Biophysical Characterization of Tryptophan Locales, Mg²⁺ Binding and Protein Folding in Gα Subunits

Matthew Najor
Loyola University Chicago, mnajor@luc.edu

Recommended Citation
Najor, Matthew, "Biophysical Characterization of Tryptophan Locales, Mg²⁺ Binding and Protein Folding in Gα Subunits" (2013). Dissertations. Paper 730.
http://ecommons.luc.edu/luc_diss/730

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2013 Matthew Najor
LOYOLA UNIVERSITY CHICAGO

BIOPHYSICAL CHARACTERIZATION OF TRYPTOPHAN LOCALES, Mg$^{2+}$ BINDING AND PROTEIN FOLDING IN $G_{\alpha}$ SUBUNITS

A DISSERTATION SUBMITTED
TO THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CHEMISTRY

BY
MATTHEW S. NAJOR
CHICAGO, ILLINOIS
DECEMBER 2013
ACKNOWLEDGMENTS

I would like to acknowledge all the people who made the completion of this dissertation possible. First and foremost, I would like to thank my graduate advisor, Dr. Duarte Mota de Freitas, for teaching me how to think critically and how to write scientifically. You are a terrific mentor and an even better friend.

I would also like to thank my dissertation committee, Dr. Miguel Ballicora, Dr. Paul Chiarelli and Dr. Ken Olsen for their guidance and valuable insight into my project. Other faculty members who have assisted me in my graduate education at Loyola University Chicago include Dr. Daniel Graham who helped me with data analysis. I would like to thank Dr. David French for his assistance in my NMR studies.

Graduate student colleagues who have made brightened my graduate tenure include Matt Bartucci, Dan Kissel, and Natalie Gumataotao. I would like to thank the undergraduate researchers who have contributed to the overall goal of the project: Steve Chukwulebe, Sean Keil, Iva Golemi, and Colin Senfelds. I also would like to acknowledge my lab mates Brian Leverage and Jessie Goosens. In addition, I would also like to thank former lab mate Dr. Guoyan Wang for teaching me the techniques of PCR and G protein cultures. Dr. Chris Malarkey deserves special thanks not only for his guidance and lab training in my first year of graduate school, but for his friendship as well.
My friends and family have been a continuous source of calm and inspiration during this time. My brothers Marc and Nicholas, my sister Courtney, and my Mom and Dad have never doubted and always believed in me now and forever. My grandmother Lyla and grandfather George, for always supporting me and encouragement in my dreams. Finally, I would like to thank my wife Lindsay for her unwavering love, patience and overall belief in me during my time in graduate school.
To my parents for all their undying love, encouragement and continuous support
TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS xii

CHAPTER ONE: INTRODUCTION 1

CHAPTER TWO: CONTRIBUTIONS OF EACH TRP RESIDUETOWARDS
THE INTRINSIC FLUORESCENCE OF THE G\textsubscript{i\alpha1} PROTEIN 13
Introduction 13
Materials and Methods 15
Expression and Protein Purification 15
Fluorescence Measurements 15
Trypsin Digestion Analyzed by SDS-PAGE 16
Modeling 16
Results 17
AMF and GTP\textsubscript{γS} Activation of WT G\textsubscript{i\alpha1} and its Trp Mutants 17
Shifts in the Emission Spectrum of Activated G\textsubscript{i\alpha1} Proteins 26
Trypsin Digestion of G\textsubscript{i\alpha1} Proteins 30
Discussion 30

CHAPTER THREE: FOLDING OF G\textsubscript{α} SUBUNITS: CHANGES IN THE
ENVIRONMENTS OF TRP AND TYR RESIDUES AND ON THE
SECONDARY STRUCTURE 41
Introduction 41
Materials and Methods 45
Expression and Protein Purification 45
Fluorescence Measurements 46
Tyrosine Assay 46
Determination of Secondary Structure 47
Refolding of G\textsubscript{α} Subunits 47
Results 48
AMF and GTP\textsubscript{γS} Activation of G\textsubscript{i\alpha1} 48
Fluorescence Emission Spectra of G\textsubscript{α} Proteins 50
Tyrosine Assays 54
Temperature–Dependence of Secondary Structure G\textsubscript{i\alpha1} 58
Discussion 64

CHAPTER FOUR: Mg\textsuperscript{2+} DEPENDENCE OF THE FOLDING OF G\textsubscript{α}
SUBUNITS 75
Introduction 75
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>78</td>
</tr>
<tr>
<td>Expression and Protein Purification</td>
<td>78</td>
</tr>
<tr>
<td>Calculation of $[\text{Mg}^{2+}]$ in the Presence of EDTA</td>
<td>78</td>
</tr>
<tr>
<td>Fluorescence Measurements</td>
<td>79</td>
</tr>
<tr>
<td>Determination of Secondary Structure</td>
<td>79</td>
</tr>
<tr>
<td>Results</td>
<td>80</td>
</tr>
<tr>
<td>Effect of $\text{Mg}^{2+}$ on the Intrinsic Fluorescence of $\text{G}_\alpha$ subunits</td>
<td>80</td>
</tr>
<tr>
<td>Changes in the secondary structure of $\text{G}<em>{i\alpha1}$ and $\text{G}</em>{s\alpha}$</td>
<td>85</td>
</tr>
<tr>
<td>Discussion</td>
<td>91</td>
</tr>
<tr>
<td>APPENDIX A: EFFECT OF DTT ON WT $\text{G}_{i\alpha1}$ ACTIVATION</td>
<td>95</td>
</tr>
<tr>
<td>APPENDIX B: $\text{Li}^+$ INHIBITION OF GTP$\gamma$S BINDING AND GTP HYDROLYSIS</td>
<td>101</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>108</td>
</tr>
<tr>
<td>VITA</td>
<td>114</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Kinetic Parameters for Activation of WT and W Mutants 21

Table 2. Solvent Exposure of W residues in WT and W Mutants 25

Table 3. Observed and Calculated Distances and $\lambda_{\text{max}}$ shifts in WT and W Mutants 29

Table 4. Interaction Energies between R208 and W211 36

Table 5. Computational Calculations for Solvent Exposure of R208 in the Molecular Models 39

Table 6. $T_m$’s Estimated for all G$\alpha$ Proteins Using Three Spectroscopic Methods 53

Table 7. Composition of WT G$i1$ Secondary Structure at Various Temperatures 61

Table 8. Composition of WT G$s1$ Secondary Structure at Various Temperatures 69
LIST OF FIGURES

Figure 1. G – protein signaling cascade 3

Figure 2. Crystal structure of WT $G_{i\alpha 1}\cdot GDP\cdot Mg^{2+}$ 5

Figure 3. Solvent effects on ANS 8

Figure 4. Model of WT $G_{i\alpha 1}$ depicting its carbon backbone 18

Figure 5. Intrinsic Tryptophan fluorescence of WT $G_{i\alpha 1}$ and its W131F, W258F and W211F mutants after activation with AlF$_4^-$ 20

Figure 6. Intrinsic Tryptophan fluorescence after GDP→GTPγS exchange 22

Figure 7. Emission spectra of WT $G_{i\alpha 1}$ and its three Trp mutants in the GDP conformation 23

Figure 8. Intrinsic tryptophan fluorescence of $G_{i\alpha 1}$ proteins 27

Figure 9. Depiction of red shifts undergone by WT and W mutants upon addition of AlF$_4^-$ or GTPγS 28

Figure 10. Trypsin digests of wild-type and various tryptophan mutants of $G_{i\alpha 1}$ 31

Figure 11. Molecular dynamic calculations of R208 solvent exposure in WT and its three Trp mutants in all conformations 40

Figure 12. WT $G_{i\alpha 1}\cdot GDP\cdot Mg^{2+}$ displaying its three tryptophan residues, Arg208 and Mg$^{2+}$ 43

Figure 12. WT $G_{i\alpha 1}\cdot GTP\gamma S$ depicting the same residues as Fig. 12 except the bound nucleotide is GTPγS 44

Figure 13. Temperature dependence of AlF$_4^-$ activation of WT $G_{i\alpha 1}\cdot GDP\cdot Mg^{2+}$ 49
Figure 14. Temperature dependence of GTPγS Activation of WT G\textsubscript{ia1}•GDP•Mg\textsuperscript{2+}

Figure 15. Fluorescence emission spectra of WT G\textsubscript{ia1}•GDP•Mg\textsuperscript{2+} as a function of temperature

Figure 16. Refolding of WT G\textsubscript{ia1}•GTPγS

Figure 17. Refolding of WT G\textsubscript{ia1} in all three conformations as monitored by fluorescence

Figure 18. WT G\textsubscript{ia1}•GDP depicting its 13 Tyr residues

Figure 19. Unfolding of WT G\textsubscript{ia1} as monitored through Tyr movement

Figure 20. Unfolding of WT G\textsubscript{ia1}•GDP•Mg\textsuperscript{2+} secondary structure

Figure 21. Unfolding of WT G\textsubscript{sα}•GDP•Mg\textsuperscript{2+} secondary structure

Figure 22. Denaturation and refolding of WT G\textsubscript{ia1}•GDP

Figure 23. Unfolding of WT G\textsubscript{ia1}•GDP in the presence of guanidinium HCl

Figure 24. Mg\textsuperscript{2+} dependence of AlF\textsubscript{4}− activation of WT G\textsubscript{ia1}

Figure 25. Mg\textsuperscript{2+} dependence of AlF\textsubscript{4}− activation of WT G\textsubscript{sα}

Figure 26. GTPγS activation of WT G\textsubscript{ia1} dependent on Mg\textsuperscript{2+}

Figure 27. GTPγS activation of WT G\textsubscript{sα} dependent on Mg\textsuperscript{2+}

Figure 28. Shift resulting from AlF\textsubscript{4}− activation of WT G\textsubscript{ia1} and G\textsubscript{sα} at various [Mg\textsuperscript{2+}]\textsubscript{F}

Figure 29. Shift resulting from GTPγS activation of WT G\textsubscript{ia1} and G\textsubscript{sα} at various [Mg\textsuperscript{2+}]\textsubscript{F}

Figure 30. Secondary structure of WT G\textsubscript{ia1}•AMF at various Mg\textsuperscript{2+} concentrations
Figure 31. Mg$^{2+}$ dependent activation of $G_{i\alpha 1}$ and $G_{s\alpha}$ with AlF$_4^{-}$ or GTP$\gamma$S

Figure 32. Mg$^{2+}$ dependent activation of $G_{i\alpha 1}$ and $G_{s\alpha}$ with AlF$_4^{-}$ or GTP$\gamma$S

Figure 33. WT $G_{i\alpha 1}\cdot GDP\cdot Mg^{2+}$ activation with AlF$_4$ at various DTT concentrations

Figure 34. Visualization of WT $G_{i\alpha 1}\cdot GDP$ polymerization with SDS – PAGE

Figure 35. Effect of Li$^+$ on GTP$\gamma$S binding to WT $G_{s\alpha}$

Figure 36. Effect of Li$^+$ on GTP Hydrolysis of WT $G_{s\alpha}$
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2YT</td>
<td>2 x yeast extract tryptone</td>
</tr>
<tr>
<td>α</td>
<td>Alpha helices</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’ –diphosphate</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>Aluminum chloride</td>
</tr>
<tr>
<td>AlF₄⁻</td>
<td>Aluminum fluoride</td>
</tr>
<tr>
<td>AMF</td>
<td>Aluminum magnesium fluoride</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilinonaphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’ –triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>Beta sheets</td>
</tr>
<tr>
<td>cAMP</td>
<td>3’,5’ -cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>Δ</td>
<td>Change in solvent exposure</td>
</tr>
<tr>
<td>ΔE</td>
<td>Change in the total interaction energies between GDP and either AMF or GTPγS</td>
</tr>
<tr>
<td>Δλₘₐₓ</td>
<td>Change in λₘₐₓ between GDP and either AMF or GTPγS</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol – bis (β – aminoethylether) – N,N,N’,N’ – tetra acetic acid</td>
</tr>
<tr>
<td>Elec (E)</td>
<td>Electrostatic interaction</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$F_{\text{max}}$</td>
<td>Maximum fluorescence intensity</td>
</tr>
<tr>
<td>$G$</td>
<td>Guanine nucleotide binding</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GDP•ALF$_4^-$</td>
<td>Guanosine 5’-diphosphate tetrafluoroaluminate complex</td>
</tr>
<tr>
<td>$G_i$</td>
<td>Inhibitory G protein of cAMP</td>
</tr>
<tr>
<td>$G_{i\alpha}$</td>
<td>$\alpha$ subunit of the inhibitory G protein of cAMP</td>
</tr>
<tr>
<td>$G_s$</td>
<td>Stimulatory G protein of cAMP</td>
</tr>
<tr>
<td>$G_{s\alpha}$</td>
<td>$\alpha$ subunit of the stimulatory G protein of cAMP</td>
</tr>
<tr>
<td>GSK – 3$\beta$</td>
<td>Glycogen synthase kinase – 3 beta</td>
</tr>
<tr>
<td>$G_{t\varepsilon}$</td>
<td>Transducin G protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>GTP$\gamma$S</td>
<td>Guanosine 5’-[\gamma-thio] triphosphate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>His$<em>6$ - G$</em>{s\alpha}$</td>
<td>N – terminus hexahistidine tagged G$_{s\alpha}$ protein</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>Wavelength of maximum fluorescence</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>Li$^+$</td>
<td>Lithium ion</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>Mg$_2$SO$_4$</td>
<td>Magnesium Sulfate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>Sodium dihydrogenphosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>RC</td>
<td>Random coil</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS – PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>T</td>
<td>Turns</td>
</tr>
<tr>
<td>Tris – Cl</td>
<td>Tris(hydroxymethyl)amino methane</td>
</tr>
<tr>
<td>UV – Vis</td>
<td>Ultraviolet visible spectrophotometry</td>
</tr>
<tr>
<td>VdW</td>
<td>Van der Waals interaction</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

Many proteins, peptides, lipids, ions and small molecules are responsible for cellular signaling. Hormones and neurotransmitters are known to transmit cellular signals, by binding to a receptor which then transduces the signal to the inside of the cell and activates kinases, phosphatases, proteases, and nucleotide binding proteins. Many of these proteins switch between an “on” and “off” phase, varying the intensity and duration of the transmitted signal. Guanine nucleotide binding proteins, $G$–proteins, are among the most prevalent found signal - transducing proteins [1].

Alfred Gilman and Martin Rodbell were awarded the 1994 Nobel Prize in physiology or medicine for their work and discovery of $G$–proteins. There are two major classes of $G$–proteins: large heterotrimeric membrane bound proteins and small monomeric proteins, such as Ras [1, 2].

Heterotrimeric $G$–proteins are associated with many hepta-helical serpentine transmembrane domain receptors, which are more commonly known as $G$–protein coupled receptors (GPCRs), and they regulate intracellular signaling cascades in response to GPCR activation. $G$ – proteins are bound to the inside surface of the transmembrane where they act as molecular switches between intracellular receptors and effectors. Heterotrimeric $G$ – proteins consist of $\alpha$, $\beta$, and $\gamma$ subunits [3]. The $\alpha$ – subunit contains the guanine nucleotide (GDP/GTP) binding site in the GTPase domain. Upon activation of the extracellular receptor, the G protein exchanges GDP for GTP in the $\alpha$ subunit.
The binding of GTP induces a conformational change in the α subunit and promotes dissociation of the α subunit from the βγ complex. The now active α – GTP subunit and the βγ complex interact with downstream effectors. Activation is terminated by hydrolysis of GTP which produces inorganic phosphate (P_i) and returns the G – protein back to its GDP conformation. Deactivation signals re-association of the α – subunit and the βγ complex to form the heterotrimer. The α – subunit consists of two domains: the GTPase domain, involved in the binding and hydrolysis of GTP, and a highly concentrated α – helical domain that buries the GTP within the core of the protein (4).

The α - subunits interact with various effector molecules, such as adenylyl cyclase, and induce different physiological and biological responses. Activated G – proteins bind to adenylyl cyclase affect the production of cAMP from ATP (Fig. 1).

There are 23 known Gα proteins encoded from 16 Gα genes in the human genome. These genes translate to proteins in the 39 – 45 kD molecular weight range, and, based on sequence conservation, can be divided into 4 subclasses of the Gα family: Gα(s/olf), Gα(i1/i2/i3/o/rod/t-cone/gust/z), Gα(q/11/14/16), and Gα(12/13) (1). Golfα is expressed in the olfactory sensory neurons. Gsα consists of four variant splice forms, two short (Gsα–S) and two long (Gsα–L). Structurally they are similar and functionally indistinguishable. Gsα is ubiquitously expressed and known to elevate levels of cAMP. The Giα family is known to inhibit various types of adenylyl cyclase and lowers the intracellular levels of cAMP. Gsα is abundantly found in the nervous system where it has been found to activate Ca^{2+} and K^{+} channels (5). Gzα is expressed in the adrenal medulla of the brain,
Figure 1. G–protein signaling cascade
neuronal cells, and platelets (6). Like $G_{i\alpha}$, $G_{z\alpha}$ inhibits adenylyl cyclases, although it displays slower kinetics in GTP hydrolysis compared to other $G_{\alpha}$ subunits (7). $G_{it\alpha}$, which includes rod and cone transducins, and gustducin are involved in sensory functions (6). Subunits of the $G_{qa}$ family, including isoforms $G_{11\alpha}$, $G_{14\alpha}$ and $G_{16\alpha}$, directly activate phospholipase C β - isozymes. Activation of phospholipase C produces inositol phosphate (IP$_3$), which in turn triggers the release of Ca$^{2+}$ ions from Ca$^{2+}$ storage in cells and diacylglycerol (DAG) (8, 9). G – proteins $G_{12\alpha}$ and $G_{13\alpha}$ are expressed ubiquitously and stimulate a multitude of downstream effectors, such as PLC A$_2$ and the Na$^+/H^+$ exchanger (10). In addition to secondary messengers, it has been demonstrated that they are directly involved with the activation of Rho GTPases (11).

The $\alpha$ subunit of G proteins is composed of two domains, an $\alpha$ - helical and GTPase domain, which contains the active site (12). GTPase domain is composed of six-stranded $\beta$-sheets surrounded by five $\alpha$-helices. The active site contains the guanine nucleotide and the binding sites for the $G_{\beta\gamma}$ dimer, membrane receptors and downstream effectors. There are five conserved sequences in the GTPase domain, the diphosphate binding loop ($P – loop$) (GXGESGKS), the Mg$^{2+}$-binding loops (RXXTXGI and DXXG), and the guanine ring-binding motifs (NKXD and TCAT) (Fig. 2). There are three flexible loops known as switches I, II and III near the $\gamma$ – phosphate in the active site. The switch regions are ordered and held in place by contacts with the $\gamma$ – phosphate of GTP (12). In contrast, switch regions II and III are unordered in the GDP-bound conformation of $G_{it\alpha}$, but not in $G_{it\alpha}$ (13). The $\alpha$ - helical domain is composed of six $\alpha$-helices that form a lid over the nucleotide-binding site in the GTPase domain.
Figure 2. Crystal structure of WT $G_{\alpha 1} \cdot GDP \cdot Mg^{2+}$ (1BOF, (13)). Displaying the GTPase domain (blue), $\alpha$ – helical domain (red), helical micro domain (orange), switch I (pink), switch II (cyan), switch III (silver), guanine nucleotide, GDP, and Mg$^{2+}$ ion (green sphere). The black brackets connecting the switch II and III regions indicate unordered structure.
This domain may also play a significant role in coupling specific G proteins to specific effectors (14). Both the amino (N) and carboxyl (C) termini of the α subunits are key determinants of receptor-binding specificity and play a critical role in G-protein activation, but their structures are not clear because they were disordered in the crystals. The N-terminus forms an α-helix that is ordered by its interaction with Gβ in the heterotrimeric structure of G proteins, however, its structure in the active conformation of the Gα subunits remains unknown (15, 16).

Unique to G<i>ia1</i> and G<i>sa</i> is the fact that they contain three and four tryptophan residues, respectively. W131 in G<i>ia1</i> and W154 in G<i>sa</i> are located in the α – helical domain, W211 in G<i>ia1</i> and W234 in G<i>sa</i> are contained in the switch II region within the GTPase domain, and W258 in G<i>ia1</i> and W277 and W281 in G<i>sa</i> are found in the GTPase domain (12, 17, 18). The W211 and W258 residues appear in close proximity as depicted in the crystal structure of G<i>ia1</i>. The presence of tryptophan residues in most proteins is scarce and they typically provide a role of stability (19). Gα subunits are unique in that the conformational changes that occur upon activation can be monitored through the change in the Trp residues environment. Addition of AlF₄⁻ or GTPγS to Gα•GDP results in a conformational change, which can be detected through the intrinsic tryptophan fluorescence of the protein. An increase in fluorescence is observed when the Trp residues move from a solvent exposed environment to one that is hydrophobic (20).

Weber and Laurence worked with numerous polycyclic aromatic compounds and found that when in water they were non fluorescent. However, upon binding to serum albumin they were highly fluorescent (21). One of the aromatic compounds
studied was 8-Anilinonaphthalene-1-sulfonic acid (ANS), a known extrinsic fluorophore used today to probe the environment of biological molecules through changes in its hydrophobic regions (20, 22). Decreasing the polarity of ANS results in a shift of the $\lambda_{\text{max}}$ to lower wavelengths (blue shift) and an increase in fluorescence intensity in the emission spectrum (20) (Fig. 3). The blue shifts are best explained through the Planck relation: $E = h\nu$, which displays a direct relationship between energy ($E$) and frequency ($\nu$). Since the speed of light ($c$), $\nu$ and wavelength ($\lambda$) are related by $\lambda\nu = c$, the equation can also be expressed as $E = \frac{hc}{\lambda}$. Excited states have an electron density that is more distorted than in the ground states. Thus, excited molecules are more inclined to interact with a polar environment so as to align the solvent dipoles and cause the emission spectrum to shift toward a higher $\lambda_{\text{max}}$ (red shift). The quantum yield or fluorescence intensity increases as the polarity of the environment decreases because the rate of intersystem crossing is reduced in nonpolar environments. The effects observed with ANS also occur with intrinsic chromophores such as tryptophan.

Gilman and co–workers (23) established this technique as an indirect method to investigate $G$–protein activity. Chabre worked with the $\alpha$–subunit of $G_i$ and was able to determine that the increase in fluorescence observed from activation was from a major contribution of W207, which is equivalent to W211 in $G_{i\alpha}$ (24). $G_{i\alpha}$ contains
Figure 3. Solvent effects on ANS
two tryptophan residues, therefore, investigation of the mutant W207F would lead to findings that W207 was or was not the major contributor to the observed fluorescence. Because G\textsubscript{ia1} has three Trp residues, the question of contribution is much more complex.

In addition to the increased fluorescence observed in G\textsubscript{ia1}\textbullet AMF and G\textsubscript{ia1}\textbullet GTP\gamma S, Chabre found a shift of the $\lambda_{\text{max}}$ to higher wavelengths (red shift) in the emission spectra (24). Hamm and co–workers (25) followed up on Chabre’s work and found evidence for a red shift in WT G\textsubscript{ia1}\textbullet AMF. A comparison between the crystal structures of G\textsubscript{ia1}\textbullet GDP and G\textsubscript{ia1}\textbullet AMF revealed a change in the distances between R204 and W207 to be 8.0 Å and 5.8 Å, respectively (25). Through the use of Trp mutants, W211C and W258F, Hamm – and co – workers (25) were able to determine that the cause of the shift was due to the close proximity of R208 and W211 in G\textsubscript{ia1}\textbullet AMF, the equivalent Arg and Trp in the active site of G\textsubscript{ia1}. However, they neglected to study the effects of the shift in the GTP\gamma S conformation and the contribution from W131.

Another indirect method for exploring the R208 – W211 bridge formation is through trypsin digestion in combination with SDS – PAGE. Trypsin cleaves on the carboxyl end of arginine and lysine residues. SDS – PAGE of WT G\textsubscript{ia1}\textbullet GDP displays a band at a molecular weight near 41kD. In the presence of trypsin the single band at 41 kDa is no longer observed, but two new bands appear at 21 kDa and 17 kDa. The two fragments correspond to digestion at R208. In contrast, WT G\textsubscript{ia1}\textbullet AMF and GTP\gamma S in the presence of trypsin continue to show a band at 41 kDa (26). Therefore,
\( \text{G}_{\text{i}1} \) is protected from tryptic digestion while activated and vulnerable to proteolysis in its GDP conformation (26).

Converting each Trp residue to a phenylalanine (F) via site direct mutagenesis produced three independent mutants, which were used to explore the contribution of each Trp residue towards the overall change in intrinsic tryptophan fluorescence for the AMF and GTP\( \gamma \text{S} \) conformations. In addition, we used the mutants to examine how the formation of the R208 – W211 bridge affects the red shift in the emission spectra of \( \text{G}_{\text{i}1} \).

The crystal structures of \( \text{G}_{\text{i}1} \cdot \text{GDP} \) (1GDD and 1BOF), AMF (1GFI), and GTP\( \gamma \text{S} \) (1GIA) conformations are available (12, 13, 17). The \( \text{G}_{\text{i}1} \cdot \text{GDP} \) displays unordered structure in the switch I and II regions, whereas both active structures show ordering of those two regions. Mg\( ^{2+} \) has octahedral geometry in the active site near the guanine nucleotide of both AMF and GTP\( \gamma \text{S} \) forms. In the GTP\( \gamma \text{S} \) form, Mg\( ^{2+} \) is bound to four equatorial ligands, the oxygen atoms of the hydroxyl groups in Thr181 and Ser47, and the oxygen atoms in the \( \beta \) – and \( \gamma \) – phosphate groups of GTP\( \gamma \text{S} \), and two water ligands occupy the axial positions. In the AMF conformation, the Mg\( ^{2+} \) coordination is identical to that of the GTP\( \gamma \text{S} \) form with the exception that one fluoride ion from AlF\( _4^- \) is bound to the equatorial position once occupied by the \( \gamma \) – phosphate. It is known that Mg\( ^{2+} \) is required for the formation of the AMF complex and for the hydrolysis of GTP. We hope to better understand the extent to which Mg\( ^{2+} \) contributes to the conformational states in which GTP\( \gamma \text{S} \), AlF\( _4^- \) or GDP are bound to \( \text{G}_\alpha \) subunits.
We plan to investigate, through circular dichroism, the secondary structure upon activation with GTPγS and AMF. In addition, we plan to gain insight into the role of Mg$^{2+}$ in the folding of $G_{i\alpha 1}$ and $G_{s\alpha}$ in the AMF and GTPγS forms.

The structure of a protein is essential to its biological function. Occurrences of mutations in DNA manifest in changes in the amino acid sequence that can lead to a plethora of problems, such as misfolding, interference of effector sites, unwanted translational modifications, etc. Ultimately this can produce incorrect function that leads to disease. Mutations in the GNAS and GNAI1 genes that encode for $G_{s\alpha}$ and $G_{i\alpha 1}$ have been implicated in numerous tumors found in the human body (27). Hot spot mutations at Q227 and R201 in GNAS have led to cancers, 10.6% and 88.1% of the time, in the biliary tract, pituitary gland, and small intestine (27). GNAI1, albeit to a much lower degree than GNAS, is associated with mutations at R208 leading to carcinomas in the large intestine (27).

Misfolding of proteins is another major contributor to detrimental neurological disorders and the occurrence of certain types of cancers (27, 28). For example, Alzheimer’s and Parkinson’s diseases arise from aggregation of a prion protein, known as β – amyloid. It is a protein comprised of a multitude of β – sheets and upon accumulation begins to aggregate and form particulate on the brain leading to neurological dysfunction (28).

We aim to examine the folding mechanism of $G_{i\alpha 1}$ and $G_{s\alpha}$ through spectroscopic techniques that include fluorescence, circular dichroism, and UV - Vis. Fluorescence spectroscopy can give us local insight by monitoring the change in the tryptophan environment. CD will provide information on alterations in the secondary
structure. Lastly, $G_{iα1}$ and $G_{sα}$ are comprised of numerous tyrosines at their surface. We also intend to gain knowledge on protein folding of the tertiary structure through UV – vis spectroscopy.

In conclusion, we hope to add important knowledge on the structural and functional aspects of $G_α$ proteins, which may lead to the development of better therapeutics for the treatment of $G_α$ subunit related - illnesses.
CHAPTER TWO

CONTRIBUTIONS OF EACH TRP RESIDUE TOWARDS THE INTRINSIC FLUORESCENCE OF THE G\textsubscript{iα1} PROTEIN

Introduction

G proteins are membrane-bound guanine-nucleotide binding proteins. Heterotrimeric G proteins consist of three subunits (α, β, and γ) that mediate transduction of extracellular signals to various intracellular effectors (29). Once a receptor protein binds to an extracellular hormone or neurotransmitter, it triggers the activation of the corresponding G protein. The activation of a G protein is accompanied by a conformational change in which GDP bound to the α subunit exchanges for GTP and the βγ dimer dissociates. The activated G protein returns to its resting, GDP-bound inactive state via hydrolysis of bound GTP and release of inorganic phosphate. GTP binding impacts the structures of three flexible loop segments located near the γ-phosphate, which are named switches I, II, and II. In the GTP-bound state, these switch regions are held in place by the γ-phosphate whereas in the GDP-bound state they are less ordered (29). G\textsubscript{iα1} and G\textsubscript{sα} are, respectively, the inhibitory and stimulatory α-subunits that are responsible for the decreased or increased production of the secondary messenger adenosine-3′,5′-monophosphate (cAMP) (30). This study focuses on the environment of tryptophan (W) residues in G\textsubscript{iα1}. W211 is located in the conformational-dependent switch II region. G\textsubscript{iα1} also contains two other tryptophans, W131 and W258, that are located in
the alpha helical and GTPase domains, respectively. X-ray crystallographic studies have provided a wealth of structural information on the different conformations of G proteins (12, 13, 18, 31-34). These static structural studies have been complemented with dynamic investigations in solution.

Radiolabeling of guanosine nucleotides with either $^{32}$P and $^{35}$S is a common method used for probing activation of G proteins by exploring the cycle of GTP→GDP exchange and hydrolysis (35). However, unlike electron paramagnetic studies using cysteine-anchored spin labeled G proteins (36, 37), radioactive assays do not give insight into the movements of the segments within Gα subunits. An indirect method of measuring protein activation in G proteins is through intrinsic tryptophan fluorescence, a tool that was pioneered by Gilman and co-workers (38). As observed for other proteins (39), when tryptophan residues move into less-solvent exposed environments the fluorescence intensity increases. Enhancements of fluorescence intensity were observed when either the active conformation was formed upon the exchange of GDP with GTPγS (non-hydrolyzable analog) or the transition state conformation was attained via addition of AlF$_4^-$ to G$_{i1\alpha}\cdot$GDP (38). Chabre et al. (24) found that for transducin (G$_t$), the change in fluorescence intensity upon activation was dependent on the presence of W207. (W207 in G$_t$ is the sequence counterpart of W211 in G$_{i1\alpha}$) When mutated to a phenylalanine, the W207F mutant of G$_t$ displayed a decrease in fluorescence (24, 40).

The polarity of the environment also affects the $\lambda_{\text{max}}$ values of W residues by shifting them to lower wavelengths, i.e., $\lambda_{\text{max}}$ undergoes a blue shift (20). However, Hamm and co-workers (25) found evidence for a π-cation interaction between arginine (R208) and
W211 in G_{iα1} based on the observed red shifts (to higher wavelengths) in the λ_{max} of the emission spectra of the activated forms of G_{iα1} (25).

The purpose of this investigation is to build on the foundation set by these previous studies and explore how each W contributes to the overall intrinsic fluorescence and to the R208-W211 electrostatic interaction in G_{iα1}.

Materials and Methods

Expression and Protein Purification

G_{iα1} was obtained and purified as described previously (41). W131F, W211F, and W258F mutants were prepared by site-directed mutagenesis using a kit provided by Stratagene (La Jolla, CA). Proteins were dialyzed overnight at 4 °C in 20 mM Tris pH 8.0 buffer containing 10% (v/v) glycerol, and 1 mM DTT and then stored at -80 °C. Protein purity was greater than 95% as estimated by SDS – PAGE.

Fluorescence Measurements

Experiments were performed using a PTI QuantaMaster fluorimeter (Photon Technologies, Inc., Mirmingham, NJ). Time-based assays were conducted with excitation and emission wavelengths set at 280 nm and 340 nm, respectively. Assays were initiated after 60 sec by addition of either AlF_{4} as a premixed solution (50 μM AlCl_{3} and 10 mM NaF) or 20 μM GTPyS to pre-incubated 500 G_{iα1} nM protein samples in buffer containing 50 mM Tris, pH 7.5, 1 mM DTT and 5 mM MgCl_{2}. Time-based assays were normalized to zero at 60 sec. Graphpad Prizm 3.0 was used for analyzing the time-dependent curves. The W211-R208 bridge formation was probed using emission spectra recorded over a wavelength range of 300-400 nm with excitation wavelength set at 280 nm. Signal integration time was 1 sec, and the bandpass for both excitation and
emission was 5 nm. In emission spectra, the maximal fluorescence intensities before and after activation with either AlF₄⁻ or GTPγS were normalized to 1.0.

Rates for biphasic activation of AMF were calculated by fitting the data to the equation:

\[ Y = Y_0 \left[ \frac{k_2 e^{-k_1 t} - k_1 e^{-k_2 t}}{k_1 - k_2} \right] \]

Rates for monophasic activation with GTPγS were calculated by fitting the data to the equation:

\[ Y = Y_{max} (1 - e^{-k_1 x}) \]

**Trypsin Digestion Analyzed by SDS-PAGE**

WT or mutant Gαi1 proteins at 15 μM were incubated for 60 min at 30 °C in 50 mM Tris, pH 8.0, 5 mM MgSO₄, 5mM DTT with either 100 μM GDP or GTPγS or 50 μM AlCl₃ and 10 mM NaF (26). Where indicated, trypsin (26 μg/ml) was added and incubated for an additional 10 min at 30 °C. SDS-containing buffer was added and samples were incubated for 10 min at 100 °C. Proteins were separated in a SDS 12.5 % polyacrylamide gel.

**Modeling**

The coordinates of GDP (1BOF, (13)), GDP-AlF₄⁻ (1GFI, (12)) and GTPγS (1GIA, (12)) derivatives of Gαi1 were downloaded from the Protein Data Bank (PDB, (42)). Missing loops in these structures were modeled using the corresponding transducin structures (1TAG, (33), 1TAD, (34) and 1TND, (32)) and Swiss Model. Each simulation box, containing one subunit, GDP or GTP, Mg²⁺, a TIP3 water box extending at least 10 Å beyond the protein in all directions and 0.1 M NaCl adjusted to neutralize
the charge in the water box, was assembled using the molecular graphics program VMD. The simulation box was then brought to equilibrium using the molecular dynamics program NAMD. The equilibration procedure involved energy minimization with and without restraints on the protein coordinates (3000 steps each), slow heating from 10 to 310 K (30,000 steps), and then pressure and temperature equilibration using a Langevin piston (10,000 steps). Finally, unrestrained dynamics for 100,000 steps was done before data was acquired. Periodic boundary conditions were used. The cutoffs for non-bonding (van der Waals and electrostatic) interactions were 12 Å. The switch distance was 10 Å, and 1.0 1-4 scaling factor was used. All calculations were done using CHARMM 27 parameters. The initial tryptophan point mutation models were generated using VMD and then subjected to the same equilibration procedure as the wild type structures. All molecular graphics diagrams were generated using VMD.

Results

AMF and GTPγS Activation of WT Giα1 and its Trp Mutants

Giα1 contains three trp residues: W131 is located in the helical domain; W211 is in the switch II region and W258 in the GTPase domain (Fig. 4). Intrinsic tryptophan fluorescence is a common method used for detecting Gα protein activity that has been well documented (38). To understand the contributions of each tryptophan towards the overall fluorescence of the protein, three mutants were prepared in which the tryptophan residues were mutated to phenylalanine (W131F, W211F, and W258F). Phenylalanine
Figure 4. Model of WT G$_{i_{a1}}$ depicting its carbon backbone. GDP nucleotide, three Trp residues, W131 (blue), W211 (orange), and W258 (green), and R208 (red).
was chosen as a replacement for tryptophan because of similar structure and size characteristics, and low quantum yield and distinct $\lambda_{\text{max}}$ value (43).

Upon addition of AlF$_4^-$, the fluorescence intensity of WT $G_{\text{i}1}\cdot\text{GDP}\cdot\text{AMF}$ reached a maximum of $40.3 \pm 0.04\%$ ($n \geq 3$) after 2 min (Fig. 5, curve C). This increase in fluorescence intensity is characteristic of an activated WT $G_{\text{i}1}\cdot\text{GDP}\cdot\text{AMF}$ protein (38). AMF activation of W131F (Fig. 5, curve A) displayed the highest fluorescence intensity ($F_{\text{max}}$), followed by W258F (Fig. 5, curve B), which exhibited a slight, but significantly larger fluorescence intensity than WT (Table 1). In contrast, W211F $G_{\text{i}1}\cdot\text{GDP}\cdot\text{Mg}^{2+}$ displayed an initial decrease in fluorescence upon addition of AlF$_4^-$ (data not shown). As binding of AlF$_4^-$ progressed, there was a gradual increase in fluorescence intensity that was too small to be viewed in figure 5, and indicated that, unlike the three other proteins, sample dilution was not offset by the large increases in fluorescence intensity associated with AMF activation. Addition of GTP$\gamma$S to WT, W131F and W258F mutants also resulted in increases in fluorescence intensity similar to the GDP•AMF counterparts (Fig. 6). As in activation with AlF$_4^-$, full exchange of GDP for GTP$\gamma$S, followed the same order of plateaus for WT and W131F and W258F mutants, and W211F exhibited no change in fluorescence intensity.

Despite the fact that W211F does not display an increase in fluorescence upon activation, the GDP conformation did exhibit significant fluorescence in its emission spectra similar to WT and W258F (Fig. 7). Interestingly, the emission of the W131F mutant in the GDP conformation was significantly smaller than in the other proteins (Fig. 7). In addition to its GDP conformation, W211F also exhibited similar fluorescence in its
Figure 5. Intrinsic Tryptophan fluorescence of WT Gia1 (C) and its W131F (A), W258F (B) and W211F (D) mutants after activation with AlF₄⁻.

Fluorescence (%) = ((F₀ - Fₜ)/Fᵢ)*100, where Fᵢ and F₀ are the fluorescence intensities in arbitrary units at the start of the activation and at time t.
Table 1. Kinetic Parameters for Activation of WT and W Mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>AMF Activation</th>
<th>GTPγS Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Rate Constant ($k^{1,3}$)</td>
<td>$F_{\text{max}}^{2,3}(%)$</td>
</tr>
<tr>
<td>WT $G_{i\alpha}^1$</td>
<td>0.03</td>
<td>40.3</td>
</tr>
<tr>
<td>W131F</td>
<td>0.2</td>
<td>73.7</td>
</tr>
<tr>
<td>W211F</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W258F</td>
<td>0.06</td>
<td>42.8</td>
</tr>
</tbody>
</table>

1 Error reported is less than 0.5%
2 Error reported is less than 0.5%
3 Average data from $n \geq 3$ experiments
Figure 6. Intrinsic Tryptophan fluorescence after GDP→ GTPγS exchange. The curves are labeled as in Fig. 5. Fluorescence % is calculated in the same manner as in Fig. 5.
Figure 7. Emission spectra of WT $G_{\text{i}1}$ and its three Trp mutants in the GDP conformation.
AMF and GTPγS conformations and activation of WT, W131F, and W258F $G_{ia1}$ also showed substantial increases in their emission spectra. However, for the active conformations, the emission of the W258F mutant was larger than in the other proteins (data not shown). AMF activation of WT, W131F, and W258F was biphasic with the initial $k_1$ rates differing by less than one order of magnitude (Table 1). However, the rates for the second step were approximately four orders of magnitude larger (data not shown). In contrast, GTPγS activation of WT, W131F and W258F was monophasic with rates that were of the same order of magnitude, but smaller than the initial $k_1$ rates observed for AMF activation (Table 1).

The crystal structures of WT $G_{ia1}$ GDP (1BOF), AMF (1GFI) and GTPγS (1GIA) are known (12, 13). However, in all three conformations many crucial amino acids are missing, including R208 and W211, which lie in the switch II region. The missing residues of WT $G_{ia1}$ were built in using homology modeling of the WT transducin ($G_t$) crystal structures (33, 34, 44). Structures of W131F, W211F, and W258F mutants in all three conformations were generated from the WT $G_{ia1}$ models. In an attempt to rationalize the experimental findings in Figs. 5 and 6, we calculated for each tryptophan residue the change in solvent exposure ($\Delta$) between the GDP and either the AMF or GTPγS conformations (Table 2). For AMF activation, W131F displayed the largest overall value of $\Delta$, followed by W258F, WT, and W211F. The same calculations were performed for GTPγS activation and the same trend was observed (Table 2). For each of the three W mutants, predicted maximum fluorescence ($F_{max}$) values were calculated by using the total $\Delta$ for each mutant and by setting WT $G_{ia1}•GDP$ with AMF or GTPγS
Table 2. Solvent Exposure of W residues in WT and W Mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conformation and Solvent Exposure</th>
<th>W131</th>
<th>W211</th>
<th>W258</th>
<th>Total Δ(^1)</th>
<th>Estimated F(_{\text{max}})</th>
<th>Observed (^2) F(_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>GDP</td>
<td>22</td>
<td>114</td>
<td>179</td>
<td>210(^3)</td>
<td>40</td>
<td>40.3 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>AMF</td>
<td>32</td>
<td>8</td>
<td>161</td>
<td>134*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{AMF-GDP}})</td>
<td>10</td>
<td>-105</td>
<td>-18</td>
<td>-114</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>51</td>
<td>7</td>
<td>157</td>
<td>143*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{GTP-GDP}})</td>
<td>29</td>
<td>-107</td>
<td>-22</td>
<td>-100</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>W131F</td>
<td>GDP</td>
<td>0</td>
<td>144</td>
<td>202</td>
<td>346</td>
<td>73</td>
<td>73.7 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>AMF</td>
<td>0</td>
<td>9</td>
<td>129</td>
<td>138</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{AMF-GDP}})</td>
<td>0</td>
<td>-135</td>
<td>-73</td>
<td>-208</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>0</td>
<td>12</td>
<td>176</td>
<td>188</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{GTP-GDP}})</td>
<td>0</td>
<td>-132</td>
<td>-26</td>
<td>-158</td>
<td>63</td>
<td>63.0 ± 0.03</td>
</tr>
<tr>
<td>W211F</td>
<td>GDP</td>
<td>21</td>
<td>0</td>
<td>166</td>
<td>187</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AMF</td>
<td>20</td>
<td>0</td>
<td>165</td>
<td>185</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{AMF-GDP}})</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
<td>-2</td>
<td>0.7</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>15</td>
<td>0</td>
<td>165</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{GTP-GDP}})</td>
<td>-6</td>
<td>0</td>
<td>-1</td>
<td>-7</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>W258F</td>
<td>GDP</td>
<td>43</td>
<td>118</td>
<td>0</td>
<td>161</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AMF</td>
<td>22</td>
<td>12</td>
<td>0</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{AMF-GDP}})</td>
<td>-20</td>
<td>-106</td>
<td>0</td>
<td>-126</td>
<td>44</td>
<td>42.8 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>20</td>
<td>13</td>
<td>0</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(_{\text{GTP-GDP}})</td>
<td>-23</td>
<td>-105</td>
<td>0</td>
<td>-128</td>
<td>51</td>
<td>55.5 ± 0.02</td>
</tr>
</tbody>
</table>

\(^1\) A negative indicates a decrease in solvent exposure of the tryptophan residue.

\(^2\) Errors less than 0.5 % for \(n \geq 3\)

\(^3\)(sum of the row) X (2/3) to normalize for contribution of two Trp residues
activation at the observed values of 40%. The obtained theoretical values were within 5%
of the actual experimental data (Table 2; last column).

**Shifts in the Emission Spectrum of Activated G_{i1} Proteins**

Fluorophores, such as (ANS), undergo an increase in fluorescence intensity as well as a blue shift as solvent polarity decreases (20). We therefore investigated whether the predicted blue shifts would be observed for WT and 
W mutants of G_{i1}. The contribution of the shift in \( \lambda_{\text{max}} \) was separated from the increase in fluorescence intensity by normalizing the relative fluorescence maximum of each spectrum to 1.0 (Fig. 8). In contrast to the predicted blue shifts, WT G_{i1} GDP•AMF displayed a red shift of 2.4 ± 0.1 nm when compared to its GDP bound counterpart (Fig. 8a). Both WT G_{i1}•GDP•AMF (Fig. 8a) and WT G_{i1}•GTPγS (Fig. 8b) exhibited similar red shifts. Upon activation with AlF_4\(^-\), W258F had the largest \( \Delta \lambda_{\text{max}} \) value of 3.2 ± 0.1 nm, followed by WT G_{i1}, and W131F at 2.1 ± 0.1 nm; in contrast to the other proteins, W211F displayed the only negative \( \Delta \lambda_{\text{max}} \) value of -0.4 ± 0.4 nm (Fig. 9). Upon exchange with GTPγS (Fig. 9), W131F G_{i1} had the largest \( \Delta \lambda_{\text{max}} \) value of 3.9 ± 0.5 nm, followed by W258F with 3.6 ± 0.1 nm and WT with 2.5 ± 0.2 nm; W211F again displayed a negative \( \Delta \lambda_{\text{max}} \) of -0.4 ± 0.2 nm.

\( \pi \)-cation interactions between lysines or arginines with the aromatic side chains of tryptophans, tyrosines and phenylalanines have been previously investigated (45, 46). Electrostatic interactions at distances less than 6 Å are found to be favorable with \( E_{\text{es}} < -2 \text{ kcal/mol} \) (25, 45). Distances between the C atom in the guanidinium group of R208 and the geometric center of the indole ring of W211 in WT and mutant G_{i1} protein models were measured for all conformations (Table 3). In the GDP conformation,
Figure 8. Intrinsic tryptophan fluorescence of $G_{i\alpha 1}$ proteins.
Emission of 0.5 $\mu$M WT $G_{i\alpha 1}$ before (blue trace) and after activation with either $\text{AlF}_4^-$ (panel A; red trace) or GTP$\gamma$S (panel B; red trace).
Figure 9. Depiction of red shifts undergone by WT and W mutants upon addition of AlF₄⁻ (left set of 4 bars) or GTPγS (right set of 4 bars).

The asterisks indicate statistically significant changes $n \geq 3 \pm$ SEM. measurements at $^* p < 0.001$
Table 3. Observed and Calculated Distances and $\lambda_{\text{max}}$ shifts in WT and W Mutants

<table>
<thead>
<tr>
<th></th>
<th>GDP</th>
<th>AMF Activation</th>
<th>GTP$\gamma$S Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model Distance (Å)$^1$</td>
<td>Model Distance (Å)$^1$</td>
<td>AMF$_{\text{exp}}$ (nm)$^2$</td>
</tr>
<tr>
<td>WT</td>
<td>8.63</td>
<td>6.61</td>
<td>2.4</td>
</tr>
<tr>
<td>W131F</td>
<td>9.71</td>
<td>6.50</td>
<td>2.1</td>
</tr>
<tr>
<td>W211F</td>
<td>N/A</td>
<td>N/A</td>
<td>-0.4</td>
</tr>
<tr>
<td>W258F</td>
<td>10.4</td>
<td>6.70</td>
<td>3.2</td>
</tr>
</tbody>
</table>

1 Distance measured in the models from the geometric center of the indole ring in W211 to the carbon atom in the guanidinium group in R208

2 Fluorescence red shifts ($\Delta\lambda_{\text{max}}$) between GDP and AMF or GTP$\gamma$S conformations
distances greater than 8.6 Å were found for all G_{iα1} proteins. In the activated AMF and GTPγS models, however, the R208 and W211 were in the 6.5 to 7.2 Å range (Table 3).

**Trypsin Digestion of G_{iα1} Proteins**

Digestion with trypsin, a protease that cleaves at the carboxyl termini of arginines and lysines, can be used as a tool to distinguish the inactive from the other conformations (47). R208 is solvent exposed in the GDP conformation, but not in the GTPγS or GDP•AMF conformations. SDS-PAGE was applied in conjunction with the digestion to visualize the hydrolysis. WT G_{iα1}•GDP has a molecular weight of 41 kDa (Fig.10, lane 2) and, upon trypsin addition, the band was no longer present (Fig. 10, lane 3). WT G_{iα1}•AMF and GTPγS showed bands at 40.3 kDa (Fig. 10, lanes 4 and 5, respectively) after exposure to trypsin, indicating that activated forms of WT G_{iα1} do not undergo proteolytic cleavage. The same hydrolytic patterns were demonstrated for W131F and W258F G_{iα1} proteins in all three conformations (Fig 10. Lanes 6-9 and 14-17, respectively). W211F•GDP displayed the same proteolytic cleavage in the GDP conformation as observed for the other three proteins (Fig. 10, lanes 10 and 11). However, that was not the case for the GDP•AMF and GTPγS conformations of the W211F mutant; complete proteolysis was found for GDP•AMF conformation (Fig. 10, lane 12), but a light band was observed with the GTPγS conformation suggesting incomplete hydrolysis (Fig. 10, lane 13).

**Discussion**

The focus of this study was to understand the contribution of each tryptophan in WT G_{iα1} toward the overall intrinsic tryptophan fluorescence of the protein and to the bridge formation between R208 and W211. Single point mutations of W → F at residues
Figure 10. Trypsin digests of wild-type and various tryptophan mutants of $G_{i\alpha1}$. Proteins were incubated with either GDP, GTP$\gamma$S, or AlCl$_3$/MgCl$_2$/NaF (AMF) and trypsin where indicated. Lane 1, marker; Lanes 2-5, WT $G_{i\alpha1}$; Lanes 6-9, W131F $G_{i\alpha1}$; Lanes 10-13, W211F $G_{i\alpha1}$; Lanes 14-17, W258F $G_{i\alpha1}$. 

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| GDP  | - | + | + | - | - | + | + | - | - | + | - | - | - | - | - |
| AMF  | - | - | - | + | - | - | + | - | - | + | - | - | + | - | - |
| GTP$\gamma$S | - | - | - | + | - | - | + | - | - | + | - | - | + | - | - |
| Trypsin | - | + | + | - | + | + | + | - | + | + | - | + | + | + | + | + |
131, 211 and 258 were used to investigate their roles. W211 was the major determinant toward the overall change in intrinsic tryptophan fluorescence of activated $G_{i1}$ proteins as confirmed by the absence of a fluorescence increase in W211F $G_{i1}$ incubated with either AMF or GTPγS (Figs. 5 and 6, trace D). However, the observed fluorescence in the emission spectra of W211F $G_{i1}$•GDP clearly indicates contributions from W131 and W258 (Fig. 7). Chabre and co-workers (24) also reported similar findings for the emission of W207F in Gt•AMF. The differences observed in the emission spectra of WT $G_{i1}$ and its W mutants in the GDP conformation can be justified through the total solvent exposure of Trp residues in each respective protein as shown in table 2; column 6. W258F observed the smallest total Δ value (161), followed by W211F (187), then WT $G_{i1}$ (210), and lastly, W131F (346). Smaller values indicate a less solvent exposed environment for the Trp residues. For the GDP form, the fluorescence intensities of the emission spectra followed the same trends as the calculated Δ values with the W258F mutant displaying the largest fluorescence followed by the W211F and WT $G_{i1}$, and the W131F (Fig. 7). In addition, the emission spectra for the AMF and GTPγS conformations of the $G_{i1}$ proteins (spectra not shown) were in agreement with the calculated Δ values (Table 2, column 6). Therefore, the observation of intrinsic fluorescence is not solely dependent on the presence of W211, but W258 and W131 also contribute to the intrinsic fluorescence of $G_{i1}$ subunits. The ranking of the contributions is W211> W258> W131.

Single point mutations can often have an effect on the kinetics of a protein. WT, W131F and W258F had very similar rate values for GTPγS activation as opposed to
what was observed in AMF activation. Previous studies have found that the rate
determining step for GDP → GTPγS exchange in G_{ia1} is the release of GDP (38, 48).
Presumably, binding of GTPγS to GDP – free G_{ia1} occurs at a rate much larger than GDP
release thus explaining the monophasic nature of the curves in Fig. 6. The AMF
activation curves are the sums of two exponentials. The mechanism of AMF activation
may therefore involve a slower step whereby AMF binds to G_{ia1}•GDP followed by a fast
conformational change to G_{ia1}•AMF. The fact that the rates for the two steps involved in
AMF activation do not differ as much for nucleotide exchange account for the biphasic
nature of the former (Fig. 5) and the monophasic appearance of the latter (Fig. 6).

Computational data from Table 2 indicate that both W131 and W258 have finite Δ
values in all conformations of the W211F mutant, thus explaining the observation of
fluorescence in the emission spectra. The data in Table 2 also show that the Δ values of
W131 and W258 in the W211F mutant do not change appreciably with either AMF
activation (20 vs. 21 and 165 vs. 166) or GTP activation (15 vs. 21 and 165 vs. 166),
which accounts for the lack of a fluorescence increase upon the AMF or GTPγS
activation. The validity of using Δ values is supported by experimental results that were
in agreement with predicted fluorescence enhancements calculated from the homology
models (Table 2).

Burial of W211 is not independent from that of W131 or W258, as indicated from
a comparison of the Δ values from WT with either W131F or W258F mutants (Table 2).
Homology models were able to rationalize the differences in % fluorescence (Table 1).
AMF activation of W131F G_{ia1} displays significant contribution from W258 towards its
overall fluorescence (Table 2). Examination of the W258 residue in the homology
models of W131F G\_iα1•GDP and W131F G\_iα1•AMF shows that upon activation W258 moves into a more hydrophobic environment in the mutant than in WT (-73 vs. -18, respectively). The contribution of W131 is best characterized from the activation of the W258F G\_iα1 mutant and the emission spectra of W131F•GDP. Relative to the GDP conformation, the environment of W131 in W258F•AMF and GTPγS is hydrophobic (-20 and -23, respectively), contrary to WT G\_iα1•AMF and GTPγS, which display an increase in solvent exposure (10 and 29, respectively). Despite lacking a Trp, W258F compensates for its fluorescence by the presence of W131, resulting in increases in fluorescence intensity upon activation that are similar to WT protein (Figs. 5 and 6). W131F•GDP exhibits a substantial loss of fluorescence in comparison to WT, W211F and W258F•GDP (Fig. 7). This lack of fluorescence is due to the absence of W131, which is present in the other G\_iα1 proteins. W207F•GDP in G\_i reported similar intrinsic fluorescence to WT G\_i•GDP in their emission spectra (24). Chabre and co-workers demonstrated that, although W207F lacked the key Trp in the GTPase domain responsible for observing the change in fluorescence upon activation with AlF\_4\^-, W127 like W131 in G\_i contributed considerably to the overall fluorescence.

ANS increases its fluorescence and is more blue shifted when exposed to increasingly polar solvents (20). Electrostatic interactions between lysines and arginines with aromatic amino acids occur when they are less than 10 Å apart (45). The presence of a red shift in the λ\_max of the emission spectra for activated WT and W258F G\_iα1 proteins was previously studied (25). Hamm and co-workers (25) reported that WT G\_iα1•GDP displayed a red shift upon addition of AlF\_4\^- or exchange with GTPγS because of the formation of a bridge between R208 and W211 (25). We further investigated how the
R208 – W211 interaction compared in WT and W mutants. The electrostatic interaction was removed by deletion of W211, as seen in the W211F mutant which displayed blue shifts in both the AMF and GTPγS conformations (Fig. 9). These observations were in accordance with previous ANS studies in which π – cation electrostatic interactions are absent (20). The blue shifts of -0.4 nm are also consistent with the shift of -0.4 observed for the R208C mutant, where the electrostatic interaction was also disrupted (25). WT and tryptophan mutants showed distinct red shifts, and to understand their relative Δλ_{max} values exploration of the structural models was necessary.

Distances were measured between the C atom on the guanidinium group of R208 and the geometric center of the indole ring in the tryptophan for each protein in all conformations (Table 3). There were, however, no apparent correlations between the shifts observed for each protein in either the transition state or active conformation and the measured distances (Table 3). We suggest that the magnitude of the red shift is dependent on the interaction energies between R208 and W211 in each protein.

Molecular dynamics calculations of the R208 - W211 salt bridge in WT, W131F, and W258F G_{iα1} proteins indicate that both the electrostatic and Van der Waals (VdW) energies become more negative (stronger) when activating from the GDP form to the AMF or GTPγS conformations (Table 4), with the effect being greater for the electrostatic than for VdW energies. The strongest electrostatic interaction in the AMF conformation was calculated for W258F, which also displayed the largest red shift.
Table 4. Interaction Energies between R208 and W211

<table>
<thead>
<tr>
<th></th>
<th>Elec (E)</th>
<th>VdW</th>
<th>Total</th>
<th>$\Delta E$ $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT GDP</td>
<td>-0.96</td>
<td>-3.38</td>
<td>-4.34</td>
<td>n/a</td>
</tr>
<tr>
<td>W131F GDP</td>
<td>-1.12</td>
<td>-3.37</td>
<td>-4.49</td>
<td>n/a</td>
</tr>
<tr>
<td>W258F GDP</td>
<td>-0.47</td>
<td>-3.12</td>
<td>-3.59</td>
<td>n/a</td>
</tr>
<tr>
<td>WT AMF</td>
<td>-2.47</td>
<td>-4.33</td>
<td>-6.80</td>
<td>-2.46</td>
</tr>
<tr>
<td>W131F AMF</td>
<td>-2.13</td>
<td>-4.64</td>
<td>-6.77</td>
<td>-2.28</td>
</tr>
<tr>
<td>W258F AMF</td>
<td>-2.89</td>
<td>-4.35</td>
<td>-7.24</td>
<td>-3.65</td>
</tr>
<tr>
<td>WT GTPγS</td>
<td>-2.85</td>
<td>-4.38</td>
<td>-7.23</td>
<td>-2.89</td>
</tr>
<tr>
<td>W131F GTPγS</td>
<td>-2.66</td>
<td>-4.42</td>
<td>-7.08</td>
<td>-2.59</td>
</tr>
<tr>
<td>W258F GTPγS</td>
<td>-2.30</td>
<td>-4.47</td>
<td>-6.76</td>
<td>-3.17</td>
</tr>
</tbody>
</table>

$^1$ values are in units of kcal/mol

$^2$ Differences in the total interaction energies, between AMF or GTPγS and GDP of the corresponding G$_{ia1}$ protein. These values are averages of 100 (or 101) steps with standard deviations of approximately 1.0.
W131F observed the weakest interaction and subsequently the smallest red shift. The same relationship trend between the electrostatic interaction and shift in the emission spectrum was observed for WT and its W mutants in the GTPγS form (Table 4). The exception was W131F•GTPγS, which displayed the largest red shift, but the lowest interaction energy. This may be explained by the large standard deviation (SD) calculated for the shift in W131F•GTPγS. All G reddiam•GTPγS proteins had a SD of 0.2 nm or lower, while W131F had a considerably high SD of 0.5 nm.

Digestion with trypsin was used as an indirect method to investigate the role of R208 in the bridge formation. Tryptic cleavage, which was visualized by using the SDS-PAGE, is conformation – dependent, as reported before (24). The conformational change of WT Giial•GDP activation with the addition of AlF4- or exchange with GTPγS protects the protein from cleavage (26). WT, W131F, and W258F all displayed similar characteristics in each of the respective conformations. However, W211F had some key differences in its AMF and GTPγS conformations. Hydrolysis of the W211F•AMF form indicates that the electrostatic interaction between R208-W211 is necessary to anchor R208 into a hydrophobic pocket, where it will not be exposed to the proteolytic activity of trypsin. Trypsinization of Giial•GDP proteins produced fragments of 23 Kd and 18 Kd, which are equivalent to the weights of residues 1-208 and 209-354, respectively, confirming that R208 is the site of proteolysis. To explain the experimental observations, we calculated the solvent exposure of R208 in the molecular models of WT and all three Trp mutants for all conformations. As expected, all proteins in the GDP conformation had relatively high Δ values, but in the AMF and GTPγS conformations, WT, W131F, and W258F, R208 was more solvent exposed (Table 5; Fig. 11). Compared to the AMF
conformations of the other proteins, the solvent exposure of R208 in W211F•AMF reveals higher Δ values, which range from 72 to 52 (Fig. 11), and larger standard deviations (Table 5). These calculations support our conclusion that, without the formation of the bridge, R208 is left solvent exposed and vulnerable to tryptic cleavage. In contrast to the agreement between observed and predicted hydrolysis for W211F•AMF, the molecular dynamics calculations for W211F•GTPγS suggested protection from hydrolysis, but partial tryptic cleavage was observed. We suggest that the molecular dynamics calculations may either not be sensitive enough or the time allowed was not sufficient to observe the effect.

Taken together, these results provide insight into the overall movement of the tryptophan residues in G<sub>ia1</sub> as studied from a comparison of the WT with the W131, W211, and W258 proteins, and that the R208-W211 bridge formation is a very important aspect of the protein’s structural integrity.
Table 5. Computational Calculations for Solvent Exposure of R208 in the Molecular Models. Solvent exposure values and standard deviations (SD) were calculated from the last 100 steps of the molecular dynamics calculations.

<table>
<thead>
<tr>
<th></th>
<th>Δ</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT G_{i\alpha}•GDP</td>
<td>133</td>
<td>11.3</td>
</tr>
<tr>
<td>W131F•GDP</td>
<td>149</td>
<td>8.39</td>
</tr>
<tr>
<td>W211F•GDP</td>
<td>197</td>
<td>7.23</td>
</tr>
<tr>
<td>W258F•GDP</td>
<td>158</td>
<td>17.4</td>
</tr>
<tr>
<td>WT G_{i\alpha}•AMF</td>
<td>41.5</td>
<td>5.40</td>
</tr>
<tr>
<td>W131F•AMF</td>
<td>42.3</td>
<td>4.18</td>
</tr>
<tr>
<td>W211F•AMF</td>
<td>52.4</td>
<td>6.01</td>
</tr>
<tr>
<td>W258F•AMF</td>
<td>41.5</td>
<td>4.57</td>
</tr>
<tr>
<td>WT G_{i\alpha}•GTPγS</td>
<td>40.1</td>
<td>4.52</td>
</tr>
<tr>
<td>W131F•GTPγS</td>
<td>41.6</td>
<td>4.57</td>
</tr>
<tr>
<td>W211F•GTPγS</td>
<td>41.8</td>
<td>4.20</td>
</tr>
<tr>
<td>W258F•GTPγS</td>
<td>41.9</td>
<td>4.27</td>
</tr>
</tbody>
</table>
Figure 11. Molecular dynamic calculations of R208 solvent exposure in WT and its three Trp mutants in all conformations.
CHAPTER THREE
FOLDING OF Gα SUBUNITS: CHANGES IN THE ENVIRONMENTS OF TRP AND TYR RESIDUES AND ON THE SECONDARY STRUCTURE

Introduction
G proteins are heterotrimeric membrane-bound guanine-nucleotide binding proteins that mediate transduction of extracellular signals to various intracellular effectors (1). Once a neurotransmitter or hormone binds to an extracellular receptor, it triggers the activation of the corresponding G protein. Activation is accompanied by a conformational change in which GDP bound to the α subunit exchanges for GTP and the βγ dimer dissociates. G_{iα1} and G_{sα} are the inhibitory and stimulatory, respectively, α-subunits that are responsible for the decreased or increased production of the secondary messenger adenosine-3,’5’-monophosphate (cAMP) from adenosine triphosphate (ATP) (30). The activated G protein returns to its resting, GDP-bound inactive state via hydrolysis of bound GTP and release of inorganic phosphate. GTP binding impacts the structures of three flexible loop segments located near the γ-phosphate, which are named switches I, II, and III. In the GTP-bound state, these switch regions are held in place by the γ-phosphate whereas in the GDP-bound conformation they are less ordered (13). In vitro activation may also occur through binding of AlF₄⁻ to the β – phosphate of GDP, with AlF₄⁻ mimicking the γ – phosphate of GTP and forming a transition state (AMF) conformation. The crystal structures of WT G_{iα1}•GTP (1GIA) and AMF (1GFI)
conformations depict similar folding patterns (12), which are significantly different from the WT G\textit{\textsubscript{ia1}}•GDP structure (1BOF) (13).

The structure of a protein is essential for its biological function. Thus, alteration in protein folding may cause loss of activity and denaturation, which most often leads to disease. Different types of cancers are associated with mutated G\textit{\textsubscript{a}} proteins. Analyses of tumors in the pituitary gland, small intestine, thyroid, and biliary tract have shown hot spot mutations of G\textit{\textsubscript{sa}} at R201 and Q227 (27). Mutations at R208 in G\textit{\textsubscript{ia1}} may also result in carcinomas in the large intestine (27). Therefore, investigating the folding pathway of G\textit{\textsubscript{a}} subunits may lead to information that may be useful to the development of possible chemotherapeutic agents.

In this investigation, we used fluorescence and UV/Vis spectroscopy and circular dichroism (CD) to monitor the folding of G\textit{\textsubscript{ia1}} and G\textit{\textsubscript{sa}} subunits. Activation of G proteins can be probed indirectly through intrinsic tryptophan fluorescence, a tool that was pioneered by Gilman and co-workers (38). As observed for other proteins (39), when tryptophan residues move into less-solvent exposed environments the fluorescence intensity increases (Figs. 12a and 12b). The polarity of the environment also affects the \(\lambda_{\text{max}}\) values of W residues by shifting them to lower wavelengths, i.e., \(\lambda_{\text{max}}\) undergoes a blue shift (20). Increases in fluorescence intensity were observed when either the active conformation was formed upon the exchange of GDP with GTP\gamma\textsubscript{S} (non-hydrolyzable analog) or the transition state conformation was attained via addition of AlF\textsubscript{4}\textsuperscript{−} to G\textit{\textsubscript{ia1}}•GDP (38). Site directed mutagenesis was performed on each tryptophan, thereby converting each residue to a phenylalanine (F). Fluorescence was used to investigate the
Figure 12. WT $G_{i1} \cdot GDP$ displaying its three tryptophan residues, Arg208, and $\text{Mg}^{2+}$ (green sphere)
Figure 12. WT $G_{\text{i}}\alpha_1\cdot GTP\gamma S$ depicting the same residues at Fig. 12 except the bound nucleotide is $GTP\gamma S$. 
microenvironments of the three tryptophan residues in G\textsubscript{ia1} (W211 located in the conformational-dependent switch II region, and W131 and W258 that are located in the alpha helical and GTPase domains, respectively). The mutants obtained (W131F, W211F, and W258F) were then used to explore the environment of each tryptophan residue and its contribution to the overall protein stability. For G\textsubscript{ia1}, the increase in fluorescence intensity upon activation depended primarily on the presence of W211, as demonstrated by a decrease in fluorescence in the W211F mutant (Najor et al., unpublished observations). Because tyrosine residues are abundant on the surface of G\textsubscript{a} subunits, UV – Vis spectroscopy allowed us to monitor protein folding at the exterior of the protein. CD helped explore the unfolding of G\textsubscript{a} through changes that occurred in the secondary structure.

The focus of this study was therefore to obtain a global understanding of the folding of G\textsubscript{a} subunits by using complimentary spectroscopic approaches.

**Materials and Methods**

**Expression and Protein Purification**

G\textsubscript{ai1} and G\textsubscript{as} were obtained and purified as described previously (41). W131F, W211F, and W258F mutants were prepared by site directed mutagenesis using a kit provided by Stratagene (La Jolla, CA). Proteins were dialyzed overnight at 4 °C in 20 mM Tris pH 8.0 buffer containing 10% (v/v) glycerol, and 1 mM DTT and then stored at -80 °C. Protein purity was greater than 95% as estimated by SDS – PAGE. After purification on a Ni\textsuperscript{2+} column, activated G\textsubscript{a} protein was prepared by adding stoichiometric concentrations of GTP\textsubscript{γS} to G\textsubscript{ia1}\textbullet GDP. Further purification on a size exclusion column was performed to separate free GTP\textsubscript{γS} from G\textsubscript{a} bound GTP\textsubscript{γS}. 
Fluorescence Measurements

Experiments were performed with a PTI QuantaMaster fluorimeter (Photon Technologies, Inc., Birmingham, NJ). Time-based assays were conducted with excitation and emission wavelengths set at 280 nm and 340 nm, respectively. Assays were initiated after 60 sec by addition of either AlF<sub>4</sub> as a premixed solution (10 μM AlCl<sub>3</sub> and 10 mM NaF) or 20 μM GTPyS to pre-incubated 500 G<sub>ia</sub> nM protein samples in buffer containing 10 mM HEPES, pH 7.5, 1 mM DTT and 2 mM MgSO<sub>4</sub>. Activation was monitored in the temperature range 4 °C → 60 °C in samples that were incubated for 5 min at each temperature studied before addition of AlF<sub>4</sub> and GTPγS. Time-based assays were normalized to zero at 60 and 65 sec for AlF<sub>4</sub> and GTPγS activation, respectively. Upon addition of the GTPγS nucleotide, there was a dramatic decrease in fluorescence intensity for an estimated 5 sec due to nucleotide induced quenching (48). Therefore, normalization at 65 sec indicates the time point at which GTPγS exchanges with GDP.

Emission data was recorded over the wavelength range of 300 - 400 nm with the excitation wavelength set at 280 nm. Signal integration time was 0.2 sec with the bandpass for excitation and emission set at 5 nm. Sample preparation was the same as for time-based assays. The denaturation experiments started at a temperature of 4 °C followed by 4 °C increments and concluding at the highest temperature before aggregation occurred. There was a 2 min equilibration for each set temperature.

Tyrosine Assay

Environment of tyrosine residues in G<sub>ia</sub> proteins were monitored on a Hewlett Packard UV – Vis spectrophotometer. All samples contained 50 mM Tris, pH 7.5, 1
mM DTT, and 2 mM MgSO₄. Prior to initiating the experiment, samples in the transition state conformation (AMF) were incubated with 2.5 μM Gₐα₁•GDP, 20 μM AlCl₃ and 10 mM NaF at room temperature for 20 min, whereas samples in the active conformation (GTPγS) were incubated with 2.5 μM Gₐα₁•GTPγS and 20 μM GTPγS at room temperature for 1 hr minimum. Temperature was varied from 20 °C to 80°C, and was increased with 1 °C increments. For each temperature studied, the samples were equilibrated for 1 min and the absorbance was monitored over the wavelength range of 220 – 300 nm.

**Determination of Secondary Structure**

Experiments were performed using an Olis DSM 20 circular dichroism spectrophotometer. All samples were measured in a cylindrical quartz cuvette with a 1 mm pathlength, and contained either 3 μM Gₐα₁•GDP or GTPγS, in 10 mM phosphate pH 7.5 buffer, 1 mM DTT, and 2 mM MgSO₄. A mixture of 50 μM AlCl₃ and 10 mM NaF was added to Gₐα₁•GDP samples to form the AMF conformation, whereas an additional 20 μM GTPyS was added to Gₐα₁•GTPγS samples to ensure saturation of the active conformation. Data was collected every 1 nm in the wavelength range of 190 – 260 nm. Time acquisition was determined as a function of high voltage. Temperature was varied from 20 °C to 100 °C with an incubation time of 3 min at each temperature studied. The OlisGlobalworks software was used to deconvolute the spectra and calculate percent of secondary structure and melting temperature.

**Refolding of Gₐ Subunits**

To test whether refolding of Gₐ proteins was reversible, fluorescence emission scans and CD were used. When results from the final temperature of an unfolding
experiment were obtained, the Ga sample was cooled down in 8 °C increments and incubation times remained the same as indicated above for each respective technique. Final temperatures varied depending on aggregation and ability to refold. All renaturation experiments were stopped at 4 °C and 20 °C for fluorescence and CD experiments, respectively.

Results

AMF and GTPγS Activation of Gi1α

Intrinsic tryptophan fluorescence is a useful technique to probe the folding of proteins. Upon the binding of AlF4⁻ or exchange of GTPγS, the G protein becomes activated and its tryptophan residues fold into more hydrophobic environments (38). We studied the ability of WT Gi1α1 and its W mutants to activate as a function of temperature.

Addition of AlF4⁻ to WT Gi1α1•GDP•Mg2⁺ causes an exponential increase in fluorescence followed by a plateau (Fmax) (Fig. 13). We performed activation assays as a function of temperature (in the 4 °C → 52 °C range). As the temperature was increased from 4 °C to 28 °C, there was an incremental increase in the rate and Fmax. Temperatures above 28 °C resulted in lower Fmax values and continued increased rates, with activation ceasing at 52 °C (data not shown). Activation that occurred after 44 °C saw a linear decay immediately after reaching its Fmax; the same trends were observed for AlF4⁻ activation in the Trp mutants (data not shown). W211F activity could not be monitored
Figure 13. Temperature dependence of AlF₄⁻ activation of WT G₁α1•GDP•Mg²⁺
because of the absence of a change in fluorescence when going from the GDP to the AMF conformation (see chapter 2 of dissertation). The same approach was used for the exchange of GTPγS with WT $G_{i\alpha 1}\cdot$GDP and its Trp mutants. It resulted in the same fluorescence trends observed earlier with the binding of AlF₄⁻, but with dramatically slower rates (Fig. 14). There were a few key differences. Maximal fluorescence was achieved at a higher temperature of 40 °C as opposed to 32 °C obtained in AMF activation, and there was an absence of exchange at 60 °C vs. 52°C (data not shown).

**Fluorescence Emission Spectra of $G_{\alpha}$ Proteins**

The time-based assays provided useful information into the temperature dependence of the activation of $G_{i\alpha 1}$, but were not helpful in determining the folding pathway of $G_{i\alpha 1}$. However, as shown below, fluorescence emission spectra of $G_{\alpha}$ provided insight into its folding through changes in the environment of all tryptophan residues present in WT $G_{s\alpha}$, WT $G_{i\alpha 1}$ and the $G_{i\alpha 1}$ W mutants.

A decrease in intensities of the emission spectrum of WT $G_{i\alpha 1}\cdot$GDP is observed with an increase in temperature (Fig. 15). This pattern continues until a change in fluorescence vanishes at 96 °C. A transition midpoint of 39.0 °C was calculated for WT $G_{i\alpha 1}\cdot$GDP; similar $T_m$’s were observed for the GDP conformation of tryptophan $G_{i\alpha 1}$ mutants and of WT $G_{s\alpha}$ (Table 6). The $T_m$’s calculated from fluorescence emission spectra for WT $G_{i\alpha 1}\cdot$AMF and its tryptophan mutants, and for WT $G_{s\alpha}\cdot$AMF were also similar to those of their respective GDP conformations. With the exception of W211F and WT $G_{s\alpha}$, the $T_m$’s for WT $G_{i\alpha 1}$ and its Trp mutants in the GTPγS conformation were ~18 °C higher than their respective GDP and AMF conformations.
Figure 14. Temperature dependence of GTPγS Activation of WT $G_{i\alpha 1}\cdot GDP\cdot Mg^{2+}$
Figure 15. Fluorescence emission spectra of WT G_{iα1}•GDP•Mg^{2+} as a function of temperature.
Table 6. $T_m$’s Estimated for all $G\alpha$ Proteins Using Three Spectroscopic Methods.$^{1,2}$

<table>
<thead>
<tr>
<th></th>
<th>CD</th>
<th>UV-Vis</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GDP</td>
<td>AMF</td>
<td>GTP(\gamma)S</td>
</tr>
<tr>
<td><strong>WT G(_{ia1})</strong></td>
<td>44.2</td>
<td>47.5</td>
<td>70.9</td>
</tr>
<tr>
<td><strong>W131F</strong></td>
<td>44.0</td>
<td>48.8</td>
<td>70.7</td>
</tr>
<tr>
<td><strong>W211F</strong></td>
<td>54.3</td>
<td>57.0</td>
<td>56.5</td>
</tr>
<tr>
<td><strong>W258F</strong></td>
<td>50.2</td>
<td>51.9</td>
<td>68.1</td>
</tr>
<tr>
<td><strong>WT G(_{sa})</strong></td>
<td>42.7</td>
<td>56.0</td>
<td>56.0</td>
</tr>
</tbody>
</table>

$^1$S.E.M $\leq 3$

$^2$All values reported in °C

$^3$Not measured
We also investigated the ability of Go subunits to refold after completion of the
denaturation process. A decrease in temperature was accompanied by an increase in the
fluorescence intensity of the emission spectra indicating that the tryptophan residues were re-
folding back into hydrophobic environments (Fig. 16). Refolding WT Giα1•GDP from
96 °C back to 4 °C exhibited no significant increase in fluorescence. However, upon
renaturation from 48 °C, the observed increase in the fluorescence intensity indicates a
refolding recovery of 21% (Fig. 17). When refolding from 32 °C, which is a temperature
below its Tm, WT Giα1•GDP exhibited the largest recovery of 72%. Unlike WT
Giα1•GDP, the AMF and GTPγS conformations experience increases in fluorescence
intensity when refolding from 96 °C. Comparison of denaturation data observed for
GTPγS at 70 °C and 44 °C indicates more refolding than for the AMF conformation (see
bars at 48 °C and 32 °C in Fig. 17). WT Giα1•AMF refolded 17% of its structure after
denaturation from 76 °C, whereas WT Giα1•GTPγS reported the most recovery by
regaining 30% of its fluorescence after unfolding to 84 °C (data not shown).

Tyrosine Assays

Trp residues become buried upon activation with either AMF or GTPγS, and thus
they probe the interior of Giα1. However, Tyr residues in Giα1 are located at its outer
surface, and therefore can be used to monitor temperature – induced changes at the
exterior of Giα1. There are 13 tyrosine (Y) residues in the amino acid sequence of Giα1
which are located throughout the surface of both the GTPase and alpha – helical domains
(Fig. 18). Tyrosine absorbs at a wavelength of 280 nm and is most commonly used to
calculate protein concentration. As the temperature was increased, the changes in the
Figure 16. Refolding of WT $G_{i1}\cdot\text{GTP}\gamma S$. Temperatures are in ($^\circ$C) and R represents refolded $G_{i1}$.
Figure 17. Refolding of WT G_{i31} in all three conformations as monitored by fluorescence. Temperatures denote the end of denaturation and beginning of renaturation process.
Figure 18. WT G_{i\alpha}•GDP depicting its 13 Tyr residues
absorption spectrum of WT \( G_{\text{ia1}} \cdot \text{GDP} \) were monitored; no changes in its absorption spectrum occurred from 20 °C \( \rightarrow \) 43 °C, but an increase in absorbance was observed at temperatures above 44 °C. As the protein was subjected to increasing amounts of heat, its Tyr residues became more solvent exposed causing the absorption at 280 nm to increase. The melting curves for \( G_{\text{ia1}} \) in the GDP and AMF states overlapped, but the GTP\( \gamma \)S curve was shifted to the right indicating increased stability for this active conformation. The same trends were observed for W mutants of \( G_{\text{ia1}} \) in all conformations (Fig. 19). The calculated \( T_m \) values from tyrosine assays are listen in Table 6.

**Temperature-Dependence of Secondary Structure \( G_{\text{ia1}} \)**

The crystal structure of WT \( G_{\text{ia1}} \cdot \text{GDP} \) (1BOF) reports the secondary structure composition as being 47% \( \alpha \) – helices and 12% \( \beta \) – sheets (13). Tanaka and co – workers (49) used CD to investigate the secondary structure of \( G_{\text{ia1}} \cdot \text{GDP} \) in solution and found that its alpha helical content was consistent with the X – ray structure; using two algorithms, CCA and SELCON, respectively, they estimated it to consist of 50.6% and 55.9% \( \alpha \) - helices with. The near UV wavelength range of the CD spectrum of \( G_{\alpha} \) proteins was used to investigate the unfolding of \( G_{\alpha} \) secondary structure as a function of temperature. A maximum at 190 nm, and minima at 205 nm and 222 nm are signatures of \( \alpha \) – helical predominant structures, but a comparatively lower maximum at 192 nm and a minimum at 215 nm are distinctive features of \( \beta \) – sheets. Spectra of WT \( G_{\text{ia1}} \cdot \text{GDP} \) at a temperature of 20 °C (Fig. 20) resembled that of an \( \alpha \) – helical dominated structure with a 39.7 % \( \alpha \) – helix composition, which is less than the reported PDB value (Table 7). The CD spectra remained unchanged in the 20 °C to 32 °C range. As the temperature
Figure 19. Unfolding of WT G_{iα1} as monitored through Tyr movement
Figure 20. Unfolding of WT G$_{\text{ial}}$•GDP•Mg$^{2+}$ secondary structure. Temperature units are (°C)
Table 7. Composition of WT G_{\text{id1}} Secondary Structure at Various Temperatures.\textsuperscript{1,2,3}

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>(\alpha)</th>
<th>(\beta)</th>
<th>RC\textsuperscript{4}</th>
<th>T\textsuperscript{4}</th>
<th>(\alpha)</th>
<th>(\beta)</th>
<th>RC\textsuperscript{4}</th>
<th>T\textsuperscript{4}</th>
<th>(\alpha)</th>
<th>(\beta)</th>
<th>RC\textsuperscript{4}</th>
<th>T\textsuperscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>41.3</td>
<td>14.7</td>
<td>26.3</td>
<td>18.0</td>
<td>41.5</td>
<td>14.3</td>
<td>19.3</td>
<td>16.8</td>
<td>44.3</td>
<td>12.3</td>
<td>25.7</td>
<td>17.7</td>
</tr>
<tr>
<td>40</td>
<td>40.3</td>
<td>14.7</td>
<td>26.7</td>
<td>18.3</td>
<td>42.0</td>
<td>14.3</td>
<td>24.5</td>
<td>18.0</td>
<td>42.3</td>
<td>13.7</td>
<td>24.0</td>
<td>20.3</td>
</tr>
<tr>
<td>52</td>
<td>27.7</td>
<td>21.3</td>
<td>29.0</td>
<td>21.7</td>
<td>28.3</td>
<td>22.0</td>
<td>26.0</td>
<td>20.5</td>
<td>41.7</td>
<td>13.3</td>
<td>24.7</td>
<td>20.3</td>
</tr>
<tr>
<td>64</td>
<td>23.3</td>
<td>28.0</td>
<td>28.7</td>
<td>21.0</td>
<td>22.8</td>
<td>26.8</td>
<td>28.6</td>
<td>22.0</td>
<td>38.7</td>
<td>16.0</td>
<td>24.3</td>
<td>20.7</td>
</tr>
<tr>
<td>80</td>
<td>19.7</td>
<td>29.3</td>
<td>28.7</td>
<td>23.0</td>
<td>19.0</td>
<td>28.8</td>
<td>32.2</td>
<td>23.8</td>
<td>23.0</td>
<td>25.7</td>
<td>26.0</td>
<td>23.7</td>
</tr>
<tr>
<td>92\textsuperscript{3}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21.5</td>
<td>29.0</td>
<td>25.5</td>
<td>24.5</td>
</tr>
</tbody>
</table>

\textsuperscript{1}S.E.M \leq 3
\textsuperscript{2}All numbers reported as percentages
\textsuperscript{3}Hyphens denote temperatures at which proteins were denatured
\textsuperscript{4}RC and T stand for random coil and turns
increased from 36 °C → 64 °C, the CD spectra showed the most dramatic changes in secondary structure. Absorption at 190 nm decreased, while the minima at 205 nm and 222 nm increased until they converged into a new minimum at 215 nm. The once mainly α – helical protein became increasingly dominated by β – sheet (Table 7). Temperatures after 64 °C displayed little change in the spectra and the protein eventually precipitated out at 84 °C. A T_m of 44.2 °C was calculated for WT G_{i1}•GDP. The W•GDP mutants of G_{i1} observed the same spectral trends with W211F affording the highest transition midpoint (Table 6). A transition midpoint of 42.7 °C was calculated for WT G_{s}\•GDP. The spectra of WT G_{s}\•GDP displayed a similar unfolding pattern as observed with WT G_{i1}•GDP, however, the increases in the absorption at 205 nm and 222 nm as G_{s} was denatured were less apparent (Fig. 21).

WT G_{s}, WT G_{i1} and all the W mutants observed the same trends in their AMF conformation as their GDP counterparts with higher transition midpoints calculated from the CD spectra (Table 6). Lastly, the GTPγS conformation was explored for WT G_{s}, WT G_{i1} and its W mutants. Except for a few important differences, the same pattern was observed in the spectra of G_{α} protein solutions in the GTPγS conformation. The G_{i1} samples were able to reach temperatures of 100 °C without aggregation and the transition midpoints were significantly higher than both the GDP and AMF conformations. At 80 °C, the secondary structure consisted of at least 4.0 % more α – helix than its GDP and AMF conformations. In contrast, WT G_{s} and W211F in the GTPγS active state displayed T_m’s closest to their AMF conformations (Table 6).
Figure 21. Unfolding of WT Gₛₐ•GDP•Mg²⁺ secondary structure. Temperature units are (°C)
Reversibility commenced at the end of the denaturation process. As WT $G_{i\alpha1}$•GDP was refolded from 76 °C to 4°C there was a subsequent increase at 190 nm and a decrease at 222 nm in the CD spectra (Fig. 22). At 76 °C, $G_{i\alpha1}$ consisted primarily of 28% $\beta$–sheets and 23% $\alpha$-helices. Refolding to 20 °C increased the alpha helical content to 28% and the percent of $\beta$–sheets decreased to 21%. Stopping the denaturation process at 52 °C rather than 76 °C resulted in recovery of more $\alpha$–helical structure (35%), while the $\beta$–sheet composition remained the same. At 80 °C, WT $G_{sa}$•GDP exhibited a similar denaturation pattern that was comprised of 26% $\beta$–sheets and 24% $\alpha$–helices. Upon renaturation at 20 °C, the alpha helical composition increased to 29% and $\beta$–sheets decreased to 22%. The refolding of the active GTPγS conformation was also investigated for $G_{sa}$. Incubation to 80 °C resulted in predominantly $\beta$–sheet (25%) secondary structure and 19% $\alpha$–helices. When refolded back to 20 °C, $G_{sa}$ was able to regain 11% of its alpha helical content and its $\beta$–sheets decreased by 1%.

**Discussion**

Activation of WT $G_{i\alpha1}$ with AMF and GTPγS observed some very distinct qualities that can be attributed to its functionality. When comparing their respective rates, GTPγS activation resulted in much slower rates than in AMF activation (Fig. 13 vs. Fig. 14). $G_{i\alpha1}$ must first release its bound GDP before GTPγS can bind; the release of GDP is the rate determining step (23). In contrast, $\text{AlF}_4^-$ can immediately bind to the $\beta$ phosphate of the already bound GDP (48). Thus, the difference in rates can be attributed to a two-step vs. one step process for GTPγS and AMF activation, respectively.
Figure 22. Denaturation and refolding of WT $G_{ia1} \cdot GDP$.
Temperatures are in (°C) and R represents refolded $G_{ia1}$ at 20 °C.
GTPγS activation resulted in an $F_{\text{max}}$ value at 40 °C as opposed to 32 °C with the addition of AlF$_4^-$\textsuperscript{-}. It is suggested that the heat capacity of the protein in the AlF$_4^-$\textsuperscript{-} conformation is lower than in the GTPγS form. This difference would explain why GTPγS can still bind and induce protein folding at temperatures above 50 °C where AlF$_4^-$\textsuperscript{-} loses its ability to bind and undergo activation. In order to gain further knowledge on the folding of $G_{i\alpha 1}$ independent of the state of activation, the emission spectra of each conformation was investigated at various temperatures.

Comparison of the $G_{i\alpha 1}$ crystal structures in the GDP, AMF and GTPγS conformations reveals that the GDP – bound structure is less compact than that of the active conformations of AMF and GTPγS (12, 13). The crystal structure of $G_{i\alpha}\cdot\text{GTPγS}$ (1AZT, (31)) displays comparable folding as observed in $G_{i\alpha 1}\cdot\text{GTPγS}$. One would predict the additional folding present in AMF and GTPγS, compared to the GDP form, would result in higher $T_m$’s for activated $G_{i\alpha 1}$, because the tryptophan residues are buried in hydrophobic pockets that require more heat to induce unfolding. The $G_{i\alpha 1}$ crystal structures of AMF and GTPγS are nearly identical, thus one would expect the $T_m$’s to be comparable, but this was a not observed for both $G_{\alpha}$ subunits. The GTPγS form was significantly more stable than both the GDP and AMF conformations as indicated by a comparison of their transition midpoints (Table 6). We suggest that at a temperature near the $T_m$ of the GDP, AlF$_4^-$\textsuperscript{-} is released from the β-phosphate of GDP and assumes the conformation of the inactive state and continues unfolding via a pathway analogous to the GDP form. The release of AlF$_4^-$\textsuperscript{-} would explain the similar transition midpoints for $G_{i\alpha 1}\cdot$ GDP and $G_{i\alpha 1}\cdot$ AMF. $^{19}$F NMR would provide a useful technique to detect whether the AlF$_4^-$\textsuperscript{-} becomes unbound, thus rendering the protein inactive in its GDP conformation. In
conclusion, we propose that the difference in $T_m$ values for the active conformations may be due to a lower heat capacity of AlF$_4^-$ vs. GTPγS. The increased rates and maximum fluorescence observed in the time-based assays of $G_{iα1}$ for both active conformations can be accounted for by a kinetic effect taking place. As the protein absorbs heat, it can bind AlF$_4^-$ or release GDP faster depending on the mechanism of activation.

Since structures of $G_{sα}$•GDP or AMF have not been solved, it is not possible to know whether $G_{sα}$ follows the same folding pattern as for $G_{iα1}$. The fact that the $T_m$’s for all conformations, including the GTPγS, are not significantly different suggest that protein folding around the Trp residues in $G_{sα}$ and $G_{iα1}$ is distinct. W211F•GTPγS mutant was the least stable of all the $G_{iα1}$ proteins and displayed similar transition midpoints in all conformations. This is contrary to what was found in the other $G_{iα1}$ proteins that showed higher melting temperatures for the GTPγS conformation. Previous work by Najor et al (unpublished work) and Hamm and coworkers (25) show that W211 forms an electrostatic interaction with R208 in WT $G_{iα1}$•AMF and GTPγS. It is suggested that this bridge is playing a crucial role in the structural stability of $G_{iα1}$. The electrostatic interaction presumably acts as an anchor stabilizing the protein from unfolding.

The tyrosine absorbance assays allowed for the visualization the global unfolding of $G_{iα1}$ from another prospective. There are thirteen Tyr residues contained in $G_{iα1}$ and many of them are located at its surface. Therefore the calculated $T_m$’s from the tyrosine absorbance spectra probe how the overall protein unfolds as compared to gleaning information about the local environments of tryptophan residues from the emission spectra. The results were consistent with what was observed in the fluorescence emission denaturation experiments. With the exception of the W258F mutant, the AMF
conformation of all $G_\alpha$ proteins had similar $T_m$’s to those observed the GDP form, while the GTPγS conformation was the most stable of all $G_{ia1}$ proteins (Table 6). However, the transition midpoints calculated for $G_{ia1}$ in all conformations observed in the tyrosine assay were consistently higher than the $T_m$’s calculated with the emission spectra. This difference may be explained through the hydrophobic interactions in $G_{ia1}$, which is a fundamental determinant of folding in all proteins. Since non-covalent interactions are the driving force in protein folding, the observed $T_m$ values would suggest that the denaturation of $G_{ia1}$ would initiate in the vicinity of tryptophan residues and then propagate to the entire protein, including the tyrosine residues.

The secondary structure of WT $G_{ia1}$ and WT $G_{sa}$ were the most stable in their GTPγS form followed by their AMF and GDP conformations. When comparing all conformations at 20 °C, there was an increase in the $\alpha$–helical content of $G_{ia1}$ upon activation with AlF$^-$ or exchange with GTPγS, but, for $G_{sa}$, only the latter was true. Activation of $G_\alpha$ subunits creates a hydrophobic pocket from the folding of the switch regions into more ordered secondary structure (2), thus explaining the increased $\alpha$-helices and decrease of random coil in secondary structure (Table 8). As $G_{ia1}$ proteins unfolded in their GDP and AMF conformations, precipitation at temperatures above 84 °C was observed. In contrast, $G_{sa}$ exhibited aggregation in all conformations at temperatures above 80°C. At this point in the unfolding process, the $G_\alpha$ subunits were rich in $\beta$–sheet composition and their random coil was fairly unchanged from the native
Table 8. Composition of WT G$_{sa}$ Secondary Structure at Various Temperatures.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>GDP$^{12}$</th>
<th>AMF$^{12}$</th>
<th>GTP$_{yS}^{12}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
<td>RC$^3$</td>
</tr>
<tr>
<td>20</td>
<td>34.8</td>
<td>17.2</td>
<td>31.9</td>
</tr>
<tr>
<td>40</td>
<td>28.8</td>
<td>20.5</td>
<td>33.2</td>
</tr>
<tr>
<td>52</td>
<td>27.6</td>
<td>22.6</td>
<td>33</td>
</tr>
<tr>
<td>64</td>
<td>26.8</td>
<td>23.9</td>
<td>33.2</td>
</tr>
<tr>
<td>80</td>
<td>23.8</td>
<td>25.8</td>
<td>33.7</td>
</tr>
</tbody>
</table>

$^1$S.E.M $\leq$ 3

$^2$All numbers reported as percentages

$^3$RC and T stand for random coil and turns
form. Denaturation generally occurs when a protein is devoid of the quaternary, tertiary, and secondary structure commonly found in its native state, but this did not occur with $G_{i1}$ or $G_{s}$. Instead, the secondary structure morphed from one high in $\alpha$–helices to one rich in $\beta$–sheet at which time it proceeded to precipitate before reaching complete random coil. Addition of a detergent such as guanidinium HCl (GuHCl) was able to further unfold WT $G_{i1}\cdot GDP$ (Fig. 23), as demonstrated by an $\alpha$–helical composition of 4% in the presence of 4 M GuHCl. At 20 °C, as expected, its random coil composition increased to 34%. However, $\beta$–sheets still represented 38% of the secondary structure.

$G_{s}\cdot AMF$ displayed a significantly higher melting temperature, in contrast, to WT $G_{i1}\cdot AMF$ (Table 1). The crystal structure of $G_{s}\cdot GTP\gamma S$ (31) depicts two electrostatic interactions between W231 and R234 in the GTPase domain, and W154 and R160 in the helical domain compared to one salt bridge between W211 and R208 in WT $G_{i1}\cdot GTP\gamma S$ (17). We suggest that the additional electrostatic interaction present between W154 and R160 increased the stability of $G_{s}\cdot AMF$ compared to the other $G_{\alpha}$ proteins.

The stability of a protein is fundamental to its biological function, because its resistance to misfolding is greater and less likely to develop disease. Therefore, investigation into the reversibility of WT $G_{i1}$ and WT $G_{s}$ can give insight into the probability of misfolding of $G_{\alpha}$ subunits. Regardless of conformation, CD spectra demonstrated that the unfolding process of $G_{\alpha}$ proteins was reversible when it was stopped at lower temperatures. In addition, when renaturation was initiated at temperatures below the transition midpoint of $G_{\alpha}\cdot GDP$, AMF and $GTP\gamma S$, there was a substantially higher recovery of secondary structure (Fig. 17). The ability of $G_{\alpha}$ subunits
Figure 23. Unfolding of WT Gia1•GDP in the presence of guanidinium HCl
to refold is dependent on its conformation. Unlike their GDP and AMF counterparts, Gα•GTPγS proteins were able to refold the most denatured protein after incubation at temperatures higher than their T_m’s (Figs. 16 and 17). Although this is true for both Gα proteins, Giα1 was able to refold the most structure compared to Gsα. This was indicated by the fluorescence emission spectra that displayed a 76% recovery of Giα1•GTPγS after denaturation to 96 °C, whereas Gsα•GTPγS was only able to refold 30% after denaturation to 84 °C. In addition, the Gsα•GDP exhibited precipitation at temperatures below 80 °C during renaturation.

The calculated T_m’s varied depending on the spectroscopic technique used. During the course of denaturation a protein may develop multiple intermediate conformations known as molten globule states. Common characteristics include a radius of gyration 10 – 30% larger than the native structure, a loosely packed hydrophobic core with non-polar side chains more exposed to solvent, and lack of tertiary structure, but well intact secondary structure consisting of α – helices and β – sheets (50). Overall, denaturation of Gα subunits showed the lowest transition midpoints with fluorescence, followed by UV – Vis and CD. It is suggested that the ranking of T_m’s can be explained through the molten globule model. The emission spectra monitored the polarity changes in the environment of the Trp residues. Oscillations centered around the non-polar side chains of tyrosine residues located throughout Giα1 and only after the disruption of the non-covalent interactions, would there be molten globules lacking tertiary structure. Once the protein starts to
unfold, molten globules will morph into proteins that have less secondary structure, which explains the higher T_m’s.

\( G_{\text{ia}1} \) and \( G_{\text{sa}} \) regulate the synthesis of the secondary messenger cyclic AMP, through their interactions with the effector adenylyl cyclase (AC). The crystal structure of a \( G_{sa} \cdot AC \) complex indicates eleven points of contact between the two proteins located at \( G_{sa} \) residues N239, Q236, R232, R231, T284, R283, L282, W281, R280, N279, and L272 (18, 31). A \( G_{ia1} \) and AC complex has not been crystallized; however, potential binding residues in \( G_{ia1} \) identified through alanine scanning mutagenesis are R208, K209, I212, K312, R313, K314, K315, T316, and E318 (31). Unfolding of \( G_{sa} \) and \( G_{ia1} \) resulted in an increased composition of \( \beta \) – sheets (Tables 7 and 8). Presumably, mutations at \( G_{a} \cdot AC \) binding sites would result in a percent increase in \( \beta \) – sheet structure in \( G_{a} \) subunits, comparable to what was observed in the thermal denaturation of \( G_{a} \) proteins. If indeed the secondary structure at the points of contact between \( G_{a} \) subunits and AC were to change, signal transduction may be disrupted, which in turn may lead to disease. Future studies would include mutations at these residues and investigation of the temperature – dependence of the secondary structure conducted with CD.

\( G_{a} \) proteins are associated with many cancers, due in large part from hot spot mutations in the amino acid sequence of \( G_{ia1} \) and \( G_{sa} \). Mutations at R231 in \( G_{sa} \) is associated with cancerous tumors in the brain and R208 in \( G_{ia1} \) is related to adenocarcinomas in the large intestine. Both respective Arg residues are also involved with the binding of AC to the respective \( G_{a} \) subunit and there is a suggested relationship between the two. Presumably, mutations at R208 and R231 change the structure of \( G_{a} \) which in turn loses its ability to bind AC and may lead to the development of tumors.
The unfolding of \( G_{\alpha1} \) and \( G_{\alpha} \) as a function of temperature lead to larger amounts of \( \beta \)–sheet formation. Thus, investigation of the secondary structure of the R208 mutant of \( G_{\alpha1} \) and of the R231 mutant of \( G_{\alpha} \), is necessary to determine if there is indeed a connection between the binding of AC effector to the \( G_{\alpha} \) subunits and cancer. The observed aggregation of \( G_{\alpha} \) and \( G_{\alpha1} \) as it unfolds is indicative of protein misfolding, which resembles the formation of \( \beta \)–amyloid fibrils in Alzheimer’s disease. Proteins rich in \( \beta \)–sheets, such as amyloid, have been known to aggregate and cause Parkinson’s, Alzheimer’s, and Prion disease (51). In conclusion, misfolding of \( G_{\alpha} \) subunits may lead to disruption in signal transduction cascades and ultimately lead to disease. Further investigation into the interaction of \( G_{\alpha} \) subunits with intracellular effectors may lead to the discovery of chemo therapeutic agents.
CHAPTER FOUR
Mg\(^{2+}\) DEPENDENCE OF THE FOLDING OF \(G_\alpha\) SUBUNITS

Introduction

\(G–\)Protein activation occurs once a hormone, chemokine, or neurotransmitter binds to an extracellular receptor and triggers the protein to release its GDP bound nucleotide and bind GTP. Upon exchange a conformational change occurs, where the \(\alpha\)–subunit concomitantly binds GTP and dissociates from the \(\beta\gamma\) subunits. The now activated \(G–\)protein binds to an intracellular effector, such as adenylyl cyclase and affect the production of cyclic AMP from ATP. This activation process is dependent on the presence of Mg\(^{2+}\).

The crystal structures of \(G_\alpha\) subunits in different conformations reveal critical information about the binding and functional roles of Mg\(^{2+}\). WT \(G_{i1}\cdot GDP\) has been crystallized in the presence and absence of Mg\(^{2+}\) (13). With the exception of Mg\(^{2+}\) bound and hexacoordinated at the active site, the apo and holo crystal structures are essentially identical (13, 17). Mg\(^{2+}\) exhibits octahedral geometry in WT \(G_{i1}\cdot GDP\cdot Mg^{2+}\). The four equatorial ligands of Mg\(^{2+}\) are the sidechain hydroxyl groups of threonine (T181), located in the switch I region, and serine (S47), oxygen of the \(\beta\)–phosphate in GDP, and a water molecule (13). The axial ligands are two water molecules. The GDP conformation has the distinction of two unordered switch regions, switch II and switch III. Activation with GTP\(\gamma\)S causes a conformational change that leads to rearrangement of ligands.
coordinated by the $\text{Mg}^{2+}$ ion. WT $G_{\text{ia1}} \cdot \text{GTP} \gamma S$ observes the same hexacoordinated ligands with the exception that the oxygen of the $\gamma$ – phosphate at the equatorial position is replaced at the site previously occupied by the water. The active conformation observes ordering of switch II and III regions. Activation may also occur through binding of $\text{AlF}_4^-$ to $G_{\alpha} \cdot \text{GDP} \cdot \text{Mg}^{2+}$ to form a transition state analog (AMF). $\text{AlF}_4^-$ binds to the $\beta$ – phosphate of GDP, and structurally mimics the GTP$\gamma S$ conformation. However, $\text{AlF}_4^-$ forms a square planar complex in contrast to the tetrahedral geometry observed in the $\gamma$ – phosphate of GTP (12). $\text{Mg}^{2+}$ displays similar octahedral coordination observed in WT $G_{\text{ia1}} \cdot \text{GTP} \gamma S$, except a ligand at the equatorial position is fluoride instead of the oxygen on the $\gamma$ – phosphate.

$\text{Mg}^{2+}$ is the second most common intracellular cation and the most abundant intracellular divalent cation (52). It plays a prominent role in biochemical and physiological processes, such as regulating cardiovascular function and ion channels, nucleic acid synthesis, and signal transduction. The transient receptor potential melastatin (TRPM) cation channel family has been identified as a $\text{Mg}^{2+}$ transporter (52). TRPM7, which is found ubiquitously, has been reported to be a signaling kinase involved with vascular smooth cell growth, apoptosis, adhesion, contraction, and migration, processes that, if interrupted, can lead to hypertension and a multitude of vascular diseases. Epidemiological data along with clinical and experimental studies indicate an inverse relationship between blood pressure and dietary $\text{Mg}^{2+}$ consumption (52). Magnesium induces vasodilation, improves blood flow, decreases vascular resistance, increases capacitance function of peripheral, coronary, renal, and cerebral arteries, attenuates agonist-induced vasoconstriction, and reduces blood pressure. In contrast, low
magnesium levels have opposite effects causing contraction, potentiation of agonist-
evoked vasoconstriction, and increased vascular tone and blood pressure (52). Low Mg$^{2+}$
levels have been implicated in other illnesses ranging from minor to severe, such as
migraine headaches, traumatic brain injuries, multiple sclerosis epilepsy, and Duchenne
muscular dystrophy (53).

To investigate the effect of Mg$^{2+}$ on the structure of $G_{i1}$, we used fluorescence
and circular dichroism (CD). Intrinsic tryptophan fluorescence is a technique that
monitors activation through protein folding. Protein activation typically observes
aromatic residues, such as tryptophan (W), moving into less solvent accessible pockets.
As the Trp residues fold into a hydrophobic pocket there is an observed increase in
fluorescence intensity and a blue shift ($\lambda_{\text{max}}$ shifts to lower wavelengths) in the emission
spectra (20). Gilman and co–workers (38) used fluorescence to investigate indirectly
the activation of $G$–proteins. Addition of AlF$_4^-$ or GTP$\gamma$S to $G_\alpha$•GDP•Mg$^{2+}$ causes an
increase in fluorescence and a subsequent red shift ($\lambda_{\text{max}}$ shifts to higher wavelengths) in
its emission spectra (24). Hamm and co–workers (25) reported the same shift for $G_{i1}$
and were able to determine the cause for the observed shift. A conformational change
occurs upon the activation of $G_{i1}$ and there is concomitant movement of W211 and
arginine (R208) that places the residues in close proximity to one another. This creates
an electrostatic interaction that causes a shift in the $\lambda_{\text{max}}$ to higher wavelengths in the
emission spectra, rather than a traditional blue shift. We exploited this finding to
investigate the dependence of Mg$^{2+}$ on the reported red shift in AlF$_4^-$ and GTP$\gamma$S
activation of $G_{i1}$ and $G_{s0}$. As reported (12, 13, 17), $G_{i1}$ exhibits ordering of its switch I,
II, and III regions upon activation with AlF₄⁻ or GTPγS. By using CD, we explored how Mg²⁺ impacted the secondary structure of Gα subunits.

Using both spectroscopic methods, we found that the structures of Gα₁ and Gα₃ proteins were indeed dependent on the amount of Mg²⁺ present. The CD data indicated that as Mg²⁺ concentration increased in active Gα subunits the α-helical content increased. This change in secondary structure may have implications for diseases associated with abnormalities in Gα proteins.

**Materials and Methods**

**Expression and Protein Purification**

Gα₁ and Gα₃ were obtained and purified as described previously (41). W131F, W211F, and W258F mutants were prepared by site directed mutagenesis using a kit provided by Stratagene (La Jolla, CA). Proteins were dialyzed overnight at 4 °C in 20 mM Tris pH 8.0 buffer containing 10% (v/v) glycerol, and 1 mM DTT and then stored at -80 °C. Protein purity was greater than 95% as estimated by SDS – PAGE.

**Calculation of [Mg²⁺]ᵣ in the Presence of EDTA**

The concentration of free Mg²⁺ in the presence of EDTA was calculated based on the following equation:

\[
[Mg²⁺]ᵣ = [Mg²⁺]ᵣ₀ + K [Mg²⁺]ᵣ₀ [EDTA] \left(1+K[Mg²⁺]ᵣ₀\right)^{-1}
\]

[Mg²⁺]ᵣ₀ is the total Mg²⁺ concentration added to the sample, [Mg²⁺]ᵣ₀ is the free Mg²⁺ concentration in the presence of EDTA, and K is the dissociation constant of the Mg²⁺•EDTA complex, which is 1 μM at pH 7.4 (54).
**Fluorescence Measurements**

Experiments were performed using a PTI QuantaMaster fluorimeter (Photon Technologies, Inc., Mirmingham, NJ). Time-based assays were conducted with excitation and emission wavelengths set at 280 and 340 nm, respectively. Assays were initiated after 60 sec by addition of either AlF$_4^-$ as a premixed solution (20 μM AlCl$_3$ and 10 mM NaF) or 20 μM GTPyS to pre-incubated 500 G$_a$ nM protein samples in buffer containing 50 mM Tris, pH 7.5, 1 mM DTT and various concentrations of [Mg]$_f$. Time-based assays were normalized to zero at 60 sec. W211-R208 bridge formation was probed using emission spectra recorded over a wavelength range of 300 - 400 nm with an excitation wavelength set at 280 nm. Signal integration time was 0.2 sec with an incremental step of 0.5 nm. The bandpass for excitation and emission was 5 nm. The maximum fluorescence intensity in the emission spectra was normalized to 1.0. G$_a$ samples were incubated for a minimum of 2 hrs at room temperature with all constituents before emission assays were conducted.

**Determination of Secondary Structure**

Experiments were performed using an Olis DSM 20 circular dichrometer. Data was collected every 1 nm, in the wavelength range of 260 - 180 nm. Time acquisition was determined by a function of high voltage. Samples were measured in their activated forms, either AMF (20 μM AlCl$_3$ and 10 mM NaF) or 20 μM GTPyS. All samples were measured in a cylindrical quartz cuvette with a 1 mm pathlength and contained 3 μM WT G$_{ia1}$ or G$_{ia5}$, 10 mM, pH 7.5 phosphate buffer, 1 mM DTT, and various concentrations of MgSO$_4$. Collected data was converted to molar extinction units. Algorithm CONTINLL
43 in the OlisGlobalworks software was used to calculate percentage of secondary structure.

Results

Effect of Mg\(^{2+}\) on the Intrinsic Fluorescence of G\(_{\alpha}\) subunits

Time-based assays were used to monitor the effect of Mg\(^{2+}\) on the folding pathway of G\(_{\alpha}\) subunits. AMF activation of G\(_{\text{i1\cdotGDP}}\) containing 5 mM Mg\(^{2+}\) resulted in a fluorescence intensity of 40% which is comparable to reported literature values (25, 48, 55), but the addition of AlF\(_4^-\) to G\(_{\text{i1\cdotGDP}}\) in the absence of Mg\(^{2+}\) produced a fluorescence intensity of 5% (Fig. 24). Increasing the Mg\(^{2+}\) concentration to 250 nM or greater resulted in a larger F\(_{\text{max}}\) and faster rate (Fig. 24). The F\(_{\text{max}}\) remained unchanged at Mg\(^{2+}\) concentrations above 1 mM, but the rates continued to increase until 5 mM Mg\(^{2+}\).

Similar trends were observed for WT G\(_{\text{s\cdotAMF}}\), however, concentrations above 2 mM afforded the largest fluorescence intensity as compared to 1 mM in WT G\(_{\text{i1}}\), and G\(_{\text{s\cdotAMF}}\) (Fig. 25).

GTP\(\gamma\)S exchange of G\(_{\text{i1\cdotGTP\(\gamma\)S}}\) and G\(_{\text{s\cdotGTP\(\gamma\)S}}\) was also investigated. G\(_{\text{i1\cdotGTP\(\gamma\)S}}\) in the presence of 8 mM Mg\(^{2+}\) displayed an F\(_{\text{max}}\) of 56%, whereas, in the absence of Mg\(^{2+}\), an F\(_{\text{max}}\) of 44% and a similar initial rate was observed (Fig. 26). Exchange of GTP\(\gamma\)S with WT G\(_{\text{s\cdotGDP}}\), containing 8 mM Mg\(^{2+}\), exhibited an F\(_{\text{max}}\) of 33% (Fig. 27). In contrast to apo G\(_{\text{i1\cdotGTP\(\gamma\)S}}\), G\(_{\text{s\cdotGTP\(\gamma\)S}}\) displayed a significantly reduced fluorescence of 3% in the absence of Mg\(^{2+}\) (Fig. 27). Activation of G\(_{\text{s\cdotGTP\(\gamma\)S}}\) with GTP\(\gamma\)S exhibited similar F\(_{\text{max}}\) trends in the presence of increasing Mg\(^{2+}\) concentrations as compared to its AMF equivalent. Both types of G\(_{\alpha}\) subunits resulted in slower rates for GTP\(\gamma\)S binding compared to their AMF conformation (Figs. 24 and 25).
Figure 24. Mg$^{2+}$ dependence of AlF$_4^-$ activation of WT G$_{i\alpha1}$
Figure 25. Mg$^{2+}$ dependence of AlF$_4^-$ activation of WT G$_{so}$.
Figure 26. GTPγS activation of WT G_{iα1} dependent on Mg^{2+}
Figure 27. GTPγS activation of WT $G_{\alpha}$ dependent on $\text{Mg}^{2+}$
The emission spectra of $G_\alpha$ samples was investigated along with the activity assays. In the absence of $\text{Mg}^{2+}$, addition of $\text{AlF}_4^-$ to WT $G_{i1}$•GDP and WT $G_{s}$•GDP, resulted in red shifts of 1.2 nm and 1.1 nm in the emission spectra compared to their GDP counterparts. Increasing the $\text{Mg}^{2+}$ concentration in both $G_\alpha$ subunits resulted in incrementally larger red shifts (Fig. 28). At 5 mM $\text{Mg}^{2+}$, $G_{i1}$ and $G_{s}$ completed their bridge formations and reached maximum red shifts of 3.3 nm and 2.5 nm, respectively.

The shifts that occurred from activation with $\text{GTP}\gamma\text{S}$ were also explored for both $G_\alpha$ subunits. Activation with $\text{GTP}\gamma\text{S}$ in the absence of $\text{Mg}^{2+}$ observed red shifts of 2.7 nm and 1.2 nm for $G_{i1}$ and $G_{s}$, respectively. $G_{i1}$ and $G_{s}$ reached their maximum shifts of 3.6 and 3.1 nm, respectively, at approximately 50 nM $\text{Mg}^{2+}$ (Fig. 29).

**Changes in the Secondary Structure of $G_{i1}$ and $G_{s}$**

Crystallization studies by Coleman and Sprang (13) discovered a conformation-dependent ordering of three switch regions in $G_{i1}$ upon addition of $\text{Mg}^{2+}$ to the GDP conformation. CD was used to probe the changes observed at the secondary structure level of $G_{i1}$ and $G_{s}$. Magnesium titration of $G_{i1}$•GDP exhibited no change in the CD spectra (data not shown). Addition of $\text{Mg}^{2+}$ up to a concentration of 2 mM to $G_{i1}$•GDP•$\text{AlF}_4^-$ displayed decreases in molar ellipticity at 210 nm and 220 nm of the CD spectra compared to its GDP counterpart (Fig. 30). The same trends were observed for the $\text{GTP}\gamma\text{S}$ conformations, with the exception of a slightly larger decrease at 209 nm and 220 nm (Fig. 31). $G_{s}$ observed similar findings for the AMF and $\text{GTP}\gamma\text{S}$ conformation as witnessed with $G_{i1}$ (Figs. 32).
Figure 28. Shift resulting from AlF$_4^-$ activation of WT G$_{i\alpha1}$ and G$_{s\alpha}$ at various [Mg$^{2+}$]$_F$.
Figure 29. Shift resulting from GTPγS activation of WT $G_{i\alpha 1}$ and $G_{s\alpha}$ at various $[\text{Mg}^{2+}]_F$.
Figure 30. Secondary structure of WT Giα1•AMF at various Mg$^{2+}$ concentrations
Figure 31. Mg$^{2+}$ dependent activation of $G_{i\alpha 1}$ and $G_{s\alpha}$ with $AlF_4^-$ or GTP$\gamma$S

![Graph showing molar ellipticity at 209 nm vs Mg$^{2+}$ concentration](image-url)
Figure 32. Mg$^{2+}$ dependent activation of $G_{i\alpha 1}$ and $G_{s\alpha}$ with AlF$_4^-$ or GTP$\gamma$S

![Graph showing molar ellipticity at 220 nm for different Mg$^{2+}$ concentrations and conditions.](image-url)
AlF₄⁻ may bind to GDP in apo Gα subunits and cause a small conformational change as observed for AMF activation at 0 mM Mg²⁺ (Fig. 1). The rate of formation of the AMF conformation increases in the presence of Mg²⁺ as indicated by the larger rates and Fₘₐₓ values (Fig. 1). Gsat was more sensitive to the presence of Mg²⁺ in AlF₄⁻ activation than Giat1 as indicated by the lower concentration needed to illicit an increase in fluorescence above the basal levels observed in the absence of Mg²⁺. However, to complete AMF folding both Giat1 and Gsat needed Mg²⁺ concentrations in the millimolar range. Presumably, in the case of Gsat, relatively lower Mg²⁺ concentrations induce more folding than with Giat1. One possibility is that the Mg²⁺ - Al³⁺ distance is shorter in Gsat•AMF than in Giat1•AMF. The crystal structure of Giat1•AMF is available (17), but that of Gsat•AMF is not. Therefore, it is not possible at this time to confirm this hypothesis.

GTPγS displayed similar increases in Fₘₐₓ at elevated concentrations of Mg²⁺ for Gα subunits as observed for the binding of AlF₄⁻. However, the GTPγS form observed slower rates than the AMF conformation. The effect of Mg²⁺ on the rate of GTPγS activation appeared to be greater in Gsat than Giat1. It is known that the rate determining step in GTPγS exchange is the release of GDP from Gα subunits (54). Our results show that magnesium promotes the release of GDP as exhibited by the increased Fₘₐₓ of holo Giat1 and Gsat•GTPγS compared to their apo equivalents, and the rate of release of GDP from Giat1 is faster than in Gsat and this step is less Mg²⁺ - dependent. It appears that, in the GTPγS activation of Giat1, the Trp residues fold in a manner that is weakly dependent on Mg²⁺ concentration. The opposite occurs with Gsat.
The observed red shifts and increases in fluorescence intensity for \( G_\alpha \) activation with either \( \text{AlF}_4^- \) or \( \text{GTP}\gamma\text{S} \) are dependent on the presence of \( \text{Mg}^{2+} \). The \( \text{GTP}\gamma\text{S} \) conformation is more sensitive to \( \text{Mg}^{2+} \) than the AMF form, as indicated by \( \text{Mg}^{2+} \) concentrations (in the nM range for \( \text{GTP}\gamma\text{S} \) binding as compared to the \( \mu \text{M} \) range in AMF activation) necessary to illicit an appreciable increase in fluorescence intensities and shifts in the emission spectra. However, \( G_{\text{sat}} \cdot \text{GTP}\gamma\text{S} \) was significantly more dependent on \( \text{Mg}^{2+} \) than \( G_{\text{int}} \cdot \text{GTP}\gamma\text{S} \), which is in agreement with the data from the time-based assays and the observed red shifts for both apo and holo \( G_\alpha \) proteins. Apo WT \( G_{\text{int}} \cdot \text{GTP}\gamma\text{S} \) displayed an increase of 44% in the fluorescence intensity, whereas holo WT \( G_{\text{int}} \cdot \text{GTP}\gamma\text{S} \) observed a 56% increase in fluorescence. Apo and holo \( G_{\text{sat}} \cdot \text{GTP}\gamma\text{S} \) resulted in activation of 3% and 33% (Figs. 24 – 29). These findings also indicated that GDP release from \( G_\alpha \) subunits is not completely dependent on \( \text{Mg}^{2+} \), but increases the rate at which GDP may release and bind \( \text{GTP}\gamma\text{S} \). In addition, the presence of \( \text{Mg}^{2+} \) is essential in trace amounts to complete the conformational change of \( G_{\text{int}} \cdot \text{GTP}\gamma\text{S} \) and \( G_{\text{sat}} \cdot \text{GTP}\gamma\text{S} \).

Incremental amounts of \( \text{Mg}^{2+} \) showed increased \( F_{\text{max}} \) values and red shifts in \( G_{\text{int}} \) and \( G_{\text{sat}} \) activation. We propose a direct relationship between the observed shifts and increased fluorescence intensities upon \( \text{Mg}^{2+} \) titration of \( G_\alpha \) subunits. \( \text{W211} \) and \( \text{W234} \), in \( G_{\text{int}} \) and \( G_{\text{sat}} \), respectively, are responsible for the considerable increase in fluorescence intensity witnessed upon activation and contribution to a bridge formation with \( \text{R208} \) and \( \text{R231} \). We suggest that incremental increases in \( \text{Mg}^{2+} \) concentrations concomitantly folded \( \text{W211} \) and \( \text{W234} \) into less solvent exposed regions, and into closer proximity with \( \text{R208} \) and \( \text{R231} \) in a stepwise fashion. Regardless of activation with \( \text{AlF}_4^- \) or \( \text{GTP}\gamma\text{S} \), the larger shift observed for \( G_{\text{int}} \) versus \( G_{\text{sat}} \), can be explained through the proximity of the
electrostatic interaction. Molecular modeling shows that R208 and W211 in \(G_{ia1}\text{•GTP}\gamma S\) has a bridge distance of 6.6 Å in comparison to R231 and W234 in \(G_{si}\text{•GTP}\gamma S\) which displays a distance of 7.2 Å. Computational work by Gallivan and Dougherty (45) found that electrostatic interactions between Trp residues and Arg residues to be considerably stronger when less than 6 Å as opposed to greater than 6 Å apart. The maximum red shifts incurred by \(G_{ia1}\) and \(G_{si}\) in the GTP\(\gamma S\) conformation are 3.5 nm and 2.8 nm, respectively. If the length of the R231 – W234 bridge in \(G_{si}\text{•GTP}\gamma S\) were to result in a weak interaction then one would expect a much smaller shift than observed. However, this may be explained through a second electrostatic interaction occurring in the \(\alpha – \) helical domain of \(G_{si}\text{•GTP}\gamma S\) between W154 and R160, which displays a distance of 4.3 Å. This bridge would indicate a significantly stronger interaction and be contributing to the overall shift displayed in the activation of \(G_{si}\).

The crystal structures of \(G_{ia1}\) depict unordered segments in its GDP conformation. These regions known as switch II and III become ordered when activated with GTP\(\gamma S\) or AMF (13). When titrated with Mg\(^{2+}\), \(G_{ia1}\text{•GDP}\) did not undergo any changes in its secondary structure. Deconvolution of \(G_{ia1}\text{•GDP}\) and \(G_{si}\text{•GDP}\) samples found the secondary structure to be 40% \(\alpha – \)helical, 15% \(\beta – \)sheets, 18% turns and 27% random coil (RC) and 38% \(\alpha – \)helical, 15% \(\beta – \)sheets, 20% turns, and 28% RC, respectively. Upon addition of AlF\(_4^-\) or GTP\(\gamma S\), a 2% increase in \(\alpha – \)helical content and subsequent 1% decrease in random coil and \(\beta – \)sheet was calculated for both \(G_{ia}\) subunits. This suggests that upon activation a fraction of the \(\beta – \)sheets and RC are refolding their structure into \(\alpha – \)helices. In addition, since the switch II and III regions are unordered in the crystal structure of \(G_{ia1}\text{•GDP}\) (\(G_{si}\text{•GDP}\) is unavailable) it may be plausible that the effects in the
secondary structure may be occurring in these protein segments. In conclusion, the CD data confirm that the secondary structure of $G_{i21}$ and $G_{sat}$ in their activated forms is dependent on $\text{Mg}^{2+}$. Understanding the role of $\text{Mg}^{2+}$ in $G_{a}$ activation may lead to possible therapeutic targets in $G_{a}$ protein associated illnesses, such as bipolar disorder and cancer (27, 56). Future studies would include crystallization of $G_{sat}$•GDP and $G_{sat}$•AMF to investigate the conformational dependent switch regions and to probe the electrostatic interaction of R231 and W234.
APPENDIX A

EFFECT OF DTT ON WT G_{i1} ACTIVATION
The amino acid cysteine contains a thiol group that is capable of disulfide bridge formation via oxidation of cysteine side chains to form cystines. A polypeptide chain can form cystines either through intramolecular interactions of two thiol groups within the chain or through intermolecular interactions of thiol groups between two subunits. A classic example of cystine formation within a subunit is Christian Anfinsen’s work with bovine ribonuclease (57). Native ribonuclease contains multiple cystines bonded intramolecularly that are required for stability and function. In the presence of a reducing agent, β-mercaptoethanol, and 8 M urea the non-covalent bonds were disrupted and ribonuclease formed a completely reduced random coil conformation. When dialyzed against buffer, in the absence of urea and β-mercaptoethanol, it spontaneously renatured and retained most of its enzymatic activity. However, when 8 M urea remained and the reducing agent was absent, ribonuclease refolded and had less than 1% enzymatic activity, because the disulfide bridges that formed were between the wrong pairs of cysteine residues. Properly folded protein typically demonstrates a specific biological function. Mis-folding or denaturation of the protein can lead to altered biological function, which in turn often leads to disease.

Disulfide bridge formation can also occur intramolecularly as demonstrated by insulin (58, 59). Frederick Sanger and co–workers (60, 61) pioneered the technique of sequencing in proteins, RNA, and DNA. Sanger’s foundational work was with insulin, the first protein ever sequenced. Insulin consists of two polypeptide chains bonded through two cystine residues. There is also an intramolecular bond between cysteine residues at positions 6 and 7 within chain A, in human insulin (62). Crystallographic studies of G\text{\alpha} suggest that oligomerization occurs between multiple \alpha subunits (17).
Thus, we investigated the polymerization of WT $G_{\alpha 1}$ based on the hypothesis that that disulfide linkage between $G_{\alpha}$ subunits occurred.

A method to express and purify high yields of $G_{\alpha}$ subunits has been well researched and documented by Lee and co–workers (41). Expression of hexahistidine tagged WT $G_{\alpha 1}$ typically leads to yields of 40 mg/ 1 L. After purification with Ni$^{2+}$ and size exclusion columns, the protein is usually greater than 95% in purity and is typically stored in 20 mM $\beta$ – mercaptoethanol or 2 mM dithiothreitol (DTT) (41, 63, 64). An indirect method to determine functionality of newly purified $G_{\alpha}$ protein is through intrinsic tryptophan fluorescence (38, 48). Using this purification method that includes DTT, activation with the addition of AlF$_4^-$ or exchange with GTP$_\gamma$S typically produces fluorescence of 40% or greater (25, 65).

WT $G_{\alpha 1}$ expression and purification was obtained as described (41). The only difference was the protein was stored and activated in the absence of the reducing agents $\beta$ – mercaptoethanol and DTT. Activation of WT $G_{\alpha 1}$•GDP•Mg$^{2+}$ with AlF$_4^-$ resulted in a fluorescence intensity of 34% (Fig. 33). In the presence of increasing concentrations of DTT, the fluorescence intensity increased with AlF$_4^-$ activation and, in the presence of 5 mM DTT, it reached a maximum fluorescence of 55%.

Oligomerization of WT $G_{\alpha 1}$•GDP was also investigated through SDS – PAGE in the presence and absence of $\beta$ – mercaptoethanol. WT $G_{\alpha 1}$•GDP in the absence of $\beta$ – mercaptoethanol displays bands at 41, 82, and 123 kDa (Fig. 34, lanes 2 and 3). In the
Figure 33. WT $G_{i\alpha 1}\cdot GDP\cdot Mg^{2+}$ activation with $AlF_4^-$ at various DTT concentrations
Figure 34. Visualization of WT $G_{i
alpha1}\cdot$GDP polymerization with SDS – PAGE.
Lane 1, Molecular weight markers; Lanes 2 and 3 depict gels of WT $G_{i
alpha1}\cdot$GDP samples in the absence (-) of $\beta$ – mercaptoethanol, and lane 4 is for a similar protein sample in the presence (+) of the reducing agent.
presence of 0.7 M β–mercaptoethanol, a single band at 41 kDa is observed for WT G\textsubscript{ia1}•GDP (Fig. 34, lane 3).

As discussed earlier, fully functional WT G\textsubscript{ia1} typically displays values greater than 40%. This agrees well with the results observed where the amount of DTT is greater than 0.1 mM. It is suggested that the presence of a reducing agent is necessary in order to obtain fully functional, monomeric WT G\textsubscript{ia1}. There are ten cysteine residues in WT G\textsubscript{ia1} and four of them are located at the protein’s surface. It is therefore possible that, without a reducing agent present, WT G\textsubscript{ia1} polymerizes through bonding of its monomers. A disulfide bridge is formed through linkage of solvent–exposed cysteine residues between two α subunits. Increasing amounts of the reducing agent resulted in increased activity due to the reduction of these polymers. As the protein was reduced its flexibility and movement was enhanced. SDS–PAGE confirmed the suggested expectations of G\textsubscript{a} polymerization. The use of β–mercaptoethanol, completely reduced the dimer and trimer bands to a single monomer band.

We therefore recommend that investigation of WT G\textsubscript{ia1} through functional assays or structure determination should be conducted in the presence of a reducing agent to ensure reliable information on the unpolymerized α subunit. To better understand the effects of DTT on G\textsubscript{a} subunits, future studies could make use of other reducing and oxidizing agents, for example (tris(2-carboxyethyl)phosphine) and glutathione. Determination of specific intramolecular cystine formation could also be explained through Mass Spectrometry and X–ray crystallography. It will also be interesting to see if disulfide bridges are present in the active GTPγS conformation of G\textsubscript{ia1}, and in both forms of G\textsubscript{so}. 
APPENDIX B

Li⁺ INHIBITION OF GTPγS BINDING AND GTP HYDROLYSIS
Bipolar disease is a severe mental illness in which the person experiences clinical episodes of depression and mania or hypomania, or mixed states (66). The most identifiable symptom is that of a person experiencing extreme changes in behavior ranging from tremendous aggressiveness to complete and utter depression. Approximately 2.5% of the population over the age of 18 in the United States suffers from bipolar disorder (67). It is a debilitating illness that can result in tremendous loss in productivity, and has been known to occasionally contribute to patient suicide. There are many drugs in the market today that are used to stabilize mood swings associated with bipolar disorder, such as anticonvulsants, antipsychotics, and benzodiazepines (68, 69). However, the most common and effective for treatment is the use of lithium salts. Li\(^+\) treatment doses are between 0.5-1.2 mM just short of the lethal doses that occur at approximately 2.0 mM. Because the toxic levels can easily be reached during treatment, patients must have continuous renal screenings to ensure safe plasma levels at all times (70). Despite these shortcomings, Li\(^+\) is still the preferred treatment for bipolar disorder. The study of the Li\(^+\) mechanism of action can lead to a better understanding of bipolar disorder and to the development of a better drug for its treatment (71).

Li\(^+\) inhibits several Mg\(^{2+}\) dependent activated enzymes that are involved in signal transduction, such as inositol monophosphatase in the phosphoinositide cascade, adenylyl cyclase, glycogen synthase kinase – 3 beta (GSK – 3β), and G – proteins (72). Overabundance and hyperactivity of guanine nucleotide binding (G) proteins have been linked to patients suffering from bipolar disease (71). In rat cortex membranes, Li\(^+\), at therapeutic concentrations, inhibits α subunits of both the G\(_i\) and G\(_s\), thus lessening the
manic and depressive states that the disease causes (73). During mania, there are increased levels of dopamine neurotransmission (74). Post synaptic actions of dopamine are mediated via G – protein coupled receptors. An increase in receptor and G - protein coupling have been found in post mortem subjects, and studies in animal models showed lower levels of dopamine when treated with lithium (74). Furthermore, it has been reported that specific subunits of G – proteins are associated with higher dopamine levels in bipolar patients and lithium controls the equilibrium of nucleotide activation and not the levels of G – proteins (74).

Li\(^+\) and Mg\(^{2+}\) exhibit similar physiochemical properties due to their fairly similar hydrated ionic radii (0.60 Å for Li\(^+\) and 0.65 Å for Mg\(^{2+}\)). They exhibit a “diagonal relationship” in the periodic table, with lithium being the first element in Group IA and magnesium the second element in Group IIA. These chemical similarities of Li\(^+\) and Mg\(^{2+}\) occur in their stable oxidation states in aqueous solution. However, their different ionic charges, and preferred coordination numbers (4 vs. 6) and ligand geometries (tetrahedral vs. octahedral) lead them to have unique properties (72). Because of all these properties it is plausible that Li\(^+\) may compete with Mg\(^{2+}\) at its binding sites. Ral, a member of the GTPase ras family, depicts two Mg\(^{2+}\) binding sites in its crystal structure (75). The crystal structures of G\(_{i1}\) (12, 13) and G\(_{sa}\) (31) display only one Mg\(^{2+}\) binding site, however crystallization was performed under high concentrations of LiCl and Li\(^+\) could be out competing Mg\(^{2+}\) at another possible binding site. Dr. Guoyan Wang and Dr. Chris Malarkey, past graduate students of the de Freitas group, investigated the possibility of two Mg\(^{2+}\) binding sites in G\(_{i1}\) and G\(_{sa}\) through the use of the Mg\(^{2+}\) specific,
fluorescence probe, furaptra (76). Their studies found evidence for two Mg$^{2+}$ binding sites, one with a high affinity and the second with a lower affinity for Mg$^{2+}$. These data suggest that Li$^+$ may out compete Mg$^{2+}$ at the lower affinity site. These findings provided the motivation to explore the effect of Li$^+$ on the activity of G–proteins.

We used radiolabeled isotopes to investigate whether Li$^+$ inhibited Guanosine–5’–triphosphate (GTP) binding to $G_\alpha$ subunits and the nucleotide hydrolysis. To investigate nucleotide binding a non–hydrolysable analog of GTP, GTP$\gamma$S$^{35}$, was used. Exploration of the hydrolysis of GTP was monitored through GTP$^{32}$. WT G$\alpha_\text{sat}$ was able to reach maximum exchange of GTP$\gamma$S with GDP and hydrolysis of GTP in the presence of 0.1 mM Mg$^{2+}$ and in the absence of Li$^+$ (Fig. 35). Increasing the concentration of Li$^+$ to 1 mM reduced the binding of GTP$\gamma$S by 70% compared to the observed binding in the absence of Li$^+$. Maximum Li$^+$ inhibition of GTP$\gamma$S binding was reached at 20 mM Li$^+$ with an IC$_{50}$ of 0.6 mM. The effect on hydrolysis of GTP was also investigated (Fig. 36). GTP hydrolysis was reduced by 15% in the presence of 1 mM Li$^+$. Maximum inhibition of GTP hydrolysis was observed at 10 mM Li$^+$ and had a calculated IC$_{50}$ of 2.0 mM.

WT G$\alpha_\text{sat}$ observed significant inhibition of GTP$\gamma$S binding and GTP hydrolysis in the presence of Li$^+$ at concentrations within the therapeutic range. Li$^+$ had a larger effect on the binding of GTP$\gamma$S than in the hydrolysis of GTP. Therefore, the conditions used for the two experiments enable us to differentiate the two steps - nucleotide exchange and hydrolysis - in the cyclic function of G–proteins. The data suggest that Li$^+$ has a direct effect on nucleotide binding to G$\alpha_\text{sat}$, and that the effect on hydrolysis is secondary and propagated by the inhibition observed in the exchange. We propose that
Figure 35. Effect of Li$^+$ on GTPγS binding to WT G$_{s\alpha}$
Figure 36. Effect of Li⁺ on GTP Hydrolysis of WT Gsα
Li⁺ is out competing Mg²⁺, at a second low affinity Mg²⁺ binding site that may regulate binding at the high affinity site, which, in turn, impacts nucleotide exchange. Li⁺ binding may cause a conformational change at the second site which may account for the regulation of the Mg²⁺ dependent exchange. The limiting factor in nucleotide exchange is the release of GDP (23). Therefore, it is necessary to further investigate whether Li⁺ is inhibiting the release of GDP or slowing the rate of binding of GTPγS.

Future experiments to be conducted would be possible crystallization of WT G_{i\alpha1} and G_{s\alpha} in the absence of LiCl to determine if there are two Mg²⁺ binding sites. Also, the use of radiolabeled α – GTP³² to investigate whether Li⁺ inhibition of GDP release occurs.

Li⁺ inhibition of nucleotide exchange and of hydrolysis was also studied with G_{i\alpha1}. No evidence of Li⁺ inhibition of G_{i\alpha1} activity was found (data not shown). The different behavior of G_{s\alpha} and G_{i\alpha1} may correlate with the reported effects of Li⁺ on G_{s}, but not in G_{i\alpha1}, in post – mortem cortex membranes, peripheral blood elements, and mononuclear leukocytes (71, 77). When Li⁺ inhibition of GSK – 3β was studied, it was found that 2.5 mM of Li⁺, which is a toxic level, was required to induce a 50% decrease in activity (78). However, in our studies, 1.0 mM of Li⁺, a concentration within the therapeutic range, caused 50% inhibition of nucleotide exchange in G_{s\alpha}. Although there is strong support for the role that GSK – 3β plays in bipolar disorder, it may not be the complete story and targeting Li⁺ interactions with G – proteins is imperative.
REFERENCES


VITA

The author, Matthew Najor, was born August 10, 1985 in Detroit, MI. In August 2003, he started his undergraduate studies at Loyola University Chicago. He played division II collegiate rugby for Loyola University Chicago. In May 2007, he received a Bachelor of Science degree in chemistry.

In August of 2007, he was admitted to the Ph.D. program in chemistry at Loyola University Chicago. He was awarded a graduate fellowship from August 2007 to December 2013. In April 2010, he was awarded first prize at the Frontiers in Life Sciences at Loyola University Chicago. The author received the chemistry department for best teaching assistant in 2010.