SPECTROSCOPIC STUDIES OF NOVEL MICROBIAL RHODOPSINS
FROM FUNGI AND BACTERIA

A Thesis

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by
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ABSTRACT

SPECTROSCOPIC STUDIES OF NOVEL MICROBIAL RHODOPSINS
FROM FUNGI AND BACTERIA

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University of Guelph, 2011

Microbial rhodopsins are widespread bacteriorhodopsin-like proteins found in many prokaryotes and lower eukaryotic groups including fungi and bacteria. They serve as photosensors, light-driven ion pumps, and light-gated channels. The main goal of this thesis was to spectroscopically characterize a new subgroup of fungal rhodopsins (so-called auxiliary group) and a new type of rhodopsin found in flavobacterium Donghaeana dokdonensis.

Towards the first goal, products of two known rhodopsin genes from the fungal wheat pathogen, Phaeosphaeria nodorum, were investigated. The two yeast-expressed Phaeosphaeria rhodopsins were spectroscopically characterized by Raman, time-resolved visible and Fourier transform infrared spectroscopy and showed to have many similarities: absorption spectra, conformation of the retinal chromophore, fast photocycling, and carboxylic acid protonation changes. It is likely that both Phaeosphaeria rhodopsins are proton-pumping, at least in vitro. Auxiliary rhodopsins are suggested to have separated from their ancestors fairly recently and have acquired the ability to interact with as yet unidentified transducers, performing a photosensory function without changing their spectral properties and basic photochemistry.

In the second project, a flavobacterial rhodopsin with highly unusual sequence (DDR2) was studied, which
was the expression functional on the protein in *E. coli* by our collaborators and produced light-induced pH increases in the spheroplast suspensions, suggesting an inward H\(^+\) transport. Using time-resolved visible spectroscopy, we revealed that the photochemical reaction cycle of DDR2 was fast and strongly Na\(^+\)-dependent, suggesting that the observed inward proton transport was a passive response to the active outward Na\(^+\) transport. Analysis of the sequence alignments, combined with visible and FTIR spectroscopic studies of the wild-type and mutant DDR2, provided the clues for Na\(^+\) binding and transport mechanism.

Finally, a new isotope labeling protocol, previously used for soluble secreted proteins, was successfully implemented and optimized to produce homogeneous samples of eukaryotic rhodopsin from *Leptosphaeria* in methylotrophic yeast. Isotope-labeling extent and functionality were verified by FTIR spectroscopy, and the obtained samples gave high-resolution ssNMR spectra suitable for structural studies. This protocol for overexpression of isotope-labeled multi-spanning eukaryotic membrane proteins in *Pichia pastoris* can be adopted for challenging mammalian targets, which often resist characterization by other structural methods.
Acknowledgements

What is the difference between a Masters and a PhD degree? Well, I used four years to get the answer. The difference is what I learn during the process of pursuing a PhD degree, a kind of attitude—holding a brave heart, facing failures with smiles and having encouragement to take another challenge. These four years are the most important time in my life.

First of all, I would like to express my greatest thanks to my supervisor Dr. Leonid Brown for his kind guidance, valuable suggestions and endless patience throughout my research and this thesis writing. Honestly speaking, without his unremitting editing and comments, I could not finish my thesis on time. His explanations are always to the point. His advice was always greatly helpful. He has been encouraging me to solve problems encountered during the studies. I really appreciate to have had this opportunity to work and finish those projects under his guidance.

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Specifically, I want to express my appreciations to the following persons: Lichi, thanks for teaching me to run the FTIR machine. And without you, it would be impossible to have those pretty NMR spectra of LR. Mylene, thanks for teaching me how to perform the Raman spectroscopy. David, thanks for all your assistance in the wet lab. Bill Morton and Steven Wilson in the Machine Shop, thanks for your help in maintenance of the machines.

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<td>7TM</td>
<td>seven-transmembrane</td>
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<tr>
<td>Altern.</td>
<td><em>Alternaria brassicicola</em></td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>Arg, R</td>
<td>arginine</td>
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<td>Asn, N</td>
<td>asparagine</td>
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<td>Asp, D</td>
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<td>ASR</td>
<td><em>Anabaena</em> sensory rhodopsin</td>
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<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
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<tr>
<td>Bipolar.</td>
<td><em>Bipolaris oryzae</em></td>
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<td>BLAST</td>
<td>basic local alignment search tool</td>
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<td>BMD</td>
<td>Buffered minimal dextrose</td>
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<td>BMM</td>
<td>Buffered minimal methanol</td>
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<td>BPR</td>
<td>blue-absorbing PR</td>
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<td>BR</td>
<td>bacteriorhodopsin</td>
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<td>CCCP</td>
<td>carbonyl cyanide 3-chlorophenylhydrazone</td>
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<td>CHES</td>
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<td>Cys, C</td>
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<td>DDM</td>
<td>N-dodecyl-β-D-maltoside</td>
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<td>DDR2</td>
<td><em>Donghaeana dokdonensis</em> rhodopsin</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<td>FTIR</td>
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<td>green-absorbing PR</td>
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<td>HOOP</td>
<td>hydrogen-out-of-plan</td>
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<td>Hyster.</td>
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<td><em>Leptosphaeria maculans</em></td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>Lys, K</td>
<td>lysine</td>
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<td>MAS</td>
<td>Magic angle spinning</td>
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<td>MALDI TOF</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight</td>
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<td>MD</td>
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<td>MES</td>
<td>2-(4-morpholino)-ethane sulfonic acid</td>
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<td>MGY</td>
<td>minimal glycerol</td>
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<tr>
<td>Mycosph.</td>
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<td>N. crassa</td>
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<td>NMR</td>
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<td>RD</td>
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<td>SB</td>
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<td>SBH+</td>
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<td>Sclerot.</td>
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<td>SDS–PAGE</td>
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<td>SOB</td>
<td>super optimal broth</td>
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<td>SOC</td>
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<td>sensory rhodopsin I</td>
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<td>SR II</td>
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<td>ssNMR</td>
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<td>TAE</td>
<td>Tris-acetate-EDTA</td>
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<td>TFB</td>
<td>standard transformation buffer</td>
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<td>tetraphenylphosphonium</td>
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<td><strong>YAG</strong></td>
<td>yttrium/aluminum garnet</td>
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<tr>
<td><strong>YPD</strong></td>
<td>yeast peptone dextrose</td>
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Chapter 1

Introduction to Microbial Rhodopsins
In the beginning of Genesis, God made light before creating the rest of the world, including life. Without light, there won’t be any life. The question is how so many diverse organisms can “see” light? The secret is in the light-sensing proteins. Such proteins produce active states under illumination and induce a series of reactions to pass on the signals to the rest of the cell. Among many different photoreceptor families in nature, rhodopsins are the forerunner for study and have been well characterized in detail [1-3].

1.1. Rhodopsins

Rhodopsins are a large family of photochemically active proteins that use vitamin-A aldehyde (retinal) as their chromophore, and are widely spread among prokaryotic and eukaryotic organisms. This light-absorbing protein family possesses several common features [3]. The most characteristic ones are that all of them present similar tertiary structure consisting of seven membrane-spanning helices, and a conserved lysine in the seventh helix, that functions as the covalent binding site for the retinal chromophore via a Schiff base. Another important feature is that the retinal chromophore undergoes isomerization induced by light-activation, which triggers a cascade of photochemical events, finally leading to conformational changes in the proteins. As the photochemically-initiated conformational changes progress, propagating from the inside (the retinal-binding site) to the outside of the protein, the absorption spectrum of the molecule changes, as it is sensitive to conformational alterations throughout the protein. Thus, Fourier transform infrared (FTIR) and visible
spectroscopy are widely used to detect different photointermediates in rhodopsins [3-5].

Analysis of amino acid sequence alignment divide the rhodopsins into two clearly distinct categories, microbial rhodopsins and animal rhodopsins, sometimes called Type I and Type II, respectively. Type I rhodopsins, or microbial rhodopsins, were first observed in the archaeon *Halobacterium salinarum* [6], but now are widely found in other two domains of life (eubacteria and eukaryotic microbes) as well [7-9]. These proteins normally bind all-trans-retinal and undergo all-trans to 13-cis photoisomerization in the photocycle. Two main functions in Type I rhodopsins are light-driven ion transport (bacteriorhodopsin and halorhodopsin [10]) and photoreception (sensory rhodopsins I and II) [11, 12]. As more and more homologs are discovered in other species, they may perform some other undiscovered functions. Type II rhodopsins are represented by visual and circadian photoreceptors found until now only in higher eukaryotes, for example, in animal eyes and in pineal gland. They are coupled with G-proteins in the process of transducing signals, and can bind either form of retinal as a chromophore (11-cis-retinal as a major form and all-trans-retinal as an auxiliary one). Thus, despite the existence of notable structural differences, functions overlap between the two Types, as both Types can serve as photoreceptors [3].
1.2. Microbial rhodopsins

In the 1970s, the first discovered microbial rhodopsins (bacteriorhodopsins) were found in the cytoplasmic membrane of *Halobacterium salinarum*, a halophilic prokaryote from *Archaea*. Later, four different archaeal rhodopsins were identified and are well characterized on the protein level (Fig. 1.1). Bacteriorhodopsin (BR) [6] and halorhodopsin (HR) [10, 13] are light-driven ion pumps, for protons and chloride, respectively, absorbing maximally in the green-orange region of the spectrum. Sensory rhodopsin I (SRI) [11] and sensory rhodopsin II (SRII) [12] are phototactic receptors involved in photophilic and photophobic responses, interacting with membrane-embedded Htr transducers and adjusting bacterial swimming behavior according to the prevalent color of ambient light. They provide great model systems for membrane protein studies with two fundamental functions (active transport and sensory signaling).

For a long time, researchers thought that Type I rhodopsins were only limited to the Archaeal domain. Over the past decade, microbial genome sequencing and environmental genomics have revealed thousands of new microbial rhodopsins, the archaeal rhodopsin homologs, in a broad phylogenetic range, in the other two domains of life, *Bacteria* and *Eukarya* [3, 7, 9, 14, 15]. As those dramatic discoveries came out, the perception of microbial rhodopsins underwent revolutionary change in functional, taxonomic, and ecological diversity aspects. From being an esoteric handful of halobacterial light-driven proton and chloride pumps and related photosensory
receptors, they have become a large widely-spread multi-functional group found not only in *Archaea*, but in many *Bacteria* and *Eukaryota*, including numerous fungi and algae [16, 17].

![Diagram of different rhodopsins](image)

**Figure 1.1.** The four different archaeal rhodopsins in *Halobacterium salinarum*. Bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I (SR I), and sensory rhodopsin II (SR II) with components of their transduction chains are shown. Image reprinted from [18]. Copyright (1998), John Wiley and Sons.

These homologs of archaeal rhodopsins in other domains demonstrate strong amino acid conservation of the retinal-binding pocket [3, 14]. Comparing primary structure information from the public sequence databases, for example, as done by basic local alignment search tool (BLAST), has resulted in the classification of the microbial rhodopsins into two major subgroups: rhodopsins (RDs) and opsin-related proteins.
(ORPs), the latter lacking the Lys residue necessary for covalent binding of retinal via the Schiff base. Many prokaryotic and eukaryotic species have multiple forms of RDs and ORPs. This multiplicity of RDs and ORPs could be achieved both by gene duplication (often, multiple) and by lateral gene transfer [7]. Discovery of the channelrhodopsins in the green alga *Clamydomonas reinhardtii* opened a new chapter for microbial rhodopsins [17, 19, 20]. The functions of microbial rhodopsins were no longer limited to ion-pumping or photosensory ones, but included light-gated ion channels as well. Further, with the expanding taxonomic diversity of rhodopsin hosts came additional new functions, including new types of photosensors, light-gated ion channels, and even light-activated enzymes (enzymerhodopsins) [21].

1.3 Brief Overview of Biophysical Methods as Applied to Rhodopsins

As the history of rhodopsin research (including this work) is intimately tied to spectroscopic techniques, it will be useful to briefly survey the most popular methods used in the field for better understanding of the presented data.

1.3.1. Visible Spectroscopy

1.3.1.1. Static visible spectroscopy

Usually, the static visible spectroscopy refers to electronic absorption spectroscopy in the visible (and near UV) spectral region. The visible light can be absorbed by proteins and other molecules if it induces electronic transitions in their chromophores. The visible absorption directly reports on the electronic structure of the chromophores,
and as such can be also sensitive to their environment. Basically, absorption of visible light often occurs in conjugated π systems and metal complexes. In the conjugated π systems, formed by alternating single and double bonds, absorbed photons produce transitions between different electronic energy levels, in the simplest case, between the ground and the first singlet excited state. Individual chromophores have their characteristic absorption maxima. However, the absorption wavelength maximum of a chromophore depends on environmental factors, such as temperature, polarity, and pH, and can serve as a valuable probe. Additionally, it can be used for protein quantification (using Beer’s law) and is useful in estimating protein purity, by comparing the ratio of the absorbance in the visible (the chromophore) and in the near UV (aromatic sidechains).

Rhodopsins have a retinal chromophore located in the core of transmembrane α-helices. Being a conjugated π system chromophore, retinal can absorb different wavelength of visible light depending on its ionization state and protein environment (so-called opsin shift). For example, the absorption $\lambda_{\text{max}}$ is around 380 nm for free retinal in solution. When incorporated into the protein environment, its $\lambda_{\text{max}}$ shows a large red-shift (e.g., to 480-500 nm, so-called opsin shift) in the protonated form (protonated Schiff base, positively charged) and smaller red-shift (to 400-420 nm) in the deprotonated neutral form [22]. Different isomers of retinal (such as all-trans, 13-cis, 11-cis, and 9-cis) have different absorption maxima as well [23]. Therefore, measuring static spectra of rhodopsins can provide information about the protonation
and conformational state. It can also report on the environment of the protein-bound retinal, as the absorption $\lambda_{\text{max}}$ is sensitive to the neighbouring protein charges, such as that of the carboxylic counterion or bound chloride ion [23]. As a result, the $pK_a$ values of various protein groups, including the retinal Schiff base itself, can be identified by spectrophotometric titrations [24].

1.3.1.2. Time-resolved visible spectroscopy (flash-photolysis)

Time-resolved spectroscopy in the visible range is an important tool to investigate the structural changes of chromophore/protein complexes, especially when their kinetics includes several intermediate steps. It has additional advantages compared to the static spectroscopy as it can follow kinetics of redistribution of electrons in the chromophore by monitoring the change of spectra in time. One can detect the structural changes in the chromophore by the shift of wavelength and/or intensity of absorption bands.

After a laser flash, the retinal chromophore will undergo a series of structural changes that finally induce the structure changes of the protein through retinal-opsin interactions. Time-resolved spectroscopy provides the opportunity to investigate this cyclic process at very high time resolution. Different structural states of rhodopsins during the photocycle are called photocycle intermediates. Conveniently, different intermediate states are distinguishable spectroscopically or kinetically [25]. By following the absorption changes at several characteristic wavelengths, one can
monitor the presence and absence of the photocycle intermediates. Thus, protonation state and isomerization of the retinal can be analyzed \textit{in situ} in real time. As proton translocation inside rhodopsin is undoubtedly related to the protonation state of the retinal chromophore, one can deduce the protonation/deprotonation state of the primary proton donor and acceptor in the photocycle as well. At the same time, one can measure proton uptake and release to the external medium with pH-indicator dyes (such as pyranine and fluoresceine) [26]. In spectroscopy of BR, absorption changes are often followed at 420 nm to monitor protonation state of the retinal Schiff base (M intermediate), at 460 nm to monitor the relaxation of the retinal to 13-cis (L intermediate), at 560 nm to measure disappearance of the initial state (ground state) and reprotonation of the proton donor (N intermediate), and at 620 nm to measure reisomerization of the retinal and deprotonation of the primary proton acceptor (K and O intermediates). With the help of site-directed mutagenesis, time-resolved visible spectroscopy can provide even more useful detailed information about the photocycle. The changes of the rise and decay rates of different intermediates can be influenced by mutations and report on the involvement of the mutated sites in the corresponding conformational or protonation changes. Any alteration of the spectra and/or kinetics provides the information about the function of the mutated residue (or residues). The difference in the visible spectra can be explained by the influence of the mutated residue on the primary proton donor/acceptor or the Schiff base. Data from many mutants in BR and other retinal proteins has greatly enriched the understanding of the proton translocation steps in the photocycle and predicted structurally or chemically
active important positions in these proteins [27, 28].

### 1.3.2. Vibrational spectroscopy

Molecules consist of atoms, which can be represented as masses connected by elastic bonds, and which are never stationary. As a result, they can perform periodic motions, as they have vibrational degrees of freedom. Polyatomic molecules with n atoms possess 3n-6 normal modes, which define their vibrational spectra. The same is true for proteins. Two main types of vibrational spectroscopy, infrared (IR) absorption and Raman, can yield similar type of information in general but some of it can be complementary. Both of them can detect infrared absorption bands of single molecular groups of a protein and their absorbance changes down to femtosecond time resolution [29]. However, the selection rules are not identical for IR and Raman spectroscopy. Infrared spectroscopy is mostly based on absorption spectroscopy within the infrared region of the electromagnetic spectrum. It is widely used in structural analysis of proteins under various conditions, such as in aqueous solutions (with some limitations related to strong water IR absorbance) and in membranes. From the IR spectra, one can obtain many characteristic vibrational bands, with the intensity dependent on the magnitude of the electric dipole moment changes, which belong to peptide groups and side chains in the protein. In contrast, Raman spectroscopy relies on inelastic scattering of a light by molecules. The incident laser light hits molecules and the photons can lose or acquire energy corresponding to the differences between vibrational energy levels. Intensity of a Raman band depends on
the magnitude of the change in bond polarizability. The vibrational spectra of proteins exhibit several relatively intense peaks which change only slightly in wavenumber and intensity from one sample to another. These bands are associated with the CONH backbone moiety. The slight variations which are, in fact, observed, particularly in terms of position, may yield information regarding the structure and conformation of the investigated molecule. There are nine prominent bands, which, in order of decreasing wavenumber, are referred to as amide A, amide B, and amide I to VII [30, 31]. Among these, only amide I, II, III, A, and B bands are observed in IR, whereas amide I, III, A, and B bands are observed in Raman spectra.

1.3.2.1. Fourier-transform infrared difference spectroscopy

As the structural biophysical techniques, such as neutron/x-ray scattering and nuclear magnetic resonance (NMR), greatly advanced in the last several decades, more and more details about the protein structures and dynamics became known. For example, the structure of the first membrane protein, photosynthetic bacterial reaction center, at atomic resolution was provided by X-ray crystallography. However, it only reported the static view of three-dimensional structure in the ground state and did not give insights at the light-induced changes in the protein-cofactors interactions [29].

Fourier transform infrared (FTIR) difference spectroscopy is an ideal and important tool for observing structure-function dynamics in real time [32, 33]. The most important and prominent bands in the infrared spectrum of a protein are amide I (C=O)
and amide II (mostly C-N-H) vibrations [30, 34]. Water has strong absorption in the mid-infrared region as well. The absorption of protein backbone and water overshadow the much smaller absorption bands of single residues. Due to this reason, difference spectra are taken between different protein states to investigate the small absorption bands of some functionally active residues, regardless of large background absorption of the protein (Fig. 1.2). Only absorption bands from those groups undergoing reactions would show up in the difference spectra and the quiescent background is subtracted to simplify the spectrum. In the Fig. 1.2, BR gives a good example of the difference spectra recorded for different intermediates. The changes in absorbance can be determined on the order of $10^{-3}$ OD unit against a background absorbance of up to 1. Highly sensitive instrumentation is desired to observe those small changes. FTIR spectroscopy, which converts raw interferogram data into the actual spectrum by a mathematical algorithm called Fourier transform, is able to reliably detect such small changes due to the multiplex and the Jacquinot advantages. The former allows shorter measuring times, while the latter reflects the higher light throughput of interferometers in comparison with dispersive instruments [29, 35].
Figure 1.2. Left: Diagram of the mechanism of reaction-induced infrared difference spectroscopy [36]. Reprinted with permission. Copyright 2001, American Chemical Society. Subtraction of a protein absorbance spectrum in state A from that of the protein in state B. Right: Difference spectra of intermediates of BR (L-BR at pH 6.6, 20°C, M-BR at pH 8.4, 20°C, N-BR at pH 8.4, 20°C and O-BR at pH 4.0, 40°C) [37]. Reprinted with permission. Copyright 1997, American Chemical Society.

Time-resolved FTIR techniques (introduction to two common and useful methods):

1. Static FTIR: This is the simplest technique and easy to operate. The data acquisition finishes in several seconds. As this technique is at a low time resolution, the stabilization of the transient intermediates for a few minutes is necessary. For example, by cooling, by pH variation, or by photostationary accumulation [29].

2. Rapid Scan: The principle is simple. The reference spectrum of the protein in the
ground state is measured first. After activating the protein (e.g., by a laser flash), the interferograms of the activated state could be acquired in times much shorter than the half-lives of the reactions [35]. The time pattern of such an experiment in the upper trace of Fig. 1.3 shows the “Take Data” signal. Its “high” state indicates interferogram recording. The lower trace schematically shows the reaction pathway. The first two reference (R) interferograms reflect the ground state (A), and the following three interferograms (1, 2, 3) are taken during the reaction pathway (B,C,D) [29, 35].

![Diagram of the rapid-scan method.](image)

**Figure 1.3.** Diagram of the rapid-scan method. Image reprinted with permission from John Wiley and Sons, copyright (1995). Two interferograms are taken as a reference, the reaction is initiated, and interferograms are recorded during the reaction from A to D. Then the difference spectra are calculated. The first difference spectrum is a control for the baseline quality [35].

1.3.2.2. Raman Spectroscopy

Compared to IR, Raman spectroscopy has its own advantages. As mentioned above, the water has large absorption in IR, while it produces very weak effect on the Raman spectrum. Different forms of samples such as crystal, powder, aqueous solution, and
gels are friendly to Raman spectra. The molecular properties of a compound could be examined by analyzing changes of the Raman intensity and shifts of frequency. Some bands which are weak or inactive in IR, can be observed by Raman. Another interesting feature of Raman spectroscopy is its resonance enhancement, which allows observing bands pertaining to the chromophore only.

1.3.2.3. Important vibrational bands

Amide bands arise from in-plane vibrations of peptide bond and the adjacent bonds. Fig. 1.4 shows vibrations responsible for amide I and amide II bands.

**Figure 1.4.** The vibration modes of peptide bonds.
1.3.2.3.1 Amide I region

Among the major protein bands, the amide I is the most useful for protein structural analysis. The amide I region of the IR spectrum is located within the range of 1600 to 1700 cm\(^{-1}\), with an absorption maximum between 1620 and 1700 cm\(^{-1}\) for most proteins [38, 39]. It arises predominantly from the C=O stretching vibrations with minor contributions from the out-of-phase CN stretching vibrations, the CCN deformation and the NH in–plane bend. The latter is responsible for the minor sensitivity of the amide I band to N-deuteration of the backbone. The extent to which the several internal coordinates contribute to the amide I normal mode depends on the backbone structure. Hydrogen bonding between carbonyl and amide groups, as well as interactions with the solvent dictates the exact nature of this vibration. So it also acts as an indicator of the secondary structure [4, 5]. The amide band frequency in this region is demonstrated to correlate with secondary structure. However, the assignments for secondary structure in IR and Raman spectra are somewhat different. Amide I bands in 1660-1650 cm\(^{-1}\) region can be attributed to \(\alpha\)-helices, 1640-1620 cm\(^{-1}\) to \(\beta\)-sheets, 1695-1660 cm\(^{-1}\) to \(\beta\)-sheets and \(\beta\)-turns, and 1650-1640 cm\(^{-1}\) to unordered structures in IR spectrum [29]. Whereas, for Raman spectra the bands at 1650-1657 cm\(^{-1}\) are assigned to \(\alpha\)-helices, bands at 1665-1680 cm\(^{-1}\) are assigned to \(\beta\)-sheets and bands at 1666-1668 cm\(^{-1}\) are assigned to unordered structures, respectively [31]. Because of the strong overlap with water vibrations, secondary structure analysis is often performed on Amide I band of proteins hydrated with heavy water (D\(_2\)O).
1.3.2.3.2. Amide II region

The amide II region is found at approximately 1550 cm\(^{-1}\) in H\(_2\)O and 1450 cm\(^{-1}\) in D\(_2\)O (named amide II’). It is the out-of-phase combination of the NH in-plane bend and the CN stretching vibration with smaller contributions from the CO in-plane bend and the CC and NC stretching vibrations. The amide II is one of the main components of IR spectra and is quite strong. In contrast, it is quite weak or not even present in Raman spectra. Like the amide I vibration, it has the correlation between secondary structure and frequency, but less straightforward than for the amide I [30, 38]. Due to its deuterium sensitivity, it is often used to probe the presence of compact secondary structures [40, 41], which produce much slower H/D exchange kinetics than the exposed disordered parts.

1.3.2.3.3. Retinal

The retinal Schiff base vibrations are located between 800 and 1700 cm\(^{-1}\) (containing, among others, HOOPs, C-C stretches, methyl rocks, C-H and N-H in-plane bends, C=C and C=N stretches). These are related to vibrations of the individual bonds of the retinal (with specific signatures available for all-trans or 13-cis configuration) [5, 42-44]. In the Fig. 1.5, the retinal vibrations such as C-C and C=C stretches, as well as other vibrations, are assigned in dark-adapted/light-adapted retinal in BR from the Raman spectra.
Figure 1.5. Resonance Raman spectrum of retinal chromophore in BR. The dominant internal coordinate contributions are indicated for the major vibrations. Left: Spectrum of all-trans-retinal in light-adapted BR₅₆₈ [43]. Reprinted with permission. Copyright 1987, American Chemical Society. Right: Spectrum of 13-cis-retinal in dark-adapted BR₅₄₈ [45]. Reprinted with permission from. Copyright 1987, American Chemical Society.

1.3.2.3.4. Amino acid side chains

Vibrations of most amino acid side chains are really difficult to distinguish in the IR spectra due to overlap in the absorption with other side chains or with protein backbone. However, as some side chains are in the core of the molecular reactions of proteins, it is useful to study the microenvironment of the side chains. With isotopic labelling and difference spectroscopy, IR can be successfully utilized to gain insights into the function of amino acid residues and ionization state of the side chains in the
protein. The most successful case is bacteriorhodopsin. Information about the carboxylic acids in BR obtained from FTIR difference spectra can be used to follow the proton translocation during the photochemical reactions [4, 46, 47]. The absorption of C=O of individual protonated carboxyl groups could be distinguished from each other and are far enough from amide I region in the spectra (Table 1-1). The other exception is side chain SH of Cys. Side chains of many other amino acids can be masked by the amide I band. In the case of Gln and Asn, their absorption is at 1660-1690 cm\(^{-1}\) in H\(_2\)O and down-shifts 30 cm\(^{-1}\) in D\(_2\)O. While the amide I band shifts up to 15 cm\(^{-1}\) in D\(_2\)O, the bands belonging to Gln and Asn can be identified by larger shifts due to H/D exchange [38, 48].

<table>
<thead>
<tr>
<th>Assignments</th>
<th>Band position/cm(^{-1}), in H(_2)O</th>
<th>Band position/cm(^{-1}), in D(_2)O</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys, Asp, Arg, Lys, Glu, Asn, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>2500</td>
<td>1649</td>
<td>Without H-bond up to 1762 cm(^{-1}) observed in proteins (Fahmy et al. 1993). Single H-bond shifts 25 cm(^{-1}) down. Above (\sim)1740 cm(^{-1}) inverse correlation of v(C=O) with (\varepsilon) (dissipation constant) (Doumas &amp; Beaven, 1995).</td>
</tr>
<tr>
<td>Gln, Asp, Arg, Phe, His, Lys, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1712 (220)</td>
<td>1706 (280)</td>
<td>Expected to be up to 30 cm(^{-1}) higher without H-bond. See also Asp v(C=O)</td>
</tr>
<tr>
<td>Arg, Asp, Glu, Lys, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1565-1605 (420-490)</td>
<td>1605-1608 (460)</td>
<td>Position depends on the salt bridge between Arg and other residues only for H(_2)O not for D(_2)O. In H(_2)O near 1625 cm(^{-1}) without salt bridge. In protonated up to 1095 cm(^{-1}) (H(_2)O) and down to 1955 cm(^{-1}) (D(_2)O) (Chungade et al. 1975; Berendes &amp; Braunstein, 1990; Budinger et al. 1995; Braunstein et al. 1995).</td>
</tr>
<tr>
<td>Glu, Asp, Lys, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1600-1668 (560-360)</td>
<td>1635-1654 (150)</td>
<td>Between 1619 and 1696 cm(^{-1}) in proteins (Hemmerich et al. 1997).</td>
</tr>
<tr>
<td>Arg, Asp, Glu, Lys, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1514-1566 (300-340)</td>
<td>1531-1536 (100)</td>
<td>Position depends on the salt bridge between Arg and other residues only for H(_2)O not for D(_2)O. In H(_2)O near 1635 cm(^{-1}) without salt bridge. In protonated down to 1576 cm(^{-1}) (Chungade et al. 1975; Braunstein et al. 1995).</td>
</tr>
<tr>
<td>Hid(_1)(^{-}), v(C==C)</td>
<td>1631 (290)</td>
<td>1600 (151), 1621 (33)</td>
<td>Only one strong band observed for 4-methylimidazole at 1615 (H(_2)O) and 1605 cm(^{-1}) (D(_2)O) (Haregno et al. 2000)</td>
</tr>
<tr>
<td>Lys, Asp, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1620-1629 (60-130)</td>
<td>1610</td>
<td>(\delta)H(_2)O band position based on shift observed for CH(_2)OH-Cl and CH(_2)N(_2)H(_4)Cl</td>
</tr>
<tr>
<td>Asp, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1614-1621 (85-150)</td>
<td>1612-1618 (160)</td>
<td>(\varepsilon) estimated relative to 1517 cm(^{-1}) band, Tyr or p-cresol</td>
</tr>
<tr>
<td>Pro, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1612-1622 (80-160)</td>
<td>1618</td>
<td>Tyr or p-cresol</td>
</tr>
<tr>
<td>Tyr, v(C==C)</td>
<td>1612</td>
<td>1612</td>
<td>Doublet due to two promonotonic resonances of His</td>
</tr>
<tr>
<td>Pro, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1599-1602 (100)</td>
<td>1603 (150)</td>
<td>May shift (-6) to (-40) cm(^{-1}) (Takken, 1985; Nara et al. 1994) upon cation chelation, in extreme cases band position as for v(C==C) (Deacon &amp; Phillips, 1980)</td>
</tr>
<tr>
<td>Glu, Asp, Lys, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1574-1579 (280-380)</td>
<td>1584 (820)</td>
<td>See Asp, v(C==O)</td>
</tr>
<tr>
<td>Glu, Asp, Lys, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1556-1560 (450-470)</td>
<td>1567 (830)</td>
<td>(\delta)H(_2)O band position based on shift observed for CH(_2)OH-Cl and CH(_2)N(_2)H(_4)Cl</td>
</tr>
<tr>
<td>Lys, Asp, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1526-1527 (76-106)</td>
<td>1570</td>
<td>Tyr or p-cresol</td>
</tr>
<tr>
<td>Asp, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1516-1518 (340-430)</td>
<td>1513-1517 (100)</td>
<td>Infrared spectrum</td>
</tr>
<tr>
<td>Pro, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1495</td>
<td>1496</td>
<td>Tyr or p-cresol</td>
</tr>
<tr>
<td>Pro, Glu, His, Pro, Val, Leu, Ile, Thr, Ser, Tyr, Phe, Trp</td>
<td>1496</td>
<td>1496</td>
<td>Tp Raman spectrum, observed in the Tp Raman spectrum at 1407 cm(^{-1}) (Grunert &amp; Vold, 1976).</td>
</tr>
</tbody>
</table>
Table 1-1. An overview of the IR absorption of amino acid side chains in H₂O and D₂O. Reprinted from [49], Copyright (2000), with permission from Elsevier.

### 1.3.2.4. Band assignments

The IR difference spectrum usually contains many different bands, which indicate the wealth of information that is encoded in the spectrum. Nearly all bands in a spectrum contain contributions from several different groups within the protein. It is thus necessary to separate these bands into their constituents and to assign these bands to specific structural groups. These band assignments are most often accomplished by...
studying model compounds, by chemical modifications of cofactors or ligands, by site directed mutagenesis, in which the removal of a specific group will remove its contribution from a spectrum (thus allowing the interpolation of its contribution), or by isotopic labeling (commonly hydrogen/deuterium exchange or $^{13}$C and $^{15}$N replacement), in which the difference of mass of these atoms alters the characteristic vibrations of the molecules, shifting the location of their absorption bands. Fig. 1.6 shows the band assignments in the M state of BR from FTIR spectra [38].

![Diagram of amide regions of protein backbone, vibrations of amino acids and retinal in BR.](image)

**Figure 1.6.** Diagram of amide regions of protein backbone, vibrations of amino acids and retinal in BR. Reprinted from [38], copyright (2002), with permission from Cambridge University Press. Positive bands correspond to the M state and negative bands to the ground state. Assignments are indicated as reviewed by Maeda [5]. The details of the C—C assignments are taken from Raman studies [43]. Measurements were performed at 20°C and pH 8.4 using Attenuated Total Reflectance (ATR) technique. For experimental details see [37].
1.4. Bacteriorhodopsin from *Halobacterium salinarum*

Bacteriorhodopsin is the most famous archaeal rhodopsin due to the fact that it is the oldest microbial rhodopsin possessing many features, which make it a highly desirable sample for biophysicists and biochemists. As it is extremely stable, easy to produce in large quantities, can be excited repeatedly and produces large optical and electric signals, almost every possible biophysical technique was applied to this protein at some point. Its essential features are also strongly conserved in the other three archaeal rhodopsins [3]. Interestingly, due to their structural similarity, haloarchaeal rhodopsins seem to be functionally interconvertible. BR and HR are able to switch functions under certain pH and in the presence of certain anions [50, 51]. Moreover, the D85T and D85S mutations in BR convert this proton pump into a chloride pump with HR-like photoreactions [52, 53]. Similarly, it was found that single mutations in SRI can give it SRII-like responses [54, 55]. In the absence of their cognate transducers, sensory rhodopsins can pump protons [56, 57]. Recently, it was shown that BR, mutated at a few strategic places, can work as a photosensor [58].

For the practical reasons mentioned above in combination with its fundamental importance in bioenergetics [59], BR has been studied extensively and these studies yielded many great insights [60, 61]. The different intermediate states in the BR photocycle are clearly described by various methods, such as FTIR [62-65] and visible spectroscopy [66], X-ray diffraction [67, 68], and solid-state NMR spectroscopy [69, 70], and each step in the transmembrane proton translocation has
been clearly described. Considering its conceptual simplicity (26.5 kDa integral membrane protein) and experimental advantages, BR is widely used as a model in the studies of novel type I rhodopsins and other photosensors and ion pumps.

The retinal chromophore in BR undergoes an all-trans to 13-cis isomerization after excitation by light and stores sufficient energy to begin a chain of thermal relaxations, which drive a series of tightly coupled conformational changes [71]. These changes finally result in protons pumping across the membrane through a series of deprotonations and reprotonations of the Schiff base and key carboxylic residues [60]. After photoisomerization of the retinal, the photocycle of BR shows several spectrally and structurally distinguishable transient states called K, L, M, N, and O, as revealed by spectroscopy and crystallography (Fig. 1.7). The total reaction can be dissected into five steps, (i) deprotonation of the retinal Schiff base and protonation of Asp-85, the proton acceptor located on the extracellular side (L \rightarrow M), (ii) the proton release to the extracellular side (M intermediate state), (iii) reprotonation of the Schiff base by Asp-96, the proton donor on the cytoplasmic side (M \rightarrow N), (iv) reprotonation of Asp-96 from the cytoplasmic surface (N intermediate state), and (v) deprotonation of Asp-85, reprotonation of the proton release site (N \rightarrow O) [72]. The final result is the movement of a proton from the cytoplasmic surface to the extracellular one.
Figure 1.7. The photocycle of bacteriorhodopsin is composed of six distinct states, BR, K, L, M, N, and O. Reprinted from [73] with permission from Elsevier. The reversible steps in the photocycle are shown with double-ended arrows. The proton release and uptake take place at the extracellular and cytoplasmic side, respectively.

The two halves of the protein, characterized by the extensive hydrogen-bonded network on the extracellular side and a large cluster of hydrophobic residues on the cytoplasmic side, play two different roles in the cycle, release and uptake of the proton, respectively [73]. Exquisite molecular details are known about the proton transfers in BR, as described in the following. In step one (L→M), the proton of the Schiff base is driven by the energy conserved in a distorted retinal backbone and in twists of two double bonds to transfer to Asp-85, the primary proton acceptor [73]. The proton may pass through Asp-212 or side-chain OH of Thr-89, and then transfers to Asp-85 via the water molecule (wat) 402 (Fig. 1.8). The Asp-85 and the proton release site at the extracellular surface are coupled by the shuttling of the side-chain of
Arg-82. In the BR state, Arg-82 is stabilized by electrostatic interactions with the anionic Asp-85 and a hydrogen-bond to the water molecule (wat) 407. In step two (M), the protonation of Asp-85 directly causes the collapse of this network and the positively charged Arg-82 shifts from near Asp-85 and Asp-212 region towards the anionic Glu-194/Glu-204 pair [72]. The Glu-194/Glu-204 pair releases the proton to the extracellular side [24, 74-76]. In the BR state, the chain of bonds begins from Ala-215 in the π-bulge [77] to Asp-96 (W182-wat501-A215-K216-wat502-T46-D96) (Fig. 1.9). The isomerization of the retinal forces the displacement of the π-bulge, which breaks the hydrogen-bond between Trp-182 and wat502. Consequently, it causes movements in the chain, finally moving Thr-46 away from Asp-96. Trp-182 is then stabilized by hydrogen-bond to Thr-178 via wat501 (T178-wat501-W182), and a new chain of three water molecules (Lys216-wat502-Thr46, wat502-wat503-wat504-Asp96) is gradually set up to work as a proton-conducting pathway on the cytoplasmic side [74]. Consequently, in step three (M→N), the proton transfers from Asp-96, the primary proton donor, to the Schiff base by this proton-conducting pathway. In step four (N), Asp-96 regains the proton with the help of the numerous acidic residues on the cytoplasmic surface (Asp-36, Asp-38, Asp-102, Asp-104, and Glu-166), which operate as buffering groups and capture the proton from the cytoplasmic side, and then facilitate funneling of the proton to Asp-96 [78]. The last step does not require structural rearrangements on either side, as it uses the existing network of water molecules to reprotonate the extracellular proton release group [73].
Figure 1.8. View of structural changes in the extracellular region (including the retinal) between the BR (a) and the M₂' (b) states. Image reprinted with permission from [79], Copyright (2004), American Chemical Society. Data from the PDB files 1M0L (a) and 1C8S (b). Color coding represents electrostatic properties, red is negative and blue is positive. Red ball represents the water molecule. Bottom is the extracellular surface, where a proton is released.
Figure 1.9. View of BR in the cytoplasmic region. Hydrogen bonds are in gold in the BR state, green in the M intermediate. Image reprinted from [74], Copyright (2000), with permission from Elsevier. The BR state is in beige color, from the E204Q mutant; earlier M state is in blue color, from the E204Q mutant; late M state is in red color, from D96N. (a) Comparison of the BR and the early M states of the E204Q mutant. (b) Comparison of the BR and the late M states of the D96N mutant.
1.5. Proteorhodopsins – homologs of BR from eubacterial domain

The first eubacterial homolog of archaeal rhodopsins was discovered by genomic analysis of marine bacterioplankton, in \( \gamma \)-Proteobacteria of the “SAR86” group, from Monterey Bay, California. The gene was later cloned and functionally expressed in \( E. \) coli by Beja and co-workers [80]. The expressed protein was named proteorhodopsin (PR), and it was found to function as a light-driven proton pump upon addition of retinal. The new bacterial rhodopsin PR has maximum absorption \( \lambda_{\text{max}} \) at 520 nm, matching well with the available irradiance in the ocean’s upper water column. PR exhibited a rapid photochemical cycle (tens of ms half-time) with intermediates and kinetic characteristics close to those of BR [81, 82]. Later, many different PR genes have been reported in marine plankton from different sea areas, including the Red Sea, the Mediterranean Sea [83, 84], and other regions [85-87], and suggested a significant phototrophic role for these globally distributed marine microbes [88]. Work done by Venter and co-workers revealed the abundance of PR genes (more than 782 new genes) in seawater samples of the Sargasso Sea near Bermuda [89], confirming this idea. Recently, it was shown that green-absorbing PR may be especially beneficial upon carbon starvation [90].

The PRs were classified into green-absorbing PRs (GPR) and blue-absorbing PRs (BPR) based on the wavelength of absorbed light [80, 91, 92]. From the analysis of the amino acid sequence of PRs, it is apparent both the primary proton acceptor (homologs of Asp-85 in BR) and the primary proton donor (replaced by Glu,
homologs of Asp-96 in BR) are conserved, but the extracellular proton-releasing glutamate complex present in BR is lost [15]. GPR was first demonstrated to have light-driven outward-directed proton transport in *E. coli* cells, but later suggested to mediate both outward and inward directed light-driven proton transport in oocytes and BLM-attached liposomes [82], even though it is still disputed [93]. Fig 1.10 shows models of the photocycles of GPR, which transport protons inward at pH 5 but outward at pH 10. The pKₐ of the primary proton acceptor in GPR (Asp-97) is about 7.5, much higher than that of BR (2.5), suggesting a different environment of Asp-97 and a higher proton affinity in GPR. The Asp-97 is much more weakly coupled to Arg-94 than Asp-85 of BR is coupled to Arg-82 in BR [94]. Previously, it was suggested that BPRs are spectrally tuned for deep ocean environment [83, 88, 92]. BPR takes an order of magnitude longer time to finish the photocycle than GPR, which suggests that BPR performs a different function [92] or has adapted to lower light intensities [95]. PRs can be arranged in three functional categories: GPR-like group functioning as fast-cycling proton-pump, BPR-like group performing slow-cycling proton-pumps with unknown function, and non-transporting photosensors [96].

It should be noted that a number of other eubacterial rhodopsins, distinct from PR, have been identified. For the sake of brevity, only a few most interesting and unusual cases will be mentioned. First, *Anabaena* Sensory Rhodopsin (ASR), a unique photochromic photosensor interacting with a soluble transducer (in contrast to
halobacterial membrane-spanning Htr transducers) was found in cyanobacteria [97, 98]. Next, a light-driven proton pump with an auxiliary carotenoid antenna, xanthorhodopsin (XR), was identified in *Salinibacter ruber* [99, 100]. Finally, a large number of freshwater homologs of XR (so-called actinorhodopsins) was found recently [101].

**Figure 1.10.** Photocycle models of GPR at pH 5 and pH 10. Image reprinted from [82], Copyright (2000), with permission from Elsevier.
Fungi give us a fine example of rhodopsin divergence based on gene duplication with subsequent specialization. The homology searches of available genome and expressed sequence tag (EST) databases indicate the presence of dozens of archaeal rhodopsin homologs in various fungi, including plant and human pathogens such as Ascomycetes *Botryotinia fuckeliana*, *Fusarium sporotrichioides*, *Gibberella zeae*, *Leptosphaeria maculans*, *Mycosphaerella graminicola*, and Basidiomycetes *Ustilago maydis* and *Cryptococcus neoformans* [14, 102]. The RDs and ORPs are widely distributed among different fungal species, as can be stated based on the knowledge of many fungal genome sequences obtained recently. Moreover, numerous fungal species possess multiple forms of RDs and ORPs [103].

The first identified homolog of BR in fungi was ORP, called YRO2 from yeast *Saccharomyces cerevisiae*, a suggested fungal chaperone protein. It is expressed during stress such as heat shock, acid shock or organic solvent shock [104, 105]. The discovery of yeast ORPs was followed by the detection [106] and characterization [107] of the first true fungal RD, *Neurospora* rhodopsin (NR) from *Neurospora crassa*. With hundreds of new opsin sequences known in fungal species, only a few novel fungal rhodopsins have yet been expressed and characterized. Sequence analysis (Fig. 1.11) provided evidence that all of these putative fungal rhodopsins show suprisingly high sequence similarity to haloarchaeal rhodopsins (e.g., BR).
Figure 1.11. A structural map of conservation of important bacteriorhodopsin residues in fungal rhodopsins (RDs) and opsin-related proteins (ORPs) based on the structural model of bacteriorhodopsin structure 1C3W. The residues in red are strongly conserved in both RDs and ORPs, while the ones in blue are strongly conserved only in RDs. The numbering of residues used here is according to the H. salinarum BR sequence. Modified from [102].

As the residues functionally important for the archaeal proton pump are conserved in RDs (an especially high conservation is found in the retinal-binding pocket), it suggests that they may also work as proton pumps [102]. However, some of the homologous haloarchaeal rhodopsins work as photosensors, so it might be also possible for fungal rhodopsins to perform photosensory functions, especially if one considers the absence of phototrophy in fungi, which contradicts the putative bioenergetic role of these proteins (as mentioned above, light-driven proton-pumping rhodopsins can provide energy to cells). The positional conservation of functionally or structurally important residues of BR is corroborated by the biophysical data on a
limited number of mutants of *Neurospora* RD (NR) [108, 109], and recently characterized *Leptosphaeria* rhodopsin (LR), which was confirmed to be a bacteriorhodopsin-like proton pump from a eukaryote [110, 111].

Combining the accumulated knowledge from the biophysical data and sequence analysis of the fungal homologs of BR (excluding ORPs), one can distinguish three subgroups with different hypothetical functions (Fig. 1.12) [15]. The first subgroup is called the NR-like group, showing high similarity of sequences to NR. Fungal rhodopsins in this subgroup may display a slow photocycle and work as photosensors. Another subgroup contains LR-like fungal rhodopsins. They are predicted to have a fast cycle and may perform light-driven proton pumping. Such functional diversification is supported by an independent recent analysis of fungal sequences [7]. Typical representatives from these subgroups will be described in greater detail below.

The last subgroup is called the auxiliary ORP-like fungal rhodopsins, comprising the second or the third form of rhodopsins in the same fungal species. All the residues structurally important in proton pumping are strongly conserved in the fungal rhodopsins in this subgroup, although their sequences are close to those of ORPs. Neither physiological nor biophysical data for this subgroup are available yet, but its members may play some new roles in fungal physiology. Characterization of this group is the main focus of Chapter 3.
Figure 1.12. Guide tree of fungal rhodopsins from *Ascomycetes* (full sequences only, no ORPs) constructed by CLUSTALW program, drawn using Treeview. Three putative rhodopsin subgroups are indicated. 0.1 indicates 10 nucleotide substitutions per 100 nucleotides.
Several genetic studies have failed to detect any phenotypic modifications in the fungal rhodopsin gene knock-outs [106, 112-114], so the physiological functions of rhodopsins in fungi from *Ascomycetes* and *Basidiomycetes* remain unknown. A rhodopsin from a Chytridiomycete (*Allomyces*) is the only one with the known physiological role, mediating the phototaxis of zoospores [115]. Two rhodopsins in fungi, NR from *N. crassa* [106] and LR from *Leptosphaeria maculans* [116] have been investigated at the genetic level and characterized as proteins biophysically, so far without any conclusive physiological data [107, 108, 110, 117]. It is known that for some fungal species, the genes encoding proteins of the auxiliary subgroup belong to the carotenoid biosynthesis cluster, even though their disruption does not interfere with the carotenoid metabolism [112].

1.6.1. *Neurospora rhodopsin* (NR)

The genome sequencing project of the filamentous fungus *Neurospora crassa* [118] revealed the first eukaryotic rhodopsin of microbial type, designated NOP-1 [106]. The discovery of *nop-1* gene opened up the stage for fungal rhodopsins studies. The *nop-1* gene was heterologously expressed in the methylotrophic yeast *Pichia pastoris* with very high yield, and the resulting membrane-associated protein *Neurospora* rhodopsin (NR) was characterized on the protein level. NR forms a green-absorbing pigment with an absorption maximum at 534 nm and the approximately 100 nm half-bandwidth after addition of all-trans retinal [107].
Sequence comparison to archaeal rhodopsins, such as BR, SRI, and SRII, shows that the retinal chromophore binding pocket motif is highly conserved and NR has approximately 80% similarity to archaeal rhodopsins. A key residue involved in proton release complex on the extracellular side (E204) and proton donating complex on the cytoplasmic side (T46 and D96) in BR, which disappear in either SRI or SRII, are conserved in NR. Furthermore, many residues known to be important for proton-pumping in BR are conserved, such as R82, Y83, and W86 in BR. For this reason, NR is more similar to BR than to SRI or SRII [107], which strongly suggested that it possesses some proton-pumping activity. However, this putative function of NR was disproved by the biophysical data as it appears to lack activity as a proton pump, and has a seconds-long photocycle from laser flash kinetic spectroscopy, similar to that of SRII but not of BR [107, 108].

The photocycle of NR was well characterized by spectroscopic methods (visible and infrared spectroscopy) and was found to contain a sequence of quasi-stable intermediates (K-, L-, M-, N-, and O-like states), which are similar to those in other retinal-proteins (BR and SRII) [107-109]. In the first half of the photocycle of NR, the Schiff base deprotonates in the submillisecond time range transferring the proton to the primary proton acceptor D131, homologous to that of BR’s D85, similar to BR and SRII. This conclusion was supported by the phenotype of the D131E mutant [108]. It also suggested that the chromophore in NR stores enough energy of the photon to move the proton from one side of the membrane to the other side. However,
steps involved in the reprotonation of the Schiff base of NR are different from those in BR but may be more similar to those in SRII [119]. The reprotonation of the Schiff base in NR is strongly pH-dependent. In contrast, BR shows pH-independence in a wide pH range due to the existence of internal proton donor D96 of the Schiff base. Accordingly, the photocycle kinetics of the E142Q mutant changed very little overall, with reprotonation of the Schiff base being even faster than in the wild-type NR at the same pH [108]. This result is surprising, as E142 was expected to act as a proton donor for the Schiff base based on homology with BR. Contrary to expectations, the carboxylate glutamate at this position (142 in NR) seems to be an even more ineffective proton donor than the unprotonatable residue glutamine (Fig. 1.13). This unexpected behavior of the carboxylic acid residue in NR suggests that the cytoplasmic half of NR does not participate in proton translocation [108]. The conformation of the retinal and retinal-binding K263 in NR was revealed to be somewhat different from that in BR as judged from the FT-Raman spectra [108]. The FTIR difference spectra showed that the changes in the carboxylic acid region are more similar to those in SRII than to those in BR [109].

In line with these findings, the results from low-temperature FTIR spectroscopy indicated that the structural changes of retinal in NR upon photoisomerization are quite different from those in BR, especially in the hydrogen-out-of-plane (HOOP) region, though the overall structure of retinal is similar. Hydrogen bonds of internal water molecules in NR are different from those in BR, which may be responsible for
Figure 1.13. Comparison of the light-induced absorption changes at 420 nm (M intermediate), reflecting deprotonation and reprotonation of the retinal Schiff base, in the wild-type and D96N mutant of BR and in the wild-type and homologous mutant E142Q in NR. Image modified from [108], and reprinted with permission from Journal of Biological Chemistry, copyright (2001).

the lack of the reprotonation switch [120]. Thus, no matter how similar it is to BR in terms of the sequence, even with both key carboxylates (the primary proton acceptor and donor) present, NR is not a proton pump but probably a sensory rhodopsin, as strongly confirmed by the results from spectroscopic studies, site-directed
mutagenesis, and proton-transport assays [108].

The exact physiological function of Neurospora rhodopsin has not been identified yet. Analysis of nop-1 transcription by Northern blot analysis indicates that there is no transcription of nop-1 in young submerged mycelia, whereas it can be strongly induced in conidia, in sexually differentiated mycelia, and in mature cultures grown under illumination. The nop-1 gene knock-out mutant does not give any detectable phenotypic alteration compared to the wild-type during the laboratory life cycle of N. crassa. Subtle differences are observed, such as a light-dependent change in colony morphology in the presence of several ATPase inhibitors, or influence on transcription levels of several genes involving in carotenoid biosynthesis [106, 117]. Photobiological role of NR is not not obvious from modulation of light-induced transcriptional changes as shown by genome wide analysis of light-inducible response on N. crassa [121]. However, NR may participate in negative regulation of several conidiation genes as suggested by light-dependent mRNA accumulation in the nop-1 gene knock-out strain [122].

1.6.2. Leptosphaeria rhodopsin

The ops gene from Leptosphaeria maculans, the fungal agent of blackleg in canola, has been characterized as the second opsin gene identified in the fungal kingdom [116]. Its product, Leptosphaeria rhodopsin (LR), is the first light-driven proton-pumping rhodopsin found in fungi and in a eukaryotic organism in general [110]. The Leptos. maculans ops gene was heterologously expressed in Pichia
*pastoris* by the method developed for NR [107]. Upon addition of all-trans-retinal, LR forms a stable red pigment with the absorption maximum at 542 nm. The red-shifted $\lambda_{\text{max}}$ of the retinal is also confirmed by Raman spectroscopy of LR in liposomes [110].

Sequence alignments on LR, NR, and BR reveal that LR is quite similar to NR (70.3%) [123] (Fig. 1.15). Most of the key residues of the retinal binding pocket of BR are conserved in LR. Previously, LR was expected to have proton pumping ability [116], but the data on NR [108] made this question complicated and not easily predictable, so that in-depth biophysical study was warranted. Studies by time-resolved visible spectroscopy showed that LR has K-, L-, M-, N-, and O-intermediates which are similar to those in BR (Fig. 1.14). This BR-like character of the LR photocycle was also supported by the results of rapid-scan FTIR difference spectroscopy. The photocycle is really fast, few tens of milliseconds, which is the characteristic feature for ion-pumps. The kinetics of the photointermediates are similar to those of BR as well [110].

The proton-pumping ability of LR was confirmed by site-directed mutagenesis, direct proton transport measurements, and low temperature FTIR spectra [110, 123]. The cytoplasmic proton donor D150 in LR was proved to be functional by observing the D150N mutant. In D150N, the reprotonation of the Schiff base is up to 100 fold slower than in the wild type protein, similar to the D96N mutant of BR [110]. This
rate can be restored to the wild-type level by sodium azide, the same way as in BR [124, 125]. This suggests that, in contrast to E142 of NR, the cytoplasmic homolog of D96 is active as a proton donor of the Schiff base of LR [110].

Figure 1.14. The kinetics of light-induced absorption changes of LR in N-dodecyl-β-D-maltoside-treated (DDM) *Pichia* membranes. Image modified from [110], and reprinted with permission from PNAS, copyright (2005). Characteristic intermediates are shown by arrows.

The kinetics of proton uptake and release in LR were recorded by the visible spectroscopy with the pH-sensitive dye pyranine. Steady-state proton pumping was confirmed by the apparent light-induced pH changes observed in the suspension of unbuffered LR L-α-phosphatidylcholine/L-α-phosphatidylserine (PC/PS) liposomes, which was abolished with addition of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), indicating active proton pumping [110]. Low
temperature FTIR spectra showed that the structural changes of retinal in LR upon photoisomerization are similar to BR but quite different from those in NR. Strongly hydrogen-bonded water molecules, which are found only in proton-pumping proteins, based on studies of BR mutants and archaeal rhodopsins, were also detected in the spectra of LR but not in those of NR [123].

As LR is so similar to NR in terms of the amino acid sequence (Fig. 1.15), the structural reasons for the dramatic differences in their photochemistry were investigated by mutating a few residues that were different, namely, T87, D150, T233, D248, and G271 [111, 126]. The phenotypes of T233A, G271P, and D248G mutants of LR did not show significant differences from the wild-type, as judged from the analysis of their photocycle kinetics [126]. However, a seemingly innocuous Asp/Glu replacement at the key position of the cytoplasmic proton donor to the retinal Schiff base (D150E) converts LR into a slow-cycling NR-like protein [111]. In the case of BR and its haloarchaeal homologs, if a homolog of D96 is Asp, it is paired with Thr [127, 128], whereas a homologous Glu/Ser pair exists in eubacterial proton pumps (PR, XR) [15, 99]. To check if the Glu/Thr mismatch was the cause of the disfunctional behavior of the D150E mutant of LR, the double mutant T87S/D150E was produced [126]. The double T87S/D150E mutant could not restore the photocycle turnover to the few tens of milliseconds, while the effect of T87S was similar to that of T46V mutant of BR [129]. This argues that D150 may be the only major determinant of the fast BR-like photochemistry of LR.
Figure 1.15. Partial alignment (manually edited CLUSTALW) of primary sequences of conserved transmembrane regions of BR, LR, and NR. Reprinted from [126], Copyright (2007), with permission from Elsevier. Residues conserved in all variants of BR are shaded, mutated residues shown by arrows.

No targeted mutagenesis has been done to produce knock-out of *ops* in *Leptos. maculans*. The transcription of *ops* gene is at high levels in mycelia grown in the presence and absence of light, different from the pattern of *nop-I* [116]. The exact physiological role of LR is still mysterious, as it is not clear whether its proton-pumping ability is related to bioenergetics. Recent electrophysiological study of NR and LR expressed in neurons confirmed their drastically different proton-pumping abilities [130].

1.6.3. Opsins from *Fusarium fujikuroi*

Finally, it is worth describing opsins from yet another fungus, which are probably the most studied physiologically. The fungus *Fusarium fujikuroi* (*Gibberella fujikuroi* mating group C) contains three opsin genes in the genome, two genes coding for putative fungal rhodopsins (*carO* and *opsA*) and another gene for an opsin-related
protein (hspO) [131]. The transcription levels of these three opsin genes are increased by heat shock. OpsA and HspO are the orthologous proteins for NR and YRO-2 from *N. crassa*, respectively, based on sequence comparisons, while CarO does not have homologs characterized physiologically or biophysically [114].

The gene *carO* is in the carotenoid gene cluster, which has two genes responsible for torulene synthesis (*carRA* and *carB*), *carO*, and *carX* coding for a retinal-forming oxygenase. It is frequent to find clusters of genes participating in the same metabolic pathway [132], e.g., seven co-regulated genes in the gibberellin cluster of *F. fujikuroi* [133-135]. CarO shows the same regulation pattern (light-induced and deregulated in carotenoid overproducing mutants) as the rest of the three carotenoid biosynthesis genes. The predicted CarO protein is highly similar to heat-shock proteins (ORPs), but is also similar to NR and LR. A *carO* mutation has no detectable phenotype [112].

OpsA is the orthologue of NR as corroborated by having 155 identical positions between OpsA and NR, while only 87 for NR and CarO out of a total sequence length of 300 residues. Transcription of *opsA* is light-induced but not enhanced in carotenoid-overproducing mutants, which is different from *carO* (Fig. 1.16). The *opsA* deletion mutant does not give any detectable phenotypic alteration either. However, a drastic decrease in the expression of structural genes of the carotenoid pathway is observed in the *opsA* deletion mutant [114].
Figure 1.16. Effect of heat shock, light, and the genetic background on expression of *Fusarium fujikuroi* genes for opsin-like proteins and the gene carB. Image reprinted from [114], Copyright (2009), with permission from Elsevier. (a) Real-time RT-PCR analyses of the indicated genes from RNA samples of the wild-type strain grown in the dark and exposed either to 38 or 42 °C for 1 h (light gray bars) and 2 h (dark gray bars) or to light for 30 min (white bars). Relative mRNA levels are referred to the value for each gene from unexposed mycelia. (b) Real-time RT-PCR analyses of the genes carB, carO, and opsA from RNA samples of the wild-type strain grown in the dark and exposed for 15 min, 30 min, 1 h, or 2 h to light. Relative mRNA levels are referred to the values from non-illuminated mycelia. A representative Northern blot experiment for opsA is shown in the inner box. (c) Northern blot and real-time RT-PCR analyses of the gene opsA from RNA samples of the wild-type and mutant strains for the indicated genes grown in the dark (gray bars) or exposed to light for 30 min (white bars). Real-time RT-PCR data represent average and standard deviation for four determinations from two independent experiments.
In summary, no proven physiological role based on green light photochemistry could be assigned to any of fungal rhodopsins, neither in vivo nor in vitro [103]. It was suggested that the true physiological role may be revealed under conditions not encountered in the laboratory settings, for example under cold or drought stress.

1.7. Research objectives

As the microbial rhodopsins world is expanding both in terms of taxonomic and ecological diversity, many different novel functions are uncovered. Based on the simple bacteriorhodopsin template, microbial rhodopsins give us a wide variety of structures and functions by replacing amino acids, sometimes just at a few sites, as well demonstrated in fungi, algae, and eubacteria. The first objective of this work was to analyse the function of auxiliary OPR-related fungal rhodopsins, previously uncharacterized subgroup of rhodopsins present as additional forms in many fungal species. The meaning of their existence in fungi, which may also shed extra light on the existence of multiple forms of rhodopsins in fungi in general, is investigated here. Next, we have tried to perform uniform isotope labeling of a fungal rhodopsin (LR) for solid-state NMR studies, as a testing ground for other eukaryotic membrane proteins. This isotope labeling protocol in Pichia pastoris will be hopefully applied to other eukaryotic membrane proteins, such as GPCRs, in the future. The third objective of this thesis was to study the novel eubacterial rhodopsin with highly unusual sequence (DDR2) from flavobacterium Donghaeana dokdondensis. This eubacterial rhodopsin was shown to mediate passive inward proton transport, which
suggested that it may have unique ion specificity. Our goal was to reveal that specificity by studying its unique photocycle under various ionic conditions and to understand the working mechanism of the ion transport by combination of spectroscopy and mutagenesis.
Chapter 2

Materials and Methods

Parts of this chapter were published in J. Biomol. NMR, 49: 151-161 [136], and other parts have been accepted to Biochim. Biophys. Acta – Bioenergetics.
2.1. Materials

The *Pichia* expression plasmid (pPICZaA) and host strains (GS115, SMD1168H) were from Invitrogen. *Escherichia coli* strain DH5α was used for plasmid propagation. Yeast nitrogen base without amino acids, yeast extract, and protease peptone for yeast cultures were from Difco (Sparks, MD, USA). Isotope-labeled components were from Cambridge Isotope Laboratories (Andover, MA). Tryptone and yeast extract for bacterial cultures were from Fisher (Fair Lawn, NJ, USA). High-fidelity thermostable *Pwo* polymerase used in PCR amplification was from Roche (Mannheim, Germany). Reagents for PCR amplification and agarose gel electrophoresis were from Fisher (Fair Lawn, NJ, USA). Restriction endonucleases were from Promega (Madison, WI, USA). Miniprep plasmid isolation kit, nucleotide removal kit, and nucleotide extraction kit were from Qiagen (Mississauga, ON, CA). All-trans-retinal and lyticase were from Sigma. Synthetic oligonucleotides (primers) for site-directed mutagenesis were from Cortec (Kingston, ON, CA) and Sigma-Aldrich (Oakville, ON, CA). Acrylamide, SDS, and other reagents employed in SDS-PAGE were also from Fisher (Fair Lawn, NJ, USA). Bio-beads SM-2 were from Bio-Rad Company (Hercules, CA, USA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Concentrators used in concentrating proteins during liposomes preparation were Amicon Ultra, from Millipore (Bedford, MA, USA).
2.2. Sample preparation for *Phaeosphaeria* Rhodopsins

2.2.1. Insertion of *Phaeosphaeria ops* genes into the expression plasmid

The *Phaeosphaeria* rhodopsin genes *ops*1 (SNOG_00807, Gene ID: 5968425, encoding PhaeoRD1) and *ops*2 (SNOG_00341, Gene ID: 5967674, encoding PhaeoRD2) were cloned between the *EcoRI* and *XbaI* sites of the pPICZαA vector. The coding DNA sequences were truncated (*ops*1 to 795 bp and *ops*2 to 822 bp) to remove the putative extra-membrane portion of the N termini according to sequence alignments with NR and LR. Such replacement of the native N-terminus with the yeast signal sequence produced robust expression and good membrane targeting in the past [107, 110, 126, 136]. *EcoRI* site was created at the 5’ ends of the rhodopsin genes while *XbaI* site was at 3’ ends, and 6-his-tag was added at the C-terminus by performing the polymerase chain reaction (PCR) with the following primers (Table 2-1). The sequences of the DNA inserts of reconstituted plasmids were verified by DNA sequencing (Univ. of Guelph sequencing facility).

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<th>Oligonucleotide</th>
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<tr>
<td>Phaeo ops1 forward</td>
<td>Phaeo reverse ops1</td>
<td>5’GGCAATTCGAATCTGGCCAGAAGACCCTC3’</td>
</tr>
<tr>
<td>Phaeo ops2 forward</td>
<td>Phaeo reverse ops2</td>
<td>5’GGCAATTCGACCATGGCTCAGACTTG3’</td>
</tr>
<tr>
<td>Phaeo ops1 reverse</td>
<td>Phaeo reverse ops2</td>
<td>5’GCTCTAGATTAATGGTGATGGTGATGGTGCGCGCGCGTCATCCTCACCGAG3’</td>
</tr>
</tbody>
</table>

Table 2-1. Oligonucleotides designed for amplifying the genes of Phaeo *ops*. 
2.2.2. Site-directed mutagenesis

Two primers (see details in Table 2-2) containing DNA for the desired mutation and high-fidelity thermostable polymerase *pwo* were employed for site-specific mutagenesis by a single-step PCR from the wild-type construct, the *Phaeosphaeria ops* genes inserted into the pPICZαA vector. To set up the PCR, the following components were added in the specified order: 5 µL 10×*pwo* polymerase buffer with 20 mM MgSO₄, 2 µL 10 mM dNTPs, 10 pmol of each primer, 20-200 ng DNA template, 0.3 µL *pwo* DNA polymerase (5 units/L), topped up with sterile H₂O to the total volume of 50 µL. Thermal cycle conditions were set up as follows: the reaction mixture was denatured at 95°C for 1 min, annealing temperature was touched down from 65°C to 55°C for 30 s and amplified 12 to 16 cycles. Each cycle had denaturation at 95°C for 30 s, annealing temperature for 30 s, and elongation at 72°C for 4 min 30 s. The final elongation cycle was 10 min at 72°C. 10 µL of the PCR products were analyzed by agarose gel electrophoresis on a 1×Tris-acetate-EDTA (TAE) buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA), 0.8% gel, and run at 120 V for 30 min.
Table 2-2. Oligonucleotides designed for site-directed mutagenesis.

| Oligonucleotide   | Sequence (5’ to 3’)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PhaeoRD2E146D-1</td>
<td>CAT TGT TGA TGA CAT CAT GAT CAT C</td>
</tr>
<tr>
<td>PhaeoRD2E146D-2</td>
<td>GAT GAT CAT GAT GTC ATC AAC AAT G</td>
</tr>
<tr>
<td>PhaeoRD2D126N-1</td>
<td>CCT TTG CTC CTG ACC AAC CTC ATG CTC ACC GC</td>
</tr>
<tr>
<td>PhaeoRD2D126N-2</td>
<td>GCG GTG AGC ATG AGG TTG GTC AGG AGC AAA GG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Product Plasmid</th>
<th>Template and Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPhaeoRD2E146D</td>
<td>Mutates Glu-146 to Asp in PhaeoRD2 protein encoded by pPICZaA-PhaeoRD2 plasmid.</td>
</tr>
<tr>
<td>pPhaeoRD2D126N</td>
<td>Mutates Asp-126 to Asn in PhaeoRD2 protein encoded by pPICZaA-PhaeoRD2 plasmid.</td>
</tr>
</tbody>
</table>

1Underlined letters represent bases that were changed relative to the original plasmid sequence.

2.2.3. Transformation into DH5α E.coli cells

The protocol of preparation of competent cells was according to the Hanahan method for high-efficiency transformation in E.coli with small modifications. E.coli DH5α cells from SOB plate were inoculated into 4 mL of SOB broth and grown at 37°C, 120 rpm, overnight. 1 mL of the overnight culture was transferred into 24 mL of SOB broth in a 250 mL baffled flask, and grown at 37°C, 120 rpm. The growth of E. coli was monitored every 30 min by UV-Visible spectrophotometer Cary-50 (Varian, Mississauga, ON, CA) and the cells were harvested when OD₆₀₀ was around 0.45-0.55. The cells were spun down at 8,000×g for 10 min at 4°C after cooling down on ice for 10 min. The cell pellet was washed with 8.3 mL of standard transformation buffer (TFB) (10 mM MES, 45 mM MnCl₂, 10 mM CaC₂, 100 mM KCl, and 3 mM
Hexamminecobalt chloride) and incubated on ice for 15 min. After centrifugation at 8,000×g for 10 min at 4°C, the cell pellet was resuspended in 2 mL TFB and then mixed with 70 µL of DMSO. After incubation on ice for 5 min, 70 µL of DTT was added into the cell mixture. The cell mixture was incubated on ice for 10 min, and then another 70 µL of DMSO was added and left on ice for another 5 min. Between 10 and 200 ng of plasmid DNA or PCR products were mixed with 200 µL of competent cells and incubated on ice for 1 h. The cell-DNA mixture was put under heat pulse for 90 s at 42°C and put back on ice for 2 min. 800 µL of SOC media was added into the mixture and cultured at 37°C for 1-2 h. 10-200 µL aliquots were plated on the low salt LB plates with 25 µg/mL zeocin (from Cedarlane). Zeocin is a water-soluble antibiotic used for positive selection of *E. coli* and *Pichia pastoris* transformants with pPICZ and pPICZα shuttle vectors. It requires low salt concentrations and neutral pH (~7.5) to remain active. Only the transformants with the plasmid DNA or PCR products can survive on the medium containing zeocin.

2.2.4. Transformation into *P. pastoris* cells

The pPICZαA-PhaeoRDs vectors were propagated in DH5α strain of *E. coli* in low salt LB medium with zeocin, isolated using Qiagen kit (QIAprep Spin Miniprep), and transformed into *P. pastoris* GS115 cells by electroporation according to the manual of the *Pichia* expression kit (Invitrogen) with small modifications. Briefly, 10 µL of stock of *P. pastoris* GS115 cells was inoculated into 25 mL of yeast peptone dextrose (YPD) medium in a 250 mL baffled flask, grown at 30°C, 300 rpm to achieve OD₆₀₀
~10 (normally, 24 h). Approximately 2–10 mL of the overnight culture was spun
down at 700×g for 2 min and resuspended with 400 mL of fresh YPD medium in a 2.8
L Fernbach flask. The culture was grown at 30°C, 300 rpm to OD$_{600}$ 1.4–2.0 (the
growth time was adjusted according to the starting value of OD$_{600}$; the doubling time
of log phase $Pichia$ in YPD was ~2.5 h). The cells were collected by centrifugation at
1,500×g for 4 min at 4°C. The cell pellet was washed twice with 300 mL of sterile
ice-cold water and centrifuged again. The same procedure was repeated using 40 mL
of sterile ice-cold 1 M sorbitol and the final pellet was resuspended in 0.5 mL of
sterile ice-cold 1 M sorbitol. Before transformation, the plasmid was linearized with
$BstXI$, and then purified by the QIAquick nucleotide extraction kit (Qiagen), desalted
by the QIA-quick nucleotide removal kit (Qiagen), concentrated by ethanol
precipitation and resuspended in a desired volume of water. 40 µL of yeast cell
suspension in sorbitol was mixed with 5 µg of desalted DNA in an electroporation 0.2
cm cuvette (Bio-Rad). After incubating on ice for 5 min, the cuvette was subjected to
a “Pie” pulse (MicroPulser, Bio-Rad). Normally, the applied voltage was ~2 kV and
the pulse duration was 5.6–5.7 ms. 1 mL of sterile ice-cold 1 M sorbitol was added
immediately after the pulse and mixed thoroughly. 100–200 µL aliquots of the
transformation mixture were spread on YPD sorbitol plates containing zeocin (at two
different concentrations: 100 and 500 µg/mL). Following 3–10 days of incubation at
30°C the transformant colonies were isolated from the plates.
2.2.5. Expression screening of PhaeoRDs in *P. pastoris*

The high expression screening was done analogously to that for the wild-type LR [110]. Usually 6-10 transformants were screened for high expression level of PhaeoRDs. The pPICZαA-PhaeoRDs transformants isolated from the YPDS/zeocin plates were grown in 25 mL of BMGY medium in a 250 mL baffled flask, shaking at 30°C, 300 rpm for 1-2 days. As OD$_{600}$ reached ~10, 2.5 mL of culture was centrifuged at 1,500×g for 5 min at 4°C, and resuspended in 25 mL of BMMY medium, then grown by shaking at 240 rpm, 30°C. After 24 h, additional 175 µl of 100% methanol (final concentration 0.7%) and 6.25 µL of 10 mM all-trans-retinal (isopropanol stock, final concentration 2.5 µM) were added into the culture. At different time points (24 h, 40 h, 42 h, 48 h, and 52 h), 1 mL of the expression culture was taken and centrifuged at 1,500×g for 5 min at 4°C in a 1.5 mL microcentrifuge tube. The expression level of the protein was evaluated by the intensity of the color of the yeast pellet, and the colonies showing the most intense red color were selected for a large scale expression.

2.2.6. Scale-up of the expression of PhaeoRDs

Large-scale protein expression followed the established shake-flask protocol for the *Pichia* expression kit (Invitrogen) with small modifications. Briefly, a small scratch from a cell colony with the highest expression level of PhaeoRDs in small-scale culture was inoculated into 25 mL of BMGY in a sterile 250 mL baffled flask. This seed culture was grown, shaking at 30°C (300 rpm) for 18–24 h, until the OD$_{600}$ exceeded 2, and inoculated into a sterile 2 L baffled flask containing 250 mL of
BMGY. This culture was shaken at 29–30°C (270 rpm) for 18–24 h, until the OD$_{600}$ reached 3.6. To induce PhaeoRDs expression, the cells were pelleted in sterile containers at 1,500×g for 5 min at 4°C, and gently resuspended in 0.8 L of BMMY, which was placed into 2.8 L Fernbach flask and shaken at 29–30°C (240 rpm). 10 mM isopropanol stock of all-trans-retinal (needed for rhodopsin regeneration, final concentration 5 µM) and 100% filtered methanol (final concentration 0.7%) were added to the growth medium after 24 h of induction. The red-colored cells were collected by centrifugation at 1,500×g for 5 min at 4°C after 40 h of induction, as the protein yield was found to be lower upon longer (48–52 h) and shorter (24 h) incubation times. The cell pellet was washed with MilliQ water twice and stored frozen at -20°C for later use.

### 2.2.7. Purification of PhaeoRDs

The cell breakage and protein purification protocols were based on those used for *Neurospora* rhodopsin (NR) and LR with small modifications [110, 120]. The cell pellet collected from the 800 mL of culture was re-suspended in one pellet volume of buffer A (7 mM NaH$_2$PO$_4$ at pH 6.5, 7 mM EDTA, 7 mM DTT, and 1 mM PMSF) and incubated with 5 mg of lyticase (from *Arthrobacter luteus*) for digestion of the cell walls, and additional 25 µM of all-trans-retinal to ensure complete rhodopsin regeneration, by slowly shaking in the dark at room temperature for 3 h. The cells were then centrifuged at 1,500×g for 5 min at 4°C and immediately resuspended in one pellet volume of buffer A. Half of the pellet volume of ice-cold acid-washed glass
beads (Fisher) (420–600 µm diameter) was added, and the cells were disrupted with four 1 min pulses using vigorous mixing with a vortex mixer. The cell debris were removed by centrifugation at 700×g for 5 min at 4°C and the cell lysate was collected. An additional half pellet volume of buffer A was added to resuspend the cell debris, and vortexing and centrifugation steps were repeated several times (usually 8 times in total) to achieve complete breakage of the cells. All cell supernatants containing the membrane fraction were combined and centrifuged at 40,000×g for 30 min (PhaeoRD1) and 150,000×g for 50 min (PhaeoRD2) at 4°C, and the membrane pellets were stored at -20°C for later use.

To purify PhaeoRDs, the pellets of frozen membranes were thawed and resuspended with solubilization buffer, then mixed with 6-His tag affinity resin (Ni²⁺-NTA agarose, Qiagen) by the batch method. We estimated the quantity of solubilized protein spectroscopically (Cary 50, Varian), assuming the molar extinction similar to that of BR, to calculate the amount of resin needed. As PhaeoRDs have different biochemical properties, the conditions for purification were different. The pellets of frozen membranes were stirred in the dark in a solubilization buffer (see details in Table 2-3), at 4°C for 3-4 h, then centrifuged at 40,000×g for 30 min at 4°C for PhaeoRD1, and stirred in the dark at 4°C overnight, then ultra-centrifuged at 38,000 rpm for 50 min at 4°C for PhaeoRD2. Solubilized PhaeoRDs were mixed with 6-His tag affinity resin and incubated in the dark at room temperature with gentle agitation to allow complete binding (usually 3 h).
Table 2-3. Buffers required for purifications of PhaeoRDs.

<table>
<thead>
<tr>
<th></th>
<th>PhaeoRD1</th>
<th>PhaeoRD2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solubilization</strong></td>
<td>1% DDM, 20 mM KH$_2$PO$_4$, 1 mM PMSF, pH 7.5</td>
<td>1% Triton X-100, 20 mM KH$_2$PO$_4$, 0.3 M NaCl, 10 mM β-mercaptoethanol, 1 mM PMSF, pH 7.5</td>
</tr>
<tr>
<td><strong>Buffer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Washing</strong></td>
<td>0.25% DDM, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 35 mM imidazole, pH 7.5</td>
<td>0.25% Triton X-100, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 1 mM DTT, pH 7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25% Triton X-100, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 35 mM imidazole, 1 mM DTT, pH 7.5</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td>0.25% DDM, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 250 mM imidazole, pH 7.5</td>
<td>0.25% Triton X-100, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 250 mM imidazole, 0.2 mg/ml <em>Pichia</em> lipids, pH 7.5</td>
</tr>
</tbody>
</table>

The role of the *Pichia* lipids in the elution buffer was to stabilize the purified PhaeoRD2, a procedure previously used for NR [108, 120]. The lipids were first extracted with methanol/chloroform from *P. pastoris* cells and then precipitated by acetone according to the protocol of Kates *et al.* [137].

The clear supernatant containing other solubilized proteins was removed after centrifugation at 4,000×g at 4°C for 2 min. The spectrum of the eluate was monitored to detect the loss of non-specifically bound cytochromes (at 410 nm) and specifically bound rhodopsin (PhaeoRD1 at 540 nm and PhaeoRD2 at 535 nm). The resin was washed more than 10 times with increasing concentrations of imidazole, until the cytochrome band disappeared from the eluate spectrum. Tightly bound proteins were
eluted from the resin with the elution buffer, after 2 min of mixing. The resin was eluted several times until the amount of bound rhodopsin was negligible. All eluates were combined together and concentrated (usually to 2-6 OD/ml) by centrifugation at 6,000×g at 4°C using a concentrator (Amicon Ultra 4).

2.2.8. Reconstitution of purified PhaeoRDs into liposomes

The lipid reconstitution protocol followed that used for LR [110, 126]. The dry powder lipids (DMPC: DMPA = 9:1 w/w) were first dissolved and mixed in warm chloroform, which was thoroughly removed by evaporation under vacuum to yield a thin lipid film. The dry lipids were rehydrated by adding a rehydration buffer (50 mM KH₂PO₄, 100 mM NaCl, pH 7.5) and agitated with vigorous shaking, mixing, and sonication to obtain lipid suspension at high concentration (usually, 10 mg/mL). Purified solubilized PhaeoRDs were added to the preformed liposomes, which were semi-solubilized (as judged by the drop in turbidity) with Triton X-100 at protein/lipids/detergent (w/w/w) ratio of 1:3:1.5, and stirred for 15 min at room temperature. The resultant semi-transparent mixture became turbid after removal of detergent by adding 400 mg of Bio-beads SM-2 (Biorad) per 1 mL of the mixture and incubation with stirring at 4°C in the dark. Next, the proteoliposome suspension was withdrawn by a syringe, after which the colored beads (with remaining bound PhaeoRDs) were washed by 0.1 M NaCl several times. The proteoliposomes were collected by centrifugation at 20,000×g for 30 min at 4°C.
2.2.9. **Preparing polyacrylamide gels containing DDM-treated fungal protein for time-resolved visible spectroscopy**

The upper part of the membrane pellet (obtained by the cell breakage as described above) was resuspended with 1 mL of deionized H$_2$O and transferred into a clean centrifuge tube. 10% N-dodecyl-β-D-maltoside (DDM) solution was added gradually into the protein suspension to decrease the size of the membranes and get rid of peripheral proteins and residual cell walls. The suspension was incubated at room temperature for 5 min and spun down at 5,000×g for 5 min at 4°C. This step was repeated several times until most of the residual pellet had turned to the original yeast color (milk-white). The maximal final DDM concentration in the mixture was 0.5% (higher DDM concentrations solubilized the membranes fully). The colored supernatant was collected and centrifuged at 20,000×g for 30 min at 4°C. After this high speed centrifugation, the solubilized protein in the supernatant was discarded, and only the membrane embedded protein from the pellet was used to prepare polyacrylamide gels (1 mL of polyacrylamide gel = 0.3 mL of 33% acrylamide + 0.3 mL of 1% bis-acrylamide + 0.4 mL of DDM-treated protein solution + 2.4 µL of 10% APS + 3 µL of TEMED). Normally, the OD value of protein for 1 mL gel was in the range from 0.2 to 0.3 (about 0.1 to 0.15 mg protein per gel). After the gel was solidified, it was washed in distilled water overnight at room temperature to get rid of the remaining APS and TEMED. The protein gels were equilibrated with the desired pH buffers (Table 2-4) for at least 2 h before the measurements.
Table 2-4. Compositions of pH buffers used for the visible spectroscopy samples.

<table>
<thead>
<tr>
<th>pH Buffers</th>
<th>Components and Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5 buffer</td>
<td>0.05 M MES, 0.05 M KH₂PO₄, 0.1 M NaCl</td>
</tr>
<tr>
<td>pH 6 buffer</td>
<td>0.05 M MES, 0.05 M KH₂PO₄, 0.1 M NaCl</td>
</tr>
<tr>
<td>pH 7 buffer</td>
<td>0.05 M KH₂PO₄, 0.1 M NaCl</td>
</tr>
<tr>
<td>pH 8 buffer</td>
<td>0.05 M Tris, 0.05 M KH₂PO₄, 0.1 M NaCl</td>
</tr>
<tr>
<td>pH 9 buffer</td>
<td>0.05 M CHES, 0.05 M KH₂PO₄, 0.1 M NaCl</td>
</tr>
</tbody>
</table>

2.2.10. Visible spectroscopy

The static visible spectroscopy was performed with a Cary 50 spectrophotometer (Varian, Mississauga, ON, CA). Time-resolved visible spectroscopy was done using our custom-built apparatus. The sample was excited by the second harmonic of a Nd-yttrium/aluminum/garnet (YAG) laser (Continuum Minilite II), ~7 ns pulse at 532 nm. A quartz tungsten 250 W halogen lamp (an Oriel Quartz Tungsten Halogen (QTH) source) was used as probing light source. The monochromatic light was obtained by this QTH source and two aligned monochromators (before and after the sample). The laser repetition rate was controlled by the Function generator FG-8002 (EZ Digital Co. Ltd). The laser artifact was minimized by the additional notch filter placed before the second monochromator. The transmitted visible light was converted to electric current by an Oriel photomultiplier and amplified with 350 MHz wide-bandwidth amplifier. The signal was sampled by a Gage analog-to-digital converter (CompuScope 12100-64M). Pre-trigger mode allowed recording the absorption level right before the photoexcitation. Light-induced absorption changes at different wavelengths (e.g., 400 nm, 460 nm, 560 nm, and 620 nm) were averaged (usually, several hundreds of traces) and converted into a quasilogarithmic time scale by the in-house software (written by
2.2.11. Fourier-transform infrared difference spectroscopy instrumentation

Absolute static and time-resolved rapid-scan difference FTIR spectra were collected as described previously [110], using a Bruker IFS66vs machine with a temperature-controlled sample holder (Harrick, Pleasantville, NY, USA) connected to a circulating water bath (Fisher, Ottawa, ON, CA). The timing of laser pulses was controlled with Digital delay generator (Berkeley Nucleonics Corporation, model 565 pulse/delay, San Rafael, CA, USA). Photochemical cycle was activated by light provided by the second harmonic of a Nd-YAG laser (Continuum Minilite II), using 7 ns pulses at 532 nm. Dry or hydrated films of the DMPC:DMPA PhaeoRDs wild-type or mutants liposomes were compressed between two CaF$_2$ windows with a 6 µm Teflon spacer, and data acquisition was controlled by OPUS software (Bruker, Milton, ON, CA).

Low-temperature FT-IR was performed in Professor Kandori’s laboratory, Nagoya Institute of Technology, by H. Ito, as described previously [111, 123]. The sample was placed in a cell in an Oxford DN-1704 cryostat mounted in the Bio-Rad FTS-40 spectrometer. The cryostat was equipped with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision. Illumination with 500 nm light at 77 K for 2 min converted PhaeoRD$_2$ to PhaeoRD$_2\kappa$. Since PhaeoRD$_2\kappa$ was completely reconverted to PhaeoRD$_2$ upon illumination with > 600 nm light for 1 min,
cycles of alternating illumination with 500 nm light and >600 nm light were repeated a number of times. The difference spectrum was calculated from two spectra constructed from 128 interferograms taken before and after the illumination. Several difference spectra obtained in this way were averaged to produce the PhaeoRD2_K minus PhaeoRD2 spectrum.

2.3. Sample preparation for *Leptosphaeria* rhodopsin

2.3.1. Insertion of *Leptosphaeria* ops gene into the expression plasmid

In the earlier work, N-terminally truncated (48 residues removed) LR with C-terminal 6-His-tag, inserted in pHIL-S1 vector was successfully transformed into GS115 strain of *P. pastoris* [110]. To maximize the yield of stable expression for isotope-labeling, pPICZαA vector (Invitrogen) with different secretion signal (α-factor) was selected with transformation into the protease-deficient strain SMD1168H, which allows selection for multiple integration events on zeocin (Cedarlane) plates. The LR encoding sequence from the pHIL-S1-LR construct (with two extra EF residues at the truncated N-terminus, C-terminal 6-His-tag and a stop codon) was inserted into the multiple cloning site of the pPICZαA vector, using EcoRI and XbaI restriction sites.

2.3.2. Site-directed mutagenesis

Two primers, N79S-1 (5’GGAAGATCCCAGTTTCCAGCGTTTG3’) and N79S-2 (5’CAAACGCCTGGAAACTGGGATCTTCC3’), and high-fidelity thermostable polymerase *pwo* were employed for site-specific mutagenesis by a single-step PCR
from the pHIL-S1-LR construct. Refer to section 2.2.2 for details.

2.3.3. Transformation into *P. pastoris*

For details please refer to section 2.2.4, but note a different host strain, SMD1168H for wild-type LR.

2.3.4. Expression screening of wild-type LR in *P. pastoris*

Following 3–10 days of incubation at 30°C, the transformant colonies were isolated from the plates and screened for high expression level of LR, by inoculating into 5 mL of BMD medium in a 250 mL baffled flask and shaking at 30°C, 300 rpm overnight. After the value of OD$_{600}$ reached ~2, another 20 mL of BMD was added to the culture, which was shaken at 300 rpm, 30°C for 18–24 h until the OD$_{600}$ was 10. This culture was centrifuged at 1,500×g for 5 min at 4°C, resuspended in 25 mL of BMM, and grown by shaking at 240 rpm, 30°C. After 24 h, additional 175 µL of 100% methanol (final concentration 0.7%) and 6.25 µL of 10 mM all-trans-retinal (isopropanol stock, final concentration 2.5 µM) were added into the culture. At different time points (24 h, 40 h, 48, and 52 h), 1 mL of the expression culture was taken and centrifuged at 1,500×g for 5 min at 4°C in a 1.5 mL microcentrifuge tube. The expression level of the protein was evaluated by the intensity of the color of the yeast pellet, and the colonies showing the most intense red color were selected for a large scale expression.
2.3.5. Expression screening of LR N79S mutant in *P. pastoris*

The high expression screening was done analogously to that for the wild-type LR (the pHIL-S1-LR construct) [110, 126]. A single colony with the gene of interest was isolated from the MD plate, inoculated into 25 mL of MGY medium in a 250 mL baffled flask, and shaken at 30°C, 300 rpm for 2 days. After the value of OD\(_{600}\) reached approx. 10, 2.5 mL of the culture was centrifuged at \(1,500 \times g\) for 5 min at 4°C, and resuspended in 25 mL of BMM medium, then shaken at 240 rpm, 30°C. After 24 h, additional 175 \(\mu\)L of 100% methanol (final concentration: 0.7%) and 6.25 \(\mu\)L of 10 mM all-trans-retinal (ethanol stock, final concentration: 2.5 \(\mu\)M) were added into the culture. After further growth for 14-17 h at 30°C, the cells were harvested by centrifuging at \(1,500 \times g\) for 5 min at 4°C. If the expression level of mutant protein is high enough, the yeast pellet would show a red or orange color, especially after breaking the cells, due to binding of retinal by LR. By then, the colonies showing the most intense color were selected for a large scale expression.

2.3.6. Large scale expression and isotope-labeling of wild-type LR protein

Large-scale protein expression followed the established shake-flask protocol for secreted soluble proteins [138] with small modifications. Briefly, a small piece from a cell colony with the highest expression level of LR in small-scale culture was inoculated into 50 mL of BMD (or \(^{13}\)C,\(^{15}\)N-BMD for isotope-labeled LR, with 0.5% \(^{13}\)C-glucose and 0.8% \(^{15}\)NH\(_4\)Cl) in a sterile 250 mL baffled flask. This seed culture was grown, shaking at 30°C (300 rpm) for 18–24 h, until the OD\(_{600}\) exceeded 2, and
inoculated into a sterile 2 L baffled flask containing 200 mL of BMD (or
\(^{13}\text{C},^{15}\text{N}-\text{BMD} \)). This culture was shaken at 29–30°C (270 rpm) for 18–24 h, until the
OD\(_{600}\) reached 10. To induce LR expression, the cells were pelleted in sterile
containers at 1,500\(\times\)g for 5 min at 4°C, and gently resuspended in 0.8 L of BMM (or
\(^{13}\text{C},^{15}\text{N}-\text{BMM} \), with 0.5% \(^{13}\text{C}\)-methanol and 0.8% \(^{15}\text{NH}_4\text{Cl} \)), which was placed into
2.8 L Fernbach flask and shaken at 29–30°C (240 rpm). The temperature was kept at
the standard value, as there was no evidence of any significant protein misfolding or
heterogeneity warranting expression at lower temperatures. 10 mM isopropanol stock
of all-trans-retinal (needed for rhodopsin regeneration, final concentration 5 \(\mu\text{M} \)) and
100% filtered methanol (or \(^{13}\text{C}\)-methanol, final concentration 0.5%) were added to the
growth medium after 24 h of induction. The 0.5% concentration of labeled methanol
(and glucose), lower than that in several labeling protocols, was used following the
recommendation of the protocol for the economical labeling [139], where it showed
additional benefits of more complete incorporation of isotopes and lack of cell lysis
by high concentration of methanol. We evaluated growth at 1% \(^{13}\text{C}\)-methanol, but did
not find any improvement of the protein yield. The red-colored cells were collected by
centrifugation at 1,500\(\times\)g for 5 min at 4°C after 40 h of induction, as the protein yield
was found to be lower upon longer (48–52 h) and shorter (24 h) incubation times. The
protein yields at 24 h were less than 20% of those at 40 h, showing much lower yield
per unit of \(^{13}\text{C}\)-methanol, making the collection at 40 h the most economical. The cell
pellet was washed with MilliQ water twice and stored frozen at -20°C for later use.
2.3.7. Scale-up of the expression of N79S LR mutant

Large-scale expression was carried out analogously to the wild-type LR (the pHIL-S1-LR construct) [110, 126]. The colonies, which showed the darkest color after complete breakage of the cells, were picked from an MD plate and grown in 25 mL of MGY medium at 30°C, shaking at 300 rpm for 2 days until the value of A₆₀₀ reached 10.0. Afterward, 4 mL of the culture was transferred to fresh 250 mL of MGY in a 2 L Erlenmeyer flask and shaken at 270 rpm, 30°C. Yeast culture grown to OD₆₀₀=3.6-4 (18-24 h) was centrifuged at 1,500×g for 5 min, and the pellet was resuspended in 800 mL of BMM in a 2.8 L Fernbach flask, which was shaken at 30°C, 240 rpm for 38~41 h. The induction began upon transfer to BMM, which contained methanol as its ingredient. Additional 560 µL of 100% methanol (final concentration: 0.7%) and 400 µL of 10 mM all-trans-retinal (final concentration: 5 µM) were added 24 h after the induction. The cells were harvested 16 h after the second methanol addition by centrifugation at 1,500×g for 5 min at 4°C, and the supernatant was subsequently removed. The pellet was washed with ice-cold water once and frozen until further treatment.

2.3.8. Preparing polyacrylamide gels containing DDM-treated LR N79S mutant protein for time-resolved visible spectroscopy

For details please refer to section 2.2.9.
2.3.9. Purification of labeled LR

The cell breakage and protein purification protocols were based on those used for *Neurospora* rhodopsin (NR) and LR [110, 120] with some modifications. For details on the cell breakage procedure please refer to section 2.2.7.

To purify LR, the pellets of frozen membranes were thawed and resuspended with solubilization buffer (62.5 mL per L of culture, 1% Triton X-100, 20 mM KH$_2$PO$_4$, pH 7.5, 1 mM PMSF), stirred overnight in the dark at 4°C, and centrifuged at 40,000$\times$g for 30 min at 4°C. Solubilized LR was purified from the supernatant using 6-His tag affinity resin (Ni$^{2+}$-NTA agarose, Qiagen) by the batch method. We estimated the quantity of solubilized protein spectroscopically (Cary 50, Varian), assuming the molar extinction similar to that of BR, to calculate the amount of resin needed. The mixture was incubated in the dark at room temperature with gentle agitation to allow complete binding (usually 3 h). The clear supernatant containing other solubilized proteins was flown through empty PD-10 column (GE Healthcare), while the resin was retained at the bottom. The washing buffer (about two times of the volume of the resin, 0.25% Triton X-100, 50 mM KH$_2$PO$_4$, pH 7.5, 400 mM NaCl, 0–35 mM of imidazole, concentration increasing in 5 mM steps between consecutive washes) was added into column and mixed for 2–3 min, then allowed to flow through. The spectrum of the eluate was monitored to detect the loss of non-specifically bound cytochromes (at 410 nm) and specifically bound LR (at 540 nm). The resin was washed 12–13 times with increasing concentrations of imidazole, until the
cytochrome band disappeared from the eluate spectrum. Tightly bound LR was eluted from the resin with the elution buffer (0.25% Triton X-100, 50 mM KH₂PO₄, pH 7.5, 400 mM NaCl, 250 mM imidazole), after 2 min of mixing. The resin was eluted several times until the amount of bound LR was negligible. All eluates were combined and concentrated to 1–2 ml volume by centrifugation at 4,000×g for 15 min at 4°C in a concentrator (Amicon Ultra 15 ml, 10 kDa cutoff). The purity of this preparation was assessed by gel electrophoresis (SDS–PAGE) and MALDI TOF mass spectrometry (University of Guelph Advanced Analysis Center).

2.3.10. Analysis of purified LR by SDS-PAGE

SDS-PAGE was carried out by using Bio-Rad vertical electrophoresis system according to manufacturer’s instructions. Gel solutions were prepared according to table 2-5 and poured between two glass plates separated by 1.0 mm comb. Separating gel was polymerized first, and then the stacking gel solution was added between the glass plates. As the gel was completely polymerized, the gel cassette was assembled into electrophoresis apparatus and submerged into the running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). The cell lysates in 5× SDS-PAGE loading buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol and 0.1% bromophenol blue) were boiled for 5 min to denature the proteins before applying to acrylamide gel. Approximately 20 µg of proteins were loaded in each well. Later, they were run in the stacking gel under 100 V for 20-30 min followed by separating gel under 120 V for 2 to 3 h until the protein dye reached
the mark 1 cm from the gel bottom. The gel was detached from the glass plates after
electrophoresis and stained in Coomassie Blue Stain (0.1% (w/v) Coomassie Blue
R-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid) for 1 h. Then the gel was
destained in Coomassie gel destain buffer (10% (v/v) methanol, 10% (v/v) glacial
acetic acid) for several hours.

Table 2-5. Composition of solutions used to prepare SDS-PAGE gels.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% Stacking Gel</td>
</tr>
<tr>
<td>30% (w/v) acrylamide/0.8% (w/v) bisacrylamide</td>
<td>0.67 mL</td>
</tr>
<tr>
<td>Stacking Gel Buffer (0.5 M Tris/0.4% SDS, pH 6.8)</td>
<td>1 mL</td>
</tr>
<tr>
<td>Separating Gel Buffer (1.5 M Tris/0.4% SDS, pH 8.8)</td>
<td>/</td>
</tr>
<tr>
<td>Water</td>
<td>2.32 mL</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>12 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 µL</td>
</tr>
<tr>
<td>total</td>
<td>4 mL</td>
</tr>
</tbody>
</table>

2.3.11. Reconstitution of purified LR into liposomes

The purified protein was reconstituted into DMPA/DMPC liposomes at the 1:1
protein:lipid ratio (by weight). The procedure was the same as described in section
2.2.8.
2.3.12. FTIR measurements.

Refer to section 2.2.11 for details. The rehydration buffer used for LR/DMPA/DMPC liposomes was 0.05 M CHES, 0.05 M KH₂PO₄ and 0.1 M NaCl, pH 9.

2.3.13. NMR experiments.

All SSNMR experiments were performed as described previously for proteorhodopsin [140], by Lichi Shi and Vladimir Ladizhansky. Additional experimental details are given in the respective figures legends. The DMPA/DMPC/LR liposomes were collected by centrifugation at 900,000 g for 3 hrs, after which the solid pellet was ready for packing into the NMR rotor. The spectra were recorded on Bruker Avance III spectrometers operating either at 800.230 MHz or 600.13 MHz, both equipped with a 3.2-mm E-free $^{1}\text{H}$$^{13}\text{C}$$^{15}\text{N}$ probe (Bruker). The MAS frequency was 14.3 kHz for experiments carried on the 800 MHz spectrometer, and 12 kHz for experiments carried on the 600 MHz spectrometer. Hydrated proteoliposomes containing approximately 7 mg of LR were center-packed in a 3.2 mm rotor. The sample temperature was maintained at 5 °C in all experiments.

2.4. Sample preparation for DDR2

2.4.1. Reconstitution of purified DDR2 wild-type and mutant proteins into liposomes

Wild-type and mutant DDR2 proteins were provided by our collaborators (Prof. K.-H. Jung laboratory, Dept. of Life Sciences, Sogang University, Seoul, Korea) either in E. coli membranes or as purified proteins. The protein purification protocol was the
same as that for PhaeoRD1 described in section 2.2.7, but with different solubilization buffer (0.8% DDM, 20 mM KH$_2$PO$_4$, 0.3 M NaCl, 10 mM β-mercaptoethanol, and 1 mM PMSF, pH 7.5). The purified protein was reconstituted into DMPA/DMPC liposomes at 1:3 protein:l lipid ratio (w/w). The procedure was as described in section 2.2.8.

2.4.2. Preparing polyacrylamide gels containing wild-type or mutant DDR2 membrane or liposomes for time-resolved visible spectroscopy

The DDR2 membranes were resuspended in deionized H$_2$O (twice the volume of the pellet). The DDR2/DMPC/DMPA liposomes were resuspended in deionized H$_2$O at a final concentration of ~0.1 mg protein per 1 mL. Obtained DDR2 membrane or liposome suspension was used for preparing polyacrylamide gel (1 mL of polyacrylamide gel = 0.3 mL of 33% acrylamide + 0.3 mL of 1% bis-acrylamide + 0.4 mL of liposome solution + 2.4 µL of 10% APS + 3 µL of TEMED). Normally, the OD value of protein for 1 mL gel was in the range from 0.2 to 0.5 (about 0.1 to 0.25 mg protein per gel). After the gel was solidified, it was washed in distilled water overnight at room temperature to get rid of the remaining APS and TEMED. The protein gels were equilibrated with the desired buffer for at least 2 h before the measurements.

2.4.3. Visible spectroscopy measurements

Refer to section 2.2.10 for details.
2.4.4. FTIR spectroscopy measurements

Refer to section 2.2.11 for details.
Chapter 3

Photochemical Characterization of a Novel Fungal Rhodopsin from *Phaeosphaeria nodorum*

Part of the material contained in this chapter was accepted to *Biochim. Biophys. Acta – Bioenergetics*, available on line from July 2011, and now in press.
Summary

Eukaryotic microbial rhodopsins are widespread bacteriorhodopsin-like proteins found in many lower eukaryotic groups including fungi. Many fungi contain multiple rhodopsins, some significantly diverged from the original bacteriorhodopsin template. Although few fungal rhodopsins have been studied biophysically, both fast-cycling light-driven proton pumps and slow-cycling photosensors have been found. The purpose of this study was to characterize photochemically a new subgroup of fungal rhodopsins, the so-called auxiliary group. The study used the two known rhodopsin genes from the fungal wheat pathogen, *Phaeosphaeria nodorum*. One of the genes is a member of the auxiliary group while the other is highly similar to previously characterized proton-pumping *Leptosphaeria* rhodopsin. Auxiliary rhodopsin genes from a range of species form a distinct group with a unique primary structure and are located in the carotenoid biosynthesis gene cluster. The amino acid conservation pattern suggests that auxiliary rhodopsins retain the transmembrane core of bacteriorhodopsins, including all residues important for proton transport, but have unique polar intramembrane residues. Spectroscopic characterization of the two yeast-expressed *Phaeosphaeria* rhodopsins showed many similarities: absorption spectra, conformation of the retinal chromophore, fast photocycling, and carboxylic acid protonation changes. It is likely that both *Phaeosphaeria* rhodopsins are proton-pumping, at least *in vitro*. We suggest that auxiliary rhodopsins have separated from their ancestors fairly recently and have acquired the ability to interact with as yet unidentified transducers, performing a photosensory function without changing their
spectral properties and basic photochemistry.

3.1. Introduction

Microbial rhodopsins are typical membrane proteins with a seven transmembrane helical bundle similar to that of G-protein-coupled receptors [3, 7, 8]. Most microbial rhodopsins are photosensitive, with all-trans-retinal as chromophore, covalently bound via the Schiff base to a Lys sidechain. Retinal photoisomerization triggers functionally important conformational changes in the protein (opsin) moiety (please see chapter 1 for general introduction on microbial rhodopsins for more details). Since the last century, our perception of the functional, taxonomic, and ecological diversity of microbial rhodopsins has undergone a revolutionary change. Previously regarded as an eclectic mix of halobacterial light-driven proton and chloride pumps and related photosensory receptors, they have emerged as a large, widespread, multi-functional group found not only in Archaea, but in many Bacteria and Eukarya, including numerous fungal and algal species [7, 9, 17, 96, 102]. New functions were defined, including new types of photosensors, light-gated ion channels, and light-activated enzymes. We now recognise that many prokaryotic and eukaryotic species possess multiple rhodopsin (RD) and opsin-related protein (ORP) genes, which may have arisen both via gene duplication (often, multiple) and by lateral gene transfer [7, 9].

There is clear evidence that fungal rhodopsins evolved via gene duplication and neofunctionalisation [16, 102, 103]. Fungal rhodopsins are clearly related to archaeal,
rather than eubacterial, ancestors, most probably originating from the light-driven halobacterial proton pump, bacteriorhodopsin (BR) [7, 15]. Some fungal RDs share the original haloarchaeal BR-like protein template and its proton pumping ability, whilst others lost the chromophore-binding lysine (these are opsin-related proteins (ORPs)), with a range of divergent forms in between [3, 102, 103]. The recent flood of genome sequences has shown that numerous fungal species possess multiple RDs and ORPs. However, few have been functionally characterised, and their photobiological role is largely unknown.

The first identified homologs of BR in fungi were ORPs from yeast and basidiomycetes. On the basis that they were expressed during stress it was suggested they act as chaperones [104, 105]. Their discovery was followed by the detection [106] and in vitro photochemical characterization [107] of Neurospora crassa rhodopsin (NR), which coexists with its ORP. Photochemical characterization of NR expressed in Pichia pastoris revealed a slow photocycle suggesting its role is photosensory rather than proton-pumping [107, 108, 120]. Phenotypic characterization of the knock-out mutants of NR (nop) (or its close homolog in Fusarium fujikuroi (opsA)) did not reveal an obvious function for NR, but implied participation in carotenoid biosynthesis regulation [114, 117]. In contrast, the closely related rhodopsin from Leptosphaeria maculans (LR) [116] had a fast photocycle and could pump protons like BR [110, 123]. Site-directed mutagenesis showed that one of the key differences responsible for the dramatically different photochemical behavior of NR and LR
originated from a seemingly innocuous Asp/Glu replacement at the key position of the cytoplasmic proton donor to the retinal Schiff base [111, 126]. Recent electrophysiological studies of NR (along with its close homolog in Podospora anserina) and LR expressed in neurons confirmed their drastically different proton-pumping abilities [130].

Thus, even the limited biochemical and physiological analysis available so far suggests multiple functions of fungal rhodopsins. Additionally, genomic information from several fungal species shows the existence of a third group of fungal rhodopsins; these have overall sequence resemblance to ORPs, but retain all the key residues of the BR-like template [15, 102]. We have tentatively called this group the auxiliary ORP-like rhodopsins, referring to their co-existence with other rhodopsin forms in the same species [15]. Auxiliary rhodopsins have been found in many fungal species, but their expression pattern has been analyzed only in Fusarium fujikuroi [112] and Bipolaris oryzae [141] (plus distant homologs from basidiomycete Ustilago maydis [142]). A knock-out mutant of the Fusarium fujikuroi gene (carO) produced no phenotypic alterations under laboratory conditions. It may be linked to carotenoid metabolism as it is found in the carotenoid biosynthesis gene cluster [112, 131]. So far, no auxiliary rhodopsin has been characterised physiologically or photochemically. Thus, one may only speculate about their role(s); a photosensory function tuned to a distinct spectral region is perhaps the most plausible hypothesis.
Here, we present photochemical characterization of an auxiliary rhodopsin using the protein from *Phaeosphaeria (Stagonospora) nodorum* (PhaeoRD2) and compare it with the LR-like homolog (PhaeoRD1) [143]. Both rhodopsins were expressed in *Pichia pastoris* and characterized spectroscopically. The two rhodopsins have similar absorption spectra, disproving the idea that the auxiliary species are needed to respond to light stimuli of different wavelengths. Spectroscopic and mutational data suggest that the auxiliary PhaeoRD2 may have some proton-pumping ability, similar to LR and PhaeoRD1.

3.2. Results and Discussion

3.2.1. Sequence-based Analysis

The *Phaeosphaeria nodorum* genome annotation [143] included two rhodopsins. Ops1 (or PhaeoRD1) is very similar to LR, while the second rhodopsin (PhaeoRD2) belongs to a new subgroup, not characterized spectroscopically [15]. We called this subgroup “auxiliary”, because most of its members were found in addition to other rhodopsin forms. Since then, many new fungal genomes have become publicly available, so that the placement of *Phaeosphaeria* rhodopsins, as well as clustering of fungal rhodopsins in general, can be reevaluated with much greater confidence. Thus, we first compared amino acid sequences of the two *Phaeosphaeria* rhodopsins to the sequences of rhodopsins (full sequences only, excluding ORPs) from other ascomycetes, using publicly available genome databases (http://blast.ncbi.nlm.nih.gov/; http://genome.jgi-psf.org/;
The results of multiple sequence alignment analysis done by CLUSTALW confirm our earlier suggestion [15] that auxiliary rhodopsins form a very distinct branch on the fungal rhodopsin tree (boxed in Fig. 3.1). The analyzed fungal rhodopsins can be divided into two large subgroups. Within each of the subgroups, the rhodopsins align with species phylogenies [144]. The first subgroup includes previously characterized putative photosensors and proton pumps such as NR and LR. Many of the fungal species found in this first group, especially those from *Pleosporomycetidae, Dothideomycetidae, Helotiales*, and *Hypocreomycetidae*, have additional, second rhodopsin forms in the second (auxiliary) subgroup. Additionally, the auxiliary subgroup contains third and fourth forms of rhodopsins of *Dothideomycetidae* and a few standalone (if we disregard ORPs) rhodopsins, e.g., from several species of *Colletotrichum* and *Verticillium* (Fig. 3.1). It is clear that, as suggested before, PhaeoRD1 is the closest homolog of LR (79% identity, 91% similarity), and as such belongs to the first subgroup, while PhaeoRD2 is a member of the auxiliary cluster, with distinct amino acid sequence (35% identity, 53% similarity to LR). This analysis suggested that the photochemical and functional properties of PhaeoRD1 would be LR-like, while those of PhaeoRD2 were unknown.

In view of the earlier finding that the auxiliary rhodopsin from *Gibberella (Fusarium) fujikuroi, carO*, was found in a carotenoid biosynthesis cluster that also contains a
carotene oxygenase \textit{carX}, phytoene synthase/cyclase \textit{carRA} and phytoene desaturase \textit{carB} [112], we explored the genomic context for the members of this group. The cluster structure is preserved in \textit{Helotiales} (Botrytis and Sclerotinia) and \textit{Hypocreomycetidae} (Gibberella zeae, Gibberella moniliformis, Fusarium oxysporum, Nectria), and selected \textit{Dothideomycetes} (Mycosphaerella graminicola and Rhytidhysteron rufulum). In the \textit{Pleosporomycetidae} (Phaeosphaeria, Leptosphaeria, Pyrenophora, Alternaria, Cochliobolus, Setosphaeria) gene orders and orientations are shuffled (as seen in many fungal gene clusters, e.g. [143]), in a local reflection of mesosynteny [145]. The clustering of auxiliary rhodopsins with carotenoid biosynthesis genes is strongly suggestive of a carotenoid-related physiological role and expression regulation as described for \textit{carO} [114, 131].

To gain further insight into the structural differences between the two major rhodopsin subgroups, we have aligned amino acid sequences of the representative members of the auxiliary subgroup (restricted to second rhodopsin forms, including PhaeoRD2) (Fig. 3.2) and compared the conservation pattern in the last six transmembrane helices (most conserved in microbial rhodopsins) with that known for BR and LR [15, 116, 127]. The full-length alignment (except for the non-conserved termini) of a broader selection of sequences is available in the appendix. The first general trend observed from the alignment is a very high degree of conservation of the BR-like template (shown yellow on black) in fungal rhodopsins of both subgroups. The conserved residues include most of the retinal-binding pocket and the majority of amino acids.
Figure 3.1. *Phaeosphaeria* rhodopsins as representatives of the two major subgroups of fungal rhodopsins. Unrooted guide tree of fungal rhodopsin sequences from ascomycetes (excluding OPRs) produced from the CLUSTALW [146] alignment and plotted using TREEVIEW [147]. Numbers after the names of fungal species indicate multiple forms of rhodopsins found in the same species. The scale bar represents number of substitutions per site (0.1 indicates 10 nucleotide substitutions per 100 nucleotides). *Phaeosphaeria* rhodopsins studied in this work are highlighted yellow, previously characterized rhodopsins are highlighted purple, and the auxiliary subgroup of rhodopsins is boxed.
implicated in the light-driven proton transport (BR’s T46, Y57, R82, D85, T89, T90, D96, D115, W182, Y185, W189, E194, E204, D212, and many others). This suggests that auxiliary rhodopsins may possess proton pumping ability similar to that observed for LR [110], as all major proton donors and acceptors of BR are conserved. It must be noted that the primary proton donor (homolog of BR’s D96) is strictly conserved as Asp in the auxiliary subgroup, as it is known that its conservative replacement by Glu can strongly impede the proton transport in NR and mutant LR [111]. From the conservation pattern of the BR template in fungi, it is impossible to reliably predict which one of the subgroups is evolutionarily closer to the archaeal ancestor, as there are almost equal numbers of cases of exclusive conservation of BR residues in each subgroup. On the other hand, our CLUSTALW analysis of the full-length opsin sequences (Fig. 3.3) places BR somewhat closer to the first subgroup, in agreement with the previous analysis [114].

Next, we analyzed the distribution of the residues uniquely conserved in the auxiliary subgroup (highlighted purple in Fig. 3.2) relative to the putative membrane core of these proteins, as defined by homology to BR structure. While most of the unique residues are located at the ends of the helices in the membrane interfacial regions, there are notable exceptions, the most striking of which is helix D. Even though there are several uniquely conserved residues in the middle of the helices E and F, they do not change the overall character of those helices, being mere changes in size of the affected hydrophobic sidechains. On the contrary, there must be a dramatic change in
Figure 3.2. Conservation of the BR template in fungal rhodopsins and unique structural features of the auxiliary subgroup. CLUSTALW alignment of partial sequences of representative members of the auxiliary subgroups (highlighted purple), restricted to the conserved transmembrane regions of the last six helices (helices B-G, see appendix for the full-length alignment). Sequences of BR, LR, and PhaeoRD1 are given for comparison. The residues conserved in BRs are yellow on black, residues most important for proton transport are numbered using BR sequence, and the residues unique for the auxiliary group are highlighted purple. Abbreviations: Leptos. – *Leptosphaeria maculans*, Pyrenoph. – *Pyrenophora tritici-repentis*, Altern. –
Alternaria brassicicola, Bipolar. - Bipolaris oryzae, Dothistr. – Dothistroma septosporum, Mycosph. – Mycosphaerella graminicola, Gibber. – Gibberella zeae, Fusar. – Fusarium oxysporum, Hyster. – Hysterium pulicare, Sclerot. – Sclerotinia sclerotiorum.

the properties of the helix D, as a result of the introduction of a polar residue with hydrogen bonding ability into the middle of the transmembrane domain, corresponding to position 116 of BR, along with a number of other changes (Fig. 3.2). The polar residue in the middle of helix D of fungal rhodopsins from the auxiliary group is usually represented by Glu, and sometimes by Trp, and follows the super-conserved homolog of Asp-115 of BR. This puts severe constraints on the possible sidechain orientation of this new polar residue. As Asp-115 is hydrogen-bonded to Thr-90 from helix C in BR [77], and this pair is preserved in all fungal rhodopsins, one may expect that the following Glu-116 will face the core of the lipid bilayer (Fig. 3.4).

This is highly unlikely, unless it is used to interact with a protein partner (either an unknown transducer or another rhodopsin molecule, leading to oligomerization). From this tentative analysis, one may speculate that rhodopsins of the auxiliary subgroup have preserved their proton-pumping ability, but have also acquired capacity to interact with membrane-bound transducers. This is reminiscent of the evolutionary relationship between BR and halobacterial sensory rhodopsins, which preserved rudimentary proton-pumping ability in the absence of their transducers and use the same conformational changes as proton pumps to perform signaling [58, 148].
Figure 3.3. Guide tree of rhodopsins from fungi (*Ascomycetes* only) showing their relationship to BR (highlighted), produced using CLUSTALW and TREEVIEW. The upper branch represents the auxiliary group, the lower branch represents LR-like and NR-like rhodopsins.
Figure 3.4. The predicted PhaeoRD2 model (without retinal) based on BR 1c3w pdb file by using Swiss-Model [149]. a. the top view of BR. b. the top view of PhaeoRD2. c. Left: the side view of BR; Right: the side view of PhaeoRD2. For BR, the retinal chromophore and Gly116 are shown in stick representation. For PhaeoRD2, Glu146 is shown in stick representation.
3.2.2. Photochemical Characterization

Both *Phaeosphaeria* opsins expressed in *Pichia pastoris* formed red pigments upon addition of all-trans-retinal. The resulting holo-proteins were stable both in the yeast membranes and upon reconstitution of the purified proteins into synthetic lipids. The dark states of the obtained chromoproteins were first characterized by visible and Raman spectroscopy (Fig. 3.5). The maxima of the visible absorption spectra of both proteins were similar and close to that observed for LR (542 nm in yeast membranes [110]). The purified solubilized proteins have absorption maxima at 540 nm for PhaeoRD1 and 535 nm for PhaeoRD2 (Fig. 3.5, left panel), and the respective maxima are at 545 nm and 538 nm in yeast membranes (not shown). No apparent light- or dark-adaptation was observed, similar to the case of LR [110, 123].

According to the Raman spectroscopy results (Fig. 3.5, right panel), which report mostly on the retinal chromophore, the dark states contain predominantly all-trans-retinal. This is obvious from the prominent pair of C-C stretching vibrations around 1202 and 1168 cm$^{-1}$, similar to those of light-adapted BR and LR [43, 110]. The location of the major ethylenic C=C stretches (at 1533 cm$^{-1}$ for PhaeoRD1 and at 1537 cm$^{-1}$ for PhaeoRD2) is consistent with their visible maxima, where higher frequency correlates with more blue-shifted visible absorption [150]. From the characterization of the dark states, we can conclude that it is unlikely that these two rhodopsin forms exist solely to respond to different wavelength of visible light, as their absorption maxima are very close to each other and both fall into the green region. It can be also argued that the retinal-binding pockets of both *Phaeosphaeria*
opsins must be similar to that of LR, which is expected from the conservation of their transmembrane regions (Fig. 3.2), as they show very close visible maxima and similar vibrational spectra of the chromophore.

Figure 3.5. Characterization of the dark states of *Phaeosphaeria* rhodopsins. (Left panel) Visible spectra of solubilized purified rhodopsins at room temperature, pH 7.5. PhaeoRD1 was solubilized in 0.25% DDM, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 250 mM imidazole, and PhaeoRD2 in 0.25% Triton X-100, 50 mM KH$_2$PO$_4$, 400 mM NaCl, 250 mM imidazole, 0.2 mg/ml *Pichia* lipids. (Right panel) Raman spectra of liposome-reconstituted dark-adapted rhodopsins suspended in 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 7, at room temperature, accumulated for 14 h with 1024 nm excitation.
Next, we characterized the photochemical cycles of both *Phaeosphaeria* rhodopsins using time-resolved spectroscopy in the visible range. The photocycle kinetics of PhaeoRD1 were measured (Fig. 3.6) at room temperature at neutral pH (7), by recording wavelength scans at 20-nm intervals from 400 to 640 nm. As expected from the high degree of sequence identity of PhaeoRD1 and LR, their photochemistry was very similar (Fig. 3.7, lower panel). At neutral pH, the photocycle of PhaeoRD1 is quite fast, finishing in a few tens of milliseconds, as expected for proton pumps [3, 15]. It has a well-defined M intermediate with the deprotonated retinal Schiff base (observed at 400 nm), which forms on a submillisecond time scale and decays in a pH-dependent manner (Fig. 3.7, upper panel), again, similar to LR [110]. At lower pH, the reprotonation of the Schiff base (M decay) is fast (a few ms), and the M intermediate is followed by a red-shifted intermediate, which disappears at higher pH, when the M decay becomes slow. The only notable difference in the photocycle kinetics of PhaeoRD1 and LR is a higher accumulation of the early red-shifted intermediate along with the early M intermediate on the tens of microseconds time scale, which may point at a somewhat shifted protonation equilibrium between the Schiff base and the primary proton acceptor. From the early parts of the 460 nm kinetics, it is also obvious that an L-like intermediate accumulates in equilibrium with the K-like and the early M states. But this difference does not affect the later parts of the photocycle of PhaeoRD1, which is consistent with the expected LR-like photochemistry of a light-driven proton pump.
Figure 3.6. The laser flash-induced photocycle kinetics of PhaeoRD1 in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl, pH 7. Photocycle kinetics were measured at 20-nm intervals from 400 to 640 nm.
Figure 3.7. The laser flash-induced photocycle kinetics of PhaeoRD1 in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES. (Upper panel) Photocycle kinetics measured at pH 5-9 at characteristic wavelength: 620 nm (K and O intermediates) – red, 540 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 400 nm (M intermediate) – blue. (Lower panel) Comparison of the photocycle kinetics of PhaeoRD1 (color-coded as in the upper panel) and LR (black) at pH 6, normalized at the minimum of the 540 nm signal. The LR data are taken from the earlier multi-wavelength dataset [110].
An additional difference between LR and PhaeoRD1 in the late stages of the photocycle is a higher accumulation of the N intermediate, obvious from the differences in kinetics at 560 nm (Fig. 3.7, lower panel) at ~10 ms delay. In attempt to explain this difference, we compared the amino acid sequences of PhaeoRD1, BR, NR, and LR in the relevant cytoplasmic regions. While most of the critical cytoplasmic residues which could be involved in regulation of the accumulation of the N intermediate (i.e., deprotonation and reprotonation of the cytoplasmic proton donor) were conserved, one site in the Helix B (Fig. 3.8) attracted our attention based on the information provided from mutagenesis of BR published previously. While the replacement of Asp-38 by Asn in BR did not make dramatic changes to the photocycle, the photocycles of D38C and D38R mutants of BR were much slower than that of the wild-type protein [78, 151]. The homolog of Asp-38 in BR is Asn-79 in LR, Ser-71 in NR and Ser-73 in PhaeoRD1, which suggests that nature of this residue may

**Figure 3.8.** Comparison of amino acid sequences in helix B and A-B loop of BR, LR, PhaeoRD1, and NR, obtained using BLAST. Residues conserved for BRs are highlighted black, for SR-IIs – red, for fungal rhodopsins – green.
contribute to that difference in the photocycle. Thus, Asn-79 in LR was replaced by serine to see if it will change the kinetics of LR into the PhaeoRD1-like one. The N79S mutant of LR expressed in Pichia well, and its pH-dependent photocycle kinetics at pH 6 are compared with that of wild-type LR in Fig. 3.9, lower panel. Against our expectations, the photocycles of the mutant and wild-type LR have the same overall characters, and the same pH dependence. This implies that, contrary to the situation in BR, the photocycles of fungal rhodopsins are not sensitive to the nature of the polar residue at the cytoplasmic top of helix B, homologous to Asp-38 of BR.

The photocycle of PhaeoRD2 is shown in Fig. 3.10, as measured by time-resolved difference spectroscopy in the visible range at room temperature at neutral pH (7), doing a wavelength scan at 20-nm intervals from 400 to 640 nm. The photocycle kinetics of PhaeoRD2 are quite different from those of PhaeoRD1 and LR (Fig. 3.11). On the one hand, the overall kinetics of the photocycle is quite fast, with the turnover characteristic time of a few tens of ms at neutral pH, which is consistent with a proton-pumping rhodopsin behavior, similar to PhaeoRD1. On the other hand, kinetics of the rise and decay, as well as relative concentrations of photointermediates, differs dramatically for the two Phaeosphaeria rhodopsins (Fig. 3.11, lower panel). The most striking feature of the photocycle of PhaeoRD2 is an extremely fast deprotonation of the retinal Schiff base, as observed by the rise of the M intermediate at 400 nm. The plateau of the M intermediate concentration is reached in less than 10
Figure 3.9. The laser flash-induced photocycle kinetics of N79S mutant of LR in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES. (Upper panel) Photocycle kinetics measured at pH 5-9 at characteristic wavelength: 620 nm (K and O intermediates) – red, 560 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue. (Lower panel) Comparison of the photocycle kinetics of N79S mutant of LR (color-coded as in the upper panel) and LR (black) at pH 6. The LR data are taken from the earlier multi-wavelength dataset [110].
microseconds, as opposed to the sub-millisecond plateau in PhaeoRD1, LR, and BR. Such extremely fast pH-independent deprotonation of the retinal Schiff base is typical for BR mutants with perturbed protonation equilibria between the Schiff base nitrogen and Asp-85, especially as found in mutants involving Arg-82 [152]. While homologs of Arg-82, as well as of other important members of the extracellular hydrogen-bonded network (Tyr-57, Glu-194, Glu-204) [77], are conserved in all auxiliary fungal rhodopsins, there are many unique residues in the extracellular loops and interfacial regions (Fig. 3.2 and appendix).

Figure 3.10. The laser flash-induced photocycle kinetics of PhaeoRD2 in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl, pH 7. Photocycle kinetics were measured at 20-nm intervals from 400 to 640 nm.
Figure 3.11. The laser flash-induced photocycle kinetics of wild-type PhaeoRD2 in the DDM-washed yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES. (Upper panel) Photocycle kinetics measured at pH 5-9 at characteristic wavelength: 620 nm (K and O intermediates) – red, 540 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 400 nm (M intermediate) – blue. (Lower panel) Comparison of the photocycle kinetics of PhaeoRD2 (color-coded as in the upper panel) and PhaeoRD1 (black, taken from Fig. 3.6) at pH 6, normalized at the minimum of the 540 nm signal.
These unique residues could interact with the sidechain of the homolog of Arg-82 in PhaeoRD2 and change its position, affecting the pKa of the primary proton acceptor (homolog of Asp-85) via the well-described coupling mechanism [74, 153, 154]. Additionally, even though the kinetics of the Schiff base reprotonation (the M decay at 400 nm) are similarly fast and pH-dependent for PhaeoRD1 and PhaeoRD2, the accumulation of the late red-shifted intermediate (observed at 620 nm) is much higher in PhaeoRD1, possibly due to its faster decay in PhaeoRD2. It should be noted that the pH-dependence of the Schiff base reprotonation in both proteins (Fig. 3.13) is much more strongly pH-dependent than that in BR (and even LR). The persistence of the fast phase of the Schiff base reprotonation in BR is usually explained by the internal nature of its proton donor, Asp-96 [155]. The absence of such phenomenon can be interpreted as a sign of a lower pK_a of its homologs in the N-like intermediates in Phaeosphaeria rhodopsins.

The fast reprotonation of the Schiff base along with the rapid photocycle turnover in PhaeoRD2 hints at the possibility that it may have some proton-pumping ability. This would be consistent with the sequence analysis presented above, which showed the presence of the conserved homolog of Asp-96 of BR, Asp-126, possibly serving as an internal cytoplasmic proton donor to the Schiff base, ensuring its fast reprotonation. To verify that idea, we replaced the putative cytoplasmic proton donor Asp-126 with non-protonatable Asn and studied the photocycle of the D126N mutant (Fig. 3.12). If Asp-126 is indeed the primary proton donor for the Schiff base of PhaeoRD2, one
would expect a dramatically slower Schiff base reprotonation (M decay at 400 nm), similar to what was observed for LR [110, 111]. Consistent with these expectations, we observed extremely slow (on the seconds time scale) pH-dependent M decay (Fig. 3.12, upper panel, and 3.13). While such dramatic deceleration of the Schiff base reprotonation is indicative of the proton-donating role of the replaced Asp-126, there is a possibility that it may occur through the global conformational effect of the D126N mutation. The latter hypothesis can be easily disproved by checking the effect of a common artificial proton shuttle, sodium azide (NaN₃), which is known to accelerate the Schiff base reprotonation in the homologous mutants of microbial rhodopsins [110, 125]. Addition of 1 mM NaN₃ (Fig. 3.12, lower panel) restored the wild-type-like kinetics of the Schiff base reprotonation (millisecond time scale), confirming the proton-donating role of Asp-126. The M decay rates of the D126N mutant, reflecting the reprotonation of the Schiff base, were obtained by the exponential fitting of the kinetic data and compared with those of PhaeoRD1 and PhaeoRD2 wild-types as shown in Fig. 3.13.
Figure 3.12. The laser flash-induced photocycle kinetics of PhaeoRD2 D126N mutant in yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes (not treated with DDM) were encased in polyacrylamide gels equilibrated with 0.05 M KH₂PO₄ and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris. (Upper panel) Photocycle kinetics measured at pH 5-8 at characteristic wavelength: 620 nm (K and O intermediates) – red, 540 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 400 nm (M intermediate) – blue. (Lower panel) Comparison of the normalized photocycle kinetics at 400 nm, representing the reprotonation of the retinal Schiff base, of PhaeoRD2 wild-type (black) and the D126N mutant at pH 5 with (red) and without (blue) 1 mM NaN₃.
Figure 3.13. pH-dependence of the rates of reprotonation of the Schiff base (M decay) in PhaeoRD1, PhaeoRD2 wild-type and D126N mutant obtained by the exponential fitting of the data. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES.

As mentioned in the sequence-based analysis section, PhaeoRD2 has a unique Glu-146 in the helix D, seemingly facing the lipid bilayer, and this (or another polar) residue is observed in all members of the auxiliary group. It is possible that this polar residue may participate in protein-protein interactions in the membrane. Here, the mutations of Glu-146 were investigated to see whether replacement of this residue affects the photocycle and the proton pump-like behaviour of PhaeoRD2. Originally, we planned to replace Glu-146 with a non-polar residue (Leu), but this protein failed
to express. Thus, we opted for more conservative replacement, changing Glu-146 into a shorter Asp. The photocycle kinetics of the E146D mutant of PhaeoRD2 was similar to that of the wild-type, having the same overall characteristics and the same pH dependence (Fig. 3.14, upper panel). There are only slight differences between the wild-type and E146D mutant photochemical cycles, clearly observed when the kinetic traces are overlapped and compared at pH 7 (Fig. 3.14, lower panel). The first difference is that E146D mutant takes slightly longer time to finish the photocycle than the wild-type PhaeoRD2, probably due to somewhat slower reprotonation of the cytoplasmic proton donor Asp-126. The second difference is that the apparent rate of the Schiff base deprotonation and reprotonation (M rise and decay at 400 nm) of E146D is somewhat faster than in the wild-type. Considering that this mutation is a conservative replacement, the similar photochemical reactions were expected.
Figure 3.14. The laser flash-induced photocycle kinetics of PhaeoRD2 E146D mutant in yeast membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes (not treated with DDM) were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris, pH 9 - 0.05 M CHES. (Upper panel) Photocycle kinetics measured at pH 5-9 at characteristic wavelength: 620 nm (K and O intermediates) – red, 540 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 400 nm (M intermediate) – blue. (Lower panel) Comparison of the photocycle kinetics of PhaeoRD2 E146D mutant (color-coded as in the upper panel) and PhaeoRD2 wild-type (black, taken from Fig. 3.6) at pH 7.
Taken together, the photocycle kinetics data obtained by visible spectroscopy on the wild-type and mutant PhaeoRD2 strongly argue for its proton-pumping ability, even though we could not verify it directly, due to the instability of PhaeoRD2 under continuous illumination in liposomes. At the same time, it is conceivable that the photocycle of PhaeoRD2 (as well as its proton-pumping ability) are different in vivo, upon interaction with its putative transducer (in the case it is a photosensory rhodopsin as hinted by the sequence analysis). As dramatic changes in the photochemistry and ion transport are known for halobacterial sensory rhodopsins [56, 57, 156], in vitro kinetic data should be treated with caution.

To obtain further insight into the molecular details of light-induced proton transfers and conformational changes of the retinal chromophore and the opsin moiety of the Phaeosphaeria rhodopsins, we employed time-resolved difference Fourier-transform infrared (FTIR) spectroscopy. The FTIR measurements on PhaeoRD1 were performed at 12°C at two different pH values (7 and 10). Low temperature and high pH value were used to slow down the photocycle and observe the earlier intermediates, as well as to shift the equilibrium between the intermediate with protonated Schiff base and deprotonated cytoplasmic proton donor (the N-like intermediate) and the M-like intermediate with deprotonated Schiff base. The predominant photointermediates observed in these spectra can be identified by the intensities of characteristic bands in the fingerprint region (C-C stretches of retinal) [37]. The difference spectra taken at two different time delays (1 ms and 25 ms) after the excitation are compared in Fig.
3.15. Consistent with the data from the visible spectra, the photocycle of PhaeoRD1 is primarily dominated by M intermediate at higher pH value (pH 10), as obvious from mostly negative retinal bands. At pH 7, a spectral comparison between spectra taken at 1 ms and 25 ms delays (Fig. 3.15 A and B) reveals that while the early spectra are dominated by M as well, more N-like features appear at the later time, as judged from the stronger intensity of 13-cis-retinal peak at 1188 cm\(^{-1}\). The carboxylic acid vibrations pattern is consistent with that, as the negative intensity corresponding to the disappearance of protonated cytoplasmic donor at 1745 cm\(^{-1}\) develops at the later times, along with stronger positive band of deprotonated carboxyl at 1390 cm\(^{-1}\). The same pattern was observed in Fig. 3.15 C and D, even though it is harder to observe due to much greater proportion of M in the M/N-mixtures at pH 10. Likewise, FTIR difference spectra of PhaeoRD2 were measured at different pH values. In Fig. 3.16, the difference spectrum taken at pH 7 is compared to that at pH 10, with the same time delay (1ms). Obviously, the photocycle of PhaeoRD2 is strongly dominated by the M intermediate at pH 7, as expected from the visible spectra (Fig. 3.11), while some N-like intermediates are observed at pH 10 in addition to still predominant M. The latter observation is consistent with the lower amplitude ratio of the signals at 400 and 540 nm (Fig. 3.11), characteristic for the accumulation of the N intermediate [157]. The D126N mutation of PhaeoRD2 allows accumulation of almost pure M intermediate (Fig. 3.12), and this allowed us to verify the FTIR spectrum of this intermediate as shown in Fig. 3.17. As expected, the three negative peaks of all-trans-retinal at 1246, 1201, and 1167 cm\(^{-1}\) without any positive counterparts are
observed in the fingerprint region of the spectrum of the D126N mutant, typical for the M intermediate [5, 37].

Figure 3.15. Time-resolved laser flash-induced difference FTIR spectra of PhaeoRD1 reconstituted into DMPC/DMPA hydrated liposomes measured at several ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 12°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see text for details. A. Liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 7, and measured at 1 ms delay after the flash. B. Liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 7, and measured at 25 ms delay after the flash. C. Liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, and 0.05 M CHES, pH 10, and measured at 1 ms delay after the flash. D. Liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 10, and measured at 25 ms delay after the flash.
Figure 3.16. Time-resolved laser flash-induced difference FTIR spectra of PhaeoRD2 reconstituted into DMPC/DMPA hydrated liposomes measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 12°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see text for details. A. Liposomes hydrated with 0.05 M KH₂PO₄, 0.1 M NaCl, pH 7. B. Liposomes hydrated with 0.05 M KH₂PO₄, 0.1 M NaCl, and 0.05 M CHES , pH 10.
Figure 3.17. Time-resolved laser flash-induced difference FTIR spectra of PhaeoRD2 D126N mutant reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 7 and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details.

If Asp-126 is the proton donor of the Schiff base on the cytoplasmic side as suggested by the visible spectroscopy (Fig. 3.12), it may not be possible to see the negative band belonging to D126, but one may detect a new band of C=O, which has about 40 cm$^{-1}$ downshift due to the Asp$\rightarrow$Asn substitution. In the Fig. 3.17, the positive peak at 1763 cm$^{-1}$ is assigned to the proton acceptor, the homolog of D85 in BR. A pair of peaks (1743+/1734- cm$^{-1}$) is attributable to Asp-145, the homolog of D115 in BR, based on the assignments in LR and PhaeoRD1. What is missing, compared to the
wild-type, is the small negative peak at 1747 cm\(^{-1}\), observed in the wild-type at high pH, where N accumulates and one can expect to see deprotonation of the cytoplasmic proton donor (Fig. 3.16b). The development of the new negative peak at 1707 cm\(^{-1}\) can be thus assigned to perturbation of Asn-126, and that at 1747 cm\(^{-1}\) in the wild-type spectrum to Asp-126, similar to its homolog in LR and PhaeoRD1 at 1745 cm\(^{-1}\).

Figure 3.18 compares difference FTIR spectra of PhaeoRD1 and PhaeoRD2 taken at pH 7, a few ms after the excitation. From the results of the visible spectroscopy, both spectra were expected to be dominated by the M intermediate, with some contribution from a later red-shifted intermediate in the case of PhaeoRD1. This is indeed the case, as can be observed from the C-C stretching vibrations region (fingerprints), which shows only negative bands [37, 158] corresponding to all-trans-retinal of the dark state for PhaeoRD2 (1201 and 1168 cm\(^{-1}\), lower panel), with a weak positive band of 13-cis-retinal of a late photointermediate at 1188 cm\(^{-1}\) for PhaeoRD1 (upper panel). The latter band becomes prominent in the PhaeoRD1 spectra taken at 25 ms delay after the flash (as discussed above, see Fig. 3.15), consistent with the expected rise of the late intermediate and decay of M. Overall, the FTIR difference spectra of PhaeoRD1 corresponding to the M intermediate (Fig. 3.18, upper panel), as well as to the late intermediate (Fig. 3.15b), are very similar to the corresponding spectra of LR (Fig. 3.19) [110]. Among the most typical and important opsin bands observed both for PhaeoRD1 and LR, one should mention those of protonation of the primary proton acceptor (homolog of D85 of BR) at 1759 cm\(^{-1}\), and the perturbation of the homolog
Figure 3.18. Time-resolved laser flash-induced difference FTIR spectra of *Phaeosphaeria* rhodopsins reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 7, and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 12°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see text for details. (Upper panel) PhaeoRD1; (Lower panel) PhaeoRD2.
of BR’s D115 at 1741/1736 cm\(^{-1}\). As mentioned above, at a later delay (25 ms, Fig. 3.15), an additional negative band assigned to the deprotonation of the homolog of the primary proton donor D96 was observed at 1745 cm\(^{-1}\). Additionally, prominent bands at 1390/1381 cm\(^{-1}\) recently assigned to deprotonated carboxylic acids in isotope-labeled LR [136] were observed in PhaeoRD1 as well. Most retinal bands were identical or very similar between LR and PhaeoRD1, consistent with the Raman data (Fig. 3.5), including C=C stretches at 1533 cm\(^{-1}\), C-C stretches (with other contributions) at 1250, 1201, 1169 cm\(^{-1}\), and putative Schiff base vibrations at 1643/1620 cm\(^{-1}\).

Surprisingly, the FTIR difference spectra of PhaeoRD2, dominated by the M intermediate (Fig. 3.18, lower panel) were very similar to those obtained for PhaeoRD1 and LR. Some minor differences in the FTIR spectra of *Phaeosphaeria* rhodopsins originate from the different mixtures of intermediates (almost pure M for PhaeoRD2 and mixture of M with a later intermediate in PhaeoRD1). Overall, in spite of the differences in the photocycle kinetics and the amino acid sequences, all the major vibrational bands of retinal and carboxylic acids discussed above for PhaeoRD1 were observed for PhaeoRD2 as well. This points to the high degree of conservation of the transmembrane core of BR in the auxiliary rhodopsin group and that the light-induced isomerization of retinal and ensuing proton transfers are very similar for the LR-like PhaeoRD1 and auxiliary PhaeoRD2. There are a number of differences between the two rhodopsins in several opsin bands in the range of Amide I and
Asn/Gln sidechain vibrations (1700-1600 cm\(^{-1}\)), which may reflect the differences in the conformational changes of the proteins’ interfacial regions expected from the differences in the primary structures, but at this point we cannot assign them.

**Figure 3.19.** Time-resolved laser flash-induced difference FTIR spectra of LR adapted from [110] and shown for comparison. Image from [110], and reprinted with permission from PNAS, copyright 2005. LR was reconstituted into DMPC/DMPA liposomes. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. The spectra were measured at 0.5°C, A: pH 9 at 65 ms delay; B: pH 8 at 10 ms delay.

To further verify if the core structure of PhaeoRD2 is similar to those of LR and PhaeoRD1, low temperature FTIR was applied to trap different early intermediates such as K, L, and M1. The early (fast) intermediates undergo conformational changes only in the vicinity of the retinal-binding pocket, and thus report on the core structure. Low-temperature FTIR was performed in the lab of Professor Kandori’s laboratory,
Nagoya Institute of Technology. PhaeoRD2$_k$ minus PhaeoRD2 difference spectrum was measured at 77 K, while PhaeoRD2$_L$ and PhaeoRD2$_M$ difference spectra were measured at 160 K and 240 K, respectively, and the results were compared with the spectra of LR and BR measured earlier [123]. The early intermediates of PhaeoRD2 were identified by the characteristic retinal peaks: twisted 13-$cis$-retinal at 1196 cm$^{-1}$ with strong hydrogen-out-of-plane (HOOPs) at 957 cm$^{-1}$ for K, relaxed 13-$cis$-retinal at 1188 cm$^{-1}$ for L, and deprotonated 13-$cis$-retinal with no positive bands besides the three negative bands of all-$trans$-retinal at 1256, 1204 and 1168 cm$^{-1}$ for M, in accord with the assignments for BR (Fig. 3.20, Fig. 3.22 and Fig. 3.23). From Fig. 3.20, the shape of the difference spectrum of PhaeoRD2 K intermediate is nearly identical to that of LR, except for the minor band at 1179 cm$^{-1}$ showing the presence of a later intermediate (KL or even L). This difference in the intermediates mixture reflects lower thermal stability of K, consistent with its faster decay, as observed by the visible spectroscopy (no positive signal in the early part of 620 nm signals in Fig. 3.11 as opposed to Fig. 3.7). In the Fig. 3.20a, the ethylenic stretching vibration at 1537(-)/1526(+) cm$^{-1}$ is the sign of a red-shifted intermediate, PhaeoRD2$_k$. Negative bands at 1256, 1204, and 1168 cm$^{-1}$ in the C-C stretching region and at 1008 cm$^{-1}$ methyl rocks were also detected in the Raman spectra (Fig. 3.5), and correspond to the disappearance of the all-$trans$-retinal of the dark state. Fig. 3.20 also compares the PhaeoRD2$_k$ minus PhaeoRD2, LR$_k$ minus LR, and BR$_k$ minus BR spectra in the 1700-1565 cm$^{-1}$ region, where most of the bands originate from vibrations of the protein. The C=N stretch of the retinal-lysine Schiff base is
D$_2$O-sensitive. In BR, it has been observed at 1641 cm$^{-1}$ in H$_2$O and 1628 cm$^{-1}$ in D$_2$O, respectively. Analogous spectral shifts were observed for LR (at 1648 and 1629 cm$^{-1}$ in H$_2$O and D$_2$O) and PhaeoRD2 (1648 and 1629 cm$^{-1}$ in H$_2$O and D$_2$O). The larger

Figure 3.20. PhaeoRD2$_k$ minus PhaeoRD2, LR$_k$ minus LR, and BR$_k$ minus BR spectra in the 1700-800 cm$^{-1}$ region measured at 77 K. The samples were hydrated with H$_2$O (solid line) or D$_2$O (dotted line). The measurements were performed by H. Ito.
IR frequency shift in LR than that in BR implies stronger hydrogen bond of the Schiff base [150]. The similar spectral features in PhaeoRD2 indicate similarly stronger hydrogen bond of the Schiff base compared to BR. Fig. 3.21 shows the PhaeoRD2\textsubscript{k} minus PhaeoRD2, LR\textsubscript{k} minus LR, and BR\textsubscript{k} minus BR spectra in the 2750-1900 cm\textsuperscript{-1} region, which contains the X-D stretching vibrations of protein and water molecules. As the samples were hydrated with D\textsubscript{2}O and D\textsubscript{2}\textsuperscript{18}O, the spectral comparison would reveal O-D stretching vibrations of water molecules affected by the retinal photoisomerization. In BR, six negative peaks at 2690, 2636, 2599, 2321, 2292, and 2171 cm\textsuperscript{-1} were assigned to six O-D stretching vibrations of the three water molecules in the Schiff base region [159]. The water vibrations in fungal rhodopsins such as NR and LR are remarkably different from those of BR. In NR, only two peaks were observed at 2688 and 2465 cm\textsuperscript{-1}, while four negative bands at 2692, 2615, 2478 and 2257 cm\textsuperscript{-1} were found in LR [123]. PhaeoRD2 kept the same pattern of spectral changes of water as that in LR. The spectral features of PhaeoRD2 are almost identical to those of LR when it comes to the intermediates L and M1 as well (Fig. 3.22 and Fig. 3.23). This confirms that the core structure and the photochemistry of PhaeoRD2 are very similar to those of LR, suggesting that \textit{in vitro} PhaeoRD2 works as a light-driven proton-pump, exhibiting a typical strongly hydrogen-bonded water molecule near the retinal chromophore identical to that of LR and BR [160].
Figure 3.21. PhaeoRD2$_k$ minus PhaeoRD2, LR$_k$ minus LR, and BR$_k$ minus BR spectra in the 2730-1950 cm$^{-1}$ region measured at 77 K. The samples were hydrated with D$_2$O (red line) or D$_2^{18}$O (blue line). Putative bands of water molecules are labeled in green. The measurements were performed by H. Ito.
Figure 3.22. PhaeoRD2_L minus PhaeoRD2, LR_L minus LR, and BR_L minus BR spectra in the 1700-800 cm\(^{-1}\) region measured at 160 K. The samples were hydrated with H\(_2\)O (solid line) or D\(_2\)O (dotted line). The measurements were performed by H. Ito.
Figure 3.23. PhaeoRD$_2$$_M$ minus PhaeoRD$_2$, LR$_M$ minus LR, and BR$_M$ minus BR spectra in the 1700-800 cm$^{-1}$ region measured at 240 K. The samples were hydrated with H$_2$O (solid line) or D$_2$O (dotted line). The measurements were performed by H. Ito.
3.3. Conclusions

We studied a new subgroup of fungal rhodopsins (termed the auxiliary group [15]), using sequence analysis of the fungal genomic data and photochemical comparison of two representative rhodopsins from *Phaeosphaeria nodorum* [29]. The bioinformatic analysis confirms that the auxiliary subgroup forms a very distinct cluster on the rhodopsin tree (Fig. 3.1) due to the unique primary structure of its members (Fig. 3.2), which are usually present in addition to other rhodopsin forms in their host species. Evidently, the auxiliary group diverged from the other rhodopsin early in the history of the ascomycota, some 400 Mya [161]. Analysis of the genomic context shows that auxiliary rhodopsins may be linked to the carotenoid biosynthesis cluster of genes. Structural analysis of the conserved regions suggests that auxiliary rhodopsins preserved the common transmembrane core of BR and LR, but have some polar residues on the hydrophobic protein periphery, which may suggest interactions with a putative transducer or other membrane partner.

Spectroscopic analysis by visible, Raman, and FTIR spectroscopy reveals some characteristic photocycle features for the auxiliary rhodopsin of *Phaeosphaeria*, but also confirms conservation of the main BR-like characteristics. Close similarity of the absorption spectra of the two *Phaeosphaeria* rhodopsins (LR-like and auxiliary) implies that they are not designed to interact with different wavelengths of light. The photocycles of both rhodopsins are fast, and show photointermediates and proton transfer steps typical for proton-pumping rhodopsins. Taken together with the distinct
phenotype of the auxiliary rhodopsin in which the cytoplasmic Schiff base proton donor is disabled, it suggests that auxiliary rhodopsins preserved their proton-pumping ability, at least in the absence of their putative transducers. We suggest that this may point to a fairly recent evolutionary separation of these putative photosensors. Whether the auxiliary rhodopsins indeed serve as photosensors remains to be seen by the future in vivo experiments.
Chapter 4

Uniform Isotope Labeling of LR for High-Resolution NMR studies

This work has been published:
Summary

Overexpression of isotope-labeled multi-spanning eukaryotic membrane proteins for structural NMR studies is often challenging. On the one hand, difficulties with achieving proper folding, membrane insertion, and native-like post-translational modifications frequently disqualify bacterial expression systems. On the other hand, eukaryotic cell cultures can be prohibitively expensive. One of the viable alternatives, successfully used for producing proteins for solution NMR studies, is yeast expression systems, particularly *Pichia pastoris*. We report on successful implementation and optimization of isotope labeling protocols, previously used for soluble secreted proteins, to produce homogeneous samples of a eukaryotic seven-transmembrane helical protein, rhodopsin from *Leptosphaeria maculans*. Even in shake-flask cultures, yields exceeded 5 mg of purified uniformly \(^{13}\)C, \(^{15}\)N-labeled protein per liter of culture. The protein was stable (at least several weeks at 5°C) and functionally active upon reconstitution into lipid membranes at high protein-to-lipid ratio required for solid-state NMR. The samples gave high resolution \(^{13}\)C and \(^{15}\)N solid-state magic angle spinning NMR spectra, amenable to a detailed structural analysis. We believe that similar protocols can be adopted for challenging mammalian targets, which often resist characterization by other structural methods.

4.1. Introduction

Multi-spanning helical membrane proteins of eukaryotes, especially seven-transmembrane (7TM) helical G-protein coupled receptors (GPCRs), have
become very attractive targets for NMR studies [162-165]. While GPCRs are extremely important medically, the specific interest of the NMR community is fueled by several additional factors. First, the recent progress of X-ray structure determination of GPCRs, even though striking, is limited, especially when it comes to their activated states and dynamics [166-168], making the use of complementary techniques such as NMR a must. Next, the ability of both solution and solid-state (ss) NMR to get structural insights (or even full structures) for membrane proteins of this size and architecture has increased dramatically [162, 169, 170]. Finally, continuous development of expression systems often allows production of isotope-labeled eukaryotic proteins in milligram quantities sufficient for NMR studies, even though the successful functional overexpression of GPCRs is sporadic [171-174].

While a large number of GPCRs can be obtained in the inclusion bodies of *E. coli* with reasonable yields and at a low cost [171], it is often difficult to achieve their proper refolding, membrane insertion, and native-like post-translational modifications (reviewed in [162, 165, 172, 173]). In addition to expression in the inclusion bodies, several GPCRs could be inserted in the inner membrane of *E. coli*, either as fusion proteins or at low temperature [175-177]. Even though some NMR studies were conducted on isotope-labeled GPCR ligands bound to *E. coli*-expressed natural abundance proteins (e.g., [178]), there are very few cases where isotope labeling of a whole GPCR in *E. coli* resulted in good quality spectra suitable for structural studies. For example, solution spectra of vasopressin V2 receptor [176], kappa opioid receptor
[162], and Y2 receptor [179], missed a large part of the expected resonances, indicating possible problems with native folding, complicated heterogeneous dynamics, and proton back-exchange. At the same time, ssNMR spectra of uniformly labeled lipid-reconstituted Y2 and cannabinoid receptors [177, 179] gave good dispersion but poor spectral resolution, even though selective $^{15}\text{N}$ labeling in cannabinoid and chemokine CXCR1 receptors gave promising results [177, 180]. Finally, cell-free expression using *E. coli* extracts gave promising results on several GPCRs, but no high-resolution NMR spectra have been reported [181].

An alternative method of expression of isotope-labeled GPCRs is in mammalian or insect cell cultures. While this method does not have problems associated with improper folding and post-translational modifications, it suffers from high costs and difficulties with uniform labeling and deuteration, even though there is a constant improvement in these techniques [171, 174, 182, 183]. As with *E. coli*-expressed GPCRs, a number of isotope-labeled ligand NMR studies were conducted with natural abundance receptors [184-186], as well as with chemically isotope-labeled receptors [187]. While interesting structural insights could be obtained from selectively isotope-labeled GPCRs [188-190], no high-resolution ssNMR or complete solution NMR spectra of uniformly labeled GPCRs amenable for structural studies have been reported so far.

Expression of GPCRs and other eukaryotic membrane proteins for structural studies
in methylotrophic yeast *Pichia pastoris* has been viewed as a promising and cost-effective alternative for some time [171, 173, 174, 191, 192]. Many GPCRs could be functionally expressed in *P. pastoris*, some of them with high yields [193-200]. Several other polytopic helical mammalian membrane proteins were overexpressed in *Pichia* and crystallized, including potassium channels, aquaporins, leukotriene synthase, and P-glycoprotein [201-206]. Protocols for efficient and economical uniform isotope-labeling (both $^{13}$C and $^{15}$N) and deuteration are well established for soluble proteins expressed in *Pichia* [138, 139, 207-209]. Nevertheless, no structural NMR studies of isotopically labeled GPCRs produced in *Pichia* have been reported to date (with the exception of a few studies of isolated extracellular domains).

The main goal of this work was to demonstrate that expression of eukaryotic membrane proteins in yeast can result in functional, structurally homogeneous, isotopically labelled samples yielding high-resolution SSNMR spectra after reconstitution in lipids. We report on a successful adoption and optimization of uniform isotope labeling protocols, previously used for production of soluble secreted proteins in *Pichia pastoris* [138], for expression of a 7TM helical eukaryotic membrane protein for high-resolution SSNMR study. As a model system, we chose a fungal microbial-type rhodopsin from *Leptosphaeria maculans* (LR) [116], the first proven case of a bacteriorhodopsin (BR)-like eukaryotic light-driven proton pump [110]. Recently, LR was functionally expressed in neurons [130], showing its promise
in optogenetics. Being architecturally similar to GPCRs, LR is a very convenient protein for the purposes of this SSNMR study, as it is known to have high expression level in *Pichia pastoris*, remains stable and functional upon reconstitution into synthetic lipid membranes, is colored, and can be tested functionally by observing its photochemistry [110]. We achieved high yield of expression (more than 5 mg of purified protein per liter of culture in shake-flasks) of uniformly doubly labeled LR, which gives stable, homogeneous, and functionally active samples in synthetic lipids at high protein concentrations. The samples produce high-resolution $^{13}$C and $^{15}$N magic angle spinning (MAS) SSNMR spectra, which are amenable to a detailed structural analysis via multi-dimensional spectroscopy. Taking into the account of recent successes in the expression of natural abundance eukaryotic membrane proteins in *Pichia*, we believe that similar isotope labeling and reconstitution protocols can be adopted for these challenging targets as well.

4.2. Results and Discussion

4.2.1. Solid-state NMR spectra

Detailed protocol of the sample preparation was given in chapter 2. After the optimization of the induction length (optimal time 40 hours, similar to that found for NR [120], the yield of the purified protein in shake-flasks exceeded 5 mg per liter of culture. Since only 0.5% concentration of $^{13}$C methanol in BMM was used, and it had to be replenished only once (after 24 hours of induction), the cost of this sample is close to that for similar bacterial proteins produced in *E. coli*, such as PR and ASR [140, 210,
The lipid-reconstituted LR gave SSNMR spectra of high resolution allowing identification of individual chemical sites as obvious both from the 1D and 2D spectra (Figs. 4.1, 4.2, 4.3, and 4.4). The estimated line width (~0.5 ppm for $^{13}$C, 0.7 ppm for $^{15}$N) is similar to that observed for *E. coli*-expressed bacterial homologs of LR [140, 211, 212], as well as for the native 2D crystals of BR [213], suggesting structural homogeneity of the sample. In the recent past, similar spectral resolution allowed us to perform 3D and even 4D SSNMR experiments leading to the assignment of the majority of resonances for PR and ASR [211, 214].

The one-dimensional $^{15}$N spectrum shows fine structure both in the backbone amide region (Fig. 4.1) and for sidechains, such as His, Arg, and Lys. LR has several His residues in the transmembrane core, and one can distinguish at least four individual resonances of protonated His at 160-170 ppm (Fig. 4.1, inset), with additional low-intensity lines at 250-260 ppm, corresponding to the bands of the deprotonated species (not shown). The protonated retinal Schiff base is the active functional center of any rhodopsin, and its resonance is readily observed at 173.2 ppm. This position is consistent with the maximum of the visible spectrum of LR being at 542 nm [110], following the well-known relationship between these two parameters [215, 216]. A minor shoulder of this band may reflect the presence of a small fraction of 13-cis-retinal (<10%) detected earlier by Raman spectroscopy [110] and HPLC of the retinal extracts [123], but only a single correlation to the Cε atom of Lys, corresponding to the all-trans-configuration, was observed in the 2D NCA spectrum (Fig. 4.3).
One-dimensional $^{13}$C spectrum (Fig. 4.2) shows similarly high resolution, comparable to that observed for PR [140]. The absence of strong resonances at 70-90 ppm implies lack of glycosylation [217], consistent with the results of mass spectrometry (see below), as well as efficient removal of the majority of glycolipids during the purification.

The 2D NCA and CC (DARR) correlation spectra (Figs. 4.3 and 4.4) show many isolated narrow peaks, which allow identification of amino acid types and indicate that the sample is suitable for the spectral assignments. For example, 2D NCA spectrum (Fig. 4.3) shows well-resolved proline correlations (130-145 ppm $^{15}$N shifts). Only three out of five Pro resonances are clearly visible, probably due to the dynamics in the loops (one more resonance is visible, but its intensity is low). Based on the comparison to those observed in BR and ASR [211, 218], two prolines with nitrogen shifts of 136.4 and 131.5 ppm likely correspond to the residues located in the TM helices (helices C and F in BR and ASR). The third proline peak with an unusually high $^{15}$N shift of 144.2 ppm is similar to that observed in BR [218] and probably corresponds to a residue in a beta-structured loop (possibly, B-C).

Some tentative functionally important information can be derived from SSNMR spectra obtained at this early stage. A pair of CG-CB correlations around 172-173/37-39 ppm (Fig. 4.4, lower panel) is very typical for protonated buried Asp sidechains of BR, Asp96 and Asp115 [219, 220], and may belong to their homologs in
LR, which are protonated, as obvious from the difference FTIR spectra (Fig. 4.5b) [110, 111].

The analysis of the intensities of well-resolved Gly and Ala regions of the 2D $^{13}$C-$^{13}$C and NCA correlation spectra (Figs. 4.3, 4.4) allows estimating the completeness of spectral coverage. Compared to TM regions, solvent-exposed loops, turns and tails are typically characterized by increased mobility, and result in lower signal intensities in dipolar-based correlation spectra. These observations have been previously made in a number of membrane-embedded systems, such as SR-II, DsbB, PR, and ASR [211, 212, 214, 221]. It should be noted that LR has longer loops than its bacterial homologs, so one can expect lower degree of the spectral coverage if these loops are mobile. In our spectra, we observe the majority of these residues. For example, glycine resonances are well-resolved in the NCA spectrum (shown in box in Fig. 4.3), and their integration accounts for 20 out of 25 glycines (with only 10 glycines expected to be in the helical regions according to the homology modeling on the BR template). Likewise, alanine resonances can be integrated in the $^{13}$C-$^{13}$C DARR spectrum (Fig. 4.4, boxed), and account for about 23 out of 36 alanines, 25 of which are expected to be in the helical regions (the lower fraction of alanines compared to glycines may be due to stronger influence of the local dynamics on sidechains as opposed to the backbone). High dispersion of Ala, Gly, and Thr peaks, along with the high variability in the peak intensity, shows the presence of $\alpha$-helical and $\beta$-strand elements, along with random coil stretches [222] in the structure of LR.
Figure 4.1. One-dimensional $^{15}$N MAS NMR spectrum of $^{13}$C,$^{15}$N-labeled LR proteoliposomes at 800 MHz. The inset shows the expansion of a spectral region where side-chains of protonated histidines and the retinal Schiff base resonate. The data were collected by L. Shi and V. Ladizhansky.

Figure 4.2. One-dimensional $^{13}$C MAS NMR spectrum of $^{13}$C,$^{15}$N-labeled LR proteoliposomes measured at 800 MHz. The data were collected by L. Shi and V. Ladizhansky.
Figure 4.3. Two-dimensional NCA MAS NMR spectrum of $^{13}\text{C},^{15}\text{N}$-labeled LR proteoliposomes at 800 MHz. Glycine resonances are shown in the box, the Schiff base peak (folded from $\sim$173.2 ppm) is marked. The time domain data matrix was 160($t_1$)$\times$1024($t_2$), with $t_1$, $t_2$ increments of 74, and 20 $\mu$s, respectively. The carrier frequency was placed at 118 ppm and 60 ppm for nitrogen and carbon chemical shift evolution, respectively. 80 scans per point were recorded, with a recycle delay of 1.8 s. Total experiment time was 6.4 h. Data were processed with Lorentzian-to-Gaussian apodization functions and zero filled to 4096 ($t_1$) $\times$ 4096 ($t_2$) prior to Fourier Transform. 24 Hz of Lorentzian line narrowing and 40 Hz of Gaussian line broadening were applied on $t_1$ 15N indirect dimension, 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening were applied on $t_2$ 13CA direct dimension. The first contour is cut at $5\times\sigma$, with each additional level multiplied by 1.2. The data were collected by L. Shi and V. Ladizhansky.
Figure 4.4. Two-dimensional DARR (20 ms mixing) MAS NMR spectrum of $^{13}$C, $^{15}$N-labeled LR proteoliposomes at 600 MHz. Alanine resonances are shown in the box. The time domain data matrix was $1436(t_1) \times 997(t_2)$, with $t_1, t_2$ increments of 7.9 and 24 $\mu$s, respectively. The carrier frequency was placed at 90 ppm for carbon chemical shift evolution. 32 scans per point were recorded, with a recycle delay of 1.8 s. Total experiment time was 23 h. Data were processed with Lorentzian-to-Gaussian apodization functions and zero filled to 16384 ($t_1$) $\times$ 4096 ($t_2$) prior to Fourier Transform. 30 Hz of Lorentzian line narrowing and 60 Hz of Gaussian line broadening were applied on both $t_1$ and $t_2$ $^{13}$C dimensions. The first contour is cut at 5×$\sigma$, with each additional level multiplied by 1.2. The data were collected by L. Shi and V. Ladizhansky.
4.2.2. Post-translational modifications, extent of the isotope-labeling, and functionality of the protein

The purity and the presence of post-translational modifications of the sample were assessed by SDS-PAGE and MALDI TOF mass spectrometry, which showed the expected product at ~30.8 kDa (for the natural abundance protein) and a minor contaminant at 21.6 kDa. The observed molecular mass (30,832±10 Da for the natural abundance protein) corresponds to the expected mass (30,836 Da) of the Na\(^+\) adduct of non-glycosylated LR. This molecular mass indicates that only a small four-residue part of the leader sequence (EAEA) is left on the N terminus, as a result of the incomplete STE13 post-translational processing [223], possibly due to the proximity of the cleavage sites to the membrane surface. The lack of glycosylation is consistent with the absence of strong signals from carbohydrates in the \(^{13}\)C SSNMR spectra (Fig. 4.4) at 70–90 ppm [217], where only minor signals were detected, possibly from non-covalently bound glycans. Although the absence of glycosylation is rather unusual, as both N-linked and O-linked glycosylation is common for \(P.\) pastoris [217, 224], glycosylation was not observed for NR, another fungal rhodopsin expressed in \(Pichia\) [107].
Figure 4.5. FTIR analysis of the extent of labelling and functionality of LR reconstituted into DMPC:DMPA liposomes. Static mid-infrared absorption spectra of dry films of DMPC/DMPA liposomes of natural abundance (blue) and $^{13}$C,$^{15}$N-labeled (red) LR showing clear isotopic shifts of amide I and II bands, confirming the high labeling extent.
Figure 4.6. FTIR analysis of the extent of labelling and functionality of LR reconstituted into DMPC:DMPA liposomes. Light-induced difference FTIR spectra (time delay 2 ms after the laser flash) of the samples hydrated with 0.05 M CHES, 0.05 M KH₂PO₄ and 0.1 M NaCl, pH 9, at 2°C. a. DMPC/DMPA liposomes with natural abundance LR (blue). b. same with ¹⁵N-labeled (green) LR. c. same with ¹³C,¹⁵N-labeled (red) LR. The characteristic bands are labeled, clear isotopic shifts of protein bands are observed (see the text for details).
The extent of isotope labeling in *Pichia* was reported to vary between 68 and 99%, depending on the exact growth protocol [139]. The high extent of isotope labeling of the expressed LR was confirmed by static and time-resolved difference spectroscopy (Fig. 4.5 and 4.6). The absolute spectra of dry films of liposomes containing natural abundance and doubly-labeled LR (Fig. 4.5) allow comparison of the positions of Amide I (mostly backbone C = O vibrations, can report on the extent of $^{13}$C labeling) and Amide II (mostly backbone C–N vibrations, can report on the extent of both $^{13}$C and $^{15}$N labeling). In the spectra of dry film of liposomes containing natural abundance LR, the bands of Amide I and Amide II are found at 1657 and 1543 cm$^{-1}$, respectively. In the $^{13}$C, $^{15}$N-labeled LR liposomes, the bands of Amide I and II are at 1614 and 1518 cm$^{-1}$, respectively. The large shifts (43 and 27 cm$^{-1}$, respectively) observed in the positions of both peaks are in a good agreement with those reported earlier for other proteins [225, 226]. A number of prominent infrared bands not showing an isotopic shift can be readily assigned to various vibrations of synthetic lipids. For example, the position of the C=O lipid esters stretching band (at 1738 cm$^{-1}$) did not shift in the $^{13}$C, $^{15}$N-labeled, and neither did a number of vibrations involving lipids phosphorus (in the 1300-900 cm$^{-1}$ range).

To verify the extent of $^{15}$N labeling, which could be masked by carbon isotopic shifts, we measured analogous FTIR spectra of $^{15}$N labeled LR (not shown) and found 16 cm$^{-1}$ downshift of Amide II band consistent with nearly complete nitrogen labeling [183]. Additionally, absolute FTIR spectra can report on the secondary structure of the
expressed protein, verifying its native fold. The position of the Amide I peak maximum at 1,657 cm\(^{-1}\) (Fig. 4.5) indicates predominantly \(\alpha\)-helical structure of LR, while the shoulder at 1,628 cm\(^{-1}\) suggests the presence of some \(\beta\)-strands, consistent with the results of NMR (see above).

While absolute FTIR spectra can give an overall estimate of the labeling extent, they cannot detect the presence of a small number of non-labeled groups as well as nonrandom absence of labeling in selected amino acids. Such lack of sensitivity is explained by the amide peaks broadness, baseline distortions, and variations in the lipid contribution to the FTIR signal. Additional investigation of the extent of isotopic labeling of specific groups in LR combined with testing of the sample functionality is possible through the analysis of time-resolved difference FTIR spectra of the hydrated LR samples. Such light-\(\text{-minu}\)s-dark difference spectra can report on the changes in chemical groups involved in the LR’s proton-pumping function [110, 111, 123, 126]. Overall character of the difference spectra (Fig. 4.6) agrees well with those observed earlier for the proteoliposomes with lower protein:lipid ratios [110], confirming the functionality of LR at high protein:lipid ratio of the SSNMR samples. For example, the key proton transfer step could be clearly observed following the protonation signal of the primary proton acceptor Asp139 (at 1,759 cm\(^{-1}\)), along with the retinal photoisomerization (bands at 1,201 and 1,188 cm\(^{-1}\)). Comparison of the FTIR difference spectra of the natural abundance with singly- and doubly-labeled LR (Fig. 4.6) reveals a number of bands, which do not show any isotopic shifts. Those
prominent bands belong to the retinal chromophore, which was added externally, and as such was not $^{13}$C-labeled. On the other hand, many protein bands display large and complete isotopic shifts, such as those of the protonated carboxyl stretching vibrations of the three previously assigned Asp sidechains (at 1,759, 1,747, and 1,736 cm$^{-1}$, all downshifted by 43 cm$^{-1}$ upon carbon labeling) [110, 111]. One can also observe downshifts of the retinal Schiff base C=N vibrations (e.g., at 1,620 cm$^{-1}$, reflecting $^{15}$N labeling of Lys sidechains), as well as prominent shifts of several symmetric COO- stretches, presumably of Asp [227], such as that at 1,392 cm$^{-1}$. These prominent isotopic shifts of vibrational bands along with that for the Amide II band (at 1,564 cm$^{-1}$, overlapping asymmetric COO- stretches) confirm the high extent of labeling of specific sidechains along with the backbone of LR. Some of the tentative assignments of the vibrational bands mentioned above were verified by measuring analogous difference spectra of $^{15}$N labeled LR (Fig. 4.6), for example, to discriminate between C–N stretches of prolines and symmetric COO- stretches of carboxylic acids.

4.3. Conclusions

We have demonstrated that expression of a eukaryotic 7TM helical protein in *P. pastoris* can produce samples suitable for structural studies by SSNMR in a cost-effective way. The samples are stable (at least several week at 5°C) and functional, have high extent of the uniform $^{13}$C, $^{15}$N labeling, and give good spectral resolution comparable to that obtained for bacterial proteins of similar fold expressed in *E. coli*. Such spectral resolution allowed observation of resonances of nuclei of individual
chemical groups and, in the case of bacterial proteins, lead to the assignment of majority of backbone and sidechain resonances, especially in the functionally important transmembrane regions [211, 214]. New developments in SSNMR of polytopic helical membrane proteins combined with the possibility of their inexpensive uniform isotope labeling will eventually result in structural breakthroughs in the field of GPCRs and other eukaryotic membrane proteins.
Chapter 5

Spectroscopic Studies of a New Type of Microbial Rhodopsin - DDR2
Summary

Microbial rhodopsins serve as photosensors, ion pumps, and channels in many prokaryotes and lower eukaryotes. A new type of rhodopsin with unique sequence was found in flavobacterium *Donghaeana dokdonensis* by our collaborators in Korea (K.H. Jung’s lab). This new rhodopsin (named DDR2, as it exists in addition to a proteorhodopsin-like pigment) was functionally expressed in *E.coli* and produced light-induced pH changes in the sphaeroplast suspensions. The initial transport data from Jung’s lab suggested that DDR2 may mediate inward-directed passive H⁺ transport.

We analyzed DDR2 spectroscopically *in vitro*, in order to understand its ion-transporting function. The *E. coli* expressed DDR2 is membrane-associated and binds *all-trans* retinal. Time-resolved visible spectroscopy revealed that the photochemical reaction cycle of DDR2 was quite fast and strongly Na⁺ dependent. This suggested that DDR2 may be a light-driven Na⁺ pump, and that active outward transport of Na⁺ was responsible for the inward passive H⁺ transport. Analysis of the sequence alignments provided the clues for Na⁺ binding mechanism, and suggested important sites for mutagenesis. We used Fourier transform infrared spectroscopy for the elucidation of structural changes of DDR2 following the retinal photoisomerization in different buffers (KCl, NaCl, and LiCl based). Three critical mutations of DDR2, of N112, D116, and D251, were designed to explore the functional mechanism of Na⁺ binding and transport. Our results suggest that N112 is
the Na\textsuperscript{+} binding site in the dark and D116 is a crucial site serving as an acceptor for the retinal Schiff base proton, transfer of which is a gate for the metal cation transport.

5.1. Introduction - Sequence analysis of DDR2

A new type of rhodopsin with unique sequence, named DDR2, as it exists in addition to a proteorhodopsin-like pigment, was found in flavobacterium *Donghaeana dokdonensis* by our collaborators in Korea (K.H. Jung’s lab). We performed bioinformatic analysis and found that close homologs of DDR2 are present in several *Flavobacteria, Cytophagales, and Deinococci*, as well as in many environmental samples and even in diatom algae (could be a bacterial contamination or true lateral gene transfer). Sequence comparison to light-activated proton pumps, such as BR and PR, showed that DDR2 cluster of rhodopsins has a very distinct amino acid sequence. Its key features are the lack of the primary proton acceptor (Asp-85 in BR) and proton donor (Asp-96 in BR) in the predicted helix C, which are replaced by non-protonable Asn and Gln, respectively (Fig. 5.1). Quite strikingly, there is a new Asp residue (D116) located between Asn-112 (the homolog of the primary proton acceptor) and Gln-123 (the homolog of the primary proton donor), which replaces conserved Thr of BR and PRs. Additionally, there are a number of new conserved polar residues throughout transmembrane regions, such as Thr, Tyr, Asn, Gln, and Arg, highlighted in gray in Fig. 5.1. Replacement of the key carboxylic acids of light-driven proton pumps with non-protonatable polar residues and introduction of new polar groups suggests that DDR2 may have a very different ion specificity, if it is an ion pump at
all. This is reminiscent of the situation with halorhodopsin, a light-driven chloride pump, where the key proton acceptor of BR is replaced by Thr, and a number of new Ser, Thr, and Arg residues are introduced [52, 127, 228]. But, as can be seen from Fig. 5.1, DDR2 is not similar to halorhodopsin either, so that one does not necessarily expect it to be an anion pump.
Figure 5.1. BLAST amino acid sequence alignment of conserved transmembrane regions (helices B-G or 2-7) of DDR2 and its close homologs with other representative microbial rhodopsins. Residues conserved in BR are highlighted black, in PR – yellow, in SR-II – red, in fungal rhodopsins – green, in ASR – brown, in DDR2 – grey. Cyan highlighting shows deviations from the BR conservation pattern other than those listed above. Insertions into the FG loop which cannot be aligned with other rhodopsins, as well as their insertion sites are shown in blue. Most important amino acid positions are labeled according to the BR numbering. Antarct, hypsal, freshhot, and GOS represent different environmental samples from oceanic...
metagenomic databases. See the list of abbreviations for the rest.

5.2. Background – Ion transport data of the wild-type DDR2 from the Jung’s and Kandori’s labs

DDR2 was functionally expressed in E. coli in K.-H. Jung’s lab. The observation of light-induced pH changes was performed in spheroplast suspensions of E. coli cells expressing DDR2. Under illumination, the pH value of the cell suspension reversibly increases, which suggests inward (towards the cytoplasm) H⁺ transport mediated by DDR2 (Fig. 5.2, upper panel). Theoretically, such inward transport of protons could be either active, caused by light-driven H⁺ pumping (inverted in direction relative to BR or PR), or passive, for example, accompanying the inward Cl⁻ pumping similar to that of HR [51, 52], as shown in Fig. 5.3 [10]. Although one could suggest that DDR2 may be an inward light-driven proton pump, the enhanced pH changes in the suspension of cells after addition of the protonophore uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydrazone) indicated that it is not the case. CCCP effectively abolishes active proton transport, by making membranes permeable for protons, but it can enhance the passive proton transport for the same reason [229]. Fig. 5.2, lower panel, shows a very significant enhancement of the proton transport by CCCP, implying that it is a passive transport in response to the light-driven active transport of other ion in DDR2 expressing cells. Accordingly, addition of K⁺ valinomycin (Jung’s lab) or TPP⁺ (tetraphenylphosphonium) (Kandori’s lab, personal communication), abolished all the pH changes (not shown), as expected from the removal of transmembrane potential gradient created by the active transport of an unknown ion,
which drove passive $\text{H}^+$ transport. This conclusion was later supported by $\text{Na}^+$ dependence of the passive $\text{H}^+$ transport, as measured both in Jung’s and Kandori’s labs (personal communications, not shown). Here, we are going to prove the identity of the transported ion and suggest the mechanism of its transport based on visible and
infrared time-resolved spectroscopic studies of the wild-type and mutant DDR2 in vitro. Thus, in this chapter, we demonstrate that this unknown ion is Na+, making DDR2 the first proven case of light-driven Na+-pumping rhodopsin.

Figure 5.3. Diagrammatic representation of proposed ion movements in *H. halobium* cell envelope vesicles from [10], shown to explain possible origin of the pH changes in Fig. 5.2. Image reprinted with permission from JBC, copyright (1982). Filled circle, bacteriorhodopsin or halorhodopsin or putative sodium pump; open circle, proton/sodium antiporter, --> passive ion fluxes. Model I, vesicles containing bacteriorhodopsin and a proton/sodium antiporter. Model II, vesicles containing a putative light-driven sodium pump. Model III, vesicles containing halorhodopsin.

5.3. Static and time-resolved visible spectroscopy of the wild type and mutants of DDR2

To reveal the ion-specificity and mechanism of transport by DDR2, we studied its photochemistry in media of the different composition. The wild-type and mutant DDR2 proteins were provided by our collaborators (Prof. K.-H. Jung’s laboratory, Dept. of Life Sciences, Sogang University, Seoul, Korea), either in *E. coli* membranes or as purified proteins, which were incorporated into DMPC/DMPA liposomes for the spectroscopic studies as described in Chapter 2. First, the photocycle of DDR2 was
studied in *E. coli* membranes (Fig. 5.4) by time-resolved single-wavelength difference spectroscopy in the visible range at room temperature and neutral pH (pH 7). We performed the wavelength scan at 20-nm intervals from 400 to 640 nm to get information on the overall character of the photocycle and to compare it with other ion-pumping rhodopsins. Fig. 5.4 shows that DDR2 has a fast photocycle turnover at neutral pH, finishing in <30 milliseconds, which is a characteristic feature for ion pumping rhodopsins as distinguished from slow-cycling photosensors [3]. The photocycle seems to be dominated by the early (microsecond, K-like) and late (millisecond, O-like) red-shifted intermediates, and is superficially similar to the photocycle of proteorhodopsin (PR) [81, 230]. Unexpectedly, an apparent M intermediate, which reflects proton transfers from and to the retinal Schiff base, was observed in the kinetics at 400-420 nm, despite the absence of the classical primary proton acceptor in the amino acid sequence (replaced by Asn-112 in DDR2, see above). This strongly suggests that there is another potential proton acceptor in the retinal binding pocket of DDR2. The most obvious candidate is the newly introduced Asp-116, which is located between Asn-112 (the homolog of Asp-85 of BR) and Gln-123 (the homolog of Asp-96 of BR). Protonation of the primary proton acceptor at low pH usually has a dramatic effect on the photocycle of proton pumps, such as BR and PR, eliminating the M intermediate along with the late intermediates following it [81, 231]. For this reason, we collected kinetic traces similar to those shown in Fig. 5.4, but monitored only at four wavelengths (420, 460, 520, and 600 nm), at pH 4 and 8, using DDR2 reconstituted in liposomes. Indeed, the
photochemical reaction of DDR2 at low pH is completely different from that at pH 7.

For the photocycle at pH 4 (Fig. 5.5a), the M intermediate (followed at 420 nm), along with the red-shifted O-like intermediate (at 600 nm), is missing in the kinetics, which is consistent with the titration of the new Schiff base counterion (primary proton acceptor). This titration was confirmed by our collaborators who observed the protonation of the counterion following the red-shift in the static visible spectrum of solubilized DDR2, with the pKₐ of 5.9 (K.H. Jung’s lab, data not shown). Such pKₐ of the Schiff base counterion is higher than that in BR, but lower than in PR, being similar to the one observed in xanthorhodopsin (XR) [232]. Fig. 5.5a also shows the absence of any positive differential absorption correlating with the late photocycle intermediates at low pH, probably due to the spectral overlap of the N-like intermediate and the parent state, similar to BR [231]. The photocycle at pH 8 in liposomes is almost identical to that at pH 7 in membranes, with a slightly longer time to finish the photocycle (Fig. 5.5b). As the pKₐ of this titratable color- and photocycle-controlling group (putative Schiff base counterion) in DDR2 is 5.9, all the subsequent measurements were taken at pH 8 to avoid mixture of the species with different protonation states. The presence of the M intermediate at higher pH suggests that even though the proton transport observed in cells is passive, the internal light-driven proton transfers in the photocycle of DDR2 may be crucial to its function.
Figure 5.4. The laser flash-induced photocycle kinetics of DDR2 in the DDM-untreated *E.coli* membranes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes were encased in polyacrylamide gels equilibrated with 0.05 M KH₂PO₄ and 0.1 M NaCl, pH 7. Photocycle kinetics was measured at 20-nm intervals from 400 to 640 nm.
Figure 5.5. The laser flash-induced photocycle kinetics of DDR2 in DMPA/DMPC liposomes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 4 - 0.05 M CH$_3$COONa and pH 8 - 0.05 M Tris. Photocycle kinetics measured at pH 4 (a) and 8 (b) at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue.
Next, we tested the hypothesis that DDR2 may function as a Cl⁻ pump, as suggested from the transport data by the passive nature of proton flux (see above). The Cl⁻ dependency test was performed using gel-encased DDR2 with Cl⁻ free buffer at pH 8, to see if the absence of Cl⁻ will affect the kinetics of DDR2. Clearly, there is no major difference between the two kinetics measured with and without Cl⁻ (Fig. 5.6a), excluding the possibility that DDR2 is a halorhodopsin-like anion transporter. This is confirmed by the transport measurements done in K.H. Jung and H. Kandori labs (personal communications, not shown), which showed Cl⁻-independence of the light-induced pH changes.

In contrast, and to our great surprise, the photocycle of DDR2 was observed to become much slower. It was also revealed other drastic changes in the kinetics, in a K⁺ based buffer, that is, in the absence of Na⁺. Overall, the photocycle turnover in the absence of Na⁺ becomes two orders of magnitude longer, taking a few seconds to complete. Fig. 5.6b shows that the rate of deprotonation of the Schiff base (M rise) is not affected by Na⁺, however, the rate of reprotonation of the Schiff base (M decay) is greatly delayed (by almost 3 orders of magnitude, from submillisecond to subsecond characteristic times). At the same time, the rise and the decay of the O-like late red-shifted intermediate (followed at 600 nm), were strongly delayed without Na⁺. The Na⁺ concentration dependency experiment was performed by measuring the photocycle kinetics using DDR2 liposome gel in a series of buffers with logarithmically increasing Na⁺ concentration. A comparison of kinetic traces at
Figure 5.6. The ion-dependence of laser flash-induced photocycle kinetics of DDR2 followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The membranes or liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH₂PO₄ and 0.05 M Tris with the addition of the following buffers: Cl⁻ free: 0.1 M Na₂SO₄, Na⁺ free: 0.1 M KCl, and normal buffer: 0.1 M NaCl. Photocycle kinetics measured at pH 8 at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue. a. Anion dependence - DDR2 in E.coli membranes not treated with DDM. b. Cation dependence - DDR2 was in DMPA/DMPC liposomes.
different Na\(^+\) concentrations (Fig. 5.7) shows that both the rate of reprotonation of Schiff base (M decay) and kinetics of the O-like intermediate are strongly Na\(^+\)-dependent. The higher the concentration of Na\(^+\), the faster is the photocycle and the M decay. Prominent O-like intermediate starts to accumulate at higher concentrations of Na\(^+\), and both its rise (which may be kinetically coupled to the M decay) and decay become faster, as clearly seen when comparing the data from 10 mM and 100 mM Na\(^+\) buffers. Such Na\(^+\) dependence of the kinetics of late red-shifted intermediates of the photocycle is similar to the Cl\(^-\) dependence observed in *pharaonis* halorhodopsin [233], and suggests that the color changes associated with the O-like intermediate may reflect movement of Na\(^+\) ion through DDR2. However, the absence of Na\(^+\) does not have an effect on the formation of the M intermediate (deprotonation of the Schiff base), as this primary proton transfer apparently does not involve Na\(^+\). Such Na\(^+\) dependent photochemical behavior combined with the transport data of our collaborators suggests that DDR2 acts as a light-driven Na\(^+\) pump. This would be the first proven case of active sodium transport by a microbial rhodopsin. In the early eighties, it was suggested that active Na\(^+\) transport by rhodopsins can exist, but it was later shown that halorhodopsin, the anion pump, was responsible for the observed effects [10]. Channelrhodopsins of green algae, on the other hand, can be passively permeable to a number of cations, including sodium, but do not act as sodium pumps [234, 235].

To further test the cation specificity of the putative Na\(^+\) pump DDR2, its photocycle
was investigated in a Li\(^+\)-based buffer. Lithium belongs to the same family of elements as Na\(^+\) and can usually substitute for it when it comes to sodium-specific channels, for example. The photocycle of DDR2 in LiCl-based buffer measured by time-resolved difference spectroscopy in the visible range at room temperature under the same conditions as used for NaCl-based buffers is compared to those in NaCl and KCl. Fig. 5.8 shows that DDR2 in Li\(^+\) buffer produces the photocycle similar to that in Na\(^+\) buffer, with slightly slower turnover. Another difference is that the rate of O rise and decay in Li\(^+\) buffer is a little slower than those in Na\(^+\) buffer, suggesting that affinity for lithium may be somewhat lower than that for sodium. The photocycle of DDR2 in K\(^+\) buffer is dramatically different from those in Li\(^+\) buffer and Na\(^+\) buffer, as discussed above, taking much longer time to complete. These data suggest that DDR2 can perform size-based selection of cations, allowing only smaller ions to pass through DDR2, similar to some sodium-specific channels.
Figure 5.7. The laser flash-induced photocycle kinetics of DDR2 in DMPA/DMPC liposomes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.05 M Tris with the addition of the following: 1 mM NaCl, 10 mM NaCl, 100 mM NaCl, and 1 M NaCl. Photocycle kinetics measured at pH 8 at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue.
Figure 5.8. The laser flash-induced photocycle kinetics of DDR2 in DMPA/DMPC liposomes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.05 M Tris with the addition of the following: 100 mM KCl (colored dotted lines), 100 mM LiCl (colored solid lines) and 100 mM NaCl (black). Photocycle kinetics measured at pH 8 at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue.

To investigate the mechanism of cation binding and transport, the sequence alignment of DDR2 and its homologs (Fig. 5.1) was studied and locations of strategic mutations which could affect possible Na$^+$ binding sites were chosen. Asn-112 of DDR2 (the homologue of Asp-85 in BR) was replaced by Asp, Asp-116 (the homologue of Thr-89 in BR) by Asn, and Asp-251 (the homologue of Asp-212 in BR) by Asn (all mutants produced in K.H. Jung’s lab). The former two mutations were made because Asn-112 and Asp-116 are polar residues in the vicinity of the Schiff base, which are
unique for DDR2 and its homologs. Interaction of the homologs of these two residues in BR (Asp-85 and Thr-89) is important for proper vectoriality of proton transport [236, 237]. Replacement of Asp-85 by Thr was shown to change the ion specificity of BR, turning it into the halorhodopsin-like chloride pump [52]. Asp-251 was chosen as the only anionic residue in the vicinity of the Schiff base (besides Asp-116). Its homolog in BR (Asp-212) serves as a part of the Schiff base counterion and participates in the proton transport [238-240].

The N112D mutation removes the unique Asn residue and restores it to Asp typical for proton-pumping rhodopsins, such as BR and PR [15, 127]. Fig. 5.9a indicates that the photocycle kinetics of N112D DDR2 mutant does not change very strongly in the absence of Na⁺. The only difference is the apparently higher accumulation of the O-like intermediate in the Na⁺ based buffer, while the reprotonation of the Schiff base and overall photocycle turnover are not affected strongly, the latter remaining very slow in both buffers. This implies that N112D mutant loses some Na⁺-dependent steps in the photocycle, suggesting that Asn-112 may be involved in sodium binding and transport. The photocycles of the mutant and the wild-type proteins were compared in the absence of Na⁺ in Fig. 5.9b. In the mutant, reprotonation of the Schiff base is apparently uncoupled from sodium transport, remaining fast (as judged from the M decay at 420 nm), in contrast to the wild-type in Na⁺ free buffer. Possible role of Asn-112 in Na⁺ binding and transport in DDR2 was further explored by FTIR as will be shown below.
Figure 5.9. The laser flash-induced photocycle kinetics of DDR2 N112D mutant in DMPA/DMPC liposomes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.05 M Tris with the addition of the following: 100 mM KCl and 100 mM NaCl. Photocycle kinetics measured at pH 8 at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue. a. Comparison of the photocycle kinetics of DDR2 N112D mutant in 100 mM NaCl buffer (solid lines) and in 100 mM KCl buffer (dotted lines). b. Comparison of the photocycle kinetics of the N112D mutant (color-coded) and the wild-type (black) in 100 mM KCl buffer.

As mentioned above, the role of the unique Asp-116 residue in the helix C may be to accept a proton from the Schiff base, leading to formation of the M intermediate. If it is the case, replacement of the anionic Asp-116 with Asn should lead to the same phenotype as that observed at low pH in the wild-type, where Asp-116 is protonated (Fig. 5.5a), as was found for counterion mutants of other microbial rhodopsins [81, 241]. If Asp-116 is indeed the new primary proton acceptor for the Schiff base of DDR2, one would expect a blockage of the Schiff base deprotonation (absence of the
observable M rise at 420 nm) and disappearance of the late red-shifted O-like intermediates kinetically coupled to M in the mutant. The photocycle of the D116N mutant was measured by spectroscopy in the visible range and compared to that of the wild type DDR2 (Fig. 5.10). Indeed, the photocycle of the D116N mutant is similar to that of the wild-type DDR2 at pH 4, lacking any appreciable positive differential absorbance signals. Additionally, the D116N mutant loses Na\(^+\) dependence of the kinetics (Fig. 5.10a). This is consistent with the transport data from K.H. Jung’s lab, which show no ion transport in the cells expressing D116N DDR2 mutant (not shown).

Taken together, this suggests that Asp-116 is a new counterion and proton acceptor for the retinal Schiff base and that proton transfer from the Schiff base to Asp-116 may serve as a gating element for Na\(^+\) transport in DDR2.

In DDR2, D251 is the superconserved homolog of Asp-212 of BR, which is usually negatively charged and consitutes a part of the Schiff base counterion [159, 219, 240]. When D251 is replaced by neutral Asn, the photocycle turnover becomes much slower than that of the wild type in Na\(^+\) buffer (Fig. 5.11). This mutant also loses its Na\(^+\) dependence and lacks the O-like intermediate. Such behaviour suggests that Asp-251 may be a part of the Na\(^+\)-binding site, together with Asn-112. The homologs of these two residues in BR and several other microbial rhodopsins are connected to each other through hydrogen bonds to water molecules [77, 242], so it is feasible that they may coordinate a sodium ion in DDR2.
Figure 5.10. The laser flash-induced photocycle kinetics of DDR2 D116N mutant in DMPA/DMPC liposomes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.05 M Tris with the addition of the following: 100 mM KCl, or 100 mM NaCl. Photocycle kinetics measured at pH 8 at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue. a. Comparison of the photocycle kinetics of DDR2 D116N mutant in 100 mM NaCl buffer (color-coded) and in 100 mM KCl buffer (black). b. Comparison of the photocycle kinetics of DDR2 D116N mutant (color-coded) at pH 8 and wild-type DDR2 (black) at pH 4 (from Fig. 5.5a) in 100 mM NaCl buffer.
Figure 5.11. The laser flash-induced photocycle kinetics of DDR2 D251N mutant in DMPA/DMPC liposomes followed by time-resolved difference spectroscopy in the visible range, measured at room temperature. The liposomes were encased in polyacrylamide gels equilibrated with 0.05 M KH$_2$PO$_4$ and 0.05 M Tris with the addition of the following: 100 mM KCl and 100 mM NaCl. Photocycle kinetics measured at pH 8 at characteristic wavelength: 600 nm (K and O intermediates) – red, 520 nm (ground state and N intermediate) – green, 460 nm (L intermediate) – cyan, 420 nm (M intermediate) – blue. (Left panel) Comparison of the photocycle kinetics of wild-type DDR2 (from Fig. 5.6) and DDR2 D251N mutant in 100 mM NaCl buffer. (Right panel) Comparison of the photocycle kinetics of wild-type DDR2 (from Fig. 5.6) and DDR2 D251N mutant in 100 mM KCl buffer (Na$^+$ free).
Depending on how far the dark putative binding site of Na\(^+\) is located from the Schiff base and Asp-116, its occupancy may affect the pK\(_a\) of the Schiff base counterion and color of the protein. Similar synergistic effects are known for Cl\(^-\) and H\(^+\) in halorhodopsin and some mutants of BR [243], but the location of negatively charged Cl\(^-\) will be most likely different from that of positively charged Na\(^+\), considering the positive charge of the protonated Schiff base. From the kinetic data on the mutants presented above, the protonation of Asp-116 was suggested to serve as a gate, displacing Na\(^+\) during the photocycle. The reverse reaction may be expected in the static titration performed in the dark, but only if no major light-induced conformational changes take place, which is usually not the case. The titration of DDR2 liposomes was performed in a pH range from 5 to 8 to check whether Na\(^+\) affects the pK\(_a\) of the potential internal proton acceptor Asp-116. As shown in the Fig. 5.12, there is no major difference between the pH titrations and absorption maxima in the presence of the two different metal cations. It suggests that the putative Na\(^+\) binding site does not strongly interact with the retinal Schiff base and Asp-116 in the dark. Additionally, maxima of the visible absorption spectra of DDR2 liposomes suspension blue-shift when the pH value increase, similar to what is observed in BR, PR, and other rhodopsins upon deprotonation of the Schiff base counterion [81, 232, 244, 245]. The spectral red-shift at acidic pH is consistent with protonation of the new counterion, Asp-116, as the spectra of D116N and D116T mutants (where anionic Asp is replaced by neutral groups) are red-shifted as well (not shown).
Figure 5.12. Upper panel: Comparison of baseline-subtracted static visible spectra of DDR2 reconstituted in DMPA/DMPC liposomes in KCl- and NaCl-based buffers from pH 5 to 8, measured at room temperature. The liposomes were equilibrated for 20 min with 0.05 M KH$_2$PO$_4$ and 0.1 M KCl or NaCl with the addition of the following buffers: pH 4 - 0.05 M CH$_3$COONa, pH 5 and 6 - 0.05 M MES, pH 8 - 0.05 M Tris. Lower panel: pH-dependence of the $\lambda_{\text{max}}$ of the spectra from the upper panel.
5. 4. Configuration of retinal in DDR2 explored by Raman spectroscopy

The effects of pH and Na\(^+\) on the retinal chromophore in DDR2 were further studied by Raman spectroscopy, which mostly reports on the chromophoric bands due to the resonance enhancement. The intensity pattern of bands in the fingerprint region (1150-1240 cm\(^{-1}\)) reports on the isomeric state of the retinal chromophore. The Raman spectra were taken at two different pH values (pH 4 and 8) and in the presence of two different metal cations (Na\(^+\) and K\(^+\) buffers), as well as after and before the H/D exchange, all shown in Fig. 5.13. The spectra at pH 8 are strongly dominated by the retinal bands and show the all-trans configuration of retinal chromophore in DDR2, as obvious from the prominent pair of C-C stretching vibrations around 1201 and 1165/1172 cm\(^{-1}\), similar to those of light-adapted BR [43]. No apparent light- or dark-adaptation was observed in DDR2 reconstituted into DMPA/DMPC liposomes from the Raman spectra, similar to the case of PR and LR [81, 110]. Comparison between the data in the Fig. 5.13a and Fig. 5.13b shows that there is no obvious spectral difference at the same pH/pD value (8) between the two different buffers (NaCl and KCl based). Thus, the configuration of retinal in DDR2 liposomes does not have Na\(^+\) dependence at pH/pD 8, consistent with the results of static visible spectroscopy (Fig. 5.12), suggesting that Na\(^+\) does not interact with the Schiff base strongly in the dark. These results agree with the hypothesis that the dark Na\(^+\) binding site must be not between Asn-112 and the Schiff base, but rather more towards the extracellular side, possibly involving Asp-251. As mentioned above, considering the positive charge of Na\(^+\), it is reasonable for it not to be close to the positively charged Schiff base.
More details about the retinal structure and environment can be extracted from the Raman spectra. In Fig. 5.13b, the frequency of the Schiff base C=N stretching vibration in D$_2$O can be seen at 1619 cm$^{-1}$, which is lower than that in BR, and at about 1644 cm$^{-1}$ in H$_2$O (Table 5-1). The magnitude of the isotopic downshift of this band reports on the Schiff base counterion strength [150, 246, 247], but the exact position of C=N-H might be somewhat different from 1644 cm$^{-1}$ because of the overlap with the residual amide I vibrations. Nevertheless, it appears that the observed large downshift suggests a stronger hydrogen bonding of the Schiff base proton to the counterion than that in BR. Another interesting feature is the position of N-D bending vibration in D$_2$O. In other microbial rhopsins it was found at around 979 cm$^{-1}$ (LR) or 977 cm$^{-1}$ (BR), but at 988 cm$^{-1}$ for 13-cis BR and 985 cm$^{-1}$ in ASR [43, 45, 110, 248]. It is even higher in DDR2, at about 992 cm$^{-1}$. This upshift of the N-D band confirms the different H-bonding pattern of the Schiff base in DDR2, consistent with the reorganization of the counterion (the move of the anionic residue from the 112th to 116th position).

The fingerprint region of C-C stretching retinal vibrations in the spectra taken at pH 4 (Fig. 5.13c) shows a strong increase in the concentration of species with 13-cis-retinal (as shown by the much stronger characteristic 1183 cm$^{-1}$ band [45]), similar to that in BR [249]. This is consistent with the broader visible spectra (because of the mixture of two species with close, but non-identical spectra) observed at low pH (Fig. 5.12). Frequency of the main ethylenic (C=C) stretch does not appreciably shift at pH 4.
compared to that at pH 8, again similar to the situation in BR. Upon the H/D exchange at pH 4, in the fingerprint region, stretching band of the retinal C_{14}-C_{15} bond in 13-\textit{cis} configuration at 1167 cm\(^{-1}\) shifts to 1210 cm\(^{-1}\), which identifies the retinal conformation as 13-\textit{cis}-15-\textit{syn} [250, 251], consistent with a new HOOP (hydrogen out of plane) band at 805 cm\(^{-1}\), and similar to other microbial rhodopsins.

**Table 5-1.** Assignment of the retinal bands in the Raman spectra of DDR2 at pH 8 compared with those done for BR and PR [43, 81, 252].

<table>
<thead>
<tr>
<th>Assignments</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>DDR2</th>
<th>BR</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=N-H</td>
<td></td>
<td>1644</td>
<td>1640</td>
<td>1654</td>
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<tr>
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<td></td>
<td>1619</td>
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<td>1631</td>
</tr>
<tr>
<td>C=C</td>
<td></td>
<td>1532</td>
<td>1527</td>
<td>1533</td>
</tr>
<tr>
<td>C12-C13 stretching+Schiff base lysine</td>
<td></td>
<td>1244</td>
<td>1255</td>
<td>1245</td>
</tr>
<tr>
<td>rocks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C all-trans isomers</td>
<td></td>
<td>1201</td>
<td>1201</td>
<td>1198</td>
</tr>
<tr>
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<td></td>
<td>1187</td>
<td>1183</td>
<td>1185</td>
</tr>
<tr>
<td>C-C all-trans isomers</td>
<td></td>
<td>1165/1172</td>
<td>1170</td>
<td>1163/1172</td>
</tr>
<tr>
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<td></td>
<td>992</td>
<td>977</td>
<td>979</td>
</tr>
</tbody>
</table>
Figure 5.13. Raman spectra of DDR2 reconstituted into DMPC/DMPA liposomes hydrated with either H$_2$O or D$_2$O, measured at room temperature. a. DDR2 liposomes were hydrated with KCl buffer at pH 8 and pD 8 (0.05 M KH$_2$PO$_4$, 0.05 M Tris and 0.1 M KCl). b. DDR2 liposomes were hydrated with NaCl buffer at pH 8 and pD 8 (0.05 M KH$_2$PO$_4$, 0.05 M Tris and 0.1 M NaCl). c. DDR2 liposomes were hydrated with NaCl buffer at pH 4 and pD 4 (0.05 M KH$_2$PO$_4$, 0.05 M CH$_3$COONa and 0.1 M NaCl).
5.5. Rapid-scan FTIR difference spectroscopy

The photocycles of the wild-type DDR2 and its mutants (N112D, D116N, D116T and D251N) were measured by time-resolved FTIR difference spectroscopy to obtain further insight into the molecular details of light-induced ion transfers and conformational changes of the retinal chromophore and the opsin moiety of DDR2. All of these measurements were performed at 2°C to slow down the photocycle to observe the earlier intermediates while using rapid-scan FTIR mode. The step-scan mode could give higher time-resolution, but was not used because of its lower signal-to-noise ratio. The predominant photointermediates observed in these spectra can be identified by the intensities and positions of characteristic bands in the fingerprint region (C-C stretches of retinal). The 1184 cm$^{-1}$ band is one of the C-C stretches of 13-	extit{cis} retinal [45], serving as a signature for the photo-isomerisation, but it is also indicative of the protonation state of the Schiff base: when there is no positive peak, the Schiff base is deprotonated (SB), when there is a positive peak, the Schiff base is protonated (PSB or SBH$^+$) [5]. Fig. 5.14 compares light-induced difference FTIR spectra (light minus dark) of DDR2 taken at pH 8 and pD 8, 1 ms after the excitation (12 ms interferogram acquisition time). In the fingerprint region, a major positive peak at 1184 cm$^{-1}$ and a negative C-C stretches at 1201 and 1165 cm$^{-1}$ indicate a predominantly 13-	extit{cis} configuration of retinal in the light-activated state, suggesting that the spectrum is primarily dominated by the N-like intermediate. On the other hand, visible spectroscopy suggested predominance of a red-shifted, O-like intermediate (Fig. 5.4), which in BR has all-	extit{trans}-retinal configuration [253].
the Fig. 5.14, the main ethylenic stretch is observed at 1554 cm\(^{-1}\) (possibly overlapping Amide-II band), which is the characteristic peak for N intermediate [37]. At the same time, a second positive ethylenic stretch is located at 1516 cm\(^{-1}\), which can be interpreted as red-shifted, O-like intermediate. This complicated, seemingly self-contradictory, situation may be similar to that observed for PR, where red-shifted N-like intermediate (possibly having a double ethylenic stretch band) was found to exist in equilibrium with the O-like intermediate [81].

As our main goal was to investigate ion movements, a special attention was paid to the 1600-1800 cm\(^{-1}\) region, where relevant carboxyl and carbonyl stretches of important sidechains, such as unique Asn-112, Asp-116, Gln-123, and possibly others, could be observed [49]. Unlike in proton-pumping rhodopsins, no major bands of protonated carboxylic acids were observed above 1725 cm\(^{-1}\), consistent with the absence of carboxylic homologs of Asp-85, Asp-96, and Asp-115 of BR, which have distinct signatures in this region [37, 46, 47, 254]. On the other hand, one might expect to see protonation signature of Asp-116, the putative new proton acceptor, even though low accumulation of the M intermediate associated with its extremely fast (submillisecond) decay would render its amplitude very low. This is what may be revealed by a minor positive band at 1722 cm\(^{-1}\), which only partially shifts upon deuteration. The partial shift indicates overlap with lipid ester perturbation bands, commonly observed at these wavenumbers [248, 255], and make exact determination of the position of this band unreliable. On the other hand, there is a prominent pair of
deuterium-dependent bands, at 1697 (-) and 1687 (+) cm$^{-1}$. These frequencies are too low to be assigned to carboxylic acids, but are quite typical for vibrations of C=O groups of proton-exchangeable Asn and Gln [256]. If Asn-112 is indeed involved in sodium-binding in the dark, and Gln-123 participates in the cytoplasmic sodium pathway, it would be very attractive to assign these bands to their perturbation.

**Figure 5.14.** Time-resolved laser flash-induced difference FTIR spectra of DDR2 reconstituted into DMPC/DMPA liposomes and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. Spectra of DDR2 liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.1 M NaCl, pH 8 buffer data shown in green and pD 8 data in red.
To further explore how the core structure of DDR2 is affected by different ions, and which groups may be responsible for proton and sodium transfers, difference FTIR spectra were taken in Na\(^+\), Li\(^+\) and K\(^+\) buffers at pH 8, 2\(^\circ\)C, with the same time delay (1 ms) (Fig. 5.15). As judged from the fingerprint region, the spectra at pH 8 in Li\(^+\) and K\(^+\) based buffers were primarily dominated by the N/O-like 13-cis-intermediate, the same as in Na\(^+\) based buffer, but its amplitude as measured at 1184 cm\(^{-1}\) is lower, especially noticeable for K\(^+\). Accordingly, the positive ethylenic stretch at 1516 cm\(^{-1}\) shows less intensity in both Li\(^+\) and K\(^+\) buffers. According to the kinetics in the visible range, the spectrum of DDR2 in K\(^+\) buffer is dominated by M/N mixture, with much less of the red-shifted O-like intermediate than in Na\(^+\). This agrees with the data in Fig. 5.15a, where the intensity of 1184 cm\(^{-1}\) greatly decreases in K\(^+\) buffer, accompanying the moderate decrease at bands of 1389 cm\(^{-1}\) (N-H in-plane) and 1294 cm\(^{-1}\) (C-H in-plane vibration), which are strong in N intermediate but not in M intermediate of BR [5, 37]. On the other hand, a greater accumulation of the M intermediate in sodium-free buffer may be reflected by the larger amplitude of protonated carboxyl band at 1727 cm\(^{-1}\), possibly belonging to Asp-116. Fig. 5.15b shows a smaller decrease of the intensity of bands at 1184 and 1516 cm\(^{-1}\) in Li\(^+\) buffer. In general, one could say that the FTIR spectra in Li\(^+\) are intermediate between those in Na\(^+\) and K\(^+\), confirming the idea from the visible spectroscopy data that DDR2 may interact with Li\(^+\) but with the lower affinity than that for Na\(^+\).

As was mentioned above, DDR2 exhibits a distinct negative band at 1697 cm\(^{-1}\) in Na\(^+\)
buffer, which we suspected to originate from perturbation of a Na\(^+\)-binding Asn or Gln. As expected, the intensity of this negative band decreases slightly in Li\(^+\) buffer, but greatly in K\(^+\) buffer (Fig. 5.15). It is thus possible that this band is related to the Na\(^+\) binding site. This idea was recently confirmed by titration of DDR2 in the dark using attenuated total reflectance (ATR) FTIR (H. Kandori lab, personal communication, not shown). Their dark difference spectra (potassium minus sodium) show a distinct pair of bands, negative at 1696 and positive at 1674 cm\(^{-1}\). This is consistent with our idea that in the dark, Na\(^+\) is coordinated by Asn/Gln absorbing at ~1697 cm\(^{-1}\) and it gets perturbed when sodium is transported during the photocycle, producing the large negative band (with the corresponding positive band of sodium-free Asn/Gln being at ~1674 cm\(^{-1}\), observed as a satellite of a larger band at 1687 cm\(^{-1}\) in Fig. 5.14).

Such explanation of the origin of the negative band at 1697 cm\(^{-1}\) is indirectly supported by the FTIR spectra of DDR2 at pH 4. In Fig. 5.16, one can observe clear decrease in the intensity of the band. From the results of the visible spectroscopy at pH 4, DDR2 loses Na\(^+\) dependency in the photocycle, consistent with the idea that the 1697 cm\(^{-1}\) band with smaller intensity in FTIR spectra of DDR2 at pH 4 should be assigned to the Na\(^+\) binding site. More solid evidence came from the FTIR spectrum of the N112D mutant measured under the same conditions. Since Asn\(\rightarrow\)Asp substitution is expected to cause an about 40 cm\(^{-1}\) upshift of C=O stretching vibrational modes [257], the disappearing band at 1697 cm\(^{-1}\) in the wild-type
The FTIR spectrum of the D116N mutant of DDR2 (Fig. 5.18) is consistent with its visible kinetics. The disappearance of the band at 1697 cm⁻¹ is correlated with the lost Na⁺ dependence of the photocycle. The FTIR results for the D116T mutant, designed to return this residue to its BR-like state, shown in Fig. 5.18, reveal the disappearance of this band as well. But the results from this mutant should not be taken at face value, as its photochemistry is compounded by the formation of 9-cis-retinal, as obvious from the positive retinal band at 1142 cm⁻¹ [258].

The FTIR results for the D251N mutant are shown in Fig. 5.19, compared with the wild-type data under similar conditions. Remarkably, the band assigned for N112 at 1697 cm⁻¹ disappeared in the spectra of D251N as well, consistent with the disappearance of sodium-dependence of the photocycle. It is also consistent with the lack of the late red shifted O-like intermediate, which we associate with Na⁺ transport, which is replaced with long-living early-rising intermediate coexisting with M at low amplitude. Accordingly, the positive retinal band is located at 1178 cm⁻¹, which is more typical for distorted all-trans rather than 13-cis-intermediate [253], and the band at 1516 cm⁻¹ is not observed. While all of this suggests that Asp-251 may participate in sodium binding along with Asn-112, the data should be treated with caution, as the
perturbing effects of homologous Asn-212 mutations on the Schiff base of BR are very strong [259].

**Figure 5.15.** Time-resolved laser flash-induced difference FTIR spectra of DDR2 reconstituted into DMPC/DMPA liposomes and measured at 1 ms delay after the flash.
(but note the 12 ms full interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. a. Comparison of FTIR spectra of DDR2 liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.05M Tris and 0.1 M NaCl (black) and with 0.05 M KH$_2$PO$_4$, 0.05 M Tris and 0.1 M KCl (green). b. Comparison of FTIR spectra of DDR2 liposomes hydrated with 0.05 M KH$_2$PO$_4$, 0.05M Tris and 0.1 M NaCl (black) and that with 0.05 M KH$_2$PO$_4$, 0.05 M Tris and 0.1 M LiCl (blue), normalized to yield identical difference absorbance amplitude of the 1533 cm$^{-1}$ band.

**Figure 5.16.** Time-resolved laser flash-induced difference FTIR spectra of DDR2 reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH$_2$PO$_4$ and 0.1 M NaCl with the addition of the following buffers: pH 4 - 0.05 M CH$_3$COONa and pH 8 - 0.05M Tris, and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. Comparison of FTIR spectra of DDR2 liposomes hydrated with pH 8 buffer (black, from Fig. 5.15) and that with pH 4 buffer (green), normalized to yield identical difference absorbance amplitude of the 1533 cm$^{-1}$ band.
Figure 5.17. Time-resolved laser flash-induced difference FTIR spectra of DDR2, comparing the wild type and N112D, reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH₂PO₄, 0.05 M Tris and 0.1 M NaCl, pH 8, and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. a. DDR2 wild type. b. DDR2 N112D. The full scale of the vertical axis is 0.0004 absorbance units for a and b.
Figure 5.18. Time-resolved laser flash-induced difference FTIR spectra of DDR2 measured for the wild type, and D116N and D116T mutants, reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH2PO4, 0.05 M Tris and 0.1 M NaCl, pH 8, and measured at 1 ms delay after the flash (but note the 12 ms full
interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see text for details. a. DDR2 wild type, b. DDR2 D116N, normalized using amplitude of the 1184 cm⁻¹ band and c. DDR2 D116T, normalized to yield identical difference absorbance amplitude of the 1533 cm⁻¹ band.

**Figure 5.19.** Time-resolved laser flash-induced difference FTIR spectra of DDR2 measured for the wild type and D251N mutant, reconstituted into DMPC/DMPA liposomes hydrated with 0.05 M KH₂PO₄, 0.05 M Tris and 0.1 M NaCl, pH 8, and measured at 1 ms delay after the flash (but note the 12 ms full interferogram acquisition time) at 2°C. Positive bands report on the photointermediates, while the negative bands report on the dark state, the characteristic bands are marked, see the text for details. a. DDR2 wild type and b. DDR2 D251N, normalized to yield identical difference absorbance amplitude of the 1533 cm⁻¹ band.
5. 6. Conclusions

In this chapter, we studied a new type of rhodopsin with unique sequence, named DDR2 because of its coexistence with a PR-like pigment (DDR1), found in flavobacterium *Donghaeana dokdonensis* by our collaborators in Korea (K.H. Jung’s lab). The uniqueness of the primary structure of DDR2 is that the key carboxylic acids of light-driven proton pumps are replaced with non-protonatable polar residues (Asn-112 and Gln-123) and it has a new Asp-116 replacing the conserved Thr in BR and PRs, located between the two former residues. Moreover, many new polar residues are introduced into the putative transmembrane region of DDR2 and conserved in homologs of DDR2 in several *Flavobacteria, Cytophagales*, and *Deinococci*, as well as in many environmental samples and even in diatom algae (Fig. 5.1). Taken together, the sequence analysis of DDR2 suggests that it may have very different ion specificity from BR and PRs.

In vitro time-resolved and static spectroscopic analysis by the visible, Raman, and FTIR spectroscopies reveals some suggestive characteristics of the photocycle of DDR2. The most striking one is a very strong Na\(^+\) dependency in the photocycle, which is sped up by increasing the concentration of Na\(^+\). It appears that DDR2 can perform size-based selection of cations, allowing only smaller ions to pass through DDR2, as demonstrated by similarity of the photocycles of DDR2 in LiCl and NaCl based buffers in contrast to that in KCl based buffer. This size-based selectivity is similar to that of some sodium-specific channels. Combined with the transport
experiments of our collaborators, these data suggest that DDR2 may be the first documented case of light-driven sodium pump. Time-resolved FTIR and visible data from the wild-type, N112D, D116N, D116T, and D251N mutants suggest that Asn-112 is the Na\textsuperscript{+} binding site in the dark and is involved in Na\textsuperscript{+} transport, Asp-116 is a new counterion and proton acceptor for the retinal Schiff base and may serve as a gating element for Na\textsuperscript{+} transport, and Asp-251 may be a part of the Na\textsuperscript{+}-binding site, together with Asn-112.

Fig. 5.20 shows the proposed transfers of sodium ions and protons in DDR2. When D116 receives a proton from the Schiff base, the resulting disappearance of the negative charge will destabilize Na\textsuperscript{+} coordinated by N112 (and possibly D251) and will displace it to the extracellular side. After the ensuing conformational change, the Schiff base can get reprotonated again, which allows movement of another sodium ion from the cytoplasmic side, possibly mediated by Q123, which needs to be tested by further mutagenesis studies.

With respect to the possible physiological role of DDR2 and its homologs, it should be noted that DDR2 and its homologs in *Krokinobacter*, *Gillisia*, and *Hymenobacter* (all from *Bacteroidetes*) co-exist with PR-like rhodopsins. At least in this class of bacteria, a light-driven outward-directed sodium pump (DDR2-like) may be always present in addition to a light-driven outward-directed proton pump (PR-like). A recent paper [260] on the light-induced changes in gene expression in *Dokdonia*, another
flavobacterium, which does not have a DDR2-like protein, is very instructive. The authors found that light enhances production of PR and retinal biosynthesis genes, which is expected if PR can help bioenergetically under carbon-limited conditions employed. Unexpectedly, they also found that sodium-pumping machinery, such as Na⁺/phosphate and Na⁺/alanine symporters, and the Na⁺-translocating NADH-quinone oxidoreductase, were also significantly upregulated by light. They suggested that sodium pumping is a critical metabolic process in the light-stimulated growth of this flavobacterium. This may explain the evolutionary advantage the DDR2-bearing bacteria may have, when they enhance their sodium gradient under illumination, supplementing the proton gradient created by PR.

**Figure 5.20.** Diagram showing putative sodium and proton translocation steps in DDR2. Left panel: proton transfers in BR. Right panel: ion transfer steps in DDR2. Green circles represent protons, red circles represent sodium ions. Blue thin arrow shows the detected passive flow of protons, wide arrows show the direction of ion transfers by rhodopsins.
Chapter 6

Conclusions and Future Directions
Summary

Despite the 40 years of intense research, microbial rhodopsins keep surprising us. Every time when one could expect that interest in this protein family should die down, there is a new twist or turn keeping the research active. At present, there are three main hotspots which attract new scientists to the field. First, the biological hotspot, a constant discovery of new functions, is accompanied by the increasing taxonomic and ecological diversity. The nature proves its plasticity and frugality by creating multiple functions on the same protein template, allowing expansion into new ecological niches. This attracts researchers specializing in evolutionary biology, microbial ecology, bioinformatics, bioenergetics, and molecular microbiology. Second, the biophysical hotspot, in which microbial rhodopsins serve as testing grounds for many cutting-edge methods of structural biology and biophysics. Here one could recall cryo-electron microscopy, atomic force microscopy, time-resolved FTIR, biological solid-state NMR (and, most recently, dynamic nuclear polarization NMR), and many others. Finally, the third is applied hotspot, where unique light-gated properties of microbial rhodopsins are used in various applications. Lately, microbial rhodopsins experience a true revival in this respect, due to their spectacular use in optogenetics and neurobiology [130, 261].

In this work, we characterized the photochemical properties of two novel fungal rhodopsins from the same species (*Phaeosphaeria nodorum*) and one bacterial rhodopsin with unique sequence and new ion specificity, from *Donghaeana*
dokdonensis were characterized. We also implemented and optimized the isotope labeling protocols to produce homogeneous eukaryotic membrane protein samples for solid-state NMR spectroscopy. All of these studies nicely fit into the main areas of the modern microbial rhodopsin research delineated above. In the following, we summarize the individual research projects and identify possible future experiments.

6.1. Fungal rhodopsins from *Phaeosphaeria nodorum*

Numerous fungal species, as revealed by their genomic sequences, possess multiple rhodopsins (RDs) and opsin-related proteins (ORPs). However, the knowledge about their photochemical properties and physiological functions is limited, especially for the bacteriorhodopsin homologs in the ORP-like auxiliary subgroup. Our work provides some insights into the photochemistry and biology of the auxiliary subgroup. The fungal wheat pathogen *Phaeosphaeria nodorum* contains two genes encoding RDs, representing two different rhodopsin groups (the LR-like and the auxiliary group). In our project, we studied an auxiliary rhodopsin from *Phaeosphaeria (Stagonospora) nodorum* (PhaeoRD2) and compared its photochemical properties with those of the LR-like homolog (PhaeoRD1) and LR itself. One of the main advantages of our approach was to minimize the artifacts of different cytoplasmic and membrane environments by using rhodopsins from a single fungal species, allowing for much more straightforward and functionally focused comparison. The two genes were successfully expressed in *Pichia pastoris*, a methylotrophic yeast. Spectroscopic analysis of the two *Phaeosphaeria* rhodopsins by visible, Raman, and FTIR
spectroscopy revealed that they show many similarities, such as absorption spectra, conformational changes of the retinal chromophore, and fast photocycle. Sequence analysis of the conserved regions suggested that PhaeoRD2 (the auxiliary rhodopsin) preserved the common transmembrane core of BR, but has some polar residues on the hydrophobic protein periphery, suggesting interactions with a putative transducer or other membrane partner. On the other hand, PhaeoRD1 is highly similar to LR, suggesting its proton-pumping function. Further, the close similarity of the absorption spectra of the two *Phaeosphaeria* rhodopsins implies that they are not designed to interact with different wavelengths of light. The photocycles of both rhodopsins are fast, and show photointermediates and proton transfer steps typical for proton-pumping rhodopsins. The homolog of Asp-96 of BR, Asp-126 in PhaeoRD2, was verified to serve as a cytoplasmic proton donor, as shown by the analysis of the D126N mutant, at least in the absence of its putative transducer. Finally, both rhodopsins have similar carboxylic acid protontation changes during the photocycle. The core structure of PhaeoRD2 is similar to those of LR and PhaeoRD1 as was revealed by low-temperature FTIR results.

Taking all these data together, we have provided direct experimental evidence suggesting that these two rhodopsins can perform light-driven proton-pumping, at least *in vitro*. We suggest that this may point to a fairly recent evolutionary separation of these putative photosensors. However, the study on the two rhodopsins did not help understand their roles in the pathogenicity or photobiology of *Stagonospora nodorum*. 
A carotenoid-related physiological role of PhaeoRD2 is strongly suggested by the location of its gene in the carotenoid biosynthesis cluster, similar to the auxiliary rhodopsin CarO from *Fusarium fujikuroi*. The future *in vivo* experiments are needed to test whether the auxiliary rhodopsins indeed serve as photosensors and find out the exact functions of two rhodopsins in *Stagonospora nodorum*.

6.2. Uniform isotope labeling of a eukaryotic membrane protein for solid-state NMR studies

The isotope labeling protocol for eukaryotic proteins in *Pichia pastoris* is well-established for soluble secreted proteins. The yeast expression system has a number of outstanding advantages compared to *E. coli*, especially when it comes to membrane proteins of eukaryotic origin, being able to perform proper folding, membrane targeting, and post-translational modification. In our project, we demonstrated this modified protocol to be applicable to eukaryotic membrane proteins by expressing and doubly isotopically labeling a eukaryotic 7TM helical protein LR (*Leptosphaeria* rhodopsin) in *Pichia pastoris*. First of all, the yield of yeast-expressed isotope-labeled LR was high (5 mg of purified protein per L of culture), even in shake-flask cultures. Much higher yield is expected with fermenter vessels. Second, the uniformly $^{13}\text{C}$, $^{15}\text{N}$-labeled LR was stable for several weeks at 5°C. The sample was functionally active as proved by FTIR spectra upon reconstitution into lipid membranes at high protein-to-lipid ratio required for solid-state NMR. Most importantly, the samples were homogenous and gave high-resolution $^{13}\text{C}$ and $^{15}\text{N}$
solid-state magic angle spinning NMR spectra, amenable to a detailed structural analysis. Reasonable price, decent expression yield, proper protein folding and post-translational modifications, and well-resolved signals in the ssNMR spectra obtained in the membrane-like environment, make this optimized protocol attractive for expression of challenging mammalian targets, such as GPCRs and aquaporins, in this system. Our future plan is to express such eukaryotic proteins using this protocol, with the view of eventually obtaining their 3D structures in the native-like environment.

6.3. New cation pump, DDR2 from *Donghaeana dokdonesis*

DDR2, a microbial rhodopsin with the unique primary structure, was found to produce very untypical light-induced pH changes in the *E. coli* sphaeroplast suspensions in the hands of our collaborators. We studied this new rhodopsin using spectroscopic analysis by the visible, Raman, and FTIR spectroscopies in the media of different ionic compositions. This bacterial rhodopsin was expressed in *E. coli*, membrane-associated, and bound all-trans-retinal as proved by the Raman spectra. The analysis on the primary structure of DDR2 suggested that it may have very different ion specificity from BR and PRs, as it replaced the key carboxylic acids of light-driven proton pumps with non-protonatable polar residues (Asn-112 and Gln-123) and had a new Asp-116 replacing the conserved Thr in BR and PRs, located between the two former residues. Moreover, DDR2 has many new polar residues in the putative transmembrane region, also conserved in its close homologs in several
Flavobacteria, Cytophagales, and Deinococci, as well as in many environmental samples and even in diatom algae. We studied its photochemistry and found that the photochemical reaction cycle of DDR2 was quite fast and strongly Na$^+$ dependent, suggesting that it may be a light-driven sodium pump. It appears that DDR2 can perform size-based selection of cations, allowing only smaller ions to pass through, as demonstrated by similarity of the photocycles of DDR2 in LiCl and NaCl based buffers in contrast to that in KCl based buffer. This size-based selectivity is similar to that of some sodium-specific channels. However, the putative Na$^+$ binding site does not strongly interact with the retinal Schiff base and Asp-116 in the dark. Further, site-directed mutagenesis of DDR2 revealed that Asn-112 is the Na$^+$ binding site in the dark and is involved in Na$^+$ transport, Asp-116 is a new counterion and proton acceptor for the retinal Schiff base and may serve as a gating element for Na$^+$ transport, and Asp-251 may be a part of the Na$^+$-binding site, together with Asn-112. Taken together, DDR2 is the first proven case of light-driven Na$^+$-pumping rhodopsin.

Both DDR2 and its homologs in Krokinobacter, Gillisia, and Hymenobacter (all from Bacteroidetes) co-exist with PR-like rhodopsins. Studies on the light-induced changes in gene expression in Dokdonia, another flavobacterium, suggest that sodium pumping is a critical metabolic process in the light-stimulated growth of this flavobacterium [260]. This may explain the evolutionary advantage the DDR2-bearing bacteria may have, when they enhance their sodium gradient under illumination, supplementing the proton gradient created by PR. However, the mechanism of sodium and proton translocation in DDR2 needs to be confirmed by
further mutagenesis studies, and its physiological role must be verified by *in vivo* experiments. This new light-switchable sodium-pumping protein may turn out to be useful in optogenetics, where it can provide fast hyperpolarisation of membranes silencing the neurons of choice.
Appendix

1. Recipes of Broth and Media

Low salt Luria Bertani Lennox (LB) broth (per 1 L)

Dissolve 10 g tryptone, 5 g yeast extract and 5 g NaCl in MilliQ-water and fill it up to 1 L. Autoclave 20 min on liquid cycle. Add zeocin when the broth cools to ~55°C.

Low salt LB agar plates (per 1 L)

Prepare low salt LB broth mentioned above and add 15 g of agar. Autoclave 20 min on liquid cycle. Add zeocin to 25 µg/mL final concentration when the broth cools to ~55°C. Pour the plates and store the plates at 4°C in the dark.

Super Optimal Broth (SOB) (per 1 L)

Dissolve 20 g of bacto-tryptone (2%), 5 g bacto-yeast extract (0.5%), 0.584 g NaCl (10 mM), 0.186 g KCl (2.5 mM), 0.952 g MgCl\(_2\) (10 mM) and 1.204 g MgSO\(_4\) (10 mM) in MilliQ-water and fill it up to 1 L.

Super Optimal broth with Catabolite repression (SOC) (per 1 L)

Add 3.803 g glucose (20 mM) in above SOB.

10× YNB (13.4%Yeast Nitrogen Base with Ammonium Sulfate without amino acids) (per 1 L)
Dissolve 134 g of yeast nitrogen base (YNB) in MilliQ-water and fill the volume up to 1 L and then filter-sterilize.

$500 \times B$ (0.02% Biotin) (per 100 mL)

Dissolve 20 mg biotin in 100 mL of MilliQ-water and filter-sterilize.

$10 \times D$ (20% Dextrose) (per 500 mL)

Dissolve 100 g of D-glucose in 500 mL of MilliQ-water and filter-sterilize.

$10 \times GY$ (10% Glycerol)

Mix 100 mL of glycerol with 900 mL of MilliQ-water. Autoclave 20 min on liquid cycle.

$1 \text{ M NaH}_2\text{PO}_4$, pH 5 (per 1 L)

Dissolve 138 g of NaH$_2$PO$_4$·H$_2$O into 950 ml of MilliQ-water. Adjust pH to 5 with NaOH and fill volume up to 1 L. Autoclave 20 min on liquid cycle.

$\text{NH}_4\text{Cl}/^{15}\text{NH}_4\text{Cl}$ (0.8%) (per 1 L)

Dissolve 4 g of NH$_4$Cl/$^{15}$NH$_4$Cl into 500 mL of MilliQ-water. Autoclave 20 min on liquid cycle or filter sterilize.

Potassium phosphate solution, pH 5 (per 1 L)
Dissolve 23 g KH₂PO₄ and 118 g K₂HPO₄ in 950 mL of MilliQ-water. Adjust pH to 5 with potassium hydroxide and fill volume up to 1 L. Autoclave 20 min on liquid cycle.

Yeast Extract Peptone Dextrose Medium (YPD) (per 1 L)
Dissolve 10 g yeast extract and 20 g of peptone in 900 mL of MilliQ-water and autoclave for 20 min at 15 lb./sq.in. Add 100 ml of 10× D solution when the solution cools to ~60°C.

YPDS agar plates (per 1 L)
Dissolve 10 g yeast extract, 182.2 g sorbitol, 20 g of peptone and 20 g of agar in 900 mL of MilliQ-water. Autoclave 20 min on liquid cycle. Mix with 100 mL of 10× D solution. Add zeocin to the mixture when it cools to ~60°C.

Minimal Dextrose Medium (MD) plates (per 1 L)
Dissolve 15 g agar in 800 mL of MilliQ-water and autoclave for 20 min at 15 lb./sq.in. Add 100ml of 10× YNB, 2 ml of 500× B and 100 ml of 10× D solution, when the solution cools to ~60°C.

Buffered Minimal Glycerol (BMM) (per 1 L)
Autoclave 700 ml MilliQ-water on liquid cycle for 20 min. Mix 100 ml of 1 M NaH₂PO₄, pH 5, 100ml of 10× YNB, 2 ml of 500× B and 100 ml of 10× GY when it
cools to ~60°C.

Buffered Glycerol-complex Medium (BMGY) (per 1 L)

Dissolve 10 g of yeast extract, 20 g peptone in 700 ml MilliQ-water. Autoclave 20 min on liquid cycle. Mix the solution with 100 mL of NaH₂PO₄, pH 5, 100 mL of 10× YNB, 2 mL of 500× B solution, 100 mL of 10× GY solution.

Buffered Methanol-complex Medium (BMMY) (per 1 L)

Dissolve 10 g of yeast extract, 20 g peptone in 800 ml MilliQ-water. Autoclave 20 min on liquid cycle. Mix with 100 mL of NaH₂PO₄, pH 5, 100 mL of 10× YNB, 2 mL of 500× B solution, 7 mL of methanol.

Buffered minimal glucose (BMD)/¹³C,¹⁵N-labeled BMD, pH 5 (per 1 L)

Mix 100 mL of potassium phosphate solution (pH 5), 25 mL of 10× YNB solution, 2 mL of 500× B solution, 25 mL of 10× D solution /¹³C of glucose solution, 100 mL of NH₄Cl/¹⁵NH₄Cl and 750 ml of sterile MilliQ water.

Buffered minimal methanol (BMM)/¹³C,¹⁵N-labeled BMM, pH 5 (per 1 L)

Mix 100 ml of potassium phosphate solution (pH 5), 25 mL of 10× YNB solution, 2 mL of 500× B solution, 10 mL of filter-stereled methonal, 100 mL of NH₄Cl/¹⁵NH₄Cl and 765 mL of sterile MilliQ-water.
2. Sequence alignment of rhodopsins from ascomycetes produced by CLUSTALW.

Members of the auxiliary group are shown on top; LR and PhaeoRD1 are shown at the bottom for comparison.

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<th>Rhodopsin</th>
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<th>Helix B</th>
</tr>
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</tr>
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<td>Bipolaris RD2</td>
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