LIPID-PROTEIN INTERACTIONS:
PHOTORECEPTOR MEMBRANE MODEL

by

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LIST OF ABBREVIATIONS

BHT; butylated hydroxytoluene

CCCP; carbonyl cyanide 3-chlorophenylhydrazone

cGMP; cyclic guanosine monophosphate

CMC; critical micelle concentration

DTAB; dodecyltrimethylammonium bromide

DHA; docosahexaenoic acid

di(Phyt)PC; 1,2-diphytanoyl-sn-glycero-3-phosphocholine

di(DHA)PC; 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine

di(14:1)PC; 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine

di(16:1)PC; 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine

di(18:1)PC; 1,2-dioleoyl-sn-glycero-3-phosphocholine

di(20:1)PC; 1,2-dieicosenoyl-sn-glycero-3-phosphocholine

di(22:1)PC; 1,2-dierucoyl-sn-glycero-3-phosphocholine

di(24:1)PC; 1,2-dinervonoyl-sn-glycero-3-phosphocholine

DMPC; 1,2-dimyristoyl-sn-glycero-3-phosphocholine

DOPE; 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

DOPC; 1,2-dioleoyl-sn-glycero-3-phosphocholine

DTT; dithiothreitol

EDTA; ethylenediamine tetraacetic acid

EFG; electric magnetic field
LIST OF ABBREVIATIONS – Continued

eggPC; egg yolk phosphatidylcholine

EPR; electron paramagnetic resonance

FSM; flexible surface model

FRET; fluorescence resonance energy transfer

G_{ta}; \alpha-subunit of transducin

GDP; guanosine diphosphate

GPCR; G-protein coupled receptor

GTP; guanosine triphosphate

HEPES; N-(2-hydroxyethyl) piperazine N’-(2-ethanesulfonic acid)

LUV; large unilamellar vesicles

MLV; multilamellar vesicles

MES; 2-(N-morpholino) ethanesulfonic acid

meta I; metarhodopsin I

meta II; metarhodopsin II

meta III; metarhodopsin III

OG; \beta-octyl-D-glucopyranoside

PDPC; 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine

POPC; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

POPE; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

PC; phosphatidylcholine
LIST OF ABBREVIATIONS – Continued

PE; phosphatidylethanolamine
PS; phosphatidylserine
PDE; phosphodiesterase

$T_m$; main phase transition temperature
ABSTRACT

G-protein coupled receptors (GPCRs) are transmembrane proteins capable of recognizing an astonishing variety of biological signals, ranging from photons of light to hormones, odorants, and neurotransmitters involved in key biological signaling processes. The aim of the current work is to identify how lipid-protein interactions involving the membrane bilayer ultimately affect such vital biological functions. The present study investigates the relationship between the bilayer thickness, hydrophobic mismatch, and protein aggregation by expanding the framework of membrane-receptor interactions in terms of a new flexible surface model. Previously, we have shown how coupling of the elastic stress-strain due to mismatch of the spontaneous curvature and hydrophobic thickness at the lipid/protein interface can govern the energetics of conformational transitions of membrane proteins, e.g., due to a protrusion from the bilayer in the longitudinal direction. This approach has now been extended to include coupling of the transverse or lateral organization of the GPCR rhodopsin to the curvature and area (hydrophobic thickness) stress and strain of the proteolipid membrane.

In this work, a large variety of well-defined membrane vesicles containing rhodopsin together with phospholipids having different combinations of zwitterionic head groups and acyl chains (length and unsaturation) were prepared. Rhodopsin was labeled with site-specific fluorophores, and a fluorescence resonance energy transfer (FRET) technique was employed to probe protein association in response to the elastic stress of the bilayer. Moreover, UV-visible spectroscopy was used for thermodynamic characterization of the
meta I–meta II conformational change of rhodopsin, encompassing different pH values and lipid compositions. Lastly, the deformation of the lipids with and without rhodopsin was probed in terms of acyl chain order parameters and relaxation rates by solid-state deuterium NMR methods, giving insight into the lipid deformation as a result of hydrophobic mismatch.

The results presented here showed that optimal receptor activation as defined by the meta I–meta II transition occurs in phosphatidylcholine bilayers of 20-carbon acyl chain length. Rhodopsin activation is promoted by more dispersed environment and by lipids having a long acyl chain length; hence we can say that metarhodopsin II is likely to adopt an elongated shape. Lipids promoting aggregation, e.g. those with short or long acyl chain lengths, or below their gel to liquid-crystalline transition temperature all favor formation of metarhodopsin I. However, the data also showed that association and activation of rhodopsin do not always correlate. This is seen for different phospholipids which promote a similar degree of rhodopsin association, but in contrast they activate rhodopsin differently. In terms of the extended flexible surface model proposed in this work, the stress due to hydrophobic mismatch is coupled via the effective number of lipids surrounding the protein due to the lateral organization of the membrane. The measured changes in rhodopsin-rhodopsin interactions and membrane influences on the conformation of rhodopsin after photoisomerization may be crucial to understanding physiological regulation of the rod disk membranes. In consequence, the findings in this dissertation are relevant to understanding
the complexity of biomembranes involved in many cellular mechanisms, including signal transduction.
CHAPTER 1
INTRODUCTION AND SIGNIFICANCE

The complexity of the biological membranes arises from the enormous number of components, including different proteins with their specific functions, phospholipids, glycosphingolipids, and cholesterol with amounts varying from cell to cell. To complicate things even more, the membrane environment surrounding a protein can be heterogeneous with respect to phospholipid composition with different head groups attached to a variety of different acyl chains.

About three decades ago, Singer and Nicolson [1] introduced the fluid mosaic model of cellular membranes. This model introduced the concept that membranes are two-dimensional dynamic structures composed of proteins (peripheral and integral) and phospholipids, which both rotate and diffuse laterally in the plane of an asymmetric fluid membrane, as illustrated in Figure 1.1. The fluid mosaic has become the standard paradigm for studies of membrane structure and function, but our view of how cell membranes are organized has been changing recently. New membrane models have appeared which include the cytoskeleton, polymorphism of the lipids, and more recently the bilayer has been proposed to be non-homogeneous medium, with lateral microdomains or lipid assemblies that segregate membrane components (so-called rafts) [2].

1.1. General Features of Biological Membranes

The membrane bilayer is responsible for facilitating or imposing certain constraints due to the lipid-protein interactions. In this regard, the most important lipid structure in
FIGURE 1.1. Schematic illustration of the plasma membrane of a representative eukaryotic cell. A fluid membrane comprising peripheral and transmembrane proteins, glycocalyx carbohydrate, cytoskeleton, and cholesterol is depicted. To complete our view of biomembranes, it is necessary to consider polymorphism of the membrane lipids, lateral domains or microdomains (rafts), and protein association.
almost all biological cell membranes is the fluid bilayer under physiological conditions. Such fluidity is necessary to accommodate the conformational changes of various transmembrane proteins in conjunction with their mechanisms of action. The fluidity of membranes depends to a large extend on both the polar head group and acyl chain regions of the lipid bilayer [3, 4]. The major lipid species found in biological membranes are phospholipids, which spontaneously form bilayers composed of two monolayer leaflets, each being only one lipid molecule thick. The apolar lipid acyl chains are oriented towards the bilayer center and the polar lipid head groups are at the two surfaces of the bilayer. The mixture of phospholipids in the cell membrane imparts specific physical properties to the bilayer, which can affect integral membrane protein function. For instance, hydrophobic residues of a transmembrane protein make contact with the interior environment of the bilayer membrane. It follows that the lipid acyl chains and their interactions with the intramembraneous hydrophobic surface of integral membrane proteins are important for their function and stabilization within the lipid bilayer.

Basically lipid membranes are elastic anisotropic materials, which are supple and can deform in response to an applied mechanical force. Any kind of deformation, such as those associated with polymorphism, hydrophobic mismatch, the melting transition temperature, and elastic curvature stress of the lipids, can all be related to a change in the free energy of the proteolipid assembly. Within this framework, one is able to obtain insights into how membrane physical properties can be modulated to minimize the energy required for stabilizing a protein in the bilayer. Therefore, to understand the basic physical principles
underlying biomembrane function, much research has been carried out using well-defined membrane systems, which possess the essential features of a biomembrane with a minimum number of components. Such recombinant membrane systems are the subject of the investigations in the present studies.

1.1.1. Polymorphism and Intrinsic Curvature of Membrane Lipids

Biological membranes contain mixtures of lipids in which a substantial fraction prefers to adopt a non-bilayer or reverse hexagonal ($H_n$) phase in water/lipid mixtures under physiological conditions [5, 6]. Lipids that form $H_n$ structures have been suggested to be important for many of the processes carried out by biomembranes [7]. The significance of the non-bilayer forming lipids is that their presence causes a curvature stress in the membrane, i.e. changes in the average properties of the membrane occur as the lamellar to hexagonal phase transition is approached [8], which can affect the activity of membrane proteins. These lipids produce packing stresses in the membrane that can influence the protein conformation. In consequence, the composition of bilayers containing lipids close to their lamellar phase boundaries are characterized by elastic stress or frustration. Some biological processes, including fusion and fission and many others, can occur as manifestations of the influences of non-bilayer phases, such as the reverse hexagonal phase which deviates markedly from the lamellar phase [9, 10].

The liquid-crystalline lamellar phase ($L_a$) corresponds to fluid lipid bilayer matrix of many biomembranes. It is formed by two opposed monolayer leaflets comprised of the amphipathic lipids. The liquid-crystalline, lamellar phase is promoted by phospholipids that
adopt a cylindrical average shape, such as those having large phosphocholine (PC) head group. By contrast, the reverse hexagonal ($H_{II}$) phase is favored by lipids with an inverted cone-like average shape, for example phosphoethanolamine (PE), whose smaller effective head group size compared to the cross-sectional area of the acyl chains results in a negative spontaneous monolayer curvature. The area at the lipid/water interface for the lamellar phase ($L_a$) is the same as the area at the ends of the chains; whereas, for the reverse hexagonal phase ($H_{II}$), the cross-sectional area at the lipid-water interface is less than the area at the chain ends. The cubic phase is another example of self-assembly, involving the water-lipid interface [5]. These non-lamellar phases tend to be formed above the lamellar gel to liquid-crystalline phase transition temperature. Figure 1.2 illustrates the molecular organization of four different phases adopted by the lipids in water mixtures, including (a) the lamellar phase ($L_a$); (b) the reverse hexagonal phase ($H_{II}$); (c) the normal hexagonal phase ($H_I$); and (d) the cubic phase ($Q$). The various nanostructures can be induced to transform to one another depending on the experimental conditions. Lastly, it is worth noting that lipids that form micellar structures such as lysophosphatidylcholine are relatively minor components of membranes. Detergent molecules forming micelles have a reverse geometry corresponding to a cone-like average shape. They are packed in geometrical arrangements in micelles, which resembles $H_I$-phase nanostructures in that they have a positive interfacial curvature.

Quantitatively the non-bilayer phases of membrane lipids can be treated in terms of their intrinsic radii of curvature and their elastic bending moduli. In this context a lipid monolayer curls so the system minimizes its internal energy. Substantial energy is required
FIGURE 1.2. Illustration of the molecular organization in various nonlamellar structures of membrane lipids. Different phases (left) together with their respective average molecular shapes (right) are shown. Four different lipid nanostructures are included: (a) the lamellar phase ($L_\alpha$); (b) the reverse hexagonal phase ($H_n$); (c) the normal hexagonal phase ($H_i$), and lastly (d) the cubic phase ($Q$). The various nanostructures can be induced to transform among each other depending on the experimental conditions. Lipid polymorphism is an important factor mediating the function of certain membrane-bound proteins.
to unbend the lipid monolayer to yield a flat structure [6, 11]. The curvature (bending) energy represents the intrinsic curvature strain within the membrane bilayer, *i.e.* it is the deformation energy that would be liberated if each monolayer of the lipid bilayer would bend to a curved geometry, *i.e.* to its spontaneous intrinsic curvature. In consequence, lipid bilayers whose composition leads to lamellar-nonlamellar phase boundaries are characterized by an elastic curvature stress, which we refer to as frustration. It follows that studies of the different macroscopic structures can play an important role in understanding the functions of proteins embedded in the bilayer membrane. A good example from the authors work of how lipid polymorphism affects the function of a membrane protein is described in reference [12].

1.1.2. Hydrophobic Mismatch in Lipid-Protein Interactions

A mismatch of the lipid bilayer to the intramembraneous hydrophobic interface of an integral membrane protein represents one possible way that lipid-protein interactions can influence biomembrane function. The extent of hydrophobic mismatch can be different for different proteins on account of their different hydrophobic interfacial lengths. Changing the composition of the membrane can alter the thickness of the bilayer, which in turn can affect the contact area between the hydrophobic regions of the protein surface with the nonpolar acyl chains of the membrane lipids. As a consequence, the protein tries to adjust to the new thickness by many different mechanisms [13, 14], which results in a energetic cost for the protein. Among the various possibilities is that the protein can react by changing the acyl chain ordering of the membrane lipids, altering the helix tilt or backbone conformation, or by associating into dimers or other oligomers.
One way to experimentally test the role of the energetic constraints generated by this hydrophobic mismatch is to monitor the function of a protein embedded in membranes containing phospholipids with different acyl chain lengths. The hydrophobic mismatch can also be probed by monitoring its effect on the gel to liquid-crystalline phase temperature of the lipids, or by its influence on the membrane curvature. Substantial research has been conducted to monitor how the hydrophobic mismatch affects the activity of different proteins, including the visual pigment rhodopsin [15-19].

1.1.3. Thermal Phase Transitions of Membrane Lipids

It is widely known that the lipid components of membranes are able to undergo order-disorder phase transitions at well-defined temperatures. The thermodynamics of the melting transition describes the lipid molecule as being in one of two main phases, viz. gel and fluid. The chain melting, i.e. the gel to fluid transition, is an endothermic process with a large latent heat, viz. transition enthalpy of 36.4 kJ K\(^{-1}\)mol\(^{-1}\) (for DPPC), and a transition entropy of about 108.78 J K\(^{-1}\)mol\(^{-1}\). Moreover, the membrane volume increases by about 4%, with a concomitant increase in the membrane lipid/water interfacial area and a decrease in the bilayer thickness. Such order-disorder transitions are associated with restricted rotation of the acyl C–C bonds having absolute energy minima for certain angles (\textit{trans} configuration), with equal excitation energies for the \textit{gauche} ± conformations. The all-\textit{trans} configuration is the predominant chain conformation in the gel phase, and it consists of extended acyl chains, parallel to each other and highly ordered. On the other hand, in the \textit{gauche} conformations the chains are more disordered and their orientational correlation is reduced. The occurrence
of such gauche defects increases with temperature, and characterizes the fluid phase. The disordered fluid chains are effectively shorter than the ordered chains in the gel phase, and they occupy more lateral area leading to an expansion of the membrane surface.

The melting transition of membrane lipids is known to be a highly cooperative process. During the melting transition temperature, the lateral viscosity of the lipids decreases by about two orders of magnitude giving rise to an enhancement of fluidity. The effects of temperature on bilayer structure are of special importance, since a reduction in flexibility or a loss of fluidity can inhibit the conformational changes of the protein affecting its biological function. Recall that membranes undergo reversible transitions from an ordered, quasi-crystalline gel phase to a disordered, liquid-crystalline state at a critical temperature. In that regard, the crystalline aspect refers to the one dimensional periodicity of stacked lamellae, designated by the $L$ notation for both gel ($L_\beta$ or $L_\beta'$) or fluid ($L_a$) phases. A change in the order of the membrane is reflected mainly in the packing arrangement of the acyl chains, in which $\alpha$ refers to disordered chains, and $\beta$ and $\beta'$ refer to ordered chains.

The phase transition of the membrane lipids also affects the hydration of the head groups, which is associated with the aqueous interfacial area occupied per molecule. Higher temperature can increase the mobility of the lipids toward the interior of the membrane, thereby affecting the bilayer permeability. Other factors such as acyl chain length, degree of unsaturation and insertion of an intrinsic protein, ionic strength, and the compositional symmetry of the acyl chains can also affect the phase transitions of phospholipids. One further aspect is that the phase transition temperature can lead to a lateral separation of lipids
below the midpoint temperature, and in this way alter lipid-protein interactions [20, 21].

1.1.4. Boundary Lipids and the Intramembraneous Protein Interface

In the characterization of complex materials such as a biomembrane, knowledge of
phase equilibria is necessary for their general understanding. Descriptions of phase
separation in condensed matter including biomembranes have so far involved two categories.
First, a solid model or fluid model has been presented [1], and second, a viscoelastic model
has been introduced to describe the phase separation of a dynamically asymmetric mixture
where fast and slow components are present [22]. This dynamic asymmetry can be induced
by a large size difference, or by a difference in the temperature transition of the material. In a
dynamic mixture including both lipids and proteins, phase separation generally leads to the
formation of a gel of relatively immobile molecules, given that attractive interactions
between the components are sufficiently strong.

With regard to biomembranes, one can consider fluid-solid phase mixtures to be
analogous to suspensions, and fluid-fluid phase mixtures to be analogous to emulsions. In
fact both can be present in proteolipid membranes, but as the bilayer is effectively a two-
dimensional nanostructure, the droplets can resemble islands. There is a need to highlight
that phase separations do not necessarily need to occur as in the case of water and oil. A
soluble solution mixture can form even in a presence of two different components such as
lipid and protein. The two components can give rise to a homogeneous mixture, even if the
component properties can be accessed separately. The occurrence of phase separation or
microdomains (rafts) will depend on the nature of the materials involved.
Now in the case of lipid-protein mixtures, one may think that the majority of the lipid molecules in contact with a transmembrane protein function as a solvent, interacting with the protein surface relatively non-specifically. On the other hand, some proteins may interact strongly with a small number of lipid molecules. In general, however, to a first approximation the total number of lipids can be separated in two regions, viz. the boundary lipids in direct contact with the intramembraneous hydrophobic surface of the protein, and the lipid regions with no direct contact with the protein surface, referred to here as bulk lipids. In a dispersed environment, the perturbation promoted by the protein will be felt in both lipid regions, boundary and the bulk, depending on the correlation lengths of the lipid-protein and lipid-lipid interactions. Another example is the case where the lipid environment promotes association of the protein into dimers or higher oligomers. Depending on the interaction of the protein with the surrounding lipids, the boundary lipids will feel the greatest effect of the protein. Knowing that proteins and lipids can interact via their hydrophobic interfacial regions, differences in their physical properties can lead to consequent changes in their miscibility. In this way, such material properties may exert a direct influence on the lateral organization of the protein in the bilayer membranes.

Let us now consider an illustrative example, which is diagrammed in Figure 1.3. If the fraction of the boundary lipids is small, then mainly bulk properties of the system would be affected. Figure 1.3 shows two different situations where a protein, e.g., rhodopsin can self associate away from the lipid, as suggested in this dissertation. In part (a) of Figure 1.3, the proteins (solute, large circles) are equally dispersed in a fluid (\(L_\alpha\)) environment (solvent).
FIGURE 1.3. Schematic illustration of the consequences of protein association suggested in this work. The large circles represent a protein surrounded by its boundary lipids (dark grey spheres), which are in the region of stress. The bulk lipids (light grey spheres) are further removed from the protein. (a) Well-dispersed proteins where boundary and bulk lipids are in the $L_\alpha$ phase. (b) A less-dispersed environment where the stress around the protein is diminished due to aggregation. An analogous situation may exist close to the phase transition temperature of the lipids, where gel and fluid phases can coexist ($L_\alpha-L_\beta$).
Part (b) depicts a situation where $L_a-L_a$ phase separation can occur in the presence of few lipids (1:50 lipid molar ratio to protein). Equivalently, the same description can be observed for systems having a melting phase transition of the membrane lipids, with fluid and gel phases separated in the membrane, as in a $L_a-L_{β'}$ coexisting phase mixture. In both cases, parts (a) and (b), only a single kind of lipid solvates the protein and promotes different structural rearrangements of the protein in the membrane environment.

1.2. The Visual Photoreceptor Rhodopsin

Rhodopsin is one of the visual pigments of rod cells in the retina of the mammalian eye. It is a seven transmembrane helix protein, and constitutes an important receptor which upon activation binds a heterotrimeric guanine nucleotide-binding protein, (GPCR). Thus it is a G protein-coupled receptor and its cognate G protein is known as transducin (Gt).

The major fraction of rhodopsin is localized within the rod disk membranes of the rod outer segments (ROS) of rod cells. Rod cells are thin, long and are divided into an inner and outer segment, connected via a thin cilium. They are aligned parallel to one another, and are densely packed in the retina. The outer segments undergo biosynthesis near the ciliary connection to the inner segment. They are in contact with the retinal pigment epithelium (RPE), which phagocytizes the rod disk membranes at the apical end of the cells. The combination of biosynthesis and degradation results in a lifetime of about 5–80 days for the individual rod disk membranes [23], depending on the species which contain hundreds of flattened disks packed closely in a discontinuous stacked array along the length of the ROS, as illustrated in Figure 1.4. The stacking of the disks is stabilized by the protein peripherin
FIGURE 1.4. Illustration of the visual system. (Top) Schematic depiction of the vertebrate eye showing the retina containing rod and cone cells. (Bottom) Representation of the rod cells (left) and cone cells (right). Note that both cell types are divided into an outer and inner segment, which are separated by a narrow region (cilium) near the middle of the cell. (Adapted from Ref. [25].)
The disk edges or rim are tightly curved and contain specific proteins, including ABCR, peripherin, and Rom-1, which are located only in this part of the disk. Rhodopsin and all the components of the visual transduction cascade are found exclusively in the flattened part of the disk [26].

Light absorption, by the chromophore 11-cis retinal of rhodopsin, causes a chemical change due to isomerization from 11-cis to all-trans retinal [27]. In the resting state of rhodopsin, the chromophore forms a protonated Schiff base with the side chain of the residue Lys296, which is located within the hydrophobic core of the seven transmembrane helix motif of opsin. After rapid isomerization of the retinal cofactor, small local changes in packing and charge distribution occur in the internal configurations of some amino acids, followed by a global rearrangement and more distant changes between two key forms, meta I and meta II, after which an equilibrium is reached. Isomerization of the chromophore leads to the formation of higher-energy rhodopsin intermediates, distinguished by their characteristic absorption spectra, including the formation of photorhodopsin, bathorhodopsin, luminorhodopsin, metarhodopsin I, and metarhodopsin II. The latter ultimately decays to the apoprotein opsin and free all-trans retinal. Each of the intermediates is spectrally defined and has been trapped at specific temperatures, as shown in Figure 1.5. During the bleaching process (loss of rhodopsin color), changes occur in the protonation and hydrogen-bonding states of the some amino acid residues [28, 29]. The retinylidene protonated Schiff base is stabilized by a negatively charged carboxylate group (Glu113). These changes influence the absorption properties of the photolyzed rhodopsin, by shifting of the absorbance maximum
FIGURE 1.5. Photothermal processes of bovine rhodopsin after absorption of a photon. Photolysis leads to chromophore isomerization from 11-cis retinal to all-trans retinal, with absorption maxima indicated for each of the intermediates. The reversible equilibrium between meta I and meta II is monitored in this work (from Ref. [30].)
from 478 nm in meta I to 380 nm in meta II, due to the deprotonation of the Schiff base.

1.2.1. Transduction Signal Cascade and The Visual Process

The conversion of meta I to meta II is a critical step for initiation of the visual transduction cascade. Meta II binds the G-protein transducin, in this way acting as a catalyst for the first stage of amplification of the visual process. The light signal received by the receptor rhodopsin initiates a transduction cascade, which converts the light into an electric potential, triggering a nerve impulse that is ultimately delivered to the visual cortex of the brain.

Vision is initiated after a sequence of changes in the conformation of the receptor, after which heterotrimeric G-protein $G_t$ (transducin) binds with high affinity to the excited state of rhodopsin, meta II. Both rhodopsin and transducin are in constant Brownian motion, diffusing randomly and colliding with each other in the two-dimensional space of the rod disk membrane surface. Only upon formation of meta II do they interact specifically and long enough to cause the exchange of GTP bound to transducin. GTP/Mg$^{2+}$ binds to the site from which GDP was released, yielding a conformational change of transducin which causes dissociation of the GTP-$G_{ta}$ subunit from meta II. Release of metarhodopsin II allows further activation of copies of the G-protein upon collision in a diffusion-controlled manner [31].

Subsequently, GTP-$G_{ta}$ activates a cGMP specific phosphodiesterase (PDE), so that the signal is amplified through the GTP-GDP exchange activity of transducin to influence a cascade of second messengers, including cyclic GMP. This constitutes the second catalytic step, where the signal due to a single photon is amplified again. Several thousand cGMP
molecules are hydrolyzed per second per PDE activated, followed by the subsequent hydrolysis of cyclic GMP. This enzymatic amplification leads to rapid depletion of the cytoplasmic cGMP, and closure of cGMP-gated cation channels located in the plasma membrane, followed by hyperpolarization of the rod and termination of synaptic transmission [32, 33].

As a final step of the visual excitation process, light-activated rhodopsin is switched off through phosphorylation by rhodopsin kinase and sequential binding of arrestin. Binding of arrestin to phosphorylated sites close to the G protein binding site sterically hinders transduction activation. Figure 1.6 illustrates a simplified depiction of the visual transduction cascade initiated by the activated rhodopsin intermediate, meta II.

1.2.2. Structural and Function of Rhodopsin

Rhodopsin is composed of 40 kDa apoprotein opsin formed by 348 amino acids. It is the only GPCR for which a high-resolution crystal structure has been determined [34] and recently refined in [35]. The ground state of rhodopsin is inactive towards catalysis of exchanged FTP for GDP bound to Gt. The crystal structure of rhodopsin reveals structural features such as several hydrogen bonded networks that limit the transmembrane helices and the interaction between the chromophore and its environment.

The meta I–meta II intermediates are in a pH-and temperature-dependent equilibrium. Meta I is the first intermediate in which large changes in the secondary structure of rhodopsin occurs, as shown by FTIR experiments [36-38]. The meta I to meta II state is accompany by protonation of the counterion Glu113, which implies transfer of a proton from a
FIGURE 1.6. Schematic illustration of the visual signal transduction cascade in rod disk membranes. (a) Activation of transducin and PDE lead to a closure of cGMP-gated channels and hyperpolarization of the rod cells. (b) The return to the dark state involves phosphorylation of serine and threonine residues by rhodopsin kinase followed by binding of arrestin. (Figure adapted from Refs. [39] and [40].)
hydrophobic environment of the protein. Another protonation step occurs at the cytoplasmic side at residue Glu134, part of the highly conserved D(E)RY motif in HIII [29, 41].

It still is not clear if the proton uptake occurs directly from one residue to another, or through intervening water molecules, or if some water molecules undergo changes in hydrogen bonding in conjunction with the formation of meta I or meta II [42, 43]. A two-dimensional structure of rhodopsin based on the crystal structure is shown in Figure 1.7. Helix VIII is terminated by palmitoylated Cys322 and Cys323. Lys296 is located in helix VII and it is bound to the chromophore via a protonated Schiff base linkage. In this state the Schiff base is weakly hydrogen-bonded, which accounts for the lower \( pK_a \) compared to the dark state. The structure of the cytoplasmic surface of rhodopsin is also altered upon formation of meta II. G-protein, rhodopsin kinase, and arrestin bind to region of rhodopsin located within the cytoplasmic loop a region. Figure 1.8 depicts the most recent X-ray structure of bovine rhodopsin, obtained at 2.2 Å resolution [35].

1.3. Lipid Composition of Rod Disk Membranes and Role of Docosahexaenoic Acid (DHA)

Rhodopsin comprises by far the major protein component in rod disk membranes. In addition, lipids account for half of the dry weight of the membranes, mainly consist of 45 mole\% phosphatidylcholine (PC), 42 mole\% phosphatidyethanolamine (PE), 16 mole\% phosphatidylserine (PS), 2 mole\% (PI), and 2 mole\% cholesterol. The ROS disk membranes are distinguished by a uniquely high amount of docosahexanoic acid (DHA) chains [44]. About 50% of all fatty acids of the ROS phospholipids are docosahexaenoic acid (DHA, 22:6\( \omega3 \)), a long chain highly unsaturated fatty acid that is essential for neural development
FIGURE 1.7. Schematic illustration of folding of rhodopsin within the membrane corresponding to the X-ray structure. The extracellular side of the membrane is at the top and the cytoplasmic side is at bottom. The extracellular side contains the glycosylated chains attached to Asn2 and Asn15. The 11-cis retinylidene chromophore is bound to helix VI via a protonated Schiff base linkage to Lys296. The counterion Glu113 is located in helix III. Cys110 and Cys 187 are disulfide bonded. The cytoplasmic side helix VIII contains Cys 322 and Cys 323 with palmitoyl ester groups due to posttranslational modification. (from Ref. [95].)
FIGURE 1.8. Crystal structure of bovine rhodopsin at 2.2 Å. A highly organized heptahelical transmembrane bundle is evident, in which the cofactor 11-cis retinal is involved in maintaining rhodopsin in the ground state. The left and right figures are related by a 90° rotation. (from Ref. [35].)
and function [45]. DHA-phospholipids are enriched in the rhodopsin-post-Golgi membrane vesicles [46]. The co-localization of DHA-phospholipids and rhodopsin remains after fusion of the vesicles with the plasma and raise intriguing questions about the preference of rhodopsin for DHA-phospholipids.

Several works have suggested that a fluid phospholipid environment for rhodopsin is important for the stability and regenerability of rhodopsin. In addition the lipid polar head group and nonpolar acyl chain composition affect the equilibrium between the meta I and meta II states [10, 47]. Such lipid regulation of protein function has clear implications for human diseases such as retinitis pigmentosa, brain development in infants, and atherosclerosis, as revealed by epidemiological studies of the dietary intake of essential and polyunsaturated fatty acids (PUFAs) [48]. One should note that insertion of rhodopsin in the native ROS lipid membranes is asymmetric, that is to say unidirectional, with the two halves of the monolayer containing a preferred composition of phospholipids, PC and PE being found at cytoplasmic side, and PC and cholesterol being more enriched at the extracellular side. By contrast, in artificial liposomes rhodopsin is assembled bi-directionally, *viz.* symmetrically.

Figure 1.9 illustrates the chemical structures of the main phospholipids studied in this work. Our hypothesis is that combinations of the non-polar acyl chains and polar head group change the elastic material properties of the bilayer membrane, and in this way modulate the functions of the membrane proteins.
FIGURE 1.9. Chemical structures of representative phospholipids employed in this work. Variation of both the head groups and acyl chains has enabled investigation of the elastic properties of the membrane and function of rhodopsin.
CHAPTER 2
THEORETICAL BACKGROUND

Here, a simple thermodynamic theory is proposed based on principles of surface chemistry, for coupling the energetics of membrane proteins to material properties of the bilayer lipids. A role of the non-lamellar forming tendency of mixtures of the lipids phosphatidylethanolamine (DOPE) together with phosphatidylcholine (DOPC) is suggested in relation to the photochemical of rhodopsin, as defined by the meta I–meta II conformational equilibrium. The free energy coupling involves the elastic stresss/strain of the bilayer, together with the local acyl chain packing energy of the lipids. In addition, the electrostatic membrane environment of rhodopsin is treated, where the negatively charged head group of phosphatidylerine stimulates formation of metarhodopsin II by lowering the local pH, while at the same time inhibits its formation by affecting the bilayer surface elasticity.

2.1. Flexible Surface Model for Lipid–Protein Interactions

The influences of lipid polymorphism and the role of the spontaneous curvature of a membrane bilayer are investigated by monitoring the meta I–meta II equilibrium of rhodopsin in binary mixtures of the electrically neutral lipids dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE). Here a flexible surface model is proposed in which the spontaneous curvature of the membrane represents a key aspect. The curvature free energy is given by

\[ g_c = k(H^L - H_0^L)^2 \] (2.1)
where \( \kappa \) is the force constant (bending rigidity), \( H^L \) is the monolayer curvature of the lipid film, and \( H_0^L \) is the natural or spontaneous curvature. A mechanism involving coupling of the elastic area or curvature stress/strain of the planar bilayer lipids (frustration) to the chain packing energy at the intramembraneous protein surface yields an energy balance that explains the lipid influences on rhodopsin-mediated functions [8, 12, 47, 49].

2.1.1. Thermodynamic Formulation

The conformational energetics of integral membrane proteins such as rhodopsin can be treated with a simple thermodynamic approach. We begin with the fundamental equation of chemical thermodynamics,

\[
dG = VdP - SdT + \sum_i \mu_i dn_i
\]

where \( G \) is the Gibbs free energy, \( V \) is the total volume, \( P \) is the total pressure, \( S \) is the entropy, and \( T \) is the temperature. The chemical potentials in terms of the standard state are

\[
\mu_i = \left( \frac{\partial G}{\partial n_i} \right)_{T,P,n_j \neq i} = \mu_i^0 + \gamma_i \left( \frac{m_i}{m_i^0} \right)
\]

in which \( n_i \) denotes the moles of the \( i \)th component, \( \gamma_i \) is the activity coefficient, \( m_i \) is the molality, and \( m_i^0 \) corresponds to the Henry’s law standard state. Dividing \( dG \) by the differential extent of reaction \( d\xi \) at constant \( T, P \) yields

\[
\Delta G = \left( \frac{\partial G}{\partial \xi} \right)_{T,P} = \sum_i \nu_i \mu_i
\]

which \( \nu_i \) are the (signed) stoichiometric coefficients of the products (+) and reactants (-).
For the overall meta I–meta II equilibrium of rhodopsin following flash photolysis,

\[ \text{MI} + \nu \text{H}_3\text{O}^+ \rightleftharpoons \text{MII} \quad (2.5) \]

the chemical potentials are given by

\[ \mu_{\text{MII}} = \mu_{\text{MII}}^0 + RT \ln [\text{MII}] \quad (2.6a) \]

\[ \mu_{\text{MI}} = \mu_{\text{MI}}^0 + RT \ln [\text{MI}] \quad (2.6b) \]

\[ \mu_{\text{H}} = \mu_{\text{H}}^0 + \nu RT \ln [\text{H}_3\text{O}^+] \]

\[ = \mu_{\text{H}}^0 - 2.303 \nu RT \text{pH} \quad (2.6c) \]

where the square brackets [ ] indicate the component activities, viz., \( \gamma_i (m_i / m^0) \). From Eqs. (2.4) and (2.6a-c), the equilibrium constant for the meta I–meta II transition can be defined as

\[ K = e^{-\Delta G^\circ / RT} = \frac{[\text{MII}]_{\text{eq}}}{[\text{MI}][\text{H}_3\text{O}^+]_{\text{eq}}} \quad (2.7) \]

corresponding to the base ionization constant, which is independent of pH. The dependence of the fraction of meta II formed (\( \theta \)) on pH is then given by

\[ \theta = \frac{[\text{MII}]_{\text{eq}}}{[\text{MI}]_{\text{eq}} + [\text{MII}]_{\text{eq}}} = \frac{1}{1 + 10^{\nu \text{pH} - pK}} \quad (2.8) \]

Now, in Eq. (2.8) \( pK = -pK_a \), where \( K_a \) is the acid ionization constant corresponding to the reverse of the equilibrium in Eq. (2.5). Note that use of the bulk solution pH in the above expression gives the apparent \( pK_a \) for the transition. However, for a titratable surface, the dependence of the local pH on the surface potential leads to a shifting and broadening of the titration curve (\( \nu \neq 1 \)). Alternatively, use of the local pH (calculated from the surface potential; \textit{vide infra}) yields a Henderson-Hasselbalch titration curve in terms of the intrinsic
Therefore we introduce the electrochemical potential for a given state \((i)\) as given by

\[
\mu_i = \mu_i + \mu_i^{\text{el}}
\]  

(2.9)

where the electrical part is

\[
\mu_i^{\text{el}} = z_i F \psi
\]  

(2.10)

In the above formula \(z_i\) is the charge, \(F\) is the Faraday constant, and \(\psi\) is the electrostatic potential. The non-electrostatic part, \(\mu_i^{\text{p}}\), of the electrochemical potential (at constant \(T, P\)) describes the dependence on concentration, and is

\[
\mu_i = \mu_i^{\text{p}} + RT \ln a_i
\]  

(2.11)

where \(R\) is the gas constant, \(T\) is the temperature, \(a_i = \gamma_i (m_i / m^o)\) is the activity of the \(i\)th component, \(\gamma_i\) is the activity coefficient, \(m_i\) is the molality, and \(m^o = 1\) m (molal) assuming the Henry's Law standard state \((^o)\). For the equilibrium \(\text{meta I} + v\text{H}_3\text{O}^+ \rightleftharpoons \text{meta II}\), it follows that the standard free energy change is \(\Delta G^o = \mu_{\text{MII}}^{\text{o}} - \mu_{\text{MI}}^{\text{o}} - v\mu_{\text{H}^+}^{\text{o}} = -RT \ln K_{\text{eq}}\), where the equilibrium constant is \(K_{\text{eq}} = [\text{MII}]_{\text{eq}} / [\text{MI}]_{\text{eq}} [\text{H}_3\text{O}^+]_{\text{eq}}\), which is independent of \(pH\).

However, the above treatment assumes a specific mechanism with respect to the stoichiometry of \(\text{H}_3\text{O}^+\) ions in the meta I–meta II equilibrium, which in fact is the subject of investigation and in principle is unknown. Therefore, an alternative is to bypass explicit consideration of the hydronium in stoichiometry and absorbs the (potentially unknown) contribution of \(\text{H}_3\text{O}^+\) into the standard free energies or the reactant and product states. Adapting this approach for photolyzed rhodopsin, the relevant chemical potentials are then...
\[ \mu_{\text{MI}}^0 = \mu_{\text{MI}}^0, \mu_{\text{MII}}^0 = \mu_{\text{MII}}^0, \text{and } \mu_{\text{H}^+}^0 = \mu_{\text{H}^+}^0 + RT \ln \alpha_{\text{H}^+}^0. \]

Here the superscript \(^0\) designates the biochemical standard state, which is a 1 M total concentration of the solute species at the reference pH, typically assumed to be pH 7. With this convention, \(\alpha_{\text{H}^+}^0\), is the bulk hydronium activity in the biochemical standard state. This gives \(\Delta G^0 = \Delta G^0 + vRT \ln \alpha_{\text{H}^+}^0 = -RT \ln K'\), where the apparent equilibrium constant \(K' = a_{\text{MII}} / a_{\text{MI}} = [\text{MII}]/[\text{MI}]\), and is now \(pH\) dependent. It follows that the standard part of the chemical potential can be expressed as

\[
\mu_i^0 = \mu_i^{0,L} + \mu_i^{0,LP} + \mu_i^{0,P}
\]

(2.12)

Here \(\mu_i^0\) is the standard chemical potential (kJ mol\(^{-1}\)) for a given state of the membrane, \(i.e.\) corresponding to the Henry’s law standard state; \(\mu_i^{0,L}\) is the part due to the membrane lipid bilayer; \(\mu_i^{0,LP}\) represents the lipid-protein interface; and \(\mu_i^{0,P}\) is due to the other “internal” protein contributions. According to Eq. (2.12), the non-ideality of the system is attributed to the lipid component of the membrane, and is divided into two parts. The first is due to the lipid bilayer itself \((\mu_i^{0,L})\) and the second to the lipid-protein interaction \((\mu_i^{0,LP})\).

As discussed below, a further source of non-ideality involves the protein-protein interactions, which are assumed to be independent of the lipid composition. The non-ideality of the protein in each of its conformational states is described by the corresponding activity coefficient \(\gamma_i\), and gives a constant term to the chemical potential, which is not considered explicitly [12].

### 2.1.2. Elastic Deformation of the Membrane Bilayer

The membrane deformation accompanying meta I-meta II conformational changes can
be treated in terms of elastic area deformation of the bilayer surface, or in terms of elastic curvature deformation due to spontaneous curvatures of the two opposed monolayer films [50].

(i) For the first term in Eq. (2.12) one can write in general

$$\mu_i^{0,L} = \varepsilon (X_i^L - X_0^L)^2$$  \hspace{1cm} (2.13)

The corresponding equilibrium value is given by $X_0^L$, and $\varepsilon$ is the force constant (elastic modulus) that relates the strain $X_i^L - X_0^L$ to the stress. In the case of area elastic stress, viz. displacement of the lipid/water interfacial area $A^L$ away from the equilibrium value $A_0^L$ in the absence of protein, one can write:

$$\mu_i^{0,L} = (\gamma_{lw} / A_i^L)(A_i^L - A_0^L)^2$$

$$= k_a (A_i^L - A_0^L)^2$$  \hspace{1cm} (2.14)

In the above formula $A_i^L - A_0^L$ is the area strain ($\AA^2$), where $A_i^L$ is the area per lipid at the aqueous interface, $A_0^L$ is the equilibrium lipid area in the absence of protein, $\gamma_{lw}$ is the interfacial tension of the lipid-protein interface, and $k_a$ is the elastic area compressibility modulus [51].

Analogously, one can consider the curvature stress/strain (frustration), in which the geometrical variable is the mean curvature $H = 1/2 (1/R_1 + 1/R_2)$ of the two opposed monomolecular films (leaflets) of the bilayer. Here $C_1 = 1/R_1$ and $C_2 = 1/R_2$ are the two principal curvatures, allowing for a saddle shape of the hypersurface describing the force balance due to the polar head groups and the acyl chains of the phospholipid bilayer [7, 52]. Displacement of the actual mean curvature $H$ from the equilibrium spontaneous curvature $H_0$ leads to a curvature elastic stress (free energy). Further generalizing Eq. (2.1) leads to
\[
\mu_i^{a,L} \propto g_{c,i} = \kappa(H_i^L - H_0^L)^2 + \kappa K
\]  \hspace{1cm} (2.15)

where \( g_{c,i} \) is the curvature free energy in the \( i \)th membrane state, \( \kappa \) is the so-called bending rigidity (the splay elastic modulus), \( \kappa \) is the modulus of Gaussian (saddle) curvature, and \( K = C_1 C_2 \) is the product of the two principal curvatures. (It is known from studies of colloidal systems that the Gaussian curvature plays a secondary role, as described by the Euler characteristic [53]). Here, the first term in the above expression is designated as the curvature free energy.

(ii) The second term in Eq. (2.12) corresponds to the acyl chain packing energy due to stretching of the lipid acyl chains to solvate the hydrophobic surface of the protein, which can be formulated as

\[
\mu_i^{a,LP} = \gamma_{LP} A_i^p
\]  \hspace{1cm} (2.16)

where \( \gamma_{LP} \) is the interfacial tension of the lipid/protein interface. The above equation represents the work needed for the membrane lipids to solvate the hydrophobic surface of the protein, with area \( A_i^p \) in the \( i \)th state.

(iii) Lastly, the “internal” free energy of the protein is designated in terms of unspecified other contributions,

\[
\mu_i^{a,P} = G_i^p
\]  \hspace{1cm} (2.17)

where \( G_i^p \) is the free energy of the “bare” protein, in the absence of the bilayer lipids.

It follows that the standard Gibbs energy change for the meta I–meta II transition can be written as
\[ \Delta G^\circ = \Delta \mu_0^{\text{L}} + \Delta \mu_0^{\text{L, P}} + \Delta \mu_0^{\text{P}} \] (2.18)

The electrostatic part, given in Eq. (2.10), does not contribute to the standard chemical potential, and is described below. In what follows, it is assumed the protein contribution is the same in both meta I and meta II states. Since the non-ideality is attributed to the lipids, the term \( \Delta \mu_0^{\text{P}} \) is independent of the membrane composition, and provides a constant term, which does not enter explicitly into the subsequent analysis. This leads to the result that

\[ \Delta G^\circ = k(H_{\text{III}}^\text{L} - H_0^\text{L})^2 - k(H_{\text{III}}^\text{L} - H_0^\text{L})^2 + \gamma_{\text{L,P}}(A_{\text{III}}^\text{P} - A_{\text{III}}^\text{P}) \] (2.19)

Assuming the hydrophobic surface of the protein \( A_{\text{III}}^\text{P} \) increases in forming meta II [54], the contribution from the last term in Eq. (2.19) is positive, as it costs free energy to form the lipid/protein interface. Consequently, if the local bilayer curvature \( H_{\text{III}}^\text{L} \) differs from \( H_{\text{III}}^\text{L} \), then variations in stress/strain can occur which are coupled with the mechanical work of the transition.

Therefore the free energy coupling between the protein and the membrane lipids is related to the bilayer strain \( (H^\text{L} - H_0^\text{L}) \). The curvature free energy is the work needed to deform an individual monolayer film of the bilayer from its spontaneous curvature, \( H_0^\text{L} \), to the actual mean curvature \( H^\text{L} \), as given by Eq. (2.15). The reduction of the elastic stress/strain for the opposed monomolecular films of the lipid bilayer, both having a negative spontaneous curvature \( H_0^\text{L} \), is due to their curvature \( H^\text{L} \) toward water, leading to a relief of curvature frustration. The decrease in elastic free energy compensates for the increased
solvation energy of the lipid/protein interface and the balance is described by Eq. (2.19).

To illustrate these new biophysical concepts, Figure 2.1 shows a cartoon of a working model for rhodopsin embedded in a bilayer membrane, representing the dark state of rhodopsin, meta I and meta II. The elastic stress/strain of the bilayer is formulated in terms of a generalized modulus $\varepsilon$, together with the corresponding geometric variable $X^L$ and its equilibrium value $X_0^L$. This conceptual framework has been explicitly tested in work reported in this dissertation.

### 2.1.3. Contribution of Curvature Free Energy of Membrane Lipids

The elastic curvature stress/strain of the lipid bilayer is given by

$$\mu^{0,L} = G_c = ANN_A g_c.$$ 

The curvature free energy per unit area is explicitly defined as $g_c$, where $A$ is the area/lipid $70 \, \text{Å}^2$, $N$ is the number of lipids per rhodopsin, and $N_A$ is Avogadro's number. According to Eq. (2.19),

$$\ln K_{eq} = \frac{-\Delta G}{RT} = -ANN_A \Delta g_c / RT - \Delta G_{other} / RT$$ (2.20)

Our hypothesis is that the curvature free change ($\Delta g_c$) between two states of the protein depends on the mole fraction of non-lamellar forming lipids in the membrane. For instance, in the case of a DOPE/DOPC mixture, the spontaneous curvature is given by

$$H_0^L = H_0^{DOPE} X^{DOPE},$$

where $H_0^{DOPE}$ is the spontaneous curvature of pure DOPE [11], and $H_0^{DOPC} \approx 0$. Hence, the change in monolayer curvature free energy for the meta I–meta II transition is given by

$$\Delta g_c = -2k(H_{\text{mix}}^L - H_{\text{mi}}^L)H_0^L + k(H_{\text{mix}}^L - H_{\text{mi}}^L)^2$$ (2.21)
FIGURE 2.1. Biomembrane model involving coupling of spontaneous curvature of lipid bilayer to the conformational energetics of an integral membrane protein. Rhodopsin is shown as an example of a G protein-coupled receptor. The increase in the lipid/protein interfacial free energy in the meta II state is balanced by a reduction in curvature elastic stress.
Equations (2.20) and (2.21) together that imply the change in Gibbs free energy $\Delta G^\circ$ for the protein conformational change depends linearly on the spontaneous curvature $H_0^L$ of the DOPE/DOPC lipid mixture. By differentiating with respect $X_{\text{DOPE}}$ we obtain the result

$$\frac{\partial \ln K_{\text{eq}}}{\partial X_{\text{DOPE}}} = \frac{2kAN}{k_BT} (H_{\text{MII}}^L - H_{\text{MI}}^L) H_0^\text{DOPE}.$$  \hspace{1cm} (2.22)

In the above expression $k_B = \frac{R}{N_A}$ is the Boltzmann constant, $k$ is the bending rigidity, $H_{\text{MII}}^L$ and $H_{\text{MI}}^L$ are the mean lipid curvatures of the meta II and meta I states, respectively, and $H_0^\text{DOPE}$ is the spontaneous curvature of pure DOPE. Equation (2.22) shows that the Gibbs free energy change depends linearly on the mole fraction $X_{\text{DOPE}}$ in the recombinant membranes.

### 2.1.4. Electrostatic Treatment of Bulk versus Surface pH

The surface potential ($\psi_0$) originates from the charge of rhodopsin and depends on the pH, as well as anionic lipids as in the case phosphoserine head group. To include a description of electrostatic influences on the meta I–meta II equilibrium, the Gouy-Chapman equation was used to calculate the surface (local) pH of the membrane [55, 56]. The above treatment can be further extended in terms of the electrochemical potential, $\mu_i$, by including the electrostatic potential $\psi$, leading to

$$\mu_i \rightarrow \mu_i^\text{el} = \mu_i + \mu_i^\text{el}$$  \hspace{1cm} (2.23)

where $\mu_i^\text{el} = z_i F \psi$ is the electrical energy, $z_i$ is the ion charge, and $F$ is the Faraday. Equating the electrochemical potential for hydronium ions, Eq. (2.23), at the membrane surface ($\psi = \psi_0$) and in the bulk solution ($\psi = 0$) yields the Nernst equation,
\[ \rho H_{\text{local}} = \rho H_{\text{bulk}} + \frac{zF\psi_0}{2.303RT} \]  

(2.24)

The electrostatic potential is related to the surface charge density (\(\sigma\)) in terms of the Poisson-Boltzmann equation, which for a planar surface gives the Gouy-Chapman formula,

\[ \sigma = \left( \frac{C^{1/2}}{A} \right) \sinh \left( \frac{zF\psi_0}{2RT} \right) \]  

(2.25)

Here \(\sigma\) is the membrane surface charge density, \(\psi_0\) is the membrane surface potential, \(C\) is the molarity of cations due to the sodium phosphate buffer, \(z = 1\) is the sodium ion charge, and \(A = 134.8 \text{ M}^{1/2} \text{ Å}^2\). Note that in Eq. (2.25) the electrical potential \(\psi_0\) is not an additive function; whereas the surface charge density can be decomposed into lipid and protein components,

\[ \sigma = \sigma^l + \sigma^p \]  

(2.26)

Alternatively, the total charge density per rhodopsin unit cell, Eq. (1.26), can be calculated using

\[ \sigma = -\sigma_{\text{PS}} + \sum_i \sigma_{\text{basic}}^{(i)} - \sum_j \sigma_{\text{acidic}}^{(j)} \]  

(2.27)

The charge density of PS is obtained from the formula

\[ \sigma_{\text{PS}} = \frac{N}{1 + \left( \frac{[\text{H}_3\text{O}^+]}{K_H} + \frac{[\text{Na}^+]}{K_{\text{salt}}} \right) \exp \left( -\frac{F\psi_0}{RT} \right)} \]  

(2.28)

where \(N\) is the surface density of the ionizable groups, assuming a surface area for a rhodopsin unit cell of 4000 Å\(^2\) at \(T=28\text{ °C}\) [57], \(K_H\) is the dissociation constant for \(\text{H}_3\text{O}^+\), \(K_{\text{salt}}=1/0.7 \text{ M}\) is the dissociation constant for binding of \(\text{Na}^+\) to PS, and \([\text{H}_3\text{O}^+]\) and \([\text{Na}^+]\) are the bulk concentrations of the hydronium and sodium ions (in the phosphate buffer.
solution) [58]. The intrinsic pKₐ value used for the PS carboxyl group was 3.6. The contribution from the charge density of the basic amino acids is given by

\[
\sigma^{(i)}_{\text{basic}} = \frac{N}{1 + 10^{pH_{\text{local}} - pK_a^{(i)}}}
\]

(2.29)

where \( K_a^{(i)} \) is the corresponding acid ionization constant of the \( i \)th residue. For the acidic amino acids,

\[
\sigma^{(j)}_{\text{acidic}} = N \left(1 - \frac{1}{1 + 10^{pH_{\text{local}} - pK_a^{(j)}}}\right)
\]

(2.30)

The Gouy-Chapman model, Eq. (2.25), and the directly calculated charge density, Eq. (2.27), yield a set of simultaneous, non-linear equations, which can be solved iteratively to eliminate the surface potential \( \psi_0 \). The local pH is then obtained from the bulk pH using the Nernst equation (Eq. 2.24).

2.2. Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance transfer energy (FRET) is a distance-dependent interaction between the electronic states of two dye molecules, in which the excitation is transferred from a donor molecule to an acceptor molecule without the emission of a photon. The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological macromolecules. It is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.

2.2.1. Primary Conditions Needed for FRET
Donor and acceptor molecules must be in close proximity (10–100 Å), and the absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor. Moreover, the donor and acceptor transition dipole orientations must be approximately parallel. Under this condition, energy is transferred non-radiatively from the donor to the acceptor with an efficiency defined by

\[ E = \frac{R_0^6}{(R_0^6 + r^6)} \]  

(Eq. 2.29)

Here \( r \) is the distance between the two fluorophores, and \( R_0 \) is the Förster distance which a characteristic parameter for every dye pair defining the distance at which the efficiency is 50%. At this distance, one-half of the donor molecules decay by energy transfer and one-half decay by the usual radiative and nonradiative rates, that is to say 50% of the excited donors are deactivated by FRET [59]. Förster distance is usually reported for an assumed value of \( K^2 = 2/3 \), which is appropriate for dynamic random averaging of the donor and acceptor. The magnitude of \( R_0 \) is dependent on the spectral properties of the donor and acceptor dyes (the overlap), the quantum yield of the donor, and the relative orientation of the donor and acceptor. Excitation of donor fluorophore in a FRET pair leads to quenching of the donor emission, and to a sensitization or increase in acceptor emission.

Energy transfer (\( E \)) is the fraction of photons absorbed by the donor that are transferred to the acceptor, and can be calculated as the ratio of the transfer rate \( K_T \) to the total decay rate of the donor, namely \( E = K_T (\tau_D^{-1} + K_T) \) where \( \tau_D \) is the lifetime of the donor in the absence of acceptors or any other quenching effector. Since (\( E \)) depends on the inverse of the sixth power of the distance \( r \), FRET has become the technique of choice to observe
protein-protein interactions and to measure distances between fluorophores [60].

2.2.2. FRET Index and Donor-Acceptor Configuration

The FRET index is a relative value that varies with changes in energy transfer associated with changes in the donor-acceptor configuration. FRET indices are useful to perform qualitative studies, or to obtain relative measures within the same experiment [61]. However, each FRET index is tuned for specific related experimental needs. A direct comparison between the results obtained with different indices can be difficult. In biological applications, this technique has been applied to qualitatively map protein-protein interactions.

Two different approaches are generally used to measure FRET: (a) emission spectral measurements and (b) acceptor photobleaching. In (a) the measurements are based on the excitation of the donor and detection of the light emitted by either the donor and/or the acceptor in the presence of the other fluorophore. When FRET occurs, the donor emission is decreased and the acceptor emission is increased. For (b) one monitors the excitation of the donor and detects the light it emits before and after acceptor photobleaching. In both approaches, values can be obtained that represent either a FRET index or the transfer efficiency. These methods are also in general use in steady-state FRET microscopy.

2.2.3. Alexa Fluorophores as Probes of Biomolecular Interactions

It is widely appreciated that fluorescent dyes are excellent probes for detecting conformational changes of biopolymers or for studying protein-protein interactions. However, it is difficult to find a good fluorophore for rhodopsin, since many fluorescent
probes are excited at wavelengths that cause bleaching of the protein.

In this work, the conjugated fluorophores Alexa 488 and Alexa 594 C5-maleimides were chosen due to their thiol-reactive sites, pH independence in the region from 4 to 10, and due to their high photostability. Alexa 488 C5-maleimide is a bright green fluorophore with the molecular formula $C_{30}H_{25}N_{4}Na_{12}S_{2}$ (Molecular Probes). The molecular weight is $MW=720.66$ g/mol and the molar absorption coefficient is $\varepsilon =72\,000\,M^{-1}cm^{-1}$. The thiol-reactive Alexa 594-C-5 maleimide (pink bright color) absorbs at $\lambda > 600$ nm and emits in a red region of the spectrum, which makes it a good combination with green fluorescent probes. The molecular formula is $C_{44}H_{45}N_{4}NaO_{12}S_{2}$, the molecular weight $MW=908.97$ g mol$^{-1}$, and it has an absorption coefficient of $\varepsilon =96\,000\,M^{-1}cm^{-1}$. Both chemical structures are illustrated in Figure 2.2.

Rhodopsin has several cysteine residues which can be used to label the protein. Among the cysteines residues of rhodopsin, Cys316 and Cys 140 are located in the cytoplasmic site H VIII and HII, respectively. They have been found to be the most reactive sites with respect to maleimide and iodoacetamide. The preference for Cys316 has been determined by limited proteolysis with thermolysin and characterized by SDS-gel electrophoresis. Labeling of Cys140 can be avoided by controlling the reaction according to the incubation time at which the protein is exposed to the fluorophore.

2.3. **Solid-State Deuterium NMR Spectroscopy of Biomembranes**

Deuterium nuclear magnetic resonance spectroscopy ($^2$H NMR) is a powerful tool for characterizing the physical properties of lipids and membranes [22, 62, 63]. It can be used to
FIGURE 2.2. Chemical structures of Alexa 594 (top) and Alexa 488 (bottom) fluorophores. The C-5 maleimide reacts with the thiol group of cysteine residues to form a thioether compound. Alexa fluorophores are incorporated into Cys316 located on the cytoplasmic side (helix VIII) of rhodopsin.
probe both the ordering of the hydrophobic acyl chains and different types of motions, such as isomerization of the flexible lipid molecules and rotational, and lateral diffusion of the membrane lipids. Because the lipids are essentially fluid under physiological conditions, \(^2\)H NMR spectroscopy can probe the motions associated with their fluidity, which are fast on the NMR time scale. For anisotropic material such as lipid membrane, the dipolar and quadrupolar interactions are not averaged to zero due to the lamellar geometry of the bilayer. These interactions are projected along the membrane surface normal, which is the symmetry axis for fluid motions. Another advantage of the NMR technique is that the selective replacement of \(^1\)H for \(^2\)H does not perturb the natural arrangement of the molecule in the membrane (unless only specific hydrogen bonding is involved). The natural abundance of deuterium is low (0.016\%), and the magnetic dipole moment of \(^2\)H is a factor of about 6 times smaller than of \(^1\)H, so that interactions with neighboring protons have little effect on the spectrum. Thus the deuterium NMR signal can be assigned to the \(^2\)H-labeled site and used to specifically probe the membrane properties.

### 2.3.1. General Deuterium NMR Theory

The Hamiltonian of a deuteron in a magnetic field can be written as

\[
\hat{H} = \hat{H}_z + \hat{H}_Q
\]  

(2.30)

where \(\hat{H}_z\) is the Hamiltonian for the Zeeman interaction between the magnetic nuclear moment \(\mu_n\) with the static magnetic field \(B_0\) described as

\[
\hat{H}_z = -\gamma_n B_0 \hat{I}_z
\]  

(2.31)
The second term $\hat{H}_Q$ is the quadrupolar Hamiltonian for the coupling of the nuclear quadrupolar moment $(eQ)$ and the electric field gradient (EFG) and is given by [64],

$$
\hat{H} = \frac{eQ}{4I(2I-1)} \left[ V_z (3\hat{I}_z^2 - \hat{I}^2) + V_y (\hat{I}_z \hat{I}_y + \hat{I}_y \hat{I}_z) + (V_x^3 \hat{I}_x^2) \right] 
$$

(2.32)

where $e$ is the elementary charge, $Q$ the quadrupolar moment, and $I$ is the nuclear spin for deuterium ($I=1$). Here $V$ is the irreducible tensor representation of the EFG tensor, and can be described in terms of its irreducible components in the molecular framework $V^{(2,m)} (m=0, \pm 1, \pm 2)$

$$
V^{(2,0)} = V_{zz}, \\
V^{(2,\pm 1)} = \pm \sqrt{2/3} (V_{xx} + iV_{xy}), \\
V^{(2,\pm 1)} = \sqrt{1/6} (V_{xx} + V_{yy}) \pm iV_{xy}
$$

(2.33)

The $V_{ik}$ is a symmetric tensor and it is diagonal in a molecular coordinate system with the components $V_{xx}$, $V_{yy}$, and $V_{zz}$ [62]. The electric field gradient $eq = V_{zz}$, and the asymmetry parameter is described as $\eta = V_{xx} - V_{yy} / V_{zz}$. Wigner rotation matrices [63] are applied to transform the principal values of the irreducible components $V^{(2,p)x}$ of the EFG tensor from its molecular axis to the laboratory frame (primed) as

$$
V^{(2,m)'} = \sum_{p=-2}^{2} D^{(2)}_{pm} (\alpha \beta \gamma) V^{(2,p)} 
$$

(2.34)

where $D^{(2)}_{pm} (\alpha \beta \gamma)$ are the elements of the Wigner rotation matrix. The quadrupolar energy is relatively small and can be treated as a perturbation of the Zeeman energy $|eQ/h| \ll |\gamma_N B_0|$. To the first order, only the secular terms in the total Hamiltonian which
commute with $I_z$ contribute to the observed lineshape. It follows that the energy is given by

$$E_m = -\gamma N B_0 m + \frac{eQ}{4I(2I-1)} V^{(2,0)} [3m^2 - I(I+1)]$$

(2.35)

From the three eigenvalues ($m=-1, 0, +1$) or energy levels for deuterium, only two single-quantum transitions are allowed according to the selection rule $\Delta m = \pm 1$. Hence only two resonance lines are observed in the $^2$H NMR spectrum, centered at the Larmor frequency $\omega_0 = -\gamma B_0$.

It follows that the two different frequencies are given by

$$h\nu_+ = E_{-1} - E_0 = \gamma B_0 m + \left(\frac{3}{4}\right) eQ V^{(2,0)}$$

$$h\nu_- = E_0 - E_+ = \gamma B_0 m - \left(\frac{3}{4}\right) eQ V^{(2,0)}$$

(2.36)

The quadrupolar splitting is then calculated as

$$\Delta \nu_Q = \nu_+ - \nu_- = \frac{3eQ}{2h} V^{(2,0)}$$

(2.37)

Thus any orientation can be calculated from the Euler angles as

$$\Delta \nu_Q(\theta, \phi) = \frac{3eQ}{2h} \left( \frac{3\cos^2 \theta - 1}{2} + \frac{1}{2} \eta_0 \sin^2 \theta \cos 2\phi \right)$$

(2.38)

In the above equation $e$ is the electronic charge, $eQ$ is the $z$-component of the electric field gradient felt at the nucleus, and $Q$ is the nuclear quadrupole moment, which is the nuclear property that describes the strength of the quadrupolar coupling to the electric field gradient at the nucleus. For the aliphatic C-$^2$H bonds the static quadrupolar constant
\( e_2 q Q / h = \chi_Q \) has a value of 170 kHz.

### 2.3.2. Deuterium NMR Spectra and Derived Order Parameter Profiles

The order profile of a lipid bilayer shows the variation of the order parameter \( S_{CD} \) with respect to the position of the segment in the chain, and is an expression of the average angular fluctuations around the bilayer normal. By labeling each segment of the hydrocarbon chain one can experimentally measure their order parameters. The quadrupolar splitting \( \Delta \nu_Q \) of a given 2H-labeled segment is decreased by a factor \( |S_{CD}| \), which can take values in the range 0 to 0.5 for methylene groups in the fluid lamellar phase membrane. Thus the order parameter indicates the disorder of the acyl chains in the system.

Figure 2.3 shows a representative powder-type \(^2\)H NMR spectrum of a fully hydrated vesicles of rhodopsin/POPC-\(d_{31}\) (1:100) mole ratio in the fluid lamellar phase, together with its deconvoluted (de-Paked) spectrum. The prominent doublet peaks in the powder-type \(^2\)H NMR spectrum correspond to \( \theta=90^\circ \) bilayer orientation, where the normal to the membrane surface is perpendicular to the magnetic field. The shoulders with twice of the quadrupolar splitting represent the \( \theta=0^\circ \) orientation, where the membrane normal is parallel to the magnetic field [63]. The residual quadrupolar splittings are directly related to the orientational order parameters of the flexible lipid molecules in the fluid state \( (L_o) \). They characterize the angular amplitudes of the motions of the various C-\(^2\)H labeled segments, so the order parameters can be defined as

\[
S_{CD}^{(i)} = \left< P_2 \left( \cos \beta \right) \right> = \frac{1}{2} \left< 3 \cos^2 \beta_{\rho 0}^{(i)} - 1 \right> \quad (2.39)
\]
FIGURE 2.3. Representative powder-type and de-Paked $^2$H NMR spectra for rhodopsin/POPC-\textit{d}_{31} recombinant membranes (1:100) mole ratio at 27 °C. The central spectrum (solid line) represents the powder-type $^2$H NMR spectrum of randomly oriented membranes. The lower spectrum (dotted) represents the de Paked spectrum obtained by numerical inversion, corresponding to the $\theta=0^\circ$ orientation of the bilayer normal parallel to the magnetic field.
Here $\beta_{P_{D}}^{(i)}$ is the angle between the $i$th C-$^2$H bond, where $(P)$ is the principal symmetry axis of the EFG tensor and the director $(D)$ is the macroscopic bilayer normal. In Eq. (2.39) $P_2$ is the well-known second Legendre polynomial. The brackets designate that motional averaging occurs over the characteristic time scale associated with the $^2$H NMR transitions, which for the lipid dispersions is $10^{-6}$ s [65]. In addition, the experimental powder-type spectra can be numerically deconvoluted to obtain the corresponding spectra for the $\theta=0^\circ$ orientation, a procedure which in the NMR community is given the name de-Pakeing (after Pake).

Lastly, for the case of powder-type $^2$H NMR spectra obtained for random membrane samples, the first two moments of the spectral lineshapes can be calculated from

\[ M_1 = \frac{\pi}{\sqrt{3}} \chi_{Q} \langle |S_{CD}| \rangle \]  

(2.40)

\[ M_2 = \frac{9\pi^2}{20} \chi_{Q}^2 \langle |S_{CD}|^2 \rangle \]  

(2.41)

Here $M_1$ and $M_2$ are related to the mean orientational order parameter and its mean-squared value, respectively [66]. $M_1$ for a rigid all-trans polymethylene chain is $3.08 \times 10^5$ s$^{-1}$, and $M_2$ has a value of $1.28 \times 10^{11}$ s$^{-1}$. For the axial rotation of the acyl chains these values are reduced to $1.4 \times 10^5$ s$^{-1}$ and $3.21 \times 10^{10}$ s$^{-1}$.

2.3.3. Equilibrium Properties of Membrane Lipids and Averaged Projected Length of Acyl Chains

In the liquid-crystalline ($L_\alpha$) state, the experimental order parameters $S_{CD}^{(i)}$ can be
further interpreted in terms of the rotational isomerizations of the acyl chain segments and corresponding bilayer properties. The probabilities of trans and gauche isomers [67, 68] can be described by a simple diamond-lattice model for the configurational statistics for polymethylene acyl chains in lipid bilayers. Here the chain is modeled as a series of virtual bonds connecting the various segments, which are parallel to the plane spanned by the two C–2H bonds of each methylene unit. The all-trans configuration is therefore the reference state for the statistical model, with the virtual bond length of \( l = 1.25 \text{Å} \). The different possible orientations of the methylene group (0°, 60°, 90°, 120°, and 180°) correspond to the angle between the virtual bond and the bilayer normal. Angles 120° and 180° are folded-back towards the aqueous interface and are neglected. The 60° segment orientation of the two deuterons are inequivalent and have angles of 35.3° and 90°.

However, these conformations cannot be identified as trans or gauche isomers since to identify them would require knowledge of the relative orientations of the additional two segments. For the terminal methyl group C–C\(^2\)H\(_3\) there is an additional axis of motional averaging parallel to the bond direction of the terminal carbon and the penultimate carbon. The contribution of the terminal methyl group is given by \( 3S_{CC}^{(n)} \) where \( l_0 = 1.25 \text{Å} \) is the length of one carbon-carbon bond in the all-trans state. The number of carbons in the acyl chain is given by \( n \), where the hydrocarbon region is considered to begin at segment \( m \). For example, in the case of the \( sn-1 \) acyl chain \( m = 2 \) and for the \( sn-2 \) acyl chain \( m = 3 \).

It can then be shown that the segmental order parameters can be related to the average length of a fatty acyl chain with \( n \) carbon-carbon bonds as [51]
Here $S^{(i)}_{CD}$ is the C–$^2$H order parameter of position $i$ in the acyl chain, which includes the influence of the fast segmental motions, and $S^{(n)}_{CD}$ is the order parameter of the methyl segment. The above equation gives the hydrophobic thickness of a monolayer, therefore, one can estimate the hydrophobic thickness of a bilayer as $d \approx 2 \langle L \rangle$ for a pure lipid membrane.

From the average projected chain length in the $L_\alpha$ phase, one can estimate the mean molecular area at the lipid/water interface [51]

$$\langle A \rangle = \frac{V_{\text{chain}}}{\langle L \rangle}$$

where $V_{\text{chain}}$ is the chain volume [69]. For a saturated chain the latter is given by $V_{\text{chain}} = n_{\text{CH}_2} V_{\text{CH}_2} + V_{\text{CH}_1}$, in which $V_{\text{CH}_1} \approx 2 V_{\text{CH}_2}$ and $n_{\text{CH}_2} = n - m$ is the number of methylene segments used to calculate the projected chain length. For example, in the case of POPC, $n_{\text{CH}_2} = 14$.

2.3.4. Nuclear Spin Relaxation and the Molecular Dynamics of Lipid Membranes

The nuclear spin relaxation rates characterize the fluctuations due to segmental, molecular, and collective motions of the lipids in the megahertz range (spectral densities of motion or power spectra). Such relaxation measurements can detect both local motions and the larger-scale dynamical behavior of membrane phospholipids [63]. The NMR relaxation rates (or spectral densities) allow one to separate the motions giving rise to the segmental order parameter $S_{CD}$. The relaxation manifests the strength of the C–$^2$H bond fluctuations as
a function of frequency, and thus can be associated with the "softness" of the membrane bilayer. It depends on the mean-squared amplitudes of the fluctuations of the C–2H labeled molecules, and the correlation times for the associated lipid reorientational motions near the nuclear Larmor frequency. Rapid local motions of the C–2H bonds can occur due to trans-gauche isomerization and out of plane displacements of lipid groups, which change the C–2H orientations with respect to the membrane normal and modulate the static EFG tensor relative to its average value. The remaining residual (slow motions) also produce a change in the orientation of the methylene segments [65].

The spin-lattice (longitudinal) relaxation rate \( R_{1Z} \) is due to the motions of the individuals C–2H bonds (the EFG) relative to the laboratory frame. It is given to second-order in the perturbing Hamiltonian by

\[
R_{1Z} = \frac{3}{4} \pi^2 \chi_0^2 \left[ J_1(\omega_D) + 4J_2(2\omega_D) \right]
\]

where \( \omega_D \) is the deuteron resonance (Larmor) frequency. This equation shows how relaxation rate measurements are connected to the membrane dynamics in terms of the spectral densities \( J_1(\omega_D) \) and \( J_2(\omega_D) \) (power spectra) of the lipid motions that produce averaging of the EFG coupling tensor.

In turn, the spectral densities of the lipid motions are directed related to the \( ^2 \)H NMR relaxation rates [22, 64]. For correspondence to the relaxation measurements, the spectral densities of motion are Fourier transforms of the correlation functions \( G_m(\tau) \) are \( J_m(\omega) \) as given by

\[
J_m(\omega) = \int_{-\infty}^{\infty} G_m(t) \exp(-i\omega t) dt
\]

(2.45)
The angular frequency is denoted by $\omega$, and $t$ is the time. Her $G_m(t)$ is the auto-correlation function of the C–$^2$H bond fluctuations for an individual segment position of the flexible lipid molecules in the liqui-crystalline ($L_a$) state. As an example of how the spin-lattice relaxation times (rates) are measured, Figure 2.4 illustrates a series of partially relaxed $^2$H NMR spectra obtained for rhodopsin/POPC-$d_{31}$ recombinant membranes (1:100) mole ratio using an inversion-recovery pulse sequence. From the rate of the inversion recovery the $T_{1Z}$ relaxation time are determined, which are the inverse of the spin-lattice relaxation $R_{1Z}$. 
FIGURE 2.4. Representative partially-relaxed de-Paked $^2\text{H}$ NMR spectra of rhodopsin/POPC-\textit{d}_{31} (100:1) recombinant membranes at $T$-27 °C and 76.77 MHz.
CHAPTER 3
EXPERIMENTAL PROCEDURES

In what follows, the effects of various membrane lipids on rhodopsin in the dark and activated states were investigated. Following isolation of the receptor and its purification in the presence of different detergents, rhodopsin was subsequently recombined in different phospholipid bilayers. Incorporation of rhodopsin into phospholipid vesicles was performed by a dialysis method. The cationic detergent dodecyltrimethylammonium bromide (DTAB) was used for the $^2$H NMR studies, and a combination of the zwitterionic detergent octylglucoside (OG) and the anionic cholate detergent was used in the case of FRET and UV-Visible spectrophotometry. For the FRET experiments, rhodopsin was selectively labeled with Alexa 488 and Alexa 594 fluorophores, before purification of the protein. In the case of the UV-visible experiments incorporation of the ionophores CCCP and Valinomycin was performed after formation of the membrane vesicles by dialysis, with the goal of equilibrating the internal and external pH.

3.1. Rod Outer Segment (ROS) Membrane Preparation

Native ROS membranes were isolated from frozen bovine retinas (W. L. Lawson Co., Lincoln, NE) by the method of Papernaster [70] in a discontinuous sucrose density gradient. All manipulations were carried out in dim red light (15 W bulb, Kodak Safelight filter no. 1). The purified ROS appears as a dense red band at the 1.11/1.13 g mL$^{-1}$ interface in the sucrose step gradient after 1 h centrifugation at 25 000 rpm using and OTD-65 centrifuge (Sorvall-Kendro Laboratory Products; Newton, CT) equipped with a AH-627 rotor. The
purified ROS membranes are stored in 67 mM sodium phosphate buffer, pH 7.0. The $A_{280}/A_{500}$ absorbance ratio is typically 2.4 ± 0.1 as determined from the absorbance spectra scanning from 700 to 250 nm. A volume of 100 μL of the ROS membranes is taken and solubilized in 900 μL of a detergent buffer solution comprising 30% (v/v) Ammonyx LO (Stepan Co.; Northfield, IL), 100 mM hydroxylamine (Sigma Chemical Co.; St. Louis, MO), and 10 mM sodium phosphate buffer, pH 7.0. The purified ROS membranes were stored at −70 °C under argon, and thawed immediately before use.

3.2. Purification of Rhodopsin and Preparation of Recombinant Membranes

3.2.1. Rhodopsin Purification and Recombination using the Cationic Detergent Dodecyl-Trimethylammonium Bromide (DTAB)

In this procedure, rhodopsin was purified on a hydroxyapatite column prepared from 13 g of BioGel HTP, DNA grade (Bio-Rad, Richmond, CA) suspended in 100 mL of 15 mM sodium phosphate buffer, pH 6.8, with gentle swirling. The gel was allowed to settle for 10 min and the fines were removed 5 times repeatedly. It was then further resuspended with the same volume of phosphate buffer (buffer/gel), and degassed for column pouring [25]. The hydroxyapatite suspension was added to a 2.5 × 6.5 cm column (Bio-Rad Laboratories; Richmond, CA) which was then equilibrated with a solution of 100 mM DTAB detergent (Sigma Chemical Co.; St. Louis, MO) 15 mM of sodium phosphate, pH 6.6, and 1 mM DTT. Before loading the the column, the ROS membranes were incubated with DTAB for 1 h at 4 °C under dim red light followed by centrifugation for 20 min at 40 000 rpm, using a T-865.1 rotor (Sorvall-Kendro Laboratory Products; Newton, CT).

ROS membranes solubilized in DTAB were loaded to the hydroxyapatite column, and
the protein was eluted with a 300 mL in a linear gradient of 0.5 M sodium chloride for 5 h at 4 °C. The flow rate was 0.4–0.5 mL/min with the elution regulated by a peristaltic pump. The fractions comprised a volume of 2.0–2.5 mL, collected using an automated fraction collector (RediFrac; Amersham Biosciences; Piscataway, NJ). A total yield of 75–85% of rhodopsin loaded was eluted from the column. The purified rhodopsin gives a spectral ratio $A_{280}/A_{500}$ of 1.6–1.7.

3.2.2. Rhodopsin Purification and Recombination with Membrane Lipids Detergent

Octyl Glucoside (OG) and Anionic Cholate Detergent

Purification was carried out using an anti-rhodopsin monoclonal IgG type antibody from mouse myeloma cells (National Cell Culture Center; Minneapolis, MN), covalently attached to carbolink gel (Pierce; Rockford, IL) modified from the original rho-1D4-Sepharose 4B [71]. The ratio was 1 mL of 1D4 slurry (packed resin + buffer) per 1 mg of protein. This kind of column is selective for the visual receptor and the purpose of using it in this work was to design a procedure compatible with cell culture expression of visual pigments and their mutants (not applied in this work). The rhodopsin-binding capacity of the 1D4-Sepharose beads used in the past was 1.2 mg mL$^{-1}$. However, in this work, the 1D4-Carbolink yield was found to be 0.2 mg mL$^{-1}$ [72]. Evidently a much better yield could be obtained from hydroxyapatite (Section 3.2.1) or for example, Concanavalin-A. The 1D-4 method provides a high purity of rhodopsin with the ratio of absorbance ($A_{280}/A_{500}$) typically around 1.6–1.7. The 1D-4 slurry before the purification procedure was placed in a Falcon tube (BD Biosciences Discovery Labware; Bedford, MA) and centrifuged to remove the buffer solution, in which the resin was kept. The OG buffer was used to equilibrate the resin
before loading the protein.

Native ROS membranes with ratio $A_{280}/A_{300}$ of 2.4–2.6 were solubilized in buffer solution containing 1.5% β-octyl-glucoside (Anagrade®-Anatrace, Inc.; Maumee, OH); 125 mm KCl, 1 mM EDTA, and 25 mM MES, 25 mM HEPES, and 25 mM KOH, with a final pH of 6.7. One tablet of complete EDTA-free protease inhibitor (Roche; Penzberg, Germany) was added and the sample was incubated for 1 h at 4 °C. To remove any impurities, the samples were centrifuged for 1 h at 32 000 rpm (Beckman model XL-90 Ultracentrifuge) in a 4 ml polycarbonate tube, placed in a 45 Ti fixed angle rotor at 4 °C. The supernatant containing rhodopsin was carefully removed before loading the solution onto the 1D4 column.

Under dim red light, the solubilized ROS membranes were poured directly on the resin (ratio 1 volume resin/2 volumes of OG buffer). The wash steps were performed exactly the same as for labeling rhodopsin (Section 3.3.3). Elutions were performed by washing the resin five times with the same OG buffer containing the epitope nonapeptide (AnaSpec Inc.; San Jose, CA) (33 μL.mL⁻¹ buffer) in a purity > 95% as determined by reverse HPLC on a C18 column. The peptide contains the sequence NH$_2$-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala-COOH, and corresponds to the C-terminal residues 339–348 of rhodopsin. The ratio of the packed resin and volume of the buffer was 1:1. The resin needed to be very well mixed, carefully, until none was found at the bottom of the Falcon tube. Each elution required 1 h of incubation time followed by desktop centrifugation to separate the resin from the elutant. For the fifth and last elution the sample was left to incubate overnight. After each elution
step, the supernatant containing the purified rhodopsin was collected separately and the absorbance checked in order to monitor the course of the elution. All supernatant/elutant was mixed and further concentrated in the centricon tubes (MW cutoff of 10 000 kDa) to about 1 mL final volume. Finally, absorbance spectra were taken to calculate the concentration of rhodopsin necessary for preparation of the recombinant membrane vesicles (Section 3.5.1).

Recombinant membranes containing rhodopsin were then prepared by detergent dialysis. The cationic detergent DTAB used for the 2H NMR studies (Section 3.2.1) and also used previously for flash photolysis [73] but could not be applied because of the strong denaturation of the protein in this detergent at temperatures higher than 4 °C, and times > 12 h. For this work, it was necessary to have a stable protein at room temperature since some of the phospholipids used, such as DMPC, POPE, and di(24:1)PC, have their melting transition temperature around room temperature.

The concentration of rhodopsin was calculated to have the correct equivalents of the protein to be recombined with the desired phospholipids. Membrane vesicles were prepared by dialyzing the solubilized mixture of rhodopsin and the phospholipids in the nonionic detergent β-octyl-D-glucopyranoside (OG), present throughout the purification, together with the anionic sodium cholate detergent solution (cmc 15mM for OG, and 2.8 mM for cholate). This combination OG/cholate detergent has been used before for the preparation of rhodopsin vesicles [17, 72, 74, 75]. The main goal of using such a detergent solution in this work was to obtain a stable lipid/protein ratio over large variations of the lipid composition. Moreover, cholate has the advantage of forming a bicelle, thus preserving the lipid-protein
environment, and avoiding phase separation between the lipid and protein. On the other hand, the OG detergent is beneficial to solubilize rhodopsin by stabilizing the protein during purification. However it limits the lipid to protein ratio (70:1) [76], which is not appropriate for the lipid-protein molar ratio experiments conducted in this work.

Each phospholipid (Avanti Polar Lipids; Alabaster, AL) was prepared in 1.5% cholate detergent, MW =430 g mol\(^{-1}\) (Anatrace-Anagrade; Maumee, OH) with immediate addition of the dialysis buffer (25 mM MES, 25 mM HEPES, 25 mM KOH, 125 mM KCl, 1 mM EDTA, pH 6.7) to make a 1% stock solution with a final concentration of 10 mg mL\(^{-1}\). The precaution of adding the dialysis buffer immediately after adding the cholate solution to the phospholipid had to be taken since cholate is basic, and it can hydrolyze the phospholipid acyl chains. For the DOPE and POPE systems it was necessary to add 3% cholate final concentration for complete solubilization of the phospholipid. If the phospholipids were solubilized in chloroform, solvent was first evaporated under N\(_2\) and exposed to high vacuum to make a film of lipid. For the polyunsaturated lipids containing docosahexaenoyl acyl chains (DHA), a solution of 30 mg BHT solubilized in 1 mL ETOH (3% BHT in 1 \(\mu\)L of 200 proof ethanol) was prepared and added to the polyunsaturated phospholipids to prevent oxidation. Preparation of the polyunsaturated lipids was performed under inert argon atmosphere in a glove box. All phospholipids were kept at \(-20^\circ\)C until ready to be used. All solutions were clear, and colorless implying no sign of oxidation.

The volume of the phospholipid stock solution to be added to rhodopsin was calculated by the relation \(V_{\text{lipid}} = \frac{n\text{moles lipid}}{C_{\text{lipid}} (\text{mM})}\). Addition of the phospholipids to
rhodopsin was performed under red dim light, and the mixture was vortexed, blown with a stream of argon gas, and incubated for 1 h at room temperature. Solubilized rhodopsin/OG samples were supplemented with 1.5% cholate before they were mixed with phospholipids. Thereafter, the samples were transferred to a dialysis cassette Slide-A-Lyzer set; (Pierce Biotechnology; Rockford, IL) in a mini dialysis unit with a MW cut off of 10 kDa, containing 25 places. The dialysis cassette unit was placed immediately in a dialysis container filled with 200–300 mL degassed buffer, for each exchange (1mL rhodopsin/200-300 mL buffer). The dialysis container was then placed in a vacuum desiccator, with six cycles of evacuation followed by an argon purge, and tightly closed to avoid oxidation of the polyunsaturated phospholipids during dialysis. The dialysis buffer was exchanged with an interval of 6 h for 2 days. Only for the first exchange was the dialysis performed at room temperature (buffer not cooled), so that the vesicles started forming without the problem of gel phase formation for phospholipids with a $T_m$ around room temperature. The subsequent dialysis exchanges were performed with the buffer solution cooled at 4 °C.

After the final buffer exchange, the resulting proteoliposomes were harvested directly from the cassettes and used for the pH assays with no further centrifugation or sonication. That is to say, the vesicles were kept intact, exactly the way they were prepared, and used like that for all experiments. Vesicles containing polyunsaturated phospholipids were collected and immediately studied in the UV-visible spectrophotometry and FRET to avoid any risk of oxidation. The resulting proteoliposomes had a symmetrical distribution of rhodopsin in the membrane, and the vesicle size was around 100 nm. All vesicles
preparations had the same osmolarity inside and outside throughout the experiments, since the same dialysis buffer was used for all assays. The homogeneity of the recombinant membrane vesicles with respect to the lipid-protein molar ratio was qualitatively characterized by linear isopycnic sucrose density centrifugation [76] which provided a visualization of bands containing the vesicles at different positions in the sucrose medium. For all of the data reported in this dissertation a single predominant band due to recombinant membranes was obtained.

Phospholipids were chosen according to the acyl chain length, acyl chain type (including degree of unsaturation), head groups (PE and PC), and charge (POPS, DOTAP, POPG). The influence of the acyl chain length employed the homologous series 1,2-dimyristoleoyl-\textit{sn}-glycero-3-phosphocholine, designated di(14:1)PC; 1,2-dipalmitoleoyl-\textit{sn}-glycero-3-phosphocholine, indicated as di(16:1)PC; 1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine, denoted di(18:1)PC; 1,2-dieicosenoyl-\textit{sn}-glycero-3-phosphocholine, indicated as di(20:1)PC; 1,2-dierucoyl-\textit{sn}-glycero-3-phosphocholine, designated di(22:1)PC; and lastly 1,2-dinervonoyl-\textit{sn}-glycero-3-phosphocholine, denoted as di(24:1)PC. Studies of the acyl chain type employed the series egg phosphocholine (egg PC), 1,2 dimyristoyl-\textit{sn}-glycero-3-phosphocholine (DMPC); 1,2-didocosahexaenoyl-\textit{sn}-glycero-3-phosphocholine, indicated by di(DHA)PC; and 1-palmitoyl-2-docosahexaenoyl-\textit{sn}-glycero-3-phosphocholine (PDPC). Investigation of head groups type employed POPE/POPC (50:50) DOPE/DOPC (25:75), DOPE/DOPC (50:50), and DOPE/DOPC (75:25).

3.3. Labeling of Rhodopsin with Fluorescent Probes
3.3.1. Determination of Alexa Fluorophore-Rhodopsin Molar Ratio

Stock solutions of the reactive fluorophores were prepared by solubilizing 1 mg of the appropriate Alexa dye (Molecular Probes; Eugene, OR) in DMSO (sure-sealed bottle 50 mg with septum; Sigma; St. Louis, MO). The final concentration of the stock solution was 20 mg mL\(^{-1}\) (22 mM) which was stored in a desiccator at -20 °C until used. To find a proper rhodopsin/Alexa molar binding ratio, rhodopsin was labeled with Alexa 488 and Alexa 594 in a series of diluted solutions, taking different volumes (\(\mu\text{L}\)) of DMSO to a fixed volume of the fluorophore. The next step was to add the native ROS membranes to the diluted solutions and incubating them for 15 h at room temperature according to [77, 78]. Quenching of the Alexa 488 and Alexa 595 reaction was accomplished by adding 1 mM DTT (6 \(\mu\text{L}\)) and the sample was then cooled on ice. The ROS membranes were solubilized in 1.5% OG, 25 mM MES, 25 mM HEPES, 25 mM KOH, 125 mM KCl, 1 mM EDTA, pH 6.7, 125 mM NaCl (pH 6.8) before loading to the 1D4-resin for further purification (for see details in Section 3.3.3). This labeling procedure resulted in incorporation of a 0.99–1.3 molar ratio of the fluorophore per rhodopsin. The amount of rhodopsin used for the first titration trial was 1.08 nmol.

The molar ratio of Alexa 488 or Alexa 594 to rhodopsin solubilized in OG buffer was checked by the UV-visible absorbance from 250–700 nm range, and it was calculated from

\[
\frac{n_{\text{Alexa/rho}}}{A_{\text{Alexa/rho}}} = \frac{A_{\text{Alexa}} - A_{\text{Alexa (baseline)}}}{\Delta A_{\text{500}} - \Delta A_{\text{500um (baseline)}}} \times \frac{\epsilon_{\text{rho}}}{\epsilon_{\text{Alexa}}}
\]

where \(n\) is the molar ratio of fluorophore to rhodopsin. The absorbance of Alexa 488 or Alexa 594 was measured after the sample was fully bleached and the baseline absorbance
value subtracted. For rhodopsin, the absorbance was taken from the difference spectrum at 500 nm subtracted from the baseline at 650 nm. A value of $\varepsilon = 40000 \text{ M}^{-1}\text{cm}^{-1}$ was used for the rhodopsin molar absorption coefficient at 500 nm [79]. The corresponding values taken for Alexa 594 and Alexa 488 were $\varepsilon = 96000 \text{ M}^{-1}\text{cm}^{-1}$ and $\varepsilon = 72000 \text{ M}^{-1}\text{cm}^{-1}$, respectively (Molecular Probes; Eugene, OR).

3.3.2 Labeling of Rhodopsin in Native ROS Membranes with Alexa Dyes

Native ROS membranes solubilized in 67 mM sodium phosphate buffer, pH 7.0, containing 4 mg of rhodopsin were divided in two equal aliquots to be labeled with Alexa 595 or Alexa 488, separately. To the first aliquot, 10.69 $\mu$L of Alexa 594 (from the stock solution previously prepared) together with 5 $\mu$L of 50×complete-EDTA free (1 tablet / 1 mL H$_2$O) were added to ROS membranes. To the second aliquot, 7.1 $\mu$L Alexa 488 was added in 182 $\mu$L of ROS membranes supplemented with 4 $\mu$L of 50×complete-EDTA free tablet. During the stoichiometric assay it was found that Alexa 488 was more reactive than Alexa 594 (perhaps due its sulfonate group which gives a more hydrophilic character), which was the reason for using a smaller volume of Alexa 488 for labeling rhodopsin.

The two aliquots of ROS membranes containing the Alexa fluorophores were incubated in a water bath at 28 °C for 12 h. The labeling reaction was quenched by addition of 1 mM (final concentration) of the reducing agent DTT, keeping the samples on ice for 30 min. The sample was then re-solubilized in 8.5 mL OG buffer (1.5% OG; 25 mM MES, 25 mM HEPES, 25 mM KOH, 125 mM KCl, 1 mM EDTA, pH 6.7; 125 mM KCl; 1 mM EDTA) with continuous agitation for 1 h at 4 °C. After this period, the sample was
centrifuged at 32,000 rpm for 1 h to remove any impurities that could be present from the ROS membrane preparation. The samples were kept separately until ready for the membrane vesicle preparation (Section 3.3.2).

3.3.3. Purification of Rhodopsin-Alexa Conjugates

The optically transparent solution prepared as described above was poured into a 4 mL packed anti-rhodopsin 1D4 resin using a 15 mL Falcon tube for each of the rhodopsin-Alexa 488 and rhodopsin-Alexa 594 derivatives. The 1D4-resin was washed 5 times with 10 mL OG buffer, each time with inversion and incubation on a nutator for 20 min at 4 °C. After the incubation step, the resin was pelleted with a desk centrifuge, and the supernatant was removed with a plastic pipette without disturbing the resin. Elution of the purified rhodopsin was performed by resuspending the 1D4 resin with the same OG buffer, supplemented with the C-terminal nonapeptide (Section 3.2.2), followed by inversion and 2 h incubation on a nutator at 4 °C for each elution step. Before the final elution, the samples were incubated overnight. Details of the purification procedure are given in Section 3.3.3 as preparation of the samples for both the FRET and UV-visible studies was the same. The labeling procedure resulted in a binding ratio ranging from 0.99–1.3 and the absorbance ratio ratio $A_{280}/A_{500}$ was 1.7.

The samples containing either rhodopsin-Alexa 488 or rhodopsin-Alexa 595 were subsequently mixed to produce a 1:1 molar ratio for the vesicle preparation. The amount of protein in each aliquot was 0.452 mg. In order to have the same amount of each Alexa fluorophore to achieve a 1:1 molar ratio to rhodopsin, a volume of 401.8 μL was taken from
the rhodopsin Alexa 594 sample (from the total solution prepared) and added to the solution of rhodopsin-Alexa 488. The total amount of rhodopsin from this preparation was 0.904 mg.

Before adding the phospholipids to rhodopsin, 1.5% of cholate detergent (29.5 \( \mu \text{L} \) from the 30% stock) was added to the total volume of the purified rhodopsin sample. Subsequently, the sample was divided into small aliquots, according to the number of recombinants to be prepared. For this work, the total volume was divided in 28 aliquots containing a volume of 620.3 \( \mu \text{L} \) each, and 0.807 nmoles of rhodopsin each, ready to be mixed with the desired phospholipid.

3.4. FRET Spectrophotometric Assay

All of the FRET measurements were carried out in the dark (red light). Samples were placed in a 1 cm path length cuvette with constant stirring and equilibrated for 10 min at 20 \(^\circ\text{C}\) in the dialysis buffer (25 mM MES, 25 mM HEPES, 25 mM KOH, 125 mM KCl, 1 mM EDTA, pH 6.7, 125mM KCl, 1mM EDTA). Excitation spectra were recorded at a fixed emission wavelength of 620 nm. Conversely, emission spectra were acquired with the excitation wavelength set to 485 nm. The quartz cuvette was treated, following the manufacture instructions, with Aqua Sil (long chain alkane) to silanize the quartz. With this procedure the fluorescence signal was stabilized. An untreated cuvette gave rise to a time-reduction or decay of the fluorescence signal, due to the binding of the vesicles to the walls of the quartz cuvette.

For samples containing polyunsaturated lipids, viz. DHA, di(DHA)PC and PDPC, 2 mL of buffer were degassed and equilibrated in the cuvette to the temperature of the experiment (20
°C) for 5 min. Then the recombinant membrane sample was added to the equilibrated buffer, with further equilibration for 5 min in the cuvette. For each measurement and for all the phospholipids membranes studied, the volume of the buffer was 2 mL, and the concentration of rhodopsin in each assay mixture was 10 μg. The purpose of having such a dilute membrane suspension in the cuvette was to isolate the vesicles from each other. With a more dilute solution, the excitation beam affects a very small fraction of the sample, and together with constant stirring, the exposed region is constantly replenished by fresh rhodopsin. Consequently, this strategy avoided bleaching of rhodopsin as would the case if it was exposed to the beam longer. In preliminary work a smaller volume of 200 μL was used, but this was less than ideal due to constant stirring of the small cuvette.

3.5. UV-Visible Spectrophotometric Experiments

The present experimental procedure had the goal of reducing the protein amount compared to an absorbance-based time-resolved flash photolysis analysis of the photoreceptor states [12]. In addition we wanted to minimize the artifacts common to absorbance measurements containing samples with light scattering and baseline instability, i.e. following addition of reagents used for the UV-visible spectrophotometric assay.

3.5.1. UV-Visible Spectral Measurements

Immediately before proceeding with the UV-visible scanning measurements, a defined amount of freshly prepared CCCP/Valinomycin was introduced to the prepared proteoliposomes. Stock solutions of both the toxic ionophores carbonyl cyanide 3-chlorophenylhydrazone (CCCP; MW=204.62 g mol⁻¹) (Sigma; St. Louis, MO) and
Valinomycin (MW = 908.97 g mol⁻¹) (Sigma; St. Louis, MO) were prepared in DMSO, whose final concentrations were 100 mM (yellow solution) and 10 mM (clear solution), respectively, they were kept at −20 °C until used. For 10 μM of rhodopsin, the final concentration of CCCP was 1 μM; whereas, for Valinomycin the concentration was 0.1 μM (much higher activity).

The two ionophores were introduced to facilitate the diffusion of hydronium ions (H₃O⁺) and potassium ions (K⁺) through the membrane bilayer. This helped to create a fast pH equilibrium between both sides of the vesicles each time the pH was changed for the titration curve measurements. This method has been used for membrane potential studies generated by plasma membrane H⁺-ATPase reconstituted vesicles [80, 81]. For the present data shown in [Chapter 5], a freshly diluted solution of each ionophore was prepared from the stock solution by taking 2.5 μL of each ionophore and diluting it with 10 mL of DMSO.

For the UV-visible absorption assay, the samples were placed in a 1-cm pathlength quartz cuvette, having a narrow dark window holding 90 to 130 μL of total solution. The sample solution was temperature controlled with a water bath set at 20 °C. A volume of freshly prepared hydroxylamine, pH 7.0 (3.5 M stock solution, pH 7.0) was added to the solubilized rhodopsin in OG detergent to reach a final concentration of 50 mM. The absorption spectrum was scanned from 700–250 nm in 50 nm steps, first in the dark, then after bleaching for 30 s with a fiber optics illuminator; (Fiber-Lite A-200 > 490 nm, Dolan Jenner Industries Inc.; Lawrence, MA) equipped with a FY-32-52 yellow filter (tempered; 25 mm × 2.5 mm). The concentration of rhodopsin was calculated from the absorption difference
spectrum taking the molar extinction coefficient at 500 nm as 40,600 M\(^{-1}\)cm\(^{-1}\).

It is important to note that all samples employed in FRET and UV-visible methods were studied under the same conditions, and the same buffer was used throughout the experiments.

3.5.2. Meta I–Meta II Equilibrium of Rhodopsin

In order to obtain functional data regarding membrane-deformation in the presence of different phospholipids, the recombinant membranes were assayed using a Perkin-Elmer-Lambda 19 spectrophotometer, containing a photomultiplier detector placed immediately adjacent to the cuvette. In this way the light scattered was decreased due to the particulate nature of the sample. Experiments were performed at 20 °C using a water bath system connected with the thermostated cuvette. Each assay mixture contained 10 μg of rhodopsin. A complete spectrum was obtained in 39 s. The equilibrium between meta I and meta II was monitored from pH 6 to 8, with addition of small volume of a KOH standard solution (1.0 N) to reach the desired pH. Volumes of 1, 2, 3, and 4 μL were added to reach approximately pH 6.5, 7.0, 7.5, and 8.0, respectively.

Initially, 130 μL of recombinant membranes containing rhodopsin together 2.5 μL of CCCP/Valinomycin and 5.93 μL of HCl were loaded into a dark narrow-window cuvette with equilibration of 5 min at 20 °C. Addition of HCl assured that initial pH of the sample was 6.0, which is the pH that promotes formation of meta II. Next, four spectra were recorded for each pH value. The first spectrum was taken in the dark state of rhodopsin. The sample was then bleached for 15 sec with the fiber optics illuminator having a yellow filter.
(λ>490 nm). Immediately thereafter, a small volume of 1.0 N KOH standard solution was added directly into the cuvette with rapid stirring, immediately followed by recording of the second spectrum. An aliquot of 30 μL was removed from the cuvette to be later checked for the experimental pH. To the remaining sample (100 μL), 100 mM of freshly prepared hydroxylamine (2 μL from 3.5 M stock, pH 7) was added to promote retinal oxime formation. The cuvette containing the sample was then removed from the spectrophotometer, and it was incubated in a water bath at 37 °C for 5 min to accelerate the reaction and the subsequent decay of meta III. This step assured that the hydroxylamine had reacted completely and retinal oxime was completely formed. After this time, the cuvette was returned to the instrument and the sample was re-equilibrated for an additional 5 min at the experimental temperature 20 °C, and the third spectrum was acquired. For the fourth and final spectrum, the sample was fully bleached for 1 min.

3.6. Solid-State Deuterium NMR Experiments

The lipid studied in this work was 1-perdeuteriopalmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, designated as POPC-\(d_{31}\) (Avanti Polar Lipids; Alabaster, AL), in which the \(sn\)-1 acyl chain was perdeuterated. Rhodopsin was isolated from rod outer segment (ROS) membranes prepared from bovine retinas (W. L. Lawson, Co., Lincoln, NE), and was purified, and recombined with POPC-\(d_{31}\) (1:100) molar ratio by detergent dialysis as described in Section 3.2.1) [12]. Recombinant membranes were concentrated by ultracentrifugation at 100,000 ×g for 1 h, and resuspended in a small volume of 67 mM sodium phosphate buffer, pH 7.0, prepared from \(^2\)H-depleted H_{2}O (Aldrich, WI), containing
1 mM EDTA. Samples were stored under argon atmosphere at -80 °C, and were used directly for the NMR experiments. The absorbance ratio $A_{280}/A_{500}$ was 1.7 ± 0.1 for the recombinant membranes, and 2.4 ± 0.1 for the ROS native membranes.

Rhodopsin/POPC membranes used for the $^2$H NMR experiments were characterized in terms of their regenerability, viz. 11-cis-retinal binding to the apoprotein opsin, as well as formation of meta II after flash photolysis [12]. Photoactivation of rhodopsin in the recombinants was determined by titration of the 478 nm absorbance from flash-photolysis spectroscopy between pH 5 and pH 8 and 27 °C, as described [8, 12]. Approximately equal amounts of the meta I and meta II photolysis products were obtained at pH 7, in agreement with previous work [82]. Moreover, the regeneration of opsin in the POPC membranes with 11-cis-retinal was comparable to the isolated native ROS membranes (95±5%) [8].

Rhodopsin recombinant membranes were analyzed for a homogenous protein/lipid ratio by isopycnic sucrose density ultracentrifugation, resulting in a single sharp band for the rhodopsin/POPC membranes. The protein/lipid ratio of these bands was determined by optical determination of the rhodopsin concentration and lipid phosphorus analysis, giving a molar ratio of 1:100 ± 10, in agreement with earlier work on rhodopsin/egg PC membranes. A rhodopsin-free sample of POPC-$d_{31}$ was prepared as a control following the same dialysis procedure. Alternatively, multilamellar lipid vesicles were prepared from ~ 30 mg of POPC-$d_{31}$ in the above buffer (66 wt %) under argon and subjected to multiple freeze-thaw-vortex cycles (cooled with liquid nitrogen and heated to 25–30°C, above the phase transition of the sample), to ensure complete dispersion of the lipid [83]. All samples were checked after the
NMR experiments by thin layer chromatography (silica gel G, 250 microns, Analtech, Inc. Newark, DE) with a solvent mixture comprising CH$_3$Cl/MeOH/H$_2$O (65:25:4). They were visualized by exposure to I$_2$ vapor and by spraying the plates with 40% H$_2$SO$_4$ in ethanol, followed by charring on a hot plate. The single spot on a TLC plate revealed no contamination or lipid degradation.

All POPC experiments were performed with a modified Bruker AMX-500 spectrometer, operating at a magnetic field strength of 11.7 T. The deuterium NMR experiments used the solid quadrupolar echo, with the sequence (π/2)$_x$-$\tau$-(π/2)$_y$-$\tau$-acquire, followed by Fourier transformation of the signal. A home-built $^2$H NMR probe was used, having an 8-nm transverse solenoidal radio frequency coil design, together with high voltage capacitors (Polyflon; Norwalk, CT). A kilowatt radio frequency boost amplifier (Model Tempo 2006, Henry Radio, Los Angeles, CA) was used in series with the spectrometer output to enable 90 ° pulse durations around 4 $\mu$s for the 8 mm radio frequency coil. Typical spectral acquisition involved an interpulse delay ($\tau$) of 40 $\mu$s, a dwell time of 2 $\mu$s (spectral width of ± 250 kHz), and collection of 2048 data points. Typically 600–2400 transients were collected, apodized by exponential multiplication (100 Hz line broadening), and Fourier transformed beginning at the maximum of the quadrupolar echo. Recycle times were 1 s, data from both quadrature channels were used, and the $^2$H NMR spectra were not symmetrized. The sample temperature was measured before and after each measurement with a thermistor inserted directly above the radio frequency coil, and varied by less than 0.5 °C.
CHAPTER 4

FLUORESCENCE RESONANCE ENERGY TRANSFER STUDIES OF MEMBRANE LIPID EFFECTS ON RHODOPSIN ASSOCIATION

In this aspect of the research, fluorescence resonance energy transfer was used to investigate the possibility of association of rhodopsin in membranes containing different lipids, in which the molar ratio of lipid to protein, acyl chain length, degree of chain unsaturation, and head group composition were all varied. The experimental data were the result of energy transfer between two fluorescent probes, the donor Alexa 488 and the acceptor Alexa 594. Both fluorophores were used to label rhodopsin separately, and the labeled proteins were mixed at a 1:1 molar ratio of rhodopsin-Alexa 488 to rhodopsin-Alexa 594. Measurements were conducted for rhodopsin in the dark state at 20 °C and pH 6.7. It was discovered for the first time that the lateral association of rhodopsin in the membrane was affected by the number of lipids per protein molecule, the acyl chain length, the chain melting transition temperature, polar group head groups such PE, and by the degree of the acyl chain unsaturation.

4.1. Results

4.1.1. Characterization of Alexa 488 and Alexa 594 Fluorophores

Prior to these experiments, the fluorescence spectra of rhodopsin-Alexa 488 solubilized in octyl glucoside micelles (OG) were recorded in a buffer solution containing 1.5% OG buffer, 25 mM MES, 25 mM HEPES, 25 mM NaOH, 125 mM NaCl, and 1 mM EDTA. The resulting fluorescence spectra, including both excitation and emission
intensities, are illustrated in Figure 4.1. The inset indicates the UV-visible absorption spectra of the sample, where spectrum (1) shows the absorption of the fluorophore-labeled rhodopsin in the dark state, (2) is the bleached spectrum after illumination of rhodopsin, and (3) is the difference spectrum. From the UV-visible absorption spectra, the stoichiometry of the rhodopsin-fluorophore derivative was calculated from (Eq. 3.1) and was found to be 0.99–1.41. The excitation spectrum was scanned in the range of 275–500 nm in 5 nm steps (slit width 0.15 nm) at a fixed emission wavelength of 520 nm (slit width of 5 nm) with an integration time of 0.5 s. The emission spectrum was scanned from 505–700 nm (slit of 5 nm) at a fixed excitation wavelength of 485 nm. As shown in Figure 4.2 the same procedure was repeated for the rhodopsin-Alexa 594 derivative. Here the excitation spectrum was recorded from 325–600 at a fixed emission wavelength of 620 nm, and the emission was scanned from 600–800 nm with a fixed excitation wavelength of 575 nm. In addition to the primary excitation, for both fluorophores there is an additional excitation in the UV-visible region, at around 315–340, and 340–400 for Alexa 488 and Alexa 594, respectively.

To substantiate that the emission intensities were not due to overlap between the two fluorophores, a control mixture of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 at a 1:1 ratio was studied in the dark in the presence of the octylglucoside detergent, as shown in Figure 4.3. One can observe a very negligible cross-talk or overlap of the Alexa 488 and Alexa 594 emission, as evident from a very small peak near 620 nm, which did not affect the analysis of the subsequent data. Solubilization of rhodopsin in OG micelles significantly lowers the effective protein concentration, reducing the proximity of fluorescent-labeled
FIGURE 4.1. Fluorescence spectra of rhodopsin labeled with Alexa 488 in octyl glucoside micelles. The sample contained 1.5 % OG, 25 mM MES, 25 mM HEPES, 25 mM NaOH, 1 mM EDTA, and 125 mM NaCl, and was in the dark state at 20 °C, pH 6.7. The excitation spectrum (left) was recorded over the range of 275–500 nm at a fixed emission wavelength of 520 nm. Emission (right) was scanned from 505–700 nm at a fixed excitation wavelength of 485 nm. The inset shows the UV-visible absorption spectra of rhodopsin-Alexa 488 in the dark (1), fully bleached (2), and their difference (3).
FIGURE 4.2. Fluorescence spectra of rhodopsin labeled with Alexa 594 in octyl glucoside micelles. The preparation included 1.5% OG, 25 mM MES, 25 mM HEPES, 25 mM NaOH, 1 mM EDTA, and 125 mM NaCl, and was in the dark state at 20 °C, pH 6.7. The excitation spectrum (left) was scanned from 325–600 nm with a fixed emission wavelength of 620 nm. The emission spectrum (right) was recorded from 605–800 nm at constant excitation wavelength of 595 nm. The inset presents the UV-visible absorption spectra of rhodopsin-Alexa 488 in the dark (1), fully bleached (2), and their difference (3).
FIGURE 4.3. Fluorescence spectra of a control mixture of separately labeled rhodopsin-Alexa 488 and rhodopsin-Alexa 594. The sample comprised a 1:1 molar ratio of the two labeled rhodopsin derivatives in the dark state at 20 °C, pH 6.7, and contained 1.5 % OG, 25 mM MES, 25 mM HEPES, 25 mM NaOH, 1 mM EDTA, and 125 mM NaCl. This important control experiment demonstrates the absence of FRET in a detergent environment. The emission spectrum (dotted line) of the Alexa 488 derivative was measured with 485 nm excitation; whereas the excitation spectrum (solid line) of the Alexa 594 derivative was obtained by monitoring the emission at 620 nm. Note that the emission and excitation fluorescence spectra of the short and long wavelength fluorophores are nearly unaltered in the mixture.
rhodopsin molecules, as compared to the higher local rhodopsin concentration in a lipid bilayer. In this control experiment, the emission spectrum (left) of Alexa 488 was measured at a fixed excitation wavelength of 485 nm and the excitation spectrum was due to Alexa 594 measured at fixed emission wavelength of 620 nm. These measurements were crucial as a control to show a good choice of the donor-acceptor pair of fluorophores.

In what follows, the experimental data were analyzed in terms of the emission spectra of both fluorophores. All fluorescence spectra were normalized such that the 515 nm peak had a value of unity. Thus, the analysis simply amounts to observing the normalized fluorescence emission intensity at 620 nm. The higher the intensity at 620 nm, the more energy is transferred from Alexa 488 to Alexa 594 and vice-versa. Since the fluorophores are covalently attached to the protein, an increase or decrease of the emission intensity indicates proximity between two labeled rhodopsin molecules in the membrane. In this way, one can see directly how the different lipid environments affect the lateral organization of rhodopsin in the membrane. Moreover, the two-dimensional density of the acceptor molecules in the membrane is accessible, as discussed subsequently in Chapter 7.

**4.1.2. Influences of the Membrane Lipid-Protein Molar Ratio in Recombinant Membranes**

Experiments were conducted for a series of lipid-protein ratios using POPC, PDPC, and POPE reconstituted membranes, with rhodopsin to lipid molar ratios of 1:50, 1:100, 1:200, and 1:400. Effectively, the results pertain to dilution of rhodopsin within the membrane, and they are summarized in Figure 4.4. Parts (a) and (b) show a gradual decrease
FIGURE 4.4. FRET experiments for a mixture of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 in recombinant membranes at different lipid-protein molar ratios. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, at pH 6.7 and 20 °C. Recombinant membranes comprising (a) POPC, (b) PDPC, and (c) POPE were tested for rhodopsin association at lipid-protein molar ratios of 50:1, 100:1, 200:1, and 400:1 in order of decreasing intensity at 620 nm. Normalized emission spectra were acquired with the excitation wavelength set to 485 nm and were recorded over the range of 500–800 nm. The peak at 517 nm is due to the Alexa 488 fluorophore, and the peak at 620 nm shows energy transfer to the Alexa 594 fluorophore. Note that the POPE membranes are least affected by changes in the lipid to protein molar ratio.
in the emission intensity at 620 nm of the normalized spectra for rhodopsin in the POPC and PDPC membranes, respectively, as the lipid to protein molar ratio increased. The dispersal of the protein in POPC was more evident than in the PDPC membranes, as evident by comparing the emission intensity at 620 nm in panel (a) to panel (b). Although PDPC also promotes dispersal of the protein, one can say that PDPC favors greater association of rhodopsin as compared to POPC.

In contrast, part (c) of Figure 4.4 shows that for membrane samples containing POPE the emission intensities at 620 nm were higher, and changed less with increasing the amount of lipid. This implies that rhodopsin is more associated in the POPE membrane environment. The reason relies on the fact that at the experimental temperature (20 °C) POPE is found in the gel phase ($T_m=24^\circ$C). In consequence, lateral phase separation of rhodopsin is promoted from the gel-phase domains. A similar behavior of rhodopsin in a gel-phase membrane has been shown previously for DMPC recombinant membranes ($T_m=24^\circ$C) [84].

4.1.3. Effect of Acyl Chain Length in Membrane Recombinants

Fluorescence emission spectral results for rhodopsin recombined with members of the acyl chain length series $\text{di}(14:1)\text{PC}$, $\text{di}(16:1)\text{PC}$, $\text{di}(18:1)\text{PC}$, $\text{di}(20:1)\text{PC}$, $\text{di}(22:1)\text{PC}$, and $\text{di}(24:1)\text{PC}$ at a 1:100 lipid/protein ratio are presented in Figure 4.5. The lowest FRET intensity was observed for the system containing rhodopsin in $\text{di}(20:1)\text{PC}$ recombinant membranes, indicating that the hydrophobic interface of rhodopsin is well solvated by phospholipids with an acyl length of 20 carbons. The highest FRET values were observed
FIGURE 4.5. Normalized fluorescence emission spectra for a mixture of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 in recombinant membranes having different acyl chain lengths. The lipid-protein molar ratio was 100:1 and the samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, at pH 6.7 and 20 °C. The excitation wavelength was fixed at 485 nm and the emission spectra were recorded over the range of 505–700 nm. The emission intensity at 620 nm corresponds to the FRET index, which increases with the degree of protein association in the membrane. Phosphatidylcholine acyl chain lengths used in this series (from top to bottom) were: di(24:1)PC, di(14:1)PC, di(16:1)PC, di(18:1)PC, di(22:1)PC, and di(20:1)PC. The membrane environment corresponding to the lowest FRET index was found for 20 carbons, indicating that for this acyl chain length the protein is least associated.
for rhodopsin in di(24:1)PC membranes, with 24 carbons length, and for di(14:1)PC with the shortest chains investigated. The di(24:1)PC membranes are in the gel phase at the experimental temperature of 20 °C, and the FRET intensity is enhanced for the same reason as for POPE. Note that there is a decrease in FRET intensity, and hence the degree of protein association as the length of the acyl chains increases, from 14 to 20 carbons. However, chains longer than 20–22 carbons led to greater association of the protein, implying that there is an optimal length for matching to rhodopsin. When the protein is well-solvated by the membrane lipids, association is minimized as was observed for di(20:1)PC recombinant membranes.

In what follows, the term FRET index refers to the fluorescence intensity at 620 nm divided by the intensity at 515 nm of the normalized emission spectra, $I_{620 \text{ nm}} / I_{515 \text{ nm}}$ (acceptor emission over donor emission). This is further described in (Section 4.1.4). A summary of the FRET index values obtained for different values of the lipid-protein molar ratio and the acyl chain length is provided in Figure 4.6. In part (a) the changes in FRET index are shown for rhodopsin in POPC, PDPC, and POPE recombinant membranes. It is clear the FRET index changes as the lipid to protein ratio increases, which is most pronounced for rhodopsin in the POPC system. A similar reduction in FRET intensity with increasing lipid-protein ratio was also observed for PDPC system. However, the FRET index values with increasing ratio of lipid to rhodopsin were higher compared to the POPC recombinant membranes. This implies that rhodopsin in PDPC membranes having polyunsaturated DHA acyl chains is more associated. By contrast, part (a) of Figure 4.6
FIGURE 4.6. Relative FRET index plotted against lipid-protein molar ratio and acyl chain length variations. (a) Comparison of the FRET index for rhodopsin in POPC, PDPC, and POPE recombinant membranes, showing variations in the degree of protein association due to the lipid-protein molar ratio. (b) Comparison of FRET index for rhodopsin in phosphatidylcholine recombinants with different acyl chain lengths. The results show that rhodopsin associates least in the presence of the di(20:1)PC phospholipids; hence this acyl length matches best to the hydrophobic interface of rhodopsin in the dark state.
shows that for the POPE membrane recombinants the association of rhodopsin was nearly independent of the lipid-protein ratio.

Note that for all three systems, at a 50:1 lipid-protein molar ratio the FRET index values are effectively the same. In the case of POPE membranes, rhodopsin associates at a 1:50 molar ratio nearly to the same extent as for POPC and PDPC, even though the membranes are in the gel state. This can be explained by the necessity of the protein being surrounded by a minimum amount of lipids required for its solvation, corresponding to a 1:50 molar ratio, where the lipids must be in the fluid phase. Thus, in the case of membrane lipids favoring the gel phase, even under strong protein association the boundary lipids around the protein-rich domains are in a fluid-like state. By adding more lipids to a gel-state membrane, the fluid boundary lipid region surrounding the protein remains unaffected. The membrane will take up the introduced lipids into the bulk region, which are in the gel phase with frozen acyl chains. Such an onset of phase separation might explain the jump in the FRET index for rhodopsin in POPE membranes on going from a 1:50 to a 1:100 lipid to protein ratio. Nonetheless, adding more lipids to the environment further enhances the segregation of the lipid between its fluid and gel phase, so that the FRET index remains largely unchanged for the POPE recombinant systems.

Now, in part (b) of Figure 4.6, the FRET index data for rhodopsin are shown according to the variation of the acyl chain length. As can be seen, there is a progressive reduction in the FRET index as the acyl length (bilayer thickness) is increased, reaching a minimum at 20 carbons, beyond there is a further enhancement. From these results, the
smallest FRET index values correspond to di(20:1)PC recombinant membranes, thus indicating 20 carbons as the optimal acyl chain length to solvate the hydrophobic interface of rhodopsin in the dark state. The comparison between parts (a) and (b) of Figure 4.6 provides an interesting aspect when it comes to understanding the experimental results for the \(^2\)H NMR order parameter profiles, to be discussed in Chapter 6 (\textit{vide infra}).

An additional comparison of the effects of the membrane properties on the organization of rhodopsin is indicated in Figure 4.7. Here the results show how the emission spectral intensities change for rhodopsin in recombinant membranes with DMPC, eggPC, and DOPE/DOPC (25:75, 50:50, and 75:25 molar ratios). It can be observed that rhodopsin in DMPC membranes is strongly associated due the lipids being in the gel state. At this point, we note that three different recombinant membranes have been used to investigate the influence of the chain melting transition temperature, \textit{viz.} POPE, di(24:1)PC, and now DMPC. The purpose was to use them as the controls for the influences of the gel-phase lipids [15] on protein association, and for the later activation work presented in Chapter 5. Figure 4.7 shows that compared to rhodopsin in gel-phase DMPC bilayers, a dramatic reduction in the FRET index is evident when it is recombined with eggPC, clearly showing the dispersal of the protein in liquid-crystalline membranes. Lastly, for rhodopsin in DOPE/DOPC reconstituted membranes, the presence of the PE head group resulted in even less association. This finding indicates that DOPE/DOPC membranes provide a lipid environment highly conducive to dispersal of rhodopsin, which may account for the effects of DOPE on rhodopsin activation [12].
FIGURE 4.7. Normalized fluorescence emission spectra for a mixture of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 in DMPC, egg PC, and DOPE/DOPC recombinant membranes. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, at pH 6.7 and 20 °C. The excitation wavelength was fixed at 485 nm and the emission spectra were recorded over the range from 505–700 nm. Emission spectra are shown for DMPC (100:1); eggPC (100:1); and DOPE/DOPC at (25:75), (50:50), and (75:25) lipid molar ratios in order of decreasing intensity at 620 nm. The DMPC recombinant showed greater rhodopsin association, as the experimental temperature was below the lipid phase transition ($T_m \approx 24$ °C).
For the next set of experiments, the effect of POPE in membrane mixtures containing POPC was examined in order to further investigate the influence of the lipid phase state. The results are shown in Figure 4.8 for rhodopsin in recombinant membranes containing POPE, POPE/POPC (50:50), and POPC in recombinant membranes. Recall that pure POPE is found in the gel phase at the experimental temperature (20 °C), which leads to substantial energy transfer and a high degree of protein association in the recombinant membranes. The inclusion of POPC in the recombinant membranes leads to a depression of the phase transition temperature of POPE, and the association state of rhodopsin becomes similar that of POPC in the fluid phase. It is important to recognize that the mixture POPE/POPC is in the fluid phase to enable comparison to DOPE/DOPC recombinant membranes, as these mixtures were used to investigate the effect of the spontaneous curvature on rhodopsin activation.

Further, the effect of unsaturation on protein association was also investigated by comparing rhodopsin in recombinant membranes with PDPC, POPC, and di(DHA), each system having a 1:100 lipid to protein molar ratio. As it can be seen in Figure 4.9, the emission intensity of rhodopsin in the di(DHA)PC recombinant membranes at 620 nm was lower than for the PDPC systems (only one DHA acyl chain). Thus there is a smaller FRET index value for rhodopsin in the di(DHA)PC recombinants compared to the PDPC membrane recombinants. The presence of two polyunsaturated acyl chains is likely to make the membrane softer, consequently allowing rhodopsin to be more dispersed within the bilayer. In the case of PDPC, there are two different acyl chains (16:0/22:6), so that the
FIGURE 4.8. Normalized fluorescence emission spectra for a mixture of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 in POPC, POPE, and POPE/POPC recombinant membranes. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, at pH 6.7 and 20 °C. The excitation wavelength was fixed at 485 nm and the emission spectra were acquired over the wavelength range of 505–700 nm. The emission spectra of the POPE, POPE/POPC (50:50 molar ratio), and POPC recombinant membranes (top to bottom) demonstrate that rhodopsin association is higher in pure POPE, since the measuring temperature corresponds to the gel phase.
FIGURE 4.9. FRET experiments showing normalized emission intensities for a mixture of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 in PDPC, POPC, and di(DHA)PC recombinant membranes (top to bottom). The lipid-protein molar ratio was 100:1 and the samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, 125 mM KCl, at 20 °C, pH 6.7. The excitation wavelength was fixed at 485 nm and the emission spectra were recorded in the range of 505–700 nm. Comparison of the emission intensities indicates the effect of polyunsaturated DHA chains. The presence of two polyunsaturated acyl chains is likely to make the membrane softer, allowing rhodopsin to be better dispersed.
flexibility of the DHA chain (22:6) is decreased by the presence of the palmitoyl chain (16:0).

In summary, the present results have investigated the association state of rhodopsin by FRET index experiments using rhodopsin that was labeled with Alexa fluorophores. The dispersal of the protein in the different membrane recombinants was interpreted based on the changes in the emission spectral intensity of the acceptor Alexa 594 at 620 nm. Thus the FRET index values could be taken as indication of the organization of rhodopsin. This novel approach was further combined with the data from UV-visible spectrophotometry [Chapter 5] to provide a new understanding of how the lateral organization of rhodopsin is related to the mechanism of its activation.

4.1.4. Data Analysis and Reduction

The resonance energy transfer fluorescence (FRET) measurements were performed using a SPEX-Fluorolog τ3 spectrofluorometer (Horiba Jobin Yvon Inc.; Edison, NJ) fitted with a grating excitation and emission monochromator. The instrument is equipped with a 450 W high pressure xenon arc lamp and with a R928 photomultiplier emission detector. The emission spectra were acquired with the excitation beam set to a fixed wavelength of \( \lambda = 485 \text{ nm} \) (slit width 0.15 nm) and scanning the emission monochromator over the range of 505–800 nm (slit width 5 nm). The excitation spectra had a fixed emission wavelength set to 620 nm (slit width 5 nm), scanning the excitation range from 325–600 nm with increments of 5 nm and a slitwidth of 0.15 nm. The integration time in both cases was 0.5 s.

The FRET index approach [61, 85] taken in this work considers the definition:
FRET index = \frac{\text{emission of acceptor}}{\text{emission of donor}} \quad (4.1)

Intensity values were taken at 515 nm, the emission maximum of the donor Alexa 488, and at 620 nm, the emission maximum of the acceptor Alexa 594. Emission spectra were recorded with the donor Alexa 488 excitation wavelength set at 485 nm in the presence of both fluorophores. In this simple FRET implementation, it is assumed that the acceptor is not excited at the donor excitation wavelength, and that there is no cross talk between the acceptor and donor, and also that the molar ratio of donor to acceptor is held fixed. These requirements are met in the case of the present experiments, as can be seen from Figures 4.1–4.3. The advantage of this simple method is that it requires only one sample in which both fluorophores are present. One is thus able to screen a large number of different lipid recombinants to give a semi-quantitative determination of the degree of rhodopsin association in the membrane.

4.2. Discussion and Conclusions

In this work, fluorescence resonance energy transfer (FRET) was used to provide a new way to analyze the lateral organization of proteins in membranes, viz., association of different rhodopsin molecules in a variety of different phospholipid environments. The manner in which rhodopsin associates in different membranes was directly observed in this work from the FRET experiments. Our results suggest that the protein can phase separate or association in membranes containing one single type of phospholipid, and can segregate into microdomains even in the fluid phase.

The fluorescence emission spectra of rhodopsin in the dark state in recombinant
membranes comprising POPC, PDPC, or POPE at 20 °C with increasing lipid-protein molar ratio (Figure 4.4) showed clear and dramatic differences in the degree of rhodopsin association. In particular, it was discovered that POPC promoted a better environment for dispersal of rhodopsin as compared to PDPC or POPE at the same lipid-protein molar ratio (100:1). However, for POPE membranes one needs to account for the effect of the temperature of the experiment, which is lower than its gel to liquid-crystalline phase transition temperature (~ 24 °C). Thus the bulk of the POPE lipids in the recombinant membranes used in the FRET assay are in the gel phase. As the lipid melts in a cooperative way, the effect of having a membrane in a gel phase is to induce a strong association of the protein. However at 50:1 lipids per rhodopsin there are a fewer lipids surrounding the protein, and the boundary lipids are most likely in the fluid phase. If only a few lipids are present it is difficult for a patch of lipid to form and then to freeze. As more lipids are added to a gel-phase membrane, as in the case of the POPE recombinant systems, one would expect that the lipids would be equally redistributed around the protein. Nonetheless the boundary lipids are kept the same, so that the additional lipids phase separate from the small fluid patches surrounding the protein. In the end, they do not affect the FRET index and the protein association as seen for the POPE results.

From the results probing hydrophobic mismatch, the influences of the acyl chain length showed clearly that the most dispersed or least aggregated environment for rhodopsin is promoted by recombinant systems containing a 20-carbon length. An increase in the FRET index values was shown for the shortest and longest acyl chain systems, di(14:1)PC
and di(24:1)PC, respectively. The increase in FRET emission intensity for the recombinants of rhodopsin with DMPC, having the shortest acyl chain length (14:0) and with di(24:1)PC, having the longest, is due to the fact that both systems were studied in the gel phase.

One interesting finding from the FRET experiments is that both of the systems POPC and PDPC were clearly distinguished with respect to the protein association in the membranes. The comparison of the FRET index results for the lipid-protein ratio with the data for the acyl chain length experiments (Figure 4.6) was done with the purpose of providing more evidence for the intrinsic effects of the lipid composition on rhodopsin association. In this way, one could examine the possibility that the differences in the FRET index between POPC and PDPC as a function of the lipid-protein molar ratio would have come from the effect of the acyl chain length. In order to do so, the FRET index value was taken for the POPC recombinant membranes and PDPC at a lipid (1:100) protein-ratio, and these values were compared to the FRET index from the acyl chain results.

Refering back to Figure 4.6, it can be seen that POPC at a 1:100 molar ratio in part (a) corresponds to the FRET index of the di(18:1)PC recombinant membranes in part (b), which differs from the optimal acyl length of 20 carbons. Likewise, at a 1:100 molar ratio PDPC was found to be comparable in its FRET index to an acyl length of 15 carbons. But in their average acyl chain lengths POPC and PDPC are about the same [83]. Therefore, if the acyl chain length would have played a role in the association of rhodopsin, the FRET index for POPC and PDPC would have had the same values. Clearly, this is not the case, suggesting that rhodopsin has an intrinsically greater propensity to associate in PDPC, perhaps due to
the acyl polyunsaturation. On the basis of these analyses, one can conclude that PDPC brings
rhodopsin together in the dark state. This finding may be relevant to understanding some of
the reasons why polyunsaturated DHA chains are present in such a high amount in the rod
disk membranes.

Paradoxically, by comparing the 1:100 molar ratio systems for the PDPC, POPC, and
di(DHA)PC recombinant membranes (Fig. 4.9) it is clear from the FRET index results that
PDPC promotes more association of rhodopsin than in either the POPC and di(DHA)PC
recombinant membranes. One could think that the DHA chain in the recombinant
membranes of rhodopsin with PDPC or di(DHA)PC tries to adjust its effective length to a
value between those for pure 16:0 and 22:6 chains, which leads to a mismatch as manifested
by the data in Figure 4.9. This leads towards the thinking that rhodopsin in di(DHA)PC
membranes will associate more, since it contains two DHA chains. On the contrary,
di(DHA)PC promotes a more dispersive environment for rhodopsin, and the protein is less
associated than in the PDPC membranes. From this analysis the double polyunsaturated
chains act to increase protein dispersal. One can say that the higher degree of
polyunsaturation of the di(DHA)PC works in the direction of promoting a more flexible
bilayer membrane. By contrast, the PDPC recombinant system contains the acyl chains
16:0/22:6; thus the two different acyl chains may restrict the movement of each other. As a
result the bilayer is not as flexible or soft as for the di(DHA)PC membrane recombinants,
and rhodopsin is correspondingly less well dispersed.

One thought that could be raised about the different behavior of rhodopsin in
membranes containing POPC or PDPC comes from the differences in their phase transition temperatures. But evaluating the $T_m$ values of both POPC and PDPC does not account for the differences [83] since the $T_m$ values are very close for POPC ($-3$ to $-5$ °C) and PDPC ($-6$ °C to $0$ °C). Therefore, POPC indeed showed some degree of rhodopsin association, and this result was important to explain the differences between the experimental $^{2}$H NMR order parameter profiles and the MD simulations [cf. Chapter 6].

Recently, a different FRET-based method was applied to study cholesterol-dependent recruitment of DHA containing rhodopsin/phospholipid recombinant membranes. These studies demonstrated preferential association of rhodopsin with polyunsaturated lipids. By contrast cholesterol associated with saturated lipids [86], or with cholesterol-dependent lateral domains, such lipid rafts. Moreover, the interpretation of spin label EPR experiments in terms of rotational correlation times [74] have also given an insight into rhodopsin association in membranes. Our work has shown that even in a mixture for example of DOPE/DOPC or POPE/POPC or in a single lipid environment and in a fluid phase membrane, rhodopsin is organized differently in the membrane, implying that phase separation or microdomains can occur in the fluid phase.

The FRET experiments involved good controls in which mixtures of rhodopsin-Alexa 488 and rhodopsin-Alexa 594 in octylglucoside micelles were shown to yield a negligible FRET signal between Alexa 488 and Alexa 594 (Figure 4.3). Comparison of the FRET index results with the UV-visible spectral data in Chapter 5 showed that phospholipids with a tendency for gel phase formation under the experimental conditions had a similar degree of
rhodopsin association, but different activation towards meta II. All the systems analyzed are under stress, which originates from properties of the lipid bilayer, that make the protein molecules cluster together in different ways, thus meriting further detailed investigation.

To expand further, distance measurements between two proteins were not calculated in this work, since there are many ways in which rhodopsin can be organized within the membrane, thus leading to a variety of possibilities for the different individual fluorophores. This distribution is difficult to quantify and it is not necessary for the qualitative analysis described here.

One should also recognize that the FRET experiments were carried out for the dark state of rhodopsin, and not for the illuminated forms, which are of particularly great interest with regard to the activation mechanism of rhodopsin. In the dark state, the chromophore 11-cis retinal is the acceptor of Alexa 488. The inset in Figure 4.1 showed that the absorption of Alexa 488 overlaps with rhodopsin at 500 nm. In the dark, transfer of energy to 11-cis retinal is kept constant, so the energy transfer from Alexa 488 to Alexa 594 as a function of the membrane composition can be measured. It follows that the influence of rhodopsin association could be extracted based on the changes in emission spectral intensity that occurred at 620 nm (emission of Alexa 594). Bleaching of rhodopsin promotes the formation of a mixture of meta I, meta II, and subsequent opsin and all-trans retinal. Similar to the situation in the dark state, excitation energy of Alexa 488 would be transferred to the meta I intermediate at 480 nm, but not to the UV-absorbing intermediate meta II. The samples would contain a lipid-dependent mixture of meta I and meta II, and consequently it
would be difficult to analyze the lipid dependency of the protein aggregation in a clear way. For this reason, FRET experiments were performed at a very low light intensity to minimize bleaching of rhodopsin.

Alternatively, one could fully bleach rhodopsin in the presence of hydroxylamine to generate the opsin state. The opsin state would lack the energy transfer from the dark state. However, opsin is known to be quite susceptible to denaturation. Some of the lipid systems employed in this study could stress the membrane so much that opsin would denature. This in turn would again complicate the interpretation of the lipid effects on protein aggregation, and therefore all experiments were performed only in the dark state of rhodopsin.

In summary, FRET index was used here as a screening method. Measurements were done in the same way for all samples with the same method of preparation. Since our FRET index is related to the ratio of the emission of the acceptor over the emission of the donor, the FRET method is rather straightforward. A further aspect is that it is valid for the high throughput vesicle preparations performed in this work, and therefore it is applicable for the investigation of a wide variety of different recombinant membranes [61].
CHAPTER 5

UV-VISIBLE SPECTROPHOTOMETRIC INVESTIGATION OF MEMBRANE LIPID INFLUENCES ON RHODOPSIN ACTIVATION

Following light absorption, rhodopsin is bleached to a series of intermediates and the most striking changes in the absorption spectrum occur in conjunction with the meta I to meta II transition. Here the absorbance spectra of the intermediates were monitored at a series of different pH values, with absorbance maxima occurring mainly at 500 nm (rhodopsin), 480 nm (meta I), and 380 nm (meta II). These changes suggest that the reversible equilibrium between meta I and meta II is affected by the different membrane lipids surrounding rhodopsin. In this work, the reversible equilibrium was characterized in terms four species as photoproducts of the activation of rhodopsin. Meta II formation implies formation of its protonated and deprotonated Schiff base forms; the same applies to the meta I intermediate. From these experiments, it was concluded that formation of the Schiff base-deprotonated form of meta II was favored by dispersal of the protein within the bilayer lipid matrix. As shown in Chapter 4, the dispersal of rhodopsin within the membrane is promoted by an optimal acyl length of 20 carbons, by a softer and more flexible bilayer, and by elastic curvature stress/strain within the bilayer membranes.

5.1. Results

5.1.1. Effect of pH on Meta II–Meta I Equilibrium

The absorbance changes for rhodopsin/phospholipid vesicles were measured based on a novel pH step method. The important point was to have the initial pH of the sample set
equal to 6, which stabilizes formation of meta II. Under this condition, the equilibrium for the transition from the meta II state to the meta I state was monitored with UV-visible spectrophotometry. This approach differs from that used in flash photolysis where the transition transition from meta I to the meta II state is measured [12].

In fact, such a pH step method has been previously used for ROS native membranes [87], in which rhodopsin is accessible only at the cytoplasmic side, since is oriented asymmetrically in the membrane. By contrast rhodopsin in synthetic vesicles is inserted symmetrically; thus only one half of the protein is accessible to changing the proton activity and such pH step procedure cannot be applied directly. To circumvent this problem, a combination of the K+ ionophore valinomycin together with the proton shuttle CCCP was incorporated in the vesicles, and used for the first time with rhodopsin-containing membranes. This new approach allowed a safe pH step with rapid pH equilibration throughout the vesicles. To increase the pH value from 6 (initial) to the desired pH value, a pre-determined volume of 1N KOH solution was added after the sample was bleached for 15 s with a fiber optical illuminator (Dolan Jenner Industries Inc.; Lawrence, MA), having a yellow filter (FY-32-52, λ > 490 nm.). Recall that Meta I is favored in a more alkaline pH; whereas meta II occurs in a more acidic pH medium.

As an example of how the experiments are performed, Figure 5.1 illustrates the spectral transitions of rhodopsin/POPC reconstituted membranes having 1:100 molar ratio at 20 °C. The top left panel shows the absorption changes monitored at pH 5.89, and on the right the absorption changes are monitored at pH 6.69, with the corresponding difference
FIGURE 5.1. UV-visible absorption spectra of rhodopsin/POPC (1:100) recombinant membranes at 20 °C. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1mM EDTA, 125 mM KCl, and included Valinomycin/CCCP and 5.93 μL of 1N HCL (Section 3.3.3). Initial concentration of rhodopsin was 2.3 ± 0.3 μM. (Top) absorption spectra at pH 5.89 (left) and pH 6.69 (right); (bottom) corresponding difference spectra. In each of the top panels the spectral sequence is the following, from top to bottom at 500 nm: (d) dark, (l) light, bleaching for 15 s with addition of defined volumes of 1N KOH, (h) addition of hydroxylamine yielding 100 mM final concentration followed by 5 min incubation; and (b) fully bleached for 1 min (cf. text). Formation of Schiff base-deprotonated meta I and meta II can be seen as a positive absorbance at 380 nm in the (l–d) difference spectra. Formation of Schiff base-protonated meta I and meta II give a positive peak (third line, top to bottom ) at 480 nm in the difference spectra (l–h). The θ values were 0.88 for pH 5.89 and 0.59 for pH 6.69.
spectra indicated below. Each set of pH measurements consists of four spectra, which are acquired according to the following sequence: dark (d), light (l), hydroxylamine (h), and fully bleached (b). The initial absorbance spectrum (black-top line) corresponds to rhodopsin in the dark state, indicated by the highest absorbance peak at 500 nm. Illumination for 15 s at pH 6 (vide supra) is followed immediately by addition of a previously determined volume of 1N KOH, and then a second UV-visible absorption spectrum is recorded. The resulting absorption spectrum (light-second spectrum from the top at 500 nm) contains meta I and meta II in equilibrium, in which the Schiff base-deprotonated species absorb at 380 nm and the Schiff base-protonated forms absorb at 480 nm. In addition unbleached rhodopsin and possibly isorhodopsin (if formed) are present. Next, a third spectrum (h) was acquired after addition of hydroxylamine, in which meta I and meta II are converted to opsin and free retinal oxime; together with unbleached rhodopsin; and isorhodopsin (if formed). Last, a fully bleached spectrum (b) was taken after 1 min exposing rhodopsin to yellow light (\(\lambda > 490\) nm) as indicated by the spectrum line with the lowest absorbance value at 500 nm and the highest absorbance value at 380 nm.

From the difference spectra, one can best observe the species that are formed and how to distinguish them. The dark minus fully bleached (d–b) difference absorbance spectrum is represented by the black-top spectrum at 500 nm. The dark minus hydroxylamine (d–h) spectrum is shown by the red-second line (top to bottom at 500 nm). The light minus hydroxylamine (l–h) and light minus dark (l–d) spectra are represented by the 3rd and 4th spectra, where the 3rd spectrum is indicated by the line with the lowest positive absorbance at
480 nm and the third negative (from the bottom) absorbance at 380 nm. The 4th spectrum (l–d) corresponds to the line with the lowest negative absorbance value at 480 nm and the highest positive peak at 380 nm. In these difference spectra, formation of the Schiff base-deprotonated species, meta I and meta II, is visualized at 380 nm as a positive absorbance (l–d spectrum). Formation of Schiff base-protonated meta I can be observed at 480 nm. In the (l–h) difference spectrum, the negative difference absorbance at 380 nm corresponds to the transition of meta II to retinal oxime. Lastly, in the (d–h) difference spectrum the negative peak at 380 nm (d–h) indicates complete retinal oxime formation.

It is important to note that isochromic forms are difficult to characterize in the UV-visible absorbance spectra. Here the isochromic forms are the meta I and meta II Schiff base-deprotonated species that absorb at 380 nm, and the meta I and meta II Schiff base-protonated species that absorb at 480 nm. The final step of the analysis of the spectra yields a pH titration curve. A probable mechanism for the interconversion of meta I to meta II is subsequently proposed in this work, which is explained in detail (cf. Section 5.1.5). From the suggested mechanism, the species formed at 380 nm are either meta II or meta I having a deprotonated Schiff base.

We also note that bleaching of rhodopsin for a duration of 15 s at pH 6 resulted in 85–95% photolysis, implying that almost of all the rhodopsin initially present was converted to an equilibrium mixture of meta I and meta II. Consequently, this approach of high bleaching at low pH assures reproducibility of the initial conditions, thereby justifying the present data analysis. At 20 °C the lifetime of meta II is relatively long, so that it is stable for
5 min or longer at lower temperatures due to the slower kinetics. As the scanning speed for each spectrum was 39 s, after 1 min the meta II intermediate could still be characterized. In other words, since meta II can be trapped for up to 5 minutes at 20 °C, [77] there was adequate time to perform the pH adjustment and record the UV–visible absorption spectra.

Transient formation of isorhodopsin 9-cis-retinal chromophore (~ 480 nm) was not considered in our experiments under conditions favoring meta II formation, which included pH values at or below 6.0. However, under conditions where the meta I intermediate is favored during bleaching at pH 6.0, as for example at low lipid to protein ratio, or under the influences of certain lipids which favor meta I, isorhodopsin can be formed. Nevertheless, any contribution from isorhodopsin does not change the calculation of the fraction of meta II_{SB} formed to any appreciable degree (vide infra; Eq. 5.3).

In the following experiments, after addition of hydroxylamine the samples were incubated for 5 min at 37 °C in a water bath, and then equilibrated for 5 min at 20 °C before recording the UV-visible spectra. The reason was to accelerate the decay of the meta III photointermediate. In preliminary experiments, a contribution to the absorption spectra near 500 nm was observed after the initial bleach, and was attributed to formation of isorhodopsin. But instead we discovered it was due to meta III, whose presence was removed by incubating the sample for an extra 5 min at 37 °C. Moreover, the final hydroxylamine concentration was increased to 100 mM to accelerate the formation of free retinaloxime.

The meta II–meta I equilibrium was probed from pH 6 to 8, a relatively limited range
compared to the whole titration curve (Section 5.1.3, Figure 5.2). This was done with the purpose of identifying around pH 7 (physiological pH) the changes caused by the lipids in the activation of rhodopsin. The titration curves were fit to an individual values of $pK_{a1}$ and $pK_{a2}$ as in the case of a polyprotic acid-base equilibrium (cf. Section 5.1.3).

5.1.2. Calculation of Fraction of Metarhodopsin II

All UV-visible spectral data were analyzed with the program ORIGIN 7.0. The initial concentration of rhodopsin $[\rho]_0$, the fraction of rhodopsin bleached ($f$), and the fraction of Shiff base-deprotonated meta II + meta I formed ($\theta$) for each of the samples were calculated from the three difference spectra (vide supra). The initial concentration of rhodopsin $[\rho]_0$ is given by

$$[\rho]_0 = \frac{(\Delta \epsilon_{\text{rhodopsin}}^{500\text{nm}} \Delta \epsilon_{\text{rhodopsin}}^{650\text{nm}})}{(\Delta \epsilon_{\text{rhodopsin}}^{500\text{nm}} \Delta \epsilon_{\text{rhodopsin}}^{650\text{nm}})}$$  \hspace{1cm} (5.1)

Here $\Delta \epsilon_{\text{rhodopsin}}^{500\text{nm}}$ is the difference absorbance, dark minus fully bleached at 500 nm; $\Delta \epsilon_{\text{rhodopsin}}^{650\text{nm}}$ is the baseline absorbance at 650 nm; and $\Delta \epsilon_{\text{rhodopsin}}^{500\text{nm}}$ is the difference molar absorption coefficient for rhodopsin minus opsin at 500 nm, where $\epsilon_{\text{rhodopsin}}^{500\text{nm}} = 40600 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{opsin}}^{500\text{nm}} = 0 \text{ M}^{-1} \text{ cm}^{-1}$ for opsin (minus the spectral difference baseline). The bleached fraction ($f$) was calculated from the relation

$$f = \frac{(\Delta \epsilon_{\text{rhodopsin}}^{500\text{nm}} \Delta \epsilon_{\text{rhodopsin}}^{650\text{nm}})}{(\Delta \epsilon_{\text{rhodopsin}}^{500\text{nm}} \Delta \epsilon_{\text{rhodopsin}}^{650\text{nm}})}$$  \hspace{1cm} (5.2)

The absorbance difference corresponded to the dark minus hydroxylamine spectra at 500 nm,
corrected for the baseline. Likewise, the denominator indicates the difference absorbance for
the dark minus fully bleached spectra at 500 nm. The fraction of rhodopsin bleached was
found nearly to be constant over the pH range investigated.

Quantification of the fraction of the Schiff base-deprotonated species formed was
derived from [8]. The deprotonated Schiff base fraction was calculated from the difference
spectra as

\[
\theta = \left( \frac{\Delta A_{d-b}^{380nm}}{\Delta A_{d-h}^{500nm}} - \frac{\Delta A_{d-b}^{426nm}}{\Delta A_{d-h}^{650nm}} \right) \left( \frac{\Delta \varepsilon^{500nm}}{\Delta \varepsilon^{380nm}_{\text{MII-MI}}} \right) + \frac{\Delta \varepsilon^{380nm}_{\text{MII-MI}}}{\Delta \varepsilon^{380nm}}
\] (5.3)

The first term indicates the light (partially bleached), minus dark difference at 380 nm
\(\Delta A_{d-b}^{380nm}\) (where the formation of meta II can be observed) minus the absorbance difference
from the light minus dark difference at 426 nm (\(\Delta A_{d-b}^{426nm}\)), corresponding to the isosbestic
point for correcting the light scattering [58]. The quantity \(\Delta A_{d-h}^{500nm}\) is the absorbance
difference at 500 nm, dark minus hydroxylamine spectra at 500 nm. The difference molar
absorbance coefficient \(\Delta \varepsilon^{500nm}_{\text{rho-opsin}}\) for rhodopsin minus opsin at 500 nm was taken as
\(\Delta \varepsilon = 40600 \text{ M}^{-1}\text{cm}^{-1}\), and zero for opsin [88]. For the difference in molar absorptivity
\(\Delta \varepsilon^{380nm}_{\text{rho-MII}}\) the value taken was 34 800 \(\text{M}^{-1}\text{cm}^{-1}\), the sum for the Schiff base-deprotonated
species, and lastly \(\Delta \varepsilon^{380nm}_{\text{rho-MI}}\) was considered as \(-7 200 \text{ M}^{-1}\text{cm}^{-1}\) [8, 58]. The differences in
the denominator give a value of \(\Delta \varepsilon^{380nm}_{\text{MII-MI}} = 42000 \text{ M}^{-1}\text{cm}^{-1}\).

The equilibrium constant \((K_{eq})\) is then given by \(K_{eq} = 1/1 - \theta\), which corresponds to
the ratio of the 380 nm and the 500 nm (d–b) in the difference absorbance spectra (l–d), as
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explained in Figure 5.1. Note that the exact absorption coefficients ($\varepsilon$) for the metarhodopsin species according to Eq. 5.3 are not known. From the whole titration curve (Figure 5.3) at pH 6.0, the fraction of $\theta$ values was found close to 1. If the wrong absorption coefficient was taken at pH 6.0 the $\theta$ values would be different. However, one can note that for meta I, at higher pH, the $\theta$ values did not reach the maximum value of one, and that might be due to the assumed $\varepsilon$ values which require further investigation.

5.1.3. Fitting of pH-Dependent Spectral Data

A complete titration curve from pH 4 to 10 for rhodopsin in POPC membranes at 20 °C is shown in Figure 5.2. As can be seen, there appear to be three separate titrations involving a total four different photoproducts, which vary in their protonation state. As discussed subsequently in Section 5.1.5, beginning at low pH we propose that the first step is the *deprotonation* of meta II$_{PSB}$, *i.e.* meta II having a protonated Schiff base (PSB) to yield meta II$_{SB}$, *i.e.* meta II with the deprotonated Schiff base (SB). The next titration step is the *protonation* of meta II$_{SB}$ to yield meta I$_{PSB}$, *i.e.* meta I with a protonated Schiff base; here a complex series of proton transfer occurs which is incompletely understood at present. The third titration step is the *deprotonation* of meta I$_{PSB}$ to give meta I$_{SB}$, *i.e.* meta I having a deprotonated Schiff base.

In the plots which follow, the solid lines for the pH titration curves are results of fitting the data according to the equation

$$
\theta = 1 - \frac{1}{1 + 10^{(pK_{a1} - pH)}} + 10^{(pH - pK_{a2})}
$$

(5.4)

The above formula corresponds to two sequential pH-dependent reaction steps, as in the case
FIGURE 5.2. pH dependence of the fraction ($\theta$) of Schiff base-deprotonated photoproducts for rhodopsin in POPC recombinant membranes. The photoproducts include metarhodopsin I and metarhodopsin II (Eq. 5.5). The lipid-rhodopsin molar ratio was 1:100 and the sample contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1mM EDTA, and 125 mM KCl, together with CCCP/Valinomycin ionophores. Measurements were performed at 20 °C in the pH range from 4 to 10. The general shape of the curve resembles a pH titration with three separate pK$_a$ values corresponding to steps (1), (2), and (3). The dotted lines delimit the pH range in which additional experiments were performed.
of an amphoteric compound. The fitting uses two parameters, $pK_{a1}$ and $pK_{a2}$, employing the program ORIGIN 7. The calculated $\theta$ value is related to the equilibrium constant $K$ for each of the reaction steps, defined as $K_1$ and $K_2$. For example, in terms of the equilibria proposed subsequently in Section 5.1.5, the equilibrium constant $K_1 = [M_{I_{PSB}}][H_3O^+]/[M_{II_{PSB}}]$ in step 2, and $K_2 = [M_{I_{SB}}][H_3O^+]/[M_{I_{PSB}}]$ in step 3, where the ratio $K_2/K_1 = [M_{II_{SB}}]/[M_{I_{SB}}]$. It follows from the Hendersen-Hasselbalch equation that the equilibrium constants can be rewritten as $K_1 = 10^{(pH-pK_a1)}$ and $K_2 = 10^{(pH-pK_a2)}$. Algebraic manipulation of $K_1$ and $K_2$ then leads to Eq. (5.4) above.

For the purpose of analyzing the absorption spectra, the lower part of the titration curve (cf. Figure 5.2) corresponding to the lower pH values (4 to 5) is not used; however one can consider the first pK on the more acidic side of the curve as $pK_{a0}$. In the fitting curve, the $pK_{a2}$ parameter was initially varied first, but it was found that the $pK_{a2}$ values for all curves were approximately the same (cf. Figure 5.4). Thus for the fitting of the data, the $pK_{a2}$ value was fixed to 7.8, the result for all the curves. Note that curve fitting allowing two parameters to vary, e.g., both $pK_{a1}$ and $pK_{a2}$, gives more uncertainty than fitting with one fixed parameter.

5.1.4. Influences of Membrane Lipid Environment on pH-Dependent Meta I–Meta Equilibrium of Rhodopsin

The pH dependent curves for rhodopsin in POPC membranes are shown in Figure 5.3. Membrane recombinants were prepared at lipid-protein molar ratio of 50:1, 100:1, 200:1, and 400:1 in parts (a–d). The $pK_{a1}$ and $pK_{a2}$ values from the curve fitting are shown in Figure 5.4, which shows that the $pK_{a2}$ is not appreciably affected by the lipid environment. Figure
FIGURE 5.3. Effect of lipid-protein molar ratio on the fraction ($\theta$) of the Schiff base-deprotonated photoproducts as a function of pH for rhodopsin/POPC recombinant membranes. The photoproducts include metarhodopsin I and metarhodopsin II. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1mM EDTA, and 125 mM KCl, together with CCCP/Valinomycin ionophores at 20 °C. The protein-lipid molar ratio was 1:50, 1:100, 1:200, and 1:400 in parts (a)–(d), respectively. The smooth curves represent a two-parameter fit according to Eq. (5.4). A rather narrow pH range from 6–8 is measured to probe activation of rhodopsin as close as possible to the physiological pH of 7.0. Calculated $pK_{a1}$ values were found to be 6.43, 6.73, 7.06, and 7.46 in parts (a)–(d), respectively. The value of $pK_{a2}$ was held constant. Note that formation of the Shiff base-deprotonated species is more favorable at the higher lipid-protein ratios where rhodopsin is more dispersed according to FRET studies (*vide infra*).
FIGURE 5.4. Plot of $pK_a$ values versus lipid-protein molar ratio for rhodopsin/POPC membranes. In general there is little or no effect on $pK_{a2}$ of the titration curve; by contrast an increase in $pK_{a1}$ is observed with increasing lipid-protein molar ratio.
5.5 and Figure 5.6 parts (a–d), respectively show the corresponding data for rhodopsin in PDPC and POPE membranes. The activation of rhodopsin in each of the systems, POPC, PDPC, and POPE, at the different lipid to protein molar ratios is summarized in Figure 5.7 parts (a–c). An important finding is that increasing the lipid/protein molar ratio from 50:1 to 400:1 in the recombinant membranes promotes greater formation of the Schiff base-deprotonated meta I and meta II intermediates. Overall the activation of rhodopsin appears similar for POPC and PDPC; however, for POPE membranes the lipid-protein molar ratio did not affect the formation of meta $\text{II}_{\text{SB}}$ due to the membrane being in the gel state.

Turning next to the influences of the acyl chain length, the pH dependence of the meta II–meta I equilibrium was further investigated for rhodopsin in phospholipid recombinants comprising the homologous series di(14:1)PC, di(16:1)PC, di(18:1)PC; di(20:1)PC, di(22:1)PC, and di(24:1)PC. The results for each of the systems are shown in Figure 5.8. The upward shift of the inverted bell-shaped curves indicates the effect of the lipid environment on the formation of meta $\text{II}_{\text{SB}}$. Better comparison of the effect of the acyl chains can be seen in Figure 5.9, where the results are summarized. It can be noted from the plots that the optimal length favoring meta $\text{II}_{\text{SB}}$ is 20 carbons.

For the next series of experimental results, pH titration curves are plotted for rhodopsin in DMPC, eggPC, POPC, and POPE recombinant membranes, as shown in Figure 5.10. As a comparison, data for these four systems are shown together in Figure 5.11. One can observe that the least favorable membrane environment for rhodopsin activity was DMPC, not only due to its short acyl chain (14:0), but also due to its higher chain melting
FIGURE 5.5. Influence of lipid-protein molar ratio on the fraction ($\theta$) of the Schiff base-deprotonated photoproducts plotted against pH for rhodopsin/PDPC recombinant membranes. The Schiff base-deprotonated photoproducts encompass both metarhodopsin I and metarhodopsin II. The samples included 25 mM MES, 25 mM HEPES, 25 mM KOH, 1mM EDTA, and 125 mM KCl, together with CCCP/Valinomycin ionophores at 20 °C. The protein-lipid molar ratio was 1:50, 1:100, 1:200, and 1:400 in panels (a)–(d), respectively. The smooth curves represent a two-parameter fit using Eq. (5.4). The pH range from 6–8 is studied to probe activation of rhodopsin as near as possible to the physiological pH of 7.0. Calculated $pK_{a1}$ values were 6.53, 6.96, 7.06, and 7.55 in parts (a)–(d), respectively. The value of $pK_{a2}$ was fixed at 7.8. Formation of the Schiff base-deprotonated species is promoted at higher lipid-protein ratios, where dispersal of rhodopsin is more favorable.
FIGURE 5.6. Effect of protein-lipid molar ratio on the fraction ($\theta$) of the Schiff base-deprotonated photoproducts versus pH for rhodopsin/POPE recombinant membranes. The Schiff base-deprotonated photoproducts include both metarhodopsin I and metarhodopsin II. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, together with CCCP/Valinomycin ionophores at 20 °C. The protein-lipid molar ratio was 1:50, 1:100, 1:200, and 1:400 in parts (a)–(d), respectively. The smooth curves represent a two-parameter fit according to Eq. (5.4). A relatively narrow pH range from 6–8 is investigated to probe activation of rhodopsin as close as possible to the physiological pH of 7.0. Calculated $pK_{a1}$ values were found to be 6.54, 6.60, 6.65, and 6.68 in parts (a)–(d), respectively. The value of $pK_{a2}$ was frozen at 7.8. Formation of the deprotonated Schiff base species is independent of the protein-lipid ratio, since the measurements are below the gel to liquid-crystalline transition temperature of POPE (24 °C).
FIGURE 5.7. Summary of the effects of the lipid molar ratio on the fraction ($\theta$) of the Schiff base-deprotonated photointermediates as a function of pH. The Schiff base-deprotonated intermediates encompass both metarhodopsin I and metarhodopsin II for rhodopsin in recombinant membranes. Data are summarized for rhodopsin reconstituted in (a) POPC, (b) PDPC, and (c) POPE membranes having protein-lipid molar ratios (from top to bottom) of 1:50, 1:100, 1:200, and 1:400 measured for the range from pH 6–8 at 20 °C. The smooth curves represent two-parameter fits according to Eq. (5.4). The value of $pK_{a2}$ was fixed to 7.8.
FIGURE 5.8. Effect of acyl chain length on the fraction ($\theta$) of deprotonated Schiff base photoproducts versus pH for rhodopsin reconstituted with different phosphatidylcholines. Schiff base-deprotonated photoproducts comprise both metarhodopsin I and metarhodopsin II. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, including CCCP/Valinomycin ionophores at 20 °C. Data are presented for di(14:1)PC, di(16:1)PC, di(18:1)PC, (20:1)PC, di22:1)PC, and di(24:1)PC membranes at a 1:100 protein-lipid molar ratio at 20 °C. The smooth curves are the results of two-parameter fitting using Eq. (5.4). Measurements were carried out from pH 6–8 to probe activation of rhodopsin in the range close to the physiological pH of 7.0. The calculated $pK_a$ values were 5.93, 6.37, 6.87, 7.14, 6.95, and 6.52 in parts (a)–(f), respectively. The $pK_a$ value was held constant at 7.8 (Section 5.1.3). The best hydrophobic match for rhodopsin in the dark state was found to correspond to a 20-carbon acyl length.
FIGURE 5.9. Summary of the effect of acyl chain length on the fraction ($\theta$) of Schiff base-deprotonated photointermediates plotted against pH for rhodopsin in recombinant membranes. Data are shown (from the lowest to the highest ($\theta$) value) for rhodopsin reconstituted in di(14:1)PC, di(16:1)PC, di(24:1)PC, di(18:1)PC, di(22:1)PC, and di(20:1)PC membranes at a 1:100 protein-lipid molar ratio from pH 6–8 at 20 °C. The smooth curves are the results of two-parameter fitting according to Eq. (5.4). The $pK_a$ value was frozen at 7.8 (Sec. 5.1.3). The best hydrophobic match was obtained for a 20-carbon acyl length.
FIGURE 5.10. Influence of acyl chain length on the fraction ($\theta$) of Schiff base-deprotonated photoproducts as a function of pH for rhodopsin recombined with (a) DMPC, (b) egg PC, (c) POPC, and (d) POPE. Schiff base-deprotonated photoproducts include both metarhodopsin I and metarhodopsin II. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, together with CCCP/Valinomycin ionophores at 20 °C. The curves are the results of two-parameter fitting utilizing Eq. (5.4). Measurements were carried out for the pH range 6–8. The calculated $\rho K_{a1}$ values in parts (a) to (d), respectively, were found to be 5.45, 6.61, 6.73, and 6.60. The $\rho K_{a2}$ value was held constant at 7.8 (Sec. 5.1.3). In the case of DMPC, the relatively short acyl chain length and higher phase transition temperature (24 °C) promote more meta I than meta II. A similar effect was also found for eggPC, POPC, and POPE, independent of the transition temperature. Note that DMPC and POPE have $\approx$ the same melting transition temperature.
FIGURE 5.11. Summary of the effect of acyl chain unsaturation on the fraction ($\theta$) of Schiff base-deprotonated photointermediates obtained for rhodopsin in recombinant membranes as a function of pH. Data for rhodopsin reconstituted with DMPC (the lowest curve), having saturated acyl chains, are compared to results for egg PC, POPE, and POPC, with unsaturated chains, at a 1:100 protein-lipid molar ratio at 20 °C. The fitted curves are the results of two-parameter fitting in terms of Eq. (5.4). The value p$K_{a2}$ was maintained fixed at 7.8 (Section 5.1.3). Measurements were conducted from pH 6–8. Calculated curves are compared to the native ROS membranes, whose p$K_{a1}$ value is 7.62.
transition temperature effect (24 °C). As a result DMPC shifts the meta I–meta II equilibrium towards meta $I_{PSB}$ intermediate.

The effect of the spontaneous curvature was also probed using the DOPE/DOPC and POPE/POPC recombinant membranes in 50:50 molar ratio mixtures per rhodopsin. The results are shown in Figure 5.12 for each of the systems separately, and the results for both systems are plotted together in Figure 5.13. The data show clearly the pronounced effect of DOPE/DOPC (50:50) towards formation of meta II$_{SB}$. From the point of view of hexagonal phase formation related to the spontaneous curvature, the DOPE transition temperature from the lamellar to the reverse (H$_{II}$)hexagonal phase is $T_H = 10$ °C. This implies that the DOPE/DOPC (50:50) mixture is close to the lamellar-hexagonal phase boundary under the temperature conditions of the experiment (20 °C). By contrast for POPE the $L_a$-H$_{II}$ transition is much higher, 60 °C. Thus the cause of such a larger effect of DOPE/DOPC on the meta I–meta II equilibrium can be attributed to the effect of the intrinsic or spontaneous curvature of the DOPE/DOPC systems. Moreover, shown previously in Chapter 4, these two systems promote rhodopsin association in a similar manner, implying that chemically specific effects of PE headgroups are not involved.

Effects of the phase transition of POPE were also investigated for mixtures of POPE/POPC (50:50), and compared to the POPC and POPE recombinant membranes for a 100:1 lipid-rhodopsin molar ratio. The results are indicated in Figure 5.14, and summarized in Figure 5.15. About the same fraction of meta II$_{SB}$ was observed for rhodopsin in the POPE/POPC, pure POPE, and POPC membranes. Note that pure POPE is in a gel phase
FIGURE 5.12. Effect of the PE head group on the fraction ($\theta$) of the Schiff base-deprotonated photoproducts versus pH for rhodopsin in recombinant membranes. Schiff base-deprotonated photoproducts comprise metarhodopsin I and metarhodopsin II. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl together with CCCP/Valinomycin ionophores. Data are included for rhodopsin reconstituted in (a) DOPE/DOPC, and (b) POPE/POPC membranes at a 50:50 protein-lipid molar ratio at 20 °C. The calculated curves are the results of two-parameter fitting utilizing Eq. (5.4). Measurements were conducted for the pH range of 6–8. The calculated pK$_{a1}$ values were 8.15 for DOPE/DOPC, and 6.53 for POPE/POPC membranes in parts (a)–(b), respectively. The value of pK$_{a2}$ was frozen at a value of 7.8. Note that chemically-specific influences of the PE head group can be ruled out, since the acyl chains of DOPE and POPE are distinct. For DOPE the transition temperature from the lamellar to reverse-hexagonal ($H_{II}$) phase is $T_H = 10$ °C; whereas for POPE the transition temperature is $T_H = 60$ °C. In consequence, DOPE leads to a higher curvature stress/strain under the temperature of the experiment.
FIGURE 5.13. Summary of the effect of the PE head group on the fraction ($\theta$) of the deprotonated Schiff base photointermediates, metarhodopsin I and metarhodopsin II, as a function of pH for rhodopsin in recombinant membranes. Data are compared for rhodopsin reconstituted in DOPE/DOPC and POPE/POPC membranes at a 50:50 molar ratio at 20 °C. The calculated curves are the results of two-parameter fitting according to Eq. (5.4). The value of $pK_{a2}$ was held fixed at 7.8. Measurements were carried out in the range from pH 6–8. The presence of DOPE in the membrane leads to a higher curvature stress at the temperature of the experiment.
FIGURE 5.14. Influence of POPE on the fraction ($\theta$) of the Schiff base-deprotonated photoproducts plotted against pH for rhodopsin in recombinant membranes. Schiff base-deprotonated photointermediates include metarhodopsin I and metarhodopsin II. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1 mM EDTA, and 125 mM KCl, together with CCCP/Valinomycin ionophores. Data are for rhodopsin in (a) native ROS membranes, (b) POPE/POPC (50:50), and (c) pure POPE (1:100). Theoretical curves were fit with two parameters, as in Eq. (5.4). The pH range from 6–8 was investigated at 20 °C. The $pK_{a1}$ value for ROS membranes was calculated as 7.62. For POPE/POPC a value of 6.53 was found, and the $pK_{a1}$ values for pure POPE and POPC were 6.60 and 6.73, respectively. Therefore POPC and POPE have nearly the same activation effect on rhodopsin. Note that the pure POPE membranes are in the gel state at the measuring temperature ($T_m=24$ °C); whereas POPC in the liquid-crystalline state ($T_m=-5$ °C).
FIGURE 5.15. Summary of the effect of POPE on the fraction ($\theta$) of the deprotonated Schiff base photointermediates, metarhodopsin I and metarhodopsin II, for rhodopsin in recombinant membranes as a function of pH. Data are shown over the pH range from 6–8 for rhodopsin reconstituted in pure POPE, POPC, and POPE/POPC 50:50 membranes, whose protein-lipid molar ratios (1:100) are compared to the native ROS membranes. Note that POPE is in the gel phase at the temperature of 20 °C, whereas POPC is in the liquid-crystalline phase.
under the conditions of the experiments, but in the presence of the POPC the liquid-crystalline fluid phase is present. One should also recall that from the FRET data (Figure 4.7) POPE/POPC and POPC showed an equivalent effect with respect to rhodopsin association in the membrane, in contrast to POPE membranes in the gel phase. Lastly, the effects of polyunsaturated chains were examined by comparing PDPC, POPC, and di(DHA)PC recombinant systems, as seen in Figure 5.16. To better compare the different effects of the membrane lipids, the curves are plotted together, as shown in Figure 5.17. It is clear that double polyunsaturated chains promote a higher fraction \( \theta \) of meta II\(_{SB} \) when compared to PDPC, which contains only one polyunsaturated chain, or to POPC (reference). The two identical acyl chains of di(DHA)PC may promote a softer membrane, which favors formation of meta II\(_{SB} \). On the other hand, PDPC has only one polyunsaturated DHA chain, so that the bilayer may be somewhat stiffer due to the restricted motions caused by the presence of the palmitoyl chain.

The results presented here demonstrate in specific detail how the bilayer properties can drive the energetics of the conformational transition from meta I to meta II. Our findings reveal that these recombinant membranes differ markedly in the way they modulate the meta I–meta II equilibrium of rhodopsin.

5.1.5. Proposed Mechanism for the Meta II–Meta I Equilibrium

Referring back to Figure 5.3, a complete titration curve (Figure 5.3) has been investigated in this work using POPC/rhodopsin (1:100) recombinant membranes for the pH range from 4–10, at 20 °C. Since the titration curve suggests the presence of three pK\(_a\)
FIGURE 5.16. Effect of polyunsaturated acyl chains on the fraction ($\theta$) of the Schiff base-deprotonated photoproducts as a function of pH for rhodopsin in recombinant membranes. The samples contained 25 mM MES, 25 mM HEPES, 25 mM KOH, 1mM EDTA, 125 mM KCl, and included CCCP/Valinomycin ionophores. Data are shown for rhodopsin in (a) di(DHA)PC, (b) PDPC, and (c) POPC membranes at a 1:100 protein-lipid molar ratio. Curves were fit with two parameters as in Eq. (5.4). Measurements were carried out in the pH range from 6–8 at 20 °C. The $pK_{a1}$ value for di(DHA)PC was calculated as 7.85; the $pK_{a1}$ value for PDPC was 6.96; and that for POPC was 6.73. Note that the effect of having two polyunsaturated (DHA) chains in di(DHA)PC is to promote activation of rhodopsin, e.g. by softening the bilayer membranes. In addition POPC and PDPC have a similar influence on rhodopsin activation, although PDPC promotes greater protein association.
FIGURE 5.17. Summary of the influence of polyunsaturated acyl chains on the fraction ($\theta$) of the Schiff base-deprotonated photointermediates as a function of pH for rhodopsin in recombinant membranes. Data are shown for rhodopsin reconstituted in di(DHA)PC, PDPC, and POPC membranes (top to bottom) at 1:100 molar ratio over the pH range from 6–8 at 20°C. Results were compared to the native ROS membranes, whose $pK_a$ was calculated as 7.62. The effect of having two DHA acyl chains affects the flexibility of the membrane, which becomes softer thereby promoting rhodopsin activation.
values, a possible mechanism is proposed comprising four different states and three sequential steps. It can be described as

$$\text{meta II}_{\text{PSB}} \xrightleftharpoons{^{-H^+}}_{(1)} \text{meta II}_{\text{SB}} \xrightleftharpoons{^{-H^+}}_{(2)} \text{meta I}_{\text{PSB}} \xrightleftharpoons{^{-H^+}}_{(3)} \text{meta I}_{\text{SB}}$$

(5.5)

where the subscript PSB indicates all-trans retinal with a protonated Schiff base, and SB denotes the deprotonated Schiff base. Here meta II and meta I designate the photointermediates metarhodopsin II and metarhodopsin I.

The mechanism proposed in Figure 5.2 highlights the presence of four species in the different pH regions. The two species meta I and meta II with a deprotonated Schiff base appear in the same wavelength region at $\lambda=380$ nm. The two others are formed at $\lambda=480$ nm, and correspond to meta I and meta II having a protonated Schiff base. The fraction of the deprotonated species formed ($\theta$) is defined as the ratio between the Schiff base-deprotonated forms, meta I$_{SB}$ and meta II$_{SB}$, to the concentration of all intermediates in their protonated and deprotonated forms as:

$$\theta(\text{pH}) = \frac{[\text{meta II}]_{SB} + [\text{meta I}]_{SB}}{[\text{meta II}]_{PSB} + [\text{meta II}]_{SB} + [\text{meta I}]_{PSB} + [\text{meta I}]_{SB}}$$

(5.6)

A reasonable explanation for the assignment of the three steps can be ascribed based on comparison to the model Schiff base in ethanol. It has been found that at low pH the protonated Schiff base gives a long wavelength absorbance maximum ($\lambda$) around 470 nm, and at high pH the deprotonated Schiff base can be distinguished by a shorter wavelength maximum, around 380 nm. Now, guided by Figure 5.3 (vide supra) one can observe that at low pH the long wavelength form (PSB) predominates. By going to a slightly higher pH
value, the protonated Schiff base becomes deprotonated (SB). By continuously raising the pH the Schiff base is protonated again, and at even higher pH it is again deprotonated. Referring to the mechanism proposed in Eq. 5.5, one can see that steps 1 and 3 correspond to a normal titration behavior. However, there is a paradoxical behavior in the case of step 2, where by increasing the pH the resultant intermediate would normally correspond to a deprotonation reaction. Moreover, the same Schiff base cannot be deprotonated in two independent pH ranges, unless is coupled with other changes due to proton movements in the protein.

Continuing further, step 2 represents the classical work of Wald et al., [89], where meta II is the deprotonated Schiff base species going on to form a protonated meta I. They found that if meta I was the protonated species it should be formed at low pH. However, it was the meta II state which was formed at low pH. Further studies concluded that the protonation of Schiff base came from an internal proton transfer within the protein, found later from mutagenesis studies to involve the Schiff base counterion Glu113. Therefore, another residue must be deprotonated from the classical meta II_{SB} to meta I_{PSB}, which most likely is the amino acid residue Glu134 at the end of helix III of rhodopsin [29].

Now, in the dark state of rhodopsin the pK_a of the retinylidene Schiff base was found to be 16, a very high value [90]. By contrast, for the meta II intermediate the protonated Schiff base a value of pK_a=4.0 was found [91]. In this work, the pK_a for the Schiff base-deprotonated form meta I (meta I_{SB}) was found to be 7.8. One can thus see how photoisomerization of rhodopsin leads first to the non-activated agonist and then to the
activated agonist form of the photoreceptor. In terms of the general behavior for G-protein coupled receptors, when a ligand is present it does not activate the receptor immediately, but partially activates it until the receptor rearranges to be fully activated.

How do all these explanations correspond to what is new for the resulting titration curve in this work? Here, a simple model is proposed describing the state of rhodopsin by two internal salt bridges, according to the sequential steps described in Eq. 5.5. In principle we consider two sites of protonation, one from the retinal-Shiff base in close proximity to the counterion Glu113 (located at H3), and the other which corresponds to the conserved residues Glu134 and Arg135 (both located at H3). Thus, all the four species, viz. meta II<sub>PSB</sub>, meta I<sub>SB</sub>, meta I<sub>PSB</sub>, and meta I<sub>SB</sub> differ from one another by the release of a hydronium ion (H<sub>3</sub>O<sup>+</sup>). In the scheme below, the two possible protonated sites are described side by side.  

The left side of the symbol (//) corresponds to the protonation of the Schiff base, and the right side of the equation describes the protonation state of the conserved residues. The symbol (•••) designates the salt bridge formed between the Schiff base with Glu113, and between Glu134 and Arg135 residues.

\[
\begin{align*}
\text{(a)  } & \text{ meta II}_{PSB}^{+2} = \text{retinal-C=NH}^+ - \text{Lys296} \quad \text{Glu113H} \quad \text{//} \quad \text{Glu134H} \quad \text{Arg135}^+ \\
\text{(b)  } & \text{ meta II}_{SB}^+ = \text{retinal-C=NH}^- - \text{Lys296} \quad \text{Glu113H} \quad \text{//} \quad \text{Glu134H} \quad \text{Arg135}^+ \\
\text{(c)  } & \text{ meta I}_{PSB} = \text{retinal-C=NH}^+ - \text{Lys296} \ldots \text{Glu113}^- \quad \text{//} \quad \text{Glu134}^- \ldots \text{Arg135}^+ \\
\text{(d)  } & \text{ meta I}_{SB}^{-1} = \text{retinal-C=NH}^- - \text{Lys296} \quad \text{Glu113}^- \quad \text{//} \quad \text{Glu134}^- \ldots \text{Arg135}^+ 
\end{align*}
\]
From (a) to (b) corresponding to step 1, the Schiff base is deprotonated, and due to a very low pH the Glu113, Glu134 and Arg135 remain also protonated. As the pH increases, the site of the protonated Schiff base becomes deprotonated, with the possibility of the proton going to the medium. In (b) to (c) step 2, at pH around 7, the Glu134 is deprotonated forming a salt bridge with the neighboring Arg135. The Schiff base is protonated, and due to the deprotonation of the Glu113, a salt bridge between the Schiff base and the counterion Glu113 is formed. Going to a higher pH, the Schiff base is deprotonated again.

Note there are other titratable groups in the protein such as some cysteines (at higher pH) or other glutamic groups (at low pH), as well as adjacent lipids, e.g., PS that can lead to further changes of the total charge of the system. This is relevant for an electrostatic treatment in terms of the Gouy-Chapman theory [49], but not central to understanding the present titration behavior. Our work has focused on step 2 (b to c), according to the pH region where the results were obtained and analyzed. An important feature is that our interpretation for the pH titration curve suggests the presence of two spectrally silent intermediates, meta I_{SB} and meta II_{PSB}, with discrete energetic forms.

5.2. Discussion and Conclusions

The experiments in this chapter are specifically aimed at monitoring the influence of the membrane lipid bilayer on the protein conformational energetics linked to receptor activation. Following light absorption, rhodopsin undergoes a series of photointermediate transitions, where the meta I–meta II equilibrium transition was used as a model to probe possible conformational adaptation of the protein in different membrane environments. A
key aspect involves investigating membrane lipid effects on the reversible modulation of the meta I–meta II transition. The recombinant systems were the same as the ones used for the FRET experiments in Chapter 4. The resulting spectral changes were analyzed in terms of the fraction \( \theta \) of meta II\(_{SB} \), calculated from Eq. 5.3. The data were fitted according to Eq. 5.4. For each of the recombinant systems, a different \( pK_{a1} \) value was found, indicating the energetic influences of the bilayer membrane on the conformational changes of rhodopsin.

From the experimental data probing the influence of the lipid-protein molar ratio in the POPC, PDPC, and POPE recombinant membranes, it was observed that formation of meta II Schiff base-deprotonated meta II was favored by a more dispersed environment. Probing hydrophobic mismatch with variation of the lipid acyl chain length, the results showed that the best system promoting meta II Schiff base-deprotonated meta II was di(20:1)PC, implying that a chain length of 20 carbons is the matching length to necessary solvate the hydrophobic interface of rhodopsin. A similar result was obtained from the FRET experiments in Chapter 4. One can say that the dominant factor affecting activation was aggregation, and in this case, the degree of protein association and activation were found to correlate. These experiments varying the lipid-protein molar ratio were also very important in terms of the flexible surface model discussed in Chapter 7.

However, a correlation between rhodopsin association and its activation does not appear to hold in general. This is shown by additional data in which recombinant systems with the short acyl chain length phospholipids DMPC and di(14:1)PC were compared. In both cases, activation of rhodopsin was the lowest of the systems studied, implying that short
acyl chains destabilize the activated state of the protein. However, DMPC (14:0 acyl chains) was shown to lead to the greatest degree of rhodopsin association. The activation was lowest for DMPC followed by di(14:1)PC membranes, where both have a short acyl length and thin the bilayer, thereby promoting greater aggregation of the protein. Note that DMPC is one of the phospholipid systems used in this work, which is found in the gel state due to its melting transition temperature of $T_m=24\, ^\circ\text{C}$. Now, comparing DMPC with di(24:1)PC, which is also frozen (in a gel state) at the experimental temperature, both have the same head groups, same lipid state, and the same degree of aggregation. Nevertheless di(24:1)PC promoted more activated rhodopsin.

In other examples investigated a relation between protein association and activation also does not hold, as seen for rhodopsin in the POPE/POPC and DOPE/DOPC recombinant membranes. Comparing these two systems, both influence the lateral organization of the protein in the same way, yet DOPE/DOPC promotes a higher fraction of meta II$_{SB}$. The reason is the curvature stress effect of DOPE. Therefore activation and rhodopsin association are independent entities, meaning that association is not always the predominant reason for effective activation.

The above results were obtained from vesicles containing phospholipids with neutral PC head groups measured with an ionic strength of 150 mM KCl. The physiological salt concentration minimizes the effect or contribution of any charged phospholipids, and in this case we could leave out the POPS effect in the present analysis. It is known that the PS head group has an effect on the local pH of the ROS native membranes, which can be treated as a
charge effect on the elastic deformation of the membrane [49]. In the pH range 6–8 the charge of rhodopsin is close to zero (pI = 6.0); hence the effect of repelling or attracting protons is relatively small. It follows that at physiological ionic strength and in the absence of charged phospholipid the charge effect is negligible. However, in the native ROS disk membranes, there is a polyanionic surface due to presence of about 10 PS molecules per rhodopsin. Therefore, the local pH can be larger under conditions of low ionic strength, as in the case of the results from flash photolysis experiments performed with 10 mM sodium phosphate [12].

The complete pH titration curve (Figure 5.3) was obtained as a pilot experiment acquired for an extended pH range of 4 to 10 with POPC/rhodopsin at 20 °C. Our proposed mechanism is just a hypothetical view of what may occur in the protein during the activation of rhodopsin. Data from FTIR experiments [38] has suggested a pKₐ value around 4.0, where the low pH region of the titration curve was extensively analyzed. This value supports the value of pKₐ₀ for the lower pH region of the titration curve in this work (vide supra). In this way, it was plausible to suggest a mechanism where the curve starts from such low pH value with formation of meta IIₚₛₜ. Furthermore, a kinetic model was proposed by Straume et al. [92], in which they described a branched model where the meta II at 480 nm leads to two 380 nm species. Thus, the kinetic mechanism proposed by these workers also supports the proposed mechanism suggested here.

Clearly, the whole titration curve does not correspond to the Henderson-Hasselbalch equation, nor does it correspond to the classical meta I–meta II equilibrium. Nonetheless the
classical model for the meta I–meta II transition can be recognized in the curve in the pH range from 5–7. It is plausible to say that the intermediate which is formed at higher pH has to be different than the intermediate formed at pH 6.0, i.e. Schiff base-deprotonated meta II because the same intermediate cannot be formed at different pH values. At pH 6.0, meta II having a deprotonated Schiff base is formed at 380 nm. At pH 7.0, Schiff base-protonated meta I is formed at 480 nm. Thus by going to a higher pH value, the new 380 nm species has to be different and cannot be the same as those formed before. In terms of the UV-visible spectral changes, the deprotonated-Schiff base meta I appears at 380 nm, and it cannot be distinguished spectrally from the Schiff base-deprotonated meta II, as they are isochromic forms. There is evidence from FTIR spectroscopy that meta I forms at higher pH (F. Siebert, personal communication). Thus the fraction \( \theta \) comprises all the Schiff base-deprotonated species present in the system (Eq. 5.3).

The thermodynamic mechanism suggested here has the stoichiometry of protons \( n=1+1 \), that is to say two protons are taken up sequentially with two different pK\(_a\)'s. The first proton uptake (pK\(_{a1}\)) comes from the Schiff base, and the second one from the amino acid Glu134 (pK\(_{a2}\)) located in helix III. Note that the stoichiometric coefficient \( n \) in the present work has not been measured directly. From the results presented here, it was observed that certain lipids can shift the pK\(_a\) of the central step (2) to values above 7.0. In this case, the classical meta I\(_{PSB}\) is hardly observed, and the pK\(_{a1}\) and pK\(_{a2}\) values falls together.

An argument against the mechanism suggested here would be that at higher or lower pH values possible denaturation of rhodopsin could occur. There are two possible scenarios
for such denaturation, *viz.* reversible and irreversible. It was experimentally observed that the species formed at pH 8.0 was formed nearly instantaneously within the temporal resolution of our experiments. Spectra at this pH were taken repetetively after illumination of the sample, and no changes were observed. The irreversibility could be also excluded based on the equilibrium formed between the meta II and meta I species. If there would be an irreversible denaturation, then the mixture formed would have not appeared as an equilibrium. The equilibrium would have shifted completely to one side, corresponding to the deprotonated or protonated Schiff base species. Moreover, for low pH (around 4.0) there is no reason to assume that the protein is denaturing as a general rule. There is also evidence from other research groups employing FTIR spectroscopy [93] demonstrating that meta II having a protonated Schiff base is formed at lower pH values.

*How can the effect of the lipids be clearly distinguished from the protonation steps?* What really undergoes the protonation change is the all-\textit{trans} retinylidene chromophore together with the salt bridge involving the ERY conserved residues. When the central titration step (2) takes place, the state of the ERY salt bridge changes, which affects the overall conformation of the protein. It is this shape that concerns the flexible surface model (FSM), since it can be mechanically coupled to the bilayer, and hence step 2 is dependent on the lipid composition. Steps 1 and 3 in (Eq. 5.5) are not dependent on the lipid because local changes of the protein are not coupled to the cytoplasmic side and are not involved in the shape of the protein. As an example from Figure 2.1 [Chapter 2] one can see two conformational changes of rhodopsin on going from the meta I to the meta II state. Meta I in
this picture can be interpreted as meta I_{SB} or meta I_{PSB}. The same interpretation goes for the meta II species, deprotonated and protonated Schiff base forms. Membrane lipids will affect both shape rearrangements, but without distinguishing between the protonated and deprotonated form. All species designated as meta I have one characteristic shape, and all species meta II have another shape. The shape is independent of the protonation state of the Schiff base.

Many experimental innovations had to be introduced to generate conditions where the meta II–meta I equilibrium could be better investigated. The new contribution of this work for measurement of the spectral changes was the incorporation of ionophores to the vesicles to provide a shuttle to equilibrate the inside and outside pH following pH adjustment. Valinomycin transports K^+ ion through the interior of the vesicle, and CCCP is a proton H^+ carrier. This idea was previously used for ATPase experiments, but was employed here for the first time for rhodopsin.

A stoichiometric complex of either Valinomycin or CCCP with both observed metarhodopsin forms can be excluded, mainly because of the molar excess of bleached rhodopsin, which is one to two orders of magnitude compared to the concentrations of the two ionophores. Even if Valinomycin/CCCP would have higher affinity for rhodopsin, having at least 10-fold molar excess of rhodopsin in the sample implies that only a minor fraction of the protein would be bound to these compounds, 1% for Valinomycin and 10% for CCCP. Therefore the effect of the two ionophores on the formation of the intermediates can be excluded from the design of the experiment. From the literature [81], the speed of
reaction of CCCP is 10 times lower than Valinomycin. Valinomycin always stays in the membrane while a large part of CCCP stays in the water, so that it has a high rate for transport of protons.

On the basis of the assay results for monitoring rhodopsin conformational changes coupled to the bilayer membranes, it appears that all phospholipids used in this research provided different contributions. Thus, from the observed spectral changes it was evident that each of them favored Shiff base-deprotonated meta II in different way, as reasoned from their material properties. To summarize briefly, formation of meta II_{SB} is favored by a dispersal of protein within the lipid matrix as observed from the lipid-protein molar ratio experiments, a softer or more flexible bilayer, by introducing a more polyunsaturated acyl chain, and by the spontaneous curvature stress/strain in the bilayer seen from the effect of DOPE/DOPC.
CHAPTER 6
SOLID-STATE DEUTERIUM NMR SPECTROSCOPY OF LIPID-RHODOPSIN
INTERACTIONS IN RECOMBINANT MEMBRANES

In this aspect of the work our interests are focused on elastic deformation of the
membrane lipids and the influences of rhodopsin as detected by solid-state $^2$H NMR
spectroscopy. First, the effects of hydration on the conformation and dynamics of POPC-$d_{31}$
multilamellar dispersions due to altering the water/lipid molar ratio was investigated, in order
to probe the elastic deformation of the membrane. From the hydration experiments, a square-
law dependence of the $^2$H NMR relaxation on the segmental order parameter was discovered,
showing that the membrane becomes stiffer upon dehydration, apparently indicating changes
in the elasticity of the membrane. These experiments provide a future framework for
investigating the influences of rhodopsin on membrane elasticity. Next, the effect of
rhodopsin on the packing of the acyl chains of POPC-$d_{31}$ bilayers was investigated in terms
of the order parameter profiles at different temperatures. Moreover, the ordering of the acyl
chains was evaluated below, between, and above the chain melting phase transitions of the
POPC-$d_{31}$ bilayer in the presence and absence of rhodopsin by the first moment ($M_1$)
analysis. Regarding the effect of rhodopsin on the POPC-$d_{31}$ bilayer, the data showed that
rhodopsin caused a disordering of the order parameter profiles towards the bilayer center for
(chain segments C$_7$–C$_{15}$), in qualitative agreement with molecular dynamics (MD)
simulations. By probing the order-disorder phase transitions of POPC-$d_{31}$ and POPC-
$d_{31}$/rhodopsin membranes, a significant broadening due to rhodopsin was found as expected
for a protein inclusion. Furthermore, the change of the membrane thickness was smallest in
the presence of rhodopsin at 37 °C, indicating matching of the bilayer thickness to the
intramembraneous hydrophobic surface of the protein.

6.1. Results

6.1.1. Profiles of Segmental Order Parameters and Relaxation Rates for POPC-\textit{d}_{31}
Bilayers in the Liquid-Crystalline State

To examine how variation of the hydration affects the quadrupolar splittings (\(\Delta v_Q\)) of
POPC-\textit{d}_{31} acyl chains in the fluid phase, different multilamellar samples of POPC-\textit{d}_{31} were
gravimetrically prepared to contain different levels of hydration water, encompasing 10, 20,
and 40 wt. % water. \(^2\)H NMR experiments were recorded at temperatures of 17, 27, and 37
°C. For POPC-\textit{d}_{31}, the deuterium labeling is on the saturated \textit{sn}-1 chain of the
phospholipids, containing 16 carbons. The order parameters were derived from the
quadrupolar splittings (\(\Delta v_Q\)) of the numerically deconvoluted (de-Paked) \(^2\)H NMR spectra,
(\textit{vide infra}; Eq. 6.5). The overall shape of the order profiles and the progressive reduction
along the chains manifest an increase in configurational freedom with depth in the bilayer,
which originates from statistical chain terminations.

The orientational order parameters for different hydration levels and at different
temperatures are summarized in Figure 6.1, and are related to the average structure of POPC-
\textit{d}_{31}. The data in the left hand column, parts (a), (c), and (e), show that for the same hydration
level, decreasing the temperature induces ordering of the hydrocarbon chains. On the other
hand, comparing the order parameter profiles at the same temperature but different levels,
parts (b), (d), and (f), we observe that increasing the water content leads to a greater
FIGURE 6.1. Order profiles for POPC-\textit{d}11 multilamellar vesicles at 10, 20, and 40 wt. %, water and at temperatures range of 17, 27, and 37 °C. Samples were prepared in 1 mM EDTA, 5 mM HEPES, pH 7.0 in deuterium-depleted water. The order parameters were derived from the quadrupolar splittings (\(\Delta v_Q\)) of numerically deconvoluted (de-Paked) \(^2\text{H}\) NMR spectra. The left-hand panels (a, c, e) indicate how the order profiles decrease with increasing temperature. The right-hand data (b, d, f) show an increase in the order parameter profiles as the multilamellar dispersions become progressively more dehydrated.
disordering of the chains. Moreover, it is noteworthy that the effect of temperature is more pronounced at lower hydration, parts (d) and (f). In all cases, the order profiles are characterized by a plateau for the first nine C²H₂ segments, followed by a monotonous decrease in the order towards the end of the chain.

The influences of hydration were further analyzed in terms of the spin-lattice relaxation rates $R_{\text{iz}}^{(i)}$ (Eqs. 2.44–2.45), which are plotted versus the square of the corresponding order parameters $|S_{\text{CD}}^{(i)}|^2$ in Figure 6.2. In the left-hand column, parts (a), (c), and (e), it can be seen that a reduction of the square-law slopes occurs with increasing dehydration (lower wt. % water) at each of the three temperatures investigated. The right-hand column, panels (b), (d), and (f), again show the clear decrease in the square-law slope with a reduction of hydration, and moreover the data for a given hydration level are superimposable for each of the three temperatures, i.e. the square-law correlation is maintained.

In terms of the theory for composite membrane deformation [94] the reduction in square-law slope as the bilayers are brought into closer apposition (smaller wt.% water) provides clear evidence for coupling of the mesoscopic fluctuations of the various individual lamellae of the lipid dispersion. Evidently the relaxation is governed by collective motions of the lipids molecules within a given bilayer, as well as motions of the lipids in different bilayers. The lipid fluctuations are correlated in terms of wave-like, quasi-elastic disturbances, which are propagated from the bilayer surface into the adjacent membranes. In this way we explain the systematic changes in the slopes of the plots, by alterations in the elastic properties of the membrane on the order of ≈bilayer thickness.
FIGURE 6.2. Spin-lattice relaxation rates as a function of the squared order parameters in the fluid phase of POPC-$d_{31}$ multilamellar dispersions at 10, 20, and 40 wt. % water, and at temperatures of 17, 27, and 37 °C. Samples were prepared in 1 mM EDTA, 5 mM HEPES, pH 7.0, in deuterium-depleted water. The left-hand panels (a, c, e) show that the slopes decrease as the membrane dispersion becomes progressively dehydrated (lower wt. % water) at each of the three temperatures. The right-hand panels (b, d, f) again reveal the decrease in slope with a reduction in wt. % water. Moreover the data obtained at the three temperatures are superimposable for a specific hydration level. This extended data set indicates that with increasing hydration the bilayer becomes softer and more deformable, according to the square law (Section 6.1.4).
To continue briefly, as the multilamellar vesicles became more hydrated (40 wt. %) the membranes became more flexible as compared to less hydrated membranes in 10 wt. % water. These new results provide a novel framework for extension to investigations of lipid-rhodopsin interactions in recombinant membrane bilayers (see below).

6.1.2. Influences of Rhodopsin on Order Parameter Profiles of POPC-$d_{31}$ Bilayers in the Fluid State

Using deuterium NMR spectroscopy, experimental order parameter profiles of POPC-$d_{31}$ in the liquid-crystalline ($L_{a}$) state at full hydration ($=66$ wt. %water) were compared to rhodopsin/POPC-$d_{31}$ membranes (1:100), comprising the dark-state of the protein at 27 °C and pH 7. The condition of hydrophobic matching was analyzed by a combination of solid-state deuterium nuclear magnetic resonance ($^2$H NMR) and molecular dynamics (MD) simulations [95]. A specific focus was to compare the acyl chain order parameters obtained by $^2$H NMR spectroscopy of deuterated POPC-$d_{31}$ bilayers in the presence of rhodopsin with the order parameters calculated from the MD simulations [83].

A comparison of POPC-$d_{31}$ acyl chain order parameters from the experimental solid-state $^2$HNMR studies with the theoretical MD simulations [95] is shown in Figure 6.3. In part (a) the simulated order parameter profile for the $sn$-1 chain of POPC-$d_{31}$ at 27 °C is shown in the presence of rhodopsin at a lipid to protein ratio of 1:100. The MD simulation assumes zero surface tension with an average cross-sectional area per lipid of 62.6 Å$^2$. As it can be seen in part (a), the orientational order of the lipid acyl chain segments C$_8$–C$_{15}$ is reduced due to rhodopsin incorporation in the MD model system. Comparison of the order parameter profiles in the absence of rhodopsin at a constant area per lipid reveals a
FIGURE 6.3. Comparison of calculated and experimental order parameter profiles for the sn-1 chain of POPC-\(d_1\) bilayer containing rhodopsin (100:1 lipid/protein ratio). (a) Simulated order parameter profile for \(sn-1\) chain of POPC at 27 °C; (-o-) presence of rhodopsin at zero surface tension with an average cross-sectional area per lipid in the acyl chain region of 62.6 Å², and (-9-) absence of rhodopsin. (b) Experimental \(^2\)H NMR \(sn-1\) chain order parameter profile of POPC-\(d_1\) in the presence (-o-) and absence (-9-) of rhodopsin at 27 °C and pH 7. Inset: numerically deconvoluted (de-Paked) solid-state \(^2\)H NMR spectra of POPC \(d_1\) in the absence (solid line) and presence of rhodopsin (dotted line). The order parameters nearest the aqueous interface show little effect of rhodopsin; whereas carbon segments C\(_8\)–C\(_{15}\) are more disordered by the protein.
significant decrease in the central region of the bilayer (corresponding to acyl segments C₈–C₁₅), i.e. the lipid segments in the bilayer center are significantly disordered by their interactions with rhodopsin. Part (b) shows the corresponding experimental ²H NMR sn-1 chain order parameter profile of POPC-d₃₁ in the presence and absence of rhodopsin at 27 °C, pH 7.0, with a lipid to protein ratio of 1:100. The inset shows the numerically deconvoluted (de-Paked) ²H NMR spectra of POPC-d₃₁ in the absence (solid line) and presence of rhodopsin (dotted line). In part (b), the effect of rhodopsin on the acyl chain order parameter resembles the simulated system, but it is significantly less, possibly due to the effects of protein aggregation as shown by the FRET experiments reported in Chapter 4. There is a small disordering effect of rhodopsin on the order parameter profiles for acyl chain segments C₇–C₁₅ which is in qualitative agreement with the MD simulations. The similarity of the experimental order parameters close to the head groups in the presence and absence of rhodopsin (part b) suggests equivalent packing in this region. Evidently, rhodopsin induces some disorder, where the largest effect is in the vicinity of chain segment C₁₀. The overall resemblance of the order parameter profiles indicates matching of the hydrophobic thickness of the bilayer to rhodopsin.

6.1.3. Effect of Rhodopsin on Order-Disorder Phase Transition of POPC-d₃₁ Membranes as Seen by Deuterium NMR Spectroscopy

In this series of experiments, ²H NMR spectroscopy was used to investigate the thermotropic phase behavior of the phospholipid POPC-d₃₁ both with and without rhodopsin. Rhodopsin was recombined with POPC-d₃₁ at a molar ratio of 1:100, and the experiments were carried out over temperatures from -41 °C to 37 °C in steps of 10 °C. For the pure
POPC-\textit{d}_{31} bilayer, $^2$H NMR measurements were conducted at temperatures from -36 °C to 37 °C, with smaller steps (around 5 °C) in the vicinity of the phase transition of the phospholipid ($T_m$) (~5 °C). Powder-type $^2$H NMR spectra of randomly oriented membranes, representative of the lipid gel to liquid-crystalline phase transition, are shown in Figure 6.4 for pure POPC-\textit{d}_{31} bilayers (left), and for the rhodopsin/POPC-\textit{d}_{31} recombinant membranes (right). With increasing temperature the $^2$H NMR spectra of both systems, pure and recombined POPC-\textit{d}_{31}/rhodopsin, show significant intensity around ± 60 kHz, indicative of either C$^2$H$_2$ which are immobile on the $^2$H NMR time scale ($\approx 10^{-5}$ s) or alternatively correspond to C$^2$H$_2$ groups in the \textit{trans} configuration, which rotates about the bilayer normal of the gel phase. Or in other words, the gel phase spectra show a large distribution of the quadrupolar splittings. As the temperature increases, the splittings become more resolved and reveal a distribution characteristic of the fluid phase ($L_a$). In the $L_a$ phase the quadrupolar splittings yield the segmental order parameters directly, as described above.

From such powder-type $^2$H NMR spectra, moments of the $^2$H NMR lineshapes were calculated and plotted for different temperatures, as illustrated by Figure 6.5. The $^2$H NMR spectral moments are directly proportional to the average of the order parameters $\langle |S_{CD}| \rangle$, as described subsequently [66, 96]. For instance, plots of the first moment ($M_1$) are presented in Figure 6.5, in which data for pure POPC-\textit{d}_{31} bilayers are compared to rhodopsin/POPC-\textit{d}_{31} (1:100) recombinant membranes. We observe that for pure POPC-\textit{d}_{31}, there is a rapid decrease of $M_1$ as the temperature is increased through the transition region, indicated by the abrupt change in the magnitude of the first spectral moment. For the POPC-\textit{d}_{31} bilayers containing rhodopsin, the transition is more gradual in the region between -16 °C to 7 °C,
a) POPC-$d_{31}$

- 37 °C
- 27 °C
- 17 °C
- 7 °C
- 3 °C
- 8 °C
- 13 °C
- 18 °C
- 28 °C
- 36 °C

b) rho/POPC-$d_{31}$

- 37 °C
- 27 °C
- 17 °C
- 7 °C
- 6 °C
- 16 °C
- 28 °C
- 41 °C

Frequency / kHz
FIGURE 6.4. Representative $^2$H NMR powder-type spectra of (a) pure POPC-$d_{31}$ multilamellar dispersion and (b) POPC-$d_{31}$/rhodopsin (100:1) recombinant membranes as a function of temperature. Samples were prepared in 67 mM sodium phosphate buffer, containing 1 mM EDTA at pH 7 in deuterium-depleted water. At lower temperatures, the powder-type spectra are characterized by large quadrupolar splittings, indicative of the gel phase. These correspond to $\theta=0^\circ$ orientation of all-trans axially rotating the C-$^2$H$_2$ groups, and/or the $\theta=90^\circ$ orientation of methylene groups whose motion is effectively static on the $^2$H NMR time scale. The central peak corresponds to the terminal methyl groups of the acyl chains, which undergo rapid rotation about their 3-fold axes. With an increase in temperature (> 7 °C), the $^2$H NMR quadrupolar splittings become narrower, as indicative of a transition to the liquid-crystalline (L$_c$) phase where the bilayer becomes more fluid.
FIGURE 6.5. Plot of the first moment ($M_1$) of the $^3$H NMR spectra as a function of temperature ($^\circ$C) obtained for a POPC-$d_{31}$ multilamellar dispersion and for rhodopsin/POPC-$d_{31}$ (1:100) recombinant membranes. The data for pure POPC-$d_{31}$ are indicated in the plots by open symbols (---), and for rhodopsin/POPC-$d_{31}$ by filled symbols (-----). The moment analysis was done using Eq. 6.5. The inset is an expansion of the region between 7 $^\circ$C to 37 $^\circ$C in the fluid phase for both systems. Note that at 37 $^\circ$C the first moment of the pure bilayer and the bilayer containing rhodopsin is the same, indicating the condition of hydrophobic matching. The gel to liquid-crystalline transition of the pure POPC-$d_{31}$ bilayer is marked by a sharp reduction in $M_1$; whereas, for the rhodopsin/POPC-$d_{31}$ (1:100) system the transition occurs more gradually. Hence there is a broadening of the phase transition (less cooperativity) of the POPC-$d_{31}$ bilayer due to the presence of rhodopsin.
due to a broadening of the POPC-\(d_{31}\) phase transition by rhodopsin. At temperatures higher than 7 °C, both systems are in the fluid phase, and the differences between them are smaller. Here, it is important to recognize that the first moment \(M_1\) is related to the bilayer thickness in the fluid (\(L_a\)) state. According to Figure 6.5 by increasing the temperature to 37 °C, the bilayer thickness of POPC-\(d_{31}\) and rhodopsin/POPC-\(d_{31}\) become the same. Hence we conclude that at this temperature the acyl chain packing of the pure phospholipid gives a bilayer thickness which matches the hydrophobic interface of rhodopsin. It follows that our experimental analysis provides a novel way of measuring the acyl packing with the aim of finding the conditions for hydrophobic matching of the POPC-\(d_{31}\) bilayer to the protein.

6.2. Analysis and Reduction of Deuterium NMR Data

6.2.1. Elastic Membrane Deformation as Described by Square-Law Dependence of Relaxation Rates and Order Parameters

The application of combined relaxation rate and the order parameter studies can be used to understand the elastic deformation of the membranes. This is explained by a model-free, square-law dependence [97, 98], where the bilayer is treated as a continuum elastic model. It also implies that the concept of elastic deformation of the membrane involving stress and strain of the bilayer is applicable. Hence the data provided in Figure 6.2 were plotted according to a square-law model-free analysis. Here the order parameters \(S_{CD}\) and the spin-lattice relaxation rate \(R_{1z}\) measurements (Sec. 6.1.1) were combined, in order to obtain information about the elasticity of the membrane at different hydration levels, for the case of POPC-\(d_{31}\) multilamellar dispersions vesicles in the liquid-crystalline phase (\(L_a\)).

The dynamics of the flexible phospholipids can encompass segmental motions,
molecular motions, and collective motions. The spin-lattice relaxation is given to the second order as defined in Chapter 2 (Section 2.3.4). The order parameter $S_{CD}$ (Eq. 2.39) can be decomposed into three contributions as

$$S_{CD} = S_f^{(2)} S_{int}^{(2)} S_r^{(2)}$$  \hspace{1cm} (6.1)

where $S_f^{(2)}$ describes the fast order parameter due to segmental motions of the lipid acyl chains, $S_{int}^{(2)}$ is a geometric factor, and $S_r^{(2)}$ corresponds to the slower motions of the remaining residual EFG tensor due to molecular and/or collective motions of the bilayer lipids.

The observed spin-lattice relaxation rate $R_{12}$ is then related to the spectral densities of the lipid motions in the bilayer in terms of Eq. 6.3. For a composite membrane deformation model, the irreducible spectral densities [99] are given by

$$J_m(\omega) = J_{m}^{c} (\omega) + J_{m}^{mol} (\omega) + J_{m}^{mol-c} (\omega)$$  \hspace{1cm} (6.2)

The first term describes collective 3-D membrane deformations, and assumes a single elastic constant $K$ for the splay ($K_{11}$), twist ($K_{22}$), and bend ($K_{33}$) fluctuations. To linear order [100], the contribution from these order fluctuations is

$$J_{m}^{col}(\omega) = \frac{5}{2} S_{CD} D_{\omega}^{-2-\frac{d}{2}} \left[ |D_{-1m}^{(2)}(\Omega_{2L})|^2 + |D_{1m}^{(2)}(\Omega_{2L})|^2 \right]$$  \hspace{1cm} (6.3)

where the $m=1,2$. The symbol $D_{m}^{(2)}(\Omega)$ denotes a Wigner rotation matrix element, and the Euler angles $\Omega_{2L}$ describe the transformation from the bilayer normal (director) ($D$) to the laboratory ($L$) frame. In the above equation, each of the modes relaxes with a single
exponential time constant. For 3-D director fluctuations, \(d=3\), yielding an \(\omega^{-1/2}\) frequency dispersion. Following [100] the viscoelastic constant is

\[
D = \frac{3k_B T (\eta/2)^{1/2}}{5\pi K^{3/2} (S_z^{(2)} \cdot \text{col}_y)^{1/2}}
\]  

(6.4)

where \(K\) is the effective elastic constant for 3-D excitations, and \(\eta\) is the corresponding viscosity. The \(J_m^{\text{med}}(\omega)\) term in Eq. 6.2 corresponds to effective rotations of the flexible lipids, and \(J_m^{\text{med-co2}}(\omega)\) is a geometrical cross-term. According to [99], the spectral densities scale in closed form with the square of the segmental order parameter \(S_{\text{CD}}\). As a result, by measuring how the observed \(R_{1Z}\) relaxation rates (Eq. 6.3) depend on the segmental order parameter \(S_{\text{CD}}\) one can test the novel hypothesis that the relaxation detects collective motions of the bilayer lipids.

6.2.2. Reduction of the Order Parameter Profiles

All of the powder-type \(^2\)H NMR spectra of the randomly oriented bilayer membrane dispersions of POPC-\(d_{31}\) obtained in the presence or absence of rhodopsin were numerically deconvoluted (de-Paked) [63, 68]. The deconvoluted spectra correspond to the \(\theta=0^\circ\) of the bilayer normal with respect to the magnetic field. Quadrupolar splittings were determined directly from the symmetry-related signals of the quadrupolar doublets in the de-Paked \(^2\)H NMR spectra. The \(\text{C-}^2\text{H}\) segmental order parameters were calculated from the observed quadrupolar splittings, given by

\[
|\Delta \nu_Q| = \frac{3}{2} \chi_Q | S_{\text{CD}} | |P_2(\cos \beta_{DL})|
\]  

(6.5)

using a static quadrupolar constant \((\chi_Q)\) of 170 kHz for the aliphatic \(\text{C-}^2\text{H}\) bonds. Here \(\Delta \nu_Q\)
is the experimental quadrupolar splitting, $P_2(\cos \beta_{\text{DE})}$ is the second Legendre polynomial, and $S_{\text{CD}}$ is the segmental order parameter, both defined in (Eq. 2.39). The assignments of the peaks are made in reversed order from the methyl terminus (center of the spectrum) towards the plateau region, corresponding to the deuterated methylene groups $\text{C}^2\text{H}_2$. The calculated $S_{\text{CD}}$ value for each segment is then plotted as a function of the number of carbons of the deuterated lipids.

6.2.3. Analysis of the Gel and Fluid Phase Deuterium NMR Spectra of POPC-$d_{31}$ Bilayers and Rhodopsin/POPC-$d_{31}$ Recombinant Membranes

The temperature dependence of the deuterium spectra was analyzed in this work (Figure 6.5) in terms of the first moment ($M_1$) of the spectral lineshapes to estimate the average C–H bond order parameter, $\langle |S_{\text{CD}}| \rangle$. For a spectral lineshape symmetric about its center, the $n$th moment of one-half the spectrum is defined as [63]

$$M_n = \frac{\int_0^\infty x^n F(x)dx}{\int_0^\infty F(x)dx}$$

(6.5)

where the $F(x)$ is the distribution function spectral (intensity), and $x = \omega - \omega_0$ is the angular offset (radians/s) versus the Larmor frequency $\omega_0$. The $n$ moment can be related to the distribution of order parameters by

$$M_n = A_n \left( \frac{3\pi}{2} \chi_0 \right)^n \langle |S_{\text{CD}}|^n \rangle$$

(6.6)

in which $A_n$ is a constant that depends on $n$.

The first moment, $M_1$, is directly proportional to the mean of the order parameter, by

$$M_1 = \frac{\pi}{\sqrt{3}} (\chi_0) \langle |S_{\text{CD}}| \rangle$$

(6.7)
where $\chi$ is the quadrupolar coupling constant (170 kHz) for the C−H bonds. Note that $|S_{CD}|$ is segmental order parameter taken as absolute value. Thus, the first moment of the spectrum gives the mean C−H bond order parameter, or the mean of the distribution of the order parameters. The moment analysis provides quantitative information on the distribution of the quadrupolar splittings when the splittings cannot be well resolved, as for example in the case of below the phase transition temperature. In the liquid-crystalline ($L_\alpha$) state, the first moment is, in turn, related to the mean acyl chain length $\langle L \rangle$ projected along the bilayer normal, and hence to the bilayer hydrocarbon thickness [51].

6.3. Discussion and Conclusions

A lipid bilayer is a complex fluid composed mainly of phospholipid molecules, which are highly flexible with many internal and external degrees of freedom. The collective thermal excitations present in a 3-D bilayer are superimposed together with the effective axial rotations of the lipids, giving a distribution of correlation times associated with quasi-coherent order fluctuations. This new paradigm explains the dependencies of the $R_{1Z}$ relaxation rates of the fluid bilayers on the degree of the segmental ordering, the bilayer orientation with respect to the external magnetic field, and on the magnetic field strength. A key aspect is that the spectral density is distributed broadly in frequency, and for a given value of the $S_{CD}$ order parameter, the segmental $R_{1Z}$ value is related to the dynamical "softness" of the bilayer.

In this work, deuterium ($^2$H) NMR spectroscopy was applied to study the structure of the bilayer manifested in the segmental order parameters, $S_{CD}$, of the flexible molecules, viz.,
for the phospholipid POPC in the presence and absence of rhodopsin. The pure POPC-\textit{d}_{31} bilayer was adopted as a control to investigate the effect of the protein on the packing of the acyl chains at different temperatures, which in turn affects the hydrophobic thickness of the bilayer (hydrophobic matching). Using POPC-\textit{d}_{31} multilamellar dispersions we could also examine the effects of hydration on the elastic deformation of the bilayer, by considering the square-law behavior.

From the results provided in Figure 6.1, the \( S_{CD} \) order parameters of the POPC-\textit{d}_{31} bilayer changed with hydration and temperature. In addition, the \( R_{1Z} \) relaxation rates vary with the hydration and temperature (results not shown). On the other hand, correlating the \( R_{1Z} \) rates with the order parameters in terms of a square-law dependence allows a number of systematic trends to become evident. Comparing the plots in Figure 6.2, one observes differences in the slopes indicating that at higher water content the bilayer becomes more flexible, or conversely the membrane becomes stiffer upon dehydration. This result is indicative of coupling of the lamellae due to the collective nature of the lipid motions. For a lipid molecule to move, water must do likewise, giving rise to correlated motions associated with entropic repulsion forces between the lamellae. Moreover, the data show a relatively weak effect of temperature on the square-law slopes, implying that the modes governing the relaxation are not strongly thermally activated. That is to say, the flexibility is a consequence of collective fluctuations involving a large number of segments within the hydrocarbon region of the membrane bilayer, each involving relatively small potential differences. Likewise, the effective axial rotations are not due to individual molecules, but rather are
properties of the relatively slow fluctuations of a large number of lipid segments, which are only weakly dependent on temperature.

Looking back to Eq. 6.4, one would like to know which of the biophysical properties are reflected in the slopes of the square-law plots of the relaxation and order parameter data. The square-law dependence absorbs the slow-order parameter $S_{2}^{(2)\text{col}}$ into the viscoelastic constant $D$ so that it cannot be determined independently of $K$ and $\eta$. Moreover it is assumed that $S_{s}^{(2)\text{col}}$ is constant (collective motions) along the entire acyl chain [97], but not necessary constant for different hydrations. Nevertheless, the relation between $R_{12}$ versus $S_{CD}^{2}$ for different percentages of water showed changes in the slopes, and therefore we conclude that the elastic properties of the membranes change from a more flexible (40 wt. % water) to a stiffer membrane (10 wt. % water). Given these observations, the data are in general agreement with the square-law.

One point to consider here is that a reduction of the water content will reduce the space between each lamellae (separated sheets of the multilamellar dispersions), whereas the stiffness of each lamellae could remain the same. Therefore it would be difficult to distinguish the elastic properties of an individual membrane. Since the experiments were performed with the same phospholipid, one can assume that $K$ is constant, and in this case the term that may contribute to changes in the slope is most likely to be $S_{s}^{(2)\text{col}}$, the order parameter for the slow collective motions. Upon increasing the hydration level, the slow collective motions would increase in their amplitude due to their increased bilayer separation. The corresponding decrease in $S_{s}^{(2)\text{col}}$ would in turn increase the viscoelastic constant $D$, and
consequently the square-law slope also increases.

Another application of $^2$H NMR spectroscopy in this work was to probe the hydrophobic matching of the lipid bilayer to rhodopsin. The acyl chain disorder of POPC-$d_{31}$ was compared to rhodopsin/POPC recombinant membranes at different temperatures in the liquid-crystalline ($L_a$) state at full hydration. As shown in Figure 6.3, part (b), the effect of rhodopsin on the experimental acyl chain order parameter profile of the lipid bilayer resembles the simulated system. There is a small disordering effect of rhodopsin on the order parameter profiles for chain segments $C_7$–$C_{15}$ which is in qualitative agreement with the MD simulations. Moreover, the similarity of the experimental order parameters close to the headgroups, in the presence and absence of rhodopsin, suggests equivalent lipid packing in the polar region. Nonetheless, rhodopsin induces some disorder deeper in the bilayer, where the largest effect is in vicinity of acyl chain segment $C_{10}$.

The results suggest that a greater lateral freedom of the acyl chain segments, due to the vase-like shape of the protein inclusion results in larger order parameter differences in the middle part of the chains. On the other hand, the overall resemblance of the order parameter profiles indicates matching of the hydrophobic thickness of the bilayer to rhodopsin. Regarding the differences of the matching temperature at 27 °C (Figure 6.3) and 37 °C (Figure 6.5) this was due to the different analysis procedure. In the first case, the analysis was done based on the matching of the order parameter plateau, so one could observe the packing of the acyl chains. In the second case, the matching was taken from the average of the order parameters (Eq. 6.7), and both of the two approaches are valid. Note that the
change of the membrane thickness with temperature is less in the presence of rhodopsin, and at 27 °C there is an apparent match of the bilayer thickness with and without the protein [95].

Let us next turn to a comparison of the experimental $^2$H NMR studies with the molecular dynamics (MD) simulations of the influence of rhodopsin on the POPC bilayer. The more pronounced differences between the order parameter profiles of POPC-$d_{31}$ and POPC-$d_{31}$/rhodopsin in the MD simulations may represent the fact that for a lipid/rhodopsin ratio of 100:1, approximately half of the lipid acyl chains are in the boundary layer that directly solvates the hydrophobic surface of rhodopsin. Therefore, the actual effects of the protein surface may be greater than indicated by the average order parameters obtained from the experimental $^2$H NMR measurements. At this juncture, it is worth noting from the FRET experiments reported in Chapter 4 that in POPC/rhodopsin (100:1) recombinant membranes rhodopsin showed some degree of association (Figure 4.9). Based on these results, one can say that due to protein-protein contacts the number of boundary lipids is in fact reduced. In this respect, it may be that in the configuration of the MD simulation the boundary lipids were overemphasized. Yet rhodopsin in POPC membranes at 1:100 showed to associate, and that could be the reason of the small effect of the protein in the experimental order parameter profiles.

In the first moment analysis ($M_1$) in Figure 6.5, the changes with the temperature represent the structural alterations in the bilayer on going from the gel to the liquid-crystalline phase ($L_a$). The first moment ($M_1$) is related to the bilayer thickness; thus in conjunction with the order-disorder transition the bilayer thickness is changed by the
presence or the absence of rhodopsin. Evidently, the environment of the membrane is heterogeneous with regard to protein-rich microdomains, which are sequestered away from the bulk membrane lipids in both the gel and fluid phase. In the temperature range from -13 °C to 7 °C, a two-phase coexistence of gel and fluid regions was found, and the major effect of the protein was to broaden the phase transition of the POPC-d31 bilayer. When both systems were in the fluid phase, the bilayer thickness of the POPC-d31 bilayer and the POPC-d31/rhodopsin membranes were found to converge with increasing temperature, and at 37 °C they were the same. Therefore, a new finding of this research is that in conjunction with the phase transition process one can monitor the adjustment of the acyl chains of the phospholipids to the intramembraneous protein hydrophobic surface.

The experimental 2H NMR investigations of the POPC-d31 multilamellar dispersions together with POPC-d31/rhodopsin membrane recombinants, both in a fully hydrated form, raises the question whether these two systems are adequately compared. On the one hand, multilamellar dispersions have a limiting hydration (for the polar PC head group), whereas on the other hand recombinant membrane vesicles are in effect swollen infinitely. As a result, the two different situations could be manifested in the order parameter, where the POPC-d31 multilamellar system appears to be more ordered compared to the POPC-d31/rhodopsin membranes (inset to Figure 6.5). One way to overcome with this paradox would be to prepare large unilamellar vesicles of pure POPC-d31, and determine if there is a decrease in the order parameters. Such a lipid system could then be better compared to the rhodopsin/POPC-d31 recombinant membranes. A consequence of having lower order
parameters for the pure POPC-$d_{31}$ system would be that the moment plot (upper curve in the inset of Figure 6.5), would decrease, and consequently matching of the bilayer thickness would appear at a lower temperature.

In this context, it is certainly worth noting that POPC membranes are thinner than the di(20:1)PC system found to yield optimal hydrophobic matching to rhodopsin at 20 °C in the FRET experiments (Chapter 4). This implies that at 20 °C the intramembraneous surface of rhodopsin is greater than the thickness of the POPC membranes. By contrast, from the moment analysis in Figure 6.5 (inset), rhodopsin leads to a thinning of the POPC-$d_{31}$ bilayer (recall that as the temperature increases, the bilayer gets thinner). The differences could possibly be reconciled by attributing them to the POPC-$d_{31}$ being prepared as a multilamellar vesicle (MLV) dispersion, whereas rhodopsin/POPC-$d_{31}$ samples are most likely in the form of large unilamellar vesicles (LUV). If the rhodopsin/POPC-$d_{31}$ samples were prepared as large unilamellar vesicles, and the pure POPC-$d_{31}$ as large unilamellar vesicles (LUV), these discrepancies, if not corrected completely, could be minimized. To conclude, these experiments have provided useful new knowledge regarding the structure of the bilayer in the presence of rhodopsin and its influence on the properties of the bilayer.
CHAPTER 7
EXTENDED FLEXIBLE SURFACE MODEL

In this last aspect of our work, the approach was to adopt the flexible surface model for coupling of the protein to the free energy of the membrane lipids. The picture so far developed implies that the elasticity of the bilayer membrane is linked to alteration of the solvation energy of rhodopsin upon its photoactivation. The dependence of the meta I–meta II equilibrium of rhodopsin on the lipid curvature energy was studied, and it was shown that the protein functions optimally when the membrane is under elastic curvature stress. Here our goal was to investigate an additional aspect, viz. the relationship between hydrophobic mismatch of the components of the proteolipid assembly and protein association. Hydrophobic mismatch can affect the protein organization in the membrane, as well as the conformational changes of rhodopsin. In this way, we are able to expand the framework of membrane-receptor interactions in terms of a new extended flexible surface model. As described in earlier chapters, the dispersal of the protein was examined by changing the protein surface density within the membrane through variation of the lipid-protein molar ratio. In addition, the effects of the acyl chain length were investigated systematically by varying the phospholipid composition.

The newly acquired data showed a clear dependence of the free energy on the acyl chain composition and on the lipid-protein molar ratio. As a result, one can conclude that the formation of meta II is favored by a more dispersed environment, as well as by increasing the length of the acyl chains. It follows that the free energy coupling of the bilayer lipids to the
meta I–meta II equilibrium of rhodopsin involves the bilayer thickness, as well as the curvature stress shown in my previous work [12]. Environments that are conducive to possible association are coupled to the protein in the membrane via the effective number of lipids surrounding rhodopsin. In terms of this new concept, the flexible surface model is extended to account for the elastic stress due to the curvature and thickness frustration associated with hydrophobic mismatch, leading to protein association with possible functional consequences.

7.1. Results

7.1.1. Local Deformation of the Membrane Bilayer

The flexible surface model proposed in Chapter 2, (Section 2.1) considers that the thermodynamic properties of membranes are coupled to the conformational changes of rhodopsin. Here the free energy coupling involves the elastic stress/strain of the bilayer, together with the local acyl chain packing of the lipid. Now, the first step towards extending the flexible surface model involves taking the deformation of the lipid as in the original approach, Eq. (2.20) and (2.21), and expressing it in terms of the relative area contributions of the lipid and the protein. From these equations, a change in standard free energy $\Delta G^\circ$ is driven by the monolayer spontaneous curvature of the proteolipid membrane. However, this treatment considers a well-dispersed lipid environment, that is to say ideal mixing of the lipid and protein components is assumed. A single protein molecule is considered, where any geometrical deformation of the lipid is compensated by a protein conformational change (meta I to meta II).
In what follows, we shall now extend the flexible surface model [12] by taking into explicit consideration the contributions from the relative areas of the lipids and the protein to the curvature free energy for a unit of the proteolipid membrane surface. That is to say, we shall parse the flexible surface into additive contributions from the protein and surrounding lipids. We note furthermore that the pivotal plane, where the lateral forces are balanced and the area is constant under deformation, may or may not correspond to the actual membrane lipid/water interface, e.g. it may be deeper in the bilayer [101]. This approach enables one to consider the curvature free energy as an extensive thermodynamic property in terms of the mixture of protein and lipid components. In this way, the correspondence of the curvature free energy to the lipid-protein molar ratio can be explicitly considered.

In order to consider the correspondence of the curvature free energy to the lipid-protein molar ratio, one must consider the relative area contributions of the lipid and the protein. It was necessary to infer the curvature deformation of the lipids from the area of the protein ($A^p$) relative to the total area of the lipids ($A^l$). As a result, the geometrical curvature of the lipids ($H^l$) in Eq. (2.21) was considered in terms of additive protein and lipid contributions, viz.

$$ H = H^p \left( \frac{A^p}{A^p + A^l} \right) + H^l \left( \frac{A^l}{A^l + A^p} \right) $$

where the term $H^p$ describes the deformation experienced by the protein. Since in a flat bilayer individual membrane monolayer has zero curvature, $H=0$ and hence after rearrangement one obtains
Here, $A^p$ is the flexible surface area due to the protein, and $A^l$ is the corresponding total area due to the lipid membrane affected by the deformation. The lipid cross-sectional area per monolayer per lipid is defined by $A^l = (N^l / 2) A_0$. In addition, $N^l$ is the total number of lipids per protein per bilayer, and $A_0$ is the cross-sectional area per lipid. This expression relates the curvature deformation of the lipid to the curvature strain of the protein, which in turn is related to the shape of its intramembraneous hydrophobic interface. Note that the curvature of the flexible surfaces attributed to the lipids and protein, $H^l$ and $H^p$ respectively, are opposite in their sign (direction).

Consequently, Eq. (2.21) of Chapter 2 can be reformulated in terms of $H^p$ as

$$\Delta G^o = 4\kappa N_A A^p (H_{\text{MII}}^p - H_{\text{MI}}^p) H_0 + 4\kappa N_A \frac{A^p}{A_0} (H_{\text{MII}}^{p^2} - H_{\text{MI}}^{p^2}) \frac{1}{N^l}$$

In the above formula, $H_{\text{MII}}^p$ and $H_{\text{MI}}^p$ denote the protein contributions to the mean curvatures of the flexible surface in the meta I and meta II states, respectively. The spontaneous curvature due to the lipids is $H_0$. As an example, for a mixture of PC and PE lipids $H_0 = H_0^{PC} + X_{PE} (H_0^{PE} - H_0^{PC}) = X_{PE} H_0^{PE}$, where PE and PC denote the head groups present in the DOPE/DOPC mixtures used as a paradigm for the influences of the spontaneous curvature (Chapter 2), and $X_{PE}$ is the molar ratio of DOPE.

Finally, the above equation can be abbreviated as

$$\Delta G^o = C_1 H_0 + C_2 \frac{1}{N^l}$$
where \( C_1 \) and \( C_2 \) are the constant terms in Eq. (7.3). Note that previously, in Eq. (2.21), the stress/strain of the bilayer was related to the spontaneous curvature of the lipids \( H_0 \). By including the area contributions of the lipids and the protein, one can see that the free energy depends not only on the curvature stress/strain, but also on the number of lipids in the membrane \( N^k \).

7.1.2. Dependence of Meta I–Meta II Free Energy Change on the Membrane Lipid-Protein Molar Ratio

The experimental test for the influences of the lipid-protein ratio was presented in Chapter 5. Rhodopsin was recombined in POPC, PDPC, and POPE membranes having protein-lipid molar ratios of 1:50, 1:100, 1:200, and 1:400. From the value of the \( pK_{a1} \) obtained from fitting the data in Figures (5.3)–(5.5), in each case the free energy change \( \Delta G^o \) was calculated using the equation \( \Delta G^o = -(2.303)RTpK_{a1} \). In Figures 7.1–7.3, the standard free energy changes (\( \Delta G^o \)) are plotted in part (a) as a function of the protein-lipid molar ratio \((1/N^k)\), and in part (b) versus the number of lipids \((N^k)\), for rhodopsin in the POPC, PDPC, and POPE membranes, respectively. These plots show how the extended flexible surface model can be applied to the meta I–meta II transition of rhodopsin by including the lipid to protein molar ratio.

The results indicate that meta II formation is favored by a more dispersive environment by increasing of the phospholipid content in the membrane. A nearly linear dependence of the free energy suggests that a dispersive environment promotes the conformational energetics of photolyzed rhodopsin, which is in agreement to Eq. 7.4, where a linear dependence of the free energy on the number of lipids was expected. Moreover, by
expressing in Eq. (2.21) in terms of $1/N^d$ one can examine if the membrane stress depends on the number of lipids, per rhodopsin molecule, thereby altering the distribution the protein within the plane of the membrane.

From the quantitative analysis (Figures 7.1–7.3) the effect of the protein/lipid molar ratio ($1/A^\infty$) on the free energy showed to be linear as expected by the relation described in Eq. 7.4. Indeed, the slopes for rhodopsin in the POPC and PDPC recombinant membrane systems were approximately the same, as shown in Figure 7.1 and 7.2, respectively. The positive slope implies that $H_{\text{II}}^p > H_{\text{II}}^L$ which according to Eq. 7.2 means that $H_{\text{II}}^L < H_{\text{II}}^L$; i.e. in the meta II state the lipids have an increased curvature towards water. By contrast, rhodopsin in the POPE recombinant membranes showed a much smaller slope versus the POPC and PDPC recombinant, and moreover the ordinate intercept was increased.

In terms of Eq. (7.3), since $H_0$ is negative for POPE both observations imply that the difference in the curvatures $H_{\text{II}}^L$ and $H_{\text{II}}^L$ is smaller. However, it is also possible that aggregation could explain the deviation, as shown in the FRET data. Since $1/N^d$ would then become effectively smaller due to the protein-protein contacts, the effective protein/lipid ratio could become $(1/N_{\text{eff}}^L) = x(1/N^L)$ where $x$ is less than a unity, perhaps in analogy with an activity coefficient. On the other hand, comparing the POPC versus PDPC data, in terms of Eq. 7.3, the second term in Equation 7.3, indicates that the slope corresponding to the number of lipids should be the same. The FRET data for PDPC indicated more rhodopsin association than for POPC, and thus one might expect a smaller slope of the free energy versus the protein-lipid ratio, therefore there is a actual effect of rhodopsin association in
FIGURE 7.1. Free energy dependence on the relative amounts of protein and lipid for rhodopsin/POPC membranes. Panel (a) shows the standard free energy $\Delta G^o$ dependence on the protein to lipid ratio $1/N^p$. In panel (b) $\Delta G^o$ is plotted versus the number of lipids $N^l$. The flexible surface model is tested using rhodopsin/POPC molar ratios ($1/N^p$) of 1:50, 1:100, 1:200, and 1:400, yielding a slope of 301 J mol$^{-1}$ lipid$^{-1}$ according to Eq. (7.4). Data are derived from Figure 5.3, where a shift in $pK_{a1}$ is observed as the amount of POPC increases in the membrane.
FIGURE 7.2. Free energy dependence on the protein-lipid molar ratio for rhodopsin/PDPC membranes. Panel (a) shows the free energy $\Delta G^o$ dependence on the protein to lipid ratio $1/N^L$. The flexible surface model is tested using rhodopsin/PDPC molar ratios ($1/N^L$) of 1:50, 1:100, 1:200, and 1:400. A slope of 276 J mol$^{-1}$ lipid$^{-1}$ is obtained according to Eq. (7.4). Panel (b) plots $\Delta G^o$ against the number of lipids per protein $N^L$. Data are from Figure 5.5, where a shift in $pK_{al}$ is found with increasing amount of POPC.
FIGURE 7.3. Free energy dependence on the protein-lipid molar ratio for rhodopsin/POPE membranes. In panel (a) the standard free energy $\Delta G^\circ$ dependence is shown as a function of the protein to lipid ratio $1/N^p$. The flexible surface model is tested using rhodopsin/POPE molar ratios of 1:50, 1:100, 1:200, and 1:400. A slope of 44 J mol$^{-1}$ lipid$^{-1}$ is found using Eq. (7.4). Panel (b) graphs $\Delta G^\circ$ versus the number of lipids per protein molecule $N^l$. Data are from Figure 5.6, where the shift due to POPE is nearly independent of the lipid ratio, since it is below its gel to liquid-crystalline phase transition temperature.
in PDPC membranes. Yet, one may think that this paradoxical effect could have also originated from the fact that the FRET experiments involved the dark state of the protein; whereas, the free energy change was calculated from the light activation data, i.e. probed for rhodopsin during the meta I–meta II transition.

7.1.3. Free Energy Dependence on Membrane Lipid Acyl Chain Composition

From the above general formulation for elastic deformation of the membrane bilayer, two alternative treatments were proposed in Chapter 2, viz. either in terms of elastic area deformation of the bilayer surface, involving area stress/strain of the bilayer lipids due to hydrophobic matching (Eq. 2.14), or alternatively in terms of curvature deformation due to the spontaneous curvature (Eq. 2.15). The latter was experimentally tested with the DOPE/DOPC mixtures (cf. Chapter 2, Section. 2.13). The role of the area stress/strain was also tested by performing experiments with recombinant membranes by systematically varying the acyl chain length from 14 to 24 using the systems di(14:1)PC to di(24:1)PC. It was observed from the FRET experiments in Chapter 4 that hydrophobic mismatch due to either short or long acyl chains induced rhodopsin association within the membrane. In addition, the effects of the hydrophobic mismatch due to acyl chain length on the meta I–meta II transition were tested, as summarized in Figure 5.8.

Now, one would like to know the effect of the acyl chains on the meta I–meta II equilibrium without the aggregation effect. The reason is that the flexible surface model considers a well-dispersed environment for the protein, and in this case, there is no protein association in the membrane. Hence to calculate the dependence of the meta I–meta II free
energy change on the acyl chain length, it was necessary to remove the free energy contribution due to aggregation. Recall that in case of aggregation, the number of boundary lipids around the protein diminishes, which is defined here as the effective number of lipids \( N_{\text{eff}}^L \). Thus, to calculate the free energy dependence on the acyl chain composition, the following steps were considered, as depicted in Figure 7.4. First, the FRET index data in Figure 4.9 part (b), as a function of the acyl chain length were taken as a starting point, shown in part (a) of Figure 7.4. To be able to calculate the effective number of lipids corresponding to each chain length, it was necessary to use a system which showed a systematic variation with the number of lipids. Thus, the FRET data index for the POPC/rhodopsin membranes (Figure 4.9) were used to calibrate and then convert the chain length to the protein density in the membrane, as shown in part (b) of Figure 7.4 (This is further described in Section 7.1.4.) From the linear fitting of the POPC data, the number of effective lipids was then calculated, and plotted as a function of the acyl chain length in Figure 7.4, part (c). The need for the graph (c) was to show the influence of the protein on the acyl chain length effect. In other words, this step of the analysis was required to separate the effect of the acyl chain length from the effect of aggregation. Note that from this result the effective number of lipids had a maximum value of 140 lipids; whereas the stoichiometry of the recombinant membranes was 1:100 (protein to lipid ratio). Therefore, this value exceeded the number of the lipids used in the experiments, and the discrepancy indicated that the POPC system was not ideal for the calibration. In the future, for a better calibration it would be better to use di(20:1)PC, and perform the studies at different protein to lipid molar
FIGURE 7.4. Test of the flexible surface model (FSM) showing dependence of the standard free energy $\Delta G^o$ on the acyl chain length and the protein to lipid molar ratio. The acyl chain length was varied from 14 to 24 carbons for the homologous phosphatidylcholine series di(X: 1)PC, and the protein to lipid molar ratio was varied from 1:50 to 1:400. Graphs (a), (b), (d), and (f) are from the experimental data in Figures 4.9(b), 4.9(a), 7.1, and 5.7, respectively, as explained in the text. Note that a nearly linear behavior is observed in graph (g), suggesting the free energy of the meta I–meta II transition depends on the acyl chain length.
ratios. Nevertheless, since these deviations affect only the scaling factor $x$, the final analysis is unaffected. From this point on in Figure 7.4, the remaining analysis includes the variation of the meta I–meta II equilibrium with respect to the lipid-protein ratio for POPC systems. The experimental data Figure 7.1 were used to provide a second calibration curve, as shown in graph (d) of Figure 7.4. From the linear fit, the aggregation free energy $\Delta G_{agg}^o$ was calculated directly, using the effective number of lipids from graph (c). The results are plotted in part (e) of Figure 7.4, showing the aggregation free energy versus the acyl chain length, due to the meta I–meta II transition. Next, the experimental $pK_{ai}$ values from Figure 5.7 were used to calculate the free energy change $\Delta G^o$ versus the chain length for the di(X:1)PC systems, and are plotted in panel (f). For the last step, to extract the actual acyl chain contributions to the aggregation effect, each point of $\Delta G^o$ from the graph (e) was subtracted from the $\Delta G^o$ values of graph (f), and the results were plotted in graph (g). *This graph shows a linear relation of the free energy change with the acyl chain length, after removal of the effect of aggregation.*

The above stepwise analysis provided a crucial test for the flexible surface model, with respect to the linear behavior on the free energy to the acyl chain length, according to Eq. 7.4. Because the FSM considers the protein in a dispersed environment, it was therefore necessary to eliminate the aggregation effect. In this way it was possible to establish for the first time a direct influence of the lipid acyl chain length (hydrophobic matching) on the free energy of the meta I–meta II conformational change of rhodopsin.
7.1.4. Implication of FRET Data for Two-Dimensional Membrane Packing of Rhodopsin

The aggregation analysis was possible based on the distribution of the acceptor in two-dimensions as in the case of a protein in bilayer membrane [59, 102]. For that it was important to calculate the acceptor surface density for each recombinant system containing a different lipid to protein molar ratio. One considers the area occupied per rhodopsin molecule in the membrane as 1000 Å² [75, 103], and the area per lipid was taken as 65 Å². As an example, for systems containing 50 lipids per rhodopsin, the area occupied by the lipids in each monolayer was calculated as 50/2 × 65 Å², giving a value of 1625 Å². For membranes containing 100, 200, and 400 lipids to protein ratios, the values were calculated as 3250, 6500, and 13000 Å², respectively. In case of 50 lipids, the total area of rhodopsin plus the number of lipids is 2625 Å². The surface density of the protein is calculated by taking the $R_\text{Q}$ value [104] as 60 Å × 60Å (for the donor and acceptor distances), giving a value of 3600 Å². Now, it is convenient to express the protein surface density as a reduced surface density, taking the characteristic length scale of FRET, the Förster radius ($R_0$), into account. However, in this work the Förster radius for the donor-acceptor pair, Alexa 488-Alexa 594, is not equal to 60 Å due to the energy transfer to the dark state of rhodopsin. Thus, the effective lifetime of the donor fluorophore is shorter as mentioned in [104]. The relevant quantity now is the number of acceptor fluorophore per unit area, rather than the Förster radius squared.

The number of rhodopsin molecules that occupies this area is then $3600 \text{ Å}^2/2625 \text{ Å}^2$, which gives a value of 1.37. Since in the experimental design we labeled rhodopsin at a 1:1
fluorophore molar ratio, one can assume that one-half of rhodopsin molecules contains the acceptor. Therefore, for the systems containing 50 lipids per rhodopsin, the acceptor density was calculated as 0.69 (1.37/2). For 100, 200, and 400 lipids per rhodopsin, the density of the acceptor was calculated as 0.42, 0.24, and 0.13, respectively. Using these reduced densities, and a conservative estimate for the distance of closest approach between the donor and acceptor, in the case of rhodopsin $R_c=44$ Å, one can expect an almost linear relation of FRET to the acceptor density [59, 102]. The $R_c$ value was calculated by taking the projected area of rhodopsin as 1500 Å², and approximating the protein as having a circular geometry ($A=\pi r^2$); thus the radius of rhodopsin was calculated as 21.9 Å.

All of these calculations were carried out prior to conducting the FRET experiments, to provide a framework valid approach for interpreting the data in terms of protein association. One needed these calculations to be able to estimate the energy transfer that would be provided in the rhodopsin systems. In this way, a random distribution of the acceptor in a two-dimensional analysis was assumed. As more lipids were added, the density of the acceptor decreased, and so the transfer energy efficiency also decreased. Therefore, FRET experiments were performed and the observable FRET index was indeed able to provide novel information about the protein association in the membrane. As a further analysis, the contribution of the aggregation to the free energy could be quantified (Figure 7.4).

The results for the FRET index for all the rhodopsin/phospholipids recombinant membranes studied are summarized in Figure 7.5. As it can be observed, the systems
FIGURE 7.5. Summary of FRET index data for all phospholipid systems studied in this work. The head groups are PC and PE with different acyl chain lengths and degrees of unsaturation.
DMPC, POPE, and di(24:1)PC, which were in the gel phase under the experimental conditions (20 °C), are the ones with the highest FRET index. For POPC and PDPC it is clearly seen that as the lipid-protein molar ratio increases, the FRET index decreases, implying that rhodopsin was more dispersed in the membrane. It is interesting to observe that from the FRET experiments, the PDPC systems containing DHA acyl chains promoted association of rhodopsin in the dark state.

A further important aspect is that for the systems POPE/POPC compared to DOPE/DOPC, the degree of association of rhodopsin is similar, which was the reason for discarding the possibility of the chemical effect of the PE head group. For the di(X:1)PC recombinant membranes, the protein was least associated when surrounded by di(20:1)PC phospholipids. Regarding the DOPE molar ratio to DOPC, the system containing the higher amount of DOPE (75:25) promotes a better dispersal of the protein. This is important in terms of the flexible surface model, since this mixture favors meta II formation due to the curvature stress/strain in the membrane [12] with corresponding dispersal of rhodopsin within the membrane.

7.2. Discussion and Conclusions

This work aims to extend the flexible surface model in terms of considering the stress/strain in the bilayer related to the packing density of the rhodopsin molecules (1/A^2). To confirm the theoretical development (Eq. 7.4), experiments were performed for rhodopsin in POPC, PDPC, and POPE recombinant membranes having a lipid-protein molar ratio of 1:50, 1:100, 1:200, and 1:400. The flexible surface model proposed earlier [12] takes into
account a well-dispersed environment, where the deformation of the lipid due to the conformation change of the protein is accompanied by a compensatory change in the surrounding lipids. With this view, the total amount of the lipids in the system is influenced by the protein, and vice-versa.

Knowing that it was possible to have two different environments of the membrane, dispersed and non-dispersed, it was important to conduct experiments to examine the theoretical approach (Eq. 7.4). In this way, we were able to obtain information about how the lateral organization of the protein and its function would be affected by increasing the amount of lipids in the membrane. As rhodopsin can associate in the membrane, the fraction of the lipid in the vicinity of protein oligomers can be more or less independent of the total amount of the lipids in the system. In other words, any additional lipid would simply add to the unperturbed bulk regions, well separated from the protein. Assuming strong association of the protein, changing the total amount of lipids would not influence the number of the boundary lipids. Thus, the effect of increasing the amount of total lipids would not appreciably affect the elastic coupling between boundary lipids and the protein intramembranous shape. The number of lipids affecting the environment of the protein was included in our previous treatment in the flexible surface model [12], which was extended in this work and further investigated experimentally.

Another purpose of this work was to investigate the role of other kinds of stress/strain involving bilayer deformation. The role of curvature stress/strain was tested previously, and the area stress was mentioned as a different approach to describing the elastic deformation of
the membrane bilayer [8], but it was not tested [12]. In this study, besides the number of lipids, we wanted to experimentally test the area stress/strain (frustration) of the bilayer lipids, due to hydrophobic matching with the protein. The experiments were performed by varying systematically the acyl chain length, in the case of recombinants of rhodopsin with the phosphatidylcholine series di(14:1)PC, di(16:1)PC, di(18:1)PC, di(20:1)PC, and di(24:1)PC.

Note that although hydrophobic mismatch induces aggregation, this general observation has nothing to do per se with the flexible surface model. What concerns the flexible surface model is how the aggregation or the lateral organization of the protein in the membrane affects the meta I–meta II conformational changes of rhodopsin. The two shapes of the two protein conformations are related to the stress/strain in the bilayer influenced by the material properties of the lipids.

For the quantitative analysis of the flexible surface model, the dependence of the meta I–meta II free energy change involves two kind of stress, curvature and hydrophobic mismatch (via acyl chain length). Both are coupled to the number of lipids, or in case of association, to the effective number of lipids, i.e. the boundary lipids due to less dispersal of the protein. Hence, it can be concluded that the flexible surface model, involving curvature frustration and hydrophobic mismatch as mechanisms for coupling the free energy of the bilayer lipids to rhodopsin, provides a satisfactory explanation for the experimental results.

7.2.1. Role of Hydrophobic Mismatch of Membrane Lipids and Rhodopsin

A key aspect is that the hydrophobic mismatch was found to affect the protein
organization in the bilayer membrane as revealed by the FRET experiments. The association of the protein in the membrane also influenced the conformational changes of rhodopsin from meta I to meta II, as evident from the UV-visible spectrophotometric studies. From the point of view of the flexible surface model, the acyl chain length, \textit{i.e.} after separation from the effect of protein association (Figure 7.4), was found to have an intrinsic influence on the free energy change due to formation of the meta II intermediate of rhodopsin. By separating the effect of the acyl chain length from the aggregation effect, we discovered that during the transition from meta I to meta II, rhodopsin needs to be surrounded by acyl chains whose length matches the hydrophobic interface of the protein. From these experiments, it was discovered that the optimal length was 20 carbons. Moreover, by elimination of the contribution from protein association to the free energy dependence on the acyl chain length, we showed that even in a dispersed environment rhodopsin requires long acyl chain for activation. This key finding suggests that meta II probably adopts an elongated shape compared to meta I intermediate as shown directly by plasmon resonance studies [54].

Other phospholipid systems were also tested to investigate how rhodopsin can organize itself in membranes containing phospholipids with different physical properties, such as the phase transition temperature, acyl chain unsaturation, and polar head groups (PE and PC), all of which can affect protein association. We found that the gel-phase membranes, DMPC, di(24:1)PC, and POPE, all promoted strong association of rhodopsin as compared to the other systems in the liquid-crystalline phase (Figure 7.5). Interesting was to observe that the gel-phase membranes favored formation of meta I more than meta II. In
terms of polyunsaturation, it was shown that di(DHA)PC compared to PDPC and POPC dispersed rhodopsin significantly in the membrane, presumably by increasing the flexibility of the bilayer due to the two polyunsaturated acyl chains. The di(DHA)PC membrane was able to disperse rhodopsin as much as the DOPE/DOPC systems, which supports the observation that rhodopsin functions better in a flexible and dispersed environment, in agreement with the treatment of the flexible surface model [12].

An additional effect on the protein association was found for rhodopsin in the PDPC membranes due to the presence of the polyunsaturated DHA acyl chain. This is a new finding, which together with the fact the DHA is the most abundant acyl chain in the rod disk membranes [44] may help in understanding the roles of polyunsaturation in the rod cells. For instance, DHA-phospholipids are enriched in the rhodopsin post-Golgi vesicles, and this colocalization of DHA remains after fusion with the plasma membrane [46]. It raises interesting questions about the role of DHA in assembling rhodopsin in the rod disk membranes. Moreover, the concept of oligomerization in general for GPCRs has been shown to affect the activation state of some receptors towards binding of ligands [105]. Therefore, from what has been observed from this work, our discovery of rhodopsin association in the dark is strongly supported by other research in the field.

7.2.2. Spontaneous Curvature and Balance of Forces

In previous work, it was concluded that the meta II state is favored by properties of mixtures of the lipids in the membrane [8]. The combined effects of the lipid polar headgroups and non-polar acyl chains point to the role of material properties of the lipids.
Moreover, the lipid effects on the light-induced activation of rhodopsin have suggested an important role of non-lamellar, reverse-hexagonal phase promoting lipids in the rod disk membranes [12], which are important for the stabilization of the active metarhodopsin II conformation. Our experiments were aimed at understanding of the spontaneous curvature by comparing rhodopsin in DOPE/DOPC mixtures to POPE/POPC recombinant membranes. Both systems promoted nearly the same degree of rhodopsin association in the dark state (Figure 7.5). Yet remarkably when these two systems were tested for the light activation of rhodopsin by UV-visible spectrophotometry (cf. Chapter 5), a distinct effect of DOPE/DOPC towards formation of meta II$_{SB}$ versus POPE/POPC was observed (Figure 5.12).

Some possible explanations for the role of PE head group have been raised, e.g. one considers that the PE head group affects the bilayer thickness, and another that the effects are chemically specific in origin. First we note that NMR experiments [106] show that the bilayer thickness increase due to the PE head group is less than 10%. As a comparison, in this work experiments were performed with different acyl chain lengths, having a 10-carbon difference in the series di(14:1)PC to di(24:1)PC, much more than the 10% increased in thickness due to the PE headgroup alone. Therefore, one can discard the possibility of a bilayer thickness effect due to the PE head group. The second alternative is the chemical effect of the PE head groups, which can form additional hydrogen-bonds with the protein as compared to PC headgroups. From the results observed in the present work, this possibility is also discarded due to the results from FRET experiments. Since POPE/POPC (50:50) promoted a similar behavior for rhodopsin in the membrane compared to DOPE/DOPC
(50:50), the chemical effect is not present.

The key experiment came from the activation of rhodopsin. One may ask: why did the presence of POPE not stimulate meta II as much as DOPE? In these experiments, both the systems POPE/POPC and DOPE/DOPC are the same with respect to the average of the acyl chain, unsaturation, head group, and molar ratio. Moreover, they both are in the fluid, liquid-crystalline phase. The curvature stress for POPE is very small since its lamellar ($L_{\alpha}$) to inverse hexagonal ($H_{\inn}$) phase transition temperature is 60 °C. By contrast, for DOPE the lamellar to hexagonal phase transition is 10 °C. Under the temperature conditions of these experiments (20 °C), POPE could not have reached a temperature approaching the non-lamellar phase transition; whereas the DOPE/DOPC mixtures were close to the non-lamellar phase boundary. Therefore it was clear that the effect on the meta I–meta II transition was from the spontaneous curvature. To expand further, the electrically neutral DHA chains in combination with PE are known to favor a "wedge shape" on average for the membrane lipids, promoting a negative monolayer curvature, and we have shown that they are important for optimal function of rhodopsin under physiological conditions. As a result, phospholipids containing PE headgroups together with DHA tend to adopt the nonlamellar, reverse hexagonal ($H_{\inn}$) phase. The native mixture of headgroups comprising PC, PE, and PS together with polyunsaturated DHA chains is close to a lamellar-nonlamellar phase boundary, and it is sufficient for maximum photochemical function of rhodopsin.0.

In this work, polyunsaturated DHA acyl chains were combined with PC headgroups (PDPC), and were shown to promote association of rhodopsin in the dark state. When
compared to POPC, as a reference, PDPC was found to promote more meta II\textsubscript{SB}. Therefore, one can say that the bulky chain of DHA favors formation of the meta II\textsubscript{SB} intermediate. In other words, DHA has a neutral effect in that it activates rhodopsin but promotes association of the protein. From the experiments performed in this work, the spontaneous curvature effect of DHA combined with PC headgroups was not observed directly. However, due to its bulky acyl chain, which provides a driving force to form the non-lamellar $H_\text{II}$ phase, one still can say that DHA has a propensity of forming the nonlamellar phase, thus driving conformational changes of rhodopsin. Nonetheless, further experimental studies of the physical properties of DHA-containing phospholipids are required.

In conclusion, the results from this work can be summarized according to the following. The activated intermediate meta II\textsubscript{SB} is favored by a more dispersed and flexible environment. The optimal chain length that favors meta II\textsubscript{SB} was found to be 20 carbons in length. In terms of the shape transition from meta I to meta II, it was observed that lipids in the gel phase increase the bilayer thickness, and promote rhodopsin association, consequently favoring a more cylindrical shape of the protein, which in our work is meta I. Lipids in the fluid-phase ($L_\alpha$) have a decrease in the bilayer thickness, favoring protein dispersion, and promoting meta II\textsubscript{SB}. The spontaneous curvature, acyl chain length, and number of lipids are all coupled to the free energy of meta I–meta II conformational energetics of rhodopsin.

Based on the FRET experimental results, a possible formation of protein-rich phases within fluid bilayers is suggested, due to a hydrophobic mismatch, implying that protein association can occur in the presence of a single lipid type. To illustrate the new
biomembrane model developed in this work, Figure 7.6 shows two rhodopsin molecules in the dark state embedded in the POPC membranes. Future crystal structures of the photointermediates might provide additional support for the conclusions in this work.

7.3. General Perspectives and Future Directions

These studies provide a general understanding of how the elastic properties of the membrane bilayer can be modulated to promote stabilization and function of integral membrane protein, including GPCRs such as rhodopsin. The multi-disciplinary results obtained from FRET, UV-Visible spectrophotometry, and $^2$H NMR spectroscopy provide an insight of how lipids and proteins interact and organize themselves in membranes. These findings relating bilayer thickness, conformational energetics of rhodopsin in different lipids, together with protein association and the effective number of lipids, are meant to be understood not in isolation, but within the context of the system as a whole. The interrelation between these factors serves as a general framework for understanding how the elastic properties of the bilayer membrane can be modulated to achieve a membrane environment conducive for the protein to function optimally.

The visual receptor system is very attractive for investigating lipid-protein interactions in membranes. Several key experiments were done to study the relevance of the lipid environment to the physiological processes associated with visual signaling. Different types of phospholipids were sampled to bring a more complete understanding of how to tune the biophysical properties of the bilayer to obtain an optimal structural organization and function
FIGURE 7.6. Molecular model of rhodopsin in POPC membranes in the dark state. The molecular dynamics simulation (from Ref. [107]) is equilibrated at 37 °C, and corresponds to the new biomembrane model suggested in this work.
of the protein. In general case of lipid membranes, it would be interesting to produce different cell types where a transmembrane protein could have a defined lipid composition, so as to test the efficiency of its behavior. More detailed studies on the aggregation effect due to either curvature or bilayer thickness is still necessary. It would be helpful to study the effects of the lateral organization of the protein on the binding of transducin, for example, or other components of the visual transduction cascade. Lastly, in this work the vesicles containing rhodopsin are assembled with different protein orientations. It would be worthwhile to find general methods to prepare the vesicles where the protein could be inserted in one direction. This could help to understand other receptors functions regarding ligand binding at one side of the receptor, which has a site specific for ligand binding, and another site for the G-protein. All these future studies could be done with the flexibility of modulating the binding according to the presence of different membrane lipids.

In conclusion, the flexible surface model was extended through a novel series of experiments. The central idea predicts that the lowest energy state of the membrane is one in which the protein is more dispersed. The curvature free energy is balanced by the solvation of the lipid/protein interface, or by the hydrophobic matching stress in the membrane, which are coupled to the effective number of lipids in the case of protein association. Biological activity could be then regulated by membrane lipids, whose spontaneous curvature or acyl chain length most closely matches the active state of the proteolipid assembly.
APPENDIX A. Gouy-Chapman calculation for the local pH of the membrane
(MATHEMATICA 4.1 program).

(* This script includes changed number of titratable
  groups, as a result,
  for a pH_{bulk} = 5.0,
  the protonation state of rhodopsin corresponds to a
  local pH =
  5.55 in PS free bilayers.
  This pH gives a clear state of protonation for Glu
  &Asp as well \ as His residues *)

modell[NPSGC_, BulkpH_, fractioncyt_, nLPGC_, cAddSalt_] :=

  AreaGC = 1000. + 70.0/2.*nLPGC;
  sigmaacid[Num_, KaGC_, pHlocalGC_] := (* anion surface
    charge density *)
    Num/AreaGC*KaGC/(KaGC + 10^(-pHlocalGC));
  sigmabase[Num_, KaGC_, pHlocalGC_] := (*
    cation surface charge density *)
    Num/AreaGC*10^(-pHlocalGC)/(KaGC + 10^(-pHlocalGC));
\[
\text{Num/AreaGC}/(1 + 10^{(-pH_{localGC})}*(1/KaGC + 1/cH_{bulk}*c_{salt}/K_{salt}));
\]

(* salt dissociation constant for PS from Hubbell 1/0.7 for Na + *)

(* salt dissociation constant for PS from Liebmann 6.7 for K + *)

\[K_{salt} = 6.7;\]

\[\text{sigma}_{cyt}[pH_{GC}, pH_{bulkGC}] := (*Cave all calculations are only for cytoplasmic*)\]

\[\text{sigma}_{base}[5, 10^{(-12.0)}, pH_{GC}](*Arg*) + \]
\[\text{sigma}_{base}[9, 10^{(-10.1)}, pH_{GC}](*Lys*) + \]
\[\text{sigma}_{base}[2, 10^{(-6.75)}, pH_{GC}](*His*) - \]
\[\text{sigma}_{acid}[2, 10^{(-4.5)}, pH_{GC}](*Asp*) - \]
\[\text{sigma}_{acid}[1, 10^{(-8.65)}, pH_{GC}](*changed from 2, Cys*) - \]
\[\text{sigma}_{acid}[7, 10^{(-4.5)}, pH_{GC}](*Glu*) - \]
\[\text{sigma}_{acid}[1, 10^{(-6.2)}, pH_{GC}](*Glu ERY*) - \]
\[\text{sigma}_{acid}[1, 10^{(-9.8)}, pH_{GC}](*changed from 2, Tyr*) - \]
\[\text{sigma}_{acid}[1, 10^{(-3.1)}, pH_{GC}](*COOH*) - \]
\[\text{sigma}_{PS}[NPSGC, pH_{GC}, 10^{(-pH_{bulkGC})}, c_{AddSalt}, 10^{(-3.6)}, K_{salt}](*PS*);\]

\[\text{sigma}_{lum}[pH_{GC}, pH_{bulkGC}] := (*Cave all calculations are only for luminal*)\]
\[ \text{sigmabase}[2, 10^{(-12.0)}, \text{pHGC}](*\text{Arg}*)+ \\
\text{sigmabase}[1, 10^{(-10.1)}, \text{pHGC}](*\text{Lys}*)+ \\
\text{sigmabase}[3, 10^{(-6.75)}, \text{pHGC}](*\text{His}*)- \\
\text{sigmacid}[7, 10^{(-4.5)}, \text{pHGC}](*\text{Glu not counting Glu113, changed from 10 Asp & Glu}*)- \\
\text{sigmacid}[2, 10^{(-4.5)}, \text{pHGC}](*\text{Asp}*)- \\
\text{sigmacid}[1, 10^{(-8.65)}, \text{pHGC}](*\text{Cys}*)- \\
\text{sigmacid}[9, 10^{(-9.8)}, \text{pHGC}](*\text{Tyr}*)- \\
\text{sigmaPS}[\text{NPSGC, pHGC, 10^{(-pHbulkGC)}}, \text{cAddSalt}, 10^{(-3.6)}, \text{Ksalt}] (*\text{PS}*) ; \\
\]

(* not considered : His211, Glu113, Glu122, Lys296, Cys219, Tyr220 *)

\[ \text{sigma}[	ext{pHGC}_-, \text{pHbulkGC}_-] := \\
\text{fractioncyt}\times \text{sigmacyt}[	ext{pHGC, pHbulkGC}] + \\
(1 - \text{fractioncyt})\times \text{sigmalum}[	ext{pHGC, pHbulkGC}] ; \\
\]

\[ \text{solGC}[	ext{pHbulkGC}_-] := \text{FindMinimum}[\text{Sqrt}[	ext{sigma}[	ext{pHGC, pHbulkGC}] - \\
\text{Sqrt}[\text{CGC}] / \text{AGC} / \\
2\times (10^{((\text{pHGC} - \text{pHbulkGC})/2)} - \\
10^{((\text{pHbulkGC} - \text{pHGC})/2)})^2] / . \{\text{CGC} \to (\text{cAddSalt}), \\
\text{AGC} \to 136.6\}, \{\text{pHGC, pHbulkGC}] ; \]
resGC = solGC[BulkpH];
(pHGC /. resGC[[2]])
);

model[NPS_, BulkpH_, fractioncyt_, cAddSalt1_] :=
    model1[NPS, BulkpH, fractioncyt, 100, cAddSalt1];

model2setup[NPS_, fraction_, Nlipid_, cAddSalt2_] := {
fn = ListInterpolation[
    Table[model1[NPS, x, fraction, Nlipid, cAddSalt2],
        {x, 0, 14, .2}],
        {{0, 14}}];
Plot[{{x, fn[x]}, {x, 0, 14}}];
}

model2[pH2_] := (x2 /. 
    FindMinimum[(fn[x2] - pH2)^2, {x2, pH2 - 0.5, 
        pH2 + 0.5}][[2]]);

model3[pH3_] := (fn[pH3]);

model2setup[0, 0.5, 100, 0.01]
Plot[model3[pH], {pH, 4, 9}]

(* Initialize the model with
   1. no negatively charged lipids (0) *)
2. a scrambled orientation of rhodopsin (0.5)
3. with 200 molecules lipid per protein (200)
4. with 150 mM potassium cation concentration (0.150)

*)

model2setup[0, 0.5, 200, 0.15]

Plot[{{pH, models[pH]}, {pH, 5.5, 8.5}}]

Plot[models[pH] - pH, {pH, 5.5, 8.5}]
APPENDIX B.I. Order Parameters and Spin-Relaxation Rates of POPC-\(d_{31}\) multilamellar dispersions at 10 wt. % water, and at temperatures of 17, 27, and 37 °C.

Table 1. Order parameters and spin-lattice relaxation rates for POPC-\(d_{31}\) multilamellar vesicles at 10 wt. % water, at 17, 27, and 37 °C. Data acquired at 76.77 MHz with a 5 mm probe coil.

| no. carbons | \(|S_{cd}|\) 17 °C | \(R_{1z}/\tilde{s}^{-1}\) 17 °C | \(|S_{cd}|\) 27 °C | \(R_{1z}/\tilde{s}^{-1}\) 27 °C | \(|S_{cd}|\) 37 °C | \(R_{1z}/\tilde{s}^{-1}\) 37 °C |
|-------------|----------------|-----------------|----------------|-----------------|----------------|----------------|
| 2           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 3           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 4           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 5           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 6           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 7           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 8           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 9           | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 10          | 0.31189        | 26.33321        | 0.29274        | 28.73675        | 0.27455        | 26.70949        |
| 11          | 0.27502        | 25.40743        | 0.253          | 28.45354        | 0.23362        | 26.70949        |
| 12          | 0.26258        | 24.98471        | 0.23577        | 23.03642        | 0.21399        | 17.688          |
| 13          | 0.23146        | 17.64286        | 0.20633        | 17.62777        | 0.18574        | 15.36098        |
| 14          | 0.19556        | 13.37366        | 0.17234        | 12.8325         | 0.15391        | 11.84854        |
| 15          | 0.14696        | 10.75503        | 0.1283         | 9.56127         | 0.11394        | 8.45277         |
| 16          | 0.04332        | 4.8143          | 0.03602        | 3.59344         | 0.03183        | 3.05479         |
APPENDIX B.2. Order Parameters and Spin-Relaxation Rates of POPC-\textit{d}_{31} multilamellar dispersions at 20 wt. % water, and at temperatures of 17, 27, and 37 °C.

Table 2. Order parameters and spin-lattice relaxation rates for POPC-\textit{d}_{31} multilamellar vesicles at 20 wt. % water, at 17, 27, and 37 °C. Data acquired at 76.77 MHz with a 5 mm probe coil.

| no. carbons | \( |S_{cd}| \) \(17 \ ^\circ\mathrm{C} \) | \( R_{1z}/s^{-1} \) \(17 \ ^\circ\mathrm{C} \) | \( |S_{cd}| \) \(27 \ ^\circ\mathrm{C} \) | \( R_{1z}/s^{-1} \) \(27 \ ^\circ\mathrm{C} \) | \( |S_{cd}| \) \(37 \ ^\circ\mathrm{C} \) | \( R_{1z}/s^{-1} \) \(37 \ ^\circ\mathrm{C} \) |
|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 2           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 3           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 4           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 5           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 6           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 7           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 8           | 0.24654          | 34.42749        | 0.22835         | 31.92421        | 0.2183          | 28.73326        |
| 9           | 0.24654          | 34.42749        | 0.21064         | 32.44553        | 0.20082         | 28.90758        |
| 10          | 0.22261          | 35.2445         | 0.20226         | 32.2439         | 0.19173         | 28.15004        |
| 11          | 0.20154          | 27.92143        | 0.18167         | 26.10843        | 0.17042         | 23.42779        |
| 12          | 0.18359          | 24.67524        | 0.16492         | 22.37246        | 0.15343         | 19.16568        |
| 13          | 0.15822          | 22.16228        | 0.1405          | 19.85653        | 0.12997         | 16.36433        |
| 14          | 0.13285          | 16.84483        | 0.11705         | 15.261          | 0.10771         | 13.11632        |
| 15          | 0.0991           | 12.33658        | 0.08641         | 10.86886        | 0.07947         | 9.32342         |
| 16          | 0.02944          | 4.45031         | 0.02561         | 3.5678          | 0.02322         | 3.14338         |
APPENDIX B.3. Order Parameters and Spin-Relaxation Rates of POPC-\textsubscript{d\textsubscript{31}} multilamellar dispersions at 10, 20, and 40 wt. % water, and at temperatures of 17, 27, and 37 °C.

Table 3. Order parameters and spin-lattice relaxation rates for POPC-\textsubscript{d\textsubscript{31}} multilamellar vesicles at 40 wt. % water, at 17, 27, and 37 °C. Data acquired at 76.77 MHz with a 5 mm probe coil.

| no. carbons \(sn-1\) | \(|S_{\text{col}}|\) \(17 ^\circ C\) | \(R_{1z}/s^{-1}\) \(17 ^\circ C\) | \(|S_{\text{col}}|\) \(27 ^\circ C\) | \(R_{1z}/s^{-1}\) \(27 ^\circ C\) | \(|S_{\text{col}}|\) \(37 ^\circ C\) | \(R_{1z}/s^{-1}\) \(37 ^\circ C\) |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 2                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 3                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 4                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 5                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 6                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 7                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 8                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 9                   | 0.21854          | 38.65702        | 0.20753         | 32.53798        | 0.1958          | 28.82748        |
| 10                  | 0.20178          | 39.0032         | 0.19005         | 31.98255        | 0.1958          | 24.99234        |
| 11                  | 0.17066          | 28.88067        | 0.15702         | 25.28812        | 0.14457         | 21.55042        |
| 12                  | 0.15582          | 25.60806        | 0.1429          | 21.00251        | 0.12997         | 17.11116        |
| 13                  | 0.13332          | 22.97911        | 0.12136         | 19.01015        | 0.10891         | 15.44594        |
| 14                  | 0.11202          | 17.52918        | 0.10149         | 13.69291        | 0.09024         | 10.72331        |
| 15                  | 0.08402          | 12.93663        | 0.07564         | 9.99404         | 0.06822         | 8.85229         |
| 16                  | 0.02537          | 4.39808         | 0.02226         | 3.41241         | 0.02035         | 2.90399         |
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