OLIGOMERS OF G PROTEINS IDENTIFIED IN LIVE CHO CELLS AND EXTRACTS OF S/9 CELLS

by

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ABSTRACT

Whereas many G protein-coupled receptors (GPCRs) are known to exist wholly or in part as functional oligomers, the oligomeric status of their attendant G proteins has received comparably little attention. In the current project, dual-color fluorescence correlation spectroscopy (dcFCS), model-based analysis of Förster resonance energy transfer (FRET), and step-wise photobleaching were used to probe for oligomers of fluorescently labelled G proteins (G\(\alpha_1\beta_1\gamma_2\)) in live CHO cells and in purified extracts of Sf9 cells. When G proteins bearing either eGFP or mCherry were co-expressed in CHO cells, the presence of a complex was indicated by high levels of specific FRET and a high degree of cross-correlation in dcFCS. Moreover, an analysis of apparent FRET efficiencies indicated that the complex contained at least four equivalents of the fluorophore. Photobleaching of single particles of eGFP-tagged G protein purified from Sf9 cells revealed 4–6 equivalents of the fluorophore; the number of steps was largely unaffected by guanyl nucleotides or by M\(_2\) receptor, but it was decreased by the combination of guanyl nucleotide and agonist-activated receptor. These results suggest that G proteins form tetramers or larger oligomers that dissociate into dimers or monomers upon activation. Signalling therefore involves a receptor-regulated interconversion between different oligomeric states.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A2AAR</td>
<td>$A_2A$ adenosine receptor</td>
</tr>
<tr>
<td>$\beta$ARK</td>
<td>$\beta$-adrenergic receptor kinase</td>
</tr>
<tr>
<td>$\beta_2$-AR</td>
<td>$\beta_2$-adrenergic receptor</td>
</tr>
<tr>
<td>$\beta_2$-AR$^*$-Gs</td>
<td>a complex of the activated $\beta_2$ adrenergic receptor and $G_s$</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>BS$^3$</td>
<td>bis(sulfosuccinimidyl)suberate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>DAG</td>
<td>diacyl glycerol</td>
</tr>
<tr>
<td>dcFCS</td>
<td>dual-colour fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EC, ECL, and ICL</td>
<td>extracellular, extracellular loop, and intracellular loop</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EMCCD</td>
<td>electron multiplying charged coupled device</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-regulated inwardly rectifying $K^+$-channel</td>
</tr>
<tr>
<td>GMP-PNP</td>
<td>guanylyl imidodiphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTP$\gamma$S</td>
<td>guanosine 5’-O-[3-($\gamma$-thio)triphosphate]</td>
</tr>
</tbody>
</table>
H8  8th α-helix of a GPCR
HEK  human embryonic kidney
HEPES  sodium N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonate
HRP  horseradish peroxidase
IB  immunoblot
IMAC  immobilized metal ion affinity chromatography
IP  immunoprecipitation
mCh  mCherry, a monomeric red fluorescent protein
M2G  a purified complex of M2 receptor and heterotrimeric G protein
Ni²⁺-NTA  nickel-nitrilotriacetic acid
PAGE  polyacrylamide gel electrophoresis
PDE  phosphodiesterase
PIP2  phosphatidylinositol 4,5-bisphosphate
PLCβ  phospholipase Cβ
RG  receptor-G protein complex
RGS  regulator of G protein signalling
SDS  sodium dodecyl sulfate
Sf9  Spodoptera frugiperda
TIRFM  total internal reflection fluorescence microscopy
TM  transmembrane domain/region
Tris  Tris(hydroxymethyl)aminomethine
CHAPTER 1 INTRODUCTION
1.1. G Protein-Coupled Receptors

1.1.1. Classification

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane signalling molecules in the human genome\(^{96}\). Although of wide structural diversity, all GPCRs possess a common structure of seven transmembrane helices (7TM) with an extracellular amino-terminus and an intracellular carboxyl-terminus. As the helices traverse the membrane seven times, three extracellular and three intracellular loops (ECLs and ICLs) are formed. The first and third ECLs are short and well conserved among different receptors. The more diverse ECL2 has been shown to play crucial roles in binding of ligands\(^{32}\). The ICLs interact with intracellular proteins such as G protein and arrestin. The ICL regions are more conserved compared to the ECLs and display significant changes in conformation upon receptor activation\(^{87}\).

The conserved features of GPCRs were initially identified based on the results of hydropathic analyses and mutation studies on various cloned receptors (e.g., reference 2). More recently, following the emergence of the first high-resolution crystal structure of inactive monomeric rhodopsin\(^{131}\), more than twenty GPCRs in both inactive and active states were resolved in the last decade\(^{87}\). Resolution of those structures allows researchers to develop a thorough understanding of the structures and functions of the receptor. A recent milestone structural achievement of the resolution of the complex of the activated \(\beta_2\)-AR and the heterotrimeric \(G_s\) allows for a better view of the receptor-G protein interaction. The structure confirmed some interactions that were previously deduced from structural and functional studies (e.g., references 31,42,64,123) and discovered some novel structural alterations of the receptor and the G protein\(^{146}\) whose functional significance has been investigated in further studies (e.g., references 3,65).

The superfamily of GPCRs, which has more than 800 members in humans, can be classified into 5 main families based on phylogenetic analysis. They are the families of rhodopsin (or Class A), secretin and adhesion (or Class B), glutamate (or Class C), and frizzled/taste2 receptors\(^{49}\). Except for the rhodopsin family, GPCRs in all the other families have long amino-termini. Moreover, many of them are rich in cysteine residues, a feature that plays key roles in ligand recognition and binding\(^{49}\).

Receptors in the secretin family have amino-termini that are approximately 60–80 amino acids in length. The \(N\)-termini often contain key cysteine residues that are important in binding
of large peptide ligands. Besides the secretin receptors, members of this family also include the corticotropin-releasing hormone receptors, the glucagon receptors, and the growth hormone-releasing hormone receptors\(^49\). Crystal structure of two receptors in this family of GPCRs are available: the glucagon receptor (GCGR)\(^1\) and the corticotropin-releasing factor 1 receptor (CRHR1)\(^7\). Receptors in the adhesion family have large amino-termini that consist of up to 2800 amino acids. These long peptide sequences often are rich in proline residues and glycosylation sites for the formation of adhesion-like motifs such as EGF (\textit{e.g.}, references 67,68). GPCRs in this family participate in cell adhesion and many of them are orphan receptors\(^11,16\). To date, no solved crystal structure is available for this family\(^49\).

The glutamate family contains the metabotropic glutamate receptors and the GABA receptors which are abundant particularly in the nervous system. Ligands for these receptors are derivatives or mimics of the excitatory neurotransmitter glutamate, such as \(\gamma\)-amino butyric acid (GABA) and \(N\)-methyl-\(D\)-aspartic acid (NMDA) and they generally bind to the amino-terminus of the respective receptors. The \(N\)-terminus of the metabotropic glutamate receptor forms a "Venus fly trap" to capture and to retain ligands in an extracellular cavity\(^52\). The \(N\)-termini of the GABA receptors are large but lack the cysteine-rich sequences found in other members of the family\(^49\). The glutamate family also includes 5 taste receptors (TAS1)\(^49\). Only the crystal structure of metabotropic glutamate receptor 1 (mGluR1)\(^19\) is currently available for this family.

The frizzled/taste2 receptor family contains two major groups, namely, the frizzled and taste (TAS2) groups. In some reports, binding of Wnt (a glycoprotein substrate) at the long \(N\)-termini (~200 amino acids long) of the frizzled receptors has been suggested to induce G protein coupling, placing them into the GPCR superfamily\(^15\). Ten frizzled receptors together with smoothened (SMOH), a human frizzled receptor, make up the frizzled group. They are involved in cell proliferation, polarity, and death\(^49\). The crystal structure of smoothened was recently solved\(^17\). Members of the taste2/TAS2 group have short amino-termini, and ligand binding therefore is likely to occur elsewhere for this group of receptors. Very little is known for this group of GPCRs except that they are found in tongue and palate epithelium and serve as bitter taste receptors\(^49\).

With more than 700 members, the rhodopsin family is the largest among the five families\(^49\). The ligand-binding site of this family is usually buried in the core of the 7TM bundle\(^7\), with the exception of the glycoprotein-binding receptors, whose ligand-binding sites are at their
amino-termini. Homology of the peptide sequences indicates that receptors of the rhodopsin family are divided into 4 groups: α-, β-, γ-, and δ-groups. Receptors in some groups can be further categorised into clusters.

Receptors in the α-group are sub-divided into five major clusters: amine receptors, adenosine receptors, melatonin receptors, opsin receptors, and prostaglandin receptors. Of the five, the amine receptors are among the most commonly studied targets in pharmacological investigations. Members of this cluster include serotonin receptors, dopamine receptors, muscarinic receptors, histamine receptors, adrenergic receptors, and trace amine receptors. They bind small amines that have a single aromatic ring. In the current study, the M₂ muscarinic receptor, which belongs to the amine cluster of the α-group in the rhodopsin family, was studied. More than half of the crystallised GPCRs are members of the α-group: rhodopsin (e.g., references 131,134), and the β₂-adrenergic receptor (e.g., references 145,146) in various states, M₂ and M₃ muscarinic receptors, β₁ adrenergic receptor (e.g., references 76,119), D₃ dopamine receptor, A₂A adenosine receptor (e.g., references 97,192), histamine H₁ receptor, and sphingosine-1-phosphate receptor. It is noteworthy that only the structures of rhodopsin were determined from material purified from native membranes.

Receptors in the β-group bind peptides at regions formed by the amino-terminus, ECLs, and extracellular (EC) regions of TM helices. Some examples of this group include the gastrin-releasing peptide receptor, the neurotension receptors, the growth hormone secretagogue receptor, and the thyrotropin releasing hormone receptor. A structural representation of the β-group is provided by the crystal of the neurotensin receptor. γ-Group receptors can be sub-divided into clusters of the somatostatin/opioid/galanin (SOG) receptors, the melanin-concentrating hormone (MCH) receptors, and the chemokine receptors. Ligands of the γ-group receptors also are peptides which bind at the EC region of the TM helices. Resolved γ-group receptor crystal structures include κ-, µ-, and δ-opioid receptors, two chemokine receptors (CXCR4 and CCR5), and the nociception/orphanin FQ peptide receptor (NOP). δ-Group GPCRs include many olfactory receptors and various types of receptors that bind glycoproteins, such as LH, TSH, and FSH. Two members of this group have been crystallised, namely, the protease-activated receptor 1 (PDB code 3VW7) and the purinergic P2Y12 receptor.
1.1.2. Conserved Motifs of the Rhodopsin Family GPCRs

Alignment of the peptide sequences among receptors in the rhodopsin family reveals 20–30 regions of conserved amino acid residues within the 7TM bundle. Two common structural regions identified are the E/DRY motif at the border between TM3 and ICL2 and the NPXXY sequence at the cytosolic end of the TM7. In the discussion of this section, the residues are numbered according to the Ballesteros-Weinstein numbering system to demonstrate the degree of conservation.

The E/DRY motif has a key arginine residue (Arg\(^{3.50}\)) that is conserved in 96% of the rhodopsin family GPCRs. It is connected to an acidic residue (either Asp\(^{3.49}\) or Glu\(^{3.49}\)) via a salt bridge. This connection has been observed in all inactive and even some active crystal structures (e.g., references 97,145,192). It remains intact in most receptor structures except in the structures of the active form of rhodopsin* and the complex form of \(\beta_2\)-AR*-G\(_\alpha\). In those two active structures, the salt bridges are broken and the guanidinium group of Arg\(^{3.50}\) changes position to interact with the C-terminus of the G\(_\alpha\) subunit instead. In rhodopsin, the side chain of Arg\(^{3.50}\) also interacts with Asp\(^{6.30}\) and forms an "ionic lock" with residues of TM6. Another important residue in the E/DRY motif, Asp\(^{3.49}\) or Glu\(^{3.49}\), also is involved in the interaction with ligands, as suggested by early mutagenesis results (e.g., reference 166). For instance, the carboxyl group of Asp\(^{3.49}\) interacts with the protonated amine group of typical agonists for the \(\beta\)-adrenergic receptors.

The NPXXY motif serves as a major micro-switch for activation and has a key tyrosine residue (Tyr\(^{7.53}\)) that is conserved in 92% of all rhodopsin family GPCRs. In the inactive state, the side chain of Tyr\(^{7.53}\) points towards TM1, TM2, and helix 8 (H8). In the active conformation, the side chain of Tyr\(^{7.53}\) points towards the core of the 7TM bundle and interacts with TM6 and TM3 residues. In the active state, the structures of rhodopsin* and \(\beta_2\)-AR* reveal a hydrogen bond between Tyr\(^{7.53}\) and Tyr\(^{5.58}\), another micro-switch which is conserved in 89% of GPCRs. On the other hand, the behaviour of the Tyr\(^{5.58}\) side chain varies among activated structures of receptors.
1.2. Heterotrimeric G Protein

1.2.1. Classification and Structural Characterisation

A G protein is a heterotrimeric protein comprising \( \alpha \), \( \beta \), and \( \gamma \)-subunits. Unlike the highly diverse family of GPCRs, the family of G proteins has fewer members. In the human genome, 16, 5, and 12 genes code for 21 \( G\alpha \), 6 \( G\beta \), and 16 \( G\gamma \) subunits, respectively\(^4\). G proteins are categorised into four classes based on the similarity of the primary structure of \( G\alpha \), namely, \( \alpha_{i/o} \), \( \alpha_s \), \( \alpha_{q/11} \), and \( \alpha_{12/13} \)\(^1\). Nine family members of \( G_{i/o} \) are involved in inhibitory pathways and interact with GPCRs such as the \( M_2 \) and \( M_4 \) muscarinic receptors, \( \alpha_{2A-C} \) adrenergic receptors, D\(_2\)-4 dopamine receptors, H\(_3\) and H\(_4\) histamine receptors, melatonin receptors, and 5-HT\(_{1A/B/D/E/F} \) serotonin receptors. \( G_{i/o} \) interacts with adenyl cyclase to inhibit the production of second messenger cAMP and blocks action of various cAMP-dependent pathways\(^1\)\(^2\). A special member of this group, transducin (\( G_t \)), is uniquely involved in relaying the signal from phot-activated rhodopsin. \( G_t \) interacts with phosphodiesterase (PDE) which breaks down the second messenger cGMP and eventually leads to hyperpolarisation of the membrane potential\(^1\)\(^3\). \( G_s \) (3 members) activates adenyl cyclase and promotes the production of cAMP, in contrast to \( G_{i/o} \). It interacts with GPCRs such as \( \beta_{1-3} \) adrenergic receptors, D\(_1\) and D\(_3\) dopamine receptors, H\(_2\) histamine receptor, and 5-HT\(_{4,6,7} \) serotonin receptors.

\( G_{q/11} \) (4 members) interacts with the \( M_{1,3,5} \) muscarinic receptors, H\(_1\) histamine receptor, and 5-HT\(_{2A/B/C} \) serotonin receptors. It activates phospholipase C (PLC) which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol trisphosphate (IP\(3)\)\(^1\)\(^6\). These products are involved in various pathways. For instance, DAG can interact with Ca\(^{2+}\) channels and lead to an increased Ca\(^{2+}\) current into the cell\(^1\). \( G_{12/13} \) (2 members) interacts with GPCRs such as the sphingosine-1-phosphate receptor 1 (SIP1) and the protease-activated receptor 1 (PAR1). They induce the functionality of a wide range of effectors such as Rho-specific guanine nucleotide exchange factors (RhoGEFs)\(^1\)\(^7\) that exchange GDP for GTP on the \( G\alpha \).

Similar to GPCRs, various mutation studies, molecular dynamic modeling, and high resolution crystal structures in various conformations have allowed for a structural characterisation of the G proteins. Some important details are discussed below along the description of the structural features of the G protein subunits.
1.2.2. Gα-subunit

The α-subunit of the G protein contains two domains: a Ras-like domain (Gα-Ras) and an α-helical domain (Gα-AH). Gα-Ras is a GTPase that hydrolyses bound GTP to GDP upon activation; Gα-AH forms a lid over the guanyl nucleotide binding pocket and buries the bound nucleotide in the core of the protein\(^{160}\). In the Gα-Ras domain, five loops formed by alternating α-helices and β-sheet strands construct the guanyl nucleotide binding pocket: the loops between β1-α1 (G1 or p-loop), α1-β2 (G2), β5-α2 (G3 or SWII), β3-α4 (G4), and β2-α5 (G5). Residues at G1 bind to the α- and β-phosphates of the guanyl nucleotide. A threonine residue in G2 coordinates the binding of Mg\(^{2+}\) ions. The G3 region is involved in binding of the γ-phosphate of GTP and Mg\(^{2+}\). Residues in the G4 and G5 regions recognise the guanine ring and the guanine base\(^{160}\).

A structural comparison of the GDP- and GTP-bound G proteins reveals conformational differences in three major regions. They are at or near the nucleotide binding pocket and are termed the switch regions (SWI–III)\(^{129}\). These regions are located at linker 2 (between αF, an α-helix in the Gα-AH region and β2, SWI), the G3 loop (SWII), and the loop between β4 and α3 (SWIII)\(^{160}\). Changes in conformation implicate those switches in accommodating binding and releasing of the guanyl nucleotide. The switches also have been postulated to support water molecules required for GTP hydrolysis, to properly orientate Mg\(^{2+}\) ions, and to facilitate the exit of free phosphate\(^{160}\). Moreover, the switch regions are crucial in arranging protein-protein interactions between Gα and its partners such as Gβγ, effectors, and regulators of G protein signalling (RGS)\(^{17}\). The Gα-AH domain, which comprises a bundle of 6 α-helices, is joined to the Gα-Ras domain via 2 linkers (L1 and L2)\(^{160}\). Due to its diverse makeup, Gα-AH has been shown to increase the affinity of guanyl nucleotides to the G protein, to increase the rate of GTP hydrolysis, and to play an important role in determining the interaction between the G protein and its specific effectors\(^{129}\).

Myristoylation (G\(_i\) only) and/or palmitoylation (all except for G\(_i\)) that occurs at the N-terminal helix (αN) of the Gα contributes to its membrane localisation\(^{129}\). In G\(_i\), the singly myristolated Gα was retained in the cytosol\(^{37,187}\). Moreover, a myristoylation-preventing mutation at the N-terminus of Gα where the glycine at position 2 is replaced by an alanine also inhibits palmitoylation\(^{50}\). The lack of lipid modifications eventually leads to accumulation of the
Gα in the cytoplasm\textsuperscript{50,120}. Other classes of G protein that are only palmitoylated or possess mutations that prevent palmitoylation are similarly retained in the cytoplasm\textsuperscript{37,186}.

Currently, crystal structures of the Gα have been obtained for the inactive GDP-bound state: Gα\textsubscript{t}(GDP)\textsuperscript{94}, Gα\textsubscript{1}(GDP)\textsuperscript{30,117}, and Gα\textsubscript{12/13}(GDP)\textsuperscript{91}; for the active GTP-bound state: Gα\textsubscript{t}(GTP\textsubscript{γ}S)\textsuperscript{126}, Gα\textsubscript{1}(GTP\textsubscript{γ}S/GppNHp)\textsuperscript{28,29}, Gα\textsubscript{α}(GTP\textsubscript{γ}S)\textsuperscript{167}; and for the state favoured by GDP-AlF\textsubscript{4}–: Gα\textsubscript{t}(GDP-AlF\textsubscript{4}–)\textsuperscript{159}, Gα\textsubscript{1}(GDP-AlF\textsubscript{4}–)\textsuperscript{28} and Gα\textsubscript{12/13}(GDP-AlF\textsubscript{4}–)\textsuperscript{91}. The crystal structures of α-subunits have also been solved with effectors, such as Gα\textsubscript{t}i(GDP-AlF\textsubscript{4}–)-RGS9-PDE\textsuperscript{γ}156, Gα\textsubscript{α}(GTP\textsubscript{γ}S) in combination with the catalytic domains of adenylyl cyclase V and II\textsuperscript{171}, Gα\textsubscript{α}/(GDP-AlF\textsubscript{4}–)-GRK2 in combination with the heterodimer of β\textsubscript{γ}172, and Gα\textsubscript{13} (GDP-AlF\textsubscript{4}–)-p115RhoGEF\textsuperscript{24}.

1.2.3. Heterodimer of Gβγ

β- and γ-subunits of the G protein are tightly coupled and dissociate from each other only under denaturing conditions\textsuperscript{63}. Gβ is a seven-bladed β-propeller in which each 40-amino acid blade or WD sequence is made of four anti-parallel strands. Gγ is composed of two α-helices joined by a loop. The amino-terminus of Gβ is also an α-helix which forms a coiled-coil structure with the N-terminal α-helix of Gγ. This coiled-coil structure is important for the proper folding of Gβ\textsuperscript{29}. The carboxyl-terminus of Gγ binds to blades 5 and 6 of Gβ\textsuperscript{58}. Lipid modifications on Gγ facilitate membrane association of the G protein. The attachment of an isoprenyl group at the C-terminus of Gγ leads to membrane association and acts as a signal for further modification of Gα such as palmitylation\textsuperscript{129}. Mutations of Gα\textsubscript{s} and Gα\textsubscript{i} that block palmitoylation prevent their association with Gβγ and result in cytosolic accumulation\textsuperscript{45}. Crystal structures of the Gβγ heterodimer also have been resolved, such as that of Gβ\textsubscript{1}γ\textsubscript{158}. The heterodimer has also been solved with effectors and regulators, such as Gβ\textsubscript{1}γ-phosducin\textsuperscript{54,55,104} and Gβ\textsubscript{1}γ2-GRK2\textsuperscript{103}.

1.2.4. Structural Interactions in the Heterotrimeric G Protein

Crystal structures of two heterotrimeric G proteins (Gα\textsubscript{4}iβ\textsubscript{1}γ\textsubscript{1} and Gα\textsubscript{4}iβ\textsubscript{1}γ\textsubscript{2}) display the interacting sites between Gα and Gβγ subunits\textsuperscript{95,178}. Two sites of interaction have been revealed between Gα and Gβ. One interface is composed of hydrophobic residues and exists between
SWI and II of the Gα and blades 5 and 7 of the Gβ. This interface buries ~1800 Å² of surface area and plays a crucial role in locking GDP in the binding pocket of Gα. The other interface is located between the N-terminus of Gα and blade 1 of Gβ and buries ~900 Å² of surface area. There is no evidence for any direct interaction between Gα and Gγ, although the N- and C-termini of the two subunits are in close proximity to each other.

1.3. G Protein-Mediated Signalling Cycle

G protein-mediated signalling is initiated when the externally stimulated receptor relays the signal to its attendant G protein; the activated G protein incorporates GTP at the nucleotide-binding pocket in the place of GDP. In this system, the G proteins bridge the receptors that discriminate among a wide range of external stimuli and the effectors which initiate downstream pathways inside the cell. Components of the G protein-mediated signalling cycle include the receptor, G protein, and effector, classification and structural features of which are discussed briefly above. Moreover, studies of various mutants and dynamic modeling, as well as structural comparisons among solved structures of the signalling components, have shined some light on our understanding of the interactions in the G protein-mediated signalling cycle. In the following section, some structural features of the key interactions and associations are discussed.

1.3.1. Ligand-Receptor Interaction

1.3.1.1. Structural Features of the Ligand-Binding Pocket

Two types of ligand-binding pocket, namely, those with an open or restricted entrance, are revealed in the ligand-bound crystal structures of GPCRs. Most members of the rhodopsin family have an extracellular entrance for ligands at ECL2. The diverse makeup of the secondary structures formed by the ECL2 is proposed to be the first step for recognising specific ligands. ECL2 also forms a disulfide bridge with a key cysteine residue (Cys325) in TM3, which is an important structure for receptor stability, possibly by limiting the degree of receptor conformational change upon activation. The second type of binding pocket, observed in the structures of rhodopsin (e.g., reference 162) and the S1P1 sphingosine receptor, is either partially or fully blocked at the extracellular regions of the receptor. In the S1P1 receptor, the crystal structure reveals a "side" opening between TM1 and TM7 for hydrophobic ligands to directly enter the pocket from the lipid bilayer.
As illustrated by various crystal structures, binding pockets containing residues from TM3, TM6, and TM7 make direct contact with ligands. TM1 has not been observed structurally to contact with the ligand but also is able to affect ligand binding, as shown in mutagenesis studies\textsuperscript{176}. Those transmembrane helices form a consensus scaffold for ligand binding, and the variability in the makeup of the scaffold contributes to ligand specificity in different receptors. Some of these residues also form inter-domain structures to ensure the proper orientation of a specific ligand in the pocket and a precise conformation of the pocket\textsuperscript{176}. One example is the network of inter-helical interactions that has been modeled for the structure of inactive $\beta_1$-AR\textsuperscript{176}.

1.3.1.2. Conformational Changes in GPCRs Induced by Ligand Binding

Binding of a ligand induces microscopic changes in the binding pocket conformation that lead to large-scale rearrangements of the transmembrane helices. These rearrangements act to facilitate association of the receptor with other proteins, such as with hetreotrimeric G proteins and arrestin\textsuperscript{146}. A structural comparison of GPCRs in the inactive and active states revealed an outward swing of TM6 as one of the most significant changes in conformation. In the structure of the adenosine A$_{2A}$ receptor, the side chain of Trp$^{6.48}$ interacts with the ligand, stabilises the shift of the residue, and leads to a corresponding displacement of TM6\textsuperscript{97,192}.

In the $\beta_2$-adrenergic receptor, the displacement of TM6 is largely dependent on the polar interaction between the agonist and serine residues (Ser203 and Ser207) in TM5. The interaction between TM5 and the ligand causes a subsequent inward shift of the EC tip of TM5. That shift leads to a conformational change in a TM3 residue (Ile121), which in turn is coupled to a side-chain movement of a residue (Phe282) in TM6\textsuperscript{165}. This eventually leads to the outward swing of TM6 (14 Å) observed in the crystal structure of $\beta_2$-AR$^*$-Gs. The displacement of TM6 allows insertion of the $\alpha_5$-helix (C-terminus) of the G$\alpha$ subunit into the cavity between TM5 and TM6\textsuperscript{146}.

In addition to the conserved regions within TM5 and TM6, the structure of $\beta_2$-AR$^*$-Gs also reveals conserved motifs in ICL2 of the receptor at the receptor-G protein interface. A conserved phenylalanine residue (Phe139) of ICL2 is posited to be in contact with a hydrophobic surface of G$\alpha$, a structure that is crucial for RG coupling\textsuperscript{146}. Mutation of the phenylalanine residue resulted in impaired coupling\textsuperscript{118}. The ICL2 region is stabilised by an interaction between ICL2 and the aspartic acid residue in the conserved DRY motif of TM3. The G protein also
makes contact with the receptor at the arginine residue of DRY, which packs against the tyrosine residue in the conserved NPXXY motif in TM7\textsuperscript{146}. It therefore appears that the ICL2 region links the G protein to the conserved regions of the receptor.

Another dynamic region within the orthosteric binding pocket of the receptor involves TM3 and TM7, and the specific conformational changes vary for different receptors. For rhodopsin, disruption of a salt bridge formed between residues in TM3 and TM7 results in an increase of 2–3Å in the distance between the helices\textsuperscript{87,162}. In contrast, in A\textsubscript{2A}AR, the ribose rings of the agonist form a strong network of hydrogen bonds with residues in TM3 and TM7, leading to decrease of \textasciitilde2Å in the distance between the helices\textsuperscript{81,87}. In β\textsubscript{2}-AR, residues of TM3 and TM7 are bridged by the hydroxyl and amino groups of agonists, but the absolute distance between the helices does not change extensively\textsuperscript{87,145,146}.

1.3.2. G Protein Activation and GDP Release

Activated GPCRs catalyse the exchange of GDP for GTP on Gα, which in turn activates the G proteins. The rate-limiting step of G protein activation is the release of GDP due to its slow dissociation rate in the absence of a guanyl nucleotide exchange factor such as an activated GPCR (R*)\textsuperscript{74}. Release of GDP is allosterically induced by R*, as the binding sites of the active receptor on Gα (N- and C-termi, as well as the loops between α\textsubscript{4}-β\textsubscript{6} and α\textsubscript{3}-β\textsubscript{5}) are some 30 Å away from the proposed nucleotide-binding pocket (G1 through G5 regions)\textsuperscript{63}. Those contact sites between the receptor and Gα have been revealed by mutagenesis (e.g., references 38,61,141), peptide mapping (e.g., references 64,112) and chemical cross-linking (e.g., references 21,80). Most of the postulated interactions were confirmed by the crystal structure of β\textsubscript{2}-AR*-G\textsubscript{s}.

Mutations in the α\textsubscript{5}-helix and β\textsubscript{6}-α\textsubscript{5} loop of Gα can increase the spontaneous release of GDP\textsuperscript{78,173} and decrease receptor-catalysed nucleotide exchange\textsuperscript{110,111}. Results from site-directed spin labeling indicate that, in the presence of an activated receptor, GDP is released upon a rigid-body rotation of the α\textsubscript{5}-helix towards the β\textsubscript{6}-strand\textsuperscript{130}. Those results suggest that the GDP release is coordinated between the α\textsubscript{5}-helix and other parts of the G protein. Not surprisingly, when the crystal structure of β\textsubscript{2}-AR*-G\textsubscript{s} is compared to the inactive form of the Ras-like domain of Gα, the α\textsubscript{5}-helix of Gα\textsubscript{s} in the complex is seen to translate and rotate towards the receptor. The major displacement of the α\textsubscript{5}-helix is also associated with changes at the β\textsubscript{6}-α\textsubscript{5} and β\textsubscript{1}-α\textsubscript{1} loops\textsuperscript{146}. In
the Gα-Ras structure, those loops interact with the guanosine ring and β-phosphate of the nucleotide, respectively. In addition, the β6-α5 loop is displaced away from the nucleotide binding pocket, potentially facilitating the exit of GDP\textsuperscript{167}.

Deuterium exchange measurements showed an increased level of exchange in the β1 strand and the N-terminus of the α5-helix upon formation of the RG complex\textsuperscript{27}. On the basis of double electron-electron resonance (DEER) distance measurements and the β6-AR*-Gs crystal structure, a hybrid model was constructed of the complex between the active form of rhodopsin and Gi. By tracking energy changes, the model showed a disruption of links between the α5-helix and the β6-α5 loop, the α1-helix, αG which is a helical structure located between the Ras-like and the helical domains of the α-subunit, and the GDP binding site. Furthermore, the loss of contact between the domains is one of the most drastic changes in the crystal structure of β6-AR*-Gs, where a 127° displacement of the α-helical domain relative to the Ras-like domain was observed\textsuperscript{146}. Moreover, the observations of increased deuterium exchange at the interface between the two domains and a large change identified by DEER spectroscopy in the distance between nitroxide probes on the two domains confirmed the inter-domain separation\textsuperscript{27}.

\textit{1.3.3. GTP Binding}

After the release of GDP, the nucleotide-free G protein heterotrimer and the activated receptor remain bound with high affinity (e.g., references 18,44). We and others (e.g., reference 146) took advantage of this phenomenon to isolate stable complexes of RG in a procedure of co-purification (Chapter 2). The tightly-bound RG complex is thought to be only a transiently occurring entity, however, owing to the rapid binding of GTP which is highly abundant in the cytosol (i.e., ~µM).

A comparison of the crystal structures of the GDP-, GDP-AlF\textsubscript{4}^- and GTPγS-bound G proteins reveals a similar structural arrangement in most regions of the G protein with exception of the three switches regions that undergo significant rearrangements when GTP replaces GDP\textsuperscript{128}. While the α- and β-phosphates of the GTP molecule bind to the α-subunit of the G protein in region of the p-loop, the γ-phosphate binds at the regions of the loops of α1-β2 and β3-α2 that overlap with switches 1 and 2. The phosphate hydrogen atom binds to the amide oxygen atom of the conserved glycine residue in switch 2, and to the hydroxyl group and amide of the
serine/threonine residue of the p-loop\textsuperscript{160}. The coordinate of the nucleotide, Mg\textsuperscript{2+}, binds to other residues, such as threonine in G2, and incorporates water molecules in the complex\textsuperscript{160}.

1.3.4. \textit{G Protein–Effector Interactions}

G protein activation is marked by the release of GDP and the binding of GTP. Subunits of the G protein subsequently interact with specific effectors depending upon the signalling cascade that they are involved in. While some G protein subunits are capable of interacting with a wide variety of effectors, others are more specific and couple only to certain effectors (\textit{e.g.}, G\textsubscript{\alpha} and cGMP phosphodiesterase \(\gamma\) (PDE\(\gamma\))\textsuperscript{129}.

1.3.4.1. G\textsubscript{\alpha}–Effector Interactions

Different types of G\textsubscript{\alpha} lead to varying signalling outcomes even via the same effector. For example, interaction with G\textsubscript{\alpha}s leads to activation of adenylyl cyclase, but interaction with G\textsubscript{\alpha}i results in inhibition of the effector. G\textsubscript{\alpha}i interacts with phospholipase C\(\beta\) (PLC\(\beta\)) and hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP2) to second messengers, and G\textsubscript{\alpha} couples to PDE\(\gamma\) and catalyses the breakdown of cGMP\textsuperscript{129}. Crystal structures of G\textsubscript{\alpha} coupled to effectors have identified interfaces between the two proteins. In general, hydrophobic side chains of the effectors insert into the cavity formed by the N-termini of the \(\alpha\textsubscript{2}\)- and \(\alpha\textsubscript{3}\)-helices of G\textsubscript{\alpha}. Additional contacts at the C-termini of the helices of \(\alpha\textsubscript{2}\) and \(\alpha\textsubscript{3}\) as well as the loops of \(\alpha\textsubscript{2}-\beta\textsubscript{4}\) and \(\alpha\textsubscript{3}-\beta\textsubscript{5}\) are thought to be involved in determining the binding specificity of effectors\textsuperscript{129}. Furthermore, studies other than crystallography also suggest the involvement of the loop of \(\alpha\textsubscript{4}-\beta\textsubscript{6}\) in the binding of G\textsubscript{\alpha}s to PDE\(\gamma\)\textsuperscript{102,144} as well as that of G\textsubscript{\alpha} to adenylyl cyclase\textsuperscript{10}.

1.3.4.2. Fate of the Heterotrimer and G\(\beta\gamma\) Heterodimer–Effector Interactions

The crystal structure of G\textsubscript{\alpha}-GTP reveals a spectrum of structural rearrangements at the switch regions. In particular, rearrangement at the region where binding of the \(\gamma\)-phosphate occurs disrupts the binding site for G\(\beta\gamma\). Therefore, it has been proposed that binding of GTP diminishes the binding surface for G\(\beta\gamma\) and leads to dissociation of the G protein heterotrimer\textsuperscript{17,56}. Subsequently, the two entities of the G protein interact with their respective effectors. However, FRET/BRET studies that monitored the resonance energy transfer efficiencies between G\textsubscript{\alpha} and G\(\beta\gamma\) upon activation in live cells yielded differing results. Whereas some studies support the
dissociation hypothesis with observations of either a decrease or complete loss of resonance energy transfer between G protein subunits (e.g., references 6,84,194), others suggest an alternative possibility of subunit rearrangement with observations of increased energy transfer (e.g., references 19,70,93). Therefore, the status of the heterotrimeric G protein subunits after activation remains to be elucidated.

Since the discovery of the first effector that interacts with Gβγ (i.e., the K+ ion channel in cardiac membranes)\textsuperscript{105}, other such effectors have been identified such as adenylyl cyclase\textsuperscript{169}, β-adrenergic receptor kinase (βARK)\textsuperscript{139}, and calcium and potassium channels (e.g., references 71,148). Mutation studies show that the interacting effectors share overlapping binding sites on Gβγ (e.g., references 47,99,132). The overall conformation of the crystal structure of the heterotrimer is generally preserved when the protein interacts with various effectors, although some loops in Gβ displace and form the binding site for the effector proteins. Moreover, the interacting surface is also the binding site for the SWII region of Gα. Thus it has been argued that binding of Gα inhibits binding of effectors on Gβγ, and this view is supportive of the dissociation model of the G protein heterotrimer\textsuperscript{129}.

1.3.5. Inactivation of G Proteins: GTP Hydrolysis

Termination of the signalling cycle is marked by hydrolysis of GTP to GDP and reassembly of the heterotrimeric G protein. Key residues and regions for GTP hydrolysis on Gα are identified by comparison of the crystal structures of Gα-GDP and Gα-(GDP +AlF\textsubscript{4}^-). In the latter structure, AlF\textsubscript{4}^- forms a tetragonal bipyramid structure with oxygen from the β-phosphate group of GDP and a water molecule\textsuperscript{28,159,160}. Comparisons of the structures of Gα uncovered crucial residues for GTPase activity. Particularly, a glutamine residue appropriately places the water molecule in the bipyramid structure, and a conserved arginine residue stabilises the negative charge on the leaving phosphate group\textsuperscript{28,126}. Moreover, the intrinsic GTPase activity of Gα is relatively slow as the turnover rate of Gα-catalysed GTP hydrolysis is in the range of 2–5 min\textsuperscript{-1} (reference 56). Typically, a class of proteins termed the GTPase-activating proteins or GAPs also are present to accelerate GTP hydrolysis and to aid in the termination of the signalling cascade. These proteins were initially found to be involved in enhancing the activity of Ras proteins in the catalytic reaction of GTP hydrolysis\textsuperscript{160}. Certain effectors also have been shown to
function as GAPs. For instance, phospholipase $\beta_1$ has been found to accelerate the GTPase activity of $G\alpha_{\gamma1}$.

A major family of GAPs named regulators of G protein signalling, or RGS, has been discovered. Members of the RGS family share a common domain that is segmented by linkers of various sizes. RGS proteins are proposed to stabilise the transition state of the reaction of GTP hydrolysis based on two types of observations. One, RGS proteins have been shown to display higher affinity for $G\alpha_{\omega i}(GDP-AlF_4)$ than for $G\alpha_{\omega i}(GTP_S)$ or $G\alpha_{\omega i}(GDP)$. Two, high-resolution crystal structures of the $G\alpha$-RGS complex, such as that of $G\alpha_{i1}(GDP-AlF_4)$-RGS4 and $G\alpha_{i}(GDP-AlF_4)$-RGS9, illustrate the interactions between the RGS protein core and the switch regions of $G\alpha$. As previously discussed, the switches are sensitive regions for binding and hydrolysis of the guanyl nucleotide. In addition, a comparison between the crystal structures of $G\alpha_{i2}(GDP + AlF_4)$ and $G\alpha_{i3}(GDP)$ reveals that a glutamic acid residue in the loop between the helices of $\alpha D$ and $\alpha E$ in the helical domain acts as a spring to separate the Ras-like and helical domains of the $\alpha$-subunit. The open space between the separated domains may be involved in the dissociation of $G\alpha$ from the bound effector; therefore, the heterotrimer of G protein can be subsequently reassembled.

1.4. A Closer Look at the Agonist–Receptor Interaction

1.4.1. Nucleotide-Sensitive Heterogeneity Revealed by Agonist Binding Assays

Radiolabelled ligands permit direct observation of the ligand-receptor interaction. Other than observing the direct binding behaviour of a radioligand to the receptor, a widely used method permits studying the binding of a wide range of unlabelled agonists by observing their inhibition pattern on the binding of a radiolabelled ligand. For example, graded concentrations of an unlabelled agonist can be used to inhibit the labelling of receptors by a fixed concentration of radiolabelled antagonist. For various types of GPCRs, such assays commonly give rise to an inhibition pattern that has a shallower slope and a Hill coefficient less than 1. The shape of the curve deviates from a rectangular hyperbola that is predictive of an interaction according to the law of mass action. Such patterns have been demonstrated in the binding of many GPCRs, such as the adrenergic receptor (e.g., references 60,88), the muscarinic receptor (e.g., references 15,16), the opiate receptor, the dopamine receptor, and the serotonin receptor. Addition of guanyl nucleotides (e.g., GTP) induces a rightward shift (GTP-shift) in the inhibition curve of an
agonist and results in a curve whose Hill coefficient is near 1. Antagonist binding results in curves with Hill coefficients near 1 and are insensitive to guanyl nucleotides (e.g., references 12,43). Partial agonists produce GTP-shifts with less deviation from 1 compared to those induced by full agonists. It has been shown that the magnitude of the GTP-shift corresponds to the efficacy of the agonist$^{43,88}$.

Empirically the binding curves with Hill coefficients less than 1 can be fitted in terms of multiple binding sites (i.e., a sum of more than one rectangular hyperbola) (e.g., references 88,113). According to the multi-site model, the heterogeneity observed in the binding pattern is viewed as a result of a mixture of sites of high and low affinities for the agonist; addition of GTP converts the sites of high affinity to sites of low affinity. However, if only one gene product is present, and if the states of the receptor do not interconvert, the multi-site model is merely a way to smooth the data points by means of curves.

1.4.2. Ternary Complex Model

Due to the inadequate explanation of the binding behaviour provided by the multi-site model, other mechanistic models have been proposed to describe the phenomenon of nucleotide-sensitive heterogeneity in agonist binding. One popular model is the ternary complex model, which regards the receptor and G protein as being monomeric entities that transiently interact during G protein-mediated signalling. The model rationalises the observed heterogeneity as a result of different affinities for a G protein-free receptor, which has a low affinity for the agonist, and for a G protein-coupled receptor, which has a high affinity for the agonist. GTP, which has a higher affinity for the uncoupled G protein, binds to the receptor-coupled G protein and promotes disassociation of the RG complex. Dissociation of the complex by GTP eventually leads to the GTP-shift as the uncoupled receptor has a lower affinity for agonist$^{35}$.

Although the ternary complex model explained many observations qualitatively, data simulated by mathematical models based on it were not in agreement with the experimentally obtained binding data (e.g., references 59,98,124). To make the simulated data agree with the observations, compromises such as changes in total G protein number were required$^{35,116}$. Furthermore, this model also has several assumptions that have been under debate. One, the receptors are mutually independent; therefore, the heterogeneity revealed in agonist binding is induced in the receptor by the G protein. Two, the G proteins are also assumed to be mutually
independent of each other, and this aspect has been largely overlooked in the literature. Three, the receptor-G protein complex is assumed to be a transient entity formed by random collision of its components.

1.4.3. Status of the Functional Unit of the Receptor-G Protein Complex

Upon binding of an agonist, the activated receptor relays its signal to the associated G protein. The complex of receptor and G protein (RG) is considered as the functional unit in the signalling cycle and its oligomeric integrity as a whole as well as that of its components has been the subject of numerous investigations. The ternary complex model assumes a transient unit of RG, formed by random collision, whose dissociation is regulated by guanyl nucleotides. On one hand, guanyl nucleotides have been shown to cause dissociation of the RG complex in solubilised preparations (e.g., references 18,56). On the other hand, the existence of a stable complex in solubilised and even purified samples also has been reported (e.g., references 85,107). In recent years, fluorescent techniques such as resonance energy transfer (RET) and fluorescence recovery after photobleaching (FRAP) have been used to probe the dynamics of the RG complex (e.g., references 51,70,125,142). For instance, under resting conditions, specific interactions were observed between α2A adrenergic or M4 muscarinic receptors and their cognate G protein subunits125. A FRAP study demonstrated a receptor-dependent decrease in the mobility of a fluorescent G protein in the complex of the M3 muscarinic receptor and Gq142. These observations pointed to "pre-coupling" of GPCR and G protein.

Moreover, in the same FRAP study, the addition of neither agonist nor inverse agonist changed the mobility of Gq. Furthermore, fluctuations in resonance energy transfer per se rather than a complete loss of BRET was reported in the complex of α2A-AR and Gα1β1γ2 after activation of the receptor51. These observations suggest a stable RG complex whose components only rearrange rather than dissociate upon activation. Meanwhile, others reported activation of receptor to lead to dissociation of the RG complex and the heterotrimeric G protein using similar techniques (e.g., references 69,70).

1.4.4. Cooperative GPCRs and Oligomeric Arrangements

The ternary complex model also assumes mutually independent receptors and implies that the observed heterogeneity in the binding pattern is a result of G protein regulation. A
biphasic pattern was observed for inhibition of the labeled antagonist [³H]AF-DX 384 by the unlabelled agonist oxotremorine-M at purified M₂ receptors devoid of G protein. This observation implies that the heterogeneity is intrinsic to the receptor and is possibly a result of different affinities of the agonist for different protomers within a cooperative oligomer of receptors. A similar observation has also been reported for purified receptors reconstituted in phospholipid vesicles. In our laboratory, quantitative analyses in terms of cooperativity suggested four or more binding sites for the agonist in a tetramer or larger oligomer of receptors. The same conclusion has been drawn from the binding patterns of M₂ receptors in native membranes and purified preparations. When M₂ receptors were co-purified with G proteins, the binding profile obtained with a labelled antagonist and graded concentrations of an unlabelled agonist became bell-shaped in the presence of GMP-PNP, a non-hydrolysable GTP analog. Bell-shaped patterns are inconsistent with non-interacting sites and can be accounted for by cooperativity.

Cooperativity implies oligomers. Although it has been shown that some GPCRs from the rhodopsin family (e.g., β₂-AR) can signal as monomers, several lines of contradicting evidence suggestive of an oligomeric arrangement also have emerged. Early studies involving hydrodynamic assays and target size analysis by radiation inactivation suggested the existence of dimers or larger oligomers (e.g., references 33, 140). Later, purified receptors from native tissues were shown to migrate as dimers and trimers during electrophoresis (e.g., reference 188). Moreover, co-immunoprecipitation (Co-IP) of differently tagged receptor purified from Sf9 cells and increased receptor size upon chemical cross-linking have also been observed (e.g., references 34, 136, 137).

The GABA_B receptor has been shown to form heterodimers of GABA_B₁ and GABA_B₂. As GABA_B₁ only binds agonist but does not couple to G protein and GABA_B₂ only couples to the G protein but does not bind agonist, a unit of both is required to transmit a signal. Another striking piece of evidence that directly revealed oligomers of a GPCR was the identification by atomic force microscopy of arrays of rhodopsin dimers arranged in a pseudocrystalline lattice in the disks of rod outer segments.

More recently, fluorescence-based techniques such as FRAP (e.g., reference 40), RET-based techniques, bimolecular fluorescence complementation (BiFC) (e.g., reference 53), and single-molecule tracking have been used to detect oligomers of GPCRs in live cells. A large
body of evidence for oligomeric GPCRs comes from RET-based (FRET and BRET) studies. Briefly, in FRET-based studies, an external excitation source irradiates the donor fluorophore, and the efficiency of non-radiative energy transfer is monitored between two closely positioned fluorescent molecules (a pair of compatible donor and acceptor). BRET follows the same principle, with the replacement of the fluorescent donor by a biluminescent donor\textsuperscript{106}. Since an early observation of homo-oligomers of \( \beta_2 \)-adrenergic receptor in HEK-293 cells\textsuperscript{5}, similar results suggesting oligomers of a wide variety of GPCRs have been reported. Details of the results of those studies have been systematically reviewed (e.g., reference 106). Whereas most studies of this nature confirmed the existence of GPCR oligomers but placed only a lower limit on their size (i.e., dimeric), a study which employed quantitative model-fitting of the FRET efficiency between GFP\textsubscript{2} and YFP-tagged M\textsubscript{2} muscarinic receptors suggested tetrameric receptors in CHO cells\textsuperscript{138}.

Other fluorescence techniques, such as fluorescence correlation spectroscopy (FCS), also have been used to determine the size of oligomeric receptors. FCS estimates the size of fluorophore-tagged receptors based on the emitted intensity as they diffuse through a small detection volume defined by the wavelength of the laser\textsuperscript{180}. Dimers have been suggested for the serotonin 5-HT\textsubscript{2C} and the \( \mu \)-opioid receptor in HEK-293 cells\textsuperscript{57,73}. In addition to single-colour FCS, dual-colour FCS (dcFCS) is made possible by the addition of another compatible fluorophore and corresponding laser\textsuperscript{180}. DcFCS revealed a substantial level of heterodimers between estrogen receptors ER\textsubscript{\( \alpha \)} and ER\textsubscript{\( \beta \)}. A combination of FRET and dcFCS led to the conclusion that the EGF and ErbB receptors pre-form homo- and heterodimers prior to activation\textsuperscript{101}. From the level of cross-correlation between fluorescent IL-4 and the co-receptor, Weidemann \textit{et al.} distinguished two types of IL-4-induced IL-4 receptor formation: one formed a complex with its co-receptor, while the other did not\textsuperscript{181}.

The above fluorescence-based techniques acquire data either from a whole cell or over a period of time from a small portion of the cell membrane. More recently, studies using total internal reflection fluorescence microscopy (TIRFM) have allowed investigators to track molecules on a single-molecule level and to count the number of fluorophore-labelled molecules that are present in an oligomeric array. Briefly, TIRFM allows for total internal reflection using a high numerical aperture (NA) objective. The reflected light subsequently generates a narrow evanescent field immediately adjacent to the interface between the coverslip and the sample.
medium. Compared to wide-field fluorescence, TIRFM produces clearer images with a high signal-to-noise ratio and thus allows for the observation of single molecules. Using TIRFM, $M_1$ muscarinic and $N$-formyl peptide receptors were observed to be either stably monomeric or transiently dimeric in CHO cells$^{72,86}$; $\beta_1$- and $\beta_2$-adrenergic receptors were reported to vary from monomers to tetramers$^{22}$.

On a structural basis, diffraction-quality crystals of oligomeric GPCRs with multiple interacting interfaces have been resolved for different receptors, including rhodopsin$^{151}$, $\kappa$- and $\mu$-opioid receptors$^{109,190}$, and CXCR4$^{189}$. Two types of symmetric receptor-receptor interfaces, termed interfaces A and B, were observed. Interface A, which has been observed in rhodopsin and $\kappa$- and $\mu$-opioid receptors, consists of helices 1, 2, and 8. Within the interface, the clusters of helix form separate interaction patches$^{109,151,190}$. This interface has also shown minimal change in conformation upon activation$^{87}$. Interface B comprises helices 5 and 6 in the $\mu$-opioid receptor and helices 3 and 4 on the intracellular side of CXCR4$^{109,189}$.

In our laboratory, the $M_2$ muscarinic receptor has been shown to be tetrameric in cardiac membranes$^{188}$, solubilized extracts from $S/f9$ cells$^{137}$, purified receptor reconstituted into phospholipid vesicles$^{108}$, and CHO cells$^{138}$ by means of mechanistic model fitting, immunoblotting, cross-linking, co-immunoprecipitation and quantitative analysis of FRET efficiencies. The observation of tetramers is not unique to the $M_2$ receptor. The $\beta_2$-AR has been shown to migrate at least partially as a tetramer on SDS-PAGE denaturing gels following chemical cross-linking$^{150}$. Tetramers of GABA$_B$ receptors that are composed of dimers of heterodimers of GABA$_{B1}$ and GABA$_{B2}$ also have been observed using FRET$^{114}$.

1.4.5. Oligomeric G Proteins

A complex of the $M_2$ muscarinic receptor and a mixture of different subtypes of $G\alpha$ purified from porcine atria displayed a ratio of roughly 1:1 between R and G$^{107}$. A similar result has been reported for the $\mu$-opioid receptor from rat brain$^{23}$. Moreover, an agonist-sensitive heterogeneity in the binding of GDP to receptor-coupled G proteins mirrors the well-known phenomenon of nucleotide-sensitive binding of agonists to G protein-coupled receptors$^{25}$. Therefore, if GPCRs are oligomers as discussed previously, it implies the functional unit of signalling also includes oligomers of G proteins.
In recent years, as researchers have devoted much effort to investigating the oligomeric status of various GPCRs. The quest to detect and characterise oligomeric G proteins was mostly disregarded with the exception of a few early in vitro studies. In a series of early hydrodynamic studies, multimers of Gs, Gi, Go, and Gq, whose sizes were comparable to those of cross-linked tubulin, were identified in solubilised extracts from native tissues. Gαo subunits purified from E. coli also were found to exist mainly as dimers, trimers, and higher order oligomers, based on their electrophoretic mobility on non-denaturing gels. Cross-linking of the sample also revealed a series of oligomeric bands. In addition, co-immunoprecipitation performed on M2 muscarinic receptor co-purified with Gαo, Gαi1-3, and caveolins from porcine atria has revealed a mixture of various combinations of homo- and hetero-oligomers of Gα subunits. Furthermore, guanyl nucleotides have been found to induce disaggregation of G protein oligomers. For instance, it has been shown on polyacrylamide gels that cross-linked Gαo exists as oligomers; such oligomers dissociate upon GTPγS activation. In addition, no apparent cross-linking has been observed in Gαo samples that were pre-incubated with the nucleotide, also indicating the possible dissociation of the G protein oligomers upon activation by a guanyl nucleotide.

The current project revisits the concept of a G protein oligomer, and it is the first attempt to directly identify such oligomers in live cells. Also of interest has been the oligomeric status of the G protein upon its activation.

1.5. Rationale and Study Proposal

The superfamily of G protein-coupled receptors is composed of a diverse collection of transmembrane molecules that are responsive to a wide range of stimuli. The G proteins bridge the externally activated receptors and the respective downstream effectors which subsequently lead to intracellular signalling events. Biochemical studies as well as the recently solved high-resolution crystal structures of various types of receptors, G proteins, and effectors have aided in the structural visualisation of the components of this G protein-mediated signalling cycle. Moreover, many of the crystal structures, as well as dynamic modeling and mutagenesis studies based on the solved structures have advanced our understanding of the interactive dynamics among those components. Of those structures, the structure of the complex between the β2-adreneric receptor and the stimulatory heterotrimeric G protein in particular has provided a direct demonstration of the interaction between an active receptor and its attendant G protein.
Despite the structural discoveries, a common mechanistic description of the G protein-mediated signalling cycle is still lacking. Early reports of radioligand binding assays described a guanyl nucleotide-sensitive heterogeneity in binding of agonists. Various models have been used to account for the phenomenon. The popular ternary complex model assumes a monomeric receptor and transient receptor-G protein complex and regards the heterogeneity as a result of G protein intervention. However, the model provides only a qualitative description of the binding pattern, and it is inconsistent with several lines of observations regarding the size of the receptor and the stability of the signalling complex. In our laboratory, the binding curve of a purified M₂ receptor reconstituted as a tetramer devoid of G protein also revealed to be heterogeneous. This indicates that the heterogeneity is intrinsic to the receptor and perhaps arises from cooperative interactions among receptors in an oligomeric setting. Various other types of GPCRs also have been shown to form oligomers in live cells by fluorescence-based techniques such as FRET, BRET and FRAP. In addition, the notion of a transient signalling complex has been debated due to reports of contradicting results (e.g., references 51,70,125,142). Although much effort has been invested in understanding the above two issues, comparably little attention has been paid to the oligomeric status of the G proteins.

An agonist-sensitive heterogeneity revealed in binding of guanyl nucleotides mirrors the more commonly known nucleotide-sensitive heterogeneity in the binding of agonist. Moreover, a stochiometry of 1:1 has been reported for the interaction between receptor and G protein (e.g., references 23,107). Therefore, if receptors exist in a cooperative oligomer, it implies the existence of an oligomer of G proteins arranged in a similar fashion. In early hydrodynamic and chemical cross-linking studies, oligomers of G proteins have been isolated and detected. In the current project, we have used fluorescence-based techniques to identify G protein oligomers in live CHO cells and purified extracts of Sf9 cells. Furthermore, the size of the oligomer purified from Sf9 extracts was monitored upon activation by guanyl nucleotide and by an activated M₂ muscarinic receptor to reveal a ligand-dependent dissociation into smaller entities of G protein.
CHAPTER 2 MATERIALS AND METHODS
2.1. Acknowledgements

Fluorescence-based experiments were completed in collaboration with Rabindra Shivanaraine and Nellie Han in our laboratory, as well as with Yuchong Li and Dennis Fernandes in Dr. Claudiu Gradinaru's laboratory in the Department of Chemical and Physical Sciences, University of Toronto (Mississauga). Models regarding the interpretation and analysis of FRET were developed by Dr. Valerică Raicu based on the binomial expansion and Förster theory. MATLAB programs used for the image processing and data analysis were written locally as follows: FRET calculation, Nellie Han and Rabindra Shivanaraine; correlation function fitting, Yuchong Li and Rabindra Shivanaraine; and step determination of the intensity-time trajectory, Rabindra Shivanaraine. The single molecule photobleaching experiments were conducted in collaboration with Dennis Fernandes.

2.2. Constructs and Vectors

A cartoon depiction of the constructs used in this project is shown in Figure 2-1. In panel A, gene sequences coding for the various proteins were inserted in the pcDNA3.1+ vector for transfection into CHO cells and subsequently used in cell imaging. In panel B, baculoviruses coding for variants of Gα1i and for the M2 receptor were used for infection of Sf9 cells. The expressed proteins were extracted and purified using a Ni²⁺-NTA column as described below. Monomers and multimers of eGFP were expressed in E.coli and purified by immobilized metal ion affinity chromatography (IMAC).

The cDNA encoding the enhanced green fluorescent protein (eGFP) or mCherry (mCh) was inserted between positions 91 and 92 of the wild-type Gα1i. Both fluorophores were cloned in a vector containing wild-type Gα1i using 2 restriction sites of BamH1 and sequences of 5’-AATGCGGATCCGAATTCAAGGGCGAGGAGGATA-3’ and 5’-AATGCGGATCCCTTGTA-CAGCTCGTCCATGC-3’ were used as forward and reverse primers, respectively. A polyhistidyl tag (His6) was added immediately preceding the fluorophore by site-directed mutagenesis. For addition of the His6 tag to the sequences for mCh-Gα1i, sequences of 5’-GGTGGTGGATCCCATCATCATCATCATGATGATGATGATGATGGGATCCACCACCACC-3’ were used as forward and reverse primers. For insertion of the His6 tag into GFP-Gα1i, sequences of 5’-GTGTTGATCCCATCATCATCATCATGATGATGATGATGATGGGATCCACCACCACC-3’ and 5’-
AGCTCCTCGCCCTTATGATGATGATGATGATGGGATCCACCACCAC-3’ were used as forward and reverse primers. The constructs for Gβ1 and Gγ2 in pcDNA3.1+ were purchased from the cDNA Resource Center, Missouri University of Science and Technology. The construct for N-terminally tagged GFP-Gβ2 was a gift from Dr. Stephane Angers. Constructs of the fusion protein of eGFP with the first 32 amino acids of Ga1 (MP-GFP) and of the M2 muscarinic receptor with an enhanced localisation sequence (α7-M2) were made previously in our laboratory by Dr. Luca Pisterzi138.

Two groups of constructs were made for in vitro experiments, namely, eGFP- and mCh-Gα1 in the pFastBac vector and various fused eGFP multimers in the pcDNA3.1+ vector. The constructs for (His6)-GFP-Gα1 and (His6)-mCh-Gα1 in pcDNA3.1+ were cloned into pFastBac1 by addition of the restriction sites Rsrl and XbaI using the following primers: 5’-ACTCGCGGTCCGCCATGGGCTGCACGCTGAGCGCCGAGACAAGGCGGCGGTG GAGCGGAGTAAGATG-3’ (forward) and 5’-CCGTAAAGCACTAAATCGGAAC-3’ (reverse). The transferring procedure was carried out according to the manufacturer's instructions (Life Technologies). Baculoviruses of the other constructs were available in our laboratory: FLAG-tagged M2 receptor was prepared by Dr. Paul Park137; (His6)-Gα1, Gβ1, and (His6)-Gγ2 were gifts from Dr. Tohru Kozasa, Department of Pharmacology, University of Illinois at Chicago.

The fusion eGFP multimers were tagged with His6 at their N-termini and connected by linkers cleavable by thrombin (thr) or tobacco etch virus (tev) protease. Individual units of eGFP were inserted consecutively by digestion and ligation using the following pairs of restriction sites: KpnI and BamHI, BamHI and EcoRI, EcoRI and NotI, NotI and XbaI. The primers used for adding the restriction sites, linkers, and the His6 tag are listed below. To insert KpnI and BamHI along with an amino-terminal His6 tag and thrombin linker, the sequences 5’-AGCTTGGTACCGCCATGCATCATCATCATCATCAAGGGCGAGGAGCTGTTCATTCAAGAGGTACCTTGCATTCATGAGCTCCATCTCCATGCAACATCCTGGGGAACAGTACAGCTCGTCCATGC-3’ and 5’-GGCCCTCTAGATTACGGCTTCATGCAACATCCTGGGGAACAGTACAGCTCGTCCATGC-3’ were used as forward and reverse primers, respectively. The second copy of eGFP was inserted between the restriction sites for BamHI and EcoRI along with the tev-sensitive linker at its C-terminus; the following sequences were used as primers: 5’-ACTGTGGATCCAGGGCGAGGAGCTGTTCATTCAAGAGGTACCTTGCATTCATGAGCTCCATCTCCATGCAACATCCTGGGGAACAGTACAGCTCGTCCATGC-3’ (forward) and 5’-ACTGTGAATTCTGATTGGAACTGAGTTTTCCTTGTACAAGTACCTTGCATTCATGAGCTCCATCTCCATGCAACATCCTGGGGAACAGTACAGCTCGTCCATGC-3’ (reverse).
CATGC-3’ (reverse). The third copy of eGFP was inserted between the restriction sites for *EcoRI* and *NotI* along with the thrombin-sensitive linker at the 3’-end; the following sequences were used as forward and reverse primers: 5’-ACTGTGAATTCAAGGGCGAGGAGCTGTTC A-3’ and 5’-TCGATGCAGCCCGCTACCTCTAGGTACTAGCTTGTACAGCTCGTCCATG C-3’. The fourth eGFP was inserted between the sites for *NotI* and *XbaI* using the following primers: 5’-ACTGTGCGGCGCCGGAGGGGAGGAGCTGTTC-3’ (forward) and 5’- GGCCCTCTAGATTACTTTGTACAGCTCGTCCATG-3’ (reverse).
Figure 2-1: Cartoon depiction of the various constructs used in this project.

Ribbon representation of the constructs used in this study is shown on the left side and their identity is shown on the right side of each panel. Constructs in panel A were transfected into CHO cells for the measurements of dcFCS and FRET. Constructs listed in panel B were expressed, extracted, and purified for \textit{in vitro} single-molecule measurements.
2.3. Multi-colour In-cell Fluorescence Techniques

2.3.1. Fluorophore Selection

Three fluorescence techniques were used in the current project for the identification and quantification of G protein oligomers in CHO cells and in purified extracts of Sf9 cells. Two fluorophores were selected based on their spectral separation and spectral overlap: enhanced green fluorescent protein (eGFP) and mCherry (mCh). Quantitative FRET requires that the two fluorophores have a considerable spectral overlap between the donor emission and acceptor excitation to allow for sufficient energy transfer. Spectral separation reduced the level of direct excitation of the acceptor following donor excitation. In dcFCS experiments, spectrally well-separated fluorophores allow for collection of the emission intensity from either fluorophore without much cross-contamination from its neighboring channel. Normalised spectra of eGFP and mCherry, which satisfy the various requirements for both studies, are shown in Figure 2-2.

![Figure 2-2: Excitation and emission spectra of eGFP and mCherry.](image)

Normalised excitation and emission spectra of the two fluorophores used in this study (*i.e.*, eGFP and mCherry) are shown in dotted and solid lines, respectively. The spectra of eGFP and mCherry are displayed in green and red, respectively.

2.3.2. Dual-colour Fluorescence Correlation Spectroscopy (dcFCS)

FCS is a technique that correlates the intensity of photon emission from fluorescent particles with respect to themselves as they move through the focal volume defined by the laser.
The focal volume is dependent upon the focusing of the laser beam, where the ratio $(s)$ of height to width reflects this focus. Values of $s$ in this study were measured to be between 4 and 6.

2.3.2.1. Correlation of Fluorescence

The fluorescence fluctuation at any time point $(\delta F_i(t))$ is compared with that of the same fluorophore at some lag time $\tau$ later, $(\delta F_i(t + \tau))$. This is normalised over the averaged fluorescence signal (Equation 2-1) and the resulting normalised quantity is termed auto-correlation, or $G_i(\tau)$, for the monitored fluorophore. Correlation of the intensities of two spectrally separated signals (colours) is a special implementation of FCS known as dual-colour FCS (dcFCS). The two-colour correlation is known as cross-correlation $(G_x(\tau))$ and is calculated according to Equation 2-2. In the equation, the subscripts $g$ and $r$ respectively refer to the emission intensities from eGFP and mCherry. To identify the diffusive properties of the auto- and cross-correlating components, various models need to be fitted to the data. Receptor and G protein at the membrane can diffuse in only two dimensions ($x$ and $y$); therefore the data were fit with a two-dimensional model that accounts for crowding (i.e., the anomalous model)\(^{46,82}\) (Equation 2-3). The parameters of the model are as follows: $G(0)$ corresponds to the amplitude of the correlation curve and $\tau_D$ is the diffusion time of the molecules through the focal volume. The anomalous factor is described by $\alpha$. Values of $\alpha$ less than 1 would indicate the diffusion of crowded molecules. In the case of auto-correlation, the average number of molecules within the confocal volume is inversely proportional to the amplitude of the fitted diffusion curve ($G(0)$).

The extent of co-diffusion of eGFP and mCherry is measured by the amplitude of the cross-correlation curves. The existence of cross-correlation would imply that the fluorophore-tagged molecules diffuse as oligomers.

\[
G_i(\tau) = \frac{\langle \delta F_i(t) \delta F_i(t+\tau) \rangle}{\langle F_i(t) \rangle^2} \quad \text{Equation 2-1}
\]

\[
G_x(\tau) = \frac{\langle \delta F_g(t) \delta F_r(t+\tau) \rangle}{\langle F_g(t) \rangle \langle F_r(t) \rangle} \quad \text{Equation 2-2}
\]

\[
G(\tau) = G(0)(1 + \left(\frac{1}{\tau_D}\right)^{\alpha})^{-1} \quad \text{Equation 2-3}
\]

2.3.2.2. Estimation of the Overlap Volume:

Co-diffusing molecules are captured only within the volume illuminated by both lasers. That volume is called the overlap volume and it is less than either of the individual focal
volumes. To accurately account for the number of co-diffusing fluorophores, a correction factor for the overlap volume of the two lasers was computed using Equations 2-4g and 2-4r. In the equations, $G_x(0)$ and $G_i(0)$ represent the amplitudes of the cross- and auto-correlation curves; $V_x$ and $V_i$ are the illuminated volumes created by the two overlapped lasers and one of the two lasers. The correction factors for the overlapped confocal volumes ($V_i/V_x$) relative to the volume for one laser were calculated from the amplitudes of a dual-fluorophore-labelled, double-stranded sequence of DNA.

To estimate the overlap volume correction factor ($V_i/V_x$), a double-stranded DNA oligonucleotide was prepared that comprised a strand labelled with fluorescein at its 5’-end and Tex615 at its 3’-end and a hybridised non-fluorescent complementary strand. The fluorophores in the former strand were separated by 40 base-pairs. Upon hybridisation, the 40 base-pairs allow for sufficient separation between the two fluorophores so that no significant resonance energy transfer occurs. Since the molecules are dually-labelled, their cross-correlation curve should overlap with either of the auto-correlation curves if the confocal volumes created by the two lasers are perfectly aligned. In practice, although a high level of cross-correlation was observed, the curve did not perfectly overlap with that of the auto-correlation. This is due to the imperfect alignment of the two lasers so that the co-diffusing molecules observed make up only a fraction of the dual-colour molecules. Therefore, a correction factor obtained by comparison of the amplitudes of the cross- and auto-correlations was applied to account for this underestimation of the co-diffusing population. The correction factor ($V_i/V_x$) was calculated according to Equation 2-4, in which $G_i(0)$ and $G_i(0)$ ($i \equiv r$ or $g$) are the amplitudes of the auto- and cross-correlation curves, and $f_g$ and $f_r$ represent the fractions of the co-diffusing eGFP and mCherry molecules. The two fluorophores are located on the same strand of DNA, and the fraction of co-diffusing molecules therefore was assumed to be one.

$$f_g = \frac{G_x(0) V_{\bar{g}}}{G_r(0) V_x} \quad \text{Equation 2-4g}$$

$$f_r = \frac{G_x(0) V_r}{G_g(0) V_x} \quad \text{Equation 2-4r}$$
2.3.2.3. Data Acquisition

CHO cells co-expressing eGFP- and mCherry-tagged proteins were simultaneously irradiated at 473 nm and 561 nm with solid-state lasers on a Nikon Eclipse Ti/Nikon A1 confocal microscope. The laser beams were focused on the sample through a water immersion objective (Apo40x, NA=1.25, Nikon). The emission signal was divided into two channels by a dichroic mirror (560DCXR, Chroma). The signal was further filtered by 2 band-pass filters (520/35 and 624/40) and was finally collected at 500–545 nm and 600–640 nm for observation of the intensities emitted by eGFP and mCherry, respectively. The emitted photons from each channel were recorded by a PicoHarp 300 TCSPC detector (PicoQuant) and fed to fluorescence lifetime imaging and correlation software (SymPhoTime 64, PicoQuant) that computes the auto- and cross-correlations of the recorded signals. A custom-written MATLAB program was used to plot the correlation (G(τ)) as a function of lag time (τ) and to fit the anomalous diffusion model to the data. The resulting amplitudes of the correlation functions were used to estimate the fraction of co-diffusing species with respect to each population.

2.3.3. Förster Resonance Energy Transfer (FRET)

FRET-based techniques can be used to characterise protein-protein interactions by quantifying the transfer of resonance energy between donor (D) and acceptor (A) fluorophores. The transfer of energy occurs only if the appropriately positioned fluorophores are aligned appropriately within 10–100 Å of each other, depending upon the Förster radius.

2.3.3.1. Estimation of the Apparent FRET Efficiency by Spectral Unmixing

CHO cells were co-transfected with the plasmids coding for eGFP-αi1, mCh-αi1, and other proteins as required. Upon excitation of the donor (eGFP), a stack of images spanning the emission spectra of both donor and acceptor (mCherry) was collected. The composite spectrum comprising both contributions was unmixed according to Equation 2-5 in which \( nE_{mD} \) and \( nE_{mA} \) are the reference spectra for eGFP and mCherry, respectively; \( k_D \) and \( k_A \) are the scaling factors for the respective contributions from eGFP and mCherry.

\[
Y = k_D \times nE_{mD} + k_A \times nE_{mA} \tag{Equation 2-5}
\]

To account for a small amount of direct excitation of the acceptor by the donor laser, the acceptor contribution was corrected using Equation 2-6. The quantity \( k'_A \) represents the corrected
value of $k_A$, and $k_{AD}$ represents the emission from mCherry when the fluorophore is excited by the donor laser; $f$ is an experimentally determined parameter obtained as the slope of the linear relationship between $k_A$ and the observed acceptor emission intensity from cells expressing different levels of mCherry. The apparent FRET efficiency was computed from $k_D$ and $k_A'$ using Equation 2-7; In the equation, $Q^A$ and $Q^D$ are the quantum yields of the donor and acceptor, respectively, and $w_D$ and $w_A$ are their elementary spectral integrals.

$$k_A' = k_A - f k_{AD} \quad \text{Equation 2-6}$$

$$E_{app}^{dq} = \frac{1}{1 + \frac{Q^A k_{DP} w_D}{Q^D k_A' w_A}} \quad \text{Equation 2-7}$$

2.3.3.2. Estimation of Donor and Acceptor Concentrations

The dependence of the apparent FRET efficiency on the relative amount of acceptor and donor ([A]_T/[D]_T) provides some insight into the number of protomers in the complex. The values of [A]_T and [D]_T were determined according to Equation 2-8a and 2-8d from the emission upon irradiation at 543 and 488 nm. $I_A$ and $I_D$ are the emission intensities from acceptor and donor channels. The constants $\epsilon^A$ and $\epsilon^D$ are the extinction coefficients at the peak of the excitation spectrum of A and D; $e^A$ and $e^D$ are correction factors for partial excitation, and $L_A$ and $L_D$ are the laser powers. To more accurately determine $[D]_T$, the effect of donor-dequenching due to FRET was taken into account by including the term $1 - E_{app}^{dq}$ in Equation 2-8d.

$$[A]_T = \frac{I_A}{Q^A \epsilon^A e^A L_A} \quad \text{Equation 2-8a}$$

$$[D]_T = \frac{I_D}{Q^D \epsilon^D e^D L_D (1 - E_{app}^{dq})} \quad \text{Equation 2-8d}$$

2.3.3.3. Pairwise FRET Efficiency and Oligomeric Size

Following the above procedures, various cells co-expressing eGFP-G$\alpha_1$ and mCh-G$\alpha_1$ were selected, and their FRET spectra were spectrally unmixed for the calculation of apparent FRET efficiency. The size of the G protein oligomer was estimated by accounting for all possible combinations of donor and acceptor within complexes of varying sizes (Equation 2-9). This model describes the dependence of the apparent FRET efficiency on the mole fractions of donor and acceptor (e.g., $P_A = \frac{[A]_T}{[A]_T + [D]_T} = \frac{[A]_T /[D]_T}{1 + [A]_T /[D]_T}$).
\[ E_{\text{app}}^{dq} = \frac{1}{n} \sum_{k=1}^{n} \frac{k(n-k)E_1}{1+(n-k-1)E} \binom{n}{k} P_D^{k-1} P_A^{n-k} \]  

Equation 2-9

The equation considers all possible combinations of D and A for a certain oligomeric size \( n \) and accounts in each case for all pathways of resonance energy transfer. The counter \( k \) is number of donors in the oligomer. The model can be fitted to the relationship between \( E_{\text{app}} \) and \([A]_T/[D]_T\) for an oligomer of fixed size \( n \) to deliver an estimate of a parameter termed the pair-wise FRET efficiency \( (E) \), which is the resonance energy transfer efficiency between a single donor and a single acceptor within the oligomer. The data were fitted by Equation 2-9 taken with different fixed values of \( n (n = 2, 3, 4, 6, \text{and} 8) \) to determine the respective pair-wise FRET efficiency for each size of oligomer.

2.3.3.4. Image Acquisition and Processing

Images were acquired on a Zeiss LSM 710 confocal microscope using a 63× oil-immersion objective (NA=1.4, Plan Apochromat). eGFP was excited using a 488 nm Ar\(^+\) laser at a power of 0.37 \( \mu \)W and with a digital gain of 1.0 and a master gain of 900. The spectrum was measured from 495 to 541 nm. mCherry was excited using a 543 nm HeNe laser at a power of 0.11 \( \mu \)W and with a digital gain of 1.0 and a master gain of 900. The spectrum was measured from 580 to 700 nm. To obtain the FRET spectrum, cells with co-expression were irradiated using the 488 nm Ar\(^+\) laser to excite eGFP. The intensities from a stack of 30 images spanning the range from 493 to 643 nm in increments of 5 nm were pooled to obtain the FRET spectrum. The collected images were processed in MATLAB using a locally written program for cell selection, for FRET spectrum construction, and for \( E_{\text{app}} \) and \([A]_T/[D]_T\) computation.

2.3.4. Cell Culture and Transfection of CHO Cells

Chinese hamster ovary (CHO) cells were grown in 1× Dulbecco’s modified Eagle’s medium containing 4.5 g/L glucose and L-glutamine (Corning Cellgro) supplemented with 10% (v/v) fetal bovine serum (Gibco), 0.1 mM nonessential amino acids (Gibco), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich). Cells were grown on 35 mm sterile dishes with a 14 mm microwell formed by a No. 1.5 glass coverslip (MatTek Corporation) in a humidified incubator at 5% CO\(_2\) and 37 °C. The cells were transfected at approximately 30–40% confluency using GeneExpressoMax Transfection Reagent (Excellgen). The DNA and the transfection reagent were used at a ratio of 1:3, as instructed by the manufacturer. Total DNA used was in the range of 1–2 \( \mu \)g. Forty-eight hours after transfection, confocal imaging was
performed in 1× Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, L-glutamine and 25 mM HEPES (Gibco).

2.4. In vitro Techniques for Quantification of G Protein Oligomers

2.4.1. Expression and Extraction of G Proteins

Baculoviruses coding for eGFP-inserted α1, wild-type β2, and wild-type or His6-γ subunits of the G protein and for FLAG epitope-bearing human M2 muscarinic receptor were expressed in Sf9 cells. The cells were cultured at 27.5 °C in Ex-Cell 400 insect medium (JRH Biosciences) containing 2% fetal bovine serum, 1% Fungizone, and 0.1% gentamycin (all from GibcoBRL). Cells growing at a density of 2 × 10^6 cells/mL were infected with one or more baculoviruses at a total multiplicity of infection of 7.5.

The G protein purification procedure used in this project was adapted from reference 90. The harvested membranes were lysed with buffer A (20 mM KH2PO4 (Sigma-Aldrich), 20 mM NaCl (Bioshop), 20 mM HEPES (Bioshop), 0.1 mM EDTA (Sigma-Aldrich), 10 mM β-mercaptoethanol (Sigma-Aldrich), and Complete Protease Inhibitor tablets (Roche), adjusted to pH 7.4 with NaOH). The lysed membranes were first washed with buffer A, and subsequently with buffer A in the absence of EDTA (buffer B). The washes with buffer B prepared the sample for subsequent purification on the nickel-nitrilotriacetic acid (Ni2+-NTA) agarose (Qiagen). Between each wash, the membrane pellets were resuspended in the appropriate buffer and homogenised by Polytron (setting 5, 10 seconds per burst for three bursts). The homogenate was centrifuged for 30 min at 100,000 g and 4 °C. The washed membranes were resuspended to approximately 3 mg/mL protein and were incubated with 1% (w/v) sodium cholate (Sigma-Aldrich) at 4 °C overnight. The solubilised supernatant was collected by centrifugation and loaded on the Ni2+-NTA column for purification.

Prior to loading the sample, the column was equilibrated with buffer E, which contains 20 mM HEPES, 20 mM NaCl, 1 mM MgCl2 (Sigma-Aldrich), 10 mM β-mercaptoethanol, and 0.2% (w/v) sodium cholate, adjusted to pH 7.4 with NaOH. After loading the sample, the column was washed twice in succession with buffers W1 and W2. The two wash buffers only differed in the concentration of NaCl, which was added to remove non-specifically associated materials from the column. They both contain 20 mM HEPES, 3 mM MgCl2, 10 mM β-mercaptoethanol, 60 mM imidazole (Sigma-Aldrich), 0.2% (w/v) sodium cholate; and 100 mM NaCl (W1) or 20
mM NaCl (W_{G2}). After the removal of non-specifically bound materials by stringent washing, the His_{6}-tagged G proteins were eluted with buffer E_{G2} (20 mM HEPES, 20 mM NaCl, 3 mM MgCl_{2}, 10 mM β-mercaptoethanol, 150 mM imidazole, and 1% (w/v) sodium cholate, adjusted to pH 7.4 with NaOH). A schematic description of the procedure is presented in Figure 2-3A.

Extraction of receptor and G protein followed steps similar to those described above. The key additions to the co-purification procedure included the incorporation of the agonist carbachol and the steps to remove bound GDP for the isolation of the complex. A depiction of the solubilisation and purification procedures is shown in Figure 2-3B. The co-infected Sf9 cell pellets were lysed with buffer A_{RG} (10 mM carbachol, 20 mM KH$_2$PO$_4$, 20 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, 10 mM β-mercaptoethanol, protease inhibitor tablets, and 200 μg/mL bacitracin (Sigma-Aldrich), adjusted to pH 7.4 with NaOH). After centrifugation, the pellets were washed three times with buffer A_{RG} supplemented with 1 mM EDTA to remove GDP (B_{RG}). The membrane was subsequently washed with buffer A_{RG} without EDTA (C_{RG}) for subsequent purification on the Ni$^{2+}$-NTA chelating column. The washed membrane was then resuspended in buffer C_{RG}, and 25 mU/mL apyrase (New England Biolabs) was added to the homogenate. The mixture was inverted gently at 4 °C for 60 min so any bound GDP could be hydrolysed by the purine pyrophosphatase apyrase. Steps for GDP removal are crucial to the isolation of a coupled RG complex as the GDP-bound G protein disrupts the stability of the receptor-G protein complex. Finally, the membrane was resuspended to approximately 5.5 mg/mL protein and solubilised in digitonin–cholate (0.86% digitonin, 0.17% cholate) in the presence of 10 mM carbachol before purification on the Ni$^{2+}$-NTA column (Figure 2-3B). The digitonin was purchased from Wako at purity near 100%.

Purification of the RG complex was similar to that of the G protein, with some minor alternations to the buffers. The equilibration buffer E_{RG1} contained 20 mM HEPES, 20 mM KH$_2$PO$_4$, 20 mM NaCl, 10 mM β-mercaptoethanol, 10 mM carbachol, and 0.22%-0.04% digitonin–cholate. The wash buffers W_{RG1} and W_{RG2} both contained the following common constituents: 20 mM HEPES, 20 mM KH$_2$PO$_4$, 10 mM β-mercaptoethanol, 10 mM carbachol, 60 mM imidazole, 0.22%-0.04% digitonin–cholate, and 100 mM NaCl (W_{RG1}) or 20 mM NaCl (W_{RG2}). The elution buffer E_{RG2} contained 20 mM HEPES, 20 mM KH$_2$PO$_4$, 20 mM NaCl, 10 mM β-mercaptoethanol, 10 mM carbachol, 150 mM imidazole, and 0.86%-0.17% digitonin–cholate, adjusted to pH 7.4 with NaOH. After purification, samples were concentrated with their
respective fresh elution buffers (E$_{G2}$ or E$_{RG2}$) without imidazole to facilitate subsequent immobilisation for photobleaching analysis.
Figure 2-3: Extraction of eGFP-tagged G proteins from S9 cells.

Schematic representations of the steps in the extraction and purification of eGFP-Gαβγ in the absence and presence of the M2 muscarinic receptor are shown in panels A and B, respectively. In the procedure for co-purification of the receptor and the G protein, additional steps were implemented to ensure the isolation of a stable RG complex.
2.4.2. Cross-linking and Immunoblotting

Aliquots of purified G protein sample were mixed with different concentrations of bis(sulfosuccinimidyl) suberate (BS\(^3\)) (Thermo Scientific) and incubated for 30 min at room temperature. The cross-linking reaction was terminated by the addition of Tris-HCl to a final concentration of 20 mM. The samples were heated subsequently at 65 °C for 5 min before being applied to a 10% polyacrylamide gel (Bio-Rad). Electrophoresis was performed as described in reference 107. The transferred proteins on a nitrocellulose membrane were incubated with anti-G\(\alpha\) (Chemicon) and Horseradish Peroxidase (HRP)-conjugated anti-mouse IgG from sheep (GE Healthcare) as primary and secondary antibodies at dilutions of 1:1000 and 1:3000, respectively. A control consisting of the non-cross-linked sample and a molecular weight ladder (Frogbio) were run in parallel. Cholate-solubilised S/9 membrane from uninfected cells was included as a negative control.

2.4.3. \([^{35}S]\)GTP\(\gamma\)S Binding Assays

Various G protein-containing samples were mixed with graded concentrations of \([^{35}S]\)GTP\(\gamma\)S (PerkinElmer). To achieve the desired concentrations of the nucleotide, the specific activity of radiolabelled GTP\(\gamma\)S was reduced to approximately 20 mCi/mmol by addition of unlabelled GTP\(\gamma\)S (Sigma-Aldrich). Non-specific binding was taken as total binding in the presence of 0.1 mM unlabeled GTP\(\gamma\)S (Sigma-Aldrich). The reaction mixture was incubated at 30 °C for 2.5 h. Free and bound radioligand were separated by chromatography on Sephadex G-50. All measurements were made in triplicate, and the values were averaged to obtain the means used in subsequent analyses. Further details regarding the assays have been described elsewhere (e.g., references 25,108).

The data were analysed according to Equation 2-10, in which \(B_{\text{max}}\) is the total amount of G protein in \(m\) classes of sites, \(B_{\text{sp}}\) is the specific binding of radiolabelled GTP\(\gamma\)S at the total concentration \([P]_t\), and \(F_j\), \(K_j\), and \(n_{H(j)}\) represent the fraction of sites of type \(j\), the affinity of \([^{35}S]\)GTP\(\gamma\)S for the sites of type \(j\), and the corresponding Hill coefficient, respectively.

\[
B_{\text{obsd}} = B_{\text{max}} \sum_{j=1}^{m} \frac{F_j([P]_t-B_{\text{sp}})^{n_{H(j)}}}{K_j^{n_{H(j)}} + ([P]_t-B_{\text{sp}})^{n_{H(j)}}} + NS([P]_t - B_{\text{sp}}) \quad \text{Equation 2-10}
\]
2.4.4. Step-wise Photobleaching

The cycle of fluorescence excitation and emission is finite. When a fluorophore stops absorbing and emitting fluorescence, it is said to have been photobleached. In a molecule containing multiple fluorophores, the intensity of emission is proportional to the number of fluorophores present, and the decrease in intensity through photobleaching can be measured. Using a sensitive detector, the number of step-wise drops in the emitted intensity can serve as a measure of oligomeric size. Therefore, the size of a fluorescent multimer can be inferred from an analysis of the intensity-time trajectory obtained for a single molecule. In this project, step-wise photobleaching analysis has been used to quantify eGFP-labelled G protein oligomers.

2.4.4.1. Data Acquisition and Analysis

Purified eGFP-tagged G proteins (His$_6$-GFP-$\alpha_i$, GFP-$\alpha_i\beta_i$-His$_6\beta_2$, and the co-purified complex of GFP-$\alpha_i\beta_i$-His$_6\beta_2$ and M$_2$ muscarinic receptor) and multimers of eGFP fused end to end were immobilised on coverslips. The surface of the coverslip was coated with a mixture of purchased 95% polyethylene glycol (PEG) and 5% PEG-biotin. The coated surface subsequently was treated with streptavidin and a biotinylated antibody that specifically binds to hexahistidyl sequence and thus anchors the His$_6$-tagged G protein samples for measurement. In some cases, G protein samples were incubated with various compounds including GDP and AlF$_4^-$, GTP$_\gamma$S, DTT, and mastoparan for 20 min prior to immobilisation. The immobilised sample was excited at 473 nm using a diode-pumped solid state laser (Cobolt Blue, MarketTech). During the course of the experiments, various levels of laser power were employed to irradiate the fusion proteins of eGFP: 60 W/cm$^2$, 180 W/cm$^2$, and 260 W/cm$^2$; eGFP-labelled G proteins were irradiated at a power of 200 W/cm$^2$. In all cases, the laser beam was focused on an observation area of 3600 $\mu$m$^2$ and an oil-immersion objective (60×, NA = 1.45, Olympus) was employed. Movies of the selected region on the coverslip were recorded for 15 to 45 s by an electron-multiplying charged-coupled device (EMCCD) camera (DU-897BV, Andor), which yields 500 images at an exposure time of 30 ms per image.

Time trajectories of the fluorescence intensities of single molecules or particles were extracted from the movies and analysed using a custom-built program in MATLAB. An intensity-time trajectory of a set of flatterned images was filtered using a modified version of the Chung-Kennedy filtration algorithm. The program determines the occurrence of a photobleaching step.
based on a comparison of the intensity drop with the running average. For each molecule, the maximum number of photobleaching steps was recorded and the results from a large number of molecules (400–3000) were summarised in a histogram.
CHAPTER 3 RESULTS
3.1. Cross-linking and Immunoblotting of Purified $\text{G}_\alpha_1$

His$_6$-tagged $\text{G}_\alpha_1$ was expressed in Sf9 cells and purified as described in Chapter 2. Aliquots of the purified material were applied to the polyacrylamide gel and subjected to electrophoresis. The contents of the gel then were transferred to a nitrocellulose membrane and blotted with a $\text{G}_\alpha$-specific antibody (Figure 3-1). A single band corresponding to monomeric $\text{G}_\alpha$ migrated with a molecular mass of roughly 45 kDa (lane 2). Samples cross-linked with bis(sulfo succinimidyl) suberate (BS$_3$) (lanes 3 and 4) showed the appearance of an additional band with a molecular mass greater than 245 kDa and a concomitant reduction in the density of the monomeric band. In lanes 3 to 4, as the applied concentration of the cross-linker was increased from 2 mM to 4 mM, the intensity of the oligomeric band increased and the monomeric band disappeared (lane 4). The samples were assayed in parallel with the solubilised extract from non-infected Sf9 membranes for detection of non-specific interactions with the antibody. None was observed on the blot (lane 1).

3.2. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ Binding Assays on G Protein Variants

The functionality of the purified G proteins was evaluated in $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assays in the absence and presence of activated M$_2$ receptors. The binding curves obtained for His$_6$-tagged wild-type $\text{G}_\alpha_1$ (His$_6$-G$\alpha$) and for the fluorophore-tagged analogue (i.e., His$_6$-XFP-G$\alpha$, where XFP represents eGFP or mCherry) are compared in Figure 3-2A. The estimated binding affinities for His$_6$-G$\alpha$ and His$_6$-XFP-G$\alpha$ are 2.19 $\mu$M and 0.50 $\mu$M, respectively. It therefore appears that the insertion of a fluorophore at position 91 of $\text{G}_\alpha_1$ did not compromise its ability to bind nucleotide; rather, the affinity was increased about fourfold. However, the presence of G$\beta\gamma$ had little if any effect on the affinity of the nucleotide for XFP-G$\alpha$ (Figure 3-2B) as the fitted values are 0.50 $\mu$M and 0.98 $\mu$M for His$_6$-XFP-G$\alpha$ and XFP-G$\alpha$$\beta$-His$_6$$\gamma$, respectively. In the case of holo-G proteins, the hexahistidyl tag was placed on the $\gamma$-subunit to ensure that all $\alpha$-subunits in the purified preparation were associated with a $\beta\gamma$-heterodimer.

On the other hand, profiles for the binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ to the stable complex of fluorophore-labelled G protein and activated M$_2$ muscarinic receptor (M2G) displayed a leftward shift of approximately two orders of magnitude compared to that of XFP-G$\alpha$$\beta$-His$_6$$\gamma$ (Figure 3-2C). As a result, M2G samples no longer exhibit the comparatively low, micro-molar affinity for
the nucleotide. The appearance of high–affinity binding and the concomitant loss of low–affinity binding indicate that all of the G proteins are associated with receptors. This observation also suggests that the insertion of a fluorophore at position 91 does not perturb the normal functioning of the G protein (i.e., coupling to an activated receptor). Therefore, data acquired from fluorophore-tagged G proteins are reflective of interactions involving G proteins in native preparations.

Hill coefficients for the binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) to the M2G complex were markedly lower than 1 \((n_{H1} = 0.66 \pm 0.03, N = 5)\) (Equation 2-10, \(m = 1\)). Whereas one component is sufficient to describe binding to His6-G\(\alpha_i\) and His6-XFP-G\(\alpha_i\) in terms of Equation 2-10 \((n_{H(j)} = 1\) for all \(j\)) \((p \geq 0.03)\), two components are required for M2G \((p < 0.0005)\). For both the G protein alone and the M2G complex, the fitted values of \(K_j\) showed little variability among different experiments (Table 3-1). In the case of M2G, however, the value of \(F_j\) varied from 0.2 to 0.95. The pooled results from M2G therefore were analysed with single values of \(K_1\) and \(K_2\) as the constrains on \(K_j\) were without appreciable effect on the sum of squares \((p = 0.62)\), but separate values of \(F_2\) for the data from different experiments. The fitted parametric values are listed in Table 3-1.

In conclusion, the binding profiles obtained with \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) indicate that the insertion of the fluorophore in G\(\alpha_i\) did not impair the ability of the G protein to bind guanyl nucleotide or to couple to the activated M\(_2\) receptor. Moreover, the heterogeneity observed in the binding curves of M2G demonstrates that the nucleotide binds to receptor-coupled G proteins with different affinities, possibly indicating the existence of oligomers. To further investigate this issue, the fluorophore-tagged G proteins were used as markers to monitor their oligomeric status in fluorescence-based experiments. Live CHO cells were transfected with the plasmids for eGFP- and mCherry-tagged G proteins along with other necessary constructs. Individual cells co-expressing both fluorophore-tagged proteins were selected for further analysis.

### 3.3. Detection of Co-diffusing G Proteins in CHO Cells by DeFCS

Fluorescence correlation spectroscopy (FCS) monitors the diffusion of fluorescent molecules through a focal volume defined by the excitation laser. The simultaneous measurement of two fluorophores allows one to monitor the co-diffusion of two spectrally compatible fluorescent molecules through the focal volume. The fraction of molecules that co-
diffuse is estimated from the amplitude of the cross-correlation curve relative to that of each individual auto-correlation curve. A large fraction implies the existence of oligomers that are at least dimeric in size.

Measurements of the co-expressed eGFP- and mCherry-tagged G proteins revealed that approximately 41% of the entire population of eGFP-G\(_{i1}\) and 39% of the entire population of mCh-G\(_{i1}\) co-diffused with the other colour (Figure 3-3A). Co-expression of a membrane-localised form of eGFP (MP-GFP) and mCh-G\(\alpha_1\) resulted in little or no cross-correlation (Figure 3-3C) and the pair served as a negative control. When mCh-G\(\alpha_1\) was co-expressed with eGFP-tagged G\(\beta\) (GFP-\(\beta\)), virtually all of the mCh-G\(\alpha_1\) co-diffused with GFP-\(\beta\) (Figure 3-3B), indicating that the heterotrimeric G protein is stable at rest. The pair was used as a positive control.

3.4. Model-based FRET Efficiency Analysis for Size Estimation of G Protein Oligomers

Spectral unmixing of the observed emission spectrum yields the emission components of the donor (\(k_D\)) and the acceptor (\(k_A'\)) (e.g., Figure 3-4A). Those values are used to calculate the overall apparent FRET efficiency (\(E_{\text{app}}\)) for a selected cell. Apparent FRET efficiencies obtained in this way are plotted against the ratios of acceptor to donor (\([A]/[D]\)) in Figure 3-4B–D. FRET between eGFP-G\(\alpha_1\) and mCh-G\(\alpha_1\) in the presence of G\(\beta_1\)\(\gamma_2\) was monitored in the absence (GG) and in the presence of M\(_2\) receptors (RGG). The latter sample (i.e., RGG) also was monitored after the addition of a muscarinic agonist carbachol (RGGag). In all three cases, values of \(E_{\text{app}}\) up to about 60% were observed between the eGFP- and mCherry-tagged G proteins and appeared to reach to a plateau when the value of \([A]/[D]\) exceeded 1.

To examine whether the observed FRET arose in a stochastic manner, \(E_{\text{app}}\) was measured in cells co-expressing mCh-G\(\alpha_1\) and a membrane-localised donor (MP-GFP). As the fused sequence added to eGFP includes the myristoylation and palmitoylation sites of G\(\alpha_1\), MP-GFP ought to localise at the membrane. There was little or no FRET between MP-GFP and mCh-G\(\alpha_1\), as indicated by FRET efficiencies near or indistinguishable from zero (Figure 3-5, black empty squares), indicating an absence of stochastic interactions. Furthermore, to test the effect of the level of expression on the distribution of apparent FRET efficiencies over the range of \([A]/[D]\), the data for GG, RGG, and RG\(_{\text{Gag}}\) were equally divided into four groups based on the total expression levels of donor and acceptor. Cells with varying expression levels were
distributed throughout the measured ranges of $[A]_T/[D]_T$ and $E_{\text{app}}$. To sum up, the low non-specific FRET between the non-interacting pair of MP-GFP and mCh-Gα1 and the independence of $E_{\text{app}}$ on the expression level indicate that the observed FRET between fluorophore-tagged G proteins was not a result of stochastic interactions between over-expressed donors and acceptors.

In Figure 3-6, the plots of apparent FRET efficiency *versus* $[A]_T/[D]_T$ were fitted to Equation 2-9 to extract values of the pair-wise efficiency ($E$) for oligomers of various sizes. As summarized in Table 3-2, expressed as percentage, the estimated values of $E$ for GFP-Gα1 and mCh-Gα1 alone (GG) are $151 \pm 2$ ($n = 2$, dimer), $90.8 \pm 1.0$ ($n = 3$, trimer), $67.0 \pm 0.8$ ($n = 4$, tetramer), $44.9 \pm 0.6$ ($n = 6$, hexamer), and $34.1 \pm 0.5$ ($n = 8$, octamer). The values of $E$ for GFP-Gα1 and mCh-Gα1 in the presence of receptor (RGG) are $133 \pm 2$ (dimer), $78.0 \pm 1.2$ (trimer), $56.7 \pm 0.9$ (tetramer), $37.3 \pm 0.7$ (hexamer), and $28.0 \pm 0.6$ (octamer). Upon the addition of carbachol (RGGag), the values are $133 \pm 1$ (dimer), $76.8 \pm 0.9$ (trimer), $55.3 \pm 0.7$ (tetramer), $36.0 \pm 0.5$ (hexamer), and $26.8 \pm 0.4$ (octamer).

Dimeric fits to all three sets of data resulted in values of $E$ above 100%. The result suggests that a dimer of G proteins is unlikely. We also determined the range of $E$ within which the fit of a dimeric model did not significantly differ from the best-fit curve. For each of the three conditions (*i.e.*, GG, RGG and RGGag), a fixed value of $E$ was varied in increments of 1% as illustrated in Figure 3-7, and the sum of squares from each fit was compared with that of the best fit using an $F$-test. In this way, the lowest value of $E$ that was not significantly different from the best fit was determined. In each case, even the lowest possible value $E$ is above 100% and a dimer therefore can be rejected definitely. The relationship between $p$ and $E$ is illustrated in Figure 3-7, where the dashed horizontal line corresponds to $p = 5 \times 10^{-4}$. Fits with $E$ above the line are not significantly different from the best fit (*i.e.*, $p > 5 \times 10^{-4}$); fits with $E$ below the line (*i.e.*, $p < 5 \times 10^{-4}$) deviate significantly from the best fit. The lowest values of the determined $E$ that do not compromise the goodness of the fit are as follows: 146%, GG; 127%, RGG; and 129%, RGGag. These results suggest that an oligomer of G proteins is at least tetrameric.

In conclusion, FRET observed between eGFP- and mCherry-tagged G proteins implies the existence of G protein oligomers. Moreover, the dependence of $E_{\text{app}}$ on the ratio of total acceptors to total donors ($[A]_T/[D]_T$) points to a tetramer or larger oligomer, based on pair-wise FRET efficiencies estimated in terms of a model for FRET between neighbouring fluorophores. Neither the addition of M2 receptor nor its activation by the muscarinic agonist carbachol had a
substantial effect on the pair-wise FRET efficiency. The latter observation may be due to the nature of the measurement, in that each cell yields a single averaged value of $E_{\text{app}}$. To determine the exact size of the G protein oligomers and to monitor their status upon stimulation, photobleaching analysis and total internal reflection fluorescence microscope (TIRFM) were used to directly count the number of fluorescent protomers in single molecules of eGFP-tagged G protein.

3.5. Direct Quantification of the Size of the G Protein Oligomer by Photobleaching Analysis

eGFP-$G\alpha_{i1}$, $G\beta_1$, and His$_6$-$G\gamma_2$ were expressed in Sf9 cells in the absence and presence of the $M_2$ muscarinic receptor. The co-expressed samples were subsequently extracted and purified on Ni$_{2+}$-NTA columns as described in Chapter 2. The purified eGFP-labelled G proteins were immobilised on a polyethylene glycol (PEG)-coated surface using an anti-histidine antibody. The immobilised eGFP-containing samples were excited and subsequently photobleached at 473 nm. The custom-built TIRF microscopy used in this study ensures sub-millisecond detection and therefore was sufficiently rapid to capture variations in intensity. A sample intensity-time trajectory is shown in Figure 3-8A, where each permanent decrease in intensity as a result of photobleaching is regarded as one step. The size of a fluorescent complex can be inferred from the maximum number of observed photobleaching steps.

The photobleaching patterns of concatenated eGFP molecules comprising one, two, and four units of the fluorophore were monitored under conditions similar to those used for purified G proteins. Each fusion protein gave rise to distributions of photobleaching steps such that the largest number of steps did not exceed the number of fluorophores (Figure 3-8B–D). For instance, irradiation of tetrameric eGFP resulted 1–4 steps, but the number of steps never exceeded 4 (Figure 3-8D). Moreover, the distribution of steps was observed to vary with the laser power (Figure 3-8D). The relative number of molecules that displayed four photobleaching steps increased as the laser power increased and decreased as the laser power decreased. In Figure 3-8D, a comparison of the distributions obtained at a medium power of 180 W/cm$^2$ (grey bars) and a higher power of 260 W/cm$^2$ (white bars) shows a rightward shift in the distribution with an increase in the power, and hence a higher occurrence of four steps. A comparison of the distributions obtained at a medium power of 180 W/cm$^2$ and a lower power at 60 W/cm$^2$ (black
bars) shows a leftward shift in the distribution with a decrease in the power, and hence a lower occurrence of four steps.

In the measurements of eGFP-tagged G proteins, a laser power of 200 W/cm² was employed. The reason for choosing such a medium level of power is two-fold: one, high laser powers are associated with rapid photobleaching thus a smaller sampling pool; two, low laser powers are not capable of exciting all fluorophores in a molecule, thus there would be an underestimation of the oligomeric size. Therefore, experiments of this nature involve a trade-off between more molecules to survey and a more accurate estimate of the number of photobleaching steps.

In Figure 3-9A, distributions of 1 through 6 steps were observed when eGFP-labelled Gα1β-His6-γ (grey bars) and the heterotrimer co-purified with the M₂ receptor (black bars) were photobleached. In the presence of M₂ receptor, the occurrence of the molecules that displayed five and six steps was markedly reduced. Similarly, addition of the reducing agent dithiothreitol (DTT) also reduced somewhat the occurrence of the fifth and sixth steps (white bars) (Figure 3-9B).

The effects of ligands on the G protein oligomeric status are shown in Figure 3-10. In the absence of the M₂ receptor, photobleaching of the G protein heterotrimer resulted in a step distribution that was essentially unchanged upon the addition of GDP plus AlF₄⁻ (Figure 3-10A, white bars). In contrast, the addition of an activated GPCR analog, mastoparan, markedly reduced the prevalence of particles with a higher number of steps (4–6) and led to a distribution that was populated predominantly by particles with 1 and 2 steps (black bars). With the co-purified complex of M₂ receptor and eGFP-tagged G protein heterotrimer (Figure 3-10B), the addition of GTPγS (black bar) or GDP plus AlF₄⁻ (white bars) reduced substantially the number of particles exhibiting a higher number of photobleaching steps (4–6) while increasing the frequency of those with fewer steps (1–3). The above observations suggest that the G protein oligomers are largely unaffected by either the agonist-ligated M₂ receptor or guanyl nucleotides when added separately but dissociate into dimers or monomers when an agonist, the receptor and the nucleotide are added together.
Figure 3-1: Effects of cross-linking on purified His$_6$-G$\alpha_i$.

Sf9 cells expressing His$_6$-G$\alpha_i$ were extracted in sodium cholate and purified on a Ni$^{2+}$-NTA column as described in ‘Materials and Methods’. Aliquots of approximately 10 nM purified His$_6$-G$\alpha_i$ were loaded in lanes 2–4 of the polyacrylamide gel. Samples in lanes 3 and 4 were pre-incubated with the cross-linker BS$_3$ at concentrations of 2 mM and 4 mM, respectively. Solubilised membrane from uninfected Sf9 cells was loaded in lane 1 as a negative control. After electrophoresis, the samples were transferred to a nitrocellulose membrane and blotted with anti-G$\alpha_i$ antibody. The concentration of G proteins was estimated from the binding of $[^{35}$S]GTP$_\gamma$S.
Figure 3-2: Binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ to $\alpha_{i1}$ and holo-G proteins purified with and without M2 receptor.

The binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ to the $\alpha_{i1}$-subunit was measured in preparations purified from Sf9 cells expressing $\alpha_{i1}$ (A), $\alpha_{i1}$ plus $\beta_1\gamma_2$ (B, C) and $\alpha_{i1}\beta_1\gamma_2$ plus M2 receptor (M2) (C). The different symbols and lines denote the different forms of $\alpha_{i1}$ as follows: His6-$\alpha_{i1}$ (half-filled symbols and dashed line), His6-XFP-$\alpha_{i1}$ (empty symbols and solid line), XFP-$\alpha_{i1}\beta_1\text{His6-}\gamma_2$ (filled symbols and dotted line), and M2XFP-$\alpha_{i1}\beta_1\text{His6-}\gamma_2$ (crossed or dotted symbols and solid lines). XFP here denotes both eGFP and mCherry. All of the data from panels A, B, and C are re-plotted for comparison in panel D. The standard error on triplicate determinations typically was less than 4% of the mean. Estimates of total binding were analysed according to Equation 2-10 ($m = 1$ or $2$) to obtain the fitted curves shown in the figure and the parametric values listed in Table 3-1. Further details are described in Table 3-1.
Table 3-1: Parametric values for the binding of [$^{35}$S]GTP\γS to G\αi1 purified alone and as holo-G\i1 with and without M2 receptor

<table>
<thead>
<tr>
<th>Sample</th>
<th>m</th>
<th>n_{H(i)}</th>
<th>log K₁</th>
<th>log K₂</th>
<th>F₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>His₆-G\α₁ (3)</td>
<td>1</td>
<td>1</td>
<td>−5.66 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>His₆-XFP-G\α₁ (5)</td>
<td>1</td>
<td>1</td>
<td>−6.30 ± 0.11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>XFP-G\α₁G\β₁His₆-G\γ₂(5)</td>
<td>1</td>
<td>&lt; 1</td>
<td>−6.01 ± 0.07</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>M₂XFP-G\α₁G\β₁His₆-G\γ₂(5)</td>
<td>2</td>
<td>1</td>
<td>−7.13 ± 0.04</td>
<td>−9.03 ± 0.04</td>
<td>0.20 ± 0.05</td>
</tr>
</tbody>
</table>

\(^a\)G\αi1 with or without a fluorophore (XFP, *i.e.*, eGFP or mCherry) was expressed in Sf9 cells, either alone or in combinations with G\β₁, G\γ₂, and the M₂ receptor (M₂) as indicated in the table. The G\αi1-subunit or subunit-containing complex was purified by means of a hexahistidyl tag fused to the N-terminus of G\α₁ (His₆-G\α₁), XFP-G\α₁ (His₆-XFP-G\α₁) or G\γ₂ (His₆-G\γ₂). The binding of [$^{35}$S]GTP\γS was measured at graded concentrations of the guanyl nucleotide after incubation for 2.5 h at 30 °C. The data from 3–5 experiments on each sample were pooled and analysed in concert according to Equation 2-10 (m = 1 or 2). Single values of Kᵢ were common to all of the data in each analysis (m = 1 or 2). In the case of M₂XFP-G\αi₁G\β₁His₆-G\γ₂ (m = 2), a separate value of Fᵢ was assigned to the data from each experiment. The number of experiments is shown in parentheses. The value of n_{H(i)} was indistinguishable from 1 throughout (p ≥ 0.03) and was fixed accordingly, except in the case of XFP-G\αi₁G\β₁His₆-G\γ₂. The data and fitted curves are illustrated in Figure 3-2.

\(^b\)The fitted value of the Hill coefficient is 0.81 ± 0.03.
Figure 3-3: Identification of G protein oligomers by dcFCS.

CHO cells co-expressing eGFP-\(\alpha_1\), mCh-G\(\alpha_1\), and G\(\beta_1\gamma_2\) were irradiated at 473 nm and 561 nm on a confocal microscope. The emission from eGFP and mCherry was collected over time at 500–540 nm and 600–640 nm, respectively. The correlation function \(G(\tau)\) was calculated according to Equations 2-1 and 2-2, and the values were fitted according to the anomalous diffusion model (Equation 2-3). Auto-correlation data and the corresponding fits are shown in green and red (symbols and lines) for the eGFP (squares) and mCherry (circles)-tagged proteins, respectively. Cross-correlation data and fits are represented by the blue triangles and corresponding lines. In the case of co-expressed mCh-G\(\alpha_1\), eGFP-G\(\alpha_1\), and G\(\beta_1\gamma_2\) (panel A), 41\% of eGFP-G\(\alpha_1\) and 39\% of mCh-G\(\alpha_1\) co-diffused as hetero-oligomers. In the case of mCh-G\(\alpha_1\) and eGFP-G\(\beta_2\) (panel B), which served as a positive control, virtually all of the less-abundant species co-diffused with the other. In contrast, there was essentially no co-diffusion of membrane-anchored eGFP (MP-GFP) and mCh-G\(\alpha_1\) (panel C), which served as a negative control.
Figure 3-4: Identification of G protein oligomers by FRET.

CHO cells co-expressing eGFP-\(\alpha_i\), mCh-\(\alpha_i\), and \(\beta\gamma_2\) in the absence and presence of \(M_2\) receptor and agonist were irradiated at 488 nm on a confocal microscope. A typical cropped image of a cell co-expressing GFP-\(\alpha_i\), mCh-\(\alpha_i\), and G\(\beta\gamma_2\) is shown in the inset to panel A where the white dotted line encircles the analysed cell. The emission was measured at a resolution of 5 nm over 30 consecutive channels from 493 to 643 nm to obtain a composite spectrum (panel A, squares), which was unmixed by linear regression to obtain the individual contributions of donor (green dashed line) and acceptor (red dotted line). The fitted spectrum is illustrated in the figure (panel A, solid blue line), and the corresponding parameters were used to calculate the apparent FRET efficiency \(E_{\text{app}}\) according to Equation 2-7. The ratio of total acceptor to total donor \([A]_T/[D]_T\) was calculated from the spectral properties of each fluorophore as described in Chapter 2. The values of \(E_{\text{app}}\) and \([A]_T/[D]_T\) were also calculated for cells co-expressing eGFP-\(\alpha_i\), mCh-\(\alpha_i\), and G\(\beta\gamma_2\) (panel B) or eGFP-\(\alpha_i\), mCh-\(\alpha_i\), G\(\beta\gamma_2\), and \(M_2\) receptor in the absence of agonist (panel C) and in the presence of carbachol (10 mM) (panel D).
Figure 3-5: Absence of stochastic FRET.

The data in panels B–D of Figure 3-4 are re-plotted in panels A–C above (A, eGFP-G\(_{\alpha_1}\), mCh-G\(_{\alpha_1}\), and G\(_{\beta_1}\)\(_{\gamma_2}\); B, eGFP-G\(_{\alpha_1}\), mCh-G\(_{\alpha_1}\), G\(_{\beta_1}\)\(_{\gamma_2}\), and M\(_2\) receptor; C, eGFP-G\(_{\alpha_1}\), mCh-G\(_{\alpha_1}\), G\(_{\beta_1}\)\(_{\gamma_2}\), and M\(_2\) receptor plus 10 mM carbachol). Different symbols are colour-coded according to the level of total expression of acceptor and donor. All of the cells are divided equally into four groups as follows, from lowest to highest expression: black circles, green squares, blue upright triangles, and red inverted triangles. Also shown in each panel are data from cells co-expressing MP-GFP and mCh-G\(_{\alpha_1}\) (black squares).
Figure 3-6: Model-base estimation of oligomeric size.

The data are re-plotted from Figure 3-5, and the lines represent the best fits of Equation 2-9 to the values obtained from cells co-expressing eGFP-Gαi1 and mCh-Gαi1 (A, eGFP-Gαi1, mCh-Gαi1, and Gβ1γ2; B, eGFP-Gαi1, mCh-Gαi1, Gβ1γ2, and M2 receptor; C, eGFP-Gαi1, mCh-Gαi1, Gβ1γ2, and M2 receptor plus 10 mM carbachol). Fitted estimates of the pair-wise efficiency ($E$) for oligomers of different size are indicated in parentheses as indicated in Table 3-2, expressed as percentages ($n = 2, 3, 4, 6, 8$).
Table 3-2: Values of the pairwise FRET efficiency for GFP- and mCh-tagged G proteins computed for oligomers of different sizes

<table>
<thead>
<tr>
<th>$n$</th>
<th>eGFP- and mCh-G$\alpha\beta\gamma$</th>
<th>$M_2 +$ eGFP- and mCh-G$\alpha\beta\gamma$</th>
<th>carbachol + $M_2 +$ eGFP- and mCh-G$\alpha\beta\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>151 ± 2</td>
<td>133 ± 2</td>
<td>133 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>90.8 ± 1.0</td>
<td>78.0 ± 1.2</td>
<td>76.8 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>67.2 ± 0.8</td>
<td>56.7 ± 0.9</td>
<td>55.3 ± 0.7</td>
</tr>
<tr>
<td>6</td>
<td>44.9 ± 0.7</td>
<td>37.3 ± 0.7</td>
<td>36.0 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>34.1 ± 0.5</td>
<td>28.0 ± 0.6</td>
<td>26.8 ± 0.4</td>
</tr>
</tbody>
</table>

$G\alpha_1$ tagged with eGFP or mCherry was co-transfected in CHO cells in the absence and presence of the $M_2$ muscarinic receptor ($M_2$). Receptor-containing cells subsequently were treated with the agonist carbachol (10 mM). The data from at least 250 cells were analysed in terms of Equation 2-9 to obtain the values of the pair-wise FRET efficiency for different values of $n$. The data and fitted curves are illustrated in Figures 3-4 and 3-5.
Figure 3-7: Rejection of dimeric G proteins.

The data shown in Figure 3-6 were compared with the predictions of Equation 2-9 (n = 2) with the pair-wise efficiency (E) fixed at different values between 1.40 and 1.50 (○, eGFP-Gα1, mCh-Gα1, and Gβ1γ2) or between 1.22 and 1.32 (□, eGFP-Gα1, mCh-Gα1, Gβ1γ2, and M2 receptor; Δ, eGFP-Gα1, mCh-Gα1, Gβ1γ2, and M2 receptor plus 10 mM carbachol). The sum of squares at each fixed value of E was compared with that from the fit at the optimal value, and the level of significance of the difference (p) was calculated from the F-statistic. The values of log p are plotted on the y-axis, and the horizontal dashed line corresponds to p = 0.0005. The lowest values of E consistent with the data at p = 0.0005 are as follows: ○, 146%; □, 127%; and Δ, 129%.
Purified eGFP and fusion proteins comprising 1, 2, and 4 units of the fluorophore were immobilised and irradiated at 473 nm on a TIRF microscope as described in ‘Materials and Methods’. The intensity-time trajectory of single particles was monitored and an example obtained with a dimeric GFP is shown in panel A. The number of photobleaching steps was determined for each particle and the distributions obtained at a laser power of 180 W/cm$^2$ are shown in grey (B, monomeric eGFP; C, dimeric eGFP; D, tetrameric eGFP). In the case of tetrameric eGFP, the photobleaching pattern and distribution of steps also was determined after irradiation at 60 W/cm$^2$ (black bars) and 260 W/cm$^2$ (white bars).
Figure 3-9: Effects of the M₂ receptor and DTT on the photobleaching pattern of heterotrimeric G₁₁.

eGFP-labelled Gα₁β₂γ₁ was expressed in Sf9 cells either alone (grey bars) or together with M₂ receptor (black bars). The heterotrimeric G protein or receptor-G protein complex was purified by means of a hexahistidyl tag fused at the N-terminus of the γ-subunit. The purified materials were immobilised and the photobleaching patterns of single particles were recorded to obtain the data plotted in panel A. The photobleaching pattern of purified eGFP-Gα₁β₂γ₁ also was recorded in the presence of 10 mM DTT to obtain the data (white bars) plotted together with the samples without DTT (grey bars) in panel B.
Figure 3-10: Effects of ligands on the photobleaching pattern of heterotrimeric G\textsubscript{i1}.

Panel A. eGFP-labelled G\(\alpha_1\beta_1\gamma_2\) bearing a hexahistidyl tag at the N-terminus of the \(\gamma\)-subunit (i.e., eGFP-G\(\alpha_1\beta_1\)His\textsubscript{6}-\(\gamma_2\)) was purified from \textit{Sf9} cells. The photobleaching patterns of single particles were recorded for the G protein alone (grey bars) and in the presence of 10 \(\mu\)M mastoparan (black bars) or 10 \(\mu\)M GDP plus 10\(\mu\)M AlF\textsubscript{4}\(^{-}\) (white bars) to obtain the shown distributions. Panel B. A complex of the M\textsubscript{2} receptor and eGFP-G\(\alpha_1\beta_1\)His\textsubscript{6}-\(\gamma_2\) was purified from \textit{Sf9} cells; the photobleaching patterns of single particles were recorded for the complex alone (grey bars) and in the presence of 10 \(\mu\)M GTP\gamma S (black bars) or 10 \(\mu\)M GDP plus 10 \(\mu\)M AlF\textsubscript{4}\(^{-}\) (white bars) to obtain the shown distributions. Purification of the receptor-G protein complex, its immobilisation on biotin-tagged PEG and photobleaching all were carried out in the presence of 10 mM carbachol.
CHAPTER 4 GENERAL DISCUSSION
4.1. Insights from the Thesis

A dispersion of affinities is observed in the binding of agonists to GPCRs and the breadth of that dispersion is reduced upon the addition of guanyl nucleotides. Different models have been used to account for this phenomenon, one of which attributes the implied heterogeneity to cooperative interactions between neighboring sites in an oligomeric array of GPCRs. When applied quantitatively, the cooperativity model has been shown to provide a good description of the binding properties of the M₂ receptor in various preparations if there are at least four interacting sites, presumably within a tetramer\textsuperscript{108,135,188}. In agreement with the model-based predictions of binding assays, oligomers of various GPCRs have been detected in recent studies involving biophysical techniques (Chapter 1).

Whereas the guanyl nucleotide-sensitive heterogeneity seen in the binding of agonists to GPCRs is a well-known phenomenon, it is mirrored by a less-known, agonist-sensitive heterogeneity in the binding of GDP to receptor-coupled G proteins\textsuperscript{25,79,175}. For example, the binding of guanyl nucleotides to sites labelled by [\textsuperscript{35}S]GTP\textsubscript{γ}S in myocardial membranes has revealed a heterogeneity indicative of multiple classes of sites; in the case of GDP, the addition of a muscarinic agonist induced a rightward shift in the binding curve that appeared as an interconversion of G proteins from a state of high affinity to a state of low affinity. The reciprocal nature of these effects suggests that essentially the same mechanism underlies the effect of guanyl nucleotides on agonists and that of agonists on GDP. Furthermore, some GPCRs have been co-purified with equimolar quantities of their attendant Gα subunits (\textit{e.g.}, the M₂ receptor purified from porcine atria\textsuperscript{23,107} and the μ-opioid receptor from rat brain\textsuperscript{23,107}). Such observations suggest that the functional unit of GPCR-mediated signalling is an oligomer comprising multiple copies of receptor and G protein at an overall ratio of 1. Ratios of receptors to G protein of 2:1 and 4:1 also have been reported in the case of rhodopsin when reconstituted into phospholipid vesicles\textsuperscript{100,182}. A tetrameric M₂ receptor therefore may be associated with a monomeric, dimeric, or tetrameric G protein.

In contrast to the much-studied oligomeric GPCRs, the oligomeric status of G proteins has been largely ignored with the exception of a few early biochemical studies. Hydrodynamic studies performed more than two decades ago identified multimers of various types of G protein in isolated native tissues\textsuperscript{83,121,122}. Such observations led to the proposal of the ‘Disaggregation Theory of Hormone Action’\textsuperscript{149}, which offered an alternative to more commonly acknowledged
schemes based on the ternary complex model\textsuperscript{56}. The disaggregation theory hypothesises the existence of two populations of G protein during the signalling cycle. Upon the binding of hormones to GPCRs, multimeric G proteins disaggregate into GTP-bound monomers that subsequently induce the activities of downstream effectors.

Oligomeric $G\alpha_o$ has been detected on western blots of non-denaturing polyacrylamide gels loaded with purified G protein subjected previously to cross-linking\textsuperscript{193,196}. In contrast, no cross-linked band was observed in a sample that was pre-incubated with GTP\textgamma\textupsilon S\textsuperscript{193}. In agreement with those results, which are consistent with the disaggregation theory, the current study found that affinity-purified $G\alpha_{i1}$ appeared as oligomers when resolved on polyacrylamide gels after cross-linking with BS\textsuperscript{3} (Figure 3-1). In the current project, we also have examined fluorescent G proteins for their oligomeric status in live CHO cells and at the level of single molecules after solubilisation and purification from S/9 cells. Oligomers were detected under the above conditions, and their disaggregation dynamics were tracked upon the addition of M\textsubscript{2} muscarinic receptor, an additional reducing agent, and various ligands.

4.1.1. Identification of G Protein Oligomers

A comparison of the binding curves obtained with $G\alpha_{i1}$ purified alone, with $G\beta\gamma$, and with $G\beta\gamma$ plus M\textsubscript{2} receptor (M2G) showed that receptor-coupled G proteins are of higher affinity for [$^{35}$S]GTP\textgamma\textupsilon S (Figure 3-2). Moreover, the lower affinities associated with receptor-free G proteins were not observed in samples of M2G; therefore, all of the G protein in those samples was coupled to receptor.

In addition, heterogeneity was observed in [$^{35}$S]GTP\textgamma\textupsilon S binding assays on M2G. The difference between the estimated affinities is about 80-fold ($K_1/K_2$). Because the S/9 cells were infected with baculovirus coding for only one type of G protein, and because no endogenous G protein was detected on western blots of material from uninfected cells (Figure 3-1, lane 1), it appears that the heterogeneity observed in binding profiles was induced in an otherwise homogeneous population of G proteins. That could arise from oligomers of G proteins with their constituent protomers interacting in a cooperative fashion. Such an arrangement would be similar to that described for oligomers of the M\textsubscript{2} muscarinic receptor (e.g., references 108,135,188). Heterogeneity revealed in the binding of GTP\textgamma\textupsilon S has been reported previously when the M\textsubscript{2} muscarinic receptor was co-purified with various subtypes of $G\alpha$ from porcine atria\textsuperscript{107}.
In two fluorescence based approaches that monitored the eGFP- and mCherry-bearing G proteins in CHO cells, large fractions of both labelled G proteins were found to co-diffuse in measurements of dcFCS, as determined from the amplitudes of the auto- and cross-correlation functions (Figure 3-3A); also, significant levels of FRET were observed between the two fluorophores (Figure 3-4). eGFP- and mCherry-tagged G proteins therefore appear to exist as dimers or larger oligomers. Several lines of evidence argue that the association is specific. There was no cross-correlation or FRET between MP-GFP and mCh-Gα1 (Figures 3-3C and 3-4); in contrast, nearly all of mCh-Gα1 co-diffused with eGFP-Gβ (Figure 3-3B), indicating the existence of a stable G protein heterotrimer in a system at rest. Furthermore, there was no relationship between the apparent FRET efficiency (E_{app}) and the expression levels of acceptor and donor (Figure 3-5). The interactions detected by dcFCS and FRET therefore do not appear to arise in a stochastic manner, suggesting that G proteins exist wholly or in part as oligomers in live CHO cells.

4.1.2. Quantification of G Protein Oligomers

FRET is not only an indication of close distance between donor- and acceptor-tagged G proteins, it also can be used quantitatively to estimate the size of an oligomer. In the current study, the dependence of E_{app} on [A]/[D] was analysed in terms of a model based on a stable oligomer to estimate the size of oligomers formed by eGFP- and mCherry-tagged G proteins (Equation 2-9). The same model has been used previously to estimate the size of oligomers formed by M2 muscarinic receptors in CHO cells. The model yields fitted estimates of the pair-wise FRET efficiency (E) for a given oligomeric size (n). The E value obtained for a dimer of eGFP- and mCherry-tagged G proteins in a large number of cells exceeded 100% under all three conditions and even the lowest fixed values of E that did not significantly compromise the goodness of fit were well above 100% (Figure 3-7). The values obtained for a trimer were physically possible (i.e., < 100%) but high nonetheless (E = 77–91%). Realistic values of E were obtained only for a tetramer or larger oligomer (i.e., E ≤ ~60%)

The model employed in this study accounts for all combinations of donor and acceptor and includes all pathways for resonance energy transfer in an oligomer of defined size. It assumes that there is no net change in oligomeric size, at least under the conditions of the measurement, and it therefore is unsuitable for dynamic systems in which the G proteins
interconvert between monomers and oligomers or between oligomers of different size. The model also assumes that the distance between fluorophores and therefore the pair-wise FRET efficiency is identical for all pairs of donor and acceptor within the oligomer. The latter assumption makes the estimated value $E$ less accurate when the fixed size of the oligomer exceeds a dimer. The rejection of a dimer and very likely a trimer on the basis of anomalously large pair-wise FRET efficiencies places a lower limit of four on the size of the oligomer in CHO cells. There was little or no effect on the value of $E$ upon the inclusion of M$_2$ receptor, either with or without agonist.

To avoid the limitations associated with efficiencies averaged over an entire cell and over the time of the measurement, step-wise photobleaching analysis was employed to track individual fluorophore-labelled G proteins at the level of single oligomers. Upon irradiation at 473 nm, purified eGFP-tagged G proteins gave intensity-time trajectories with successive photobleaching steps, the maximum step of a large amount of sampled molecules formed a distribution. A series of multimeric eGFP fusion proteins of different sizes also gave distributions of photobleaching steps, but the number of steps never exceeded the number of fluorophores in the fusion protein (Figure 3-8). Laser power also plays a role in shaping the step distribution (Figure 3-8D). Employment of a high laser power, which can potentially excite and photobleach all fluorophores in a complex, has been shown to increase the occurrence of traces with a higher number of steps. Higher power therefore provides a more accurate indication of the true size of the photobleached protein. It also leads to rapid photobleaching, however, and thus to fewer molecules that can be surveyed. The choice of laser power therefore is a trade-off between obtaining more accurate results with a higher power and observing more molecules before they are photobleached with a lower power. A medium level of power therefore was used to examine the size of G protein oligomers in the present study.

Photobleaching of eGFP-tagged G proteins revealed distributions spanning 1 through 6 steps, with a predominance of 4 steps (Figure 3-9). The presence of the M$_2$ muscarinic receptor and the addition of DTT both reduced the occurrence of traces with 5 or 6 steps (Figure 3-9). These observations imply the existence of mostly tetrameric G proteins. Monomers and dimers cannot be ruled out, however, and particles that displayed 5 or 6 steps may be a result of non-specific association between tetramers and the smaller complexes. If so, those aggregates appear to be broken apart or otherwise disfavoured by reducing agents or the M$_2$ receptor. We therefore
conclude that G proteins at rest exist as oligomers that likely comprise four heterotrimeric units, both in the absence and in the presence of M₂ receptor. Taken together with previous results from our laboratory, which demonstrated the existence of a tetrameric M₂ muscarinic receptor in various preparations, it appears that the functional unit of M₂ receptor and Gα₁β₁γ₂ comprises four copies of each.

4.1.3. Size of G Protein Oligomers upon Activation

In the absence of the M₂ muscarinic receptor, the addition of guanyl nucleotides induced little or no change in the distribution of photobleaching steps of the G protein (Figure 3-10A). It follows that the oligomeric status of a tetrameric G protein is insensitive to stimulation solely by a guanyl nucleotide. In contrast, in the presence of an agonist-activated M₂ muscarinic receptor, the addition of a guanyl nucleotide led to a leftward shift in the photobleaching step distribution (Figure 3-10B). The addition of an active GPCR mimic, mastoparan, resulted in a similar re-distribution (Figure 3-10A). It therefore appears that the dissociation of G protein oligomers requires stimulation from both an activated GPCR and a guanyl nucleotide.

Results of early biochemical studies and the data collected in the current project argue for a stable oligomeric G protein under resting conditions. The oligomeric size also seems unaffected by guanyl nucleotide alone. However, the combination of activated M₂ receptor and guanyl nucleotide appears to cause dissociation of the oligomer as indicated by the lower number of steps observed. It therefore appears that a constitutive oligomer of G proteins undergoes dissociation upon the activation of a coupled receptor by an agonist and of the G protein by a guanyl nucleotide. Such an arrangement is consistent with the disaggregation theory proposed initially by Rodbell⁴⁹.
5.1. Minimising the Occurrence of Residual Oligomers

Observations from the current study suggest the existence of two types of G protein oligomer: a stable oligomer that is insensitive to stimulation by guanyl nucleotides and a transient GPCR-coupled oligomer that dissociates into smaller entities upon the addition of guanyl nucleotides. The presence of both types of oligomer was inferred from the results of photobleaching studies on immobilised G protein samples. This single-molecule technique takes advantage of the proportional relationship between the intensity of fluorescent emission and the number of fluorophores present in a multimeric molecule. A step-wise decrease in intensity that follows photobleaching is used as a means to determine the size of the fluorescent oligomer.

With oligomers of G\textsubscript{i1} in the absence of the receptor, the addition of guanyl nucleotides did not affect the distribution of the number of photobleaching steps, thus indicating a stable oligomer. With oligomers of G\textsubscript{i1} coupled to agonist-activated M\textsubscript{2} receptors, the addition of guanyl nucleotides led to a shift in the distribution from one centred on four steps to one that was composed predominately of one and two steps. This shift implies a dissociation of tetrameric or larger G proteins to monomers or dimers, but the same distribution also contained an appreciable number of particles with three photobleaching steps. The retention of such larger particles in the presence of guanyl nucleotides may be a result of the incomplete dissociation of the original oligomer or the non-specific formation of smaller units of the G protein.

To examine this further, various parameters of the measuring system will be optimised to yield a more definitive result, particularly the elimination of particles showing three or more photobleaching steps after addition of guanyl nucleotide. First, the current time of 20 min for incubation of the purified proteins with various ligands may not be sufficient for all of the sampled particles to be affected. Therefore, by increasing the incubation time prior to photobleaching, one would expect to observe a lower retention of the larger species. Second, a more robust activation may be achievable with a constitutively active mutant of G\textsubscript{\alpha}1 in which GTP hydrolysis is blocked by the point mutation Q204L\textsuperscript{89}. The mutant itself may show fewer particles with three or more photobleaching steps, and the addition of a guanyl nucleotide may lead to fewer still. Combinations of the mutant, mastoparan and guanyl nucleotides (\textit{e.g.}, GTP\textsubscript{\gamma}S or GDP plus AlF\textsubscript{4}\textsuperscript{-}) also may reduce the number of steps. Third, larger residual oligomers may arise from non-specific interactions between smaller units such as monomers and dimers, even in the presence of \textbeta-mercaptoethanol. The reducing agent DDT has been shown to reduce the
occurrence of G protein oligomers that displayed 5 and 6 photobleaching steps (Figure 3-9B), but DTT itself may have undergone oxidation under the conditions of the assays\textsuperscript{164}. This will be tested by replenishing the sample with additional DTT over the course of the measurements described above.

### 5.2. Oligomeric Status of the M\textsubscript{2} Receptor

As discussed previously, the oligomeric status of functional GPCRs and the composition of the signalling complex formed by GPCRs and G proteins have been topics of great interest and much debate. Single-particle photobleaching therefore will be used to investigate the oligomeric status of the M\textsubscript{2} muscarinic receptor, both alone and in a complex with G\textsubscript{i1}.

To estimate the size of the receptor alone, M\textsubscript{2} receptor bearing a His\textsubscript{6} tag and eGFP at its amino terminus will be expressed in S\textsubscript{f9} cells and purified in digitonin and sodium cholate as previously described (e.g., reference 136). The baculovirus encoding the construct is currently available in our laboratory. The extracted and purified receptor will be immobilised on biotin-containing PEG, and the photobleaching step patterns will be recorded by means of TIRF microscopy. The reducing agent DTT will be included throughout the purification and subsequent measurements to minimise non-specific aggregation of the receptor. At least four photobleaching steps are expected on the basis of evidence that the M\textsubscript{2} receptor is a tetramer. To examine the effect of agonists on the oligomeric status of the receptor, a saturating concentration of the muscarinic agonist carbachol will be added to the receptor prior to immobilisation. If the distribution of photobleaching steps remains largely unchanged, it would suggest that the receptor exists as a stable oligomer; alternatively, a shift to fewer steps would argue in favour of a transient oligomer that dissociates upon activation.

To monitor the oligomeric status of the receptor in a complex with the G protein, the eGFP-bearing M\textsubscript{2} receptor will be co-purified with heterotrimeric G\textsubscript{i1} bearing a hexahistidyl tag at the N-terminus of the \(\gamma\)-subunit. The purification will be carried out as described in Chapter 2, with the same steps taken to ensure the association of the receptor and the G protein (Figure 2-3B). The immobilised complex will be subsequently examined by single-particle photobleaching. Placement of the fluorophore at the N-terminus of the M\textsubscript{2} receptor and His\textsubscript{6} on the \(\gamma\)-subunit of the G protein will ensure that all measurements will be of receptors coupled to heterotrimeric G proteins. Measurements in the absence and presence of guanyl nucleotides will provide information on the oligomeric status of the receptor within the complex before and after
activation of the G protein. A nucleotide-dependent decrease in the number of immobilised fluorescent particles would suggest that activation causes dissociation of the receptor from the G protein.
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