this results in a large enhancement in the signal to noise and allows for extremely small sample sizes, on the order of micro liters\(^\text{85}\).

Samples used for Raman spectroscopy were prepared as a wet paste of the proteoliposomes hydrated with desired buffer. The Raman spectra were collected using FRA106/s accessory to the Bruker IFS66vs spectrometer, with excitation at 1024 nm, at a 2 cm\(^{-1}\) resolution, using OPUS software.
2.4) Proton transport assays

2.4.1) Sample preparation

Proton transport assays for PspR in the whole *E. coli* cells and spheroplasts were performed according to the published protocol. The cells from 200 mL culture grown as described above were collected at 2400×g and 4 °C for 10 min. Half of the cells were washed three times with unbuffered solution (10 mM NaCl, 10 mM MgSO₄·7H₂O, 100 μM CaCl₂), and then resuspended for proton transport measurements. The other half of the cells was used to prepare spheroplasts, right-side-out membrane vesicles embedding WT PspR. Once prepared and collected, the spheroplasts were washed three times with unbuffered solution and then resuspended for proton transport measurements.

2.4.2) Experimental setup

A glass electrode (Accumet Microprobe Extra Long Calomel Combo Electrode) was used to monitor pH changes of the cells or spheroplasts suspended in unbuffered solution with gentle stirring. A digital oscilloscope (Agilent Technologies DSO 1052B Digital Storage Oscilloscope) was used for recording the pH. The sample was illuminated (Cole Parmer 9741-50 illuminator) with yellow light (570–590 nm) using a glass filter.
If light induced proton translocation in the extracellular direction was to occur, it would be detected as a decrease in pH of the solution. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a control to ensure that the pH changes that were measured were not the result of passive transport in response to electrogenic transport of a different ion\textsuperscript{87}. CCCP is a protonophore which causes the cell membrane to be more permeable to protons. It can cross the membrane in its neutral protonated form, and this neutral molecule dissociates at the cytoplasmic membrane interface where the proton is released\textsuperscript{88}. Of course, the proton can cross the membrane unaided, but this process is extremely slow as there is a large energy penalty because cell membranes are hydrophobic (have low dielectric constant).

3) RESULTS AND DISCUSSION

3.1) Identification of the new group of proteobacterial rhodopsins

Growing diversity of eubacterial rhodopsins has been recognized and analyzed in a number of recent publications\textsuperscript{35,89–95}. An updated phylogenetic tree of selected eubacterial rhodopsins (see Figure 3.1, halobacterial BR-like proton pumps included as a reference) shows the established groups of rhodopsins as well as a new, previously uncharacterized group. Roughly, the tree can be broken into the two main branches, with the lower branch being dominated by PR-like and XR-like proton pumps (the latter includes actinorhodopsins) as well as chloride and sodium pumps (NTQ and NDQ types). This branch also includes the unique proton-pumping rhodopsins from *Exiguobacterium* (ESR) mentioned above, in which the carboxylic
Figure 3.1: Relationship of the new group of proteobacterial rhodopsins (marked with DTG???) to the known groups of eubacterial rhodopsins and their haloarchaeal homolog BR. Clustal Omega⁹⁶ unrooted neighbor-joining tree (rendered by TreeView) of selected eubacterial rhodopsins from public genome databases (http://www.ncbi.nlm.nih.gov/ and http://img.jgi.doe.gov) shows the major protein clusters as well as ESR, which is distinct from the major PR-like and XR-like groups. The new group was named DTG based on its helix C motif, in line with the same convention used to name the NTQ and NDQ chloride and sodium pumps. The scale bar represents the number of substitutions per site (0.1 indicates 10 nucleotide substitutions per 100 nucleotides).
Figure 3.2: Multiple sequence alignment of the channel-forming helices (B, C, F, and G) for representatives of different groups of microbial rhodopsins, in which the typical conserved residues of each group are highlighted. BR — bacteriorhodopsin from *Halobacterium salinarum* (conserved residues shown in purple), GPR — proteorhodopsin from gamma-proteobacterium EBAC3108 (yellow), ASR — *Anabaena* sensory rhodopsin from *Nostoc* PCC7120 (tan), NDQ — sodium-pumping rhodopsin from *Nonlabens dokdonensis* (green), PspR — rhodopsin from *Pseudomonas putida* (blue). Positions corresponding to the functionally important residues of BR are marked by red arrows and numbered according to the BR sequence (Thr46—proton donor partner, Asp-85—proton acceptor, Asp-96—proton donor, Asp-212—Schiff base counterion, Lys-216—retinal Schiff base). Polar cytoplasmic regions of PspR are underlined red, note that the proton donor of BR is replaced by Gly and its hydrogen-bonding partner is replaced by His.
The upper branch, which is more closely related to archaeal rhodopsins (represented by BR-like proton pumps) contains ASR-like photosensors (also known as xenorhodopsins)\textsuperscript{42} and another unknown group, labelled with three question marks. From the high degree of conservation of the retinal binding pocket (see Figure 3.2), it is obvious that this group comprises true rhodopsins rather than opsin-related proteins, which do not have the retinal-binding lysine.

This previously uncharacterized group of microbial rhodopsins contains representatives of several genera of gamma and alpha proteobacteria, including \textit{Pseudomonas}, \textit{Pantoea}, \textit{Methylobacterium}, \textit{Sphingomonas}, \textit{Sphingopyxis}, and \textit{Asaia} (see Figure S1 of the Supplementary Information for the full list of sequences). The rhodopsin genes are usually found in tandem with the key genes needed for retinal production, that of β-carotene oxygenase\textsuperscript{6,97}. In a recent phylogenetic analysis\textsuperscript{89}, some members of this group were included into the same cluster as ASR-like xenorhodopsins, however, a closer look at the conserved features of both groups (see Figure 3.2 and/or Figure S1) shows that while they have higher sequence similarity to each other and BR than to their eubacterial counterparts (PR-like and XR-like proteins), they are clearly two distinct groups.

Xenorhodopsins contain many unique polar residues in their cytoplasmic halves, most of which are not conserved in the new group, even though the predominantly polar character of this domain is preserved. Most importantly, one of the main distinctive features of ASR-like proteins is the unique structure of the Schiff base counter ion (homolog of anionic Asp-212 of BR), which is replaced by a neutral residue (usually Pro) in xenorhodopsins. This is in stark contrast to the
situation in the new group of rhodopsins in which the carboxylic counter ion is preserved at this position (see Figure 3.2).

The overall sequence identity between PspR (chosen as a typical representative of the new group) and other microbial rhodopsins is somewhat low, ~30%. Close inspection of the sequences (see Figure 3.2 and/or Figure S1) indicates that several residues known to be essential for BR function are not conserved in PspR and its close homologs, or occur in a different sequence context. As summarized below, important sequence changes likely to influence protein dynamics and water interactions are largely concentrated in the cytoplasmic proton transfer hemi-channel.

On the extracellular side of the retinal moiety of PspR, the members of the extended retinal counter ion are conserved (Arg-82, Asp-85, and Asp-212 in BR, see Figure 3.3). The sequence contexts in which these key amino acid residues occur (i.e., groups adjacent in the sequence) are also largely conserved. The two hydrogen-bonding partners of BR's Asp-212, helix B Tyr-57 and helix F Tyr-185 are both conserved in the new rhodopsin family as well.

The cytoplasmic half of PspR is markedly different from that of BR, such that overall the putative proton transfer hemi-channel is more polar in PspR. The L-barrel and F-lids of BR are severely disrupted in the new group, often being replaced by polar amino acids. One helical turn away from the Schiff base Lys (BR Lys-216), a Tyr replaces Phe-219 (BR numbering) in most of
Figure 3.3: Illustration of protein regions likely important for proton transfers in PspR. (A) The cytoplasmic proton transfer channel of PspR has regions markedly different from BR. The molecular graphics depicts BR (PDB ID: 1QHJ\textsuperscript{98}) with retinal and functionally important groups shown as bonds. Carbon atoms are colored cyan, oxygen — red, and nitrogen — blue. For transmembrane helices B, C, F, and G, purple segments show where the amino acid sequence is significantly different in BR vs. PspR: helix B K40–A44 and T46–L48, helix C L95–D102, helix F F171–N176, and helix G F219–L224. For clarity, helix B is shown transparent. (B) Illustration of a cluster of polar amino acid residues on the cytoplasmic side of PspR. Starting from the crystal structure of BR (PDB ID: 1QHJ), the F42H/T46H/T47A/D96G/F171Y/F219Y/L224E mutant was created using CHARMM\textsuperscript{99} with an all-atom representation of the protein\textsuperscript{100,101} and retinal molecule\textsuperscript{75,102,103}, and TIP3P water\textsuperscript{104}; the coordinates of the mutated amino acid side chains were geometry-optimized by fixing all other atoms. The helical regions colored violet contain Ser or Thr. Collaborator, Dr. Ana-Nicoleta Bondar (of the Department of Physics, Freie University, Berlin, Germany) used VMD\textsuperscript{105} to prepare the molecular graphics, the molecule was rotated for better view as indicated by the arrow.
the new rhodopsins (Tyr-213 in Figure 3.3). Closer to the cytoplasmic side of helix G, the hydrophobic stretch I\textsubscript{222}LL of BR is missing, being replaced by a TSE motif at the cytoplasmic tip of helix G in \textit{Pseudomonas} rhodopsins. Since hydroxyl groups of Thr/Ser can make intra-helical hydrogen bonds\textsuperscript{106–108} and alter local helix dynamics\textsuperscript{109}, the conserved TS motif may be important for the local interactions and dynamics of helix G in \textit{Pseudomonas} rhodopsins. The most dramatic amino acid replacement observed in all new rhodopsins is that of the BR's proton donor Asp-96 in helix C and its hydrogen-bonding partner Thr-46 in helix B by Gly (and occasionally Ala) and His, respectively (Gly-84 and His-37 in Figure 3.3). To reflect the striking lack of the carboxylic proton donor on the cytoplasmic side, we called the new group DTG rhodopsins, in line with the nomenclature used for NTQ chloride pumps and NDQ sodium pumps\textsuperscript{35,89,90}. The His residue, His-37 in PspR, appears to be a part of a larger complex of polar and charged groups contributed by helices B, C, F, and G. His-33, which is present in a large number of the new rhodopsins, replaces Phe-42 of BR (See Figure 3.3). Close to His-33 and His-37, helix B contains Gly, Ala, or Thr amino acid residues that could contribute to the local dynamics of helix B. There are several additional amino acid replacements in PspR (when compared to BR) that may be important for the local dynamics at the cytoplasmic proton donor site. In helix C, for example, one helical turn away from BR's Asp-96, the hydrophobic L\textsubscript{99}L segment is replaced by SS in \textit{Pseudomonas} rhodopsins. On helix F, YRRN replaces F\textsubscript{171}KVL (See Figures 3.2, 3.3). A key outcome of these amino acid replacements is that, at least in \textit{Pseudomonas} rhodopsins, a cluster of polar and charged groups largely replaces the hydrophobic cluster at the tips of helices B, C and G of BR (F42, L100, and I229).

Based on the considerations above, we suggest that the proton donor region of PspR contains a complex cluster of intra- and inter-helical hydrogen bonds. The proximity of the polar
cluster to the cytoplasmic bulk indicates that water molecules likely participate in hydrogen-bonding. Indeed, preliminary MD computations on a PspR homology model appear to suggest that the cytoplasmic region of PspR could host numerous water molecules.

The introduction of His-37 (conserved for all members of the DTG group, see Figure S1) into the position homologous to Thr-46 of BR, the hydrogen-bonding partner of the proton donor, concomitant with the loss of the carboxylic proton donor, could be hypothetically interpreted in two major ways. The first hypothesis is that these rhodopsins are sensory, and the conserved His residue on helix B may serve as a proton acceptor, similar to the situation in halobacterial SR1110. The second hypothesis is that the DTG group of rhodopsins comprises light-driven proton pumps with the unique proton donating complex centered around the histidine (His-37) on helix B. To verify these hypotheses experimentally, we expressed one representative of the DTG group, from Pseudomonas putida (PspR) in E. coli, and characterized it spectroscopically and by proton transport assays. Characterization of a second representative member of this group, Pantoea ananatis (PaR), by low-temperature Fourier transform infrared spectroscopy (FTIR) and proton transport assays was performed by collaborators in Japan (Yohei Shibata, Shota Ito, Keiichi Inoue and Hideki Kandori), and the data is available in the supplementary information (See Figure S2, S3, S4 & S5).

3.2) Characterization of the dark state by visible and Raman spectroscopies

E. coli-expressed PspR has red color (absorption maxima of DDM-solubilized protein is 535 nm, see Figure S5). To investigate retinal's conformation and the environment, Raman
spectra of PspR in crude *E. coli* membranes in lysis buffer were recorded (See Figure S6). The observed position of the main ethylenic C=C stretch at 1532 cm\(^{-1}\) correlates with the absorption maximum in the visible range\(^{111}\), while the major fingerprint C–C stretches at 1200 and 1165 cm\(^{-1}\) and the lack of an observable band at 1184 cm\(^{-1}\) indicate that PspR contains mainly all-trans-retinal\(^{85}\). The retinal composition as detected by Raman has not changed upon light-adaptation (by orange light) and subsequent prolonged (up to 48 h) dark adaptation, in great contrast to BR and ASR\(^{112}\). As many minor vibrational bands were obscured by strong background signals from other proteins and lipids present in the *E. coli* membrane, Raman spectroscopy has been repeated on purified lipid-reconstituted PspR (See Figure 3.4).

The Raman spectra of purified lipid-reconstituted PspR confirmed the main features detected in the *E. coli* membranes, such as the positions of ethylenic and fingerprint peaks, predominant all-trans-retinal conformation and the lack of light- and dark-adaptation. Increased signal quality allowed for the detection of vibrations of the Schiff base and their isotopic shifts, such as N–D wag vibration at 977 cm\(^{-1}\) and the 21 cm\(^{-1}\) shift of C=NH stretch from 1646 to 1625 cm\(^{-1}\), which is believed to be proportional to the strength of hydrogen-bonding\(^{113}\). The observed isotope shift is somewhat larger than that for BR, very similar to the one found in ASR\(^{114,115}\), but smaller than those in PR and XR-like *Gloeobacter* rhodopsin (GR)\(^{116,117}\). This indicates fairly strong hydrogen-bonding of the Schiff base, which was further investigated with low-temperature FTIR by observing N–D stretching vibrations (See Figure S3).
Figure 3.4: Raman spectra of purified lipid-reconstituted PspR hydrated with H$_2$O- (red) and D$_2$O- (blue) based lysis buffer (0.15 M NaCl, 0.05 M Tris base, 1 mM MgCl$_2$, pH = 7.2), vertically offset for clarity. Positions of the C=N Schiff base stretching vibrations, ethylenic C=C stretches and fingerprint C–C stretches are shown, along with N–D wag of the Schiff base (from left to right). Isotopic shift of the C=N Schiff base stretching vibration is shown with the black arrow.

3.3) Photocycle characterization by time-resolved spectroscopy in the visible range

To characterize the photocycle of PspR, which can provide information on its physiological role, time-resolved laser spectroscopy in the visible range was applied to crude
membranes of PspR-expressing *E. coli* encased in polyacrylamide gels (See Figure 3.5). At first glance, the photocycle seems to be consistent with the proton-pumping function of PspR, as its turnover is relatively fast (tens of ms), and a strong signal of fast Schiff base deprotonation (the M intermediate at 400–420 nm) is observed, both typical for proton pumps\(^3\,^3^5\). One can also clearly observe fast and slow red-shifted intermediates (K-like and O-like).

Global multi-exponential analysis of the kinetic traces collected every 20 nm revealed additional details of the photocycle, which could be reliably represented by 5 statistically valid exponential processes (See Figure 3.6). The “Initial” spectrum represents a snapshot of the formation of the red-shifted K-like intermediate, and progression of the photocycle from this point can be followed by looking at the decay-associated spectra, where positive amplitudes represent decay and negative amplitudes represent formation of intermediates or their mixtures\(^8^2\). The first time constant \((\tau_1 = 3 \, \mu s)\) is likely to represent a decay of the red-shifted K-like intermediate into the blue-shifted L-like intermediate and its early equilibration with the M-like intermediate (analogous to the \(~1 \, \mu s\) component of the BR photocycle)\(^1^1^8\,^1^1^9\). The next kinetic component \((\tau_2 = 23 \, \mu s)\) is the main phase of the M-like intermediate formation (Schiff base deprotonation) from the K/L mixture. The third component \((\tau_3 = 0.8 \, ms)\) is likely to represent M1 to M2 sub-state transition\(^1^2^0\), in which M1 equilibrated with an L-like intermediate converts into M2 in equilibrium with an O-like intermediate. This is supported by the spectral shape of this component, which looks almost spectrally silent for the short wavelength region but shows growing absorption at the long wavelengths at the expense of the disappearing absorption at around 500 nm. The last two processes \((\tau_4 = 6 \, ms\) and \(\tau_5 = 27 \, ms)\) correlate to the two phases of the M decay (Schiff base reprotonation), the first of which is also the main decay of the O-like
Figure 3.5: Kinetics of the light-induced absorption changes in the photocycle of wild-type PspR in *E. coli* membranes encased in polyacrylamide gel and incubated at pH 6, 50 mM KH$_2$PO$_4$, 50 mM MES, room temperature.

intermediate. It appears that the O-like intermediate may not be the last intermediate of the photocycle, which has been observed for other retinal proteins$^{121,122}$. It should be noted that it is referred to as O-like here solely based on its red-shifted absorption, while its molecular structure may be more similar to the N intermediate of BR.

Based on the above data, four characteristic wavelengths have been selected for further characterization of the photocycle of the wild-type and mutant PspR under different conditions (420 nm for the M intermediate, 460 nm for the L-like intermediate, 540 nm for the dark state,
Figure 3.6: Spectra of the kinetic components of the PspR photocycle extracted by global multi-exponential analysis of the data in Figure 3.5. The spectrum labeled “Initial” represents extrapolation of the fit to zero time point. Characteristic times of the spectral components are shown in the inset and discussed in text. The negative absorption means formation of intermediate(s), whereas the positive absorption corresponds to their disappearance.

and 620 nm for the K-like and O-like states). To verify the proton-pumping function of PspR and to get insight into the mechanism of proton translocation, we studied cation and anion dependence, kinetic isotope effect, and pH-dependence of its photocycle. As expected for a proton pump, the photocycle was not strongly changed by the addition of 0.5 M sodium chloride, potassium chloride or potassium acetate to the 50 mM potassium phosphate/MES buffer (See Figure S7), but was strongly proton-dependent (See Figures 3.7 and 3.8).
First, a strong kinetic isotope effect (KIE) on the photocycle of PspR was observed upon the replacement of H$_2$O with D$_2$O (See Figure 3.7). More specifically, both deprotonation and reprotonation of the Schiff base (M rise and decay), as well as kinetics of the O-like state rise and decay show significantly slower rates. Large KIEs, such as the one observed for M formation of BR are often associated with proton transfers in hydrogen bonded networks$^{123,124}$. While the KIE observed for the overall M rise of PspR (~4.5) is similar to that observed for BR (~5), the KIE for the overall M decay (~3.5) is larger than in BR (~1.8)$^{124}$, and similar to the one observed for ESR$^{61}$. This is consistent with a mechanism of Schiff base reprotonation different...
from the classical BR-like mechanism with a carboxylic proton donor, and possibly involving hydrogen-bonded networks, in line with the polar nature of the cytoplasmic half of PspR.

Figure 3.8: pH-dependence of the photocycle of wild-type PspR in *E. coli* membranes encased in polyacrylamide gel measured at 420 nm (blue), 460 nm (cyan), 540 nm (green), and 620 nm (red). pH is 4, 5, 6, 7, 8, and 9 going from left to right.

Second, the photocycle of PspR was found to be strongly pH dependent (See Figure 3.8), in a way that is typical for known proton pumps. While the M rise, which is likely to represent transfer of the Schiff base proton to the primary proton acceptor Asp-73 (homolog of Asp-85 of BR), is virtually pH-independent, the reprotonation of the Schiff base (the M decay) has a
Figure 3.9: Photocycle of wild-type PspR in *E. coli* membranes encased in polyacrylamide gel measured at 420 nm (blue), 460 nm (cyan), 540 nm (green), and 620 nm (red). pH 3 is at the top left, pH 2 is the top right, and pH 1 is on the bottom. Notice that the vertical scales are variable.

pH-dependence consistent with the presence of an internal proton donor (See Figure S9). Such pH-dependencies tend to be shallow at low pH, where the internal proton donor is protonated, but become steeper at higher pH values\(^{48}\). It should be noted that the pKa of the primary proton acceptor of PspR should be very low, as a significant M formation was observed at pH values as low as 1 (See Figures 3.9), but it could not be measured reliably as the spectral titration was not
possible to complete without denaturing the protein. Notice that the amplitude of the M-intermediate decreases with decreasing pH, which indicates that Asp-73 is likely becoming protonated at low pH.

The photocycle of purified WT PspR reconstituted in DMPC/DMPA mixture used for Raman spectroscopy and FTIR was also investigated and the data are available in the supplementary information (See Figure S8). The signal quality is poor, likely due to scattering from the large liposomes which were easily observed through rapid sedimentation. DMPC/DMPA lipids appear to supply a near native environment (or one can conclude that the photocycle is not strongly lipid-dependent) as the rate of photocycle turnover is comparable to that of PspR in the E. coli membrane; the rate of M-rise and M-decay are only altered slightly, by a factor of ~0.9 and ~1.5, respectively.

3.4) Proton transport assays

While characteristics of the photocycle of PspR presented above are suggestive of its proton-pumping role, the direct verification of proton transport capability is necessary to confirm its putative physiological function. Thus, light-induced pH changes were measured in cell suspensions of PspR (See Figure 3.10) and PaR (See Figure S4). Robust light-induced acidification was observed in both cases, consistent with BR-like outward proton transport. To verify the active nature of the proton transport, the cells were treated with the protonophore CCCP (10 μM concentration), which greatly reduced the light-induced pH changes, as expected. An additional experiment aimed at eliminating possible cell-related artifacts was conducted on
spheroplasts made from *E. coli* cells expressing PspR, and the result was qualitatively similar to the one observed in cell suspensions, confirming the proton-pumping ability of PspR.

Figure 3.10: Light-induced pH changes in suspensions (left) and spheroplasts (right) of *E. coli* cells expressing PspR.

3.5) Investigation of proton transport mechanism by site-directed mutagenesis

To identify main participants in the mechanism of proton transport by PspR, several mutants suggested by the sequence analysis (see section 3.1) were expressed and characterized by time-resolved spectroscopy in the visible range. First, Asp-73, homologous to the primary proton acceptor Asp-85 of BR, was replaced by Asn. As expected from the phenotype of the homologous D85N mutant of BR\(^{67,125,126}\), the resulting protein was blue (strongly red-shifted absorption maximum, see Figure S10), and existed in pH-dependent equilibrium with a yellow form with deprotonated Schiff base. The spectral red-shift and the strong decrease of the pKa of the Schiff base are consistent with Asp73 being a part of the Schiff base counter ion and being
deprotonated. As expected from its role as a primary proton acceptor, the replacement of Asp-73 with non-protonatable residue resulted in a dramatic decrease of the M intermediate amplitude and much slower rate of M formation (See Figure 3.11), again similar to the respective mutant of BR. Thus, one can conclude that in PspR, similar to other proton-pumping rhodopsins, the primary proton transfer occurs between the Schiff base and a homolog of Asp-85 of BR, Asp-73.

Figure 3.11: The photocycle of the D73N mutant of PspR compared to that of the wild-type. The photocycle was measured in E. coli membranes encased in polyacrylamide gel, at pH 6.

As described above, the homolog of the cytoplasmic proton donor (Asp-96 of BR) is Gly-84 (See Figure 3.2), while its hydrogen-bonding partner (Thr-46 in BR) is replaced by histidine (His-37 in PspR), conserved in the whole DTG group. To test the hypothesis that His-37 may be the new proton donor, moved from helix C to helix B, we replaced His-37 by Asn, Tyr, and Arg,
chosen to preserve polarity, aromaticity, and charge, respectively, at the same time removing the ability to donate protons. If His-37 is the new proton donor, it is expected that replacing it with a non-protonatable residue will result in much slower reprotonation of the Schiff base (M decay), which is the case for H37R and H37Y mutants where the overall rate of M decay (in a simplistic

Figure 3.122: The photocycle of the H37R mutant of PspR at pH 6. Blue (420 nm), red (620 nm), cyan (460 nm) and green (540 nm).

single-exponential approximation) is slowed down ~11- and ~200-fold, respectively (See Figure 3.12 and 3.13 respectively, and Table 3.1). Please note that WT PspR data are available for comparison (See Figure 3.7 & 3.11). Surprisingly, M decay rate in the H37N mutant was affected only slightly (~1.7-fold slower than the wild-type), which suggests that His-37 is not a
sole proton donor, but rather part of a H-bonded proton-donating complex and Asn (but not Tyr or Arg) can fulfill its role almost as efficiently (See Figure 3.14). Polar complexes containing water molecules are familiar in the context of proton pumping microbial rhodopsins. This situation is similar to the PRC in BR described earlier (See section 1.1.5) where Arg-82, Glu-204 and Glu-194 form a complex with several water molecules to facilitate a proton release to the extracellular surface. Taken together with the observed KIE in D₂O (See Figure 3.7) it is reasonable to suggest that His-37 is likely part of a cytoplasmic proton-donating complex containing water molecules. It is interesting to note that the pH dependence of the M decay in the

Figure 3.133: The photocycle of the H37Y mutant of PspR at pH 6. Blue (420 nm), red (620 nm), cyan (460 nm) and green (540 nm). Notice the change in the scale of the x-axis.
H37N mutant (See Figure S11) is similar to that observed for the wild-type, which suggests that it does not originate from deprotonation of His-37. Another interesting observation is that deprotonation of the Schiff base (M rise) is somewhat slowed down in H37N and H37Y (1.9- and 1.5-fold, respectively), but not in H37R (See Table 3.1), which may suggest that a positive charge at this position has a long-range effect on the Schiff base. Finally, it should be noted that the H37N mutation produces another long-range effect, resulting in apparent redistribution between the K and L intermediates, which results in a dramatic decrease of the absorption changes at 620 nm in the microsecond range with simultaneous appearance of the positive kinetic feature at 460 nm.

The polar cytoplasmic patch, GHT, on helix B (Figure 3.2) contains His-33 (homologue of F42 in BR), which is conserved among most members of the new group (as mentioned earlier in section 3.1). Since it was found that H37 is likely part of a polar cytoplasmic proton donating complex, it is reasonable to ask whether H33, which is in close proximity to H37, participates in forming this complex. Similar to the H37 mutants, H33Y mutant was expressed and characterized (See Figure 3.15). This mutation was not perturbing to the reprotonation of the SB (M decay) or to SB deprotonation (M rise). When compared to WT PspR, again in a simplistic single-exponential approximation, the M-rise is only slightly faster (~0.9 fold) and the M-decay is insignificantly delayed (~1.1 fold). While the tyrosine mutation of His-37 (H37Y) produced a profound effect on the rate of M decay (~200 fold), the similar mutant, H33Y only has a moderate effect on the rate of M-decay and therefore His-33 is not likely a participant in the proton donating complex.
The proton donor partner in BR, threonine in position 46 on helix B (Thr-46) is H-bonded to the proton donor Asp-96, as mentioned earlier (section 1.1.6). In the case of PspR, His-37 is in place of Thr-46 and Gly-84 is in place of Asp-96. In an attempt to create a BR-like eubacterial proton pump with PspR as a scaffold, a double mutant (H37T/G84D) was expressed and characterized (See Figure 3.16). The rate of M rise is fast compared to the WT PspR (~0.4 fold) and the M decay is delayed (~4.4 fold slower). When the rate of M decay of WT BR at pH 7 is fit to two exponentials, there is a fast and slow component of ~2 ms and ~7 ms\(^{127}\). Here the H37T/G84D double mutant is between ~5 and ~15 fold slower than BR at pH 7, despite being measured at pH 6. As mentioned earlier (section 1.1.6), the mechanism of proton translocation in
Figure 3.15: The photocycle of the H33Y mutant of PspR at pH 6. Blue (420 nm), red (620 nm), cyan (460 nm) and green (540 nm).

BR is complex and relies on the presence and properties of many specific residues. It is thus not surprising that this double mutant is less efficient at reprotonating the SB when compared to BR or PspR. The largely polar cytoplasmic half of PspR is markedly different from the cytoplasmic half of BR, and this polar nature would be conserved in the double mutant. The proton donor, Asp-84 in the double mutant likely doesn’t possess the same pKa as Asp-96 of BR, because the hydrophobic region comprised of a leucine barrel and phenylalanine lids is largely absent in PspR, for example.
Figure 3.16: The photocycle of the H37T/G84D mutant of PspR at pH 6. Blue (420 nm), red (620 nm), cyan (460 nm) and green (540 nm).

In summary, through the use of site directed mutagenesis, we showed that Asp-73 is the proton acceptor for PspR and that His-37 is very likely part of a polar cytoplasmic proton donating complex containing water molecules. The results of the kinetic analysis of all of the mutants are summarized in Table 3.1.

After this work was published in *BBA – Bioenergetics*, Sudo and co-workers characterized three new members of the DTG group, namely, rhodopsins from *Pantoea vagans* C9-1 (PvR), *Spingomonas* sp. PAMC 26621 (SpR), and *Asaia platycodi* JCM 25414T (ApR). Robust light-induced acidification was observed in proton transport assays for all three
Table 3.1: Single-exponential approximation of the M-rise and M-decay of all PspR mutants.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>M-rise (μs)</th>
<th>Ratio to WT</th>
<th>M-decay (ms)</th>
<th>Ratio to WT</th>
</tr>
</thead>
<tbody>
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<td>-</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>WT D2O</td>
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<td>4.5</td>
<td>24</td>
<td>3.5</td>
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<td>1.5</td>
<td>1336</td>
<td>194.5</td>
</tr>
<tr>
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<td>1.9</td>
<td>12</td>
<td>1.7</td>
</tr>
<tr>
<td>H33Y</td>
<td>19</td>
<td>0.9</td>
<td>7</td>
<td>1.1</td>
</tr>
<tr>
<td>H37T/G84D</td>
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<td>0.4</td>
<td>30</td>
<td>4.4</td>
</tr>
<tr>
<td>D73N</td>
<td>288</td>
<td>14.5</td>
<td>11</td>
<td>1.7</td>
</tr>
</tbody>
</table>

rhodopsins, and the signal was impaired in the presence of CCCP, as expected. Through high performance liquid chromatography experiments on PvR, a high proportion of all-trans retinal was observed (>90%), consistent with the Raman spectroscopy findings in this work. Again in PvR, they approximated the pKa values of the proton acceptor (Asp-73) and the retinal SB (Lys-211) as 2.1 and 13.5, respectively (closer to BR 2.6 and 13.3 than eubacterial proton pump PR 7.0 and ND). This relatively low pKa of the acceptor is consistent with the PspR behaviour at low pH (See Figure 3.9). M-formation occurs at pH as low as 1, albeit with smaller amplitudes indicating that some portion of the Asp-73 population become protonated in the dark at this pH.
4) CONCLUSIONS

4.1) Summary

A new group of proteobacterial rhodopsins with proton-pumping ability was discovered (and was named DTG rhodopsins). The DTG group is most similar to ASR-like xenorhodopsins in terms of the primary structure of its members, but is distinct by not sharing the conserved features of ASR. Instead, all members of the DTG group possess a unique His residue in place of Thr-46 of BR and lack the carboxylic proton donor (Asp-96 of BR). It was proved that two proteins from this group can pump protons under illumination by using proton transport assays in suspensions of *E. coli* cells. Further, a number of features, such as fast photocycle turnover, fast Schiff base deprotonation and reprotonation (the M intermediate formation and decay), its proton dependence (both pH-dependence and kinetic isotope effects), and presence of a strongly-bound water molecule in the Schiff base vicinity, are all consistent with the proton-pumping function of the new DTG group. Site-directed mutagenesis data suggest that the unique conserved histidine residue on helix B (His-37) may be part of the proton-donating complex, but is unlikely to be the sole proton donor. The histidine in position 33 is not likely part of this complex. As expected, Asp-73 was confirmed as the carboxylic proton acceptor.
4.2) Future work

These findings further expand the range of structures and mechanisms of microbial rhodopsins and offer new scenarios of light-driven proton transport across the cell membrane. It remains to be found why the DTG group of proton pumps employed a completely different mechanism of reprotonation of the Schiff base. It is believed that the reason must be related to physical parameters of their habitat, as most of proteobacteria harboring PspR-like proteins are soil- and plant-associated, unlike the other, mainly aquatic, bacteria expressing different microbial rhodopsins. This theory is supported when one compares PspR to ESR where the situation on the cytoplasmic side of retinal is quite similar. Both proteins have atypical residues in the proton donor positions (Gly and Lys) and are both highly polar in this region as well. *Exiguobacterium sibiricum* lives in Siberian permafrost and as such, its access to water is likely limited. *Halobacterium salinarum* however, is an aquatic organism with ample access to water. Perhaps PspR and ESR have largely polar regions on the cytoplasmic side (in great contrast to BR) for drought resistance. The risk of backflow of protons in the dark is much less apparent for these proteins when compared to BR, and so PspR and ESR may be able to resist drought without the risk that would present if BR were to employ such polar regions. Investigating the polar cytoplasmic patches (SSAY and TSE) of helix C and G through site directed mutagenesis would provide further evidence for participants in the His-37 based cytoplasmic cluster, and possibly lead to an understanding of the benefit of the new proteobacterial cytoplasmic proton donating scheme.
5) REFERENCES


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6) SUPPLEMENTARY INFORMATION

6.1) Materials and methods – Standard operating procedures (SOPs)

6.1.1) SOPs for time-resolved spectroscopy in the visible range

Turn on the computer, the (Oriel Instruments) photomultiplier, the (Thermo Oriel) high voltage power supply (HVPS) and the (Spectra Physics) lamp power supply. Prepare your sample while these components warm up for approximately 20 min. If the difference between the starting and ending pH values of your gel is just 1, then incubate the gel sample in approximately 20 mL of desired buffer for 20 minutes; replace the buffer and incubate for another 20 mins. If the pH difference is 2 or greater, then incubate the gel in approximately 20 mL of desired buffer for approximately 1 hour; again replace the buffer and repeat incubation for another 30 mins. Transfer the sample into the 9SOG cuvette and hydrate with approximately 1 mL of buffer. Load the sample into the sample compartment on the spectrometer. Orient the cuvette such that the lamp light has the shortest path length (4 mm), and the laser light has the longest path length (10 mm). Turn on the Nd-YAG laser (Continuum Minilite II), open the shutter and turn on the (EZ Digital Co., Ltd.) pulse generator. It is good practice to inspect the laser cooling system for leaks or bubbles at this stage. Set the frequency to approximately 0.5 Hz (unless the desired frequency is known). Open GageScope and turn on the lamp. Locate the channel 1 signal in GageScope. Increase the offset voltage on the HVPS until channel 1 signal is offset to y = 0. Ensure that the depth under the Depth heading is set to 1,000,064 and 29,998,080 points for pre and post trigger, respectively. Set the timebase such that the whole signal is in the window (usually approximately 1 s/d). Set vertical scale for channel 1 (individual signal) and channel 3 (averaging) to 5 mV (or
something convenient). Set the sample rate (under the CS12100 heading) as high as possible without losing any of the later part of the signal. It is ideal to have high quality data (large sample rate) while avoiding any cut off. To test if there is any cut off, it is advantageous to record a short run (~100 averages), at a desired sample rate (50 MS/s), process the data and check manually if the data is cut off. If the data has been cut, repeat the run at a lower sample rate (20 MS/s). Repeat procedure until the largest sample rate without data cutting is determined; use this sample rate. Once the sample rate, depth, timebase and the vertical scale are set, save this “setup” on GageScope. When returning to perform subsequent experiments on the same or similar samples, this setup can be loaded on program start-up.

Prior to starting an experiment, the actinic laser pulse frequency must be optimized. Carefully observe channel 1 signal. Ideally, the signal should “update” for each laser pulse. If the frequency is too high, the signal will update but not for every laser pulse. Reduce the frequency slowly until the signal updates just after each laser pulse. If the frequency is too low, the signal will update but there will be a delay between laser pulse and update. While fine tuning the frequency, attempt to minimize the delay.

Once the frequency has been optimized an experiment can be performed. For PspR, the four characteristic wavelengths are 420, 460, 540 and 620 nm. Set the monochromators to 540 nm. Adjust the offset voltage on the HVPS until channel 1 signal is offset to y = 0, this represents the absorbance of the ground state at 540 nm. Set the number of averages (click Ch 03
tab, then select the Averaging tab); will usually be between 600 – 1500 accumulations. To initiate a run, click reset until Ch 03 updates. Calculate the run time.

Once the run completes, save the file (make sure it is channel 3). Repeat for each wavelength. Collect a control spectrum of 540 nm using 10% of the number of averages used for the original 540 nm trace to evaluate possible bleaching of the sample by laser.

Collect all the files into one folder. Drag and drop Gage100T.exe into the folder. Double click on Gage100T.exe. The software will prompt the user for certain information, including “trigger shift relative to laser” which is 0.00000015 s. Converted traces are plotted using Origin.

6.1.2) SOPs for Raman spectroscopy

Begin preparing the Raman (Bruker FRA 106/S) spectrometer for measurement by filling the 25 L Liquid Nitrogen (LN₂) dewar with approximately 10 L of LN₂. Carefully transfer LN₂ to the dewar on the detector, 1 L at a time (approximately 10 L total). Allow approximately 2 hours for the detector to cool. The detector is ready to use when the red LED on the dewar turns off. Nitrogen lasts for about 7 days. Change the beam-splitter to SICA (the beam-splitter is inside the case on the top of the FTIR machine). Also change the beam-splitter option in OPUS program to SICA (in the “optic” heading). Turn on the vacuum pump. Set aperture = 3 mm. Load DTGS detector (in the “basic” heading). Check signal and perform the auto-alignment (intensity ~3092 after alignment). Turn the MIR source off. Load Raman XPM (basic). Turn the
laser on. Press RESET if the orange LED does not turn on. Change the source setting to “Laser; 9394.0 cm$^{-1}$; 525 mW” (optic). Load the sulfur sample from the blue Bruker case and make a fine auto-align. Start evacuating the chamber (“EVAC. IF / VENT SM” on the Raman machine). Check signal: sulfur spectrum should have a maximum amplitude between 12 and 15. Load the sample of interest and set the number of scans to 100. Perform this short measurement to check signal quality; if adequate, select the number of scans and start the experiment. Example of scans: 15773 = 14 h of measurement, 3976 = 3 h and 30 min, 568 scans = 30 min, 190 scans = 10 min.

6.1.3) SOPs for proton transport assays

Transfer 3 mL of cells in suspension in unbuffered solution to cuvette (10 mm x 10 mm, with a small stir bar). Turn on the oscilloscope and pH meter, and insert the pH electrode into the cuvette. Allow the pH to stabilize. Adjust settings on the oscilloscope; select 20 s/d as the timebase, and 2 mV/d as the vertical scale. Turn on the lamp 20 s into the run, and turn it off at 3 min. Press Stop, then Save/Recall and switch the format to csv. Save the file onto a usb. Add 3 μL of 10 mM CCCP (carbonyl cyanide m-chlorophenyl hydrazone) and repeat the experiment as a control. Repeat whole procedure a few times, with fresh cells each time.

6.2) Supplementary figures

Figure S1: Clustal Omega$^1$ sequence alignment for the new group of rhodopsins from proteobacteria. The sequences were obtained from public genome databases
(http://www.ncbi.nlm.nih.gov/protein and http://img.jgi.doe.gov). ASR and BR are shown on top for comparison. Conserved histidine on helix B and Gly in place of the carboxylic donor on helix C are highlighted red for PspR and cyan for PaR. The “Magnetospirillum” sequence most likely belongs to some species of *Methylobacterium*.
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Figure S2: The light-induced difference FTIR spectra of the primary K intermediate minus the parent state for PspR, PaR and BR at 77 K in the 1800–850 cm\textsuperscript{-1} (A), 1750–1600 cm\textsuperscript{-1} (B) and 1000–920 cm\textsuperscript{-1} (C) regions. The samples were hydrated with H\textsubscript{2}O (solid lines) or D\textsubscript{2}O (dotted lines). In the hydrated films, PspR and PaR molecules are oriented randomly, while BR
molecules are highly oriented. The spectrum of BR is reproduced from a previous work where the sample window is tilted by 53.5°. One division of the y-axis corresponds to 0.01 for (A) and 0.005 absorbance units for (B) and (C), respectively. Performed by collaborators [Yohei Shibata, Shota Ito, Keiichi Inoue, Hideki Kandori (a: Department of Frontier Materials, Nagoya Institute of Technology, Nagoya, Japan b: PRESTO, Japan Science and Technology Agency, Japan)].

Figure S3: The light-induced difference FTIR spectra of the primary K intermediate minus the parent state for PspR, PaR, and BR at 77 K in the 2750–1950 cm\(^{-1}\) region. The sample was hydrated with D\(_2\)O (red lines) or D\(_2\)\(^{18}\)O (blue lines). Spectrum of BR is reproduced from a previous work. Green-labeled frequencies correspond to those identified as water stretching vibrations. In BR, the underlined frequencies are N–D stretches of the Schiff base. One division of the y-axis corresponds to 0.001 absorbance units. Performed by collaborators [Yohei Shibata, Shota Ito, Keiichi Inoue, Hideki Kandori (a: Department of Frontier Materials, Nagoya Institute of Technology, Nagoya, Japan b: PRESTO, Japan Science and Technology Agency, Japan)].
Figure S4: Light-induced pH changes in suspensions of *E. coli* cells expressing PaR. Performed by collaborators [Yohei Shibata\(^a\), Shota Ito\(^a\), Keiichi Inoue\(^{a,b}\), Hideki Kandori\(^a\) (\(a\): Department of Frontier Materials, Nagoya Institute of Technology, Nagoya, Japan \(b\): PRESTO, Japan Science and Technology Agency, Japan)].
Figure S5: Absorption spectra of DDM-solubilized PspR (red, in 5 mM NaCl, 10 mM Tris, 0.05% DDM, pH=8) and PaR (blue, in 100 mM NaCl, 50 mM MES, 0.1% DDM, pH = 6). PaR measurement was performed by collaborators [Yohei Shibata\textsuperscript{a}, Shota Ito\textsuperscript{a}, Keiichi Inoue\textsuperscript{a,b}, Hideki Kandori\textsuperscript{a} (a: Department of Frontier Materials, Nagoya Institute of Technology, Nagoya, Japan b: PRESTO, Japan Science and Technology Agency, Japan)].
Figure S6: Raman spectrum of *E. coli* membranes containing PspR hydrated with lysis buffer (0.15 M NaCl, 0.05 M Tris base, 1 mM MgCl$_2$, pH = 7.2). Positions of the main characteristic peaks of C=C ethylenic stretches and fingerprint C-C stretches are shown (from left to right).
Figure S7: The photocycle of WT PspR at pH 6 measured in the presence of 0.5 M C$_2$H$_3$KO$_2$ (A), 0.5 M KCl (B), and 0.5 M NaCl (C) at 420 nm (blue), 460 nm (cyan), 540 nm (green) and 620 nm (red).
Figure S8: Photocycle of WT PspR reconstituted into DMPC/DMPA lipids, pH 6 at 420 nm (blue), 460 nm (cyan), 540 nm (green) and 620 nm (red). Quality is relatively poor likely due to large size of liposomes causing light to scatter.
Figure S9: pH-dependence of the apparent transition rates of the photocycle of wild-type PspR in *E. coli* membranes encased in polyacrylamide gel. The data were obtained by the global five-exponential fitting of the four-wavelength kinetics from Figure 3.8. Time-constants T1 and T2 reflect M formation, while T4 and T5 reflect M decay. The intermediate time constant T3 most likely reflects M1 to M2 transition and the corresponding spectral component has a different sign of its amplitude depending on pH.
Figure S10: Spectral properties of the D73N mutant of PspR. Spectra were taken on the DDM-solubilized purified samples, the wild-type spectrum from Figure S5 is given for comparison.
Figure S11: Comparison of the pH-dependencies of the apparent transition rates of the photocycle of wild-type PspR (from Figure S9) and its H37N mutant in *E. coli* membranes encased in polyacrylamide gel. The data were obtained by the global five-exponential fitting of the four-wavelength kinetics. See Figure S9 for more details.
7) SUPPLEMENTARY REFERENCES

