Use of Fluorescence Resonance Energy Transfer for Detection of Lattice Formation in ASR

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Abstract

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Anabaena sensory rhodopsin (ASR) is a microbial sensory rhodopsin which is known to form trimers in lipids and detergent. Furthermore, recent SAXS results suggested that the trimers assemble into the BR-like hexagonal lattice. This project aimed to investigate the hexagonal lattice formation of ASR by developing and applying Fluorescence Resonance Energy Transfer method. When the hexagonal lattice formation occurs the distances between monomers in different trimers will be short, resulting in strong intertrimer FRET signals. Mutant S26C ASR was successfully labelled with FRET pair, Alexa Fluor 555 and Alexa Fluor 647, at non-native cysteine C26 and strong FRET was detected. The effects of environmental factors, packing density and fluidity of lipids, on lattice formation of ASR were investigated. As lipid content was increased, relative FRET efficiency reported a modest increase in the intermolecular distances between monomers in different trimers without complete lattice dissolution, while the fluidity of lipids had no significant effect on the intermolecular distances.
Acknowledgements

First, I would like to thank my advisors, Dr. Leonid Brown, for his help, support, patience and guidance over the past few years. I would also like to thank Dr. Vladimir Ladizhansky and Dr. Rickey Yada, for being on my advisory committee. In addition I would like to thank Dr. Robert Wickham as chairing my examination committee.

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List of Abbreviations

**Bacteria**

*E. coli. Escherichia coli*

**Chemicals**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-Thiogalactopyranoside</td>
</tr>
<tr>
<td>Ni^{2+}-NTA</td>
<td>Nickel-Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>DDM</td>
<td>n-Dodecyl β-D-maltoside</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>CHES</td>
<td>2-(N-Cyclohexylamino) ethane Sulfonic Acid</td>
</tr>
<tr>
<td>MMTS</td>
<td>methyl methanethiosulfonate</td>
</tr>
<tr>
<td>IAEDANS</td>
<td>5-((2-[(iodoacetyl)amino]ethyl)amino)naphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>EDANS</td>
<td>(5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>Dabcyl</td>
<td>4-((4-(dimethylamino)phenyl)azo)benzoic acid</td>
</tr>
</tbody>
</table>

**Proteins**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
</tr>
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</table>

**Lipids**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPA</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>PC</td>
<td>L-α-phosphatidylcholine (from Egg, Chicken)</td>
</tr>
<tr>
<td>PS</td>
<td>L-α-phosphatidylserine (from Brain, Porcine)</td>
</tr>
</tbody>
</table>
**Biophysical Terms**

OD  Optical Density

w/w  Weight over weight ratio

v/v  Volume over volume ratio

P/L  Protein to Lipid ratio

L/P  Lipid to protein ratio

**Biophysical method**

CD  Circular Dichroism

FRET  Fluorescence Resonance Energy Transfer

NMR  Nuclear Magnetic Resonance

SAXS  Small Angle X-ray Scattering

CA  Alpha carbon

CB  Beta carbon
Chapter 1. Introduction

1.1 Membrane proteins

Membrane proteins are proteins that interact with biological membranes. Some of these proteins that span the membrane and have segments facing both the interior and exterior surfaces are classified as integral membrane proteins or transmembrane proteins. Other proteins, called peripheral membrane proteins, are found only on one side of the membrane. Often, peripheral membrane proteins are attached to integral membrane proteins.

Membrane proteins are essential for survival of organisms. They are responsible for cell-to-cell signaling and for making physical connections between cells. Also, certain membrane proteins act as enzymes and ion (and other solute) transporters across the plasma membrane [1].

1.2 Rhodopsins

Rhodopsins are integral membrane proteins, which are found throughout animal and microbial kingdoms as receptors for light [2]. Rhodopsins are classified as microbial (prokaryotic and lower eukaryotic) rhodopsins (type I rhodopsins), and animal rhodopsins (type II). Type I rhodopsins act as light-dependent ion pumps, channels and sensors. Type II rhodopsins have been finely tuned for vision, and light sensing for nonvisual reasons such as circadian rhythms, pupillary constriction, body colour changes, determining the horizon, seasonal reproduction, and direct utilization for the isomerization of retinal [3–7].
Rhodopsins consist of seven alpha-helices and retinal as a chromophore. Retinal is covalently bound to the opsin, retinal-free form of rhodopsin, via lysine residue located on the seventh helix. When the protein absorb the light, retinal undergoes photoisomerization and its conformational change activates the protein to perform designated functions.

Halobacterial light-driven proton pump bacteriorhodopsin, BR, is one of the best studied rhodopsins and its structure and function are well known. In contrast, other microbial rhodopsins such as eubacterial proton pump proteorhodopsin or Anabaena sensory rhodopsin have much less known about them. In this thesis, Anabaena sensory rhodopsin is studied using a new biophysical approach.

1.3 *Anabaena* sensory rhodopsin (ASR)

*Anabaena* sensory rhodopsin (ASR) is an example of type I rhodopsin. It is the first eubacterial sensory rhodopsin found from the freshwater cyanobacterium *Anabaena* sp. PCC7120 [8]. Just like the other rhodopsins ASR also consists of seven alpha helices and retinal. However, ASR differs from other type I rhodopsins in its chromophore configuration, as its dark-adapted state is predominantly all-trans (~80%) which changes upon light-adaptation in the direction opposite from that found in BR. Moreover, ASR exhibits light-induced interconversion between stable 13-cis and all-trans states of retinal. The ratio of its cis and trans retinal forms depends on the wavelengths of light. Thus, ASR has the ability to signal the colour of absorbed light, and by doing this, ASR is believed to induce the chromatic adaptations, which may be mediated by a soluble transducer (ASRT), which can bind to promoter regions of DNA [2, 9-12]
ASR is a 261-residue, 27 kDa protein. Wild-type ASR contains three native intramembrane cysteine residues at positions 134 and 137 in helix E, and 203 in helix G (Fig. 1-1), none of which is reactive, unless a very high concentration of the reactant is used. This was previously verified by attempting a reaction of wild-type ASR with methyl methanethiosulfonate (MMTS), a polar diamagnetic label with high cysteine reactivity [13]. Both the chemical shifts of the CA and CB cysteine atoms of the reaction product, and the relative intensities of CA/CB cross peaks remained unchanged, indicating that all three native cysteines were nonreactive [13]. Thus, mutant ASR, S26C ASR with one non-native reactive cysteine residue was used as a site for dye labelling in this thesis. This non-native cysteine is introduced by replacing a serine on the cytoplasmic end of helix A (Fig. 1-1).

Figure 1-1. Amino acid sequence and topology of ASR. Native cysteines, C134, C137 in helix E and C203 in helix G are shown in blue. Cys26 was introduced by mutagenesis and is shown in red (from Ref. 13 with permission).
1.4 Previous studies on ASR structure

Since ASR was discovered its structure had been studied by various methods such as Solid-State NMR, X-ray diffraction, visible CD spectroscopy, FTIR and Raman spectroscopy, and more [10, 13, 34]. From these data ASR is known to form tight trimers in lipids and detergents, but not in 3D crystals. For example, SDS-PAGE showed that substantial portion of solubilized ASR run as a high molecular weight complex at 60 kDa while monomers run at 20 kDa (Fig. 1-3) [13]. In addition, visible CD spectra indicated a bilobe shape for both ASR in detergent and in lipids, suggestive of a trimer formation (Fig. 1-4) [13].

Figure 1-2. Trimeric ASR structure in lipids as determined by solid-state NMR viewed from the periplasmic side. Monomers are shown in different colors with α-helices represented by cylinders. Retinals are represented by sticks (from Ref. 41 with permission).
Figure 1-3. SDS-PAGE of solubilized wild type ASR in DDM. Lane 1: protein markers; lane 2: non-boiled solubilized ASR sample in DDM without mixing with SDS (from Ref. 13 with permission), showing a strong presence of trimers.

<table>
<thead>
<tr>
<th>250KDa</th>
<th>150KDa</th>
<th>100KDa</th>
<th>75KDa</th>
<th>50KDa</th>
<th>37KDa</th>
<th>25KDa</th>
<th>20KDa</th>
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<td>Monomer</td>
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Figure 1-4. Visible CD of solubilized wild-type ASR in pH 8.0 buffer (pH 8.0, 5 mM Tris, 10 mM NaCl, 0.05% DDM), and wild type ASR reconstituted in DMPC/DMPA lipids suspended in 70% glycerol (pH 8.0, 5 mM Tris, 10 mM NaCl, 70% glycerol (v/v)) (from Ref. 13 with permission), The bilobe shape is consistent with trimerization.

Furthermore, Small Angle X-ray Scattering (SAXS) measurements has shown that these trimers may assemble into the bacteriorhodopsin-like hexagonal lattice (a 2D crystal) (Figure 1-5) [39]. If one assumes a BR-like arrangement of trimers, it is possible that some distances
between the same residues in monomers of the adjacent trimers are much shorter than the respective distances between monomers in the same trimer (shown with short and long red arrows for helices A in Fig. 1-5). The existence of such short intertrimer distances lays a foundation for our experimental approach described in the next section.

Figure 1-5. Lattice formation of BR and ASR. A: BR trimers in lattice form (from K. Schulten’s group website with permission), red arrows show typical intertrimer and intratrimer distances between helices A; B: SAXS measurements of lipid-reconstituted ASR suggesting BR-like 2D crystallinity, shown by black arrows (from Ref. 14 with permission).

1.5 Experimental strategy

In this thesis, developing a simple and reliable method for lattice formation detection and studying the lattice formation of ASR in different environments were the main goals. When ASR forms BR-like lattice, the distances between different trimers can be even shorter than
distances between monomers of the same trimer (Fig. 1-5). Therefore, Fluorescence Resonance Energy Transfer (FRET) could be applied to achieve our goals, as FRET is distance sensitive, robust, and simple method, which, unlike SAXS does not require hard-to-obtain access to specialized facilities. Mutant ASR, S26C, was fluorescently labelled with two dyes (FRET pair) at the non-native cysteine residue in the AB loop. Several pairs were tested and the labeling conditions were optimized. By detecting the FRET signals between these two dyes, lattice formation of ASR could be detected, since FRET occurs only if the distance between the two dyes is short, in the range of <10 nm. With this strategy of FRET, the environmental effects (temperature, protein/lipid ratio) on lattice formation of ASR were investigated. The fluidity of lipids depends on temperature and nature of the lipids tails (saturated or unsaturated), and hydrophobic thickness of the lipid bilayer, which changes with the phase transition, may affect the tilts and length of individual helices, interhelical packing, side chain conformations and protein dynamics, and through these effects influence the functional properties of proteins and their oligomerization state [14–18]. In the experiment, two different types of liposomes which the nature of lipid tails are different were mixed with protein in various protein to lipids ratios and studied over a broad range of temperatures.
Chapter 2 Fluorescence Resonance Energy Transfer

2.1 Fluorescence Resonance Energy Transfer (FRET)

When radiation falls on a chromophore, the excited state of the molecule is formed, which can return to its ground state by emission of energy via several pathways. The Jablonski diagram (Fig. 2-1) shows some of these processes. When light energy is absorbed by a chromophore, the energy can be re-emitted by fluorescence, which is defined as an emission of a photon by a singlet excited state, normally on a nanosecond time scale. However, other possibilities exist, for example, the excited molecule can dissipate its energy thermally (without radiation) or pass it to another fluorophore which in turn fluoresces. This phenomenon is called fluorescence resonance energy transfer or FRET [19].

Figure 2-1. Jablonski diagram explaining the nature and time scale of fluorescence (from Ref. 19 with permission).
The Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between an excited donor molecule (D) and ground-state acceptor molecule (A) over a range of distances, typically 10-100 Å. The energy transfer involves no emission or absorption of photon (non-radiative), but it is possible by long-range dipole-dipole interactions. The theory behind the energy transfer is based on the concept of treating an excited fluorophore as an oscillating dipole that can undergo an energy exchange with a second dipole having a similar resonance frequency. If the energy transfer occurs, the quenching of donor fluorescence and increase of acceptor fluorescence intensity will happen. Quenching of donor fluorescence refers to the process in which the emission intensity of a donor is decreased due to the energy loss (Fig. 2-2). As the energy is transferred to the acceptor, its fluorescence intensity increases as a result. In addition, quenching of donor emission can also occur without increasing the acceptor fluorescence. Such an event is known as self-quenching of donor. Self-quenching originates from interaction between the molecules of the donor themselves without involving the acceptor molecules, and can happen when the concentration of donor molecules in a sample is high (Fig. 2-3).
Figure 2-2. Excitation and fluorescence spectra of an ideal donor-acceptor pair. Bright blue coloured region is the spectral overlap between the fluorescence spectrum of donor and excitation spectrum of acceptor (from Ref. 20). Intensity changes caused by FRET are shown with arrows and dotted lines.

Figure 2-3. Example of self-quenching; Fluorescence intensity of Calcein as a function of its concentration is shown, displaying decrease of the intensity at high fluorophore concentration caused by self-quenching (from Ref. 21 with permission).
The efficiency of FRET is strongly dependent on the D-A distance and is characterized by the Forster radius $R_0$, a unique parameter for each D-A pair. $R_0$

$$E_{\text{FRET}} = \frac{1}{1 + \left(\frac{R}{R_0}\right)}$$  \hspace{1cm} (2.1)

where $R$ is a distance between donor and acceptor.

When the D-A distance is $R_0$, the efficiency of energy transfer is 50%. Once $R_0$ is known, the D-A pair can be used as a molecular ruler to determine the distance between sites labelled by D and A. The magnitude of $R_0$ is dependent on the spectral overlap ($J$) and mutual orientation ($K$), as well as refraction index $n$ and donor’s quantum yield of fluorescence $QY_D$.

$$R_0 = [8.8 \times 10^{23} \cdot k^2 \cdot n^{-4} \cdot QY_D \cdot J(\lambda)]^{1/6}$$  \hspace{1cm} (2.2)

where $K^2$ is a dipole orientation factor which ranges from 0 to 4.

The spectral overlap can be calculated from the degree of overlap between the normalized emission spectrum of donor and absorption spectrum of acceptor.

$$J(\lambda) = \int \varepsilon_A (\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda \text{ cm}^3 \text{M}^{-1}$$  \hspace{1cm} (2.3)

where $\varepsilon_A$ is extinction coefficient of acceptor and $F_D$ is fluorescence emission intensity of donor as a fraction of the total integrated intensity (normalized emission spectrum of donor).

Because FRET efficiency depends on the inverse sixth power of a distance between donor and acceptor, FRET is very distance-sensitive tool making FRET useful in investigations both intermolecular and intramolecular interactions. Recent advances in the technique have led
to qualitative and quantitative improvements, including increased spatial resolution, distance range and sensitivity. FRET does not occur spontaneously under any arbitrary conditions. Instead, there are four conditions that have to be for FRET to occur. The first condition is that the normalized fluorescence emission spectrum of the donor molecule must significantly overlap with the absorption (or excitation) spectrum of the acceptor chromophore (Fig. 2-2 and equation 2.3) [22].

The larger overlap of the spectra is, the better the donor can transfer energy to the acceptor. The second condition for FRET is that the two fluorophores (donor and acceptor) have to be in a close proximity to one another (typically 1 to 10 nanometers). They have to be placed close to each other to transfer the energy efficiently. The third condition is that the transition dipole moments of the donor and acceptor must be approximately parallel to each other for the highest efficiency of interaction (Fig. 2-4). Their mutual orientation is usually described by the orientation factor $K$

$$K^2 = (\cos \theta - 3 \cos \theta_D \cdot \cos \theta_A)^2 \quad (2.4)$$

Figure 2-4. The schematic diagram of the mutual orientation of transition dipole moments (from Ref. 20).
Table 2-1. Examples of typical values of $R_0$ (from Life Technologies)

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>$R_0$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>TAMRA</td>
<td>55</td>
</tr>
<tr>
<td>IAEDANS</td>
<td>Fluorescein</td>
<td>46</td>
</tr>
<tr>
<td>EDANS</td>
<td>Dabcyl</td>
<td>33</td>
</tr>
</tbody>
</table>

The orientation factor depends upon the relative orientations in space of the donor emission dipole and the acceptor absorption dipole, and can range from zero to 4. A value of 1 corresponds to parallel transition dipoles, while a value of 4 results from dipoles that are both parallel and collinear. The value of $\kappa^2$ is often assumed to be $2/3$. This value means that both dyes are freely rotating and can be considered to be isotropically oriented during the excited state lifetime. If one of the dyes is fixed or not free to rotate, as may happen in membrane proteins, then $\kappa^2 = 2/3$ will not be applicable. In most cases, however, even modest reorientation of the dyes results in enough orientational averaging so that assuming $\kappa^2 = 2/3$ does not result in a large error in the estimated energy transfer distance due to the one sixth power dependence of $R_0$ on $\kappa^2$. Even when $\kappa^2$ is quite different from $2/3$ the error can be associated with a shift in $R_0$ and thus determinations of changes in relative distance for a particular system are still valid [23]. The last condition is that the fluorescence life-time of the donor molecule must be of sufficient duration to allow FRET to occur. The fluorescence life-time refers to the average time the molecule stays in the excited state before the emission of energy.
When all of these conditions are satisfied, FRET, one of the few biophysical methods which can measure the nanometer scale distances, occurs. This useful distance-sensitive technique is applied to studying the structure and dynamics of proteins and nucleic acids, in the detection and visualization of intermolecular association and in the development of intermolecular binding assays [24]. In addition, it is being used more and more in biomedical research and drug discovery today [42].

2.2 Measuring FRET Efficiencies

The energy transfer efficiency $E$ can be calculated from either fluorescence intensity or lifetime measurements, by combining data for donor alone (D) and donor in the presence of acceptor (D+A). When the fluorescence intensity is measured to calculate $E$, it could use either donor fluorescence intensity or the acceptor fluorescence intensity data. In addition, there is another important method called ratiometric FRET to measure $E$, which is calculated from the ratio of donor and acceptor fluorescence intensities. For the energy transfer between two chromophores to take place, the donor has to be fluorescent while acceptor could be non-fluorescent or fluorescent (letter F of FRET stands for Forster for the case when acceptor is non-fluorescent, but if both chromophores are fluorescent, F stands for Fluorescent). If the acceptor is non-fluorescent, FRET efficiency can be calculated from using donor fluorescence intensity and excited lifetime measurements.
For the case when FRET efficiency is measured by energy transfer using donor fluorescence intensity data, the knowledge of fluorescence quantum yield (QY), emission efficiency, of donor is required for a sample with both donor and acceptor (D+A) and for an identical reference sample without acceptor (D-only). These values can be input into the equation below [25-26]:

\[
E = 1 - \frac{QY_{DA}}{QY_D}
\]

where \(QY_{DA}\) is fluorescence quantum yield of sample with D+A, and \(QY_D\) is the fluorescence quantum yield of sample with D-only.

Figure 2-5. Cartoon scheme of emission intensity-based FRET studies. The example of FRET pair for this illustration is Cy3/Cy5. \(F_{DA}\) has the same meaning as \(QY_{DA}\) from the equation 2.5 (from Ref. 27 with permission).

Measuring the QY, however, is rarely practical. If the absorbance of donor at the excitation wavelength is exactly the same in the two samples (e.g., if the excitation wavelengths...
and fluorophore concentrations are the same) and the same gain and slit bandwidths are used for the two emission measurements then \( E \) can be calculated as follows [25-26]:

\[
E = 1 - \frac{I_{DA}}{I_D}
\]  

(2.6)

where \( I_{DA} \) and \( I_D \) are the total donor fluorescence intensities in presence and absence of A, respectively.

The disadvantage of the method presented above is that oftentimes the concentration of donor cannot be ensured to be exactly the same for different samples. In theory, if the absorbance at the excitation wavelength is lower than 0.1 OD (preferably 0.02-0.07 OD) the concentration difference between the donor-acceptor sample and the donor-only sample can be accounted for using the equation below [25-26]:

\[
E = 1 - \frac{A_D I_{DA}}{A_{DA} I_D}
\]  

(2.7)

where \( A_D \) and \( A_{DA} \) are the absorbance values at the excitation wavelength in the D-only and D+A samples, respectively.

In the case when acceptor is fluorescent, acceptor fluorescence intensity can be used to determine the FRET efficiency as mentioned earlier. This method is very similar to the method that uses donor fluorescence intensity. However, this method requires an acceptor fluorescence intensity not to be influenced by the presence of donor. It can be measured by exciting acceptor at such wavelength where donor does not have any influence or by obtaining acceptor fluorescence intensity from the sample that does not contain donor at all. If the acceptor intensity is obtained by using a sample with donor and acceptor, the FRET efficiency is determined as
\[ E = \frac{I_{AD}A_{AA} - I_{AA}A_{AD}}{I_{AA}A_{DD}} \]  

(2.8)

where \( I_{AD} \) is the acceptor fluorescence intensity following donor excitation, \( I_{AA} \) is the acceptor fluorescence intensity following acceptor excitation, \( A_{AA} \) is the acceptor absorbance at the acceptor excitation wavelength, and \( A_{AD} \) and \( A_{DD} \) are the acceptor and donor absorbance values, respectively, at the donor excitation wavelength.

In the case where direct acceptor excitation is impossible without exciting the donor, a sample with the acceptor only can be used as a reference. In this case the same excitation wavelength is used for both measurements and the FRET efficiency is then calculated as

\[ E = \frac{I_{AD}A_{AA} - I_{AA}A_{AD}}{I_{AA}A_{DD}} \]  

(2.9)

where \( I_{AD} \) and \( I_{A} \) are the intensities of acceptor fluorescence in the presence and absence of donor, respectively, \( A_{A} \) is the absorbance of acceptor in the acceptor-only sample at the wavelength of excitation, and \( A_{AD} \) and \( A_{DD} \) are the absorbance values of acceptor and donor, respectively, in the D+A sample [25-26].

The disadvantage of this method is exactly the same as for the first method described above (using donor fluorescence intensity). The concentration of acceptor may be different in every sample, which means that the intensities are measured under different conditions and cannot be referenced properly. In contrast, there is a method which uses donor’s excited lifetimes rather than the emission intensities of fluorophores to determine the FRET efficiency (Fig.2-6). The lifetime of donor is measured for a sample with D+A and for an identical reference sample with D-only, and the energy transfer efficiency is calculated as follows:
\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]  

(2.10)

Figure 2-6. An example of fluorescence lifetime-based FRET measured between EYFP (acceptor) and Cerulean (donor). A: The fluorescence decay of Cerulean; B: The fluorescence decay of EYFP-Cerulean. The fluorescence decay of the Cerulean moiety in the hybrid protein (EYFP-Cerulean) was accelerated due to FRET. Compared to Cerulean the fluorescence lifetime decreased to 2.0 ns from 3.0 ns (from Ref. 28 with permission).

Figure 2-7. An example of FRET measured between Cy3 and Cy5 attached to visual rhodopsin (Rh). Green curves shows FRET signal for sample that are liposome reconstituted together after labeling with Cy3 and Cy5. Red curve indicates the control (a summation of individually labeled and reconstituted Rh–Cy3 and Rh–Cy5) measured at the same concentrations and conditions (from Ref. 27 with permission).
Since the lifetime is independent of donor concentration, this method is less prone to errors introduced due to differences between reference and sample. The disadvantage is that when the donor is characterized by more than one lifetime (and it is usual for FRET dyes attached to biomolecules) there is no simple, universal expression for the FRET efficiency since this will depend on the origin of the different lifetimes measured.

The last method to measure FRET efficiency is called ratiometric FRET, which uses donor/acceptor emission intensities ratios in the case where acceptor is fluorescent. Because FRET can result in both a decrease in fluorescence of the donor molecule (quenching) as well as an increase in fluorescence of the acceptor, a ratiometric determination of the two signals can be made (Fig. 2-7). The advantage of this method is that a measure of interaction can be found that is independent of the absolute concentration of the sensor. Because obtaining the exact value of absorbance of donor and acceptor can be a very difficult task due to overlapping donor and acceptor absorption spectra and often imperfect baselines, if the concentration or absorbance of the sample are used in order to calculate the FRET efficiency, the measurement is likely to be inaccurate unless extra care is taken. In contrast, the ratio between the donor and acceptor intensities depends not only on the value of FRET efficiency, but also on the fluorescence quantum yields of the two dyes, so that ratiometric FRET is usually a relative measure of FRET and should only be used for qualitative purposes or for monitoring relative changes in the FRET efficiency (e.g., kinetic measurements). In ratiometric FRET, the "relative" FRET efficiency, also known as the proximity ratio, is given by
\[ E_{\text{rel.}} = \frac{I_A}{I_D + I_A} \]  \hspace{1cm} (2.11)

where \( I_A \) and \( I_D \) are the total fluorescence intensities of acceptor and donor, respectively, both following donor excitation [25-26].

If \( E_{\text{rel}} \) is used to find actual FRET efficiency, it additionally requires at least two correction factors: the contribution from direct acceptor excitation to \( I_A \) and the ratio between the donor and acceptor fluorescence quantum yields. In this thesis, ratiometric FRET efficiency method was used to detect lattice formation of ASR. Since the focus of this thesis is to detect the lattice formation of ASR and study the environmental effects on it, and not to measure actual intermolecular distances between the monomers, monitoring relative efficiency is an ideal method.
3. Materials and Methods

3.1. Materials

Common chemicals of reagent grade were purchased from either Fisher Scientific (Unionville, Ontario, Canada) or Sigma-Aldrich (Oakville, Ontario, Canada). The Ni\(^{2+}\)-NTA (nitrilotriacetic acid) agarose resin was purchased from Qiagen (Mississauga, Ontario, Canada). Lipids (DMPC, DMPA, egg PC and brain PS) were purchased from Avanti Polar Lipids (Alabaster, AL). MTS-4-Fluorescein ((2-[(5-Fluoresceinyl)aminocarbonyl]ethyl methanthiosulfonate) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). MTS-TAMRA (MTS-5(6)-carboxytetramethylrhodamine, mixed isomers), MTS-CF543 and MTS-CF640 were obtained from Biotium (Hayward, CA). Alexa Fluor 555 and Alexa Fluor 647 were obtained from Life Technologies (Burlington, Ontario, Canada).

3.1.1 Cell Disruption and Protein Purification buffers

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

Solubilization buffer: 1 % (v/v) DDM, 5 mM Tris, pH 7.5

10X Binding Buffer: 3 M NaCl, 0.5 M Tris, pH 8

Washing Buffer: 0.05 % (v/v) DDM, 0.3 M NaCl, 0.05 M Tris, 40 mM Imidazole, pH 8

Elution Buffer: 0.05 % (v/v) DDM, 0.5 M Imidazole, 0.3 M NaCl, 0.05 M Tris, pH 8

Reconstitution Buffer: 0.05 % (v/v) DDM, 10 mM Tris, 5 mM NaCl, pH 8

NMR Buffer: 25 mM CHES, 10 mM NaCl, pH 9
High Salted Buffer: 0.05 % (v/v) DDM, 10 mM Tris, 300 mM NaCl, pH 8

3.2 Expression and purification of S26C ASR

The DNA encoding S26C ASR mutant with accessible cysteine introduced into the cytoplasmic loop between helices A and B was produced as described earlier by our collaborators (S.Y. Kim and K.H. Jung, Sogang University, Seoul, South Korea) [29]. S26C ASR protein was expressed in BL21-Codonplus-RIL E. coli cells according to the previously described protocol [30-31]. Cells were cultured in M9 minimal medium containing two antibiotics, ampicillin and chloramphenicol, at 30°C. Ammonium chloride (1 g/L culture) and glucose (4 g/L) were used as nitrogen and carbon sources. IPTG (1 mM) was used to induce expression of ASR in the cells when the cell density of the culture reached OD of around 0.35 at 600 nm. Retinal was added at the same time as IPTG to a final concentration of 7.5 μM to regenerate expressed opsin. Cells were incubated for 21 hours (240 rpm at 30°C) after induction with IPTG and retinal, and then collected by centrifugation (5000 rpm, 15 min.) using a Beckman Coulter Centrifuge (GA-6 fixed-angle rotor). The collected cells were pre-treated with lysozyme (12 mg/L of culture) and DNAse I (600 units/L culture) for three hours at room temperature in the dark. Then cells were ready for lysis, which was accomplished through sonication (Fisher Model 500 Sonic Dismembrator using the pulse mode program of three times at 25% for 2:30 min. with 30 second intervals). The membranes were collected by ultracentrifugation at 150,000 g for 50 min. Membranes were solubilized in solubilization buffer with 1% DDM (v/v) for four hours at 4°C and insoluble debris were removed by ultracentrifugation at 150,000 g for 50 min. The supernatant (solubilized protein) was mixed with 5 mL of Ni^{2+}-NTA resin and 2.5 mL of binding
buffer for 18 hours at 4°C allowing His-tagged ASR to bind to the resin. Purification was carried out using a vacuum filter flask to remove impurities using washing buffer containing 1% DDM as the solubilizing detergent. The resultant purified protein was treated with elution buffer (0.05% DDM) to extract the protein from resin. To lower the imidazole concentration, the purified sample in filter units (Amicon Ultra-15 Centrifugal Filter Units) was concentrated from 15 mL to 1.5 mL by centrifugation (5,000 rpm, 3×25 mins.) using Beckman Coulter Centrifuge (GA-6 fixed-angle rotor). The final yields of S26C ASR were approximately 3.5 mg per litre of culture. Protein concentration was determined by measuring the absorbance of opsin-bound retinal using an extinction coefficient of 48,000 M$^{-1}$ cm$^{-1}$.

### 3.3 Fluorescent tag labeling

In all instances, fluorescent labelling was done in the reconstitution buffer (pH 8). All the dyes were attached at non-native cysteine residue (C26) on the cytoplasmic end of helix A, as the three native cysteines are not solvent accessible and do not label under normal conditions. Excess dye was repeatedly washed out using Amicon Ultra-15 Centrifugal Filter Units (5,000 rpm, 3×25 min. by Beckman Coulter Centrifuge, GA-6 fixed-angle rotor). The labelled samples were sent for liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-Q-TOF-MS) at the Advanced Analysis Centre of the University of Guelph to test the extent of labelling and screen labelling conditions for the optimal efficiency.
3.3.1 MTS-Fluorescein and MTS-TAMRA

The MTS reagents, label thiols such as cysteine side chains in proteins, reversibly, selectively, and quickly, and as such are ideal for S26C ASR. The dyes were attached to the non-native cysteine C26 and its sulfhydryls groups was converted to a disulfide as a result.

![Figure 3-1](image1.png)

Figure 3-1. The general reaction of MTS dyes with protein with thiol functional group [32-34].

![Figure 3-2](image2.png)

Figure 3-2. Chemical formula of the first pair of dyes [32]. A: MTS- Fluorescein; B: MTS-TAMRA (from Biotium website).

MTS- Fluorescein (donor) and MTS- TAMRA (acceptor) were the first FRET pairs used in the research. Fluorescein absorbs at 492 nm and emits at 516 nm at pH 7.0. The dye is pH-dependent, which gives greater absorption/emission at higher pH (Fig.3-3). TAMRA, the acceptor dye, which is pH-insensitive, absorbs at 540 nm and emits at 565 nm (Fig. 3-4).
MTS-Fluorescein and MTS-TAMRA were dissolved in dimethyl sulfoxide (DMSO) to the final concentration of ~20 mM and ~18 mM respectively. These dyes were mixed with solubilized S26C ASR separately in 3:1 molar ratio, and stirred with a small magnetic bar for 1 hour at room temperature. These conditions resulted in poor labelling of donor dyes. The mass spectra indicated that only 22% of protein was labelled with donor while 80% of protein was labelled with acceptor dyes. To enhance the labelling efficiency of the donor, the molar ratio of dyes to protein was increased to 10:1. Also, different labelling condition, at 50°C for 1 and 1.5
hours, at 35°C for 2 and 24 hours, and at 4°C for 24 hours, were tested (see the detailed results in Chapter 4).

3.3.2 MTS-CF543 and MTS-CF640

The second FRET pair used in this research project was another pair of MTS dyes, MTS-CF543 (donor) and MTS-CF640 (acceptor). MTS-CF543 is an orange-colored fluorescent dye with absorption and emission maxima at 541 and 560 nm, respectively. MTS-640, the acceptor dye for this pair absorbs at 642 nm and emits at 662 nm (Fig. 3-5). MTS-CF543 and MTS-CF640 were dissolved in DMSO to the final concentration of ~9 mM and ~10 mM respectively. These dyes were mixed with solubilized protein separately in 3:1 molar ratio, and left at 4°C for 24 hours. The mass spectra of protein with donor indicated that the degradation of protein had occurred (see the detailed results in Chapter 4). To verify that it was not due to the experimental error this process was repeated with a new batch of proteins. The outcome indicated that the peaks at the lighter molar weight than 27264 kDa, the expected molar weight of N-terminally formylated S26C ASR disappeared, but the other unidentified peaks were observed. As the chemical structures of these dyes were confidential, it was difficult to determine what was the cause. In contrast, the protein was well labelled by MTS-CF640 under the same conditions (4°C for 24 hours).
Figure 3-5. Absorption and emission spectra of CF 543 and CF 640 dyes (from Biotium company website).

3.3.3 Alexa Fluor 555 and Alexa Fluor 647

The third FRET pair, Alexa Fluor 555 (donor) and Alexa Fluor 647 (acceptor) were maleimide reagents (Fig. 3-6). Alexa Fluor 555 is pH-insensitive from pH 4 to pH 10. It absorbs at 555 and emits at 580 nm. In contrast, Alexa Fluor 647, the acceptor dye for this pair absorbs at 650 nm and emits at 665 nm (Fig. 3-7).

Figure 3-6. Reaction of a thiol with a maleimide (from Life Technologies company website).
Figure 3-7. Absorption and emission spectra of Alexa Fluor 555 (above), and Alexa Fluor 647 (below) (from Life Technologies company website).

The donor and acceptor dyes were dissolved in dimethylformamide (DMF) to the final concentration of ~16 mM and ~15 mM, respectively. They were mixed separately with solubilized protein in 10:1 molar ratio and stirred at two different conditions, at 4 °C for 24 hours and at room temperature (22 °C) for 2 hours. The mass spectra showed that samples labelled at 4 °C had much greater labelling efficiency than the samples labelled at room temperature. The donor labelling efficiency was close to 96% while acceptor dye had ~92% labelling efficiency (see the detailed results in Chapter 4).
3.4 Reconstitution of protein with lipids

Two different liposomes, DMPA/DMPA and PC/PS, were prepared for reconstitution. DMPC and DMPA liposomes were prepared by hydrating dried DMPC and DMPA mixed at 9:1 ratio (w/w). The liposomes were mixed with labelled S26C ASR (0.107mL of 0.085 mM ASR, 0.25 mg using a mixture of donor and acceptor labelled protein in 1:1 ratio (w/w)) at a protein to lipid ratio of 2:1, 1:1, 1:2, 1:5, and 1:10 (w/w). The samples were stirred at 4°C for 24 hours before the addition of Bio-beads SM (Bio-rad) to remove detergent. Bio-beads were added (0.6 g/mL) to the samples and stirred for another 24 hours, and proteoliposomes were collected in NMR buffer (pH 9) by ultracentrifugation for one hour at 150,000 g. Proteoliposomes made using PC/PS lipids were prepared following the above protocol. However, they were mixed in protein to lipids ratio of up to 1:40 (w/w). On top of these samples, two control samples were prepared for each set of proteoliposomes in 2:1 protein to lipids ratio. The control samples were: the sample labelled with donor dye only, and the sample labelled with acceptor dye only. The samples resuspended in NMR buffer were scanned by fluorescence spectroscopy.

3.5 Components of a fluorescence spectrometer

Fluorescence spectrometer measures the emitted fluorescence from the excited samples. There are two general type of instruments exist. They are filter fluorometers and spectrofluorometers. Filter fluorometers use filters to isolate the incident light and fluorescent light, and spectrofluorometers use diffraction grating monochromators to isolate the incident
light and fluorescent light. The type of instrument used in this thesis was the PTI QuantaMaster fluorescence spectrofluorometer.

In fluorescence intensity measurements with this equipment, the light from an excitation source (lamp) passes through a monochromator first, and strikes the sample in a cuvette placed into the sample holder. Once the sample absorbs the light energy, some of the molecules in the sample transition to the excited state and can emit fluorescence before returning to the ground state. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.

![Schematic representation of a spectrofluorometer](image)

Figure 3-8. Schematic representation of a spectrofluorometer (from PTI QuantaMaster 40 brochure); 1. Xeon lamp: light source; 2. Adjustable slits: determines the resolution and spectral purity; 3. Monochromator: form of wavelengths filter that accepts the polychromatic light and passes only a very narrow band of wavelengths; 4. Grating: serve as both diffraction and focusing element; 5. Sample compartment: cuvette holder; 6. Excitation correction: used to correct for the spectral distribution of the excitation channel as well as for intensity fluctuations of the lamp; 7. Detector: produce an electrical current which is proportional to the power of light detected.
This instrument is equipped with FelixGX software and can measure emission and excitation spectra. To record an excitation spectrum, the emission monochromator is set at the desired wavelength, generally the emission maximum. The excitation monochromator is then scanned through the absorption bands of the fluorophore. In contrast, emission spectra are recorded by choosing an appropriate excitation wavelength and scanning wavelength with the emission monochromator. These fluorescence measurements can be applied to numerous studies in protein-protein interaction, Fluorescence Resonance Energy Transfer, DNA binding, protein folding, polarization/anisotropy, chemiluminescence, and many more. In this thesis, FRET was measured with this instrument.
Chapter 4 Results and Discussion

4.1 Dye labeling optimization and mass spectrometry

The first step in the use of FRET is the successful labelling of the protein with cysteine reactive dyes constituting FRET pairs (see chapter 3 for their basic description). The addition of the covalently bound label changes the molecular mass of the protein by an amount detectable using high-resolution mass spectrometry (LC-ESI-Q-TOF-MS). The expected molecular mass of N-terminally formylated C-terminally truncated and 6xHis-tagged S26C ASR is 27264 Da. As each dye adds its molecular mass to the weight of protein, by comparing the amplitudes of the mass peaks present at the expected labelled protein mass to the amplitudes at the unlabelled protein mass the extent of labelling can be estimated.

4.1.1 MTS-Fluorescein and MTS-TAMRA

The first FRET pair we tried, MTS-Fluorescein (donor) and MTS-TAMRA (acceptor), have molecular weights of 513 Da and 567 Da, respectively. If they successfully label S26C ASR, the expected molecular weights are 27698.54 Da (protein + fluorescein) and 27752 Da (protein + TAMRA) (the 79 Da difference is explained by the departure of sulfinic acid from MTS) (Fig. 3-1 and 3.2).

Two samples were tested with mass spectrometry for this FRET pair. The protein was labelled with each dyes, MTS-Fluorescein and MTS-TAMRA, so that the labeling conditions could be optimized. The first labelling condition, incubation for 1 hour at room temperature,
generated very poor labeling efficiency (~23%) for the donor dye, while labeling with the acceptor dye had much better efficiency (~80%) (Fig. 4-1 and 4-2).

![Mass spectrogram of ~0.10 mM S26C ASR labelled with MTS-Fluorescein in 1:3 molar ratio; stirred for 1 hour at room temperature.](image1)

**Figure 4-1.** Mass spectrogram of ~0.10 mM S26C ASR labelled with MTS-Fluorescein in 1:3 molar ratio; stirred for 1 hour at room temperature.

![Mass spectrogram of ~0.10 mM S26C ASR labelled with MTS-TAMRA in 1:3 molar ratio; stirred for 1 hour at room temperature.](image2)

**Figure 4-2.** Mass spectrogram of ~0.10 mM S26C ASR labelled with MTS-TAMRA in 1:3 molar ratio; stirred for 1 hour at room temperature.

To enhance labelling efficiency of these dyes, the second condition, stirring the protein with the dyes for 24 hours at 4 °C, was tried. This condition improved the labelling efficiency of
both dyes: donor efficiency increased significantly, to 56.5% from 22.3%, and acceptor efficiency increased only marginally, to 80.4 % from 79.5% (Fig. 4-3 and 4-4).

Figure 4-3. Mass spectrogram of ~0.10 mM S26C ASR labelled with MTS-Fluorescein in 1:3 molar ratio; stirred for 24 hours at 4°C.

Figure 4-4. Mass spectrogram of ~0.10 mM S26C ASR labelled with MTS-TAMRA in 1:3 molar ratio; stirred for 24 hours at 4 °C.

These results showed that the acceptor dye has an acceptable labelling extent in both cases while MTS-Fluorescein was not at its optimal efficiency under these conditions. The first hypothesis to explain this was the electrostatic charge of the protein. The electrostatic
interactions with the dye due to the charges on protein surface could hinder the labelling. Thus, the labelling in high salt buffer (300 mM NaCl, 10 mM Tris) was tested. The high concentration of salt was expected to screen the protein charge and enhance the labelling efficiency. However, the results showed that the labelling efficiency was even lower than previously, standing at 40% (Fig. 4-5).

Figure 4-5. Mass spectrogram of ~0.06 mM S26C ASR labelled with MTS-Fluorescein in 1:3 molar ratio; stirred for 24 hours at 4 °C; reacted in high salt buffer.

Then, the new conditions, with increased reaction temperature (50 °C) and molar ratio (1:10), were tested. The high temperature is expected to accelerate the reaction and to render protein more open and accessible to the dye, which can lead to better labelling. The tested conditions were: 1) stirring for 1 hour at 50 °C, 2) stirring for 2 hours at 50 °C, and 3) stirring for 3 hours at 50 °C (Fig. 4-6, 4-7, and 4-8).
Figure 4-6. Mass spectrogram of ~0.004 mM S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 1 hour at 50°C.

Figure 4-7. Mass spectrogram of ~0.004 mM S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 2 hours at 50°C.

Figure 4-8. Mass spectrogram ~0.004 mM S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 3 hours at 50 °C.
As expected, the resulting mass spectra showed much greater labelling efficiency for fluorescein. The best labelling was obtained if the protein was stirred with the donor dye for 2 hours at 50°C (~88%). To ensure that these conditions are reliable and reproducible this labelling process was repeated (Fig. 4-9). Unexpectedly, the repeated labelling showed three major peaks at 27264.1 Da, 27697.5 Da, and 28131.1 Da, representing protein itself, protein labelled with fluorescein, and protein labelled with two fluorescein molecules (doubly labelled), respectively, suggesting labeling of one of the internal native cysteines.

Figure 4-9. Mass spectrogram of ~0.09 mM of S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 2 hours at 50 °C. Red coloured labelling efficiency indicates the protein +Fluorescein and blue coloured labelling efficiency indicates the protein + 2 Fluoresceins.

The doubly labelled protein was dominant over the other peaks with ~60% of labelling efficiency. Having a second dye attached to the protein at an unknown location is undesirable, as it will make FRET data uninterpretable. A few different conditions were then tested to solve the double labelling issue, hoping to minimize the exposure of an internal cysteine: 1) label at 50°C for shorter reaction time (1.5 hours), 2) label at 35°C for 2 hours, 3) label at 35°C for 24
hours. From these three conditions tested, the last condition, stirring the protein for 24 hours at 35°C, had the greatest labelling efficiency (~100%), with negligible double labeling at the same time (Fig. 4-10, 4-11, and 4-12).

Figure 4-10. Mass spectrogram of ~0.04 mM S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 1.5 hours at 50°C.

Figure 4-11. Mass spectrogram of ~0.04 mM S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 2 hours at 35°C.
Labelling under these conditions was repeated to ensure the result was reproducible. In addition, the labelling of MTS-TRAMRA with protein in 1:10 molar ratio was tested to further improve the labelling efficiency. The new results showed good reproducible (~85%) labelling efficiency for the donor and improved (~100%) labelling efficiency for the acceptor without appreciable double labeling (Fig. 4-13 and 4-14).

Figure 4-13. Mass spectrogram of ~0.04 mM S26C ASR labelled with MTS-Fluorescein in 1:10 molar ratio; stirred for 24 hours at 35°C (2nd trial).
4.1.2 MTS-CF543 and MTS-CF640

The second FRET pair tested, MTS-CF543 (donor) and MTS-CF640R (acceptor) (Fig. 3-5), have approximate molecular weights of ~1024 Da and ~969 Da respectively, according to the manufacturer (Biotium). These numbers should be taken with caution, as the exact chemical formulae are confidential, so that the actual molecular weights may be different. If successful labelling of S26C ASR has occurred, the expected molecular weights of the products would be 28209 Da (protein + CF543) and 28154 Da (protein + CF640) (the 79 Da difference is explained by the leaving sulfinic acid of MTS) (Fig. 3-1).

Two samples were tested with mass spectrometry for this FRET pair: the protein labelled with each dye, MTS-CF543 and MTS-CF640. The first labelling conditions, where the reaction mixtures were stirred for 24 hours at 4°C, produced very poor labeling efficiency (~45%) for the acceptor dye, while labelling with the donor dye generated uninterpretable results (Fig. 4-15 and 4-16).
The mass spectra of protein labelled with CF543 had major peaks at 26653.44 Da and 27339.16 Da (Fig. 4-15). These molecular weights could not be explained, especially for the 26653.44 Da peak. This weight was even lighter than the weight of the unmodified protein (27264 Da). In addition, the acceptor mass spectra had major peaks at 26578.31 Da (lighter than the unmodified protein weight), 27264 Da (unmodified protein), and at 28154.02 Da (tentatively, Protein + CF640, considering the molecular weight uncertainty mentioned above) (Fig.4-16). These latter results indicated that the protein degradation may have occurred during the labelling process. An alternative explanation would be that the protein was not purified well.
and the sample was dirty. To verify the cause of such peak, the labelling process was repeated one more time but with a high salt concentration buffer (Fig. 4-18, and 4-19). Because low ~45% labelling efficiency for the acceptor dye could have resulted from the interaction of electrostatic charges of the dye and the protein, the excess salts were expected to screen those interactions. Additionally, the solubilized purified unmodified protein was also checked by mass spectroscopy to ensure that protein is clean (Fig. 4-17), and found to be pure and clean.

![Mass spectra of ~0.03 mM S26C ASR.](image)

**Figure 4-17.** Mass spectra of ~0.03 mM S26C ASR.

![Mass spectrogram of ~0.03 mM S26C ASR labelled with MTS-CF543 in 1:3 molar ratio; stirred for 24 hours at 4 °C; reacted in high salt buffer.](image)

**Figure 4-18.** Mass spectrogram of ~0.03 mM S26C ASR labelled with MTS-CF543 in 1:3 molar ratio; stirred for 24 hours at 4 °C; reacted in high salt buffer.
As can be seen above, the high salt buffer did not improve the labelling efficiency for the acceptor, but rather decreased it to ~33% from ~44% using the previous result with the low salt buffer. In contrast, in the mass spectra of both donor and acceptor, the peaks located at molecular weight which was lighter than 27264 Da disappeared. However, the unidentified bands were still observed in the mass spectra of donor-reacted ASR. The peak at 27339.92 Da was dominant over the other peaks with molecular weights 27264 Da (unmodified protein), 28153 Da, and 30218 Da and consistently appeared in every sample in which protein was mixed with MTS-CF543. Because the chemical formula for these CF dyes are confidential, it was difficult to determine what was causing this extra band. Thus, the CF dyes were abandoned and a new FRET pair was tested instead.

4.1.3 Alexa Fluor 555 and Alexa Fluor 647

Alexa Fluor 555 (donor) and Alexa Fluor 647 (acceptor) maleimides have approximate molecular weights of ~1250 Da and ~1300 Da, respectively, according to the manufacturer.
(Invitrogen). As with the CF dyes above, these numbers should be taken with caution, as the exact chemical formulae are confidential, so that the actual molecular weights may be quite different. If S26C ASR were successfully labelled, the expected molecular weights would be 28514 Da (protein + Alexa Fluor 555) and 28564 Da (protein + Alexa Fluor 647), respectively (Fig.3-6).

The standard labeling protocol (from Life Technology) for these maleimide-based dyes suggests that the protein be incubated with the dye in 1:10 molar ratio for 2 hours at room temperature or for 24 hours at 4 °C. Therefore, these two conditions were tested for each dye to find the best labelling condition (Fig. 4-20, 4-21, 4-22, and 4-23).

Figure 4-20. Mass spectrogram of ~0.04 mM S26C ASR labelled with Alexa Fluor 555 in 1:10 molar ratio; stirred for 2 hours at room temperature.
Figure 4-21. Mass spectrogram of ~0.04 mM S26C ASR labelled with Alexa Fluor 647 in 1:10 molar ratio; stirred for 2 hours at room temperature.

Figure 4-22. Mass spectrogram of ~0.04 mM S26C ASR labelled with Alexa Fluor 555 in 1:10 molar ratio; stirred for 24 hours at 4 °C.

Figure 4-23. Mass spectrogram of ~0.04 mM S26C ASR labelled with Alexa Fluor 647 in 1:10 molar ratio; stirred for 24 hours at 4 °C.
As mentioned above, the expected molecular weights of labelled proteins are 28514 Da and 28564 Da, for donor and acceptor, respectively, based on information from the manufacturer. However, the resulting mass spectra of labelled samples had no peaks at such weights. In contrast, the acceptor labelled samples from two different conditions generated the same major peaks at 28245 Da (Fig. 4-21, and 4-23). These results indicated that the free dye Alexa Fluor 647 may have a molecular weight of 981 Da. The chemical formula of Alexa Fluor dyes were confidential so it was difficult to determine how the labelling mechanism works. To verify the molecular weight of free dye of Alexa Fluor 647, a literature search was conducted and the previous research, where the mass spectroscopy of free dye of Alexa Fluor 647 has been done, where it was determined that the actual weight of Alexa Fluor 647 is 981 Da [36]. The mass spectra of Alexa Fluor 647 from two labelling conditions were then compared for their labelling efficiencies. The samples labelled at room temperature for 2 hours showed ~66% labelling efficiency while the samples labelled at 4 °C for 24 hours showed ~86% labelling efficiency. In contrast, the sample which was labelled with the donor for 2 hours at room temperature generated many abnormal bands (Fig. 4-20). There were also peaks representing the unmodified protein and labelled protein, but they were too small to make any conclusion. However, the sample labelled with donor under different conditions (24 hours at 4 °C) generated an excellent labelling efficiency (~100%, Fig. 4-22). From this result, the actual molecular weight of Alexa Fluor 555 was determined to be 955 Da. Thus, for both dyes the best labelling efficiency was obtained when they were reacted for 24 hours at 4 °C. The labelling process under these conditions was repeated with both dyes. The donor-labeled mass spectra
showed ~96% labelling efficiency and the acceptor-labeled mass spectra showed ~100% labelling efficiency (Fig. 4-24 and 4-25).

Figure 4-24. Mass spectrogram of ~0.04 mM S26C ASR labelled with Alexa Fluor 555 in 1:10 molar ratio; stirred for 24 hours at 4°C.

Figure 4-25. Mass spectrogram of ~0.04 mM S26C ASR labelled with Alexa Fluor 647 in 1:10 molar ratio; stirred for 24 hours at 4°C.

4.1.4 Summary of labelling results and choice of the FRET pair

The S26C ASR mutant was labelled with three different FRET pairs, and two of these pairs, MTS-Fluorescein and MTS-TAMRA, and Alexa Fluor 555 and Alexa Fluor 647 maleimides, successfully labelled the protein with good labelling efficiency (>80%). No successful labelling
was accomplished for the MTS-CF dyes, and therefore, will not be discussed further. The MTS-
Fluorescein had the highest efficiency when stirred with protein for 24 hours at 35 °C. All other
dyes, MTS-TAMRA, Alexa Fluor 555, and Alexa Fluor 647, labelled with the highest efficiency
when stirred for 24 hours at 4 °C. For further studies with fluorescence spectroscopy, Alexa
Fluor 555 and Alexa Fluor 647 maleimides were chosen over the MTS-fluorescein and MTS-
TAMRA pair for several reasons discussed below. Both FRET pairs have similar \( R_0 \) (55 Å for
fluorescein/TAMRA vs 51 Å for Alexa Fluor 555/647 according to the Invitrogen manual), but
differ in other respects.

Table 4-1. Summary of fluorophore’ characteristics

<table>
<thead>
<tr>
<th></th>
<th>( \lambda_{ex} / \lambda_{em} ) (nm)</th>
<th>Extinction coeff. (M(^{-1}) cm(^{-1}))</th>
<th>pH-dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTS-Fluorescein</td>
<td>492/516</td>
<td>85,000</td>
<td>pH-sensitive</td>
</tr>
<tr>
<td>MTS-TAMRA</td>
<td>540/565</td>
<td>95,000</td>
<td>pH-insensitive</td>
</tr>
<tr>
<td>MTS-CF543</td>
<td>541/560</td>
<td>100,000</td>
<td>pH-insensitive</td>
</tr>
<tr>
<td>MTS-CF640</td>
<td>642/662</td>
<td>105,000</td>
<td>pH-insensitive</td>
</tr>
<tr>
<td>Alexa Fluor 555</td>
<td>550/580</td>
<td>158,000</td>
<td>pH-insensitive from pH 4 to pH 10</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>650/665</td>
<td>265,000</td>
<td>pH-insensitive from pH 4 to pH 10</td>
</tr>
</tbody>
</table>

Alexa Fluor dyes are brighter than Fluorescein and TAMRA. The brightness of dyes can
be deduced from their extinction coefficients (Table 4-1) in combination with quantum yields of
their fluorescence. The extinction coefficient refers to the capacity for light absorption at a
specific wavelengths. Thus, fluorescence output of dye, the brightness, is proportional to its
extinction coefficient. Since Alexa Fluor dyes have much greater extinction coefficients, it
indicates that Alexa Fluor dyes could be brighter than Fluorescein and TAMRA if the quantum
yields are comparable. This result was true for the acceptors (0.23 for TAMRA vs 0.33 for Alexa Fluor 647), but not for the donors (0.93 for fluorescein vs. 0.1 for Alexa Fluor 555). Nevertheless, fluorescein suffers from photobleaching and is pH-sensitive, which, in combination with relatively low extinction would make it a second choice.

Figure 4-26. The spectral overlap between Fluorescein and TAMRA. The absorption spectra of donor (-), and acceptor (- - -), and the emission spectra of donor ( . . . ) and acceptor (---) are presented; all measured in 100 mM NaCl, 10 mM sodium phosphate at pH 7.4 (from Ref. 37 with permission).

Secondly, the absorption and emission spectra of Alexa Fluor dyes were far apart (Fig. 3-7). In contrast, fluorescein and TAMRA had greatly overlapping absorption and emission spectra (Fig. 4-26). This is a serious issue for ratiometric FRET because this method uses the ratio of donor and acceptor fluorescence intensities to determine the relative FRET efficiency. When the absorption spectrum of the acceptor dye covers a wide range of wavelengths where the donor absorption spectrum is present, an attempt to excite the donor dye ends up exciting the
acceptor as well, giving the false (non-FRET) emission intensity of the acceptor. The emitted fluorescence intensity from the acceptor should be caused by the energy transferred from donor only. Otherwise, the FRET signal is not reliable. Since both absorption and emission spectra of TAMRA cover a broad range of wavelengths, obtaining a reliable emission and excitation amplitudes is difficult. In contrast, Alexa Fluor 555 and Alexa Fluor 647 have absorption and emission spectra far apart. When Alexa Fluor 555 was excited at 555 nm, Alexa Fluor 647 was hardly affected (Fig. 4-27).

The third reason is that the energy transfer between Fluorescein and TAMRA can be compromised is due to the presence of the chromophore of ASR, retinal. Retinal has an absorption maximum of ~ 550 nm, which is between the emission spectra of Fluorescein and TAMRA. Thus, there is a chance that the retinal may quench Fluorescein emission and dissipate it non-radiatively. This phenomenon was not confirmed by us experimentally, but the extent of FRET observed by us for this pair, even though appreciable, was low (Fig. 4-28). Alexa Fluor 555 and Alexa Fluor 647 seem to be a reasonable choice since they did not appear to experience the latter issue, with donor emission being red-shifted relative to retinal absorption, which was confirmed by much higher FRET efficiencies (see below). In summary, for these three reasons the Alexa Fluor FRET pair was chosen for further studies.
Figure 4-27. Normalized Emission and excitation spectra of controls samples (≈0.006 mM S26C ASR) by dye concentration; A: MTS-Fluorescein (donor) and MTS-TAMRA (acceptor); Yellow represents the excitation of donor-only sample detected at acceptor emission wavelength (565 nm), and Red represents the emission of acceptor-only sample excited at donor absorption wavelength (492 nm). B: Alexa Fluor 555 (donor) and Alexa Fluor 647 (acceptor); Yellow represents the excitation of donor-only samples detected at acceptor emission wavelength (675 nm), and Red represents the emission of acceptor-only sample excited at donor absorption wavelength (540 nm). All samples were had at 0.5 lipid/protein ratio (DMPC/DMPA).

Figure 4-28. FRET signals obtained from MTS-Fluorescein and MTS-TAMRA pairs. The emission spectra of three samples excited at 492 nm. Yellow, Red, and Orange represent the donor-only, acceptor-only, and sample with both dyes at 0.5 lipid/protein ratios (DMPC/DMPA), respectively.

4.2 Fluorescence intensity measurements

In all instances, to measure the concentration of protein and dyes of the samples were measured by absorption spectroscopy (Cary 50, Varian), and by fluorescence
spectroscopy to measure the emission and excitation spectra. The emission and excitation spectra were corrected for donor and acceptor concentration in the samples.

Fluorescence excitation and emission spectra of the samples were recorded with a PTI spectrofluorimeter, using excitation and emission slits of 4 nm. The wavelengths used for recording emission and excitation spectra of the labelled proteins were 540 and 675 nm, respectively, chosen to minimize overlap between donor and acceptor. All the measurements had been made with aliquots of samples (0.1 mL, ~0.003 mM S26C ASR), under steady state conditions, with corrected excitation and emission mode.

4.2.1 Packing density (protein/lipid ratio) effects on lattice formation of ASR

Proving that detection of lattice formation by ASR is possible by FRET requires preparing the control samples, such as solubilized ASR, ASR reconstituted in lipids with 1:2 protein/lipid (w/w) ratio, and resolubilizing ASR. These three controls ensured that FRET was a useful technique to detect the lattice formation of ASR (Fig. 5-1). ASR is known to form trimers both in detergent and lipids, but ASR in lipids tends to form tightly packed 2D crystals which should be detectable by FRET as described in chapter 1. It is known that monomers do not exchange between trimers in detergent from Solid-State NMR studies, thus we expected that all monomers in each trimer would be labelled with the same dye (either donor or acceptor), and then would be randomly mixed upon lipid reconstitution, so that ~50% of donors would be very close to acceptors in the neighbouring trimers (Fig. 1-4A) if the position of the label was chosen properly.
Figure 5-1. Data from the four control samples demonstrating existence of strong FRET in lipids, but not in detergent, consistent with lattice formation. A: Absorption spectra of labeled ASR in detergents and lipids; B: Emission spectra of labeled ASR in detergents and lipids (excited at 540 nm); C: Excitation spectra of labeled ASR in detergents and lipids (emission at 675 nm); Red colour: solubilized ASR, Yellow: ASR in DMPC/DMPA with 1:2 protein/lipids ratio (w/w), Green: ASR in PC/PS with 1:2 protein/lipids ratio (w/w), Blue: Resolubilized DMPC/DMPA, Purple: resolubilized PC/PS. D: Relative FRET efficiency measurements at 21°C; Yellow: $E_{\text{rel}}$ measured from emission spectra, Red: $E_{\text{rel}}$ measured from excitation spectra.

As can be seen from the data above, the relative FRET efficiency of solubilized protein was approximately 0.2, while the reconstituted samples had much stronger FRET, both in saturated (DMPC/DMPA, gel phase) and unsaturated (PC/PS, fluid phase) lipids. This result indicated that the idea of detecting the lattice formation of ASR by FRET was successful.

As an application, the effect of packing density (protein/lipids ratio) on lattice formation of ASR was investigated. The first set of samples prepared were the series of ASR in
DMPC/DMPA liposomes with various protein/lipid ratios, at 2:1, 1:1, 1:2, 1:5, and 1:10 (w/w).

From the previous research results from SAXS measurements (samples prepared by M. Ward, measured by H. Liang with coworkers), the lattice formation was not influenced by different packing densities (Fig. 5-2). The 2D crystal formation (as well as formation of ASR trimers as detected by CD spectroscopy performed by M. Ward, Ref. 14) was observed in all samples regardless of the packing density (protein/lipid ratio).

![Figure 5-2. SAXS measurements of ASR in DMPC/DMPA liposomes at different protein/lipid ratios (samples prepared by M. Ward, measured by H. Liang with coworkers). Black arrows indicate the Bragg peaks showing crystallinity (periodicity) (from Ref. 14).](image)

The results from the FRET measurements on these samples were in general consistent with the SAXS measurements (Fig. 5-3 and 5-4A). Even though the strengths of FRET signal
decreased somewhat as lipid to protein ratio increased, the lattice formation of ASR was not disrupted completely, judging from the strength of FRET which was still substantially higher than in solubilized samples (Table 5-1).

These results indicated that higher lipid concentrations likely increased the intermolecular distance between two monomers in different trimers, but lattice formation of ASR was never disrupted. This may be consistent with a recent finding that ASR in *E. coli* membranes may form a different, tetragonal, type of lattice which is more loosely packed [14]. To verify this result, three more sample sets were prepared. The second set of samples contained the exact same series as the first set, while second and third sets contained samples which were reconstituted at 0.5, 2, and 10 lipid to protein ratio. In general, results from all these samples were consistent with the first set of results regarding FRET strength as a function of lipid/protein ratio, which showed the same decreasing trend (Fig. 5-4B-D). Nevertheless, the values of relative FRET efficiencies and their differences between 0.5 and 10 lipid/protein ratio samples varied from set to set. A possible cause was differences in sample preparation conditions, i.e., the proteins labelled at different times may have different protein concentrations, labelling efficiencies, donor to acceptor ratios, possible light scattering, differences in refraction index, and various dye arrangements resulting in differences in the location of donor labelled trimers and acceptor labelled trimers in the lattice. Also, the quality of the lattice formed could depend on the reconstitution conditions and affect FRET. All of these factors may affect the measurements of relative FRET efficiency. Additionally, in some datasets (especially 5-3A), the background changes were noticeable, which most likely originated from differences in light scattering. Finally, the two samples among the four were
observed to have quite different EM (determined from emission spectra) and EX (determined from excitation spectra) FRET efficiencies, while the other two were not. The reason behind such difference is not clear, but it could be induced by the different dye concentration ratios. The samples which showed these differences tended to have higher donor concentration relative to acceptor (not all but most of them). Thus, the analyzed spectra suggest that emission fluorescence intensities may be substantially affected by donor concentration and the ratio between donor and acceptor dye.
Figure 5-3. Absorption spectra, emission spectra, and excitation spectra of each set of DMPC/DMPA samples. A: 1\textsuperscript{st} set - ~0.08 mM ASR at 0.5, 1, 2, 5, and 10 lipid/protein ratio; B: 2\textsuperscript{nd} set - ~0.009 mM ASR at 0.5, 1, 2, 5, and 10 lipid/protein ratio; C: 3\textsuperscript{rd} set - ~0.01 mM ASR at 0.5, 2, and 10 lipid/protein ratio; D: 4\textsuperscript{th} set - ~0.02 mM ASR at 0.5, 2, and 10 lipid/protein ratio; Red, yellow, green, blue and purple represent 2:1, 1:1, 1:2, 1:5, and 1:10 protein/lipid ratios, respectively.
Figure 5-4. Relative FRET efficiencies of samples reconstituted with DMPC/DMPA as a function of lipid/protein ratio. A: 1st set - ~0.08 mM ASR at 0.5, 1, 2, 5, and 10 lipid/protein ratio; B: 2nd set - ~0.010 mM ASR at 0.5, 1, 2, 5, and 10 lipid/protein ratio; C: 3rd set - ~0.010 mM ASR at 0.5, 2, and 10 lipid/protein ratio; D: 4th set - ~0.02 mM ASR at 0.5, 2, and 10 lipid/protein ratio; Yellow: $E_{rel.}$ determined from emission spectra, Red: $E_{rel.}$ determined from excitation spectra.

Table 5-1. Changes of FRET signal between 0.5 and 10 lipids/protein ratio (DMPC/DMPA samples)

<table>
<thead>
<tr>
<th>Set of ASR (DMPC/DMPA)</th>
<th>Change of FRET signal between 0.5 and 10 lipids/protein ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Emission spectra</td>
</tr>
<tr>
<td>1st set of ASR</td>
<td>-30.86%</td>
</tr>
<tr>
<td>2nd set of ASR</td>
<td>-26.86%</td>
</tr>
<tr>
<td>3rd set of ASR</td>
<td>-14.31%</td>
</tr>
<tr>
<td>4th set of ASR</td>
<td>-22.80%</td>
</tr>
</tbody>
</table>
After the effects of packing density on lattice were investigated with DMPC/DMPA liposomes (saturated lipids in gel phase), the same effects induced by different lipids, PC/PS ((unsaturated, in fluid phase), were studied as well. Liposomes of DMPC/DMPA and PC/PS have different characteristics of lipids tails resulting in different fluidities. DMPC/DMPA liposomes have saturated tails which is somewhat more rigid and stable than unsaturated tails of PC/PS. Thus, the lattice formation of ASR reconstituted with PC/PS lipids was expected to have lower FRET efficiencies than the samples with DMPC/DMPA lipids under the same conditions, especially at high lipid to protein ratios. In other words, the lattice formation of ASR was expected to have greater dependence on the packing density in PC/PS. To test this hypothesis, a series similar to those with DMPC/DMPA samples were prepared (Fig. 5-5) with PC/PS samples reconstituted at upto 40 lipid/protein ratio. As can be seen, the results from PC/PS samples had some variability, but in general showed low dependence on the lipid/protein ratio. The general pattern is that the higher lipid to protein ratio resulted in lower FRET efficiency, even though there were few exceptions, and beyond some point, the packing density had no substantial effect on FRET efficiency.
Figure 5-5. Absorption spectra, emission spectra, and excitation spectra of each set of PC/PS samples. A: 1st set - ~0.01 mM ASR at 0.5, 1, 2, 5, and 10 lipid/protein ratio; B: 2nd set - ~0.01 mM ASR at 5, 10, 20, 30 and 40 lipid/protein ratio; C: 3rd set - ~0.04 mM ASR at 0.5, 1, 2, 5, 10, 20, and 30 lipid/protein ratio; D: 4th set - ~0.01 mM ASR at 0.5, 2, and 10 lipid/protein ratio; E: 5th set - ~0.02 mM ASR at 0.5, 2, and 10 lipid/protein ratio; Red, yellow, green, blue, purple, brown, black and dark brown represent 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, and 1:40 protein/lipid ratios, respectively.
Figure 5-6. Relative FRET efficiencies of samples reconstituted with PC/PS as a function of lipid/protein ratio. A: 1\textsuperscript{st} set - ~0.009 mM ASR at 0.5, 1, 2, 5, and 10 lipid/protein ratio; B: 2\textsuperscript{nd} set - ~0.009 mM ASR at 5, 10, 20, 30 and 40 lipid/protein ratio; C: 3\textsuperscript{rd} set - ~0.04 mM ASR at 0.5, 1, 2, 5, 10, 20, and 30 lipid/protein ratio; D: 4\textsuperscript{th} set - ~0.01 mM ASR at 0.5, 2, and 10 lipid/protein ratio; E: 5\textsuperscript{th} set – ~0.02 mM ASR at 0.5, 2, and 10 lipid/protein ratio; Yellow: $E_{\text{rel.}}$ determined from emission spectra, Red: $E_{\text{rel.}}$ determined from excitation spectra.
Table 5-2. Changes of FRET signal between the lowest and highest lipids/protein ratio (PC/PS samples)

<table>
<thead>
<tr>
<th></th>
<th>Change of FRET signal between the lowest and the highest lipids/protein ratio</th>
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<tbody>
<tr>
<td></td>
<td>Emission spectra</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; set of ASR (PC/PS)</td>
<td>-25.02%</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; set of ASR (PC/PS)</td>
<td>-34.97%</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; set of ASR (PC/PS)</td>
<td>-3.03%</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; set of ASR (PC/PS)</td>
<td>+14.27%</td>
</tr>
<tr>
<td>5&lt;sup&gt;th&lt;/sup&gt; set of ASR (PC/PS)</td>
<td>+11.7%</td>
</tr>
</tbody>
</table>

In contrast, the FRET efficiencies from some samples resulted in slightly stronger FRET signals as the lipid to protein ratio increased (Fig. 5-6D and E). These two sets of samples were prepared at the same time with the 3<sup>rd</sup> and 4th set of DMPC/DMPA samples, respectively (Fig.5-4C and D). When the FRET efficiencies of the 4<sup>th</sup> PC/PS set of samples were compared to those of the 3<sup>rd</sup> DMCP/DMPA samples, the PC/PS samples were observed to have lower FRET efficiency at lower lipid to protein ratios while they had stronger FRET signals at higher lipid to protein ratios. The overall tendency for the DMPC/DMPA samples was a decline in FRET efficiency at higher lipids content, but these particular PC/PS samples had the greatest FRET efficiency at 1: 10 protein to lipids ratio. These results were contrary to the original hypothesis that the lattice may be disrupted in fluid lipid phase, although similar results were observed in other sets. When the 4<sup>th</sup> set of DMPC/DMPA and the 5<sup>th</sup> set of PC/PS samples were compared to each other, the FRET efficiency were found to be greater in PC/PS samples than in
DMPC/DMPA samples. In addition, the PC/PS samples’ FRET efficiencies determined from each kind of spectra behaved differently. While the FRET efficiencies calculated from the emission spectra were proportional to lipid to protein ratio, the excitation-based FRET efficiency slightly declined as lipid to protein ratio increased. This result again supports the possibility that the emission spectra are greatly influenced by the dye concentration, especially donor concentration, and are not reliable. If only the FRET efficiencies calculated from the excitation spectra were compared between the PC/PS and DMPC/DMPA samples, the packing density changes induced by both lipids could be deemed comparable as they both increased the distances between the monomers of different trimers at higher lipid content. Thus, the hypothesis that the unsaturated tails of PC/PS lipids might generate a less rigid lattice in ASR and result in weaker FRET signals was not confirmed.

4.2.2 Temperature effects on lattice formation of ASR

Temperature, an environmental factor which might affect the lattice formation of ASR was investigated as well. Since the fluidity of liposomes is highly dependent on the temperature, especially if the phase transition temperature is included in the experimental range, DMPC/DMPA liposomes were expected to become more fluid, and behave more like PC/PS lipids (possibly, have a weaker FRET efficiency, although contrary to the above) at higher temperatures. In contrast, PC/PS liposomes were already very flexible due to unsaturated lipid tails, so that they were not expected to be dependent on temperature. These hypotheses were tested by measuring the FRET efficiencies at four different temperatures, 21°C, 30°C, 40°C, and
50°C (the expected transition temperature for DMPC/DMPA mixture is above 23°C). The previously described samples (Fig. 5-4 and 5-6) were measured at these temperatures. The results were quite surprising again (Fig. 5-7), but not if one takes into the account the results presented above. As expected, the FRET efficiencies of samples in PC/PS liposomes had no substantial temperature effects. However, temperature effects on DMPC/DMPA samples were not significant (Fig. 5-7A) as well. Temperature effects seemed to be greater at higher lipid/protein ratio samples, but the FRET efficiency increased proportional to the temperature rather than decrease. At first, these results were questionable since the fluorescence of the acceptor control (ASR labelled with only acceptor) sample tended to decrease at higher temperatures (Fig. 5-8). Thus, the stronger FRET efficiency was suspected to be induced by reduced fluorescence of acceptor rather than real FRET. However, the donor control sample (ASR labelled with only donor) showed a similar tendency (Fig. 5-8) (decreased fluorescence at higher temperature), although there was a greater decline in fluorescence. These finding indicated that the resulting FRET efficiency for DMPC/DMPA samples may have increased at higher temperatures artificially due to the decreased fluorescence at donor wavelength. Overall, the weak temperature dependence of FRET in DMPC/DMPA samples is consistent with the small difference in FRET efficiencies between DMPC/DMPA and PC/PS reported in the previous section.
Figure 5-7. Representative plots of temperature effects on lattice formation derived from the excitation spectra; A: ~0.08 mM ASR reconstituted at varied lipids/protein ratio (DMPC/DMPA, 1st set); B: ~0.04 mM ASR reconstituted at varied lipids/protein ratio (PC/PS, 3rd set).
Figure 5-8. Effects of temperature on fluorescence of donor and acceptor. A: donor-only and acceptor-only samples - reconstituted at 2:1 protein/lipid ratio (PC/PS); B: donor-only and acceptor-only samples - reconstituted at 2:1 protein/lipid ratio (DMPC/DMPA); Red, yellow, green, and blue represents fluorescence measured at 21°C, 30°C, 40°C, and 50°C, respectively; C: normalized fluorescence (by fluorescence measured at 21 °C) of each sample as a function of temperature. Red represents donor-only in PC/PS lipids, Pink represents acceptor-only in PC/PS lipids, Dark green represents donor-only in DMPC/DMPA lipids, and Green represents acceptor-only in PC/PS lipids.
4.2.3 Summary of environment effects on lattice formation of ASR

The ability to detect the lattice formation of ASR in lipids using FRET was validated. This task was far from being trivial, as measuring FRET for membrane proteins in lipids is known to be difficult. The FRET signals were observed only in ASR in lipids while no substantial signals were obtained from solubilized ASR. However, the effects of environmental factors, packing density and temperature, behaved contrary to expectations, partially based on the behavior of bacteriorhodopsin, whose lattice could be successfully diluted in fluid lipids [38]. The greater lipid to protein ratio and higher fluidity of liposomes were expected to increase the intermolecular distances in 2D crystals of ASR in lipids, resulting in weaker FRET. The fluidity effects on lattice formation of ASR were not confirmed in the thesis. The comparison of PC/PS and DMPC/DMPA samples showed that PC/PS samples, which were more fluid, generated somewhat stronger FRET efficiency than DMPC/DMPA samples. In addition, increasing the fluidity of DMPC/DMPA lipids by heating the samples had no substantial effect on FRET efficiencies and if it had any effects (at higher lipid/protein ratio), it resulted in stronger FRET efficiency, not weaker, which is consistent with stronger FRET in PC/PS. Likewise, the dilution of the lattice at higher lipid content was not observed.

To properly compare the samples at different lipid/protein ratios and in different lipids, the same concentration of protein, and same concentration of donor and acceptor dyes were essential. However, these factors were difficult to control. Even though the samples were prepared from the same batch, the absorption and fluorescence spectra of
these samples had large differences in the amplitudes at different ratios and lipids (DMPC/DMPA and PC/PS). These differences were partially due to the scattering effect, which was found to be very dependent on protein/lipids ratio. The scattering was much greater at higher lipid to protein ratio which made reading the actual concentration of dyes hard. In addition, the loss of protein during reconstitution was also found to be dependent on protein/lipid ratio, similar to what was observed in our lab for human aquaporin-1 when BioBeads were used (S. Emami and L. Brown, unpublished). The higher lipid to protein ratio samples had greater protein concentrations after reconstitution when started with the same amount of protein. Thus, the changes in amplitudes of emission and excitation spectra were substantial. Also, the amplitudes of emission and excitation spectra could be affected by the self-quenching of dyes (Fig. 5-9). The emission spectra of acceptor-labeled ASR samples normalized by the acceptor concentration were expected to have the same fluorescence intensities if there were no self-quenching of the acceptor. However, samples with DMPC/DMPA lipids clearly showed the self-quenching was substantial at low lipid to protein ratios. In contrast, the PC/PS samples indicate the self-quenching was not always influenced by the lipid to protein ratio monotonically, but it still strongly occurring in the samples with 0.5 and 1 lipid to protein ratios. Furthermore, the PC/PS and DMPC/DMPA which have different refractive indices, n, also affected the FRET efficiency measurements according to the equation 2.2. The effect of a small difference in refractive index is not that influential to FRET efficiency measurements since the Forster radius is proportional to the $1/6$ power of the refractive index. The less densely packed lipids are known to have smaller refractive index values, so that PC/PS
lipids would have smaller refractive index value compared to DMPC/DMPA lipids, which means that DMPC/DMPA would have a greater impact on FRET efficiency compared to PC/PS under the same conditions, although this difference was negligible. Likewise, proteins have lower refractive indices than lipids, so that different lipid content could affect $R_0$ somewhat. But even if one assumes the most extreme case of pure protein ($n = 1.34$) and pure lipid ($n = 1.46$), the effect would be at most 1.5%, which is not significant.

Figure 5-9. Self-quenching of the acceptor dyes at room temperature; A: ~0.04 mM ASR labelled with acceptor in DMPC/DMPA lipids; B: ~0.04 mM ASR labelled with acceptor in PC/PS lipids; 1st column: Absorption spectra normalized by protein concentration; 2nd column: Emission spectra exited at 650 nm (normalized by protein concentration); 3rd columns: Excitation spectra emitted at 675 nm (normalized by protein concentration). Red, Yellow, Green, Blue, and purple represent the 0.5, 1, 2, 5, and 10 lipid to protein ratio samples, respectively.
Chapter 5. Final Summary and Future Studies

In this thesis, we have developed a new method for lattice formation detection and studied the lattice formation of ASR in different environments. The applied technique is a distance-sensitive tool, FRET, which requires a proper fluorescent tag labeling. Once the ASR is successfully labelled with FRET pair of fluorescent dyes, the donor and acceptor, the effects of different types of lipid, lipid content, and temperature on lattice formation in ASR were investigated.

Challenges in finding the ideal FRET pairs and labelling conditions were identified and only two of the three FRET pairs investigated could be attached (MTS-Fluorescein and MTS-TAMRA, Alexa Fluor 555 and Alexa Fluor 647) at non-native cysteine (C26) residue of the mutant ASR. Among these two FRET pairs, Alexa Fluor pair was chosen, because they have greater extinction coefficient, which results in good brightness. Also, Alexa Fluor 555 and Alexa Flour 647 have far apart emission and absorption spectra which decreases bleeding, and energy transfer between them is not interrupted by retinal, chromophore of ASR. The fluorescence parameters of labelled samples which were reconstituted under different environmental conditions were measured to obtain ratiometric FRET efficiency. Ratiometric FRET efficiency does not provide the actual distances, but it provides the relative FRET efficiency, which reflects the changes in intermolecular distances between two monomers in different trimers. Using this method, increasing the lipid content during reconstitution process was found to increase the intermolecular distances, resulting in lower ratiometric FRET efficiency, even though no complete lattice dissolution was observed at the ratios tested. However, other environmental
effects such as temperature changes, and fluidity of lipid were found not to affect the lattice formation in ASR.

The benefit of using FRET was its simplicity, unlike in SAXS measurements, which has been used to previously study the lattice formation in ASR. However, the resulting FRET efficiency measurements were different between the samples from different sample batches, showing the variability of the method in these early stages of its development. This phenomenon could be caused by differences in the labelling efficiency, the ratios between donor and acceptor dye concentrations, and structure of 2D crystals formed. In addition, the light scattering was found to highly depend on protein/lipid ratio, which makes it hard to analyze the data in a consistent manner. The self-quenching occurring at lower lipid content was another factor causing the differences in amplitudes of emission and excitation spectra. Future studies should be made to find the method to control such factors during the fluorescence measurements or analysing the data so that better distance constraints are obtained. We believe that this method should be applicable to other membrane proteins.
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