Lipid Modulation of Dynamics of a Seven-Helical Transmembrane Protein, Proteorhodopsin

by

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ABSTRACT

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Membrane proteins which comprise approximately a third of all proteins are classified for their roles in specific cell signalling, catalysis of metabolic reactions and transport of ions and molecules. One specific membrane protein, called proteorhodopsin (PR) belongs to the family of microbial rhodopsins and functions as a light-driven proton pump. Its lysine residue (Lys231) on helix G forms a Schiff base (C=N) with retinal, its chromophore which photo-isomerizes from the all-trans to the 13-cis form. Photo-isomerization initiates a photocycle, with distinct intermediates (K, M, N, and O). This study tries to emphasize the importance of interactions occurring between the membrane bilayer and PR by examining the kinetics of its photocycle and structure of the retinal chromophore using time-resolved spectroscopy in the visible range and static Raman spectroscopy. Some of the parameters of the membrane that were found to be important include protein to lipid ratio, bilayer thickness, bilayer fluidity and surface charge. The main conclusion is that PR has a very fast photocycle in negatively charged membranes, but a slower photocycle in positively charged ones, as well as in more rigid, thicker membranes. These slower cycles can originate from 1) suppression of conformational changes by the rigid bilayer or dehydration; 2) lack of available protons due to surface charge and 3) impeded isomerization.

Keywords: bio-spectroscopy, membrane proteins, protein dynamics, biological membranes, retinal proteins
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List of Abbreviations

Rhodopsins

PR  Proteorhodopsin
GPR  Green-absorbing Proteorhodopsin
BPR  Blue-absorbing Proteorhodopsin
BR  Bacteriorhodopsin
HR  Halorhodopsin
SR  Sensory Rhodopsin
ChR2  Channelrhodopsin-2
ASR  Anabaena Sensory Rhodopsin
XR  Xanthorhodopsin
AR  Acetabularia Rhodopsin
CSR  Chlamydomonas Sensory Rhodopsin
NR  Neurospora Rhodopsin
LR  Leptosphaeria Rhodopsin
GR  Gloeobacter Rhodopsin
PyrR  Pyrocystis Rhodopsin
KR1  Krokinobacter Rhodopsin 1
KR2  Krokinobacter Rhodopsin 2

Other Proteins/Molecules

GPCR  G-Protein Coupled Receptor
HtrI  Halobacterial transducer I
HtrII  Halobacterial transducer II
CheA  Histidine Kinase A
CheB  Histidine Kinase B
CheR  Histidine Kinase R
CheY Histidine Kinase Y
CheW Histidine Kinase W
ASRT *Anabaena* Sensory Rhodopsin Transducer
ATP Adenosine Triphosphate
ADP Adenosine Diphosphate

**Bacteria**

*E. coli. Escherichia coli*

**Chemicals**

DNase Deoxyribonuclease
IPTG Isopropyl-β-D-Thiogalactopyranoside
Ni$^{2+}$-NTA Nickel-Nitrilotriacetic Acid
DM $n$-Dodecyl β-D-maltoside
TX-100 Triton X-100
CHES 2-(N-Cyclohexylamino)ethane Sulfonic Acid
DiC7PC 1,2-diheptanoyl-$sn$-glycerol-3-phosphocholine

**Lipids**

DMPC 1,2-dimyristoyl-$sn$-glycerol-3-phosphocholine
DMPA 1,2-dimyristoyl-$sn$-glycerol-3-phosphate
DOPC (18:1 PC) 1,2-dioleoyl-$sn$-glycerol-3-phosphocholine
DOPS 1,2-dioleoyl-$sn$-glycerol-3-phospho-L-serine
DOTAP 1,2-dioleoyl-3-trimethylammonium-propane
POPC 1-palmitoyl-2-oleoyl-$sn$-glycerol-3-phosphocholine
POPS 1-palmitoyl-2-oleoyl-$sn$-glycerol-3-phospho-L-serine
14:1 PC 1,2-dimyristoleoyl-$sn$-glycerol-3-phosphocholine
20:1 PC 1,2-dieicosenoyl-$sn$-glycerol-3-phosphocholine
22:1 PC 1,2-dierucoyl-\textit{sn}-glycero-3-phosphocholine
24:1 PC 1,2-dinervonoyl-\textit{sn}-glycero-3-phosphocholine

**Polymers**

- **PBD** Poly(butadiene)
- **PDMS** Poly(dimethylsiloxane)
- **PS** Polystyrene
- **P4MVP** Poly(4-vinyl-N-methylpyridine iodide)

**Biophysical/Instrumental Terms**

- **FTIR** Fourier Transform Infrared
- **NMR** Nuclear Magnetic Resonance
- **Nd-YAG** Neodymium-doped- Yttrium Aluminum Garnet
- **OD** Optical Density
- **HOOP** Hydrogen Out Of Plane
- **P/L** Protein to Lipid
- **H/D** Hydrogen/Deuterium
- **w/w** weight over weight
- **v/v** volume over volume
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Chapter 1

Background on Microbial Rhodopsins
Rhodopsins are a family of proteins found in all three taxonomic kingdoms of life, Archaea, Bacteria, and Eukaryota. They are light sensitive proteins which absorb light using their chromophore, retinal (vitamin-A aldehyde). They perform a multitude of physiological functions including cell signalling and ion transport. In general, rhodopsins share many common structural features. Their secondary structure is usually dominated by alpha helices, which are clustered around retinal. The retinal chromophore is bound to a conserved lysine residue through a C=N double bond called the Schiff base. Upon illumination, retinal isomerizes around a C=C bond which drives a series of conformational changes in both the retinal and apoprotein (opsin) (Spudich et al., 2000; Klare et al., 2008).

1.1 Type I and II Rhodopsins

Rhodopsins are classified into two main groups, Type I and Type II. Type II rhodopsins have been found only in higher eukaryotes as photoreceptors important for controlling circadian rhythm and vision (Spudich et al., 2000). Retinal which is bound to lysine of type II rhodopsins via a Schiff base (C=N) is initially in the 11-cis, 15-anti form. Upon irradiation retinal isomerizes into the all-trans form. Type II rhodopsins generally have seven transmembrane alpha helices and exhibit large hydrophilic loops which interact with cytoplasmic proteins such as G-proteins, receptor kinases, arrestins, and other signalling proteins. Type I microbial rhodopsins are a group of membrane proteins found in archaea, eubacteria, and lower eukaryotes. These proteins share significant sequence homology with all members having seven transmembrane alpha helices which are connected by short, non-functional loop regions. Unlike type II rhodopsins, retinal in these proteins is initially in the all-trans, 15-anti configuration which photoisomerizes into the 13-cis, 15-anti form (Figure 1.1) initiating structurally distinct intermediate states in a sequence called a
photocycle. Despite their structural similarity a wide number of microbial rhodopsins differ in functional properties being either pumps, sensors or channels. This research will focus on one member from type I rhodopsins.

Figure 1.1. All-trans and 13-cis configurations of retinal in Type I microbial rhodopsins. In the dark, Type I microbial rhodopsins usually contain both all-trans, 15-anti and 13-cis, 15-syn isomers, and the former isomerizes to 13-cis, 15-anti upon excitation by light. The main regions of retinal are highlighted in all-trans, 15-anti retinal. Modified image from Herzfeld and Lansing (2002) with permission from the Annual Review of Biophysics and Biomolecular Structure, Volume 31 © 2002 by Annual Reviews www.annualreviews.org.
1.2 Archaeal (Type I) Rhodopsins

1.2.1 Ion Pumps

Bacteriorhodopsin (BR) and Halorhodopsin (HR) are two important archaeal rhodopsins responsible for light-driven ion transport. Despite their discovery in a common halophilic archaeon, *Halobacterium salinarum*, their photochemical properties and physical function differ significantly. BR is the most extensively studied microbial rhodopsin and is an outward directed (cytoplasm to extracellular space) proton pump (Blaurock and Stoeckenius, 1971; Oesterhelt and Stoeckenius, 1971) whereas HR is an inward directed chloride pump (Schobert and Lanyi, 1982). Under anaerobic conditions, the free energy stored in the proton gradient is used to metabolically produce adenosine triphosphate (ATP) from inorganic phosphate (P\(_i\)) and adenosine diphosphate (ADP) while the chloride gradient is used to maintain osmotic balance across the membrane. Both undergo light-dark adaptation but the ratio of 13-*cis* to all-*trans* retinal is different. Dark adapted BR samples contains 33% all-*trans* compared to ~45% all-*trans* in dark-adapted HR. The ratio is shifted to 100% all-*trans* for BR and ~ 75% all-*trans* for HR upon illumination (Zimanyi and Lanyi, 1997; Scherrer et al., 1989). In BR all-*trans* retinal is covalently bound (via the protonated Schiff base) to the ε-amino group of Lys-216 with an absorption maximum at 570 nm. In HR of *H. salinarum*, retinal is bound to Lys-242 with maximal absorption at 578 nm (Dencher, 1983).
1.2.2 Sensory Receptors

It was discovered in the 1980s that sensory rhodopsins originally found in *H. salinarum* were responsible for phototactic behaviour of bacteria (Spudich and Spudich, 1982; Sperling and Schimz, 1980; Bogomolni and Spudich, 1982). One popular model suggests that the attractant and repellent responses can be triggered by one and two photon processes in the same protein. Spudich and Bogomolni (1984) showed that the first excitation (> 500 nm) of a rhodopsin-like pigment (sensory rhodopsin I (SRI)) initiated the photocycle and the attractant response. However, when a second photon was absorbed (≈ 370 nm) the photophobic response was activated. On the other hand, another sensory rhodopsin, sensory rhodopsin II (SRII), mediates a repellent (photophobic) response by a single photon absorption. Sensory rhodopsins are also found in other microbial organisms (i.e. *Natronomonas pharaonis* (NpSRII), *Haloarcula vallismortis*) with similar processes for signal transduction (Seidel et al., 1995).

1.2.2.1 Signal Transduction

The signal for phototaxis and chemotaxis is transferred through various pathways, determining the rotational direction of the motor-driven helical flagella. Clockwise rotation of the flagellar motor leads to forward swimming whereas counter-clockwise rotation pulls the bacterium in the opposite direction (Hildebrand and Dencher, 1975; Spudich and Stoeckenius, 1979; Marwan and Oesterhelt, 1990). First, archaeal sensory rhodopsins transfer the light signal to cognate transducer proteins which usually belong to the family of halobacterial transducer proteins (i.e., HtrI, HtrII). These proteins usually consist of two domains with their secondary structure composition being dominated by alpha helices. The N-terminal transmembrane domain consists of two helices followed directly by two HAMP domains (Aravind and Ponting, 1999). In eubacteria, transducer proteins contain only one HAMP domain. The transducers then relay the
signal to a cytoplasmic signal transduction cascade, a two-component system (Figure 1.2) containing proteins with specific functions. Upon HtrI/II activation, CheA, a histidine kinase, binds to signalling domains of the sensory receptor via CheW coupling. CheA then increases or decreases auto-phosphorylation activity depending on the signalling state of the receptor which depends on the amount of attractant or repellent external stimuli. A phosphoryl group is then transferred from CheA to CheY/CheB, a response regulator allowing CheY to bind to the motor switch complex. High concentration of CheY~P increases the reversal swimming frequency, whereas low levels lead to prolonged forward swimming. Other important proteins include CheR and CheB which are used for adaptation to constant stimuli. CheR is a methyltransferase which adds methyl groups to glutamic residues of the activated transducer, whereas CheB is a response regulator. When methylated by CheA, CheB removes methyl groups when the transducer is in its ground state.

Figure 1.2. Schematic representation of the two component signal transduction cascade in sensory rhodopsin II from *Natronomonas pharaonis*. See text for a detailed description of the components and interactions. Image taken from Klare et al. (2004) with permission from Elsevier, Copyright (2004).
1.3 Eubacterial Rhodopsins

The first eubacterial rhodopsin discovered was Green Proteorhodopsin (GPR) (Beja et al., 2000) which is described in detail in section 1.6. Since then, many more eubacterial rhodopsins have been found, some of which are described in this section.

*Anabaena* Sensory Rhodopsin (ASR) found in the cyanobacterium *Anabaena* is the first retinal-binding photoreceptor found in the eubacterial domain (Jung et al., 2003). Photoisomerization of retinal occurs upon green-light illumination with an absorption maximum at 543 nm. Compared to other microbial rhodopsins it is more efficient in converting between all-*trans* and 13-*cis* retinal states, being fully photochromic (Vogeley et al., 2004). Its cytoplasmic side is also highly hydrophilic, networked by water molecules (Vogeley et al., 2004; Shi et al., 2006). This hydrophilic nature enables the protein to interact with the soluble transducer protein, *Anabaena* Sensory Rhodopsin Transducer (ASRT), which forms a complex with ASR in a 4:1 ratio and is the only example of a soluble transducer which transmits a signal from a sensory rhodopsin type receptor. ASRT is believed to be an important downstream signalling molecule that regulates a diverse range of metabolic operons, demonstrating light sensory behaviour when bound to ASR.

Another eubacterial rhodopsin called Xanthorhodopsin (XR), found in *Salinibacter ruber*, is a light-driven ion pump (Balashov et al., 2005). It is unusual because it uses two chromophores, retinal and salinixanthin to harvest light energy. Light energy absorbed by one chromophore is transferred to the other, creating a broad spectral region for efficient energy production. Because of the two chromophores, XR has two absorption maxima at 487 nm and 519 nm (Balashov et al., 2006).
1.4 Eukaryotic (Type I) Rhodopsins

1.4.1 Proton Pumps and Channels

Many well-known rhodopsins are found in marine algae and fungi. For example, Acetabularia rhodopsin (AR) is a light-driven proton pump found in the giant algae, Acetabularia acetabulum (Tsunoda et al., 2006). Protein kinetics in these rhodopsins are influenced by the external pH, which can possibly affect the direction of ion transport similar to proteorhodopsin (Friedrich et al., 2002).

The retinal binding protein, chlamyrhodopsin was found in eyespot membranes of the green alga Chlamydomonas reinhardtii (Deininger et al., 1995). The high abundance of polar and charged residues in the protein led scientists to believe that it functions as a light-gated ion channel rather than a receptor for phototaxis. Its structure, which does not contain the typical seven alpha helices, makes it hard to believe that it shares important DNA sequences with archaeal rhodopsins.

Later, certain rhodopsins of Type I were also found to be responsible for phototactic behaviour in green algae. For example, Chlamydomonas sensory rhodopsins A and B (CSRA and CSRB) were shown to be receptors for phototaxis through in vivo experiments (Nagel et al., 2002, 2003). These receptors contain two domains called the N-terminal domain (~300 residues) and membrane-associated domain (~400 residues). A characteristic feature of these algal rhodopsins is that light activation leads to photoreceptor currents. In the case of CSRA, high light intensity leads to a fast receptor current, and in CSRB low light intensity leads to a slow receptor current. CSRA and CSRB also function as channels for protons and other cations (giving them another name of channelrhodopsins), but this role is carried out by the N-terminal domain. Signal transduction occurs across the cytoplasmic domain required for phototaxis.
A final important eukaryotic rhodopsin to mention is *Neurospora* rhodopsin (NR) from the fungus *Neurospora crassa*. It is believed that NR functions as a sensory rhodopsin (Brown and Jung, 2006). Other fungal rhodopsins have been identified as proton pumps. Such activity was first observed in *Leptosphaeria maculans* with *Leptosphaeria* rhodopsin (LR) (Waschuk et al., 2005; Magyari et al., 2007). There are many other fungal rhodopsins whose functions are not yet fully understood.

Figure 1.3. Four important types of Type I rhodopsins. These include proteorhodopsin (PR, proton pump), halorhodopsin (HR, chloride pump), sensory rhodopsin I and II (SRI and SRII, phototaxis receptors), *Anabaena* sensory rhodopsin (ASR, photosensor), and *Chlamydomonas* CSRA and CSRB (phototactic receptors, proton and cation channels). The colors of the rhodopsins approximately represent their respective natural colors (Spudich and Jung, 2005). Reproduced with permission from Wiley-VCH, Copyright (2005).
1.5 Bacteriorhodopsin: Ideal System for Studying Rhodopsins

Bacteriorhodopsin is the most extensively studied microbial rhodopsin in the past 40 years, whose amino acid sequence is highly conserved in other rhodopsins. It has been studied by a variety of biophysical techniques (i.e., FTIR, visible spectroscopy, X-ray diffraction, NMR) producing high quality data with large optical and electrical signals. BR has also become a model protein because of its importance in bioenergetics, and for practical reasons such as its high stability and ability to be produced in large quantities.

Bacteriorhodopsin is a 26 kDa light-driven membrane protein found in Archaea that live in hypersaline environments and salt beds. It contains seven transmembrane alpha helices (A-G) with four main residues important in proton transport (Lanyi, 2004). Aspartic acid 85 is the proton acceptor on the extracellular side, aspartic acid 96 is the proton donor on the cytoplasmic side and glutamic acid 204 and 194 form the proton release group on the extracellular side (see Figure 1.4). Retinal, its chromophore, is covalently bound to lysine 216 (helix G) and undergoes photoisomerization from all-trans to 13-cis.
Figure 1.4. Structure of bacteriorhodopsin. Arrows indicate the proton transfer steps and numbers refer to the sequential order of steps occurring from the beginning to the end of the photochemical cycle (Neutze et al., 2002). Reprinted with permission from Elsevier, Copyright (2002).
### 1.5.1 Steps in the Bacteriorhodopsin Photocycle

Upon absorption of light, BR undergoes a sequence of structural changes called the photocycle (Figure 1.5) with six structurally distinguishable states (BR, K, L, M, N, O). The photocycle can be explained in five steps, 1) protonation of Asp-85 and deprotonation of retinal Schiff base (C=N) (L to M), 2) proton release to the extracellular side (M intermediate state), 3) reprotonation of Schiff base by Asp-96 (M to N), 4) reprotonation of Asp-96 from the cytoplasmic side (N intermediate state) and 5) deprotonation of Asp-85 and reprotonation of proton release site (N to O). The result of completing steps 1 to 5 is the transport of a proton from the cytoplasmic to extracellular side.

![Figure 1.5. The photocycle of bacteriorhodopsin containing six structurally distinct and spectroscopically identifiable states (BR, K, L, M, N and O). Reversible steps are denoted by double-ended arrows. The proton is released to the extracellular bulk solvent and taken up at the cytoplasmic side (Cartailler and Luecke, 2003). Image reprinted with permission from the Annual Review of Biophysics and Biomolecular Structure, Volume 32© 2003 by Annual Reviews www.annualreviews.org.](image-url)
1.5.2 Important Structural Changes during the Photocycle

During the photocycle, key structural events occur during the L, M and N intermediate states (Figure 1.6). According to one of the models, during the L state, helix C becomes kinked making it easier for the Schiff base to transfer a proton to Asp-85. The proton may pass through Asp-212 or the side chain of Thr-89 before being transferred directly via a water molecule. During late M intermediate helix F becomes outwardly tilted and helix G becomes inwardly tilted. This opens the cytoplasmic half of the pump allowing an entrance for water molecules required for reprotonation of the retinal Schiff base. The helix F movement may be facilitated by an interruption of the hydrogen bond to water(wat)501, which is hydrogen bonded to Ala-215 on helix G, resulting in a disconnection of both helices. Asp-96 is reprotonated in the N intermediate possibly with the help of acidic residues (Asp-36, Asp-38, Asp-102, Asp-104 and Glu-166) which funnel the proton to Asp-96. Helices F and G then tilt in the opposite direction at the end of the photocycle.
Figure 1.6. Important intramolecular changes to bacteriorhodopsin during its photocycle. Colors represent electrostatic properties (red-negative, blue-positive). Main structural changes occur in helices F and G in the N and late M intermediates with retinal bound in the space between the seven helices (A-G) and in the 13-cis, 15-anti configuration (Lanyi and Schobert, 2004; Kuhlbrandt, 2000). Reprinted with permission from American Chemical Society, Copyright (2004) and Nature Publishing Group, Copyright (2000).
1.6 Proteorhodopsin - Eubacterial Homolog of Bacteriorhodopsin

Proteorhodopsin (PR) from γ-proteobacteria is a seven transmembrane alpha helical membrane protein found in marine bacterioplankton (Beja et al., 2000). Like BR it functions as a light-driven proton pump with the primary acceptor, donor and Schiff base linkage corresponding to Asp-97, Glu-108 and Lys-231 in PR (Figure 1.8). The two types of PR absorb blue and green light with absorption maxima at ~490 nm and ~520 nm respectively (Sineshchekov and Spudich, 2004; Wang et al., 2003). Despite 26% sequence identity, PR is a bit different from BR, showing higher sequence homology to sensory rhodopsin. For instance, the pKₐ of the proton acceptor in PR is five pH units higher, around 7.5, indicating that the functional photocycle is initiated at a much more alkaline condition (Friedrich et al., 2002).

Figure 1.7. Phylogenetic tree based on microbial rhodopsins’ amino-acid sequences. KR1 is a proton pump, while KR2 is a sodium pump both from the marine flavobacterium, Krokinobacter eikastus. Taken from Inoue et al. (2013) with permission from MacMillan Publishers, Copyright (2013).
Figure 1.8. Structure of proteorhodopsin. The main amino acids and proton transfer steps are indicated with the net result being the transfer of a proton from the cytoplasmic to extracellular side. Image taken from Stehle et al. (2012), “Characterization of the Ground State Dynamics of Proteorhodopsin by NMR and Optical Spectroscopies” (supplemental material). Note: L231 (Leucine) should be K231 (Lysine).

1.6.1 Steps in the Proteorhodopsin Photocycle

The photocycle of PR is similar to that of BR with both containing almost the same number of intermediates at physiological pH. Upon photoexcitation, retinal isomerizes from the all-trans to 13-cis configuration leading to the first intermediate, the K state. The transition from the ground state to the K state occurs very fast, around 0.5 picoseconds (Neumann et al., 2008). The next intermediate is the M state which is characterized by a deprotonated Schiff base. During the K to M transition a proton is transferred to the proton acceptor, Asp-97 from the Schiff base. The rise and decay of the M state are usually in the microseconds and milliseconds range, respectively. The M intermediate then transitions to the N state which arises upon reprotonation of the Schiff base.
from the proton donor, Glu-108. Protonation of the proton donor, Glu-108 and reisomerization of retinal from 13-cis to all-trans lead to the O intermediate. The final transition to the ground state (pR) is accompanied by proton release to the extracellular side.

**1.7 Difference between the BR and PR Photocycle**

The photocycles of PR and BR contain some important differences. At alkaline pH, PR contains mixtures of intermediates whose equilibria are temperature dependent (Figure 1.9). The photocycle of PR is described by the sequence, PR-hv-K-(L?)-M-N-O (Dioumaev et al., 2002). The L intermediate cannot be detected by time-resolved UV-Vis spectroscopy because it might not accumulate during the K to L and L to M transitions. Another reason might be that the K and L intermediates cannot be spectrally distinguished. Some other differences include: 1) in PR, processes occurring before deprotonation of the Schiff base include either a red-shifted L state or a K/L equilibrium strongly shifted toward K; 2) the amount of M state accumulation is much less in PR because reprotonation of the Schiff occurs much faster; 3) in PR, proton uptake precedes proton release, which occurs without a proton release complex; 4) reprotonation of the Schiff base produces a red-shifted absorption maximum for the N intermediate; 5) the O intermediate has an absorption maximum much closer to that of the unphotolyzed state (Dioumaev et al., 2002).
1.8 Lipid-Protein Interactions

Lipid-protein interactions play an important role in controlling biological activity of certain membrane proteins and in modifying physico-chemical properties of biological membranes. For example, the composition of the bilayer can influence lateral pressure differentially acting on proteins (Figure 1.10). Factors which combine to determine the nature and extent of lipid-protein interaction free energy include bilayer thickness, head group interactions prevailing in the interfacial region, and bilayer fluidity which depends on temperature and the nature of lipid tails (saturated or unsaturated).
1.8.1 Hydrophobic Mismatch

If the thickness of hydrophobic region of the protein ($d_P$) is different from the thickness of the lipid bilayer ($d_L$), then neighbouring lipid chains should either stretch out (if $d_P > d_L$) or compress (if $d_P < d_L$) (Figure 1.11) in order to minimize the contact area between hydrophobic groups and the surrounding aqueous solution. In the first case ($d_P > d_L$), the interaction free energy is further increased due to the enhanced stretching of the lipid tails. In the latter case ($d_P < d_L$) chain flexibility (entropy) of lipids increases but the contribution to interaction free energy is compensated by the increase in interfacial free energy of lipids ($\Delta F_h$) (Figure 1.12). If the thickness of the hydrophobic region of the protein and bilayer are the same ($d_P = d_L$), the lipid deformation energy ($\Delta F = \Delta F_t + \Delta F_h$) is due entirely to the loss of conformational entropy experienced by the lipid tail ($\Delta F_t$) (Ben-Shaul, 1995).
Figure 1.11. Schematic illustration of the lipid-protein interaction model (Fattal and Ben-Shaul, 1993). Negative hydrophobic mismatch (left) results in bilayer compression and hence an increase in the average area per lipid head group near the protein. Positive mismatch (right) results in chain stretching in the vicinity of the protein. Reproduced with permission from Biophysical Society, Copyright (1993).

Figure 1.12. Lipid-protein interaction free energy as a function of the hydrophobic mismatch (Fattal and Ben-Shaul, 1993). In a, the tail ($\Delta F_t$, triangles) and interfacial ($\Delta F_s + \Delta F_h$, squares) contributions to the total lipid deformation free energy (solid circles), as a function of hydrophobic mismatch. In b, the total lipid-protein interaction free energy with no head group repulsion is shown (open circles). Reproduced with permission from Biophysical Society, Copyright (1993).
1.9 Objective and Approach

Microbial rhodopsins play an important role in controlling the flow of energy in marine ecosystems. They have many wide-scale applications from fields such as optics and biotechnology used to create things such as holograms, to helping produce biofuel and desalinize seawater. Proteorhodopsin (PR), a eubacterial rhodopsin, was discovered fairly recently and shares many structural features with bacteriorhodopsin (BR). Many residues in BR are conserved in PR, including those important for their proton pumping function. For example, the homologues of the active site residues Arg-82, Asp-85 (proton acceptor), Asp-212 and Lys-216 (retinal Schiff base binding site) in BR are conserved as Arg-94, Asp-97, Asp-227 and Lys-231 in PR. However, there are many differences in the photocycle turnover rate and structure of intermediate states. Since BR has already been extensively studied under a variety of conditions studying the behaviour of PR will help understand similarities and differences between these two proteins. The goal of this study is to highlight the importance of membrane-protein interactions, specifically the influence of membrane bilayer composition on the photochemistry and structure of green-light absorbing proteorhodopsin (GPR). Among the parameters tested will be different protein to lipid (w/w) ratios, different bilayer thickness (tail lengths) to explore hydrophobic mismatch, bilayer flexibility (saturated vs. unsaturated lipid tails) and surface charge (different headgroups). Resonance Raman Spectroscopy will be used for its sensitivity towards structural changes and flexibility of use under a variety of environments. Time-Resolved Visible Spectroscopy, helpful in distinguishing photo-intermediates will be used to investigate photocycle time constants.
1.10 Thesis Outline

Chapter 1 provides some general information on microbial rhodopsins and an explanation on the structure and function of bacteriorhodopsin and its homolog, proteorhodopsin. In chapter 2 information on sample preparation is provided along with an explanation on Raman and time-resolved spectroscopy experiments. The results and discussion will be presented in Chapters 3-7. In chapter 3, the influence of P/L ratio on the photochemistry and structure of PR will be discussed, supplemented with results from Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy. Chapter 4 will deal with results from PR reconstituted in membranes with different flexibility and fluidity. Chapters 5 and 6 will deal with the effects from membranes with different thickness and surface charge, respectively. Chapter 7 will present results for PR in synthetic matrices (polymersomes). Conclusions and important features from the results will be presented in chapter 8.
Chapter 2
Materials and Methods
2.1 Materials

All reagents used for bacterial cell cultures and buffers came from Fisher Scientific. All-
trans retinal, lysozyme, and DNase I used for cell lysis came from Sigma-Aldrich. IPTG, used for
induction, was from Fisher. Ni\(^{2+}\)-NTA resin was purchased from Qiagen and centrifugal filters for
concentrating protein from Amicon Ultra, Millipore. All lipids used in experiments were purchased
from Avanti Polar Lipids. Isotopically labeled compounds were purchased from Cambridge
Isotopes Laboratories.

Some protein samples (proteo-polymersomes) used in this work have been prepared in
collaboration with the group of Hongjun Liang from the Colorado School of Mines, United States
of America. The expression of wild-type PR was performed in our laboratory. The plasmid used
to replicate the wild-type PR gene was pKJ900-PR+oxygenase and *E. coli* BL21 Codonplus RIL
was used for gene expression with the wild-type PR gene containing a C-terminal 6-His tag. These
were provided by our collaborator Kwang-Hwan (Kevin) Jung from Sogang University, Seoul,
South Korea.

2.2 Sample Preparation

2.2.1 Protein Expression

Wild-type PR-His\(_6\) was expressed in *E. coli* BL21-Codonplus-RIL. Transformed *E. coli*
cells were grown in M9 minimal medium (6 g Na\(_2\)HPO\(_4\), 3 g KH\(_2\)PO\(_4\), 1 g NH\(_4\)Cl, 0.5 g NaCl per
liter) with 1 mM MgSO\(_4\), 0.1 mM CaCl\(_2\) and 2000x trace metal solution containing FeCl\(_3\), FeSO\(_4\),
ZnSO\(_4\), CuSO\(_4\), H\(_3\)BO\(_3\), MnSO\(_4\), Na\(_2\)MoO\(_4\) and HCl. Starter cultures were supplemented with 20%
(w/v) glucose (final concentration 4 g/L), 100x BME vitamins solution, Ampicillin (100 μg/mL)
and Chloramphenicol (50 μg/mL) before being shaken in 15 ml test-tubes at 30°C and 240-275 revolutions per minute (rpm) in an incubation shaker. After 24 hrs, cells were transferred to 250 mL baffled flasks with a starting OD$_{600nm}$ of 0.1. After about 15 hours cells were transferred to a bigger culture flask (975 mL) and overexpression was induced by addition of 1 mM (final concentration) IPTG at an OD$_{600nm}$ of approximately 0.4. All-trans retinal (5-10 μM) was added simultaneously. After induction, cells were cultivated for 21 hours at 275 rpm. Cells were then harvested via centrifugation (5000 rpm, 4°C, 15 minutes) in a Beckman Coulter Centrifuge with a GA-6 fixed-angle rotor before being resuspended in lysis buffer containing 150 mM NaCl, 50 mM Tris, 1 mM MgCl$_2$, 2μg/mL DNAse I and 0.2 mg/mL lysozyme. Cells were then shaken on a IKA VIBRAX vibrator at 400 rpm, in the dark at room temperature for 3 hours and then stored at -20°C for later use.

### 2.2.2 Cell Disruption and Protein Purification

The following buffers were used during purification and reconstitution of PR:

A 1 % (v/v) Triton X-100, 5 mM Tris, pH 7.5 (solubilisation buffer)

B 1 % (v/v) Triton X-100, 3 M NaCl, 0.5 M Tris, 50 mM Imidazole, pH 8 (10× binding buffer)

C 1 % (v/v) Triton X-100, 0.3 M NaCl, 0.05 M Tris, 30-40 mM Imidazole, pH 8 (washing buffer)

D 0.05 % (w/v) DM, 0.5 M Imidazole, 0.3 M NaCl, 0.05 M Tris, pH 8 (elution buffer)

E 0.05 % (w/v) DM, 10 mM Tris, 5 mM NaCl, pH 8 (reconstitution buffer)

F 25 mM CHES, 10 mM NaCl, pH 9 (NMR buffer)

G 10 mM Tris, 5 mM NaCl, pH 8 (reconstitution buffer)
Resuspended *E. coli* cells were disrupted using a Fisher Model 500 Sonic Dismembrator (accessorized with a macrotip) using the pulse mode program 5 × (30 seconds on, 30 seconds off) with 25% power. The cells were divided into 30 mL aliquots using plastic beakers and sequentially sonicated four times each on ice. PR containing membranes were then sedimented (38,000 rpm, 4°C, 1 h) using a Thermo Scientific Sorvall WX Ultra Series centrifuge (F50L-8X39 rotor) and solubilized in buffer A at 4°C overnight in the dark to avoid photobleaching. After another centrifugation step (38,000 rpm, 4°C, 1 h), the supernatant containing solubilized PR was loaded into a Ni^{2+}-NTA-agarose-column mixed with buffer B to improve protein binding. Before binding with PR, the Ni^{2+}-NTA resin was washed with 150 mM NaCl to remove ethanol, a denaturant. During binding, the histidine-tagged proteins bind to Ni^{2+} metal ions forming a chelate complex. Molecules that un-specifically and loosely bind to the nickel-matrix can then be removed by extensive washing using buffer C which contains imidazole, a competitive binder. To thoroughly remove cytochrome and other integral membrane proteins, the sample must be washed 3-4 times with washing buffer containing 1% (v/v) TX-100 and then 2-3 times with 0.05% (w/v) DM. In each run, the resin solution is stirred for 30 mins on ice in the dark, replenished with fresh washing buffer using a 500 ml Nalgene sterile filter. After purification, the sample is eluted from the column using buffer D, with a high concentration of imidazole to substitute the sample in the Ni^{2+}-NTA-matrix. The resin is stirred for ten minutes and then centrifuged (4,500 rpm, 10 minutes, 4°C) using a Beckman Coulter Centrifuge (GA-6 fixed-angle rotor) to collect the supernatant. This is done repetitively until the resin is completely bleached. Protein purity can be estimated by the absorption ratio of the chromophore and aromatic amino acids at ~520 nm and 280 nm (expected to be ~0.5 in pure preparations), respectively, using a UV-VIS spectrophotometer (see Figure 2.1).
To lower the imidazole concentration, the purified sample in filter units (Amicon, 10 kDa cutoff, 15 mL) is concentrated from 15 mL to 1.5 mL by centrifugation (4,000 rpm, 2×15 mins) using a swinging bucket rotor (Surespin 630). Four refillings with buffer E is sufficient to lower the imidazole concentration below 0.1 mM. Imidazole is thoroughly removed because it has an absorption maximum at ~320 nm and can interfere with the purity assays and reconstitution efficiency. The total amount of PR can be determined from measuring the absorbance of 1 mL at ~520 nm, knowing its molar extinction coefficient (~0.6 mg corresponds to 1 OD unit in 1 mL).

Figure 2.1. Appearance of wild-type GPR and its static UV-Vis spectrum after purification. The free retinal peak is located at ~370 nm, retinylidene peak at ~530 nm, and the aromatic sidechains reporting on total protein content at ~280 nm. The static UV –Vis spectrum was collected using a Cary 50 spectrophotometer.

2.2.3 Reconstitution into Lipids

During reconstitution, solubilized PR is mixed with lipids at an appropriate protein to lipid (w/w) ratio. Standard procedures were carried out for preparing liposomes. Lipids in chloroform were first placed in 25 mL round-bottomed flasks and homogenized by vigorously vortexing for
thirty minutes. The chloroform was then removed by evaporation under vacuum to yield a lipid film. A high concentration of lipids was obtained by rehydrating with buffer G and vigorously shaking and mixing. Once liposomes were ready, the tertiary mixture made up of protein, lipid and detergent was stirred for six hours before adding detergent-adsorbing Bio-beads (SM2, Bio-Rad). After twenty-four hours, beads were completely rinsed with buffer F and proteoliposomes collected using a syringe with 27 ½ G needle. The proteoliposomes were then pelleted by centrifugation (38,000 rpm, 4°C, 1 h) using a Thermo Scientific Sorvall WX Ultra Series centrifuge (F50L-8X39 rotor), either being left in pellet form for Raman spectroscopy experiments or resuspended in buffer F for time-resolved visible spectroscopy.

2.2.4 Sample Preparation for FTIR Measurements

The samples were first centrifuged under low speed to remove any salts and then hydrated with milli-Q water. A 0.1 mg aliquot of the sample was deposited on a CaF$_2$ window and dried under mild vacuum before being compressed between two windows with appropriate spacers.

2.2.5 Sample Preparation for Raman and Visible Spectroscopy Measurements

For time-resolved spectroscopy, hydrated proteoliposome samples each with approximately 1-1.5 mg of protein were placed in 1 cm path length, 1.5 mL cuvettes. Samples were diluted with reconstitution buffer to decrease scattering and turbidity and increase the signal to noise ratio. For Raman spectroscopy measurements, samples were pelleted in 1.5 mL eppendorf tubes under low speed centrifugation. Pelleted samples were then transferred using glass pipettes to special quartz cuvettes. Each sample for Raman spectroscopy contained around 2-4 mg of PR.
2.3 Biophysical Techniques

2.3.1 Time-Resolved Visible Spectroscopy

Absorbance of visible light occurs when electrons in a molecule are excited to a higher electronic quantum level. Visible spectroscopy reports on the distribution of electrons in the molecule of interest. Rhodopsins are ideal for visible spectroscopy measurements because they contain retinal, which can absorb green light. Time-resolved visible spectroscopy can provide high signal-to-noise and time resolution data used for spectral and kinetic characterization of rhodopsin photointermediates. Visible spectroscopy measurements are easy to carry out under different environmental factors such as temperature and pH and can be used to examine biochemically or genetically modified proteins (Lozier, 1982). The complication with this technique comes when analyzing kinetics from different wavelengths. It is not unusual for a measured absorbance change to represent a mixture of several different intermediates. Since it is unlikely to find at any time a signal due to just one intermediate, transient states can be isolated only mathematically (Xie et al., 1987; Dioumaev, 1997). For PR, the absorption change at 420 nm follows the protonation state of the retinal Schiff base (M intermediate), at 500 nm follows the disappearance and reappearance of the initial state (ground state), at 600 nm tracks the re-isomerization of retinal and deprotonation of primary proton acceptor, Asp-97 (N and O intermediates). The photocycle of PR can be characterized through time-constants which are extracted from global multi-exponential fitting of these traces. For this project, data from time-resolved visible spectroscopy were normalized to have the same maximum/minimum amplitude value for a given wavelength trace within each figure.
2.3.1.1 Components of a Time-Resolved Visible Spectrometer

Time-resolved visible spectroscopy measurements were carried out using a custom-built flash-photolysis apparatus (Figure 2.2 and A1 in the Appendix). Photocycle of PR was started by exciting its retinal chromophore with the second harmonics of a Nd-YAG (yttrium/aluminum garnet) pulsed laser (Continuum Minilite II, 7 ns pulse duration). The monochromatic light was obtained from a quartz tungsten 250 W halogen lamp and a wavelength was chosen using two aligned monochromators (before and after the sample). An Oriel photomultiplier was used to convert the transmitted light into an electrical current with any stray light being discarded by the notch filter. The signal was amplified with a 350 MHz wide-bandwidth amplifier and sampled by a Gage analog-to-digital converter. Several hundreds of traces were averaged, each containing up to 8 million points. Traces were converted into a quasilogarithmic time-scale using homemade software and multi-exponentially fit using FITEXP (Dioumaev, 1997). 1000 averages were taken for the 500 and 600 nm absorption traces but 8,000-10,000 averages for the 420 nm trace, mainly due to higher scattering and lower light output of the source at this wavelength.

Figure 2.2. Schematic representation of a time-resolved visible spectrometer. Main components are labelled.
2.3.2 Raman Spectroscopy

Scattering occurs when incident light is deflected from the direction it was propagating. It is a result of interactions of the electric field (from UV, visible or near IR light) with the electrons of a compound. Such interactions produce oscillating electric dipole moments in the molecule of interest and are responsible for elastic and inelastic scattering. Elastic (Rayleigh) scattering occurs when the scattered light has the same frequency or wavelength as the incident light. On the other hand, inelastic scattering occurs when the scattered light has a lower (Stokes) or higher frequency (anti-Stokes) (Figure 2.3). In Raman spectroscopy, inelastically scattered radiation is collected at 90° or 180° to the monochromatic light source with the frequency difference between the scattered and incident light used to produce a vibrational Raman spectrum.

![Energy level diagram for Raman scattering (Stokes and anti-Stokes).](image)

A Raman spectrum provides information on the energies of molecular normal modes of vibration. The advantages of Raman spectroscopy include its use to study aqueous solutions due to the weak interference from water and its ability to provide information on the protein backbone and environment of side chains. A Raman band is generated when there is an associated change in
polarizability near the vibrating nuclei. Two important features studied are the shape and shifts of bands. Band shifts are a result of changes in the bond order (twist, stretch, or charge nearby) or changes in hydrogen bonding while band broadening or narrowing is a consequence of a bond existing in different conformations or environments. Vibrations of multiply-bonded groups (C=N, C=C, C=O) generally produce more intense bands than vibrations from singly-bonded groups (C-C, C-H). Important vibrational regions in the Raman spectra of rhodopsins include the Amide I, II and retinal regions.

Amide I Band

The amide I vibrational band is caused by peptide carbonyl stretching (C=O of –CONH-) and in-plane N-H bending (see Figure 2.4). The position of the Amide I band can be used to determine protein secondary structure elements such as α-helix, 3_10 helix, and β-sheet. These structures are formed through hydrogen bonding between carbonyl oxygens and amide hydrogen atoms. In Raman spectra, the bands from α-helix, antiparallel β-sheet and unordered structures appear at 1650-1657 cm\(^{-1}\), 1665-1680 cm\(^{-1}\) and 1666-1668 cm\(^{-1}\) respectively.

Amide II Band

The Amide II vibrational band, found between 1510 and 1580 cm\(^{-1}\) is caused by N-H bending and C-N stretching of the peptide backbone (–CONH-). It is very sensitive to hydrogen/deuterium exchange and is used to check for solvent accessibility of the protein core and to distinguish between unordered and helical conformations.
Figure 2.4. Amide I and II vibrational modes of the peptide backbone.

Retinal Region

Vibrations of individual bonds from retinal are represented by bands between 800-1700 cm\(^{-1}\) (see Figure 2.5 and Table 2.1). These mainly come from changes to important groups in retinal (hydrogen out-of-plane wags, C-C stretches, methyl rocks, C-H and N-H in-plane bends, C=C and C=N stretches) with some bands used to signify the presence of all-\textit{trans} and 13-\textit{cis} retinal. Importantly, Raman scattering from retinal is much stronger than that from the rest of the protein, due to resonance enhancement.

2.3.2.1 Components of a Raman Spectrometer

The FT-Raman mode was used to collect Raman spectra using the Bruker FRA 106/s accessory to the IFS66vs spectrometer (Figure 2.6 and A2 in the Appendix). A concentrated protein sample was excited at 1024 nm using a Nd:YAG laser with 2 cm\(^{-1}\) resolution. The energy
provided was both low enough to prevent excess fluorescence or initiation of the photocycle, and high enough to prevent significant fall-off in scattering intensity (Hallmark et al., 1988). A key component to Raman experiments is the interferometer with a beamsplitter and detector used to analyze Stokes-shifted scattering. The time of measurement varied between 14 and 24 hours (15,770 and 27,034 scans) depending on the signal to noise ratio. Data from Raman spectroscopy were normalized according to the C=C (ethylenic) stretch amplitude.

Figure 2.5. A resonance Raman spectrum for retinal from a color visual pigment. Spectrum shows localized hydrogen out of plane (HOOP) wags and Schiff base stretch and delocalized fingerprint (C-C) and ethylenic (C=C) stretches. Hydrogen/deuteronium exchange can provide additional information on the environment and strength of hydrogen bonding of the Schiff base (C=N) (Kochendoerfer et al., 1999). Image reprinted with permission from Elsevier Science, Copyright (1999).
Table 2.1. Frequencies and assignments for retinal from $^{15}$N labelled, wild-type PR (2to1 P/L, w/w ratio) (Dioumaev et al., 2002; Krebs et al., 2003; Kralj et al., 2008).

<table>
<thead>
<tr>
<th>Frequency (cm$^{-1}$)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>807</td>
<td>Hydrogen out-of-plane (HOOP) wag of C$_{14}$ and lysine (13-$cis$-15-$syn$)</td>
</tr>
<tr>
<td>960</td>
<td>Hydrogen out-of-plane (HOOP) wag</td>
</tr>
<tr>
<td>1007</td>
<td>In-plane C$_{19}$-CH$<em>3$ and C$</em>{20}$-CH$_3$ methyl rocks</td>
</tr>
<tr>
<td>1040</td>
<td>Out-of-plane C$_{19}$-CH$<em>3$ and C$</em>{20}$-CH$_3$ methyl rocks</td>
</tr>
<tr>
<td>1165</td>
<td>C-C stretches and in-plane hydrogen rocks</td>
</tr>
<tr>
<td>1171</td>
<td>C-C stretches (all-$trans$)</td>
</tr>
<tr>
<td>1185</td>
<td>C-C stretches (13-$cis$)</td>
</tr>
<tr>
<td>1200</td>
<td>C-C stretches (all-$trans$)</td>
</tr>
<tr>
<td>1235, 1242, 1254</td>
<td>Retinylidene-lysine $^{15}$N-C-H rocks</td>
</tr>
<tr>
<td>1274</td>
<td>C=C stretch and in-plane hydrogen rocks</td>
</tr>
<tr>
<td>1301, 1319, 1337, 1381</td>
<td>In-plane hydrogen rocks</td>
</tr>
<tr>
<td>1454</td>
<td>C$_{19}$-CH$<em>3$ and C$</em>{20}$-CH$_3$ deformations</td>
</tr>
<tr>
<td>1536</td>
<td>C=C stretches</td>
</tr>
<tr>
<td>1579, 1584</td>
<td>C=C stretches</td>
</tr>
<tr>
<td>1636</td>
<td>C$_{15}$=$^{15}$N-H (Schiff base) stretch</td>
</tr>
</tbody>
</table>
Figure 2.6. Schematic representation of a Raman spectrometer. Main components are labelled for the Bruker FRA 106/s accessory for Raman spectroscopy. From Bruker users’ manual.
Chapter 3
Effect of Packing Density (P/L ratio) on Proteorhodopsin Dynamics
3.1 Intermolecular and Intramolecular Changes from Varying P/L Ratio

Protein to lipid (P/L) ratio does not affect the overall secondary structure of PR, reconstituted in DMPC/DMPA 9:1 mixture (Figure 3.1). The FTIR static absorption spectra were taken at four P/L ratios (1:1, 2:1, 4:1, low lipid <8:1) and show no changes in the positions of the Amide I and II bands, representing C=O and C-^15N- H stretches of the protein backbone. Since the structures of these groups do not change, hydrogen bonding between the two maintains the general alpha helical structure of PR, consistent with the observed position of the Amide I at 1657 cm⁻¹. It can be argued that the P/L ratio may cause more structural changes to lipids than to the protein. For example, by looking at the changes in position of lipid bands in Figure 3.1, the amount of protein relative to lipid has a greater effect on the structure of the lipid’s acyl chains and its CH₂ groups (~1475 cm⁻¹) than on the lipid headgroups producing vibrational bands of R(C=O)OR (~1750 cm⁻¹), PO₂⁻ (~1228 cm⁻¹ and 1085 cm⁻¹), COPOC (~1050 cm⁻¹) and N⁺(CH₃)₃ (975 cm⁻¹) in DMPC (Tamm and Tatulian, 1997).

One-dimensional NMR spectra of ^15N-labelled PR show significant intra-molecular lipid-induced changes of dynamics of important amino acids (Figure 3.2). The major band around 120 ppm in the 1D spectra represents backbone amide for most of amino acid types, with the minor bands on the left representing proline residues, and minor bands on the right representing glycine and asparagine residues (Wang and Jardetzky, 2002). The major and minor bands in lower protein to lipid ratio samples (1:1 and 2:1 P/L) are well structured and highly resolved, but become broader with higher P/L content (4:1 and especially 8:1 P/L). These NMR results may correlate with proteorhodopsin’s ability to oligomerize into 2D crystals at low lipid content (Shastri et al., 2007; Klyszejko et al., 2008), restraining protein dynamics. A varying degree of oligomerization has
been observed in detergent-solubilized PR as well (Hoffmann et al., 2010). Thus, 1D NMR results show that some minimal amount of lipid is required to maintain proper protein dynamics.

Figure 3.1. Static FTIR spectra of wild-type PR at different protein to lipid ratios. PR was reconstituted in DMPC/DMPA 9:1 mixture with four different P/L ratios: 1:1 (red), 2:1(green), 4:1(blue), and high P/L ratio above 4:1 but <<8:1(pink). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). P/L are determined by comparing ratio of the integrated intensity of the lipid ester carbonyl band near 1740 cm\(^{-1}\) with the protein amide I band near 1650 cm\(^{-1}\) (daCosta and Baenziger, 2002). Spectrum of the pure lipid is given as a reference.
3.2 Conformational Changes in Retinal from Membranes with Different P/L Ratios

Raman spectra, which are dominated by retinal bands due to resonance enhancement, show some minor structural changes in retinal between high and low protein to lipid ratio samples. Changes take place in three regions: 800-1000 cm\(^{-1}\), 1160-1200 cm\(^{-1}\), and 1300-1385 cm\(^{-1}\) (Figure 3.3). The fingerprint region between 1160 and 1200 cm\(^{-1}\) representing C-C stretches reports on the isomeric state of the chromophore. Since the intensity of the 1199 cm\(^{-1}\) band is much greater than
the 1184 cm\(^{-1}\) band, retinal is mainly in the all-\textit{trans} configuration in dark-adapted PR, similar to what was observed by other groups in different PR preparations (Dioumaev et al., 2002; Kralj et al., 2008; Krebs et al., 2003). This is unlike BR, where a significant amount of 13-\textit{cis} retinal is present, as indicated by the large amplitude of C-C stretch band at 1183 cm\(^{-1}\) and a downshift of another C-C stretch from 1201 cm\(^{-1}\) to 1167 cm\(^{-1}\) (Smith et al., 1987a). This band is upshifted two units to 1165 cm\(^{-1}\) in 9:1 P/L from 1163 cm\(^{-1}\) in 1:1 P/L suggesting some fine changes to the retinal skeleton structure.

Vibrations from hydrogen out-of-plane (HOOP) wags are shown in the 800-1000 cm\(^{-1}\) region of the spectrum. The 9:1 P/L sample contains two more HOOP wag bands at 719 cm\(^{-1}\) and 898 cm\(^{-1}\). This increase in number of hydrogen out-of-plane (HOOP) wags in 9:1 P/L might suggest a greater degree of twisting in retinal (Smith et al., 1987a).

The 1300-1385 cm\(^{-1}\) region represents in-plane hydrogen rocks from retinal. The vibrational band found at 1319 cm\(^{-1}\) in the 1:1 P/L sample is shifted to 1317/1321 cm\(^{-1}\) in the 9:1 P/L PR sample and is much broader. This is a consequence of these C-H bonds existing in different conformations or environments, which may also correlate with a different degree of retinal twist observed in the HOOP region.

Other structural changes include an additional band at 1608 cm\(^{-1}\) possibly coming from deprotonated Schiff base (C=N) stretches (Argade et al., 1981). This band is rarely seen in PR but is found in other rhodopsins such as BR, when the Schiff base deprotonation is induced by light. Its presence may be a result of thermal isomerization of retinal in the dark (leading to deprotonation) but this is an unlikely possibility considering it is found only in the 1:1 P/L sample. A more probable explanation is that PR in the 1:1 P/L sample has a weaker hydrogen binding strength for its Schiff base due to its lower pK\(_a\) value. An alternative explanation for the origin of
this band is an isolated C=C stretch of retinal due to steric interactions forcing a different degree of retinal twist around a C-C bond (Smith et al., 1987b). Additional changes occur in the 1451 cm\(^{-1}\) methyl band, which is shifted to 1453 cm\(^{-1}\) in the 9:1 P/L sample and is significantly narrower due to deformations in one or more of the methyl groups (CH\(_3\)) of retinal. The minor C=C stretch band at 1574 cm\(^{-1}\) in the 1:1 P/L sample is shifted to 1577/81 cm\(^{-1}\) in the 9:1 sample. The major C=C (ethylenic) stretch band coming from the remaining C=C bonds is located at 1538 cm\(^{-1}\) in both samples, but was found to shift by one or two units in 2:1 and 4:1 P/L samples. The 4:1 P/L sample (see appendix, Figure A3) has a broader ethylenic stretch band, implying a shift in the proportion of all-trans, 15-anti to 13-cis, 15-syn species in the dark-adapted sample, but such change in isomeric composition is not confirmed by the increase in the 1185 cm\(^{-1}\) fingerprint band of 13-cis retinal, so the source of this broadening is not clear. There is also an additional vibrational band at 973 cm\(^{-1}\) possibly coming from a hydrogen wag. A final difference comes from the position and shape of the protonated C=N (retinal Schiff base) band. In the 1:1 P/L sample, it is represented by a double band located at 1634 and 1640 cm\(^{-1}\) but is much narrower in the 9:1 P/L sample centered around 1644 cm\(^{-1}\). However, the exact position of this band is not accurate since it overlaps with the weak Amide I band of the protein (backbone C=O), and a much lower amount of protein and lipid was used to get the vibrational spectra for 9:1 and 4:1 P/L samples.
3.3 Additional Insights from $^{15}$N and $^{15}$N, $^{13}$C labelled PR

Additional $^{13}$C labelling of PR (carbon source, 2-glycerol) causes significant changes in the protonated C= N stretch and the retinylidene-lysine $^{15}$N-$^{13}$C-H rock bands (Figure 3.4). Since these bonds are directly connected to lysine-231 from the protein the $^{13}$C atom causes a downshift of the lysine band from 1254 to 1250 cm$^{-1}$. Since the 1235 cm$^{-1}$ band stays unchanged and the 1242 cm$^{-1}$ band is too small, these selective, isotope labelling experiments suggest that the band at 1250/1254 cm$^{-1}$ is mainly responsible for lysine rocks in PR. Another important observation was that the protonated C=N band which might be represented as a double band (1637/43 cm$^{-1}$) in $^{15}$N,
\(^{13}\)C PR did not change substantially when PR was labelled with the heavier \(^{13}\)C atom, as expected from the fact that the carbon atom of this bond belongs to retinal, which is not labelled isotopically. A final significant change to mention occurred in the methyl bands, with the in-plane rock bands shifting from 1007 to 1005 cm\(^{-1}\), and the deformation bands shifting from 1454 to 1450 cm\(^{-1}\) in \(^{15}\)N, \(^{13}\)C labelled PR. These vibrations mainly come from methyl groups from C\(_{19}\) and C\(_{20}\) which have been found to closely interact with important spectral tuning residues in PR (Kralj et al., 2008), but it is possible that the observed shifts come from changed contributions from amino acid sidechain methyl groups upon labeling. These changes provide additional evidence for the utility of Raman spectroscopy for PR studies, supporting the sensitivity of these groups to slight changes in the protein structure and isotopic composition.

Figure 3.4. Resonance Raman spectra for \(^{15}\)N and \(^{15}\)N, \(^{13}\)C labelled, wild-type PR reconstituted in DMPC/DMPA 9:1 lipid mixtures. PR was reconstituted in a 2:1 P/L (w/w) ratio and placed in a buffer containing 25 mM CHES and 10 mM NaCl at pH 9 and room temperature.
3.4 Kinetic Changes in the Photocycle of PR in Membranes with Different P/L Ratios

Global multi-exponential analysis of single-wavelength kinetic traces taken at characteristic wavelength reveals four statistically valid time constants corresponding to transitions between mixtures of intermediates. The transition from the K intermediate to a mixture of K/M intermediates is represented by the $\tau_1$ time constant. The $\tau_2$ time constant corresponds to the transition from the M intermediate to a mixture of N/O intermediates. The final two time constants, $\tau_3$ and $\tau_4$ represent the decay of N/O to the ground state (pR), involving reaction rates between N and O, O and pR.

The P/L ratio has some significant effects on the photocycle of PR. First, increasing the P/L content leads to a faster transition from K to M and a slower transition from N/O to pR (Table 3.1). Equilibrium between K and M is shifted increasingly towards M at higher P/L samples with less accumulation of N/O. Second, equilibration between M, N, and O intermediates becomes greater with the increase of the P/L ratio, with all signals decaying on the same timescale in very high P/L samples (Figure 3.5). This is the outcome of a faster first half of the photocycle (see $\tau_1$ and $\tau_2$) combined with slower proton transfer in the second half of the photocycle (see $\tau_3$ and $\tau_4$) in higher P/L ratio samples. However, the time to complete the photocycle is approximately the same. These changes might be influenced by the Schiff base properties, whose vibrational band is upshifted in the 9:1 P/L sample. This is supported by the presence of the Schiff base lysine vibrational band (1254 cm$^{-1}$) only in the 1:1 P/L sample. The results from this work are consistent with those gathered on the photocycle of wild-type PR solubilized in detergent molecules DM and diC7PC (Stehle et al., 2012), with the latter being the shorter molecule. In this study a greater accumulation of the late red-shifted intermediates (N/O) of PR was found in DM as opposed to the equilibrated mixture of M$_2$, N, and O states in diC7PC. Changes in the protein dynamics can be
caused by the physical characteristics of lipids/detergents (charge, chain length) and their ability to form membrane bilayers/micelles that exert different lateral pressures. Thus, the lipid membranes from this work and the detergent micelles from Stehle et al. (2012) might exert similar pressures on proteorhodopsin.

Table 3.1. Time constants (in milliseconds) for the kinetic components representing transitions between photo-intermediate mixtures of $^{15}$N labelled, wild-type PR in different P/L ratios. Errors associated with time constants are a measure of deviation of the absorption traces from their multi-exponential fits.

<table>
<thead>
<tr>
<th>P/L (w:w)</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$\tau_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.906 ± 0.086</td>
<td>4.02 ± 0.20</td>
<td>34.4 ± 1.49</td>
<td>302 ± 18.9</td>
</tr>
<tr>
<td>2:1</td>
<td>0.853 ± 0.062</td>
<td>3.64 ± 0.16</td>
<td>29.4 ± 0.96</td>
<td>241 ± 10.4</td>
</tr>
<tr>
<td>4:1</td>
<td>0.863 ± 0.227</td>
<td>2.91 ± 0.49</td>
<td>54.4 ± 4.22</td>
<td>405 ± 33.7</td>
</tr>
<tr>
<td>8:1</td>
<td>0.364 ± 0.140</td>
<td>2.57 ± 0.40</td>
<td>44.4 ± 3.21</td>
<td>303 ± 17.7</td>
</tr>
<tr>
<td>16:1</td>
<td>0.323 ± 0.178</td>
<td>2.62 ± 0.64</td>
<td>45.8 ± 3.93</td>
<td>338 ± 22.2</td>
</tr>
</tbody>
</table>
Figure 3.5. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR for different P/L (w/w) ratios. PR was reconstituted in DMPC/DMPA 9:1 lipid mixtures and photointermediates were followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines.
3.5 Summary of Effects of Protein Packing Density on Proteorhodopsin Dynamics

Even though protein density causes hardly any changes in the overall structure of the protein core of PR, as judged from FTIR, some changes occur to specific residues dynamics (e.g., proline, glycine, asparagine) and others found throughout the protein, according to solid-state NMR results. Protein crowding leads to much more significant structural and photochemical changes in the photocycle of PR. These might originate from the Schiff base, but may come from the all-trans retinal, which shows increased twist (distortions) in higher P/L samples. Schiff base hydrogen binding strength may be lower in less crowded protein samples and in other lipid samples (see below). These changes affect the photocycle, with the time constants of the first and second half being almost inversely related to protein packing density. This leads to a greater accumulation of the early intermediates (K and M) and lower accumulation of the late intermediates (N and O). These results are very similar to those in detergent micelles suggesting that densely packed membranes and short-chain detergent micelles exert similar pressures on proteorhodopsin.
Chapter 4
Effect of Membrane Flexibility and Fluidity on Proteorhodopsin Dynamics
Two typical phospholipids, POPC and DOPC, were used to investigate the influence of bilayer flexibility on the PR photocycle and structure of its chromophore, retinal. Both lipids are neutrally charged and differ only in the number of double bonds on their acyl chain. POPC has one saturated chain (16:0) and one unsaturated chain (18:1) whereas DOPC has two unsaturated chains (18:1), making membranes made of POPC somewhat more rigid. Additionally, two phospholipids with shorter fatty acid tails were used, either fully saturated (DMPC, 14:0) or having two unsaturated chains (14:1), with a different phase state at room temperature.

4.1 Conformational Changes in Retinal from Membranes with DOPC and POPC

Changes in bilayer flexibility cause major changes in the 800-1000 cm\(^{-1}\) region representing HOOP wags (Figure 4.1). There are several more HOOP wag bands (808 and 972 cm\(^{-1}\)) observed in the DOPC sample. The 808 cm\(^{-1}\) band is assigned to hydrogen wags from C\(_{14}\) and the lysine residue (Lys231) covalently bound to 13-cis-15-syn retinal whereas the 972 cm\(^{-1}\) band is assigned to hydrogen wags from all-\(\text{trans}\) retinal, and may also come from a protein group (Argade et al., 1981). This band might indicate the greater degree of retinal twisting seen in DOPC with some possible contribution from close-by residues. Another major change occurs in the methyl groups of retinal. The broad methyl deformation band at 1448 cm\(^{-1}\) in POPC is shifted to 1457 cm\(^{-1}\) in DOPC, becoming narrower. There also seems to be a band at 1038/46 cm\(^{-1}\) only found in the DOPC sample. The 1038 cm\(^{-1}\) band is assigned to out-of-plane methyl rocks of C\(_{19}\) of 13-\(\text{cis}\) retinal, and the 1046 cm\(^{-1}\) band may come from C-N stretch of the Schiff base bound residue, Lys231 (Smith et al., 1987a; Smith et al., 1987b). A final important change occurs to the protonated Schiff base (C=N) whose band is broader and upshifted three units to 1641 cm\(^{-1}\) in
DOPC. These changes to the Schiff base stretch mode and conformation may be due to changed conformation of Lys-231, whose bands at 808 cm\(^{-1}\) and 1046 cm\(^{-1}\) are only found in DOPC.

Minor changes occur in the 1300-1385 cm\(^{-1}\) region representing in-plane hydrogen rock bands. The band at 1318 cm\(^{-1}\) in POPC is shifted to 1321 cm\(^{-1}\) in DOPC. The 1380 cm\(^{-1}\) band in POPC is represented as a double band at 1377 and 1383 cm\(^{-1}\) in DOPC. The amide I band seems to be slightly shifted from 1655 cm\(^{-1}\) in DOPC to 1653 cm\(^{-1}\) in POPC suggesting some changes to the protein backbone.

Figure 4.1. Resonance Raman spectra for \(^{15}\)N labelled, wild-type PR reconstituted in DOPC or POPC lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and was measured in a buffer containing 25 mM CHES and 10 mM NaCl (pH 9, room temperature).
4.2 Kinetic Changes in the Photocycle in Membranes with DOPC and POPC & DMPC and 14:1 PC

Even though bilayer flexibility causes significant structural changes in retinal, these changes are not felt in the photocycle of PR. The time constants for PR in DOPC and POPC are not much different. The first half of the photocycle (K to M) is a bit slower in POPC but faster in the second half (Table 4.1). Both samples have very similar accumulation of M, N, and O intermediates (Figure 4.2). The results suggest that the photocycle of PR might more strongly be affected by other properties of its membrane such as its fluidity.

Table 4.1. Time constants (in milliseconds) for the kinetic components representing transitions between photo-intermediate mixtures of $^{15}$N labelled, wild-type PR in DOPC and POPC.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$\tau_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>0.323 ± 0.029</td>
<td>3.07 ± 0.12</td>
<td>20.7 ± 0.853</td>
<td>250 ± 19.3</td>
</tr>
<tr>
<td>POPC</td>
<td>0.367 ± 0.038</td>
<td>2.49 ± 0.101</td>
<td>17.7 ± 0.81</td>
<td>211 ± 18.5</td>
</tr>
</tbody>
</table>
Figure 4.2. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR reconstituted into DOPC and POPC lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and photointermediates followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines.
To investigate the combined effect of membrane flexibility and fluidity on kinetics of the PR photocycle the lipids, DMPC (14:0) and 14:1 PC were used. At room temperature, DMPC is in the gel phase while 14:1 PC is in the liquid phase. The photocycle of PR is faster in 14:1 PC compared to DMPC (see Table 4.2 and Figure 4.3). The K/M equilibrium mixture is shifted more to M in 14:1 PC. There is also a greater accumulation of M and N/O in 14:1 PC because of a faster rate of K and M decay (faster $\tau_1$ and $\tau_2$ constants). Compared to membrane bilayers, the photocycle of PR in nanodiscs (DMPC) is very similar, suggesting that protein dynamics are not strongly influenced by membrane assembly (Roos et al., 2012; Mors et al., 2013). These results show that both the phase state and rigidity are important characteristics of membranes for PR to function efficiently as a proton transporter.

Table 4.2. Time constants (in milliseconds) for the kinetic components representing transitions between photo-intermediate mixtures of $^{15}$N labelled, wild-type PR in DMPC and 14:1 PC.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$\tau_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>0.606 ± 0.100</td>
<td>4.00 ± 0.416</td>
<td>32.80 ± 3.34</td>
<td>321 ± 55.3</td>
</tr>
<tr>
<td>14:1 PC</td>
<td>0.290 ± 0.021</td>
<td>2.64 ± 0.096</td>
<td>14.6 ± 0.66</td>
<td>216 ± 19.6</td>
</tr>
</tbody>
</table>
Figure 4.3. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR reconstituted into 14:1 PC and DMPC lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and photointermediates were followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines.
4.3 Summary of Membrane Flexibility and Fluidity Effects on Proteorhodopsin Dynamics

Decreasing flexibility leads to structural changes in retinal in two main regions: methyl groups and the Schiff base. The methyl groups are most significantly affected, with changes in both their rocking mode and surrounding environment. The Schiff base is also affected in conformation and bond order, being affected by its bound lysine residue (Lys231). These changes have minimal if not no effect on the PR photocycle and the relative amounts of K, M, N, and O intermediates. Having said this, increasing membrane fluidity leads to a faster photocycle with greater accumulation of M, N and O intermediates. In conclusion, having a fluid membrane with greater number of unsaturated lipid tails leads to more efficient proton transport as a result of faster photocycle turnover.
Chapter 5

Effect of Membrane Thickness on Proteorhodopsin Dynamics
A series of PC lipids (14:1-24:1) was used to study the dependence of proteorhodopsin dynamics dependence on the bilayer thickness. Each of these neutrally charged lipids have two monounsaturated acyl chains and differ only in the number of carbon atoms on each chain, ranging from fourteen to twenty-four carbons. Pure lipid membranes made from each of the PC lipids were used to control membrane thickness.

5.1 Conformational Changes in Retinal from Membranes with Different Thickness

Comparison of the resonance Raman spectra for $^{15}$N PR in 14:1 PC and 24:1 PC lipids reveals significant changes occurring mainly in the fingerprint region (1160-1200 cm$^{-1}$) and in the Schiff base and lysine-231 bands of proteorhodopsin. The highest intensity band in the fingerprint region is found at 1199 cm$^{-1}$, but the intensity of the 1171 cm$^{-1}$ C-C stretch (all-trans) band is greater in 14:1 PC than in 24:1 PC, similar to the D97N mutant and acidic form of PR (Dioumaev et al., 2002) as well as the O intermediate of BR (Smith et al., 1983). This increase in intensity may be a general feature of the protonated all-trans retinal Schiff bases with the protonated carboxylic counterion, suggesting that the counterion pK$_a$ of PR in 14:1 PC is lowered. Another difference is in the intensity and location of the C-C stretch band which also comes from all-trans retinal. This band is downshifted four units to 1163 cm$^{-1}$ in 24:1 PC and may be consistent with conformational changes to the Schiff base (C=N) of all-trans retinal whose band varies in shape and position in all PC samples (see Table 5.1), upshifting eight units to 1640 cm$^{-1}$ in 24:1 PC (Figure 5.1). The band representing the residue directly attached to retinal (Lys231) is upshifted two units to 1236 cm$^{-1}$ in 14:1 PC. Another major change occurs in the methyl groups of retinal. The methyl deformation band located at 1449 cm$^{-1}$ in 14:1 PC is shifted to 1446 cm$^{-1}$ in 24:1 PC.
Minor changes occur in the 1300-1385 cm\(^{-1}\) and 800-1000 cm\(^{-1}\) regions representing in-plane hydrogen rocks and HOOP wags from retinal. The 718 cm\(^{-1}\) band is found only in 24:1 PC and possibly may represent a hydrogen wag. However, the position of this band is at an unusually low frequency, so that it may arise from the lysine-231 residue to which it is attached. Changes in hydrogen rock vibrations occur for two bands. The 1302 and 1320 cm\(^{-1}\) bands in 14:1 PC are downshifted two units to 1300 and 1318 cm\(^{-1}\) in 24:1. A final change occurs in the isolated C=C stretch band which is shifted from 1576 cm\(^{-1}\) in 14:1 PC to a double band at 1574/1578 cm\(^{-1}\) in 24:1 PC. The main ethylenic C=C stretch band broadens and downshifts by one unit to 1536 cm\(^{-1}\). Broadening is consistent with the idea of the presence of species with both deprotonated and protonated counterion in 14:1 PC samples. The latter type of species gives red shifted visible absorbance with their Raman bands shifted to a lower vibrational frequency (Kakitani et al., 1983).

A change worth mentioning is in the intensity of the Amide I band of the protein. Compared to 14:1 PC, the 24:1 PC sample has a more intense Amide I band at 1652 cm\(^{-1}\), which might come from a greater amount of retinal-less protein. The amplitude of this band was seen to fluctuate in different samples (Figure 5.1 and A4 in the appendix) and might be influenced by the type of lipid used for making the membrane bilayer (Table 5.2). Another band which may also come from the protein is the 1062 cm\(^{-1}\) band found only in 24:1 PC. This band, previously seen in the FTIR spectra of the proton pump, *Pyrocystis* rhodopsin (PyrR) (Miranda et al., unpublished) may be assigned to C-N vibrations of tryptophan (Barth, 2007), but it is unlikely to gain a detectable amplitude in Raman spectra. However, this band was also seen in resonance Raman spectra of pure PC lipids, arising from their all-trans C-C stretching (Nack et al., 2009).

The effects of membrane thickness (hydrophobic mismatch) and protein/lipid molar ratio have been studied on a number of rhodopsins. In one study (Botelho et al., 2006), the self-
association of visual rhodopsin (a GPCR) was promoted by a reduction in membrane thickness and increase in protein to lipid ratio. Crowding of receptors led to a reduction of accumulation of the MII state responsible for signalling. This observation supports the idea that the MI to MII transition involves an increase in partial molar volume in these signalling receptors.

Table 5.1. Position of the C=N stretch retinal band from resonance Raman spectra of $^{15}$N labelled, wild-type PR reconstituted in PC (1:2 P:L, w/w ratio). See appendix for Raman spectra of PR in 20:1 and 22:1 PC (Figure A4).

<table>
<thead>
<tr>
<th>Lipid (PC)</th>
<th>Schiff Base Stretch Band Position (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:1</td>
<td>1632</td>
</tr>
<tr>
<td>18:1</td>
<td>1641</td>
</tr>
<tr>
<td>20:1</td>
<td>1639</td>
</tr>
<tr>
<td>22:1</td>
<td>1638/43</td>
</tr>
<tr>
<td>24:1</td>
<td>1640</td>
</tr>
</tbody>
</table>

Table 5.2. Ratio of amide I (protein backbone, C=O) amplitude to main ethylenic (C=C) stretch amplitude of retinal from resonance Raman spectra of $^{15}$N labelled, wild-type PR reconstituted in different types of lipids (1:2 P:L, w/w ratio).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>0.0481</td>
</tr>
<tr>
<td>DOPC</td>
<td>0.0679</td>
</tr>
<tr>
<td>DOPS</td>
<td>0.0880</td>
</tr>
<tr>
<td>DOTAP</td>
<td>0.0833</td>
</tr>
<tr>
<td>14:1 PC</td>
<td>0.0546</td>
</tr>
<tr>
<td>24:1 PC</td>
<td>0.0983</td>
</tr>
</tbody>
</table>
Figure 5.1. Resonance Raman spectra for $^{15}$N labelled, wild-type PR reconstituted in 14:1 and 24:1 PC lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and was measured in a buffer containing 25 mM CHES and 10 mM NaCl (pH 9, room temperature).

5.2 Kinetic Changes in the Photocycle in Membranes with Different Thickness

There are no clear trends in the kinetic changes for samples containing membranes made of 14:1, 18:1, 20:1 and 22:1 PC. The time constant, $\tau_1$ decreases and $\tau_2$, $\tau_3$, $\tau_4$ constants increase as the length of the membrane increases by four carbon units (14:1 to 18:1 PC). However when the membrane is further increased (18:1 to 22:1 PC) the time constants decrease (Table 5.3). The photocycle only becomes significantly slower in membranes with 24:1 PC. Due to a slower decay of K and M intermediates, accumulation of M and N/O states is lowest in 24:1 PC membranes (Figure 5.2). Results suggest a critical point for membrane thickness, a point above which the PR photocycle might start to get incrementally slower.
Table 5.3. Time constants (in milliseconds) for the kinetic components representing transitions between photo-intermediate mixtures of $^{15}$N labelled, wild-type PR in different PC lipids.

<table>
<thead>
<tr>
<th>Lipid (PC)</th>
<th>$\tau_1$ (ms)</th>
<th>$\tau_2$ (ms)</th>
<th>$\tau_3$ (ms)</th>
<th>$\tau_4$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:1</td>
<td>$0.371 \pm 0.030$</td>
<td>$2.23 \pm 0.083$</td>
<td>$15.1 \pm 0.59$</td>
<td>$207 \pm 16.7$</td>
</tr>
<tr>
<td>18:1 (DOPC)</td>
<td>$0.348 \pm 0.033$</td>
<td>$2.92 \pm 0.11$</td>
<td>$21.0 \pm 0.81$</td>
<td>$245 \pm 17.7$</td>
</tr>
<tr>
<td>20:1</td>
<td>$0.360 \pm 0.035$</td>
<td>$2.35 \pm 0.11$</td>
<td>$16.1 \pm 0.83$</td>
<td>$209 \pm 21.5$</td>
</tr>
<tr>
<td>22:1</td>
<td>$0.303 \pm 0.039$</td>
<td>$2.43 \pm 0.15$</td>
<td>$17.6 \pm 0.93$</td>
<td>$194 \pm 19.3$</td>
</tr>
<tr>
<td>24:1</td>
<td>$0.495 \pm 0.092$</td>
<td>$3.07 \pm 0.27$</td>
<td>$29.6 \pm 1.67$</td>
<td>$279 \pm 23.1$</td>
</tr>
</tbody>
</table>

5.3 Summary of Membrane Thickness Effects on Proteorhodopsin Dynamics

Major structural changes occur in all-trans retinal, specifically located in the protonated C=N bond, whose band shifts the most from all sample comparisons, especially in the shortest lipid. The resonance Raman spectra for PR reconstituted in 24:1 PC showed two unique bands. The 718 cm$^{-1}$ band found only in 24:1 PC has not been assigned in other rhodopsins. Because of its location, it might arise from a hydrogen wag from the retinal bound lysine residue, Lys231. This may be consistent with changes in the lysine rock band. The band at 1062 cm$^{-1}$ most probably originates from the lipid. Effects on the photocycle dynamics are less significant, showing no clear trends in 14:1 to 22:1 PC samples. However, the photocycle becomes slower by some degree in 24:1 PC with much less accumulation of M, N, and O intermediates. Further experiments need to be carried out for PR in 24:1 PC samples to identify the residues involved in these changes.
Figure 5.2. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR in 14:1 to 24:1 PC. PR was reconstituted in a 1:2 P/L (w/w) ratio and photointermediates were followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines.
Chapter 6
Effect of Membrane Surface Charge on Proteorhodopsin Dynamics
To control for membrane surface charge, the lipids DOPS, POPS and DOTAP were used. The first two lipids have negatively charged headgroups but have structurally different acyl chains. DOPS contains two unsaturated chains (18:1) whereas POPS contains only one unsaturated chain (16:0-18:1). DOTAP has a positively charged headgroup with two unsaturated acyl chains (18:1). Pure DOPS, POPS and DOTAP (refer to Figure A5 in appendix for structure) lipids were used to either form positively or negatively charged membrane bilayers for studying proteorhodopsin dynamics.

6.1 Conformational Changes in Retinal from Membranes with DOPS and DOTAP

Negatively charged membranes cause major changes in the C=N Schiff base, C=C bonds, C-H groups and methyl groups of retinal (Figure 6.1). The HOOP wag bands (808 and 972 cm\(^{-1}\)) and the 1038 cm\(^{-1}\) out-of-plane methyl rock band found in DOPC are absent in DOPS. The weak deprotonated C=N stretch band present at 1606 cm\(^{-1}\) is found in DOPS and not in DOPC. The protonated Schiff base stretch band is also shifted from 1641 cm\(^{-1}\) in DOPC to 1643 cm\(^{-1}\) in DOPS which might be consistent with changes in the position of the lysine rock band which is downshifted two units to 1234 cm\(^{-1}\) in DOPS. Other major changes include the position of the methyl deformation band which is shifted from 1457 cm\(^{-1}\) in DOPC to 1447 cm\(^{-1}\) in DOPS. It has been shown that a leucine residue at position 105 in Green-absorbing Proteorhodopsin (GPR) interacts closely with the retinal C\(_{13}\) group and the Schiff base, whose environments are altered when this residue is replaced (Kralj et al., 2008). Another spectral tuning residue at position 178 (alanine), located on the cytoplasmic surface, also affects the retinal chromophore via long-range effects, with the 13-methyl carbon being the nearest atom. Replacing this residue changes the retinal absorption wavelength and pK\(_a\) of the Schiff base counterion, Asp97 in GPR (Yamada et
Thus, it is feasible that lipid headgroups can affect retinal, including its methyl groups. The 1321 cm\(^{-1}\) in-plane hydrogen rock band in DOPC is shifted to 1317 cm\(^{-1}\) in DOPS with the minor C=C stretch band upshifted four units to 1577 cm\(^{-1}\) in DOPS.

Positively charged membranes cause much more significant changes in retinal than negatively charged bilayers. Similar to results in DOPS, some HOOP wag (808 and 972 cm\(^{-1}\)) and methyl rock (1038 cm\(^{-1}\)) bands are absent in DOTAP. The position of the methyl deformation band is downshifted from 1457 cm\(^{-1}\) to 1450 cm\(^{-1}\) in DOTAP. The putative deprotonated C=N stretch band is located at 1608 cm\(^{-1}\) with the protonated band downshifted seven units to 1634 cm\(^{-1}\) in DOTAP. The C-C stretch band from all-trans retinal is upshifted three units to 1166 cm\(^{-1}\) in DOTAP. The position of this band was also found to shift for PR in 14:1 and 24:1 PC and might be related to major conformational changes to the C=N Schiff base and lysine-231, the retinal bound residue whose band was downshifted three units (1233 cm\(^{-1}\)) in DOTAP compared to DOPC. A final significant change occurs in the C=C bonds of retinal. The major C=C stretch band is upshifted two units to 1539 cm\(^{-1}\) with the minor C=C band shifted six units to 1579 cm\(^{-1}\) in DOTAP. In BR and PR, the double bonds of retinal include C\(_5\)=C\(_6\), C\(_7\)=C\(_8\), C\(_9\)=C\(_10\), C\(_11\)=C\(_12\) and C\(_13\)=C\(_14\) with the intensity of five of these contributing towards the major C=C band and two contributing towards the minor C=C band (Smith et al., 1987a; Smith et al., 1987b). It has been shown that shifts in the main ethylenic (C=C) stretch band of PR are highly influenced by spectral tuning residues such as Leu105 (helix E) and Ala178 (loop of helix E and F) (Yamada et al., 2010) which enable it to absorb different wavelengths of light and function at different depths of the ocean. Since the frequency of the major ethylenic (C=C) stretch band is inversely correlated to the absorption maximum of the electronic transition (Kakitani et al., 1983), PR in DOTAP has the lowest absorption maximum (\(\lambda_{\text{max}}\)) giving it a somewhat different color.
Figure 6.1. Resonance Raman spectra for $^{15}$N labelled, wild-type PR reconstituted in DOPC and DOPS (top) and DOPC and DOTAP (bottom) lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio, and was placed in a buffer containing 25 mM CHES and 10 mM NaCl (pH 9, room temperature).
Results from this work can be tied to studies on bacteriorhodopsin, one of PR’s close relatives. Studies have shown that lipid charges significantly affect BR’s orientation when inserted into lipid membranes. Wang et al. (2008) showed that in liposomes containing anionic lipids, BR was more likely to take on a natural orientation as in living cells. In neutral or positively charged liposomes, BR tended to reversely assemble with an inside out orientation. The detailed molecular mechanism is still unclear but the study suggests that BR might have specific interaction with lipid charges to favor one orientation or the other. Their flash photolysis experiments further revealed that proton permeability across the membrane and decay kinetics of the photocycle intermediates were affected.

6.2 Additional Insights from Isotopic Changes in Retinal in D$_2$O

To study the effect of deuteration, PR was suspended in the same buffer (25 mM CHES, 10 mM NaCl) but with D$_2$O instead of H$_2$O (Figure 6.2). On deuteration, the Schiff base vibration of PR in DOTAP membranes downshifts from 1634 cm$^{-1}$ in H$_2$O to 1622 cm$^{-1}$ in D$_2$O. Since the deuteron replaces the hydrogen atom on the Schiff base, the coupling of the C=N stretch mode with C=N-H in-plane bending mode is eliminated leaving only C=N vibration in place and giving the strength of hydrogen bonding of the Schiff base proton (Kakitani et al., 1983; Baasov et al., 1987; Rodman-Gilson et al., 1988). Since this band downshifts by fourteen units in D$_2$O the strength of hydrogen bonding is similar to that of BR but weaker compared to PR reconstituted in membranes with a neutral charge (Table 6.1). The Schiff base band of PR also becomes broader in D$_2$O, contrary to the situation in BR and ChR2, where it narrows indicating the presence of a water molecule that acts as a hydrogen bonding acceptor to the Schiff base (Nack et al., 2009). Other H/D-exchange dependent bands include the in-plane N-D bending vibrations of the Schiff
base located at 979 cm\(^{-1}\) (Smith et al., 1987a; Smith et al., 1987b), and the combination C-C stretch and lysine rock band (1233 cm\(^{-1}\) in H\(_2\)O) which loses its intensity in D\(_2\)O. The deprotonated Schiff base represented at 1608 cm\(^{-1}\) is also H/D-exchange dependent, suggesting its strong interaction with residues near the retinal binding pocket. This new insight can provide a basis for carrying out spectroscopic experiments on specific PR mutants to determine the main residues involved in the interaction. Other major changes include an upshift of the major C=C stretch (1539 to 1541 cm\(^{-1}\)) and a downshift of the minor C=C stretch (1579 to 1568 cm\(^{-1}\)) bands which come from conformational changes in different regions of the retinal molecule.

Figure 6.2. Resonance Raman spectra for \(^{15}\)N labelled, wild-type PR in DOTAP in H\(_2\)O and D\(_2\)O. PR was reconstituted in a 1:2 (w/w) ratio and placed in a buffer containing 25 mM CHES and 10 mM NaCl (pH 9/8.6, room temperature). The apparent pH measured in D\(_2\)O buffer was adjusted to 8.6 to produce a pD of 9.
Table 6.1. H/D isotopic shifts of the Schiff base (C=N) band analyzed in the following microbial rhodopsins: channelrhodopsin-2 (ChR2) (Nack et al., 2009), sensory rhodopsins I and II (SRI and SRI, respectively) (Mironova et al., 2005; Gellini et al., 2000; Fodor et al., 1989), proteorhodopsin (PR) (Kralj et al., 2008), *Gloeobacter* rhodopsin (GR) (Miranda et al., 2009), bacteriorhodopsin (BR) (Smith et al., 1985), and halorhodopsin (HR) (Maeda et al., 1985).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Function</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\Delta\text{H/D}_{\text{C=N-H}}$ (cm$^{-1}$)</th>
<th>Hydrogen Bonding</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChR2</td>
<td><em>C. reinhardtii</em></td>
<td>Channel/sensor</td>
<td>470</td>
<td>28</td>
<td>Very strong</td>
</tr>
<tr>
<td>SR II</td>
<td><em>H. salinarum</em></td>
<td>Sensor</td>
<td>487</td>
<td>23</td>
<td>Very strong</td>
</tr>
<tr>
<td>SR II</td>
<td><em>Natronobacterium pharaonis</em></td>
<td>Sensor</td>
<td>497</td>
<td>25</td>
<td>Very strong</td>
</tr>
<tr>
<td>PR</td>
<td>$\gamma$-Proteobacteria</td>
<td>Pump</td>
<td>518</td>
<td>23</td>
<td>Very strong</td>
</tr>
<tr>
<td>GR</td>
<td><em>Gloeobacter violaceus</em></td>
<td>Pump</td>
<td>543</td>
<td>26</td>
<td>Very strong</td>
</tr>
<tr>
<td>BR</td>
<td><em>H. salinarum</em></td>
<td>Pump</td>
<td>568</td>
<td>17</td>
<td>Strong</td>
</tr>
<tr>
<td>PR*</td>
<td>$\gamma$-Proteobacteria</td>
<td>Pump</td>
<td>520</td>
<td>14</td>
<td>Weak</td>
</tr>
<tr>
<td>HR</td>
<td><em>H. salinarum</em></td>
<td>Pump</td>
<td>578</td>
<td>12</td>
<td>Weak</td>
</tr>
<tr>
<td>SR I</td>
<td><em>H. salinarum</em></td>
<td>Sensor</td>
<td>587</td>
<td>8</td>
<td>Very Weak</td>
</tr>
</tbody>
</table>

*PR in positively charged membranes (DOTAP) from this project.*
6.3 Kinetic Changes in the Photocycle in Membranes with DOPS, POPS and DOTAP

The photocycle of PR becomes much faster as a result of negatively charging the membrane bilayer. Accumulation of M and N/O intermediates is much greater in POPS and DOPS because the respective time constants ($\tau_1$ and $\tau_2$) are almost two times faster (Table 6.2). However, decreasing membrane rigidity in this system leads to an even faster photocycle. Compared to POPS, the accumulation of M intermediates is much greater in DOPS with lesser accumulation of N/O intermediates because of a slower M decay (see Figures 6.3 and 6.4). This slower M decay is not obvious from the time constants in Table 6.2 because of the different contribution of slower components into the total M decay amplitude.

The shape of the 420 nm kinetics in POPS suggests equilibration between K, M$_1$ and M$_2$ intermediates (Varo et al., 2003). The photocycle of PR can be broken down into three parts. First, roughly between 1 and 10 μs (decay of 600 nm / rise of 420 nm trace) there is equilibration between K and M$_1$. Then M$_1$ goes to M$_2$ in an irreversible process seen by a strong decay of K at 600 nm. Roughly between 10 and 100 μs the K/M$_1$ mixture goes to M$_2$ with very little change in M at 420 nm. Finally, M$_2$ starts decaying strongly which happens so fast that it overlaps with the previous reaction contributing to the complex kinetics and relatively low amplitude of M.

Positively charging the membrane bilayer has an opposite effect on the photocycle. Compared to DOPC, the photocycle in DOTAP is much slower with the absorption signal decaying on a second timescale (see Table 6.2 and Figure 6.5). Because of much slower time constants compared to neutrally charged (DOPC) and negatively charged lipids (POPS and DOPS), it was not possible to analyze the accumulation of M and N/O intermediates in DOTAP with the time-resolved spectroscopy setup described in Ch. 2. Instead, the overall kinetics of the photocycle turnover was followed in a conventional Cary50 spectrophotometer after turning off the
continuous illumination. The resulting kinetics shows non-exponential character as obvious from the very close time constants with different amplitude signs, possibly arising from the mixture of different states under the continuous illumination. Studies on BR also showed that lipid charges significantly affected its M intermediate kinetics, especially the slow component in M intermediate decay. The half-life of $M_{412}$ increased significantly in BR in liposomes containing cationic lipids, while decreasing in BR in anionic liposomes (Wang et al., 2008).

Table 6.2. Time constants (in milliseconds/seconds) for the kinetic components representing transitions between photo-intermediate mixtures of $^{15}\text{N}$ labelled, wild-type PR in different lipids.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$\tau_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>0.367 ± 0.038</td>
<td>2.49 ± 0.101</td>
<td>17.7 ± 0.81</td>
<td>211 ± 18.5</td>
</tr>
<tr>
<td>POPS</td>
<td>0.208 ± 0.012</td>
<td>1.06 ± 0.02</td>
<td>7.59 ± 0.130</td>
<td>96.2 ± 6.3</td>
</tr>
<tr>
<td>DOPC</td>
<td>0.323 ± 0.029</td>
<td>3.07 ± 0.12</td>
<td>20.7 ± 0.853</td>
<td>250 ± 19.3</td>
</tr>
<tr>
<td>DOPS</td>
<td>0.156 ± 0.009</td>
<td>0.868 ± 0.02</td>
<td>7.22 ± 0.149</td>
<td>83.9 ± 6.2</td>
</tr>
<tr>
<td>DOTAP*</td>
<td>-</td>
<td>-</td>
<td>46.1 ± 0.014 s</td>
<td>49.7 ± 0.014 s</td>
</tr>
</tbody>
</table>

*Non-exponential, the amplitudes for the two phases have different sign.
Figure 6.3. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR in POPC and POPS lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and photointermediates followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines. The higher noise in the fast time range is related to the conversion to log time scale.
Figure 6.4. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR in DOPC and DOPS lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and photointermediates followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines.
Figure 6.5. Kinetics of light-induced absorption changes of $^{15}$N labelled, wild-type PR in DOPC and DOTAP lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and photointermediates followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). The kinetics in DOTAP were measured in a conventional spectrophotometer after turning off continuous illumination. Multi-exponential fits are shown with dotted lines.
6.4 Summary of Membrane Surface Charge Effects on Proteorhodopsin Dynamics

In charged membranes some retinal species have a small fraction of deprotonated Schiff bases due to their weaker hydrogen binding strength. The methyl groups are also affected and have been shown to be a result of a different retinal-binding pocket and pK$_a$ of the Schiff base counterion, Asp97. A main difference is that positively charged membranes lead to a greater change in the C=C stretch bands which have been shown to be influenced by spectral tuning residues such as Leu105 (helix E) and Ala178 (loop of helix E and F). These changes translate into dramatically opposite photodynamics for PR in charged membranes. Negatively charged membranes have a faster PR photocycle with much greater accumulation of intermediates. This allows resolving kinetics of the important intermediate substates, M$_1$ and M$_2$. On the contrary, positively charged lipids produce dramatic deceleration of the photocycle turnover.
Chapter 7
Proteorhodopsin Dynamics in Synthetic Matrices

Part of this chapter has been submitted to *J. Am. Chem. Soc.* as a manuscript titled “Spontaneous and Functional Reconstitution of a Light-Driven Proton Pump into “Frozen” Amphiphilic Block Copolymer Membranes” by Kuang, Liangju; Fernandes, Donald; O’Halloran, Matthew; Zheng, Wan; Jiang, Yunjiang; Ladizhansky, Vladimir; Brown, Leonid; Liang, Hongjun.
A number of polymersomes were used to study the influence of synthetic membrane-like matrices on the photochemistry and structure of PR. These were tri-block copolymers, the middle being a hydrophobic block (PBD_{22}, PDMS_{70}, or PS) and the two hydrophilic sides being P4MVP blocks. For example, for PBD_{22}, the synthetic membrane is represented as P4MVP_{28-b-PBD_{22}-b-P4MVP}_{28} where \( b \) stands for block (Hua et al., 2011). The PS series (PS_{26}, PS_{38}, PS_{42}) contain polymersome matrices of varying thickness, with the subscript representing number of polymer units. The P4MVP blocks also slightly vary in size for different polymersomes and were not controlled because of different synthetic conditions.

Figure 7.1. Chemical structure of the amphiphilic tri-block copolymer P4MVP-b-PBD-b-P4MVP. Image taken from Hua et al. (2011), “Self-directed reconstitution of proteorhodopsin with amphiphilic block copolymers induces the formation of hierarchically ordered proteopolymer membrane arrays” (supporting information).

7.1 Conformational Changes in Retinal from Synthetic Membranes

This was the first time that PR was studied in the polymersome matrices, based on PS_{23}, using resonance Raman spectroscopy. The spectrum is similar to that of PR reconstituted in lipids (DMPC/DMPA, 2:1 P/L) (Figure 7.2). The hydrogen wag band at 960 cm\(^{-1}\) in DMPC/DMPA is represented as a double band (959/964 cm\(^{-1}\)) in polystyrene (PS_{23}). There are also changes in the
position of methyl rock bands. The 1007 cm\(^{-1}\) band in DMPC/DMPA representing in-plane C\(_{19}\)-CH\(_3\) and C\(_{20}\)-CH\(_3\) methyl rocks is downshifted two units to 1005 cm\(^{-1}\) in PS\(_{23}\). Also the methyl deformation band at 1454 cm\(^{-1}\) in DMPC/DMPA is downshifted ten units in polystyrene. The C-C stretch bands at 1165, 1171 and 1200 cm\(^{-1}\) from all-\textit{trans} retinal are shifted two units in the synthetic matrix. This might be related to a different electrostatic environment of all-\textit{trans} retinal in PS\(_{23}\). The Schiff base band at 1645 cm\(^{-1}\) in PS\(_{23}\) is significantly shifted to 1636 cm\(^{-1}\) in DMPC/DMPA due to \(^{15}\)N labelling of the protein. Other important bands such as the lysine rock band and some HOOP wag bands could not be located in PS\(_{23}\) due to the spectral quality (low signal to noise because of the small sample size available) and excess fluorescence from the sample, represented as a large band around 800 cm\(^{-1}\). The broad band between 1000 and 1100 cm\(^{-1}\) and the small band around 1603 cm\(^{-1}\) may come from the polymersome, and not from the retinal molecule (Bayari and Yurdakul, 2000).

### 7.2 Kinetic Changes in the Photocycle in Synthetic Membranes

Time-resolved visible spectroscopy shows different kinetics of the photocycle between different polymersome samples. There is not much of a difference between PBD and PDMS except for a faster decay of K to M in PBD (Table 7.1, Figure 7.3). In the polystyrene series, increasing polymersome membrane thickness leads to a slower photocycle (increasing time constants). These trends are similar to those seen in PR reconstituted in membranes with different thickness. This might suggest that PR behaves similarly with respect to membrane thickness and rigidity in both polymersomes and liposomes, without regarding the chemical identity of the matrix surrounding the hydrophobic core. While PR samples in membranes made of PDMS, PBD, and the thinnest PS
are all kinetically close and similar to those in DMPC/DMPA, the thicker (and more rigid) PS samples further suppress protein dynamics resulting in much slower photocycles.

Figure 7.2. Resonance Raman spectra for $^{15}$N labelled, wild-type PR at a 2:1 P/L ratio in DMPC/DMPA 9:1 lipid mixture and from natural abundance PR in PS$_{23}$-based polymersomes. Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature).

Table 7.1. Time constants (in milliseconds) for the kinetic components representing transitions between photo-intermediate mixtures of non-isotopically labeled, wild-type PR in polymersomes.

<table>
<thead>
<tr>
<th>Polymersome</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$\tau_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(butadiene) (PBD)</td>
<td>0.375 ± 0.109</td>
<td>4.81 ± 0.26</td>
<td>15.5 ± 2.04</td>
<td>139 ± 10.1</td>
</tr>
<tr>
<td>poly(dimethylsiloxane) (PDMS)</td>
<td>1.08 ± 0.222</td>
<td>3.99 ± 0.15</td>
<td>24.6 ± 1.35</td>
<td>134 ± 7.31</td>
</tr>
<tr>
<td>PS$_{26}$ (polystyrene)</td>
<td>0.915 ± 0.173</td>
<td>4.65 ± 0.17</td>
<td>26.5 ± 1.52</td>
<td>153 ± 6.81</td>
</tr>
<tr>
<td>PS$_{38}$</td>
<td>1.77 ± 0.652</td>
<td>8.58 ± 0.859</td>
<td>50.4 ± 10.9</td>
<td>289 ± 46.9</td>
</tr>
<tr>
<td>PS$_{42}$</td>
<td>2.82 ± 0.331</td>
<td>25.1 ± 5.21</td>
<td>112 ± 8.37</td>
<td>878 ± 54.1</td>
</tr>
</tbody>
</table>
Figure 7.3. Kinetics of light-induced absorption changes of non-isotopically labeled, wild-type PR in polymersomes. Photointermediates were followed at their characteristic wavelengths: M at 420 (blue), N/O at 600 (red) and the ground state at 500 nm (green). Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature). Multi-exponential fits are shown with dotted lines.
Chapter 8
Conclusions and Future Directions
In this work, the photochemistry and structure of the transmembrane, green-absorbing protein proteorhodopsin (GPR) was studied using resonance Raman and time-resolved visible spectroscopy. PR belongs to the family of microbial rhodopsins, functioning as an outward directed proton pump. Its DNA sequence has been fully identified and is 26% identical to bacteriorhodopsin (BR), the most extensively studied rhodopsin. Even though PR is a eubacterial homolog of BR, our results reconfirm major changes in the configuration of retinal especially in the Schiff base (C=N) and C=C double bonds. These changes are felt in the kinetics of the photocycle as well. PR generally has a fast photocycle with far less accumulation of M intermediates compared to other rhodopsins. Having said this, comparing the dynamics of different rhodopsins was not the primary goal of this project considering the wealth of information already present.

The main goal of this study was to highlight the importance of membrane-protein interactions. The focus was on the influence of membrane bilayer composition on the photochemistry and structure of PR. Synthetic membranes were also investigated giving for the first time new insights into PR dynamics. If studied thoroughly, PR can have interesting applications in many fields such as biotechnology. By knowing the conditions for efficient transport, planktonic bacteria can possibly serve as important alternative fuel sources or can function to desalinize water. PR can be engineered to transport protons efficiently only through a better understanding of its photochemistry and structure using the same type of experiments as in this investigation.

Findings can be summarized into four sections with changes in PR dynamics originating from changes in membrane packing density, flexibility, thickness, and surface charge. Optimal conditions for efficient proton transport include having thin, negatively charged membranes with
low protein packing density. Major changes in retinal which lead to faster turnover times include those in the methyl groups, Schiff base (C=N) and its bound lysine residue, Lys231. In faster photocycles there seems to be a greater proportion of retinal species with deprotonated Schiff bases, indicating weaker hydrogen binding strengths. The strength of hydrogen bonding in charged membranes is similar to that of BR, but weaker compared to PR reconstituted in neutrally charged membranes. To better correlate strength of the Schiff base with the speed of the photocycle, H/D-exchange experiments are required for PR in other lipids especially in negatively charged membranes (DOPS, POPS). In slower photocycles, two unique bands were located at 718 cm\(^{-1}\) and 1062 cm\(^{-1}\) coming from hydrogen wags of Lys231 and possibly other C-N stretches of residues from the protein. In the future, further experiments with site-directed mutagenesis are required to identify specific residues involved in causing changes in the PR’s photocycle. Finally, the trends in dynamics for PR in synthetic matrices are similar to those seen in PR in membranes suggesting that PR behaves similarly towards membrane thickness/rigidity in both polymersomes and liposomes. In conclusion, PR has unique characteristics in its photocycle that separates it from other microbial rhodopsins. Whether this uniqueness applies towards its response to changes in membrane composition is yet to be determined.
References


Appendix

Figure A1. Image of a custom-built time-resolved visible laser spectrometer.
Figure A2. Image of Bruker FRA 106/s accessory to the IFS66vs spectrometer. View of the sample compartment (above) and its expanded view (bottom).
Figure A3. Resonance Raman spectrum for $^{15}$N labelled, wild-type PR reconstituted in DMPC/DMPA 9:1 lipid mixture at 4:1 P/L (w/w) ratio. Buffer conditions: 25 mM CHES and 10 mM NaCl (pH 9, room temperature).
Figure A4. Resonance Raman spectra for $^{15}$N labelled, wild-type PR reconstituted in 20:1 and 22:1 PC lipids. PR was reconstituted in a 1:2 P/L (w/w) ratio and was measured in a buffer containing 25 mM CHES and 10 mM NaCl (pH 9, room temperature).
Figure A5. Structures of DMPC, DMPA, DOPC, POPC, DOPS, POPS, DOTAP, 14:1 to 24:1 PC lipids. Taken from Avanti Polar Lipids, Inc., http://avantilipids.com/.