2001

Receptor- G Protein Interactions in the Visual System: A Study of Structures and Mechanisms That Couple the Cytoplasmic Surface of Rhodopsin to the Nucleotide-Binding Pocket of Transducin

Ethan P. Marin

Follow this and additional works at: http://digitalcommons.rockefeller.edu/student_theses_and_dissertations

Part of the Life Sciences Commons

Recommended Citation
http://digitalcommons.rockefeller.edu/student_theses_and_dissertations/349

This Thesis is brought to you for free and open access by Digital Commons @ RU. It has been accepted for inclusion in Student Theses and Dissertations by an authorized administrator of Digital Commons @ RU. For more information, please contact mcsweej@mail.rockefeller.edu.
Receptor-G protein Interactions in the Visual System:
A study of structures and mechanisms that couple the cytoplasmic surface of rhodopsin to the nucleotide-binding pocket of transducin

Ethan P. Marin

A thesis presented to the faculty of THE ROCKEFELLER UNIVERSITY in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

June, 2001
"The great thing is to last and get your work done and see and hear and learn and understand and write when there is something that you know; and not before; and not too damned much after."

-Ernest Hemingway
Acknowledgments

The work described in this thesis would never have been completed without the contribution, both direct and indirect, of many people. Credit is due to my thesis advisor, Dr. Thomas P. Sakmar, who was resolute in his policy of treating students as colleagues— which meant never telling me what to do. In addition, I am appreciative of the members of my thesis committee for their input on my projects.

I would also like to acknowledge all the members of the Sakmar laboratory with whom I have worked during my stay there, especially the people who helped me get started during my first years in the lab: Steve Lin, Cecille Unson, Tanya Zvyaga, Lenore Snyder, Steve Gravina, May Han, Manija Kazmi and the two students who came before me, Aaron Cypess and Chris Min. Thanks are due also to Carol Valli and Cliff Sonnenbrot, who helped keep the lab running smoothly.

Several people contributed directly to the work in this thesis. Tanya Zvyaga performed the rhodopsin-derived peptide competition experiment described in Chapter 3. The photoregeneration work in Chapter 4 was done in Berlin, in the lab of Dr. K. P. Hofmann, in collaboration with one of his graduate students, Christoph Meyer. I am grateful to Dr. Hofmann and his group for allowing me into their Institute to complete those studies. I am also grateful to the Alexander Mauro Travel Fellowship which supported a portion of my stay in Germany. Dr. Steve Graber (West Virginia University) provided purified recombinant Goᵳ, and guidance on expression and purification procedures. Vincent Archambault, a graduate student, performed some of the early experiments with in vitro expression of Goᵳ during a rotation in the Sakmar Lab. Eugene Simuni, a high school student, spent three summers in the lab working on analyzing the interdomain interface of G proteins, and helping with experiments related to that project. Wing-Yee Fu, a technician in the lab, provided tremendous assistance with large-scale preparations of rhodopsin mutants. I am particularly indebted to A. Gopala Krishna, a post-doc in the Sakmar Lab who helped me with many of the experiments described in Chapters 5 and 6, and also performed the studies on peptide binding to transducin subunits in Chapter 3.

Finally, I would like to thank my wife and family for supporting me through this endeavor.
Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>3</td>
</tr>
<tr>
<td>The Phototransduction Cascade</td>
<td>3</td>
</tr>
<tr>
<td>The biochemistry of the phototransduction cascade: an historical</td>
<td>4</td>
</tr>
<tr>
<td>overview</td>
<td></td>
</tr>
<tr>
<td>The Central Role of Rhodopsin-(G_t) Interactions</td>
<td>7</td>
</tr>
<tr>
<td>Rhodopsin structure and function</td>
<td>7</td>
</tr>
<tr>
<td>Rhodopsin structure</td>
<td>7</td>
</tr>
<tr>
<td>Opsin-Chromophore Interactions</td>
<td>9</td>
</tr>
<tr>
<td>(R^*) interactions with (G_t)</td>
<td>11</td>
</tr>
<tr>
<td>(G_t) structure and function</td>
<td>13</td>
</tr>
<tr>
<td>(G_t) interactions with (R^*)</td>
<td>16</td>
</tr>
<tr>
<td>The mechanism of (R^*) catalyzed nucleotide exchange</td>
<td>17</td>
</tr>
<tr>
<td>Advantages of rhodopsin and transducin as a model system</td>
<td>20</td>
</tr>
<tr>
<td>Outline of the Thesis</td>
<td>22</td>
</tr>
<tr>
<td>Chapter 2: Materials and Methods</td>
<td>31</td>
</tr>
<tr>
<td>Reagents</td>
<td>31</td>
</tr>
<tr>
<td>Preparation of rhodopsin-derived peptides</td>
<td>31</td>
</tr>
<tr>
<td>Preparation of (G_t)-derived peptides</td>
<td>31</td>
</tr>
<tr>
<td>Preparation of (G_t, G\alpha_t, G\beta\gamma_t), and rhodopsin from</td>
<td>32</td>
</tr>
<tr>
<td>Bovine Retinas</td>
<td></td>
</tr>
<tr>
<td>Measurement of Intrinsic Fluorescence of (G_t, G\alpha_t, G\beta\gamma)</td>
<td>34</td>
</tr>
<tr>
<td>Site Directed Mutagenesis of Rhodopsin</td>
<td>34</td>
</tr>
<tr>
<td>Table of Contents (con't)</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>Fluorescence $G_t$ Activation Assay ............................................... 35</td>
<td></td>
</tr>
<tr>
<td>Measurement of [$^3$H]Palmitic Acid Incorporation .................................. 36</td>
<td></td>
</tr>
<tr>
<td>Instrumentation for Measurement of Photoregeneration Traces ...................... 36</td>
<td></td>
</tr>
<tr>
<td>Photoregeneration Experiments ................................................................. 37</td>
<td></td>
</tr>
<tr>
<td>Numerical Fitting Procedures ..................................................................... 37</td>
<td></td>
</tr>
<tr>
<td>Site-Directed Mutagenesis of $G_{ct}$ and $G_{cq}$ ........................................... 38</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> Translation of $G_{ct}$ ................................................................. 39</td>
<td></td>
</tr>
<tr>
<td>Trypsin Proteolysis and Analysis ................................................................. 40</td>
<td></td>
</tr>
<tr>
<td>Control Reactions for Trypsin Proteolysis of $G_{ct}$ ...................................... 40</td>
<td></td>
</tr>
<tr>
<td>Uncatalyzed Activation Time Course of $G_{ct}$ ............................................. 41</td>
<td></td>
</tr>
<tr>
<td>Rhodopsin/$G_{ct}$-Catalyzed Activation Time Course of $G_{ct}$ ......................... 41</td>
<td></td>
</tr>
<tr>
<td>Data Analysis of Trypsin Proteolysis Patterns ............................................ 41</td>
<td></td>
</tr>
</tbody>
</table>

Chapter 3: The Amino Terminus of the Fourth Cytoplasmic Loop of Rhodopsin

Modulates Rhodopsin—Transducin Interaction ........................................ 43

Summary ................................................................. 43

Introduction ............................................................. 44

Results ................................................................. 45

Inhibition of $G_t$ Activation by Synthetic Peptides ......................... 45

A Synthetic Peptide Derived From C4 of Rhodopsin, rho(310-321), Alters the Fluorescence Emission Wavelength Maximum of $G_{ct}$ But Not of $G_{ct}$ ......................... 45

Preparation of Substitution Mutants in C4 of Rhodopsin ......................... 46

Activation of $G_t$ by Solubilized Purified Recombinant Pigments ................ 48
Characterization of Pigment-catalyzed GTPγS Uptake by Go as a
Function of Gβγt Concentration ........................................ 49
Palmitoylation of CTr1, Ctr2, and CTr4 ............................. 50
Amino Acid Sequence Analysis of Vertebrate Opsins and Biogenic Amine Family Receptors ........................................ 50
Discussion ........................................................................ 59
Loop C4 of Rhodopsin Is Involved in the Activation of Gt .......... 59
Factors Affecting the Structure of the C4 Loop Region .......... 61
The Role of the C4 Loop Involves Modulation of Rhodopsin-Gt Interactions ......................................................... 62

Chapter 4: Mutation of the Fourth Cytoplasmic Loop of Rhodopsin Affects Binding of Transducin and Peptides Derived from the Carboxyl-terminal Sequences of Transducin α and γ Subunits ........................................ 64
Summary ........................................................................ 64
Introduction ..................................................................... 65
Results .......................................................................... 66
The Effect of Gt and Gt-derived Peptides on the Photoregeneration of Rhodopsin ......................................................... 66
Specificity of the Effects of α(340-350) and γ(50-71)-far Peptides .... 67
Photoregeneration Assay of Recombinant Rhodopsin and Loop C4 Mutants .............................................................. 69
Discussion ........................................................................ 80
Photoregeneration is Sensitive to Interactions with Gt and Certain Gt-derived Peptides .................................................... 81
The Role of a Conserved Region at the Amino Terminus of Loop C4 of Rhodopsin in Gt Binding .......................................... 82
Table of Contents (con’t)

Possible Role of GyT-farnesyl in Docking of Gt to the Active Receptor 84

Chapter 5: The Function of Interdomain Interactions in Controlling Nucleotide Exchange Rates in Gαt ................................................................. 86

Summary .............................................................................. 86
Introduction ....................................................................... 88
Results .............................................................................. 90

Expression of Gαt in vitro and Trypsin Digestion Assay of Nucleotide Binding and Exchange ...................................................... 90

Analysis of Single Amino Acid Replacements in the Interdomain Interface of Gαt ............................................................... 93

Analysis of Double Amino Acid Replacements ...................... 94

Analysis of Gαt mutants ..................................................... 95

Discussion ......................................................................... 108

Analysis of Trypsin-Digest Products of in vitro Translated Gαt to Evaluate Nucleotide Exchange Kinetics ........................................ 108

Site-Directed Mutation of K273 or K276 Increases Basal Nucleotide Exchange Rates .......................................................... 110

Interdomain Interactions in Gαt Do Not Affect Basal or Rhodopsin-Catalyzed Nucleotide Exchange Rates ........................... 112

The function of residues at the interdomain interface differs among Gαt, Gαi, and Gαs .......................................................... 113

Chapter 6: Rapid Activation of Transducin by Mutations on the α5 Helix Distant From the Nucleotide-Binding Site: Evidence for a Mechanistic Model of Receptor-Catalyzed Nucleotide Exchange by G Proteins .................. 115

Summary .............................................................................. 115
Table of Contents (con’t)

Introduction ................................................................. 117

Results ............................................................................. 119

Proline Scanning Mutagenesis of the α5 Helix ......................... 119

Deletions and Insertions in the α5 Helix and Carboxyl Terminus of Goαt .................................................. 119

Alanine Scanning Mutagenesis of the α5 Helix ....................... 120

Mutagenesis of the β6/α5 Loop and of Residues Interacting with the α5 Helix ................................................... 121

Engineering of Disulfide Bridges Between α5 and Other Parts of Goαt .......................................................... 122

Discussion ........................................................................... 135

Alterations of the Structure of the α5 Helix by Site-Directed Mutagenesis Disrupted R*-Catalyzed Activation ................................................................. 135

Mutation of the β6/α5 Loop Increased Basal Nucleotide Exchange Rates .................................................................. 138

Identification of Nucleotide-Release Control Microdomain on the α5 Helix .......................................................... 139

Interactions Between the α5 Helix and the Rest of Goαt .......... 141

A Mechanistic Model of R*-Catalyzed Nucleotide Exchange .................. 142

Conclusions ........................................................................ 145

Chapter 7: Perspectives .......................................................... 146

The fourth loop of rhodospin in context of the rhodospin crystal structure .................................................. 146

Rhodospin — Go interactions: the big picture ............................ 150

References .......................................................................... 154
<table>
<thead>
<tr>
<th>List of Figures</th>
<th>Number</th>
<th>Title</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-1</td>
<td>The G\text{t} activation cycle</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>Crystal structures of the G\text{t} heterotrimer and rhodopsin</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>Primary and secondary structure of rhodopsin</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>1-4</td>
<td>The structure of G\alpha\text{t}</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>Primary and secondary structures of G\alpha\text{t}</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>3-1</td>
<td>Schematic representation of bovine rhodopsin</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>3-2</td>
<td>Peptides derived from the second, third, and fourth intracellular</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>loops of rhodopsin inhibit activation of G\text{t} by rhodopsin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-3</td>
<td>Effect of rho(310-321) on the intrinsic fluorescence of G\alpha\text{t}</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and G\beta\gamma\text{t} subunits</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td>Amino acid sequences of the fourth loop of bovine rhodopsin,</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>human \beta2 AR, human m1 MR, and fourth loop mutants of rhodopsin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-5</td>
<td>Rates of G\text{t} activation catalyzed by solubilized, purified</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>recombinant pigments</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-6</td>
<td>The relative rates of pigment-catalyzed GTP\gamma S uptake by G\alpha\text{t} as a function of G\beta\gamma\text{t} concentration are similar for both rhodopsin and CTr2</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>3-7</td>
<td>The amino-terminal half of the fourth loop of rhodopsin is more</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>conserved than the carboxyl-terminal half within the family of</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vertebrate opsins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-1</td>
<td>Amino acid sequences of recombinant rhodopsins and G\text{t}-derived</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>peptides</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>4-2</td>
<td>Photoregeneration of rhodopsin in the presence of increasing concentrations of $G_\alpha_t$ or $G_\gamma$-derived peptides</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>4-3</td>
<td>Altered peptides do not inhibit photoregeneration</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>4-4</td>
<td>The effect of $G_\alpha$, $\alpha(340-350)$ and $\gamma(50-71)$-far on photoregeneration of heterologously expressed rhodopsin and C4 loop mutants</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>Quantitation of the effects of $G_\alpha$, $\alpha(340-350)$, and $\gamma(50-71)$-far on the photoregeneration of recombinant rhodopsin and rhodopsin mutants</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>5-1</td>
<td>Structure of the interdomain interface of GDP-bound $G_\alpha_t$</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>5-2</td>
<td>Determination of nucleotide exchange rates by analysis of trypsin digestion patterns</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>5-3</td>
<td>Plots of uncatalyzed and catalyzed nucleotide exchange time courses derived from analysis of trypsin digest patterns of <em>in vitro</em> translated $G_\alpha_t$</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>5-4</td>
<td>Time courses of activation of mutants of residues located on the helical domain</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>5-5</td>
<td>Time courses of activation of mutants of residues located on Switch III (a.k.a. Insert 2)</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>5-6</td>
<td>Time courses of activation of mutants of residue 228 located on Switch III (a.k.a. Insert 2)</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>5-7</td>
<td>Time courses of activation of mutants of residues located on the $\alpha_G$ region (a.k.a. Insert 3)</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>5-8</td>
<td>Time courses of uncatalyzed activation of double mutants</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>5-9</td>
<td>Uncatalyzed activation of $G_\alpha_1$ and mutants of $G_\alpha_1$</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>Structure of the interdomain interface of GDP-bound $G_\alpha_1$</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>6-1</td>
<td>Structure of the $\alpha_5$ helix of $G_\alpha_t$</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-2</td>
<td>Nucleotide exchange time courses of proline mutants in $\alpha_5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-3</td>
<td>Truncation and insertion mutants of $G\alpha_t$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-4</td>
<td>Uncatalyzed nucleotide exchange time courses of alanine mutants in $\alpha_5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-5</td>
<td>Trypsin digest analysis of wild-type, A322S, and the rapidly activating alanine mutants (T325A, V328A and F332A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-6</td>
<td>Rhodopsin/(G\beta\gamma_t)-catalyzed nucleotide exchange time courses of alanine mutants in $\alpha_5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td>Nucleotide exchange time courses of mutants of residues near $\alpha_5$ and in the $\beta_6/\alpha_5$ loop</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-8</td>
<td>Schematic representation of the phenotypes of proline mutants in $\alpha_5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-9</td>
<td>Schematic representation of the phenotypes of alanine mutants in $\alpha_5$: a model for $R^*$-catalyzed $G_t$ activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-1</td>
<td>Close-up of Helix 8 (H8) of rhodopsin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Number</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-I</td>
<td>Biochemical Characterization of Rhodopsin Fourth Loop Mutants</td>
<td>51</td>
</tr>
<tr>
<td>4-I</td>
<td>$G_t$ activation and photoregeneration data</td>
<td>72</td>
</tr>
<tr>
<td>5-I</td>
<td>Rate constants for basal nucleotide exchange measured for $G\alpha_t$ and $G\alpha_t$ mutants.</td>
<td>97</td>
</tr>
<tr>
<td>6-I</td>
<td>Rate constants of uncatalyzed nucleotide exchange of $G\alpha_t$ and $G\alpha_t$ mutants.</td>
<td>124</td>
</tr>
</tbody>
</table>
Abbreviations

α5 ................................................................................................................. the α5 helix of Gαt
AR .................................................................................................................. adrenergic receptor
C1, C2, etc. ...................................................................................... First, second, etc. cytoplasmic loop of rhodopsin
DM .............................................................................................................. n- dodecyl-β-D-maltoside
E1, E2, etc. ...................................................................................... First, second, etc. extracellular loop of rhodopsin
Gt .................................................................................................................... holo-transducin
Gαt .............................................................................................................. α subunit of transducin
Gβγt .......................................................................................................... βγ heterodimer subunit of transducin
Gβt .............................................................................................................. β subunit of transducin
Gγt .............................................................................................................. γ subunit of transducin
GTPγS .................................................................................................. guanosine -3-O-(thio)triphosphate
GPCR .................................................................................................. G protein coupled receptor
IVT .............................................................................................................. in vitro translated
MII ........................................................................................................ metarhodopsin II
MR ......................................................................................................... muscarinic receptor
R* ........................................................................................................... signaling active state of rhodopsin
Ras ........................................................................................................ Ras p21ras
RM ......................................................................................................... reverted metarhodopsin
TM .............................................................................................................. transmembrane

Mutants proteins are designated by the amino acid present in the wild-type protein (using the single letter code) and its position, followed by the amino acid present in the mutant. For example, a mutant in which phenylalanine 332 is replaced by alanine is designated F332A.
Abstract

The intermolecular interaction between the photoreceptor rhodopsin and the heterotrimeric G protein transducin (Gt) initiates the vertebrate phototransduction cascade. This interaction also serves as a model system for the study of the molecular basis of related G protein-coupled receptor mediated signal transduction systems. Photoactivated rhodopsin (R*) activates Gt by catalyzing the exchange of bound GDP for GTP on its α subunit (Gαt). The structure of the R*-Gt complex and the mechanism of nucleotide exchange are unknown. We studied the function of the fourth cytoplasmic loop (C4) of rhodopsin in interactions with Gt. Chimeric mutants of rhodopsin were characterized in which regions of C4 were replaced with amino acid sequences from the β2 adrenergic receptor or the m1 muscarinic receptor. Chimeras in which the amino terminus of C4 was altered were defective in catalyzing Gt activation. A spectroscopic photoregeneration assay was used to demonstrate that mutants of the amino terminus of C4 were defective in binding holo-Gt, a peptide derived from the carboxyl terminus of Gαt, and in certain circumstances a peptide derived from the carboxyl terminus of Gγt. These results suggested that C4 mediated Gt binding and activation and that C4 interacted specifically with the carboxyl termini of Gαt and possibly Gγt. We next studied how R* induces nucleotide exchange by Gt at a distance. We tested the validity of two longstanding hypotheses: 1) that R* induces opening of the interdomain cleft of Gαt, and 2) that R* communicates with the nucleotide binding pocket via the α5 helix of Gαt. We developed an expression and assay system to characterize a large number (>50) of site-directed mutants of Gαt designed to test these hypotheses. The mutants were expressed in vitro in rabbit reticulocyte lysate and the kinetics of both basal and R*-catalyzed nucleotide exchange were determined by quantitative analysis of trypsin digest patterns. Mutations in a series of residues at the interface between the two domains of Gαt had only minor effects on the basal and catalyzed activation rates. In contrast,
mutations in a cluster of residues on the buried face of the α5 helix, 0.7-1.5 nm from the nucleotide, greatly (up to 165-fold) accelerated nucleotide exchange. Mutations of residues on the adjacent solvent-exposed surface of α5 disrupted R*-catalyzed activation, as did substitution of α5 residues with prolines. These results provided evidence that R* induced nucleotide exchange primarily by perturbing the structure of buried residues on α5 and not by opening the interdomain cleft. Structural analysis and biochemical data were used to propose a mechanistic model for receptor-catalyzed G protein activation.
Chapter 1: Introduction

The Phototransduction Cascade

The phototransduction cascade of the vertebrate rod cell can be defined as the set of regulated molecular interactions responsible for photon detection and subsequent neuronal signaling (Wald, 1968; Stryer, 1988). Extensive investigation by biochemists, biophysicists and physiologists over many decades has yielded a detailed understanding of phototransduction, which has emerged as a model for how cells detect and respond to a wide variety of extracellular stimuli. In this introductory chapter the basic features of phototransduction are discussed and a brief historical review of the key studies that unraveled the central biochemical cascade is presented. Subsequently, the structure and function of two important proteins in the cascade, rhodopsin and transducin, are examined in more detail. Finally, an overview of the thesis research, which examines the molecular mechanism of interactions between rhodopsin and transducin, is provided.

Rod cells are specialized dim-light photosensor cells. They are sensitive enough to detect single photons (Baylor et al., 1979) as a result of high signal amplification and low thermal background noise. They are one of two photoreceptor cell types in the retina, the laminated layer of neural tissue at the back of the vertebrate eye. The second photoreceptor cell type, cone cells, are specialized for bright light and color vision. In addition to the photoreceptors, the neural retina consists of four neuronal cell types (bipolar cells, ganglion cells, horizontal cells, and amacrine cells) as well as glial cells (Cepko et al., 1996). The simplest neuronal signaling pathway in the retina involves transmission from photoreceptors to bipolar cells to ganglion cells, whose axons form the optic nerve which exits the retina and travels to the brain (Sakmar, 2001).

The initial step in rod cell phototransduction takes place in the rod outer segment (ROS). The ROS is filled with stacks of disc-shaped membranes and contains the
molecular machinery of phototransduction. Rhodopsin, the photoreceptor pigment molecule, is an integral membrane protein localized primarily to the disc membranes. Rhodopsin consists of an apoprotein called opsin and a covalently attached chromophore derived from 11-cis-retinal (Wald, 1968). Upon absorption of a photon, the 11-cis-retinylidene chromophore isomerizes to all-trans-retinylidene (ATR) (Wald, 1968). Subsequent biochemical reactions lead to the hyperpolarization of the rod cell. Hyperpolarization alters synaptic transmission between rod cells and adjoining neurons in the retina, initiating a signal that is ultimately transmitted to the brain. The biochemical mechanisms that couple chromophore isomerization (which is the only light dependent step in phototransduction) and rod cell hyperpolarization were elucidated by a series of key experiments in the 1970s and early 1980s.

*The Biochemistry of the Phototransduction Cascade: an Historical Overview*

In the early 1970s Bitensky and co-workers identified a key intermediate in the phototransduction pathway when they demonstrated that light could regulate the levels of cyclic guanosine monophosphate (cGMP) in photoreceptor cells by activation of a light-dependent cGMP phosphodiesterase (PDE) activity (Miki et al., 1974). Activation of a single molecule of rhodopsin was found to result in the hydrolysis of $10^5$ molecules of cGMP (Yee and Liebman, 1978). cGMP was shown to play a crucial role in phototransduction by Miller and Nicol who demonstrated that injection of cGMP into rod cells induced depolarization of the rod cell and increased the latency for hyperpolarization by illumination (Miller and Nicol, 1979; Nicol and Miller, 1978).

Interestingly, the PDE activity demonstrated a requirement for GTP, and appeared to be regulated by a light-sensitive GTPase activity in the ROS (Wheeler and Bitensky, 1977). In 1979, Godchaux and Zimmerman reported the isolation of a protein with GTPase activity that interacted with isolated ROS membranes (Godchaux and Zimmerman, 1979). They showed that the protein could bind GDP or GTP. The activity
was purified and found initially to involve two polypeptide chains. Hermann Kühn expanded on these findings by reporting in 1980 that three proteins of molecular mass 37, 35, and 6 kDa bound to isolated ROS membranes only following exposure to light (Kühn, 1980). The two larger proteins were apparently the same as those observed by Godchaux and Zimmerman. Furthermore, Kühn showed that the three proteins could be eluted specifically from the photolyzed membranes with GTP. These observations allowed for the easy purification of the guanine nucleotide binding protein and its subsequent characterization.

At about the same time, Fung and Stryer reported that photoactivated rhodopsin (R*) catalyzed nucleotide exchange -- the release of GDP followed by the binding of GTP-- by the guanine nucleotide binding protein (Fung and Stryer, 1980). Significantly, they showed that a single molecule of R* could catalyze the exchange of several hundred nucleotides.

By 1981, the stage was set for a key paper by Stryer and co-workers that tied together the previous observations (Fung et al., 1981). Using purified preparations of the heterotrimeric guanine nucleotide binding protein, they showed that the nucleotide bound exclusively to the largest polypeptide, which they termed the α subunit. The binding of GTP induced the separation of the α subunit from the two smaller subunits, β and γ. Second, they showed that R* could stimulate the replacement of GDP with GTP in the absence of PDE. Finally, they demonstrated that GTP-bound α subunit (but not GDP-bound α) could activate PDE in the absence of R*. Based on these data, Stryer proposed the key steps of the rhodopsin-transducin cycle (Fig. 1-1). Absorption of a photon by rhodopsin causes chromophore isomerization, which leads to conformational changes that create R*. R* catalyzes the exchange of GDP for GTP by the α subunit of transducin (Gαt). GTP-bound Gαt dissociates from rhodopsin and Gβγt and then activates PDE. Activated PDE rapidly hydrolyzes cGMP to GMP, resulting in decreased [cGMP] in the
rod cell. Hydrolysis of bound GTP to GDP by the intrinsic GTPase activity of \(G\alpha_t\) returns the protein to the inactive conformation, whereupon it disengages PDE and again binds \(G\beta\gamma_t\). Since the nucleotide binding protein subunit could transduce a signal from activated rhodopsin to PDE, Stryer named it "transducin" (\(G\lambda\)).

The final piece of the cascade—the connection between decreased intracellular [cGMP] and hyperpolarization of the rod cell—was discovered in 1985. Fesenko and colleagues demonstrated using isolated patches of ROS plasma membrane that cation channels in the membrane required cGMP to remain open (Fesenko et al., 1985). It had been known previously that a "dark" current, carried primarily by Na\(^+\) ions, flowed into the ROS through plasma membrane cation channels in the absence of light (Yau, 1994). Na\(^+\) ions were simultaneously ejected from the rod cells by pumps in the inner segment. In the dark, the membrane potential of the rod cell is \(~-35\text{mV}\) (Yau, 1994). Thus, following the light-induced decrease in [cGMP], the cation channels closed, blocking the inward cation current and causing rod cell hyperpolarization.

Interestingly, at about the same time as the rod cell biochemistry was elucidated, Gilman, Rodbell and others studying the hormonal regulation of adenylyl cyclase activity were uncovering the importance of a GTP-binding protein in transducing signals from agonist-occupied hormone receptors to the enzyme adenylyl cyclase (Ross and Gilman, 1980). It eventually became clear that the stimulatory GTP binding protein of the hormonal system, \(G_S\), and the GTP-binding protein of the rod cell, \(G_t\), were both members of the same superfamily of guanine nucleotide binding regulatory proteins, or \(G\) proteins. The molecular cloning of rhodopsin (Nathans and Hogness, 1983) and of the \(\beta_2\) adrenergic receptor (Dixon et al., 1986) would reveal that the receptors shared amino acid sequence (and presumably structural) similarity. The close resemblance of the photon and hormone signal transduction systems was a surprising example of the
conservation of a basic mechanism in biology for the execution of similar tasks in different contexts.

**The Central Role of Rhodopsin-G_t Interactions**

As the experiments described above revealed, G_t plays a central role in the phototransduction cascade. It couples together two separate highly specialized proteins: the photon detector rhodopsin and the efficient second messenger modulator PDE. G_t activation by R* represents a key amplification step in the cascade in that a single R* can catalyze the activation of hundreds of G_t molecules (Heck and Hofmann, 2001; Fung and Stryer, 1980). This amplification is crucial to the sensitivity of the system. In addition, G_t exhibits a low rate of basal (uncatalyzed) nucleotide exchange that contributes to the sensitivity by maintaining low background noise. Finally, G_t provides an important site of regulation in the cascade. The rates of GTP loading and GTP hydrolysis by G_t greatly affect the amplitude and the temporal resolution of the resulting signal.

The molecular mechanisms that underlie the interaction between rhodopsin and transducin have been studied intensively over the past 20 years since Kühn, Bitensky, Zimmerman, Stryer and others worked out their central role as the first intermolecular interaction in the phototransduction cascade (Fig. 1-2) (for recent reviews, see (Bourne, 1997; Sakmar, 1998; Menon et al., 2001)).

**Rhodopsin Structure and Function**

*Rhodopsin Structure*

The crystal structure of dark state rhodopsin was recently solved at 2.8 Å resolution (Fig. 1-2) (Palczewski et al., 2000). The structure confirmed many of the topological and structural features that had been predicted by models based on sequence, biochemical and cryoelectron microscopy analyses (e.g., (Baldwin et al., 1997; Shieh et al., 1997). The
primary and secondary structures of rhodopsin are depicted schematically in Fig. 1-3. The tertiary structure reveals seven transmembrane helices (TM), most of which are tilted and kinked, arranged in a bundle surrounding the chromophore. The chromophore is covalently attached to K296 of TM7 via a protonated Schiff base. E113, the experimentally determined counterion to the protonated Schiff base (Sakmar et al., 1989), lies 0.35 nm away. Side chains of amino acid residues from a number of helices, especially TM3, TM6 and TM7 interact closely with the chromophore. There are two solvent exposed regions of rhodopsin, the cytoplasmic and intradiscal surfaces. (The intradiscal surface is often referred to as the extracellular surface in analogy to related receptors which are localized to the plasma membrane.) The extracellular surface consists of three interhelical loops (E1, E2 and E3), as well as the amino terminal tail. The cytoplasmic surface consists of three interhelical loops (C1, C2 and C3) and the carboxyl terminal tail. In addition a "fourth" cytoplasmic loop (C4) is created by the membrane insertion of palmitoyl groups attached to two adjacent cysteines (C322 and C323) in the carboxyl terminus (Ovchinnikov et al., 1988; Moench et al., 1994). The solvent exposed surfaces of rhodopsin were found to have a considerable amount of ordered structure. The extracellular surface is notable for a distorted β sheet consisting of sequence elements from the amino-terminal tail and the E2 loop. The E2 loop forms a “plug” that contacts the chromophore at the base of the helix bundle. The structure of the extracellular surface is supported by a disulfide bond between C110 and C187. The cytoplasmic surface reveals that helices extend from TM3 and TM6 beyond the apparent border of the lipid bilayer. The C2 loop projects outward from the helical bundle in an extended conformation. C3 is partially disordered, which is consistent with the conformational flexibility previously suggested by site directed spin labeling (Altenbach et al., 1996). Unexpectedly, much of C4 is an amphipathic helix (designated helix 8, or H8), which extends outward from the helical bundle along the apparent surface of the membrane. Together with C2, this structure increases the size of the cytoplasmic surface
Chapter 1: Introduction

beyond what would be predicted from the diameter of the transmembrane helical bundle alone.

Opsin-Chromophore Interactions

In the dark state, 11-cis-retinal acts pharmacologically as an inverse agonist that keeps rhodopsin in the signaling "off" state (Han et al., 1997). The first step in the phototransduction, the isomerization of the chromophore to a distorted ATR, is accomplished within 200 fsec (Schoenlein et al., 1991), qualifying it as one of the fastest biological events known. Following isomerization, the chromophore-opsin complex thermally relaxes. During this relaxation, photolyzed rhodopsin sequentially assumes a number of spectrally defined transient conformations. These intermediates were originally identified in low temperature trapping experiments, and subsequently a similar set of intermediates have been observed by time resolved spectroscopy of photolyzed rhodopsin samples (Sakmar, 1998). Within milliseconds, an equilibrium between two metastable intermediates, metarhodopsin I (MI) and metarhodopsin II (MII), is reached. This equilibrium is sensitive to environmental conditions including temperature, pH, membrane or detergent composition, and the binding of G. A subconformation of MII defined by the spectrally silent uptake of two protons from the aqueous environment, MIIib, is thought to be identical with the signaling active conformation of rhodopsin, R* (Kibelbek et al., 1991; Arnis and Hofmann, 1993). ATR acts as a pharmacological agonist which favors formation of R*.

The conformational changes that follow chromophore isomerization are induced in part by steric interactions between the all-trans chromophore and the opsin protein (Shieh et al., 1997). A recent study using a retinal derivative with a crosslinking group on the six-membered ring of the retinal mapped significant changes in the ring-protein interactions following photolysis (Borhan et al., 2000). In the dark state, the modified ring crosslinked to W265 of TM6. Following photolysis at room temperature, crosslinks
formed with A169 of TM4. The importance of steric interactions between the chromophore and the TM helices is suggested by the reduction in the formation of R* in artificial pigments created by the regeneration of opsin with retinal analogs that lack either the methyl group attached to C-9 of the polyene chain (Han et al., 1998) or the ring (Nukada et al., 1986).

Extensive research has gone into understanding the conformational and chemical changes of rhodopsin that define the formation of R*. The protonation state of several residues changes. UV-visible spectroscopy revealed that the Schiff base is deprotonated (Fahmy and Sakmar, 1993b), and FTIR studies indicate that the ground state counterion E113 becomes protonated (Jager et al., 1994). In addition, time-resolved spectroscopic measurements with pH-sensitive dyes indicate that two protons are taken up by the protein from the bulk solvent upon R* formation (Arnis and Hofmann, 1993). One of the proton acceptors appears to be E134, part of a highly conserved E/D-R-Y sequence at the cytoplasmic end of TM3 (Arnis et al., 1994). The importance of protonation of E134 in R* formation is also supported by the finding that the E134Q mutant of rhodopsin had increased ability to activate Gt at alkaline pH relative to wild-type rhodopsin (Fahmy and Sakmar, 1993a). The site of the second proton acceptor is unknown. There are also a number of documented conformational changes involved in formation of R*. Obviously, the chromophore is isomerized to the all-trans conformation. Additionally, TM3, TM6, and possibly TM7 appear to move apart from one another upon R* formation. This "helix movement" model is supported by a number of observations, including: (1) naturally occurring tryptophan residues on TM3 and TM6 experienced altered microenvironments following MII formation as detected by UV difference spectroscopy (Lin and Sakmar, 1996); (2) site-directed spin labeling studies showed that photoactivation increases the distance between spin labels on the cytoplasmic ends of TM3 and TM6 (Farrens et al., 1996); (3) the formation of disulfide crosslinks between
cysteine residues engineered into the cytoplasmic ends of TM3 and TM6 prevented activation of Gt (Farrens et al., 1996); (4) Zn$^{2+}$ inhibited Gt activation by rhodopsin mutants containing Zn$^{2+}$ binding sites in the form of His residues engineered into the cytoplasmic ends of TM3 and TM6 (Sheikh et al., 1996); and (5) replacement of G121 on TM3 with larger residues caused constitutive activity in the dark whereas substitution of F261 on TM6 with smaller residues suppressed the effects of the G121 mutants (Han et al., 1997; Han et al., 1996).

It is not understood how steric interactions in the chromophore binding pocket may be propagated to the cytoplasmic surface of rhodopsin (Shieh et al., 1997). It has been proposed that structural changes of TM3 and TM6 at the cytoplasmic surface involve rigid body movements of these helices (Farrens et al., 1996), which suggests a possible mechanism. However, the finding that the TM helices are highly kinked and irregular (Palczewski et al., 2000) raises the issue of the extent to which they can move individually as rigid bodies. As would be expected, the structural connection between the chromophore binding pocket and the cytoplasmic surface runs in both directions. Not only is the structure of the chromophore binding pocket communicated to the cytoplasmic surface, but binding of Gt to the cytoplasmic surface of rhodopsin affects the conformation of the chromophore binding pocket (Arnis and Hofmann, 1995; Ernst et al., 2000). This phenomenon is exploited as the basis of a Gt-binding assay described in Chapter 4.

**R* Interactions With Gt**

A variety of techniques has been used to identify the regions of R* that interact with Gt. Involvement of the second (C2) and third (C3) intracellular loops of rhodopsin are suggested by site directed mutagenesis (Franke et al., 1992; Franke et al., 1990) and peptide competition studies (König et al., 1989). In particular, the highly conserved R135 in C2 appears to be required for proper activation of Gt (Acharya and Karnik,
In the crystal structure of dark state rhodopsin, R135 interacts with E134. Protonation of E134, which is suggested to occur upon R* formation (see above), could free R135 to assume alternate conformations. The importance of the amino terminus of the fourth loop (C4) is discussed in detail in Chapters 3 and 4. The data regarding regions of R* that interact with G\textsubscript{t} fit well with the data relating to the conformational changes that occur at the cytoplasmic surface during formation of R*. TM3 and TM6, which are thought to move upon R* formation, are linked to C2 and C3. These helix movements may expose the otherwise occluded determinants of G\textsubscript{t} activation located at the juxtacytoplasmic regions of C2, C3, and C4.

R* activity is reduced by phosphorylation on the carboxyl terminus (Miller et al., 1986). Phosphorylation is catalyzed by rhodopsin kinase, which specifically recognizes MII. Multiply phosphorylated rhodopsin is bound by arrestin with high affinity (Wilden et al., 1986). Thus arrestin sterically interferes with G\textsubscript{t} binding. Eventually, R* decays to the apoprotein opsin and free ATR.
Gi structure and function

Crystal structures of a variety of conformations of Gi have been solved to date, including the GTPγS (Noel et al., 1993), the GDP (Fig. 1-4) (Lambright et al., 1994), and the GDP/AlF₄⁻-bound structures of Gαt (Sondek et al., 1994), as well as the GDP-bound heterotrimer (Fig. 1-2) (Lambright et al., 1996), and free Gβγt (Sondek et al., 1996). More recently, the structure of the ternary complex of Gαt bound to its effector PDE γ and RGS9 was reported (Slep et al., 2001). These structures have provided detailed information on a number of G protein mechanisms, including the nature of the conformational change induced by GTP binding, the mechanism and regulation of GTP hydrolysis, and the nature of interactions between Gαt and Gβγt.

Gαt, a 350 amino acid protein, consists of two domains (Fig. 1-4): the Ras-like domain, named due to its homology with the structure of the monomeric G protein, p21ras (Ras); and the helical domain, named to reflect its composition of 6 α helices (αA-αF). The nucleotide is bound in a cleft between the domains. The Ras-like domain consists of a central mixed 6-stranded β-sheet (designated β1-β6), surrounded on either side by a total of 6 α-helices (designated α1-α5, plus αG). The majority of the direct contacts to the nucleotide originate from conserved regions of the Ras-like domain, which map to loops emanating from the strands of the central β-sheets. These loops are homologous to canonical nucleotide-binding domains of the monomeric G proteins (Sprang, 1997). The Ras-like domain contains four regions not homologous to Ras, called Inserts 1-4. These are discussed in more detail in Chapter 5. The primary and secondary structures of Gαt are presented in Fig. 1-5.

The conformational changes that accompany the exchange of GDP for GTP are localized to three regions, denoted Switch I, II, and III. Switch I (S173-T183) and II (F195-T215) are similar to Switch regions described in Ras; Switch III (D227-R238) is unique to the heterotrimeric G proteins (Fig. 1-4). Switch I and II respond directly to the
presence of the γ phosphate of GTP, whereas Switch III appears to move in response to reorganization of the Switch II (Lambright et al., 1994). The conformational changes in Switch II involve a partial rotation of the α2 helix, which leads to the movement of several amino acid side chains from exposed to partially buried positions. These changes serve as the basis of assays of Goi activation. In particular, the movement of W207 is detected as a large increase in fluorescence emission intensity (Faurobert et al., 1993) (see Chapter 3), and the burial of R204 protects it from cleavage by trypsin (Fung and Nash, 1983) (see Chapter 5).

Goi-GTP activates PDE, a tetrameric enzyme consisting of α, β, and γ subunits in a 1:1:2 stoichiometry, by removing the inhibitory constraints that the γ subunits exert upon the catalytic α and β subunits. The binding site for PDEγ on Goi was recently determined by X-ray crystallography, and found to reside between the α2 helix of the Switch II region and the adjacent α3 helix (Slep et al., 2001).

Goi returns to its inactive GDP-bound state by hydrolyzing the bound GTP to GDP. Rapid turn-off of the cascade is essential for the temporal resolution of the signal (He et al., 1998). GTP hydrolysis is accelerated by the simultaneous binding of the effector, cGMP γ subunit, and a second protein, regulator of G protein signaling 9 (RGS9) (He et al., 1998). Goi(GDP) recombines with Gβγt, and can then be activated anew by R*.

Gβt is a 340 amino acid protein, constructed from an amino terminal α helix, followed by a β propeller structure (Sondek et al., 1996). The β propeller consists of seven “blades” of 4 β sheets each. Each blade is roughly related to the others by rotational symmetry (Fig. 1-2). At the sequence level, Gβt is notable for 7 WD40 domains, sequence repeats of roughly 40 amino acids that frequently end with Trp-Asp (WD in the single letter code). Each WD40 repeat corresponds to the fourth strand of one propeller blade, and the first three strands of an adjacent blade. Gβt is a member of a
large family of proteins containing WD40 repeats, which perform a variety of functions; all are thought to fold into β propeller structures (Neer et al., 1994b; Garcia-Higuera et al., 1996). GBț is also a member of a larger family of proteins which fold into β propeller structures, many of which do not share significant sequence homology.

GYț is the shortest transducin subunit, consisting of only 74 amino acids. It contains an amino terminal α helix, which interacts with the amino terminus of GBț in a coiled-coil conformation (Fig. 1-2). Interestingly, a study conducted prior to the determination of the structure showed that peptides derived from the helical regions of GBț and GYț do not associate with each other in solution (Marin and Neubig, 1995). The remainder of GYț wraps around GBț in an extended conformation. GBț and GYț can be dissociated from one another only under denaturing conditions, and physiologically they function as a single entity.

The structure of the Gt heterotrimer (Lambright et al., 1996) reveals two distinct sites of interaction between Gαț and Gβγț: between the amino-terminal helix of Gαț and the side of the GBț propeller, and between the Switch I / Switch II region of Gαț and the top of the GBț propeller structure (Fig. 1-2). Direct contacts between Gαț and GYț are not observed in the structure, although interactions between lipids attached to each subunit (see below) have been proposed. The structure of free Gβγț compared with Gβγț in the heterotrimer reveals that Gβγț is virtually unchanged by the binding of Gαț (Sondek et al., 1996). However, the structure of Gαț is altered in the conformationally flexible Switch I/II binding region, which makes contacts with GBț (Lambright et al., 1996). The structure of the amino terminal helix of Gαț is also likely altered by the binding of Gβγț.

A number of important post-translational modifications of Gt have been described. Gαț is heterogeneously acylated, primarily with saturated C12 and C14 lipids, at its amino terminal glycine (Kokame et al., 1992). This modification is thought to be
important for interactions with \( \text{G} \beta \gamma_t \) (Kokame et al., 1992), and possibly with membranes and rhodopsin (Min, 1996). Recently, a report describing the phosphorylation of \( \text{G} \alpha_q \) at Y142 by the tyrosine kinase Src in rod outer segments has been published (Bell et al., 2000). The significance of this modification is not yet understood. \( \text{G} \gamma_t \) is modified in a three step process that includes farnesylation of a cysteine in the carboxyl-terminal CAAX motif, followed by cleavage of the three carboxyl terminal amino acids, and carboxymethylation of the free carboxyl terminus (Wedegaertner et al., 1995). Farnesylation has been found to be important for interactions with \( \text{G} \alpha_q \) (Matsuda et al., 1998) as well as with rhodopsin (Scheer and Gierschik, 1995; Kisselev et al., 1994). There is evidence that rhodopsin can discriminate between farnesyl (C15) and geranylgeranyl (C20) lipids (Kisselev et al., 1995a), suggesting the existence of a specific prenyl binding site on rhodopsin.

\( \text{G} \alpha_q \) Interactions With \( \text{R}^* \)

A variety of biochemical and biophysical techniques have been used to identify sites on \( \text{G} \alpha_q \) that interact with membranes and with \( \text{R}^* \). The involvement of the carboxyl terminal 11 amino acids of \( \text{G} \alpha_q \) (a.a. 340-350) in interactions with \( \text{R}^* \) is suggested by many studies, including: (1) the finding that Pertussis toxin catalyzes the ADP-ribosylation of C347, which uncouples \( \text{G} \alpha_q \) from rhodopsin; (2) a peptide corresponding to amino acids 340-350 can uncouple rhodopsin from \( \text{G} \alpha_q \) and can itself bind to rhodopsin and mimic the effects of \( \text{G} \alpha_q \) (Hamm et al., 1988); (3) site directed mutagenesis (Garcia et al., 1995; Osawa and Weiss, 1995); and (4) the demonstration in related \( \text{G} \) proteins that specificity of coupling to particular receptors resides in the carboxyl terminus (Conklin et al., 1993). In addition, peptide and site-directed mutagenesis studies have suggested the involvement of the \( \alpha4/\beta6 \) loop of \( \text{G} \alpha_q \), which lies adjacent to the carboxyl terminus, in interacting with \( \text{R}^* \) (Hamm et al., 1988; Natochin et al., 1999). Experimental evidence suggests that \( \text{G} \beta \gamma_t \) is also in direct contact with rhodopsin (Phillips and Cerione, 1992;
The specific contacts between Gβγt and R* involve the carboxyl terminus of Gγt, as suggested by studies with peptides derived from that region (Kisselev et al., 1994; Kisselev et al., 1995a) and possibly the seventh propeller blade of Gβγt (Taylor et al., 1996).

All of the structures of Gt thought to participate in interactions with rhodopsin or the membrane cluster to a common face on the structure of Gt, and identify a putative rhodopsin interacting surface (Lambright et al., 1996; Bohm et al., 1997) (Fig. 1-2). However, in the crystal structure of the heterotrimer, neither the carboxyl terminus of Gαt nor that of Gγt is included. Thus the structure of the specific rhodopsin-interacting regions is unclear. A partial remedy has been provided by an NMR study of a peptide derived from the carboxyl terminus of Gαt in its rhodopsin bound conformation; these data suggest that it forms a helical extension of the α5 helix of Gαt (Kisselev et al., 1998) (Fig. 6-1).

**The Mechanism of R*-Catalyzed Nucleotide Exchange**

The photoisomerization of 11-cis-retinal to ATR leads to local structural alterations in the chromophore binding pocket of rhodopsin. These structural changes are propagated to the cytoplasmic surface of rhodopsin, and following binding of Gt, on to the nucleotide binding pocket of Gαt where GDP is released. In this way, the chromophore binding pocket of rhodopsin is allosterically coupled to the nucleotide binding pocket of Gαt approximately 5 nm away (Fig. 1-2). This conformational coupling transfers information that the chromophore of rhodopsin has absorbed a photon an equal distance.

Several key observations characterize the process of R*-catalyzed nucleotide exchange. In the absence of a catalyst, the rate limiting step in nucleotide exchange is release of GDP from Gαt to form empty-pocket Gt, Gαt(e)βγt. Gαt(e) is by itself very unstable. R* catalyzes nucleotide exchange by inducing GDP release and stabilizing the
reaction intermediate, \( \text{Go}_{\text{a}}(\text{e})\beta\gamma_{\text{f}} \). The empty pocket \( \text{Gt} \) can be dissociated from \( \text{R}^* \) by either GDP or GTP; \( \text{R}^* \) and nucleotide binding are mutually exclusive. GTP binding is nearly irreversible since conformational changes in the Switch II region destroy the \( \text{G}\beta\gamma_{\text{f}} \) binding site and induce dissociation of \( \text{Go}_{\text{a}}(\text{GTP}) \) from \( \text{G}\beta\gamma_{\text{f}} \) and \( \text{R}^* \). \( \text{R}^* \) interacts specifically with heterotrimeric \( \text{Gt} \); \( \text{G}\beta\gamma_{\text{f}} \) appears to be absolutely required for efficient \( \text{R}^* \)-catalyzed nucleotide exchange on \( \text{Go}_{\text{a}} \) (Fung, 1983). It is unclear whether \( \text{G}\beta\gamma_{\text{f}} \) plays a mechanistic role in the catalysis process (Iiri et al., 1998) or whether it merely facilitates binding between rhodopsin and \( \text{Go}_{\text{a}} \) (Phillips et al., 1992). Binding of \( \text{Gt} \) to \( \text{R}^* \) and dissociation of GDP appear to be distinct steps; rhodopsin mutants have been described which bind \( \text{Gt} \) but do not induce GDP release (Franke et al., 1990; Ernst et al., 1995).

The molecular mechanism by which \( \text{R}^* \) induces GDP release from \( \text{Gt} \) is the least understood step in the \( \text{Gt} \) signaling cycle. Despite a great deal of data regarding structures of \( \text{Gt} \) and rhodopsin that interact with each other, little is known about the detailed structure of the complex (Liu et al., 1995). The structure of \( \text{R}^* \) is not known, and the conformational changes, if any, that occur in \( \text{R}^* \) and \( \text{Gt} \)-GDP upon complex formation are not known. Very few sites of point-to-point contacts between \( \text{R}^* \) and G protein have been reliably identified (Acharya et al., 1997), and those that have been found do not greatly constrain possible geometric alignments of the two proteins in the complex. Crystallographic analysis of the \( \text{R}^* \)-\( \text{Gt}(\text{e}) \) complex may prove difficult due to the instability of \( \text{R}^* \). The alignment of the interacting surfaces in the structures of rhodopsin and \( \text{Gt} \) produces a hypothetical low resolution model of the complex (Bourne, 1997) (Fig. 1-2). These analyses, while lacking details, do suggest clearly that the cytoplasmic loops of rhodopsin, which are roughly 1.5 nm long (at most) are too short to contact directly the nucleotide-binding pocket of \( \text{Go}_{\text{a}} \), which is at least 2.5 nm from the
rhodopsin-binding surface of G\textsubscript{T}. Consequently, R* must act "at-a-distance" to induce nucleotide exchange in G\textsubscript{\alpha} \textsubscript{T} (Iiri et al., 1998).
Advantages of Rhodopsin and Transducin as a Model System

Rhodopsin and \( G_t \) offer a number of distinct advantages as a model system for the study of the molecular basis of signal transduction. First, both rhodopsin and \( G_t \) are available in large quantities from a natural source, the retina. Milligram quantities of both proteins can be purified from bovine retinas using established techniques (Kühn, 1980). Rhodopsin and \( G_t \) purified from bovine retinas are integral to the studies presented in this thesis, particularly the fluorescence \( G_t \)-activation assays presented in Chapter 3, the \( G_t \)-binding photoregeneration assays in Chapter 4, and the rhodopsin-catalyzed nucleotide exchange assays described in Chapters 5 and 6.

Second, rhodopsin is suited to spectroscopic and biophysical studies since it absorbs visible light (\( \lambda_{\text{max}} = 500\text{nm}, \varepsilon = 47,500\ \text{M}^{-1}\text{cm}^{-1} \)). The \( \lambda_{\text{max}} \) is sensitive to the environment of the chromophore, which is determined by the conformation of the surrounding protein. In particular, the \( \lambda_{\text{max}} \) shifts dramatically from 480 nm to 380 nm upon deprotonation of the Schiff base, which occurs during the transition from MI to MII. Therefore, visible spectroscopy is a powerful monitor of rhodopsin conformation. A spectroscopic assay is used in Chapter 4 to probe rhodopsin conformations. \( G\alpha_t \) conformation is effectively assayed by both fluorescence spectroscopy (Chapter 3) and trypsin proteolysis (Chapters 5 and 6), both of which rely on changes in conformations of residues in the Switch II region (see above).

Third, synthetic genes have been developed for both rhodopsin (Ferretti et al., 1986) and \( G\alpha_t \) (Sakmar and Khorana, 1988) allowing for facile preparation of site-directed mutants. Robust methods for the heterologous expression and purification of recombinant rhodopsin (Sakmar et al., 1989) allow for the study of site directed mutants in Chapters 3 and 4. Although \( G_t \) is difficult to express heterologously, \textit{in vitro}
expression methods have been developed for the characterization of recombinant samples, as described in Chapters 5 and 6.

Finally, rhodopsin and $G_t$ are each members of large families of related proteins. Rhodopsin is a prototypical member of the superfamily of seven transmembrane G protein coupled receptors (GPCRs). Members of this family, of which there are hundreds, have evolved for the detection of a tremendous array of external stimuli, from hormones, to neurotransmitters, ions, odorants, and tastants. Likewise, as the pioneering work of Rodbell and Gilman suggested, $G\alpha_t$, $G\beta_t$ and $G\gamma_t$ are members of families containing at least 20, 6, and 12 subtypes, respectively (Hamm, 1998). These proteins are widely expressed and transduce signals between a wide variety of GPCRs and effectors. Two well-studied subtypes of the $\alpha$ family are $G\alpha_i$ and $G\alpha_s$, which cause the inhibition and stimulation of adenylyl cyclase activity, respectively. Membership in large families provides two advantages to the study of rhodopsin and $G_t$ as a signal transduction model. First, comparison and contrast between the sequences, structures, and functions of these closely related proteins provides insight into their molecular mechanisms. Chimeric proteins constructed from different subtypes have proven to be powerful tools to localize domains responsible for specific functions in both GPCRs (Liu et al., 1995; Eason and Liggett, 1996) and in G proteins (Skiba et al., 1996; Remmers et al., 1999). Chimeric constructs between rhodopsin and two related receptors, the $\beta_2$ adrenergic receptor, and the m2 muscarinic receptor, are used in Chapters 3 and 4 to identify regions of rhodopsin specifically involved in $G_t$ interactions. Second, the molecular mechanisms by which rhodopsin binds and activates $G_t$ likely apply to interactions between the huge variety of GPCRs and G proteins which mediate numerous important functions.
Outline of the Thesis

In this thesis the role of several structures thought to be involved in the coupling of the chromophore binding pocket of rhodopsin to the nucleotide binding pocket of $G_t$ are evaluated. The work begins at the cytoplasmic surface of $R^\ast$. In Chapter 3, the role of the fourth cytoplasmic loop of rhodopsin (C4) in interactions with $G_t$ is evaluated using peptides, site-directed mutagenesis, and fluorescence $G_t$-activation assays. In Chapter 4 a highly sensitive spectroscopic assay is used to measure the specific binding of $G_t$ and peptides derived from the carboxyl termini of $G_\alpha_t$ and $G_\gamma_t$ to the cytoplasmic surface of a subset of the mutants in C4. This assay exploits the chromophore as a monitor of the conformation of rhodopsin. Together, the results in Chapters 3 and 4 provide evidence for the involvement of the amino terminus of C4 in rhodopsin-$G_t$ interaction, and suggest a specific point-to-point interaction between C4 and the carboxyl terminus of $G_\alpha_t$. The work in Chapters 3 and 4 on C4 of rhodopsin was completed and published (Marin et al., 2000; Ernst et al., 2000) before the determination of the crystal structure of rhodopsin (Palczewski et al., 2000). The structure does not alter any of the key conclusions reached in those chapters; however, in Chapter 7 those experiments are re-addressed in light of the new structural information.

In Chapter 5, the development of a sensitive and accurate method of assaying the rates of both uncatalyzed and $R^\ast$-catalyzed nucleotide exchange of $G_\alpha_t$ and recombinant $G_\alpha_t$ mutants expressed in vitro is described. These methods are used to examine a long-standing hypothesis regarding the mechanism of nucleotide exchange that suggests the involvement of interdomain interactions between the Ras-like and the helical domains of $G_\alpha_t$. The data demonstrate that contrary to what would be predicted by the hypothesis, disruption of interdomain interactions by site-directed mutations does not affect either basal or $R^\ast$-catalyzed nucleotide exchange rates. In Chapter 6, similar methods are used
to test a specific hypothesis regarding the mechanism by which C4-mediated R* interactions with the carboxyl terminus of Go\textsubscript{t} could affect the nucleotide binding pocket "at-a-distance". This hypothesis suggests a key role for the \( \alpha 5 \) helix of Go\textsubscript{t}. The \( \alpha 5 \) helix connects the carboxyl-terminus to the \( \beta 6/\alpha 5 \) loop, which lies adjacent to the nucleotide. The results identify a cluster of residues on the \( \alpha 5 \) helix, which when mutated, dramatically accelerate nucleotide exchange. A mechanistic model for R*-catalyzed nucleotide exchange is presented based on these data. Finally, in Chapter 7, a discussion of the thesis research as a whole is presented.
FIG. 1-1. The $G_t$ activation cycle. Rhodopsin (R) is activated by light, and forms $R^*$. $R^*$ binds inactive, GDP-bound $G_t$ heterotimer ($G\alpha_t(GDP)\beta\gamma_t$). GDP is released, and a complex between $R^*$ and empty pocket $G_t$ ($G\alpha_t(e)\beta\gamma_t$) is formed. Subsequently, GTP binds and activates $G\alpha_t$. $G\alpha_t(GTP)$ then dissociates from $R^*$ and from $\beta\gamma_t$, and interacts with its effector, PDE. Activated PDE catalyzes the hydrolysis of cGMP to GMP. GTP is hydrolyzed by an intrinsic GTPase activity of $G\alpha_t$, leading to re-formation of the inactive, GDP bound heterotimer. Those protein molecules for which crystal structures have been determined are boxed.
FIG. 1-2. Crystal structures of the $G_t$ heterotrimer and rhodopsin. Top, The crystal structure of dark state rhodopsin (PDB code 1f88) is shown with the cytoplasmic surface at the bottom, and the extracellular surface facing up. The chromophore, 11-cis-retinal, is magenta. The transmembrane helices are labeled TM1-TM7. The second (C2), third (C3) and fourth (C4) cytoplasmic loops are labeled; helix 8 (H8) is part of C4. Unordered segments of the cytoplasmic surface are not shown. The approximate position of the disc membrane surfaces are indicated by black lines. Bottom, The structure of GDP bound $G_t$ (PDB code 1got) is shown with the presumed rhodopsin-interacting surface facing up. The Ras-like domain of $G\alpha_t$ is blue, the helical domain of $G\alpha_t$ is red, GDP is magenta, the $\alpha5$ helix of the Ras-like domain is yellow, and the Switch II region of the Ras-like domain is green. $G\beta_t$ is orange, and $G\gamma_t$ is cyan. The carboxyl-terminal 7 residues of $G\alpha_t$, and 5 residues of $G\gamma_t$, are not present in the structure. The amino and carboxyl termini of $G\alpha_t$ and $G\gamma_t$, and the amino terminus of $G\beta_t$ are labeled. Structures thought to interact with rhodopsin and/or the membrane, including the amino and carboxyl termini of $G\alpha_t$ and the carboxyl terminus of $G\gamma_t$ cluster on a common surface of $G_t$. The relative orientation of the cytoplasmic surface of rhodopsin and the rhodopsin-binding surface of $G_t$ is arbitrary. Upon formation of the $R^*-G_t$ complex, the chromophore binding pocket becomes allosterically coupled to the nucleotide binding pocket of $G\alpha_t$, approximately 50 Å away (see text). The structure of the complex is unknown.
FIG. 1-3. Primary and secondary structure of rhodopsin. This figure is adapted from Menon et. al. (2001). The seven transmembrane helices are indicated by colored cylinders; the membrane associated H8 is shown as a red cylinder.
FIG. 1-4. The structure of Goα. A, GDP-bound Goα (PDB code 1tag). The structure includes residues 27-340. The ras-like domain is blue, the helical domain is red, the nucleotide is magenta, and the α5 helix of the ras-like domain is yellow. The Switch I region is cyan, the Switch II region is green, and the Switch III region is orange. B, The structure of GTPγS-bound Goα (PDB code 1tn1). The structure includes residues 26-342. The coloring is the same as in panel A. Note the GTP-induced conformational changes in the Switch regions relative to the GDP-bound structure in panel A.
FIG. 1-5. **Primary and secondary structures of Gα4.** The aligned sequences of Gα4, Gαb, and Gαi are presented. Tick marks are placed after every 10th residue in the Gα4 sequence. Secondary structure elements, deduced from the structure of GDP-bound Gα4, are presented above the sequence. Certain regions, such as the Switch regions and the Insert (I) regions (see Chapter 5) are presented below the sequence. The helical domain consists of helices αA - αF; the rest of the molecule contributes to the Ras-like domain. The linker regions which connect the Ras-like and helical domains are indicated above the sequence. Residues mutated in Chapters 5 are colored blue, and those mutated in Chapter 6 are colored red. This figure is adapted from Lambright, et. al., 1994.
Chapter 1: Introduction

Gαc (1) MGA-GAS----AEE----KHSRELEKKIEDAEEKDARTVVLLLGAGE
Gαs (1) MGCGLNSKTEDQRNEEKAQREANKKIEKQLQKDYQVRATHRLLLLAGE
Gαi (1) MGC-TLS----AEDKAVERSKMDRNLREDGEKAAREVKLLEGAGE

Gαc (40) SGKSTIVKMITEHQDGYESLE--ECKQYKAVVSYNTL
Gαs (51) SGKSTIVKMRILHVNGFNGEGGDGFQABARSNDGKATKVQDIKNL
Gαi (44) SGKSTIVKMKIEADKQYKDYQINQKLVK

Gαc (75) QSILAIVRAMTLNITQGDSARGDDAR-KLMHMADTIIEGTMPSMIDII
Gαs (101) EAIETIVAAMSLVPPTELAPNQFQVDIYLSVMNPDPDFDPEFYEHA
Gαi (79) QSIIAIRAMGRKIDFGSDUADDAR-QLFVLAGAEEGFMATAELAGVI

Gαc (124) QRLWDQIGACPDRAEYQDLSNYAGYESLDLRLVTQGVPTQDVLR
Gαs (151) KALWEDBIEVRSYQXIDCAQYFLDKIDVIQDDYVPVPSDQDOLRC
Gαi (128) QRLWDQIGACPDRAEYQDLSNYAGYESLDLRLVTQGVPTQDVLR

Gαc (174) RVKTTGITEFIQFSDKLNFMDVVGQSRERKKWICFGVTCIFIAAL
Gαs (201) RVLTSGIFETHQFSDKLNFMDVVGQSRERKKWICFGVTAIFCVAL
Gαi (178) RVKTTGITEFIQFSDKLNFMDVVGQSRERKKWICFGVTAIFCVAL

Gαc (224) SAYDMVILVEDEVPRMHESLHLFSICNHRCYATTISVILFLNKKDFVSEK
Gαs (251) SSYMVIREDNQTNLFGQALNLFKISWNWLRATIVSITFLNKQDLLAEK
Gαi (228) SDYDVLALAEDEEMNMHESMKLFDICNNKFWTDNTLISILFLNKKDLEEK

Gαc (274) I---KKAHLSIICFDPYNGPNPTEFA----GNYIKVQFLELN
Gαs (301) VLAGKSICEDYFPFARFARYTFPEDATPEPGEAPVTRAKFIRDFELRIST
Gαi (278) I---KKSPHTICYEYAGSNYEEA---AAYIQCFQFELNK

Gαc (309) RRDKVKE---IYSHMCTATDTQNVK/FDAVDI/IIIKNLKDCLGF
Gαs (351) ASGDRHCYPHFCTAVDENTIRRVFNDCRDIQRHMLQYELL
Gαi (313) RKDTKE---IYTHFTCATDTKNQVFDFADTV/IIKNNLKDCGLF

30
Chapter 2:
Materials and Methods

Reagents

Buffers and salts were from Sigma or Boehringer Mannheim. Nucleotides and protease inhibitors were from Roche. The TnT Quick Coupled Transcription/Translation kit (rabbit reticulocyte lysate) for in vitro expression of Go\(_t\) was purchased from Promega. \(^{35}\text{S}\)Methionine was purchased from New England Nuclear. TPCK-treated trypsin was from Worthington Biochemicals. Synthetic oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer, or were purchased from Genelink, Inc. DNA sequencing was carried out using BigDye Terminator Cycle sequencing in the DNA sequencing core facility at the Rockefeller University.

Preparation of Rhodopsin-derived Peptides

Peptides used in Chapter 3 were synthesized at the Rockefeller University Protein/DNA Technology Center and HHMI Biopolymer Facility by solid phase technique using FMOC chemistry. All peptides were prepared with free amino termini, and were amidated at the carboxyl termini. The peptides were purified by HPLC and characterized by mass spectrometry. The names and amino acid sequences of the peptides used in Chapter 3 are the following: rho(132-144), AIERYVVVCKPMS; rho(240-252), SATTQKAEEKVTR; rho(310-321), NKQFRNCMVTTL; rho(313-321), FRNCMVTTL; rho(310-321)scr, TLTVNMKCQNF; rho(310-321)SPD, SPDFRNCMVTTL.

Preparation of Go\(_t\)-Derived Peptides

The synthetic peptides used in Chapter 4 are listed in Fig. 4-1B. Peptides were synthesized by Dr. P. Henklein, Humboldt University, Berlin, using the Fmoc strategy with HBTU activation (Fastmoc 0.1 mmol small-scale cycles) on an ABI Model 433A
peptide synthesizer. Farnesylation of Gγ-t-derived peptides was carried out by dissolving pure peptide (60 mg) in 5 ml of a solution of 50% (v/v) 1-propanol containing 35 mmol sodium carbonate. The resulting solution was saturated with nitrogen and 0.6 ml of a freshly prepared 10% (v/v) farnesyl bromide solution in 1-propanol was slowly added under vigorous stirring while pH was adjusted to >9. The solution was flushed with nitrogen again and incubated for 24-48 hrs with shaking. The farnesyl peptides were purified by reverse phase HPLC.

Preparation of Gi, Gαγ, Gβγ, and Rhodopsin from Bovine Retinas

Gγ was prepared from frozen bovine retinas (Lawson, Inc., Lincoln, NE) using standard techniques (Kühn, 1980; Fung et al., 1981). In a typical preparation, 200 retinas were thawed and combined with 120 ml of 30% sucrose solution in isotonic buffer (70 mM potassium phosphate, pH 6.8, 1 mM magnesium acetate, 1 mM DTT, 0.1 mM PMSF, and 1 μg/ml E-64). All subsequent steps were performed in a cold room under ambient light. The rod outer segments were released by vortexing the solution for 1 min. The resulting solution was centrifuged for 6 min at 5000 rpm in a Sorvall SS-34 rotor. The supernatant was collected. Each pellet was resuspended in 15 ml of 30% sucrose solution, and centrifuged again. The supernatants were pooled and diluted with an equal volume of isotonic buffer, and then centrifuged in a Sorvall SS-34 rotor for 10 min at 10,000 rpm. The supernatants were discarded, and the pellets were resuspended in 100 ml of 15% sucrose solution in isotonic buffer by passing the pellets through a 23 gauge needle 3 times. The resuspended pellets were distributed into 8 centrifuge tubes, and underlayed with 10 ml of 0.64 M sucrose in isotonic buffer. The samples were centrifuged for 10 min at 10,000 rpm, and the supernatants were discarded. The pellets were resuspended in 0.64 M sucrose solution by passage through a 23 gauge needle. The solution was overlayed onto a discontinuous sucrose gradient consisting of 9 ml each of 1.2 M, 1.0 M, and 0.78 M sucrose. The tubes were centrifuged a Beckman SW-28 rotor.
Chapter 2: Materials and Methods

for 30 min at 25,000 rpm in a Beckman ultracentrifuge with the acceleration and
deceleration set to 6. The rod out segments (ROS) formed a band at the interface of the
0.78 M and 1 M sucrose solutions; the band was collected using a syringe. The collected
ROS were diluted to a total volume of 200 ml using isotonic buffer. The ROS was
pelleted by centrifugation for 20 min at 28,000 rpm in a Beckman Ti-45 rotor. The
pellets were resuspended in 200 ml isotonic buffer by passage through a syringe, and
pelleted and resuspended twice more. The pellets were resuspended in 200 ml hypotonic
buffer (5 mM Tris, pH 7.5, 5 mM magnesium acetate, 1 mM DTT, 0.1 mM PMSF, and 1
µg/ml E-64). The resulting suspension was photolyzed for 10 min using a Dolan-Jenner
lamp equipped with a >495 nm filter and a fiber optic attachment. The ROS were
pelleted as before, and resuspended in hypotonic buffer a total of three times. Transducin
was eluted from the ROS by resuspending the membrane pellet in 100 ml hypotonic
buffer supplemented with 0.1 mM GTP. The membranes were pelleted, and the
supernatant collected. The membranes were extracted with GTP a total of three times.
The eluted transducin was further purified and concentrated by hexyl agarose
chromatography. The GTP extracts (300 ml) were loaded onto a 10 ml hexyl agarose
column overnight at a flow rate of <0.3 ml/min. The column was washed with 50 ml of
hypotonic buffer, followed by 50 ml of hypotonic buffer with 75 mM NaCl. Transducin
was eluted with hypotonic buffer with 300 mM NaCl. The eluted transducin, generally 3-
10 ml, was dialyzed overnight into storage buffer (10 mM NaHEPES, pH 7.5, 2 mM
MgCl₂, 1 mM DTT, 5 µM GDP, and 50% glycerol). Typical yields were from 1-8 mg.

Specific activities of Gᵣ samples were determined by spectrofluorometric titration, as
described previously (Fahmy and Sakmar, 1993a). Gᵣ used in the photoregeneration
studies was purified as described (Heck and Hofmann, 1993). Gβγᵣ and Gαᵣ were
isolated from holo-Gᵣ essentially according to published methods (Shichi et al., 1984)
using a Hitachi LC-organizer HPLC system with 1 ml Hi-Trap Blue Sepharose column
Chapter 2: Materials and Methods

(Amersham Pharmacia Biotech). The proteins were eluted from the column by applying a 0-2 M NaCl gradient. Protein concentrations were determined using the Bio-Rad protein assay reagent according to manufacturer’s instructions. The subunits were stored at \(-20^\circ C\) in a 50% glycerol buffer until use. Urea-washed disc membranes, the gift of Dr. K. C. Min, were prepared as described elsewhere (Min et al., 2000). The membranes were solubilized in 1% (w/v) n-dodecyl \(\beta\)-d-maltoside (DM), and insoluble material was removed by centrifugation. The resulting solubilized rhodopsin, used in Chapters 5 and 6, displayed a \(A_{280}/A_{500}\) spectral ratio of <1.8 and a concentration of \(\sim 8 \mu M\).

**Measurement of Intrinsic Fluorescence of \(G\_t\), \(Ga\_t\), and \(G\beta\_t\)**

Fluorescence measurements were done on a Spex Fluorolog 3-11 \(\tau 3\) spectrofluorometer equipped with a 450 W Xenon arc lamp. All fluorescence experiments were performed in 10 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl, 2 mM MgCl\(_2\), 1 mM DTT, 5 \(\mu M\) GDP and 0.01% (w/v) dodecyl maltoside (DM). Spectra were recorded at \(10^\circ C\) in a 4 mm x 4 mm quartz cuvette. Protein fluorescence was obtained by exciting at 295 nm and monitoring emission from 315 to 450 nm. The excitation and emission slit bandpass were 1.5 and 5 nm, respectively. Titration experiments were typically performed by adding 10 \(\mu l\) aliquots of the peptide from a stock solution of 250 \(\mu M\) to a protein solution of 200 nM.

**Site Directed Mutagenesis of Rhodopsin**

Site directed mutagenesis was achieved primarily by using restriction fragment replacement (Lo et al., 1984) in a synthetic rhodopsin gene (Ferretti et al., 1986) cloned into a eukaryotic expression vector (Franke et al., 1988). Mutants CTr1 and CTr3 were constructed by substituting the \(BspE I - Sal I\) restriction fragment with a synthetic duplex containing the desired codon alterations; mutant CTr2 involved a similar substitution of the \(Apa I - Sal I\) fragment. Mutants CTr4 and the K311 point mutations involved
substitution of an *Apa I-BspE I* fragment. The mutant CysXV (C322S/C323S) was constructed by substituting the *Xho I-BstE II* fragment of the rhodopsin gene into CysXIII (C140S/C316S/C322S/C323S). CysXIII was prepared by substituting the *BspE I-Sal I* fragment of a C140S mutant (Karnik *et al.*, 1988) with a synthetic fragment that contained the appropriate codon alterations for C316S, C322S, and C323S. The combination mutant CTr4/CysXV was prepared by cloning the *Xho I-BstE II* fragment of CTr4 into a *Xho I-BstE II* digested CysXV vector. Cell culture, transfection, and immunoaffinity purification procedures have been described elsewhere (Sakmar *et al.*, 1989; Min *et al.*, 1993; Han *et al.*, 1996). For samples used in photoregeneration studies, the samples were concentrated ~10-fold using Centricon-30 filtration devices (Amicon) following purification. Membranes from transfected cells were prepared prior to regeneration with 11-cis-retinal using sucrose density gradient centrifugation, as described previously (Han and Sakmar, 2000).

**Fluorescence Gf Activation Assay**

The assay was performed essentially as described (Fahmy and Sakmar, 1993a). A Spex Fluorolog instrument was used with a 150 W xenon arc lamp. A solution of 250 nM Gf was prepared in fluorescence buffer (100 mM NaCl/10 mM Tris, pH 6.9/2 mM MgCl₂/1 mM DTT/0.01% DM). 1.5 ml of solution was placed in a semi-micro cuvette, and loaded into the thermojacketed cuvette holder equipped with a magnetic stirrer at 10°C. The cuvette was continually illuminated with a HeNe laser and a fiber optic attachment. Protein fluorescence was excited at 300 nm with 2 nm bandpass, and emission intensity was collected at 345 nm with a 12 nm bandpass. At 100 s, 50 µl of rhodopsin or rhodopsin mutant was injected into the cuvette using a Hamilton syringe to a final concentration of 1 nM. At 200 s, the activation reaction was initiated by injecting a 50 µl solution of GTPγS to a final concentration of 5 µM. Activation of Gf was observed as an increase in the intensity of tryptophan fluorescence emission that results from

35
conformational changes in W207 that occur upon binding GTP. Peptide competition assays were performed with 200 nM Gt and 1 nM of purified COS cell-expressed rhodopsin from which 1D4 peptide introduced in the purification procedure was removed by gel filtration on a G-50 Nick column (Pharmacia Biotech). The appropriate concentration of peptide was added from a 15 mM stock solution and pre-incubated with Gt for 30 min before the start of the assay.

Measurement of $[^3H]$Palmitic Acid Incorporation

Opsin was metabolically labeled with $[^3H]$palmitic acid (New England Nuclear, Boston, MA) essentially as described previously (Karnik et al., 1993). Briefly, 48 h post-transfection, COS cells were grown for 8 h in serum-free media. The cells were then incubated for 30 min in 1% serum, followed by 2 h in 1% serum supplemented with 100 μCi/ml $[^3H]$palmitic acid (43 Ci/mmol). Cells were washed with phosphate-buffered saline, harvested, and solubilized in 0.1% (w/v) DM solution. The detergent extracts were incubated overnight with resin conjugated with 1D4 monoclonal antibody as used in the standard rhodopsin purification procedure (Oprian et al., 1987). The resin was washed extensively, as monitored by the decreasing tritium counts present in successive washes. Opsin was eluted from the resin by incubation with wash buffer containing the 1D4 peptide. The relative amounts of $[^3H]$palmitic acid incorporated into the eluted samples were analyzed by scintillation counting.

Instrumentation for Measurement of Photoregeneration Traces

Time-resolved absorption traces of rhodopsin samples were recorded on a custom-built single-wavelength absorption photometer in the laboratory of Dr. K. P. Hofmann, Humboldt University, Berlin. Light from a 150-W halogen light source passes through a Jobin-Yvon HR460 monochromator (focal length 460 mm, 1200 lines/mm, slit width set to 1 mm) tuned to 543 nm, and from there through the cuvette (4-mm optical pathlength)
and a bandpass interference filter onto a large surface PIN photodetector. The output is
nullled and amplified twice, filtered with a 500 μs electronic low-pass filter and recorded
using a modified Nicolet 2090-IIIA digital oscilloscope.

Photoregeneration Experiments

The rhodopsin photoregeneration assay was performed as reported (Amis and
Hofmann, 1995) with adaptation for recombinant pigments as follows. All samples
contained 2 μM pigment in a volume of 0.26 ml of 200 mM Na₂HPO₄ (pH 8.0), 10 mM
NaCl, and approximately 0.03% (w/v) dodecyl maltoside (DM). Due to the concentration
procedure required for recombinant samples, the final DM concentration was not
precisely known, but was estimated not to exceed 0.035% (w/v). After equilibrating the
sample cuvette to 13 °C, the sample was illuminated for 30 s with a green HeNe laser
(543.5 nm, 5 mW, Melles Griot) to cause quantitative formation of MII. Absorption at
543 nm was recorded continuously. After 50 ms, a flash of blue light (412 ± 7 nm, ca. 20
μs duration) was applied to the sample and formation of photo-regenerated pigment was
measured at 543 nm for an additional 200 ms. Discharge of the flashlamp affected the
sensitive electronics of the detector, causing a brief artifactual negative deflection. Four
records induced by four separate flashes were collected from each sample with 30 s
intervals between the recordings. Starting with the initial illumination, each experiment
took approximately 140 s. The four records were averaged to produce experimental data
traces as presented in Fig. 4-2. The experimental photoregeneration signal traces are
depicted as absorbance changes at 543 nm versus time (i.e., a rising signal indicates a
proportional increase of absorbance due to reprotonation of the Schiff base).

Numerical Fitting Procedures and Determination of Initial Slope Values of
Photoregeneration Traces

The photoregeneration signal comprises a fast phase, which is not resolved, and a
slow phase, which is monitored for 200 ms (see Fig. 4-2). Data points obtained 4.5–7.0
ms after the blue flash were averaged and used as an estimate for the amplitude of the fast phase. The relative amplitude of the fast phase of the photoregeneration of the recombinant pigments was the same as that of rhodopsin. The initial slope of the slow phase of a photoregeneration trace was determined from the numerical fit of a simple exponential-rise function offset by the amplitude of the fast phase. Values for relative slope are presented (Table 4-1) to demonstrate the effect of G\textsubscript{t} or G\textsubscript{t}-derived peptides on the initial slope of the photoregeneration signal. Relative slope is defined as the ratio of the initial slope of the slow phase of the photoregeneration trace in the presence of G\textsubscript{t} or G\textsubscript{t}-derived peptides versus the initial slope in their absence. A relative slope of 1.0 indicates no effect, whereas a slope of <1.0 indicates inhibition of photoregeneration. The relative slope for an experiment with rhodopsin and G\textsubscript{t} (3 \mu M) was typically about 0.7.

**Site-Directed Mutagenesis of G\alpha\textsubscript{t} and G\alpha\textsubscript{i}**

The parent for all G\alpha\textsubscript{t} constructs was pGEM2sTo\alpha, the synthetic bovine G\alpha\textsubscript{t} gene cloned into the pGEM2 plasmid under control of a SP6 promoter. Point mutations and the 25-amino-acid deletion from the carboxyl terminus were prepared using the QuickChange method (Stratagene). For each mutant, two complementary primers were designed that coded for the desired mutation as well as 10-15 bases of complementary sequence on either side of the mutation site. Most amino acid substitutions could be accomplished with two or less nucleotide changes. The total length of each primer was 20-30 bases. The mutagenesis reaction (50 \mu L final) consisted of 5ng of template plasmid, 1\mu L of cloned Pfu polymerase (2.5 units/\mu L)(Stratagene), 5 \mu L of 10x Pfu buffer (Stratagene), 250 nM of each primer, 800 \mu M of dNTP mix. The reactions were thermocycled in a GeneAmp 9600 (Perkin Elmer Cetus) thermocycler with the following program: 3 min at 95°; 14 cycles of 30 s at 95°, 1 min at 55°, 8 min at 68°; and then 10 min at 72°. Amplification of the mutated plasmid was verified by running 1\mu L of the
reaction on a 1% agarose gel. The parental plasmid was selectively digested using 1 µL of the restriction enzyme DpnI (New England Biolabs) per reaction for 1.5 h at 37°C. Since DpnI digests only methylated DNA the wild type template plasmid was restricted. The DpnI-treated mutant plasmid was transformed into chemically competent bacteria (subcloning efficiency DH-5α (Gibco) or OneShot TOP10 (Invitrogen)). Generally the ratio of recombinant transformants from QuickChange reactions done with primers compared with control reactions run without primers was >50:1. All constructs were verified by automated DNA sequencing of the entire coding region of Goq.

The insertion mutants α5 ala3, α5 ala4, and CT ala4 as well as the deletion mutants CT del10 and CT del5 (Fig. 6-3) were prepared using cassette mutagenesis. DNA duplexes corresponding to the BstEII/XbaI fragment of the synthetic Goq gene were synthesized to encode the appropriate mutation. The duplexes were ligated into BstEII/XbaI digested pGEM2sTα. The pGEM2sTα was prepared from a dam bacterial strain to prevent methylation of a GATC sequence that partially overlaps the XbaI restriction site.

The parent for all Goq1 constructs was pGEM2Goq1, the Goq1 gene cloned into the pGEM2 plasmid under control of a SP6 promoter. To improve expression, a 217 bp segment of the 5' untranslated region was removed by QuickChange mutagenesis.

In vitro Transcription and Translation of Goq

Recombinant Goq subunits were prepared using the TNT Quick Coupled rabbit reticulocyte lysate transcription/translation kit (Promega). For each translation, 20µl of lysate mix was combined with 4 µl of DNA (0.5 µg total) and 1 µl of ~9µM [35S]methionine (New England Nuclear) at a specific activity of approximately ~1250 Ci/mmol. The reactions were incubated at 30°C for 90 min. Subsequent manipulations were performed on ice or at 4°C. The translated products were passed over BioSpin 6 gel
filtration spin columns (BioRad) twice consecutively to remove excess nucleotides. The volume of each reaction was then adjusted to 100 µl in a buffer of 5 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 0.01% (w/v) DM. If the reaction was to be studied in a rhodopsin catalyzed assay, Gβγt was added to a final concentration of 30 nM. Every experiment was performed on freshly translated material.

**Trypsin Proteolysis and Analysis**

The digestion procedure was adapted from Garcia et al., 1995. Aliquots (8 µl) of the in vitro expressed protein were withdrawn and mixed with 1.5 µl of digest buffer (5% Lubrol, 2 mM GDP, 1 mg/mL TPCK trypsin) or digest control buffer (5% Lubrol, 2 mM GDP). The reactions were incubated on ice for 30 min (or 5 min for Gα₁₁ samples). Digestion was terminated by the addition of 2.5 µl of 10 mg/ml aprotinin/10 µM phenyl methyl sulfonyl fluoride (PMSF), followed by 6 µl of 3x SDS sample buffer (New England Biolabs). Samples were occasionally stored at -20° for up to 48 hrs. Subsequently, the samples were boiled for 3 min and the protein fragments were resolved by polyacrylamide gel electrophoresis on 15% pre-cast Tris-HCl minigels (BioRad). Following electrophoresis, the gels were fixed for >30 min in 50% MeOH/ 10% acetic acid and then soaked for 5 min in 7% methanol/ 7% acetic acid/ 2% glycerol. The gels were vacuum-dried onto filter paper (Whatman) and then exposed to a storage phosphor screen (Molecular Dynamics) for 1 to 7 days.

**Control Reactions for Trypsin Proteolysis of Gαt**

Control reactions were performed on each sample of expressed Gα₁ in order to check the quantity and apparent molecular weight of the expressed protein, as well as the digest patterns following incubation with GDP and GDP/AlF₄⁻. 30 µl of each sample was combined with GDP to a final concentration of 100µM. One 8 µL aliquot was removed and digested ("+GDP"); another 8 µl aliquot was removed and mock digested with digest
control buffer ("not digested"). The remaining 14 µl was combined with a final concentration of 0.17 mM AlCl$_3$ and 10 mM NaF added from separate 30x stock solutions. Following a 10 min incubation at room temperature, a 8 µl aliquot was removed and digested ("+GDP/AlF$_4$"").

Uncatalyzed Activation Time Course of $G\alpha_t$

Samples (70 µl each) of in vitro translated $G\alpha_t$ were quickly warmed to room temperature in a water bath and GTPyS was added to a final concentration of 100µM. Aliquots were withdrawn at 1, 2, 3, 4, and 6 h following GTP$_\gamma$S addition, and digested. In mutants that activated slowly, the activity of the protein following 6 h incubation at room temperature was investigated by addition of rhodopsin and G$\beta\gamma_t$ (30 nM each, final concentration) and incubation under room light for 20 min. A final 8 µl aliquot was then removed and digested. For certain mutants which activated very quickly, aliquots were taken at 10, 20, 30, 60, 90, and 120 min, and the rhodopsin/G$\beta\gamma_t$ mix was not added.

Rhodopsin/G$\beta\gamma_t$-Catalyzed Activation Time Course of $G\alpha_t$

Samples (70 µl each) were quickly warmed to room temperature in a water bath. A mixture of rhodopsin and GTP$_\gamma$S (4 µl) was added to the sample yielding a final concentration of 30 nM rhodopsin and 14 µM GTP$_\gamma$S. The rhodopsin was first photolyzed by illumination for 15 s with a fiber optic cable connected to a Dolan Jenner lamp equipped with a >495 nm long-pass filter. The samples were incubated at room temperature under the illumination. Aliquots were withdrawn and digested at 1, 2, 3, 5, 10, and 20 min following addition of the rhodopsin/ GTP$_\gamma$S mix.

Data Analysis of Trypsin Proteolyis Patterns

Following exposure to the dried gels, the phosphor storage screens were scanned using a Molecular Dynamics Storm Imager machine (Molecular Dynamics) at 200 micron resolution. The resulting images of the gels were analyzed using ImageQuant software.
(Molecular Dynamics). Lines were drawn down the center of each lane on the gel, perpendicular to the bands in the lane. The intensity of each pixel along the line was determined as the average of 5-10 pixels on either side of the line. In this way, most of the width of each lane was considered. Any defect in the gel was avoided by careful placement of the lines. The intensity of each pixel was plotted as a function of position along the line. The result was a graph of intensity as a function of distance down the lane; where the bands on the gel correspond to peaks on the graph. The baseline of the graph was set so as to exclude nonspecific background intensity. The areas of the ~23- and ~34-kDa peaks were then calculated and recorded in an Excel spreadsheet. The fraction of Gαq activated in a given lane was determined by the formula: \( \frac{\text{area of } \sim34\text{-kDa peak}}{1.4 \times \text{area of } \sim23\text{-kDa peak} + \text{area of } \sim34\text{-kDa peak}} \). The area of the ~23-kDa peak was multiplied by 1.4 to normalize for the smaller number of methionines in the smaller fragment relative to those in the ~34-kDa fragment. This coefficient was adjusted (to 1.3) for analysis of M228 mutations. Activation kinetics were analyzed by plotting the fraction of Gαq activated as a function of time. In uncatalyzed assays, the data were fit to a single exponential rise to a maximum equation of the form: \( \text{percent activated} = c + 100(1-\exp(-kt)) \). The apparent rate constants derived from the fits are presented in Table 5-1 and 6-1.

For experiments conducted with Gαi, the intensity of the GDP-dependent band could not be determined reliably due to nonspecific background intensities in the region of the gel where the GDP band migrated. This background was present even in undigested samples of Gαi. Since the ratio of the GTP dependent band to the sum of the GTP and GDP bands could not be determined, the intensity of the GTP band was expressed as a fraction of the total intensity in each lane. Activation time courses are plotted as the change in this intensity over time. For fully activated Gαi, the GTP band accounted for roughly 35% of the intensity in each lane.
Chapter 3:
The Amino Terminus of the Fourth Cytoplasmic Loop of Rhodopsin Modulates Rhodopsin—Transducin Interaction*

Summary

Rhodopsin is a seven-transmembrane helix receptor that binds and catalytically activates the heterotrimeric G protein transducin (Gt). This interaction involves the cytoplasmic surface of rhodopsin, which comprises four putative loops and the carboxyl-terminal tail. The fourth loop connects the carboxyl end of transmembrane helix 7 with C322 and C323, which are both modified by membrane-inserted palmitoyl groups. Published data on the roles of the fourth loop in the binding and activation of Gt are contradictory. Here, we attempt to reconcile these conflicts and define a role for the fourth loop in rhodopsin—Gt interactions. Fluorescence experiments demonstrated that a synthetic peptide corresponding to the fourth loop of rhodopsin inhibited the activation of Gt by rhodopsin and interacted directly with the α subunit of Gt. A series of rhodopsin mutants was prepared in which portions of the fourth loop were replaced with analogous sequences from the β2 adrenergic receptor or the m1 muscarinic receptor. Chimeric receptors in which residues 310-312 were replaced could not efficiently activate Gt. The defect in Gt interaction in the fourth loop mutants was not affected by preventing palmitoylation of C322 and C323. We suggest that the amino terminus of the fourth loop interacts directly with Gt, particularly with Goαt, and with other regions of the intracellular surface of rhodopsin to support Gt binding.

Considerable evidence has implicated C2 and C3 of rhodopsin as participating in the complex with Gt (König et al., 1989; Franke et al., 1990; Franke et al., 1992; Borjigin and Nathans, 1994). However, the literature addressing the role of C4 (Fig. 3-1) in interactions with Gt is contradictory. Studies have shown that peptides derived from C4 can disrupt the stabilization of MII by Gt (König et al., 1989), interfere with rhodopsin stimulated GTPase activity of Gt (Takemoto et al., 1986), and bind directly to a fluorescently labeled Gβγt and prevent Gβγt-rhodopsin interactions (Phillips and Cerione, 1992). In contrast, truncation of rhodopsin following N315, in the middle of C4, does not impair Gt activation (Weiss et al., 1994). Since truncation at the beginning of C4 precluded proper expression and/or processing of rhodopsin, a follow-up study examined a series of single and double mutations in the amino-terminal half of C4, from N310 through N315. None of the mutations was found to disrupt Gt activation, leading to the conclusion that C4 is not required for productive interactions with Gt (Osawa and Weiss, 1994).

In this Chapter, we have carefully re-examined and defined the role of the C4 loop in rhodopsin—Gt interactions. We used fluorescence spectroscopy to demonstrate that a synthetic peptide corresponding to C4 of bovine rhodopsin, rho(310-321), binds to Gt and free Gαt. Furthermore, we demonstrate the potent inhibition of rhodopsin-catalyzed Gt activation by rho(310-321). We also prepared and characterized a series of site-directed mutants of bovine rhodopsin with alterations of the C4 loop. These data show that when either the entire C4 loop or a tripeptide (N310-K311-Q312) at the amino terminus of the loop is replaced with the analogous sequence of the β2 adrenergic receptor (β2 AR), the Gt-activating function of rhodopsin is diminished. Neither replacement of the carboxyl-terminal half of the loop, nor removal of the palmitoylation
sites disrupt $G_t$ activation. We conclude that the C4 loop is involved in mediating interactions between rhodopsin and $G_t$.

**Results**

*Inhibition of $G_t$ Activation by Synthetic Peptides Corresponding to Cytoplasmic Loops of Rhodopsin*

Peptides derived from the C2, C3, and C4 loops of rhodopsin have been shown to disrupt the ability of $G_t$ to stabilize MII as measured by an extra-MII assay (König *et al.*, 1989). An experiment done by a post-doc in the Sakmar lab, Tatanya Zvyaga, examined whether similar peptides could also disrupt the activation of $G_t$ by catalytic amounts of solubilized rhodopsin in a fluorescence activation assay. In Fig. 3-2, a dose-dependent decrease in the rate of $G_t$ activation is observed in the presence of synthetic peptides derived from the amino terminus of C2 (rho(132-144)), the carboxyl terminus of C3 (rho(240-252)), and C4 (rho(310-321)). The effective concentration at 50% inhibition (IC$_{50}$) for all peptides was in the 0.1-0.3 mM range; all peptides inhibited activation completely at concentrations $\leq$1 mM. A C1-derived peptide, rho(61-75), only modestly inhibited $G_t$ activation at 1 mM (not shown).

*A Synthetic Peptide Derived From C4 of Rhodopsin, rho(310-321), Alters the Fluorescence Emission Wavelength Maximum of $G\alpha_t$ But Not of $G\beta\gamma_t$*

In an effort to characterize the interactions of C4 with $G_t$, a postdoc in the Sakmar Lab, A. Gopala Krishna, studied the intrinsic fluorescence emission spectra of $G\alpha_t$ and $G\beta\gamma_t$ in the presence of increasing concentrations of rho(310-321). A significant red shift ($7.8\pm0.3$ nm, n=4) in the $\lambda_{max}$ of tryptophan emission of $G\alpha_t$ was observed in the presence of 45 $\mu$M peptide (Fig. 3-3A). The shift was accompanied by a modest ($\sim$10%) increase in intensity. These spectral changes are indicative of a change in the molecular environment of at least one of the two tryptophans of $G\alpha_t$ caused by the binding of the
The extent of the red shift was dependent on the concentration of peptide (Fig. 3-3A, inset). In contrast, the \( \lambda_{\text{max}} \) of tryptophan fluorescence emission of \( \beta\gamma_t \) was only minimally (1.0±0.3 nm, \( n=4 \)) affected by the peptide (Fig. 3-3B). These data suggest that the peptide does not bind to free \( \beta\gamma_t \), but they do not rule out binding in a manner that does not alter the molecular environment of enough of its 8 intrinsic tryptophans to allow for spectroscopic detection. The emission spectrum of holo-\( \gamma_t \) was red shifted by approximately 4 nm in the presence of the peptide (data not shown), which is consistent with interaction with \( \alpha_t \) but not \( \beta\gamma_t \) in the context of the heterotrimer. The effects of three additional peptides on the \( \lambda_{\text{max}} \) of \( \alpha_t \) emission were examined. The peptides, which were derivatives of rho(310-321), were: (a) rho(313-321), in which residues 310, 311, and 312 were not present; (b) rho(310-321)\( \text{scr} \) in which the order of the amino acids was scrambled; and (c) rho(310-321)\( \text{SPD} \) in which the first three positions of the peptide were changed from NKQ to SPD. The sequence of rho(310-321)\( \text{SPD} \) is derived from the rhodopsin mutant CTr4 (Fig. 3-4). The peptides rho(313-321) and rho(310-321)\( \text{scr} \) did not affect the \( \lambda_{\text{max}} \) of \( \alpha_t \) emission, whereas rho(310-321)\( \text{SPD} \) caused a ~4-nm red shift, with no change in fluorescence intensity (data not shown). As an additional control, the \( \lambda_{\text{max}} \) of the emission spectrum of bovine serum albumin was shown to be insensitive to the presence of rho(310-321) (data not shown).

**Preparation of Substitution Mutants in C4 of Rhodopsin**

Three rhodopsin mutants were prepared in which portions of C4 were replaced with sequences from analogous segments of the \( \beta_2 \) AR or the m1 muscarinic receptor (m1 MR) (Fig. 3-4). These two receptors were chosen because they have fourth loops of similar lengths to that of rhodopsin and at least one cysteine homologous to C322 or C323 of rhodopsin. The \( \beta_2 \) AR has been shown to be palmitoylated (O'Dowd et al., 1989), while this modification in the m1 MR is inferred to be very likely because of the
presence of a Cys residue at the required location. Furthermore, these receptors bind G protein types not related to $G_t$, which is a member of the $G_{i/o}$ class. The m1 MR couples to $G_q$, and the $\beta_2$ AR couples to $G_S$.

In mutants CTr1 and CTr2, portions of C4 are replaced with sequence derived from the $\beta_2$ AR (Fig. 3-4). CTr1 involves replacement of the carboxyl-terminal half of the loop, while the entire fourth loop is replaced in CTr2. Only a carboxyl-terminal replacement was constructed with the m1 MR (CTr3), since the amino-terminal halves of the C4 loops of rhodopsin and m1 MR are nearly identical. The chimeric C4 mutant approach offers several advantages. For example, since the replacements are relatively long, and the substituted sequence is derived from receptors that couple to $G_S$ or $G_q$, the sensitivity of observing a relevant disruption in G protein coupling is high. Additionally, since the fourth loops of the $\beta_2$ AR, m1 MR and rhodopsin are of comparable length, the expression, folding, and palmitoylation of the chimeric fourth loop mutants should not be disrupted. Therefore, the confidence of attributing a loss-of-function phenotype to a specific defect in $G_t$ coupling is high.

The analysis of CTr1, CTr2, and CTr3 described below pointed toward the involvement of the amino-terminal part of C4 in $G_t$ interactions. To further examine this region, mutant CTr4 was constructed in which only those positions that differ between CTr1 and CTr2 (i.e., 310, 311, and 312) were replaced (Fig. 3-4). In addition, a series of point mutations in which K311 was replaced by residues with a variety of physicochemical properties were constructed: K311P, K311S, K311R, K311W. Position 311 lies in the center of a proposed helical extension of TM 7 (Altenbach et al., 1999; Yeagle et al., 1996; Altenbach et al., 1999). These mutants were designed specifically to test and control for the possible role of a helix-altering proline in the 311 position in mutant CTr2. Two additional mutants were constructed to assess the role of palmitoylation in the function of C4. In CysXV (C322S/C323S) the sites of
palmitoylation were removed, and in CTR4/CysXV, the CTR4 and CysXV replacements were combined. The mutant CysXV has been previously described and characterized in a detergent solubilized Gt activation assay (Karnik et al., 1993). The mutant CTR4/CysXV was used to test whether the effects of preventing palmitoylation were different in the background of a mutated C4 as compared with rhodopsin.

The mutants were transiently expressed in COS cells and regenerated with 11-cis-retinal to yield pigments. The mutant pigments were either purified in DM detergent or isolated in cell membrane preparations. UV-visible spectra taken on purified samples in the dark showed that each mutant pigment had a $\lambda_{\text{max}}$ value of 500 nm, identical to that of rhodopsin prepared under that same conditions (Table 3-1). Upon illumination, mutants CTR1, CTR2 and CTR3 formed MII-like pigments with $\lambda_{\text{max}}$ values of 380 nm. Acid denaturation of the photolyzed pigments revealed that the Schiff base bonds of the mutants were at least as stable as that of rhodopsin (data not shown).

**Activation of Gt by Solubilized Purified Recombinant Pigments**

The ability of the C4 loop substitution mutants to activate purified bovine Gt was measured in a kinetic fluorescence assay. The activation of Gt was observed as an increase in the intrinsic tryptophan fluorescence of Gt upon binding of GTPγS (Fahmy and Sakmar, 1993a). The initial rate of GTPγS uptake by Gt catalyzed by each mutant was normalized to that of rhodopsin (Fig. 3-5). Mutants CTR1 and CTR3, in which the carboxyl-terminal half of the loop was replaced, displayed similar initial rates to that of rhodopsin. However, CTR2, in which the entire loop was replaced, displayed a reduced initial rate. The CTR4 mutant, in which only a tripeptide in the amino-terminal part of the loop was replaced with $\beta_2$ AR sequence, was also deficient in activating Gt. The level of activity was comparable to that of CTR2. None of the K311 point mutants was defective
in $G_t$ activation. The non-palmitoylated CysXV mutant exhibited similar activity to that of rhodopsin in the detergent assay. When assayed in membranes, CysXV was slightly hyperactive (data not shown). The activity of the combination mutant $CTr4/CysXV$ was similar to that of $CTr4$.

Characterization of Pigment-catalyzed GTPγS Uptake by $G\alpha_t$ As a Function of $G\beta\gamma_t$ Concentration

Efficient activation of $G\alpha_t$ is known to require the presence of $G\beta\gamma_t$ (Fung, 1983). A previous study of a peptide derived from the C4 loop of rhodopsin suggested that this region binds to $G\beta\gamma_t$ (Phillips and Cerione, 1992). Therefore, the reduced activation of $G_t$ by the mutant $CTr2$ might be a result of disruption of the $G\beta\gamma_t$ binding site on rhodopsin. To test this hypothesis, the rate of $G_t$ activation by solubilized, COS-cell expressed rhodopsin (1 nM) or $CTr2$ (3 nM) was measured as a function of the concentration of $G\beta\gamma_t$. Higher concentrations of $CTr2$ were necessary in this experiment due to its reduced activity. Fig. 3-6 shows the change in fluorescence over time due to rhodopsin- or $CTr2$-catalyzed GTPγS uptake by $G\alpha_t$ in the presence of different concentrations of $G\beta\gamma_t$. The intrinsic tryptophans of $G\beta\gamma_t$ affected only the background level of fluorescence, which is normalized in Fig. 3-6. If the defect in $CTr2$ were solely attributable to decreased binding of $G\beta\gamma_t$, then the concentration of $G\beta\gamma_t$ at which half-maximal activity was observed would likely be significantly higher for $CTr2$ than for rhodopsin. Additionally, one might expect the relative defect in activation rate of $CTr2$ to be reduced at high concentrations of $G\beta\gamma_t$. The data do not reveal a significant difference between rhodopsin and $CTr2$ in the effect of $G\beta\gamma_t$ concentration on $G\alpha_t$ activation, nor does the activity of $CTr2$ approach that of rhodopsin even at a 2:1 ($G\beta\gamma_t:G\alpha_t$) stoichiometric excess (Fig. 3-6).
Palmitoylation of CTr1, Ctr2, and CTr4

We investigated whether replacing portions of the fourth loop with β2 AR sequence disrupted palmitoylation of mutants CTr1, CTr2, and CTr4. The incorporation of [3H]palmitic acid present in the cell media during transfection into CTr1, CTr2, CTr4, and rhodopsin was comparable (Table 3-1). The levels of incorporation were several fold higher than the incorporation associated with CysXV, which has been reported not to be palmitoylated (Karnik et al., 1993).

Amino Acid Sequence Analysis of Vertebrate Opsins and Biogenic Amine Family Receptors

The sequence alignments and analyses available in the G Protein Coupled Receptor Database (GPCRDB) at http://swift.embl-heidelberg.de/7tm/ were used to examine the conservation of C4 residues in GPCRs (Horn et al., 1998). Amongst the 86 vertebrate opsins in the database, the residues in the amino-terminal half of C4 were found to be nearly 100% conserved (Fig. 3-7). In contrast, the carboxyl-terminal half of the loop is only ~65% conserved.
### Table 3-I: Biochemical Characterization of Rhodopsin Fourth Loop Mutants

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\lambda_{\text{max}}$, Abs (nm)</th>
<th>Transducin activation rate$^a$</th>
<th>Incorporation of [${}^3$H]Palmitic Acid$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho</td>
<td>500</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>CTr1</td>
<td>500</td>
<td>121±11.2 (5)</td>
<td>1.14±0.20 (4)</td>
</tr>
<tr>
<td>CTr2</td>
<td>500</td>
<td>20±5.5 (5)</td>
<td>1.34±0.47 (4)</td>
</tr>
<tr>
<td>CTr3</td>
<td>500</td>
<td>82±9.6 (4)</td>
<td></td>
</tr>
<tr>
<td>CTr4</td>
<td>500</td>
<td>25±6.2 (3)</td>
<td>1.10±0.20 (4)</td>
</tr>
<tr>
<td>CysXV</td>
<td>500</td>
<td>97±15 (4)</td>
<td>0.24±0.10 (4)</td>
</tr>
<tr>
<td>CTr4/ CysXV</td>
<td>500</td>
<td>38.8±5.5 (3)</td>
<td></td>
</tr>
<tr>
<td>K311P</td>
<td>500</td>
<td>85±6.7 (8)</td>
<td></td>
</tr>
<tr>
<td>K311S</td>
<td>500</td>
<td>87±0.9 (3)</td>
<td></td>
</tr>
<tr>
<td>K311R</td>
<td>500</td>
<td>123±13.5 (3)</td>
<td></td>
</tr>
<tr>
<td>K311W</td>
<td>500</td>
<td>74±11.7 (3)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Activation rates are normalized to that of rhodopsin and are presented as mean±S.E.M. (n).

$^b$The level of incorporation of [${}^3$H]palmitic acid for each sample is normalized to that of rhodopsin, after subtraction of nonspecific counts associated with samples prepared from mock transfected cells. Values are presented as mean±S.E.M. (n).
FIG. 3-1. Schematic representation of bovine rhodopsin. Seven putative TM helices are depicted as for previous models of GPCRs. The amino terminus and intradiscal surface are toward the bottom, and the carboxyl terminus and cytoplasmic surface is toward the top of the figure. The intradiscal and cytoplasmic loops are not drawn to scale. The loop C4 is defined as the 12 amino acids beginning with N310, at the membrane border of the TM helix 7, and ending with C322 and C323. Both of these cysteines are palmitoylated (Papac et al., 1992; Ovchinnikov et al., 1988), and the palmitoyl groups are inserted into the membrane (Moench et al., 1994).
FIG. 3-2. Peptides derived from the second, third, and fourth intracellular loops of rhodopsin inhibit activation of $G_t$ by rhodopsin. Each panel shows the relative initial rate of $G_t$ activation as a function of peptide concentration. Activation rates were determined using a fluorescence assay of $G_t$ activation (Fahmy and Sakmar, 1993). The peptide used in each experiment is described in the lower left hand corner of the corresponding panel. All three peptides completely inhibited activation of $G_t$, with IC$_{50}$ values in the 0.1-0.3 mM range. A peptide derived from the first intracellular loop only moderately inhibited $G_t$ activation at a concentration of 1 mM (not shown). Each panel represents data from a single set of experiments, which was repeated at least twice with similar results.
FIG. 3-3. Effect of rho(310-321) on the intrinsic fluorescence of G\(\alpha_t\) and G\(\beta\gamma_t\) subunits. Fluorescence emission spectra were collected using an excitation wavelength of 295 nm. A, Fluorescence emission spectra of G\(\alpha_t\) (200 nM) before (solid line) and after (dashed line) incubation with the rhodopsin C4 peptide, rho(310-321) (45 \(\mu\)M). The inset shows the fluorescence emission \(\lambda_{\text{max}}\) of G\(\alpha_t\) as a function of rho(310-321) concentration. The mean maximum \(\Delta\lambda_{\text{max}}\pm\text{S.E.M.}\) was 7.8±0.3 nm (n=4) B, Fluorescence emission spectra of G\(\beta\gamma_t\) (200 nM) before (solid line) and after (dashed line) incubation with rho(310-321) (45 \(\mu\)M). Inset shows the fluorescence emission \(\lambda_{\text{max}}\) of G\(\beta\gamma_t\) as a function of rho(310-321) concentration. The mean maximum \(\Delta\lambda_{\text{max}}\pm\text{S.E.M.}\) was 1.0±0.3 nm (n=4). Data shown are representative of at least four independent and reproducible experiments.
FIG. 3-4. Amino acid sequences of the fourth loop of bovine rhodopsin, human β2 AR, human m1 MR, and fourth loop mutants of rhodopsin. The amino acid sequence of each position in the fourth loop is shown using the standard single letter amino acid code. The numbering of the positions is from bovine rhodopsin. Regions that were replaced or altered in the creation of mutants are highlighted in gray.
FIG. 3-5. Rates of $G_t$ activation catalyzed by solubilized, purified recombinant pigments. Samples of rhodopsin and C4 rhodopsin mutants were expressed in COS cells, solubilized in DM, and purified by an immunoaffinity procedure as described in Chapter 2. The rates of $G_t$ activation catalyzed by each sample were determined by linear regression through the first 30-60 s of data collected in a fluorescence activation assay (Fahmy and Sakmar, 1993). Each assay contained 1 nM rhodopsin or mutant, 250 nM $G_t$, and 5μM GTPγS in a volume of 1.5 ml. The bars represent the mean rate, normalized to that of rhodopsin. Error bars depict the standard error of the mean. The data are presented numerically in Table 3-1. Those mutants in which residues 310, 311, and 312 of rhodopsin are replaced with the analogous sequence of the β2 AR, (i.e., CTr2, CTr4, and CTr4/CysXV) are deficient in $G_t$ activation.
FIG. 3-6. The relative rates of pigment-catalyzed GTP\(\gamma S\) uptake by G\(\alpha_t\) as a function of G\(\beta\gamma_t\) concentration are similar for both rhodopsin and CTr2. The rate of G\(_t\) activation by solubilized, COS-cell expressed pigment was measured as a function of the concentration of G\(\beta\gamma_t\). The top panel shows fluorescence activation traces of 250 nM G\(\alpha_t\) in the presence of 25 - 500 nM G\(\beta\gamma_t\), and catalyzed by 1 nM rhodopsin. Each trace depicts the change in fluorescence emission intensity following the addition of GTP\(\gamma S\) at 200 s. The background fluorescence emission is normalized to zero. The concentration of G\(\beta\gamma_t\) in each trace is indicated in the column at right, in the same order that the traces are displayed. The inset is a plot of activation rate, determined from the initial slopes of the activation traces, versus concentration of G\(\beta\gamma_t\). The data are fit with a two-parameter hyperbolic function. The bottom panel is identical to the top, except that the experiments were conducted with 3 nM CTr2. The data are from a single experiment that was repeated twice with similar results. The similarity of the G\(\beta\gamma_t\) concentration dependency for Rho and CTr2 argues against the hypothesis that the defect in CTr2 is attributable solely to disruption of the G\(\beta\gamma_t\) binding site (see text for further discussion).
FIG. 3-7. The amino-terminal half of the fourth loop of rhodopsin is more conserved than the carboxyl-terminal half within the family of vertebrate opsins. The conservation of each position within loop C4 of rhodopsin was analyzed in 86 vertebrate opsin sequences. The alignments and the determination of the variability at each position were obtained from the G Protein Coupled Receptor Database (GPCRDB) at http://swift.embl-heidelberg.de/7tm/ (Horn et al., 1998). The labels on the x-axis indicate the consensus amino acid at each position, using bovine rhodopsin numbering. A variability index of 100 means 100% conservation; the lower the number, the greater the variety of residues found at that particular position. The residues in the amino-terminal half of the loop are nearly 100% conserved, whereas those at the carboxyl-terminal half are only ~65% conserved. This pattern of conservation corresponds to the importance of the amino-terminal half of the loop in G\textsubscript{t} activation.
Discussion

Significant efforts have been directed toward elucidating the regions of rhodopsin involved in binding and activating $G_t$ (for reviews, see (Helmreich and Hofmann, 1996)) (Wess, 1997; Bourne, 1997). A variety of experiments using peptide competition, mutagenesis, and antibody-based approaches have defined the importance of the intracellular surface, and in particular, loops C2 and C3 in mediating interactions with $G_t$. Published reports regarding the role of loop C4 are contradictory. Studies based on peptides derived from C4 have suggested the importance of this region (König et al., 1989; Takemoto et al., 1986), but a combination of site-directed mutagenesis and truncation of C4 appeared to rule out an important function for the region in $G_t$ activation (Osawa and Weiss, 1994; Weiss et al., 1994). Our data demonstrate and characterize the importance of the amino-terminal part of the fourth loop, and suggest the role it plays in rhodopsin-$G_t$ interactions.

_Loop C4 of Rhodopsin Is Involved in the Activation of $G_t_

Several different experimental approaches in this report corroborate the importance of the fourth loop of rhodopsin in interactions with $G_t$. A peptide derived from C4, rho(310-321), can inhibit the catalysis of $G_t$ activation by rhodopsin. Previously, an identical peptide has been reported to inhibit the binding of $G_t$ to rhodopsin as measured by an extra-MII assay (König et al., 1989). Our results extend and confirm this observation by demonstrating that the peptide is active at similar concentrations in the fluorescence activation assay (Fig. 3-2). Significantly, the potency of rho(310-321) was comparable to that of peptides derived from very well characterized G protein-interacting regions, including the highly conserved Glu-Arg-Tyr sequence in the C2-derived
rho(132-144) peptide. Additionally, rho(310-321) induces a red shift in the fluorescence emission spectrum of Gαt (Fig. 3-3), which is evidence that the peptide can bind directly to the Gαt subunit. The relevance of studies with the isolated C4-derived peptide to the function of C4 in the intact receptor is supported by the report that a rho(306-348) peptide assumed a defined structure in solution (Yeagle et al., 1996).

The results of the mutagenesis data strongly support and clarify the involvement of the fourth loop in Gt activation. In particular, the data localize the important region to the amino terminus of C4, as shown by the striking decrease in the rate of Gt activation by the mutants in which residues 310-312 are replaced with β2 AR sequence (Fig. 3-5). Osawa and Weiss (Osawa and Weiss, 1994) argued against involvement of this region based on the wild-type phenotype of N310A, K311A, and Q312A point mutants. However, a recent report (Cai et al., 1999) corroborates our findings by identifying a mutation in the amino terminus of C4 (N310C) that can disrupt Gt activation. The precise role of N310 in Gt coupling appears complex.

Sequence analysis also supports the importance of the amino terminus of the C4 loop in receptor function. The amino terminus of C4 is nearly 100% conserved within the vertebrate opsins, whereas the carboxyl-terminal half of the loop is only ~65% conserved (Fig. 3-7). Furthermore, the asparagine in position 310 is very highly conserved within the large biogenic amine family of receptors, with the exception of the non-α2 ARs, where it nearly always is a serine. This latter observation suggests that the amino-terminal part of the fourth loop may be important in GPCRs other than opsins. Indeed, mutagenesis experiments in this region of the fourth loop identified it as critical for receptor-G protein interactions in the β2 AR (O'Dowd et al., 1988; Liggett et al., 1991).

Several reports have suggested that accessibility to regions in the fourth loop are specifically modulated by photoactivation of rhodopsin, an observation consistent with
the regulated involvement of C4 in $G_{T}$ activation. The cytoplasmic end of TM helix 7 has been shown to become more accessible following light activation of rhodopsin (Abdulaev and Ridge, 1998), and spin labels attached to positions 316 (Resek et al., 1993) and 313 (Altenbach et al., 1999) in the middle of C4 undergo increased mobility after photolysis. Additionally, a spin label at C316 was reported to move apart from a label at position 65, at the amino terminus of the TM helix 1, upon MII formation (Yang et al., 1996).

Factors Affecting the Structure of the C4 Loop Region

We examined several factors that potentially affect the structure of C4, including the palmitoylation of C322 and C323, the integrity of a proposed helical extension of TM7, and the presence of a membrane environment. The palmitoylation of C322 and C323 does not appear to affect the function of the amino-terminal part of the loop. Both CTr4 and CTr2 are palmitoylated (Table 3-1) despite alterations in amino acids in the vicinity of the acylation site. However, the non-palmitoylated combination mutant CTr4/CysXV is essentially indistinguishable from CTr4 in detergent assays. In membranes, CysXV is slightly more active than rhodopsin, and CTr4/CysXV is slightly more active than CTr4 (not shown). Both observations corroborate previous reports that a nonpalmitoylated mutant was similar to rhodopsin in detergent solution (Karnik et al., 1993), and that chemical depalmitoylation increased activity of rhodopsin when assayed in rod outer segment membranes (Morrison et al., 1991). Given that residues 310-312 are located adjacent to the membrane border of the TM helix 7, it seems plausible that their structure is unaffected by the membrane anchoring of C322 and C323. The same reasoning might explain why truncation of rhodopsin following N315 does not diminish $G_{T}$ activation (Weiss et al., 1994). Furthermore, C4 may form a loop in the absence of palmitoylation, as suggested by the NMR structure of a fourth loop peptide (Yeagle et al., 1996). The
mechanism of hyperactivity of non-palmitoylated rhodopsin that has been observed in membranes appears to be unrelated to the function of the amino terminus of C4.

The TM helix 7 is likely to extend beyond the membrane border, based on experimental (Yeagle et al., 1996; Altenbach et al., 1999) and theoretical (Ben-Tal et al., 1996) grounds. The TM2 (Farahbakhsh et al., 1995), TM4, and TM5 (Altenbach et al., 1996) helices have all been reported to extend into the aqueous phase. The proline in position 311 of CTr2 and CTr4 might disrupt a helical extension of TM helix 7, leading to the observed phenotype. But the K311P point mutation does not resemble the 310-312 mutation of CTr4 in Gt activation assays, indicating that perturbation of the proposed helical extension does not impair G protein activation.

The Role of the C4 Loop Involves Modulation of Rhodopsin-Gt Interactions

We propose that the role of C4 is to modulate, in conjunction with other structures, the binding site for Gt. We favor this interpretation rather than those in which C4 serves as the sole binding site or participates mechanistically in the catalysis of nucleotide exchange because: (a) residual Gt activation was observed with the CTr2 and CTr4 mutants, (b) point mutations at the 311 position did not affect activation, (c) the aminoterminal C4 sequence of rhodopsin is highly, but not absolutely, conserved in other opsins and in certain other GPCRs (Fig. 3-7), and (d) the substitution of β2 AR sequence into C4 does not allow rhodopsin to activate Gs (data not shown). The modulatory functions of C4 appear to be mediated both by contacts with Gt and by allosteric interactions with other regions of the receptor.

Specific interaction between C4 and Gt is indicated most directly by the rho(310-321)-Gαt interaction observed by fluorescence emission spectroscopy (Fig. 3-3). Direct contacts are also suggested by the inhibition of rhodopsin-catalyzed Gt activation in the
presence of rho(310-321), an observation plausibly explained by binding of the peptide to the G protein, and occupancy of a receptor contact site (Fig. 3-2). Which part of the heterotrimer is binding to the C4 loop? A C4 derived peptide, rho(310-324), has been reported to bind Gβγt (Phillips and Cerione, 1992). However, the similarity of the activation rate versus Gβγt concentration profiles for rhodopsin and CTr2, and the failure of high concentrations of Gβγt to fully rescue CTr2 activity (Fig. 3-6), argue against the fourth loop as acting solely as a Gβγt binding site. Furthermore, the direct binding of rho(310-321) to Goαt (Fig. 3-3) demonstrates that C4 is involved with Goαt binding in addition to, or even instead of, Gβγt binding. Perhaps C4 binds Goαt directly, and allosterically regulates other regions of the receptor involved in Gβγt binding.

The data in this report demonstrate a conclusive role for the amino terminus of C4 of rhodopsin in Gt interactions. We suggest that this interacts directly with Gt, particularly with Goαt, and with other regions of the intracellular surface to support Gt binding. In the following chapter, we study a subset of mutant receptors described here using a biophysical assay that detects binding of Gt or peptides derived from Gt subunit sequences.
Chapter 4:
Mutation of the Fourth Cytoplasmic Loop of Rhodopsin Affects Binding of Transducin and Peptides Derived from the Carboxyl-terminal Sequences of Transducin α and γ Subunits#

Summary

The role of the putative fourth cytoplasmic loop of rhodopsin in the binding and catalytic activation of the heterotrimeric G protein, transducin (Gt), is not well defined. We developed a novel assay to measure the ability of Gt, or Gt-derived peptides, to inhibit the photoregeneration of rhodopsin from its active metarhodopsin II state. We show that a peptide corresponding to residues 340-350 of the α subunit of Gt, or a cysteiny1-thioetherfarnesyl peptide corresponding to residues 50-71 of the γ subunit of Gt, are able to interact with metarhodopsin II and inhibit its photoconversion to rhodopsin. Alteration of the amino acid sequence of either peptide, or removal of the farnesyl group from the γ-derived peptide, prevents inhibition. Mutation of the amino-terminal region of the fourth cytoplasmic loop of rhodopsin affects interaction with Gt (Marin et al., 2000). Here, we provide evidence that this segment of rhodopsin interacts with the carboxyl-terminal peptide of the α subunit of Gt. We propose that the amino-terminal region of the fourth cytoplasmic loop of rhodopsin is part of the binding site for the carboxyl terminus of the α subunit of Gt and plays a role in the regulation of βγ subunit binding.


(*authors contributing equally)
Introduction

A variety of experimental approaches, including proteolysis, chemical modification, peptide competition and site-directed mutagenesis in combination with biochemical and biophysical assays, have been employed to map the sites of rhodopsin responsible for the binding and activation of $G_t$. The salient results have indicated that loops C2 and C3 are involved in $G_t$ binding and activation (Franke et al., 1990; Franke et al., 1992). In addition, recent studies indicate a role for the loop C4 in $G_t$ activation (Marin et al., 2000; Cai et al., 1999; Altenbach et al., 1999). Despite these studies and the availability of a high-resolution crystal structure for the $G_t$ holoprotein (Lambright et al., 1996), there is little information concerning: 1) the key functional intramolecular interactions on the cytoplasmic surface of rhodopsin that form and regulate the catalytic site for $G_t$, 2) $G_t$ subunit specificity for binding to particular cytoplasmic regions of rhodopsin, 3) the molecular mechanism of rhodopsin-catalyzed nucleotide release by $G_t$.

In Chapter 3, a region was identified at the amino terminus of loop C4 of rhodopsin that most likely interacts with the $\alpha$ subunit of $G_t$, $G\alpha_t$. Here, we studied the interaction of site-directed mutants of rhodopsin with $G_t$ and peptides derived from the carboxyl-terminal sequences of $G\alpha_t$ and $G\gamma_t$ (Ernst et al., 1999; Kisselev et al., 1999; Arnis and Hofmann, 1995). We used a novel assay in which the all-trans-retinal in metarhodopsin II (MII) is photoconverted to the cis configuration using blue light. The flash-induced photoregeneration of rhodopsin from MII can be followed spectroscopically with millisecond time resolution (Arnis and Hofmann, 1995). Since stabilization of $R^*$ by $G_t$ or $G_t$-derived peptides inhibits the rate of photoregeneration, the assay can be used to monitor the interaction of $R^*$ with $G_t$ (Ernst et al., 1999; Kisselev et al., 1999). We show that peptide $\alpha(340-350)$, corresponding to the carboxyl-terminal undecapeptide of $G\alpha_t$, and peptide $\gamma(50-71)$-far, corresponding to the carboxyl-terminal cysteinyl-
thioetherfarnesyl peptide of Gγt, stabilized R*. Alteration of the amino acid sequence of either peptide, or removal of the farnesyl group of the Gγt-derived peptide prevented stabilization of R*. Gt failed to stabilize mutant rhodopsins with alterations of the amino terminus of loop C4 near the TM helix 7 border. The Gαt-derived peptide also failed to stabilize these mutants, suggesting that loop C4 comprises part of a binding site for the carboxyl-terminal tail of Gαt.

**Results**

*The Effect of Gt and Gt-derived Peptides on the Photoregeneration of Bovine Retinal Rhodopsin*

A “photoregeneration” assay was employed that measures the kinetics of photoconversion of MII to rhodopsin (Arnis and Hofmann, 1995). The assay takes advantage of the conformational coupling of the cytoplasmic surface of the active state of rhodopsin, R*, to the chromophore-binding pocket in the membrane-embedded domain of the receptor. The slow phase of the photoconversion kinetics essentially monitors the reprotonation of the retinylidene Schiff base as a function of time after a sample of R* is subjected to a blue flash. Photoconversion of R* (λ<sub>max</sub> = 380 nm) to rhodopsin (λ<sub>max</sub> = 500 nm) is inhibited if R* is bound to Gt or certain Gt-derived peptides. R* that is not bound in a stabilizing complex with Gt or Gt-derived peptides is more readily photoconverted.

The effect of Gt on the photoconversion of R* was studied first (Fig. 4-2A). The change in absorbance at 543 nm is plotted as a function of time. The blue flash is applied to the sample at 50 ms. Superimposed upon an initial rapid change in amplitude, the slow phase of the trace represents the back conversion of R* to rhodopsin. The experiment was repeated with identical rhodopsin samples in the presence of increasing concentrations of Gt (0, 0.5, 1.0, 2.0, 3.0, 5.0 µM). The traces are superimposed to show
a clear dose-dependent inhibition of the photoregeneration reaction by $G_t$. The experimental traces were fit to an exponential function in order to calculate values for initial slopes. The calculated fits are shown as dashed lines in Fig. 4-2. The initial slopes of the photoregeneration traces are plotted as a function of $G_t$ concentration in the inset. A satisfactory hyperbolic fit yielded an effective concentration at 50% inhibition ($IC_{50}$) value of 2.56±2.0 μM. This value effectively represents a binding constant for the interaction between $R^*$ and $G_t$ under the conditions of the assay.

The effects of two peptides corresponding to the carboxyl-terminal regions of $G\alpha_t$ and $G\gamma_t$ on the photoconversion of $R^*$ was studied next (Fig. 4-2, B and C). The amino acid sequences of $\alpha(340-350)$ and $\gamma(50-71)$-far are presented in Fig. 4-1B. The $\gamma(50-71)$-far peptide carries the post-translational isoprenylation that is characteristic of $G\gamma_t$. Fig. 4-2B shows six superimposed photoregeneration traces obtained from identical rhodopsin samples in the presence of increasing concentrations of $\alpha(340-350)$ (0 to 1000 μM). The traces show a clear dose-dependent inhibition of the photoregeneration of $R^*$ by $\alpha(340-350)$. The initial slopes of the photoregeneration traces are plotted as a function of $\alpha(340-350)$ concentration in the inset to Fig. 4-2B. A satisfactory hyperbolic fit yielded an $IC_{50}$ value of 49.5±6.0 μM for the interaction between $R^*$ and $\alpha(340-350)$.

Fig. 4-2C shows six superimposed photoregeneration traces obtained from identical rhodopsin samples in the presence of increasing concentrations of $\gamma(50-71)$-far (0 to 1000 μM). The traces show a clear dose-dependent inhibition of the photoregeneration of $R^*$ by $\gamma(50-71)$-far. Plotting the initial slopes of the photoregeneration traces as a function of $\gamma(50-71)$-far concentration (inset to Fig. 4-2C) permitted a satisfactory hyperbolic fit that yielded an $IC_{50}$ value of 285±74 μM for the interaction between $R^*$ and $\gamma(50-71)$-far.

*Specificity of the Effects of $\alpha(340-350)$ and $\gamma(50-71)$-far Peptides*
The specificity of the effect of the $G_t$-derived peptides was studied by performing control experiments with altered peptides. The peptides are shown in Fig. 4-1B. One control peptide, $\alpha(340-350)\ K341R/L349A$, was studied to evaluate the specificity of the carboxyl-terminal sequence of $G\alpha_t$ in $R^*$ interaction. This peptide failed to show peptide-$R^*$ interaction (Nishimura et al., 1998; Fahmy, 1998) and the substitution of Leu$^{349}$ by alanine in $G\alpha_t$ was reported to abolish coupling to active rhodopsin (Osawa and Weiss, 1995; Garcia et al., 1995). As shown in Fig. 4-3, the $\alpha(340-350)\ K341R/L349A$ peptide failed to inhibit the photoregeneration of $R^*$. Similarly, the requirements for length, primary structure and farnesylation of the $G\gamma_t$-derived peptide were tested. Peptides $\gamma(60-71)$-far and $\gamma(50-71)$-far both inhibited photoregeneration similarly (Fig. 4-3). Positions 64 and 67 in $G\gamma_t$ have been reported to be critical for interaction with MII, as observed with the altered peptide $\gamma(60-71)$-far F64T/L67S (Kisselev et al., 1995b). The $\gamma(60-71)$-far F64A/L67A peptide did not inhibit photoregeneration (Fig. 4-3). In addition, the $\gamma(60-71)$ peptide, which lacked cysteinylfarnesylation, did not inhibit photoregeneration (Fig. 4-3). This finding is consistent with earlier results showing that lack of farnesylation prevented MII stabilization by $G\gamma_t$ derived peptides (Kisselev et al., 1994; Kisselev et al., 1995a). In other control experiments, the 1D4 peptide, corresponding to the carboxyl-terminal 18 amino acids of rhodopsin, did not affect photoregeneration, nor did it affect the inhibition of photoregeneration by $G_t$ and the $G_t$-derived peptides (data not shown).

It has been reported that detergent concentration has an influence on the activation rate of $G_t$ by $R^*$ (Franke et al., 1992; Han et al., 1998). The effect of varying concentrations of DM on the photoregeneration kinetics of rhodopsin and on the inhibition of photoregeneration by $G_t$ (3 $\mu$M), $\alpha(340-350)$ (200 $\mu$M) or $\gamma(50-71)$-far (500 $\mu$M) was studied. Varying DM concentrations from 0.01% to 0.10% (w/v) had no effect on the photoregeneration kinetics of rhodopsin, and no effect on the inhibition of
photoregeneration by $\alpha(340-350)$ (data not shown). However, the inhibition of photoregeneration by $G_t$ and $\gamma(50-71)$-far was reduced by increasing DM concentrations from 0.01% to 0.10% (data not shown). This effect mirrors the reduction in the rate of $G_t$ activation by $R^*$ in the presence of increasing [DM], as previously reported (Franke et al., 1992; Han et al., 1998). The final DM concentration under the standard conditions of the photoregeneration assay using heterologously-expressed and purified mutant pigments is estimated to be 0.01-0.035%, a range in which detergent effects were found to be modest. Furthermore, the final DM concentration in assays of each of the recombinant samples is virtually identical.

**Photoregeneration Assay of Recombinant Rhodopsin and Loop C4 Mutants**

Photoregeneration assays were carried out on wild-type recombinant rhodopsin and four mutant rhodopsins (Fig. 4-1A). Representative photoregeneration traces are presented in Fig. 4-4. In each panel, the change in absorbance at 543 nm is plotted as a function of time. The blue flash is applied to the sample at 50 ms. Superimposed upon an initial rapid change in amplitude, the slow phase of the trace represents the back conversion of $R^*$ to rhodopsin. The black trace shows the result with pigment alone. The red trace shows the result with pigment in the presence of $G_t$ (3.0 $\mu$M), $\alpha(340-350)$ (200 $\mu$M) or $\gamma(50-71)$-far (500 $\mu$M) as indicated. The behavior of COS-cell expressed rhodopsin was similar to that of bovine rhodopsin in the photoregeneration assay. Fig. 4-4 shows typical experimental traces obtained with purified recombinant rhodopsin in the presence of $G_t$, $\alpha(340-350)$ and $\gamma(50-71)$-far. Typical traces obtained with bovine rhodopsin are presented in Fig. 4-2. These results confirm that the photoregeneration assay can be employed to study recombinant pigments prepared in relatively small quantities in a heterologous expression system.

Four mutant pigments with alterations of the amino acid sequence of the C4 loop were prepared (Fig. 4-1A). Mutants CTr1, CTr2 and CTr4 are essentially chimeric
receptors in which parts of the C4 loop of rhodopsin are replaced by sequences from the β2 adrenergic receptor (β2 AR). Mutant CysXV (C322S/C323S) was designed to evaluate the effect of receptor palmitoylation on the photoregeneration kinetics. The mutant opsin genes were expressed in COS cells, treated with 11-cis-retinal and purified in DM detergent solution. The levels of palmitoylation of the expressed mutant pigments CTr2 and CTr4 were similar to that of the wild-type receptor expressed in parallel; CysXV was not palmitoylated (Table 3-I) (Karnik et al., 1993; Karnik et al., 1993). The ability of each of the mutant pigments to activate Gt was evaluated using a fluorescence Gt activation assay (Table 4-I). Mutant pigments CTr2 and CTr4 were significantly defective in their ability to activate Gt.

In the absence of Gt or Gt-derived peptides, the photoregeneration kinetics of rhodopsin and the four C4 mutants were essentially identical (Fig. 4-4, black traces). This result suggests that the C4 loop mutations do not affect the photoregeneration reaction. The effects of Gt or Gt-derived peptides on the photoregeneration of the mutant pigments are shown in Fig. 4-4 (red traces). The effects can be conveniently evaluated by comparing relative slopes (Fig. 4-5A). The relative slope is defined as the initial slope of the slow phase of the photoregeneration trace in the presence of Gt, α(340-350) or γ(50-71)-far divided by the initial slope of the trace in the absence of Gt or Gt-derived peptide. The relative slope provides a quantitative measure of the effect of Gt or a Gt-derived peptide on the slow phase of the photoregeneration kinetics. A relative slope of 1.0 indicates no inhibition of photoregeneration, and relative slopes of <1.0 indicate a progressive inhibition of photoregeneration. Average values for relative slopes are presented in Table 4-I.

Photoregeneration of mutant CTr1 was inhibited by Gt and the Gt-derived peptides. The degrees of inhibition were identical to those seen with wild-type rhodopsin. The behavior of mutants CTr2 and CTr4 was different from that of rhodopsin. Gt and the
peptide $\alpha(340-350)$ did not inhibit photoregeneration of CTr2. This result is best appreciated in Fig. 4-5A, where the relative slopes for CTr2 in the presence of $G_t$ and $\alpha(340-350)$ are $\sim 1.0$. However, the $\gamma(50-71)$-far peptide was able to inhibit photoregeneration of CTr2 to the same extent observed with rhodopsin. The photoregeneration of mutant CTr4 was not affected by $G_t$, $\alpha(340-350)$ or $\gamma(50-71)$-far. This result is best seen in Fig. 4-5A, where the relative slopes for CTr4 in the presence of $G_t$ and the $G_t$-derived peptides are $\sim 1.0$. Photoregeneration of mutant CysXV was inhibited by $G_t$ and the $G_t$-derived peptides. However, the inhibition by peptide $\gamma(50-71)$-far was relatively more pronounced for CysXV than for rhodopsin.

Each of the peptides and $G_t$ inhibit the photoregeneration of rhodopsin to different degrees. Therefore, the ability of each peptide and $G_t$ to affect a particular mutant cannot be compared directly using relative slopes. However, by normalizing the relative slope of a mutant to that of rhodopsin, such a comparison can be made. This expression, termed the normalized inhibition, is obtained from the following equation: $(1 - \text{mean relative slope})_{\text{mutant}} / (1 - \text{mean relative slope})_{\text{rhodopsin}}$. The normalized inhibition for rhodopsin is defined to be 1.0. A value of zero indicates no inhibition of the photoregeneration of the mutant pigment by a particular ligand. Data are plotted in Fig. 4-5B and listed in Table 4-I. Mutant CTr1 is similar to rhodopsin with respect to inhibition of photoregeneration by $G_t$, $\alpha(340-350)$ and $\gamma(50-71)$-far. Mutant CTr2 shows essentially normal interaction with $\gamma(50-71)$-far, but fails to be affected by $G_t$ and $\alpha(340-350)$. Mutant CTr4 is unaffected by $G_t$ and both $G_t$-derived peptides. The photoregeneration of mutant CysXV is inhibited by $\alpha(340-350)$ and $G_t$ normally, but displays an enhanced sensitivity to $\gamma(50-71)$-far.
### Table 4-1: Gt activation and photoregeneration data

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Activation of Gt</th>
<th>3 μM Gt</th>
<th>200 μM α(340-350)</th>
<th>500 μM γ(50-71)-far</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n°</td>
<td>relative slope°</td>
<td>normalized inhibition°</td>
</tr>
<tr>
<td>WT-Rho</td>
<td>100</td>
<td>2</td>
<td>0.67±0.05</td>
<td>1.00±0.15</td>
</tr>
<tr>
<td>CTr1</td>
<td>121±11</td>
<td>2</td>
<td>0.73±0.06</td>
<td>0.82±0.16</td>
</tr>
<tr>
<td>CTr2</td>
<td>20±5.5</td>
<td>2</td>
<td>0.98±0.02</td>
<td>0.06±0.04</td>
</tr>
<tr>
<td>CTr4</td>
<td>25±6.2</td>
<td>2</td>
<td>1.03±0.04</td>
<td>-0.08±0.09</td>
</tr>
<tr>
<td>CYS XV</td>
<td>97±15</td>
<td>2</td>
<td>0.61±0.12</td>
<td>1.19±0.30</td>
</tr>
</tbody>
</table>

a Previously reported (Table 3-I) (Marin et al., 2000)

b The number given for n refers to the number of independent samples studied. Each kinetic trace (Fig. 4) resulted from four separate photoregeneration experiments per sample.

c The relative slope is defined as the initial slope of the slow phase of the photoregeneration trace in the presence of Gt, α(340-350) or γ(50-71)-far divided by the initial slope of the trace in the absence of Gt or Gt-derived peptide. The initial slope of the slow phase of the photoregeneration trace was determined from the numerical fit of a simple exponential-rise function offset by the amplitude of the fast phase. The data are presented as mean ± S. E. M.

d The normalized inhibition is defined as: \( \frac{\text{mean relative slope}_{\text{mutant}}}{\text{mean relative slope}_{\text{rhodopsin}}} \). The propagated S. E. M. values are also presented.
FIG. 4-1. Amino acid sequences of recombinant rhodopsins and G\textsubscript{T}-derived peptides. A, Amino acid sequence of the loop C4 of wild-type rhodopsin (WT Rho) and mutant opsins (CTr1, CTr2, CTr4 and CysXV). In bovine rhodopsin, this region extends from position 310 at the intracellular junction of the TM helix 7 to Cys322 and Cys323, which are palmitoylated and inserted into the membrane. Changes from the wild-type sequence are highlighted in gray. In CTr1, CTr2 and CTr4, portions of the C4 loop have been replaced with analogous sequences from the β2 AR. In CysXV, the palmitoylation sites are replaced by serine residues. These mutants represent a subset of those in Fig. 3-4. B, Amino acid sequences of peptides were derived from the carboxyl termini of bovine G\textsubscript{αt} and G\textsubscript{γt} subunits. The α(340-350) peptide corresponds to the native sequence of G\textsubscript{αt}, and the γ(50-71)-far corresponds to the native sequence of G\textsubscript{γt}, which is post-translationally modified by cysteiny1 thioetherfarnesylation. Peptides with alterations of primary structure, or a lack of carboxyl-terminal farnesylation, were used as controls. The carboxyl termini of the G\textsubscript{γt}-derived peptides were amidated. The carboxyl termini of the G\textsubscript{αt}-derived peptides, and the amino termini of all the peptides, were unmodified.
FIG. 4-2. Photoregeneration of rhodopsin in the presence of increasing concentrations of Gt or Gt-derived peptides. The presence of Gt, α(340-350), or γ(50-71)-far causes a dose-dependent reduction in photoregeneration. Each trace shows a representative individual experiment and the time-dependent increase in absorbance at 543 nm. The photoregeneration signal is initiated by a flash at 50 ms. Fits to the slow phase of the signal are shown as dotted lines. Insets show the initial slope of the slow phase of photoregeneration versus added concentration of Gt or peptide as indicated. These plots were fit using a hyperbolic function with additive offset. IC50 values and errors are derived from the fits to the dose-response data and presented in the insets. A, Photoregeneration of rhodopsin in the presence of increasing concentrations of Gt (IC50 = 2.56 ± 2.0 μM). B, Photoregeneration of rhodopsin in the presence of increasing concentrations of α(340-350) peptide (IC50 = 49.5 ± 6 μM). C, Photoregeneration of rhodopsin in the presence of increasing concentrations of γ(50-71)-far peptide (IC50 = 285 ± 74 μM).
Chapter 4: Photoregeneration Studies of Fourth Loop Mutants

**A**

Transducin

- 2.6 μM

![Graph with lines and markers for different concentrations of Transducin.](image)

- Time (msec) from 0 to 250

**B**

α(340-350)

- 49.5 μM

![Graph with lines and markers for different concentrations of α(340-350).](image)

- Time (msec) from 0 to 250

**C**

γ(50-71)-far

- 285 μM

![Graph with lines and markers for different concentrations of γ(50-71)-far.](image)

- Time (msec) from 0 to 250
FIG. 4-3. Altered peptides do not inhibit photoregeneration. Photoregeneration experiments were performed as described in Fig. 4-2 in the presence of altered peptides (amino acid sequences are shown in Fig. 4-1). Concentrations were 200 μM for the Gαl-derived peptides and 500 μM for the Gγt-derived peptides. The inverse of intensity change at the detector in arbitrary units is plotted as a function of time. Data collected in the absence of peptide were normalized to a value of 1.0 at 250 ms; data collected in the presence of peptides were scaled to data collected with the same rhodopsin sample in the absence of peptide. An arbitrary unit is approximately 0.15 mOD units at 543 nm. Inhibition of photoregeneration is abolished by the conservative substitution of two amino acids in the α(340-350) or γ(60-71)-far peptide, or by removal of the farnesyl moiety in the γ(60-71) peptide. The longer γ(50-71)-far peptide shows functional identity to γ(60-71)-far.
FIG. 4-4. The effect of Gt, α(340-350) and γ(50-71)-far on photoregeneration of heterologously expressed rhodopsin and C4 loop mutants. Experiments were carried out as in Fig. 4-2. Concentrations of Gt, α(340-350) and γ(50-71)-far were 3 μM, 200 μM and 500 μM, respectively. The vertical scale bar, which depicts 0.075 mOD at 543 nm, applies to all traces. Black traces show photoregeneration of pigment alone. Red traces show photoregeneration of each pigment in the presence of Gt (Transducin), α(340-350) or γ(50-71)-far as indicated at the top of each column of panels. The sample used in each row is indicated in the labels at left. Each pair of traces is representative of at least two sets of experiments performed on different samples. WT, CTr1, and CysXV, show distinct effects of Gt or Gt-derived peptides. Photoregeneration of CTr4 is not influenced by Gt or Gt-derived peptides, and CTr2 shows an effect with γ(50-71)-far, a minor effect with Gt holoprotein and no effect with α(340-350).
FIG. 4-5. Quantitation of the effects of $G_t$, $\alpha(340-350)$, and $\gamma(50-71)$-far on the photoregeneration of recombinant rhodopsin and rhodopsin mutants. The slow phases of the photoregeneration traces were fit with exponential-rise equations, and the initial slopes were determined. A, Relative slopes. Bars represent the average ratio of the initial slope of the slow phase of photoregeneration of pigment in the presence of $G_t$ (Transducin), $\alpha(340-350)$ or $\gamma(50-71)$-far to the initial slope with pigment alone. The error bars display the standard errors. The photoregeneration of mutants CTr2 and CTr4 is unaffected by $G_t$ and $\alpha(340-350)$. Only mutant CTr4 photoregeneration is insensitive to $\gamma(50-71)$-far. B, Normalized inhibition. The vertical bars indicate the ability of $G_t$ and $G_t$-derived peptides to inhibit photoregeneration of mutant pigments relative to their effect on wild-type rhodopsin (WT). The normalized inhibition values were determined from the following equation: $(1 - \text{mean relative slope})_{\text{mutant}} / (1 - \text{mean relative slope})_{\text{rhodopsin}}$. The error bars depict the propagated standard errors from the determination of relative slope. Numerical values are given in Table 5-1. This analysis allows for direct comparison of the effects of each peptide and $G_t$ on each mutant. The effect of $G_t$ and $G_t$-derived peptides on CTr1 and CysXV are similar to their effect on rhodopsin. The photoregeneration of CTr4 does not show an effect of $G_t$ or either $G_t$-derived peptide. This behavior is consistent with a failure of CTr4 to bind $G_t$, $\alpha(340-350)$ and $\gamma(50-71)$-far. Replacement of the entire C4 loop in CTr2 shows a graded effect: CTr2 is essentially insensitive to inhibition by $\alpha(340-350)$ and $G_t$, but shows effects with $\gamma(50-71)$-far nearly identical to those of wild-type rhodopsin.
Chapter 4: Photoregeneration Studies of Fourth Loop Mutants

A Transducin α(340-350) γ(50-71)-far

B

3 µM Transducin
200 µM α(340-350)
500 µM γ(50-71)-far
Chapter 4: Photoregeneration Studies of Fourth Loop Mutants

Discussion

Several lines of evidence suggest that the conformation of the cytoplasmic surface of the active state of rhodopsin, R*, is coupled to the conformation of the chromophore-binding pocket in the membrane-embedded domain of the receptor (Sakmar, 1998; Helmreich and Hofmann, 1996). In analogy to GPCRs with diffusible ligands in which G protein binding stabilizes a receptor conformation with a high affinity ligand binding site, MII is stabilized at the expense of its tautomeric forms by the binding of Gt or Gt-derived peptides. This stabilization of MII is the basis of the “extra-MII” assay (Emeis and Hofmann, 1981; Hamm et al., 1988). This assay however, can only be applied under conditions of a dynamic equilibrium between metarhodopsin I and MII, which is exquisitely sensitive to membrane environment, pH, temperature, ionic strength, etc. Therefore, an assay was developed that measures the kinetics of photoconversion of MII to rhodopsin in detergent solution. The assay uses the fact that photoconversion of MII (λ_max = 380 nm) to rhodopsin (λ_max = 500 nm) following a blue actinic flash is inhibited if the MII molecule is bound to Gt (Arnis and Hofmann, 1995), or certain Gt-derived peptides (Kisselev et al., 1999; Kisselev et al., 1999) as a result of the coupling between the conformation of the cytoplasmic surface and that of the chromophore-binding pocket.

The initial step in photoregeneration, the photo-isomerization of the retinal to its cis conformation, may be compared to loading a spring that subsequently drives the protein, including its cytoplasmic domain, back to the ground state conformation (Arnis and Hofmann, 1995). The product formed in this initial step, termed “reverted meta (RM),” is characterized by a MII-like protein conformation and a cis retinal with a deprotonated Schiff base; it is spectrally indistinguishable from MII. RM rapidly converts to a rhodopsin-like species characterized by a rhodopsin-like protein conformation and a cis
retinal with a protonated Schiff base. The presence of $G_t$ does not affect RM formation, indicating that the isomerization of the retinal itself is unaffected. However, bound $G_t$ prevents RM from converting to rhodopsin, by stabilizing the MII-like conformation of RM. Dissociation of $G_t$ from RM by GTPγS treatment allows RM to revert to rhodopsin quantitatively (Amis and Hofmann, 1995). The effects, and presumably the mechanism of action, of certain $G_t$-derived peptides on photoregeneration are similar to those of $G_t$ itself (Kisselev et al., 1999).

Photoregeneration is Sensitive to Interactions with $G_t$ and Certain $G_t$-derived Peptides

$G_t$ interacts with $R^*$ to stabilize the active signaling state such that photoregeneration to rhodopsin is effectively blocked (Amis and Hofmann, 1995). Recently, a peptide corresponding to the carboxyl terminus of $G_{yt}$ and a peptide analogue related to the carboxyl terminus of $G_{mt}$ were demonstrated to mimic the effect of $G_t$ by inhibiting photoregeneration of $R^*$ (Kisselev et al., 1999). Here we showed that synthetic peptide $\alpha(340-350)$ could cause the same effect as $G_t$ (Fig. 4-2). In addition, the effect of $\alpha(340-350)$ was specific to its primary structure since a mutant peptide had no effect (Fig. 4-3). Synthetic peptide $\gamma(50-71)$-far also inhibited photoregeneration of $R^*$ (Fig. 4-2). The effect was specific to its primary structure and to the presence of cysteinyl thioetherfarnesylation (Fig. 4-3). Using single peptides that represent small regions of $G_t$ provides a powerful probe of subunit- and domain-specific interactions.

The potencies of the $\alpha(340-350)$ and $\gamma(50-71)$-far peptides are about 20- and 100-fold less than that of $G_t$, respectively (Fig. 4-2). This finding is reasonable considering that the tertiary structure of a short peptide is less defined, so that a higher binding energy, and thus concentration, is needed for the “induced fit.” Also, the cytoplasmic surface domain of $R^*$ comprises multiple interaction sites for $G_t$ binding, including the loops C2 and C3 (König et al., 1989; Franke et al., 1990; Franke et al., 1992). $G_t$ also has at least two, and probably more, sites that interact with the receptor during binding and
activation. Peptide α(340-350) showed a clear inhibition of photoregeneration with an almost complete suppression at saturating concentrations (Fig. 4-2B). The peptide γ(50-71)-far showed a lower efficacy to inhibit photoregeneration. Although the inhibitory effect saturated at high concentrations with a normal first-order-binding isotherm, there was not a complete suppression of the photoregeneration effect (Fig. 4-2C). The binding of γ(50-71)-far to R* is likely to be quite complex due to specificity which arises from both the farnesyl moiety and the peptide sequence (Fig. 4-3). The carboxyl-terminal region of Gγt was also studied using a MII difference spectroscopy assay with similar findings (Kisselev et al., 1994; Kisselev et al., 1995a).

The Role of a Conserved Region at the Amino Terminus of Loop C4 of Rhodopsin in Gt Binding

We used the photoregeneration assay to probe the effects of Gt and Gt-derived peptides on expressed rhodopsin and rhodopsin mutants. The rhodopsin loop C4 mutations did not significantly affect the signal transmission path itself, as is seen from the similar kinetics of the photoregeneration signals in the absence of Gt and Gt-derived peptides (Fig. 4-4). The results in Figs. 2 and 3 show that Gt, and peptides α(340-350) and γ(50-71)-far are specific probes of rhodopsin signaling. Therefore, inhibition of photoregeneration by Gt or Gt-derived peptides is interpreted as the specific interaction of these reagents with the intracellular surface. A lack of inhibition due to alteration of either the peptide or the C4 loop is interpreted as a disruption of interaction. An advantage of the photoregeneration assay is that only productive binding interactions that stabilize specific conformations of the protein are reported.

In theory, a particular mutation might have the effect of uncoupling the conformation of the cytoplasmic surface from that of the chromophore-binding pocket. For example, an E134Q mutant has been shown to assume a partially activated conformation at the cytoplasmic surface while the chromophore and surrounding structures remain in the
dark, inactive conformation (Kim et al., 1997). This type of mutant might give misleading results, as photoregeneration (monitored by structural rearrangements surrounding the chromophore) could proceed unhindered, even as Gt or peptides bound normally to the cytoplasmic surface. The rhodopsin loop C4 mutants showed no evidence of any uncoupling between the chromophore-binding pocket and cytoplasmic surface conformations. All of the mutants showed similar photoregeneration kinetics in the absence of peptide (Fig. 4-4, black traces). This suggests that the effects of the mutations are localized to the cytoplasmic surface, and do not affect the photoregeneration process itself. However, the mutant E134R/R135E photoregenerated with kinetics that were distinctly different from that of rhodopsin (data not shown), and therefore was not considered further in this study. Of course, mutants could exist that would foil all assays that rely on the detection of binding events at the intracellular surface resulting from conformational changes that are induced elsewhere in the protein. The extra-MII assay commonly used for rhodopsin, and the GTP-induced agonist affinity shift assay extensively used for other GPCRs have the same potential limitations and are much less sensitive and specific.

Taken together, the results in the preceding chapter and the biophysical analysis of selected rhodopsin mutants herein strongly suggest that the amino terminus of C4 plays an important role in Gt binding and activation. Both Gt and α(340-350) binding are disrupted when a tripeptide in this region is replaced by a sequence from the β2 AR (Fig. 4-5). The most straightforward interpretation of the data is that the amino-terminal region of loop C4 directly influences or is part of a direct binding site for the carboxyl-terminal tail of Gαt.

It is surprising that CTr4, in which residues 310-312 are replaced with analogous sequence from the β2 AR, binds neither α(340-350) nor γ(50-71)-far. Can the two peptides bind to overlapping sites on the receptor, each of which includes the 310-312
region? This seems unlikely, because the carboxyl termini of $\gamma_\alpha_t$ and $\gamma_\gamma_t$ are located at a significant distance apart from each other in the structure of the heterotrimer (Lambright et al., 1996). Two potential explanations, which are not mutually exclusive, arise: 1) the peptides bind to different sites on the receptor and the sites are allosterically coupled; 2) $\gamma_t$ undergoes a large conformational change on contact with $R^*$ to bring the carboxyl termini of $\gamma_\alpha_t$ and $\gamma_\gamma_t$ into close proximity with the interaction domain near residues 310-312. A conformational switch in $\gamma_t$, induced by the contact with $R^*$ may be identical to the switch in $\gamma_\beta \gamma_t$ that was suggested earlier (Kisselev et al., 1995a).

**Possible Role of $\gamma_\gamma_t$-farnesyl in Docking of $\gamma_t$ to the Active Receptor**

The relevance of the data to the binding site of $\beta \gamma_t$ is more subtle. The observation that CTr2, but not CTr4, binds $\gamma(50-71)$-far highlights the complexity of the binding interaction between $\gamma(50-71)$-far and rhodopsin. Further evidence of this complexity, as noted above, is that $\gamma(50-71)$-far fails to fully inhibit the photoregeneration reaction, even at saturating concentrations. This behavior contrasts with that of $\alpha(340-350)$ (Fig. 4-2). In addition, both the farnesyl moiety and the peptide itself are required for binding to $R^*$ (Fig. 4-3). Each is likely to have a distinct binding site that may be differently altered in the mutants studied. The binding of $\gamma(50-71)$-far to CTr2, but not CTr4, suggests that the structural integrity of the fourth loop is disrupted by substitution of 310-312 with $\beta_2$ AR sequence, but that the substitution of the entire loop restores the structural determinants required for $\gamma(50-71)$-far binding. In this scenario, the tertiary, but not necessarily the primary structure of C4 would be critical for $\gamma(50-71)$-far binding.

In summary, we developed a novel biophysical assay to probe the $\gamma_t$-binding domain of rhodopsin and expressed rhodopsin. $\gamma_t$ and peptides corresponding to the carboxyl-terminal regions of $\gamma_\alpha_t$ and $\gamma_\gamma_t$ specifically bind to $R^*$ and stabilize the active state of the receptor. We conclude that the amino-terminal region of loop C4 acts as part of the binding site for $\gamma_\alpha_t$ and modulates the $\gamma_t$-binding domain of $R^*$. Future work is
required to reconcile the various models for allosteric regulation of the $G_l$-binding surface of $R^*$, especially concerning the binding of $G\beta\gamma_t$. 
Chapter 5:
The Function of Interdomain Interactions in Controlling Nucleotide Exchange Rates in Transducin*

Summary

The intramolecular contacts in heterotrimeric G proteins that determine the rates of basal and receptor-stimulated nucleotide exchange are not fully understood. The α subunit of heterotrimeric G proteins consists of two domains: a Ras-like domain with structural homology to the monomeric G protein Ras and a helical domain comprised of six α-helices. The bound nucleotide lies in a deep cleft between the two domains. Exchange of the bound nucleotide may involve opening of this cleft. Thus interactions between the domains may affect the rate of nucleotide exchange in G proteins. We have tested this hypothesis in the α subunit of the rod cell G protein transducin (Gαt). Site-directed mutations were prepared in a series of residues located at the interdomain interface. The proteins were expressed in vitro in a reticulocyte lysate system. The rates of basal and rhodopsin-catalyzed nucleotide exchange were determined using a trypsin digestion assay specifically adapted for kinetic measurements. Charge-altering substitutions of two residues at the interdomain interface, K273 and K276, increased basal nucleotide exchange rates modestly (5-10 fold). However, we found no evidence that interactions spanning the two domains in Gαt significantly affected either basal or rhodopsin-catalyzed nucleotide exchange rates. These results suggest that opening of the interdomain cleft is not an energetic barrier to nucleotide exchange in Gαt. Experiments

with $G\alpha_1$, in conjunction with other published results, suggest that the organization and function of the interdomain region differ among various G protein subtypes.
Introduction

Physiologically, the detection of dim light requires that the basal nucleotide exchange rates of $G_t$ be very low to reduce background noise, and that $R^*$-catalyzed exchange be very efficient, to ensure consistent detection and amplification of light signals. In the preceding two chapters, we characterized the function of C4 of $R^*$ in binding and supporting rapid activation of $G_t$. In this chapter, we begin an analysis of the structures of $G_t$ that normally function to maintain low nucleotide exchange rates but which might be perturbed by the binding of $R^*$ to induce rapid nucleotide exchange.

$G\alpha_t$ consists of two domains: a Ras-like domain, which is structurally similar to the monomeric G protein p21$^{ras}$ (Ras), and a helical domain, which is unique to the heterotrimeric G proteins (Noel et al., 1993). The bound nucleotide lies in a deep cleft between the two domains (Fig. 5-1). Although the discovery of this arrangement initially prompted speculation that nucleotide exchange would involve opening of the interdomain cleft (Noel et al., 1993), and that interactions between the domains might affect the rate of nucleotide exchange, the intramolecular contacts in $G\alpha_t$ that determine the rates of nucleotide exchange remain to be elucidated.

There is evidence that structures that do not directly interact with the nucleotide can modulate both the basal and the receptor-catalyzed rates of nucleotide exchange. For example, although the direct contacts between the protein and the nucleotide are virtually the same in closely related subtypes of G protein, the rates of basal nucleotide exchange vary widely. Furthermore, $R^*$ tremendously accelerates nucleotide exchange, yet available evidence indicates that it does not directly contact the nucleotide binding site (Iiri et al., 1998).
Chapter 5: Interdomain Interactions in Go\textsubscript{t}

One of the regions of the G protein hypothesized to control nucleotide release rates without directly contacting the nucleotide is the interdomain interface. The interface is composed of contacts adjacent to the nucleotide, and also interactions that are distant from the nucleotide (Fig. 5-1). These latter interactions involve residues located on the \(\alpha D-\alpha E\) loop (amino acid residues 139-147 of Go\textsubscript{t}) of the helical domain, and the Switch III region (residues 227-238) and the \(\alpha G\) region (residues 269-277) of the Ras-like domain (Fig. 1B). These interactions have been implicated in mediating the lower rate of dissociation of GTP\textsubscript{y}S relative to GDP in Go\textsubscript{11} (Mixon et al., 1995), and in affecting the basal nucleotide exchange rates in Go\textsubscript{11} (Remmers et al., 1999) and Go\textsubscript{s} (Warner et al., 1998; Warner and Weinstein, 1999). Additionally, studies in Go\textsubscript{s} have suggested that interdomain interactions are involved in mediating rapid nucleotide exchange catalyzed by the \(\beta_2\) adrenergic receptor (Marsh et al., 1998; Grishina and Berlot, 1998).

We have studied the function of several residues of Go\textsubscript{t} that are located at the interdomain interface but do not contact the nucleotide. A number of site-directed mutations of these residues were constructed. The well-documented difficulties in expressing and purifying recombinant Go\textsubscript{t} were overcome by expressing the mutant proteins in vitro in a rabbit reticulocyte lysate system. The rates of basal and R*-catalyzed nucleotide exchange were measured using a trypsin digestion assay specifically adapted for kinetic measurements. Alteration of two conserved lysine residues, K273 and K276, increased the rate of spontaneous nucleotide exchange 5-10 fold. However, in contrast to what would be predicted based on published structural and biochemical studies, we found no evidence that interactions that span the domain interface were important in either maintaining the low rate of basal nucleotide exchange or in supporting the high rate of R*-catalyzed exchange in Go\textsubscript{t}. Experiments with Go\textsubscript{11} demonstrated that conserved lysine residues serve different roles in Go\textsubscript{11} than in Go\textsubscript{t}. In general, the function of the interdomain region appears to differ among various G protein subtypes.
Chapter 5: Interdomain Interactions in Go1

Results

A series of site-directed mutants of Go1 with replacements of residues located at the interdomain interface was prepared. Sites were selected for mutation based on their position in the crystal structure of GDP-bound Go1 (Lambright et al., 1994) (Fig. 1), as well as their importance in Go11 and GoS suggested by published studies (see below). Selected residues were replaced with alanine, or with the amino acid present in the homologous position of either Go11 or GoS, and/or with amino acids reported to cause altered phenotypes in Go11 or GoS. For each mutant Go1, the rates of both basal (i.e., uncatalyzed) and R*-catalyzed nucleotide exchange were measured.

Expression of Go1 in vitro and Trypsin Digestion Assay of Nucleotide Binding and Exchange

All Go1 constructs were expressed in vitro in a coupled transcription/translation rabbit reticulocyte lysate system. Time-course experiments confirmed that in vitro expression was maximal in 90 min (not shown). Typical reactions with plasmids encoding Go1 or Go1 mutant genes yielded one major protein band at the expected molecular mass of ~40-kDa (Fig. 5-2). Generally 80-90% of the total intensity in the lane was in the one band. Expressed Go1 was digested with trypsin following various treatments. Inactive, GDP-bound Go1 was prepared by incubating in vitro translated Go1 with 100μM GDP. Trypsin proteolysis of this sample resulted primarily in the formation of a ~23 kDa fragment (Fig. 5-2). The active conformation was prepared by incubating Go1 with GDP and AlF4-. AlF4- is known to bind to Go1-GDP and simulate the presence of the γ phosphate of GTP. Therefore, a conformation nearly identical to the activated GTP-bound conformation is induced (Sondek et al., 1994). Digestion of the AlF4- activated Go1 yielded a ~34-kDa band and no ~23-kDa band. Similarly, activation of
Chapter 5: Interdomain Interactions in G\(\alpha_t\)

G\(\alpha_t\) with GTP\(\gamma\)S yielded an identical \(~34\)-kDa band following trypsin digestion (Fig. 5-2).

In all cases, trypsin proteolysis produced a variety of lower molecular weight fragments. Some of these were the smaller polypeptides that were cleaved to produce the \(~23\)- and \(~34\)-kDa fragments. Others likely resulted from extensive proteolysis of protein that was not properly folded. This is consistent with the large number of potential trypsin sites present in the primary structure of G\(\alpha_t\) and the relatively small number of accessible sites in the properly folded tertiary structure. The fraction of the total pool of translated G\(\alpha_t\) that was properly folded and functional was estimated from the ratio of the intensities of the \(~34\)-kDa band following GDP/AIF\(_4\) treatment (\(i.e.,\) the properly folded, activatable pool) to that of the \(~40\)-kDa band in the undigested sample (\(i.e.,\) the total pool of translated full-length protein). This ratio was generally about 0.15 for wild-type G\(\alpha_t\) and several-fold higher for G\(\alpha_i1\). This result parallels the functional expression levels of G\(\alpha_t\) and G\(\alpha_i1\) that have been observed in other heterologous expression systems. Thus, \textit{in vitro} expression might serve as a rapid and useful predictor of the expression level of a given G protein construct in other systems.

The rate of trypsin proteolysis of GDP- and GDP/AIF\(_4\) -treated G\(\alpha_t\) and the stabilities of the resulting fragments were investigated by digestion time-course experiments. Both the \(~23\)- and \(~34\)-kDa bands formed completely by 20 min and remained stable until at least 40 min in the presence of trypsin (data not shown). We therefore chose to stop the digestion after 30 min in all experiments. Similar experiments with G\(\alpha_i1\) indicated that digestion occurred more quickly. Accordingly, digests were run for 5 min with G\(\alpha_i1\) samples.

Three major sites of trypsin proteolysis in properly folded G\(\alpha_t\) have been identified: K18, R204, and R310 (Mazzoni \textit{et al.}, 1991). Using site-directed mutagenesis, we
investigated which of these sites were contributing to the fragments produced under the digestion conditions used in this study. The mutants K18A, R204H, and R310A were prepared, expressed \textit{in vitro}, and digested following treatment with either GDP or GDP/AlF$_4^-$.

Formation of the \(\sim 23\)-kDa band following treatment with GDP was altered only by the R204H mutation (not shown). R204 is the site at which cleavage occurred in the GDP form to yield the \(\sim 23\)-kDa fragment, but which was protected from digestion in the GTPyS-bound conformation. R204 is located in the Switch II region of Go$_t$ and crystal structures confirm that it moves from a surface exposed to a more buried position upon activation (Noel et al., 1993; Lambright et al., 1994). Formation of the \(\sim 34\)-kDa band following activation with GDP/AlF$_4^-$ was affected only by the R310A mutation (not shown). Thus R310 appears to be the site at which trypsin proteolysis occurred to yield the \(\sim 34\)-kDa fragment. The K18A mutant was indistinguishable from wild-type; digestion at K18 does not appear to contribute to the formation of either fragment under the conditions used (not shown). Using these results, the ratio of the number of methionines in the \(\sim 34\)-kDa band relative to the \(\sim 23\)-kDa band was determined to be 1.4. This factor was used to normalize the intensity of the \(\sim 23\)-kDa band in calculating the fraction of activated Go$_t$ in each aliquot.

The fraction of Go$_t$ in the active conformation in a partially activated sample was calculated as the intensity of the \(\sim 34\)-kDa band (\textit{i.e.}, the activated Go$_t$) divided by the sum of the intensities of the \(\sim 23\)- and \(\sim 34\)-kDa bands. The sum of the intensities of the \(\sim 23\)- and \(\sim 34\)-kDa bands is indicative of the total pool of functional Go$_t$ in the sample. This calculation is therefore internally normalized to the total amount of functional Go$_t$ in each aliquot and does not require comparison with the \(\sim 34\)-kDa band of a separate sample (such as one in a completely activated lane).
Chapter 5: Interdomain Interactions in \( \alpha_{t} \)

The rate of nucleotide exchange of each sample was determined by monitoring the fraction of \( \alpha_{t} \) in the active conformation at specific times following addition of GTP\( \gamma \)S. In the basal exchange rate assay, \( \alpha_{t} \) was 31% activated at 6 h following GTP\( \gamma \)S addition (Fig. 5-2A). The activity of \( \alpha_{t} \) following the 6 h incubation was confirmed by demonstrating that addition of rhodopsin and G\( \beta \gamma_{t} \) could fully activate the remaining \( \alpha_{t} \) (Fig. 5-2). In comparison, \( \alpha_{t} \) A322S (a mutant known to display high nucleotide exchange rates in G\( \alpha_{q} \) (Posner et al., 1998) and G\( \alpha_{s} \) (Iiri et al., 1994)) was 100% activated in less than 1 h.

In R*-catalyzed assays, \( \alpha_{t} \) was nearly 100% activated in 20 min (Fig. 5-2B). Under the conditions of the assay, the rate of \( \alpha_{t} \) activation was found to be dependent on the concentration of rhodopsin from 0-100 nM and sensitive to the presence of added G\( \beta \gamma_{t} \) (Fig. 5-3B). Some residual activation was observed in the absence of added G\( \beta \gamma_{t} \), probably as a result of small quantities of G\( \beta \gamma \) present in the reticulocyte lysate or the rhodopsin preparations. No rhodopsin-catalyzed activation was observed in the dark (data not shown). Additionally, mutant G348P, which was previously reported to be unable to bind rhodopsin (Osawa and Weiss, 1995), was not activated by R* in this assay (Fig. 5-3B). To our knowledge, the is the first report in which trypsin proteolysis of \textit{in vitro} translated \( \alpha_{t} \) has been used to measure the kinetics of R*-catalyzed nucleotide exchange.

\textit{Analysis of Single Amino Acid Replacements in the Interdomain Interface of} \( \alpha_{t} \\

Two residues in the helical domain, S140 and Q143, extend toward and interact with residues from the Ras-like domain. S140 was replaced with alanine, arginine (the homologous residue in G\( \alpha_{q} \)), and asparagine (the homologous residue in G\( \alpha_{s} \)). Q143 was mutated to alanine and was also combined with S140A in a S140A/Q143A double mutant. None of these mutations substantially altered the rate of basal or R*-catalyzed nucleotide exchange (Fig. 5-4, Table 5-1).
Several residues in the Switch III region of G\(\alpha_t\) were altered by site-directed mutagenesis. The crystal structure suggests that D227 participates in hydrogen bonds with both S140 and K276 (Fig. 1). This residue was replaced with both asparagine (the homologous residue in G\(\alpha_q\)) and with alanine. The adjacent M228 was replaced with alanine, leucine (the equivalent in G\(\alpha_{11}\)), and glutamine (the equivalent of a mutation in G\(\alpha_{11}\) reported to increase the GDP release rate (Remmers et al., 1999)). V231 was replaced with alanine and with tryptophan. The V231W mutant was prepared to simulate a naturally occurring mutation in the homologous residue of G\(\alpha_S\), R258, which was found in a patient with Albright's hereditary osteodystrophy (Warner et al., 1998). None of these mutations was found to substantially alter either the basal or the R*-catalyzed activation rates (Fig. 5-5, 5-6 and Table 5-I). However, several mutations caused slight (~2 fold), but reproducible reductions in the basal nucleotide exchange rate. These mutations include D227A, D227N, V231A and M228L (Table 5-I).

Three lysine residues in the \(\alpha G\) region of G\(\alpha_t\) were studied. K273 and K276 are oriented toward D227 of Switch III (Fig. 1). In addition, the crystal structure of G\(\alpha_t\)-GDP indicates that K276 forms hydrogen bonds with S140 of the helical domain. K275 extends out toward the solvent. K273, K275, and K276 were each replaced with alanine. In addition, K276 was replaced with glutamic acid and with arginine. The K273A, K276A, and K276E mutations all resulted in significantly increased rates of basal nucleotide exchange, from 5- to 10-fold above wild-type (Fig. 5-7A). The K276R mutation did not affect nucleotide exchange rates, nor did mutation of K275. None of the mutations substantially altered the rate of R*-catalyzed nucleotide exchange (Fig. 5-7B).

**Analysis of Double Amino Acid Replacements**

In order to probe for functional interactions between pairs of residues, a series of double amino acid replacements was prepared by site directed mutagenesis (Table 5-I).
The K276A/D227N, K276A/D227A, and K273A/D227N double replacements all displayed slower basal rates of nucleotide exchange than the corresponding single replacements of K276 or K273 (Fig. 5-8). The double mutants displayed faster exchange kinetics than wild-type G\(_{\alpha_t}\), however. Combining amino acid replacements at positions K276 and S140 revealed that the effects on basal exchange rates of each individual mutation were roughly additive (Fig. 5-8). A combination of amino acid replacements at positions 140 and 227, S140A/D227N had similar exchange kinetics to that of wild-type G\(_{\alpha_t}\) (Table 5-1).

**Analysis of G\(_{\alpha_{i1}}\) Mutants**

G\(_{\alpha_{i1}}\) is 66% identical to G\(_{\alpha_t}\) at the primary structure level and very similar at the tertiary structure level (Fig. 5-10). However, the basal nucleotide exchange rate of G\(_{\alpha_{i1}}\) has been reported to be significantly higher than that of G\(_{\alpha_t}\) (Skiba et al., 1996). We confirmed this observation in studies with recombinant G\(_{\alpha_{i1}}\) and retinal G\(_{\alpha_t}\) in a fluorescence activation assay (Fig. 5A). The rate of basal nucleotide exchange as monitored by increases in fluorescence was much greater in G\(_{\alpha_{i1}}\) than in G\(_{\alpha_t}\) at 25°C. However, the rate and the magnitude of fluorescence change was comparable when each protein was fully activated with excess AlF\(_4^\text{-}\).

G\(_{\alpha_{i1}}\) was expressed *in vitro* and studied by trypsin digestion. SDS-PAGE analysis indicated that a ~38-kDa band was produced following full activation with either GTP\(_{\gamma}\)S or GDP/AlF\(_4^\text{-}\), and that a smaller fragment resulted from digestion in the presence of GDP. The intensity of the smaller GDP-dependent band of G\(_{\alpha_{i1}}\) could not be accurately quantitated due to reproducible nonspecific background in that portion of the gel. Therefore, the method for determining the fraction of *in vitro* translated G\(_{\alpha_{i1}}\) activated in an aliquot was modified from the "ratio" method used for G\(_{\alpha_t}\). In each aliquot, the intensity of the ~38-kDa band was determined as a fraction of the total
intensity in the lane and the time course of activation was plotted as a change in this fraction over time (Fig. 5B). This analysis suggests that basal nucleotide exchange of GDP for GTPγS by \( \text{G}\alpha_{i1} \) was complete in 1 h, significantly faster than that by \( \text{G}\alpha_t \), which was only 32% complete in 6 h. The \( t_{1/2} \) for activation of \( \text{G}\alpha_{i1} \) (~20 min) was comparable in both the fluorescence and the trypsin protection assays (Fig. 5-9).

A series of site-directed mutants was prepared in \( \text{G}\alpha_{i1} \) to probe for similarities between the functions of interdomain residues in \( \text{G}\alpha_{i1} \) and \( \text{G}\alpha_t \). The equivalent of K273 and K276 of \( \text{G}\alpha_t \) are conserved in \( \text{G}\alpha_{i1} \) as K277 and K280, respectively (Fig. 5-10). Both were replaced with alanine, expressed \textit{in vitro}, and the basal nucleotide exchange rates of the resulting mutants were determined. Neither of these mutations (K277A and K280A) increased the basal activation rate of \( \text{G}\alpha_{i1} \) appreciably (Fig. 5-9B).

Additionally R144 of \( \text{G}\alpha_{i1} \), the homolog of S140 in \( \text{G}\alpha_t \), was replaced with serine. Since the S140 of \( \text{G}\alpha_t \) forms hydrogen bonds with K276, it was hypothesized that in \( \text{G}\alpha_{i1} \) the replacement of R144 with serine might alter the position of K280 (K276 in \( \text{G}\alpha_t \)) to resemble the \( \text{G}\alpha_t \) conformation and lower the basal rate of nucleotide exchange. However, the opposite was observed. The \( \text{G}\alpha_{i1} \) R144S mutant exhibited accelerated nucleotide exchange rates (Fig. 5-9B), consistent with previous reports of mutagenesis at the R144 position (Remmers \textit{et al.}, 1999).
Chapter 5: Interdomain Interactions in Go,.

### Table 5-1. Rate constants for basal nucleotide exchange measured for Go, and Ga, mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$k_{aPP}$</th>
<th>fold inc.</th>
<th>Mutant</th>
<th>$k_{aPP}$</th>
<th>fold inc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>8.6 ± 0.7</td>
<td>1.0 ± 0.1</td>
<td>D227N</td>
<td>3.4 ± 0.6</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>S140A</td>
<td>14.7 ± 4.6</td>
<td>1.7 ± 0.6</td>
<td>M228L</td>
<td>4.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>S140N</td>
<td>7.8 ± 2.1</td>
<td>0.9 ± 0.3</td>
<td>V231A</td>
<td>4.6 ± 0.6</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>S140R</td>
<td>12.1 ± 2.6</td>
<td>1.4 ± 0.3</td>
<td>D227A</td>
<td>5.0 ± 1.1</td>
<td>0.6 ± 1.1</td>
</tr>
<tr>
<td>Q143A</td>
<td>6.1 ± 1.4</td>
<td>0.7 ± 0.2</td>
<td>Q143A</td>
<td>6.1 ± 1.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>D227A</td>
<td>5.0 ± 1.1</td>
<td>0.6 ± 1.1</td>
<td>M228Q</td>
<td>6.2 ± 0.6</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>D227N</td>
<td>3.4 ± 0.6</td>
<td>0.4 ± 0.1</td>
<td>V231W</td>
<td>6.4 ± 1.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>M228A</td>
<td>14.6 ± 0.7</td>
<td>1.7 ± 0.2</td>
<td>S140A/D227N</td>
<td>7.4 ± 0.7</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>M228L</td>
<td>4.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>S140N</td>
<td>7.8 ± 2.1</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>M228Q</td>
<td>6.2 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>wild-type</td>
<td>8.6 ± 0.7</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>V231A</td>
<td>4.6 ± 0.6</td>
<td>0.5 ± 0.1</td>
<td>K276R</td>
<td>8.8 ± 2.5</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>V231W</td>
<td>6.4 ± 1.1</td>
<td>0.7 ± 0.1</td>
<td>S140A/Q143A</td>
<td>10.4 ± 4.7</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>K273A</td>
<td>39.0 ±22.6</td>
<td>4.6 ± 2.7</td>
<td>K275A</td>
<td>12.0 ± 1.4</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>K275A</td>
<td>12.0 ± 1.4</td>
<td>1.4 ± 0.2</td>
<td>S140R</td>
<td>12.1 ± 2.6</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>K276A</td>
<td>45.1 ± 7.5</td>
<td>5.3 ± 1.0</td>
<td>D227N/K273A</td>
<td>14.5 ± 2.7</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>K276E</td>
<td>98.8 ±29.1</td>
<td>11.5 ± 3.5</td>
<td>M228A</td>
<td>14.6 ± 0.7</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>K276R</td>
<td>8.8 ± 2.5</td>
<td>1.0 ± 0.3</td>
<td>S140A</td>
<td>14.7 ± 4.6</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>S140A/Q143A</td>
<td>10.4 ± 4.7</td>
<td>1.2 ± 0.6</td>
<td>D227A/K276A</td>
<td>14.8 ± 7.3</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>S140A/K276A</td>
<td>67.3±14.0</td>
<td>7.9 ± 1.8</td>
<td>D227N/K273A</td>
<td>20.3 ± 4.7</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>S140R/K276A</td>
<td>48.0±14.6</td>
<td>5.6 ± 1.8</td>
<td>K273A</td>
<td>39.0±22.6</td>
<td>4.6 ± 2.7</td>
</tr>
<tr>
<td>D227A/K276A</td>
<td>14.8 ± 7.3</td>
<td>1.7 ± 0.9</td>
<td>K276A</td>
<td>45.1 ± 7.5</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>D227N/K273A</td>
<td>14.5 ± 2.7</td>
<td>1.7 ± 0.3</td>
<td>S140A/K276A</td>
<td>67.3±14.0</td>
<td>7.9 ± 1.8</td>
</tr>
<tr>
<td>D227N/K276A</td>
<td>20.3±4.7</td>
<td>2.4 ± 0.6</td>
<td>K276E</td>
<td>98.8±29.1</td>
<td>11.5 ± 3.5</td>
</tr>
</tbody>
</table>

*aThe apparent rate constants were derived from fits of each data set to the exponential rise equation, $y = c + 100(1-exp(-kt))$. Each mutant was assayed at least 3 times (WT Go, was assayed 26 times), and an independent fit was made to each data set. The values reported are the mean $k_{aPP}*10^4$ min$^{-1}$ ± 2*S.E.M. The catalyzed activation rate of wild-type Go, was determined to be 5390 ± 802 in the presence of 30nM photoactivated rhodopsin, 30nM Gβγ, and 14μM GTPγS. This is a 629±107 fold increase over the basal (uncatalyzed) rate.

*bThe fold increase in the rate of the mutant relative to that of wild-type is calculated at the $k_{aPP}$(mutant)/$k_{aPP}$(wild-type).
FIG. 5-1. Structure of the interdomain interface of GDP-bound Gα₄. The Ras-like domain is blue, the helical domain (Insert 1) is red, and the GDP is magenta. Sidechains of residues which have been mutated in this study are shown in ball-and-stick representation. Sidechains of residues in the helical domain are yellow, those from Switch III are green, and those from the αG region are orange. A, Overall view of the protein. The nucleotide resides in a cleft between the two domains. The highlighted sidechains potentially interact with each other across the interdomain interface, but do not directly contact the nucleotide. B, The protein has been rotated 90° about the horizontal axis relative to panel A and the interdomain region is enlarged. Hydrogen bonds are shown as dotted lines. The position of K276 in the GTPγS-bound Gα₄ structure is shown as an outline.
FIG. 5-2. Determination of nucleotide exchange rates by analysis of trypsin digestion patterns. Gαt was expressed in vitro in a rabbit reticulocyte lysate system and metabolically labeled with [35S]methionine. Translated material was treated with various conditions as indicated. Aliquots (8 μl) were removed and digested with trypsin for 30 min on ice, except where indicated otherwise. The resulting fragments were analyzed by SDS-PAGE, and visualized by phosphorimaging. A. Uncatalyzed activation. Translated Gαt was mixed with 100 μM GTPγS. Aliquots were removed and digested at 1, 2, 3, 4, and 6 h. Following collection of the 6 h aliquot, photoactivated rhodopsin and GβγT were added to a final concentration of 30 nM each. After a 20 min incubation, an aliquot was removed and digested (+rho/βγ). Control reactions show undigested Gαt and Gαt digested following incubation with 100 μM GDP or GDP/AlF4−. B. Rhodopsin/GβγT-catalyzed activation. Translated Gαt was mixed with 30 nM photoactivated rhodopsin, 30 nM GβγT, and 14 μM GTPγS. Aliquots were removed and digested at 1, 2, 3, 5, 10, and 20 min. Controls reactions were performed as in panel A.
FIG. 5-3. Plots of uncatalyzed and catalyzed nucleotide exchange time courses derived from analysis of trypsin digest patterns of in vitro translated Gαt. The percent of Gαt activated at each time point is determined as the ratio of the intensity of the ~34 kDa band to the sum of the intensities of the ~34 and ~23 kDa bands. A. Uncatalyzed activation time course. The rates of wild-type and the mutant A322S were determined. The results are similar to previously published data determined with other methods (see text). The data points depict the mean of 26 (wild-type) or 3 (A322S) individual determinations; error bars depict 2*SEM. B. Rhodopsin-catalyzed activation time course. The rate of rhodopsin-catalyzed activation of in vitro-translated Gαt was determined in the presence of different concentrations of rhodopsin and Gβγt. A mutant of Gαt, G348P, which was previously reported to be resistant to rhodopsin, is not activated in this assay. Data shown are representative of an experiment repeated twice.
FIG. 5-4. Time courses of activation of mutants of residues located on the helical domain. The percent activation at each time point was determined by analysis of trypsin digestion patterns. The time-zero data point is calculated from protein mixed with 100 μM GDP for 10 min. Each data point is the average of 3-5 independent experiments. Error bars depict ± 2*SEM. A, Uncatalyzed activation. Goα and mutants were translated in vitro and combined with 100 μM GTPγS. The solid lines represent fits to one-component exponential rise function. B, Rhodopsin/Gβγ-catalyzed activation. Goα and mutants were translated in vitro and combined with 30 nM Gβγ, 30 nM photoactivated rhodopsin, and 14 μM GTPγS.
FIG. 5-5. Time courses of activation of mutants of residues located on Switch III (a.k.a. Insert 2). The experiments were performed as in Fig. 5-4. A. Uncatalyzed activation. B. Rhodopsin/Gβγ-catalyzed activation.
FIG 5-6. Time courses of activation of mutants of residue 228 located on Switch III (a.k.a. Insert 2). The experiments were performed as in Fig. 5-4. A, Uncatalyzed activation. B, Rhodopsin/Gβγ-catalyzed activation.
FIG. 5-7. Time courses of activation of mutants of residues located on the αG region (a.k.a., Insert 3). The experiments were performed as in Fig. 5-4. A, Uncatalyzed activation. B, Rhodopsin/GBPγ1-catalyzed activation.
FIG. 5-8. Time courses of uncatalyzed activation of double mutants. The experiments were conducted as described in Fig. 5-4. A, The K276A mutation was combined with mutations of residues that are indicated in the crystal structure to interact with K276. The rates of the uncatalyzed activation were determined. Data for K276A and D227N are re-plotted for comparison with the double mutants. B, The K273A mutation was combined with the D227N mutation, and the uncatalyzed activation rate determined. K273A and D227N are re-plotted for comparison.
FIG. 5-9. Uncatalyzed activation of Gα11 and mutants of Gα11. A. Fluorescence activation trace of Gα11 (expressed and purified from Sf9 cells) and Gαt (purified from bovine retinas). 250 nM of each protein in a total volume of 500μL was placed in a stirred cuvette at 25°C. Fluorescence was excited at 300 nm, and emission was measured at 345 nm. At 200 s, 15μL of GTPγS was added to the cuvette to a final concentration of 5μM. At the times indicated by the arrows, AlCl3 and NaF were added from separate stock solutions to a final concentration of 0.05mM and 3mM respectively. B. Noncatalyzed activation of Gα11 and mutants expressed in vitro and assayed by analysis of trypsin digestion products. The intensity of the GTP-dependent band is expressed as a percent of the total intensity in the lane. Each point represents the mean of at least three independent experiments ± 2*S.E.M.
FIG. 5-10. Structure of the interdomain interface of GDP-bound $G\alpha_{11}$. View and coloring is similar to Fig. 5-1B. $G\alpha_{11}$ residues are labeled, and the corresponding residues in $G\alpha_{t}$ are noted in parentheses (see Fig. 5-1B). K280, which is equivalent to K276 of $G\alpha_{t}$, is oriented toward the solvent whereas K276 of $G\alpha_{t}$ is oriented toward the equivalent of D231. Residues 234-239 are not ordered in this structure.
Discussion

Analysis of Trypsin-Digest Products of in vitro Translated Go\textsubscript{t} to Evaluate Nucleotide Exchange Kinetics

Since Go\textsubscript{t} is refractory to expression in bacteria (Skiba et al., 1996), and is cumbersome to express in insect cells (Min et al., 2000) we chose to express Go\textsubscript{t} in vitro, and analyze nucleotide exchange rates using trypsin digestion. The pattern of proteolytic fragments resulting from trypsin digestion directly reflects the conformation of Go\textsubscript{t}, and therefore the identity of the bound nucleotide (Neer et al., 1994a; Fung and Nash, 1983). Trypsin proteolysis of expressed Go\textsubscript{t} yielded ~23-kDa fragments following incubation with GDP and ~34-kDa fragments following activation with either AlF\textsubscript{4}\textsuperscript{-} or GTP\textgamma{}S (Fig. 5-2). These observations are consistent with previously published results (Mazzoni and Hamm, 1996; Osawa and Weiss, 1995).

Trypsin proteolysis of in vitro translated Go\textsubscript{t} allowed for the precise quantitation of basal and R*-catalyzed nucleotide exchange rates. By using both the ~23- and the ~34-kDa bands, the calculation of nucleotide exchange rates was internally normalized and took into consideration both the GDP- and the GTP\textgamma{}S-bound fractions. As a result, the data were very reproducible and samples in which only a small fraction of the total expressed protein was functional could be analyzed. Additionally, the methodology was rapid enough to allow for the characterization of relatively large numbers of mutants in parallel.

The kinetic parameters for Go\textsubscript{t} activation derived from the trypsin digestion assay are consistent with data reported using other traditional methodologies (Ramdas et al., 1991; Skiba et al., 1996) as well as with analysis of retinal Go\textsubscript{t} studied with the fluorescence activation assay (Fig. 5-9). Additionally, analysis of the mutant A322S by trypsin
proteolysis indicated that the basal rate of activation was >60-fold greater than that of wild-type G\(\alpha_t\) (Fig. 5-3A). This result is also in agreement with published data using different methodologies on the analogous mutation in G\(\alpha_S\) and in G\(\alpha_{11}\) (Iiri et al., 1994; Posner et al., 1998).

Several control reactions demonstrated the fidelity of the R*-catalyzed assay. The rate of rhodopsin-dependent activation was sensitive to light (not shown), to the concentration of rhodopsin from 0-100 nM and to the presence of added G\(\beta_\gamma_t\) (Fig. 5-3B). A mutation near the carboxyl terminus of G\(\alpha_t\) (G348P) that was previously reported to disrupt R*-G\(t\) interactions (Osawa and Weiss, 1995) was not activated (not shown).

The system does present several potential limitations. The concentrations of the expressed G\(\alpha_t\) cannot be easily controlled or readily determined. Other components of the reticulocyte lysate may affect the behavior of G\(\alpha_t\) and rhodopsin. Certain mutations of G\(\alpha_t\) (such as R204H) directly alter the pattern of fragments produced by trypsin proteolysis, and therefore interfere with determination of the activation state. Despite these limitations, we show here that the analysis of in vitro translated G\(\alpha_t\) by trypsin proteolysis allowed for the rapid and accurate quantitation of activation kinetics of expressed G\(\alpha_t\) in both uncatalyzed and rhodopsin-catalyzed assays.

This expression and assay system offers additional flexibility not explored in the present work. Expressed recombinant rhodopsin could be used in place of retinal rhodopsin to test the combined effects of mutation of rhodopsin and transducin (unpublished observation). Other proteins (e.g., G\(\beta_\gamma_t\) mutants, regulator of G protein signaling proteins, etc.), could be co-translated with G\(\alpha_t\) in the in vitro system (Neer et al., 1994a). Furthermore, this system will likely prove useful in the kinetic
characterization of other G protein subtypes, such as cone transducin, which are difficult to express heterologously.

Site-Directed Mutation of K273 or K276 Increases Basal Nucleotide Exchange Rates

Replacement of K276 or K273 with alanine increased the basal rate of nucleotide exchange ~5 fold in Goq (Fig. 5-7A, Table 5-1). Replacement of the adjacent K275, which in the crystal structure is oriented toward the solvent (Fig. 5-1), had no effect. Replacement of K276 with a negatively charged glutamic acid increased basal nucleotide exchange rates more dramatically (~10 fold) than the neutral alanine replacement mutant. However, mutation to a positively charged arginine did not alter the activation rate. Together, these results suggest that the function of K276 and K273 is dependent on positive charge, and orientation toward the interior of the protein. Interestingly, in the activated, GTPγS-bound structure of Goq, K276 is rotated outward toward the solvent relative to the GDP-bound conformation (Fig. 5-1B). The K276A mutant may anticipate this active conformation.

K276 and K273 both lie in the third of four regions of Goq for which there is no homologous sequence in the monomeric G protein Ras. The four regions are known as Insert 1 through Insert 4 (Noel et al., 1993). Specific functions have been attributed to Insert 1 (the helical domain), Insert 2 (the Switch III region) and Insert 4 (which may interact with heptahelical receptors). The present work is the first to identify a functional role for residues in Insert 3 of Goq.

The structure of GDP-bound Goq reveals that K276 and K273 lie near to and possibly form ionic interactions with D227. This observation suggests that the reason for accelerated nucleotide exchange rates caused by mutations of K276 and K273 might involve disruption of interactions with D227. To test this hypothesis, the K276A and K273A mutations were combined with mutation of D227N to produce the K276A/D227N and K273A/D227N double mutants. If the effects of K276A were due
solely to breaking of an ionic interaction with D227, then the K276 mutation should not increase the rate of nucleotide exchange in the context of D227N or D227A mutants. Indeed, the increase in the basal rate of activation caused by the K276A and K273A mutations was reduced (from ~5 fold to ~2 fold relative to wild-type) when combined with D227N (Fig. 5-8). However, since the rate of the D227N mutant alone is 2 fold slower than that of wild-type, the effect of the K276A mutation is roughly the same (i.e., a ~5 fold increase in rate) whether introduced into a wild-type or a D227N background. Similar results were obtained with a D227A mutation. Thus, the origin of the increase in basal nucleotide exchange rates by the K276A and K273A mutation is not merely due to disruption of interactions with D227.

K276 also appears to interact across the interdomain interface with SMO. Replacement of SMO with alanine, which would have disrupted hydrogen bonding to K276A, did not affect the rate of activation. Thus, breaking of the putative SMO-K276 interaction does not fully explain the effects of the K276A mutation. There appear to be other unidentified requirements for positively charged sidechains in the αG region in order to maintain low basal rates of nucleotide exchange.

Mutation of D227 slows the rate of basal nucleotide exchange, both in the context of the wild-type protein as well as in the K276A mutant (Fig. 5-8). This is surprising since Goq has an extremely low rate of basal nucleotide exchange, as is demanded by the low background noise required for sensitive light detection by photoreceptors. Other mutations in the Switch III regions also appear to slightly reduce the basal rate of nucleotide exchange, including M228L and V231A (Fig. 5-5, 5-6; Table 5-1). The origins of these effects, which are relatively small, are unclear. Previously, it has been demonstrated that the entire Switch III region can be deleted from Goq without disrupting the ability to bind nucleotides (Li and Cerione, 1997). The rates of nucleotide exchange
in these Switch III-deleted constructs were not characterized, but such studies might illuminate the role of Switch III in facilitating or impeding nucleotide exchange.

**Interdomain Interactions in \( \alpha_t \) Do Not Affect Basal or Rhodopsin-Catalyzed Nucleotide Exchange Rates**

When the structure of \( \alpha_t \) was determined, the nucleotide was found to reside in a deep cleft between the Ras-like domain and the helical domain (Noel *et al.*, 1993). It was proposed that rhodopsin might accelerate the nucleotide exchange rate by opening the cleft (Noel *et al.*, 1993; Bourne, 1993). Similarly, interactions between these domains could control the rate of basal nucleotide exchange. \( \alpha_t \) has a very low rate of basal nucleotide exchange as compared to related G proteins and a very high rate of \( \text{R}^* \)-catalyzed exchange. If interdomain interactions were important mediators of either of these processes, one might expect nucleotide exchange rates in \( \alpha_t \) to be particularly sensitive to mutation of residues involved in those interactions. However, none of the \( \alpha_t \) mutants characterized in this report significantly affected either basal or \( \text{R}^* \)-catalyzed rates.

Close analyses of G protein structures indicate that opening of the interdomain cleft is not necessarily an energetic barrier to nucleotide release. The helical domain does not contribute many contacts to the nucleotide binding pocket and certain monomeric G proteins, which do not have a helical domain at all, release GDP more slowly than some heterotrimeric G proteins subtypes (Sprang, 1997). A crystal structure of the \( \alpha_{11} \) mutant A326S, which releases GDP ~250-fold faster than wild-type \( \alpha_{11} \), does not reveal any alteration in the interdomain interactions, suggesting that an open cleft is not a prerequisite of fast nucleotide exchange (Posner *et al.*, 1998). In addition, the reported increases in nucleotide release rates resulting from mutations at the interdomain interface of \( \alpha_{11} \) and \( \alpha_s \) are relatively modest (~5-10 fold) as compared with mutations in other
regions of G proteins hypothesized to be involved in regulating nucleotide exchange rates. For example, we observed up to 165-fold increases in nucleotide exchange rates in mutations of certain residues of the α5 helix of Goq, a structure implicated in the mechanism of rhodopsin-catalyzed activation (Chapter 6). In summary, the opening of the interdomain cleft may not necessarily be a rate-determining step in nucleotide exchange in G proteins.

The Function of Residues at the Interdomain Interface Differs Among Goq, Goq1 and Goq3

Many of the residues mutated in this study, such as S140, Q143, M228, and V231, have been previously found to alter basal or receptor-catalyzed nucleotide exchange rates in the related G proteins, Goq3 and Goq1. In Goq3, a mutation in the Switch III region, R258W (corresponding to V231 in Goq), was found in a patient with Albright hereditary osteodystrophy (Warner et al., 1998). Biochemical studies indicated that replacement of R258 to tryptophan and to alanine, as well as alteration of a proposed interacting residue, Q170 of the helical domain (corresponding to Q143 in Goq), led to increases in the basal nucleotide exchange rate (Warner et al., 1998; Warner and Weinstein, 1999). These mutations were hypothesized to widen the interdomain cleft. Other studies in Goq3 found that mutation of R258 or N167 (corresponding to S140 in Goq) disrupted receptor catalyzed activation (Grishina and Berlot, 1998), suggesting that the receptor induces structural changes that are communicated across the interdomain interface. In Goq1, mutation of either L232 or R144 (corresponding to M228 and S140, respectively, in Goq) increased the basal nucleotide exchange rate by disrupting a proposed interdomain hydrophobic interaction (Remmers et al., 1999). The effects of mutating R144 were corroborated by the results of the R144S mutant in the current work (Fig. 5B).
The residues analogous to those proposed to interact with each other across the interdomain interface in G\(\alpha_S\) and G\(\alpha_{11}\) are also potentially interacting in G\(\alpha_t\) (Fig. 5-1B). In many cases, however, the amino acids are not conserved. For example, V231 and Q143 of G\(\alpha_t\), which correspond to the proposed interaction between R258 and Q170 in G\(\alpha_S\), are adjacent. S140 and M228 (corresponding to R144 and L232 of G\(\alpha_{11}\)) and S140 and D227 (corresponding to the proposed interaction of N167 and N254 of G\(\alpha_S\) (Grishina and Berlot, 1998)) are similarly adjacent. However, in contrast to the results with G\(\alpha_{11}\) and G\(\alpha_S\), replacement of these residues in G\(\alpha_t\) did not affect nucleotide exchange rates. These results suggest that the interdomain interface residues are functionally different in G\(\alpha_t\) than in G\(\alpha_{11}\) and G\(\alpha_S\). Counterintuitively, G\(\alpha_{11}\) and G\(\alpha_S\), which exchange nucleotides faster than G\(\alpha_t\), appear to have tighter and more sensitive interactions across the interdomain interface than those of G\(\alpha_t\).

Both K273 and K276 of G\(\alpha_t\) are conserved in G\(\alpha_{11}\). However, the structure of GDP-bound G\(\alpha_{11}\) reveals that K280 (cognate to K276 of G\(\alpha_t\)) is oriented toward the solvent instead of toward the Switch III region as in G\(\alpha_t\) (Fig. 5-10). Functionally, mutation of K280 and K277 to alanine did not lead to increases in nucleotide exchange rates in G\(\alpha_{11}\), as was observed in G\(\alpha_t\). Thus, conserved residues, K276 and K273 of G\(\alpha_t\), are found to serve different roles and to assume different structures in closely related G proteins.

In conclusion, the data in this report ascribe a role to K273 and K276 in the \(\alpha_G\) region of G\(\alpha_t\) in maintaining low basal rates of nucleotide exchange. However, unlike in G\(\alpha_{11}\) and G\(\alpha_S\), interactions which span the interdomain interface do not appear to be important in regulating either basal or rhodopsin-catalyzed nucleotide exchange rates. Differences exist in the organization of the interdomain interface among G\(\alpha_t\), G\(\alpha_{11}\), and G\(\alpha_S\) -- and even between conserved residues in G\(\alpha_{11}\) and G\(\alpha_t\).
Chapter 6:
Rapid Activation of Transducin by Mutations on the α5 Helix Distant From the Nucleotide-Binding Site: Evidence for a Mechanistic Model of Receptor-Catalyzed Nucleotide Exchange by G Proteins

Summary

Rhodopsin, a prototypical G protein-coupled receptor, catalyzes the release of GDP from the α subunit of transducin (Gαt), a heterotrimeric G protein. Hypothetical models of the complex between photoactivated rhodopsin (R*) and transducin (Gt) suggest that R* does not directly contact the nucleotide-binding pocket of Gαt. Instead, R* is thought to act "at-a-distance" through an unknown mechanism. The α5 helix of Gαt connects the carboxyl terminal region, which binds R*, to the nucleotide-interacting β6/α5 loop. Thus the α5 helix has been proposed to mediate communication between R* and the nucleotide binding pocket. We investigated the role of the α5 helix in R*-catalyzed activation of Gαt by extensive site-directed mutagenesis of α5 and the surrounding region. The mutants were expressed in vitro and the kinetics of uncatalyzed and R*-catalyzed nucleotide exchange were determined by quantitative analysis of trypsin digests of Gαt. A set of conserved residues (T325, V328 and F332) was identified that constitutes a nucleotide-release control microdomain on the buried surface of the α5 helix about 0.7-1.5 nm from the nucleotide. Mutations of these residues did not disturb the folding of the protein, interactions with R*, or binding of GDP, GTPγS and GDP/AlF4-. However, their mutation greatly (up to 165-fold) increased nucleotide exchange rates in the absence of rhodopsin. Mutation of a series of hydrophobic residues on the opposite face of α5 reduced R*-catalyzed activation, as did replacement of α5

residues with prolines. The data suggest that the mechanism by which R* catalyzes GDP release "at-a-distance" involves perturbation of the residues in the nucleotide-release control microdomain on α5. The structural basis of this perturbation may involve the binding of R* directly to α5.
Introduction

In chapters 3 and 4, evidence was presented supporting a role for the amino terminus of the C4 loop in the binding and activation of Gt, and specifically in binding a peptide derived from the carboxyl terminus of Gt. The carboxyl terminus of Gt is 2.5 nm away from the nucleotide (Fig. 1-2); available evidence suggests that rhodopsin is unlikely to directly contact the nucleotide binding pocket (Chapter 1). It is not clear how the C4-mediated binding of Gt by the cytoplasmic surface of R* affects the nucleotide binding pocket. In Chapter 5, it was demonstrated that the mechanism is not likely to involve opening the interdomain cleft.

The α5 helix connects the carboxyl-terminal tail of Gt (residues 340-350) to the β6/α5 loop (residues 321-324) (Fig. 6-1). As discussed in Chapter 1, the carboxyl-terminal tail is a well characterized rhodopsin-binding domain of Gt (Hamm et al., 1988; Ernst et al., 2000). The β6/α5 loop directly contacts the guanine ring of the nucleotide. Certain mutations in this loop have been shown to dramatically increase GDP release rates in G proteins (Iiri et al., 1994; Posner et al., 1998). Consequently, it has been proposed (Onrust et al., 1997; Iiri et al., 1998) that R* might achieve GDP dissociation by binding to the C-terminus of Gt and communicating with the β6/α5 loop via the α5 helix.

We examined in detail the role of the α5 helix in R* catalyzed activation of Gt by extensive mutagenesis of the α5 helix and the surrounding region. Using in vitro expression of Gt coupled with quantitative analysis of trypsin digestion patterns, we characterized the kinetics of spontaneous and R*-catalyzed nucleotide exchange in a large number (>30) of relevant mutants.

We found that mutation of three residues on the buried surface of α5 (T325, V328 and F332) mimicked the receptor-bound state in that the rate of GDP release was
Chapter 6: The α5 helix of Gα₁

dramatically (up to 165-fold) increased, even though the site of the mutation was distant from the nucleotide-binding pocket.

Consistent with the proposed role of α5 we also found that mutation of a hydrophobic, solvent exposed surface of α5 disrupted R*-catalyzed nucleotide exchange. Furthermore, structural perturbation of the α5 helix induced by the replacement of individual residues with proline or by the insertion of alanines disrupted R* -catalyzed exchange.

These data support an important role for the α5 helix in determining the rates of basal and R* -catalyzed nucleotide exchange. We suggest that R* does not use α5 merely to communicate with the β6/α5 loop, but that R* perturbs the conformation of residues on the buried surface of α5 itself and thus accomplishes rapid GDP release "at-a-distance". We speculate that the structural basis by which R* perturbs the α5 helix might involve binding of rhodopsin directly to an exposed surface of α5.
Chapter 6: The α5 helix of Go

Results

Proline Scanning Mutagenesis of the α5 Helix

In an effort to test the role of the α5 helix in mediating communication between R* and the nucleotide binding pocket of Go, we introduced structural perturbations into the helix. Each residue (amino acids 325-339) was individually replaced by proline. Each of the resulting constructs yielded proteins of an appropriate molecular weight and expression level, as judged by SDS-PAGE analysis of undigested samples (not shown). Several of the mutants were severely impaired in nucleotide binding, including N327, V328, K329, V331, F332 and V335. In these cases, the trypsin digestion patterns generally indicated that GDP binding was absent and that GTPγS binding was compromised. GTPγS binding was less defective than GDP binding, likely due to the extra binding energy contributed by contacts involving the γ phosphate. Additionally, AlF4−-induced activation in these mutants was greatly attenuated, presumably due to reduced GDP binding.

The proline mutants which could bind GDP were characterized in both R*-catalyzed and uncatalyzed activation assays (Fig. 6-2). In the uncatalyzed assay, one mutant located in the first turn of the helix, T325P, exhibited an extremely fast basal activation rate. Several other proline substitution mutants exhibited moderately accelerated rates relative to that of Go, including D333P, A334P, T336P, D337P and I338P. Three proline mutants, Q326P, F330P and I339P, were similar to wild-type Go. In the R*-catalyzed nucleotide exchange assay, the proline mutants (with the exception of T325P) exhibited defects in activation of varying severity.

Deletions and Insertions in the α5 Helix and Carboxyl Terminus of Go

In a further effort to evaluate the effects of structural alterations in α5, deletion and insertion mutants were prepared (Fig. 6-3). Three deletion mutants were constructed in
which 5, 10 or 25 amino acids were deleted from the carboxyl terminus of Gαt. None of these constructs yielded proteins which could bind nucleotides as judged by trypsin digest analysis.

Four insertion mutants were prepared. In three of the mutants, the insertion point was immediately following I339. This position was chosen to minimize disruptions between the α5 helix and the rest of the protein. Three alanines, four alanines or four isoleucines were inserted at this location in three different constructs, called α5 ala3, α5 ala4, and α5 ile4, respectively. In the fourth mutant, four alanines were inserted following residue 345 in the carboxyl-terminal tail (CT ala4). Of these constructs, only the two with insertions of four alanines yielded proteins that were functional (CT ala4 and α5 ala4), and in these cases only a small fraction of the protein could bind GDP. These two constructs could be activated by GDP/AlF₄⁻; however, they were unresponsive to R*-catalyzed nucleotide exchange (Fig. 6-3C).

Alanine Scanning Mutagenesis of the α5 Helix

In order to evaluate the effects of less drastic mutations in α5, each non-alanine residue of the helix was individually replaced by alanine. Each of these mutants expressed well in vitro, could bind GDP, and could be activated by AlF₄⁻ as judged by trypsin proteolysis. A significantly reduced level of functional protein was observed only in the case of I339A. The rates of uncatalyzed and R*-catalyzed nucleotide exchange were determined for the resulting mutant proteins. In the uncatalyzed assay, the majority of the mutants behaved similarly to wild-type Gαt. However, three mutants showed dramatic increases in the rate of uncatalyzed GTPγS uptake: T325, V328 and F332. Additionally, the N327A and I339A mutants showed accelerated kinetics of uncatalyzed activation (Fig. 6-4, Table 5-1). The rapid rate of nucleotide exchange observed in these mutants was not the result of global misfolding of the proteins. The conformations of
these proteins were similar to wild-type Goalpha_i in the presence of GDP, GDP/AlF_4^- or GTPgammaS as judged by trypsin digest analysis (Fig. 6-5).

In the R*-catalyzed assay (Fig. 6-6), T325A, N327A, V328A, and F332A were activated faster than wild-type Goalpha_i. The estimated times (in units of min) for half-maximal activation (t_{1/2}) for these mutants were: T325A, 0.4; V328A, 0.4; F332A, 0.2. In comparison, the estimated t_{1/2} for wild-type was 1.1. Four mutants exhibited activation rates that were reduced 3-4 fold relative to wild-type. These mutants, and the estimated values for t_{1/2} were: F330A, 4.1; V331A, 3.2; D337A, 4.6; and I338A, 4.0. The remaining mutants displayed activation rates that were similar to wild-type: Q326A, 1.3; K329A, 1.2; D333A, 1.2; V335A, 1.5; and T336A, 1.4.

**Mutagenesis of the beta6/alpha5 Loop and of Residues Interacting with the alpha5 Helix**

The beta6/alpha5 loop immediately precedes the alpha5 helix. Mutations in the analogous region in Galpha_i and Galpha_s were previously shown to increase nucleotide exchange rates (Iiri *et al.*, 1994; Posner *et al.*, 1998). In order to test the role of this region in Galpha_i, several site directed mutations were made. The mutants A322S, A322G and C321A all caused substantial increases in the rates of uncatalyzed activation and modest increases in rhodopsin-catalyzed activation (Fig. 6-7). The results of the A322S mutant were previously reported in Chapter 5 (Fig. 5-3) as a control for the trypsin digest assay.

In order to examine the function of interactions between alpha5 and the rest of the protein, five additional residues, which interact with the alpha5 helix and are located elsewhere on Galpha_i, were mutated to alanine. The F185A and F192A mutants did not form the typical ~23-kDa band when digested by trypsin following incubation with GDP (Fig. 6-7). Under these conditions, the F185A mutant generally yielded no bands >20-kDa, and the F192A mutant produced a ~21-kDa band. The absence of a ~23-kDa band could indicate lack of GDP binding. However, it appears that F185A and F192A did indeed
bind GDP since activation by AlF₄⁻, which requires binding of GDP, was normal. Additionally, the kinetics of GTPyS uptake required at least 20 min for completion, as judged by the intensity of the ~34-kDa band at times following GTPyS addition. If the Gα₅ mutants were not binding GDP, then much more rapid GTPyS uptake would be expected.

The F185A and F192A mutations seem to interfere not with GDP binding, but with the production of a ~23-kDa fragment by trypsins proteolysis that is otherwise characteristic of the GDP bound form. As a result, the fraction of mutant Gα₅ activated in a given aliquot of the F185A and F192A mutants cannot be accurately determined, since this calculation depends on the intensities of both the ~34- and ~23-kDa bands. However, an examination of the time course ~34-kDa bands following GTPyS treatment for mutants F185A and F192A clearly shows that uncatalyzed GTPyS uptake is accelerated in these mutants (Fig. 6-7). Similarly, the catalyzed activation rates of F185A and F192A are at least as fast as wild-type Gα₅, if not faster (not shown).

A third mutant protein in which a phenylalanine was replaced with an alanine, F187A, formed a ~23-kDa band normally when digested with trypsins following incubation with GDP. F187A caused a very slight retardation in both the catalyzed and uncatalyzed activation reactions. Two other mutations of residues predicted to interact with α5, K188A and Q168A, caused moderately increased rates in both catalyzed and uncatalyzed assays (Fig. 6-7).

Engineering of Disulfide Bridges Between α5 and Other Parts of Gα₅

The effects of constraining the conformation of the α5 helix were explored by attempts to engineer disulfide cross links between the α5 helix and the rest of the protein. The residue I339 of the α5 helix lies near to V30 (Natochin et al., 2000), and a naturally occurring cysteine, C216. Mutants I339C, V30C and I339C/V30C were constructed.
Chapter 6: The α5 helix of Gα₄

Disulfide formation was monitored by trypsin proteolysis of the GDP-bound and GDP/AlF₄⁻ bound Gα₄ and analysis by nonreducing SDS-PAGE. Since trypsin cleaves GDP-bound Gα₄ at R204 (Chapter 5), disulfide bonds between 339 and 30 (in the I339C/V30C mutant) would be expected to yield larger fragments following tryptic cleavage of the GDP-bound form. Similarly, since trypsin digests GDP/AlF₄⁻ bound Gα₄ at R310, disulfide bonds between C339 and C216 (in a I339C mutant) would be expected to result in a larger fragments following cleavage of the GDP/AlF₄⁻ -bound Gα₄.

The data did not reveal any evidence of disulfide formation in either the I339C or I339C/V30C constructs (not shown). Analysis was complicated, however, by the poor quality of electrophoresis performed under nonreducing conditions, as well as by the low levels of functional I339C produced.
Chapter 6: The α5 helix of Go<sub>t</sub>

Table 6-1. Rate constants of uncatalyzed nucleotide exchange of Go<sub>t</sub> and Go<sub>t</sub> mutants.

<table>
<thead>
<tr>
<th>Position</th>
<th>mutant&lt;sup&gt;d&lt;/sup&gt;</th>
<th>( k_{app}^a )</th>
<th>fold inc.&lt;sup&gt;c&lt;/sup&gt;</th>
<th>mutant&lt;sup&gt;d&lt;/sup&gt;</th>
<th>( k_{app}^a )</th>
<th>fold inc.&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type</td>
<td></td>
<td></td>
<td>catalyzed&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q168</td>
<td>A</td>
<td>26.4 ± 5.2</td>
<td>3.1 ± 0.7</td>
<td>F187</td>
<td>629 ± 107</td>
<td>5390 ± 802</td>
</tr>
<tr>
<td>F187</td>
<td>A</td>
<td>4.8 ± 0.8</td>
<td>0.6 ± 0.1</td>
<td>K188</td>
<td>5.7 ± 1.6</td>
<td>48.5 ± 13.1</td>
</tr>
<tr>
<td>K188</td>
<td>A</td>
<td>48.5 ± 13.1</td>
<td></td>
<td>C321</td>
<td>4.3 ± 0.8</td>
<td>36.5 ± 5.9</td>
</tr>
<tr>
<td>C321</td>
<td>A</td>
<td>36.5 ± 5.9</td>
<td>4.3 ± 0.8</td>
<td>A322</td>
<td>62.6 ± 13.7</td>
<td>536 ± 109</td>
</tr>
<tr>
<td>A322</td>
<td>S</td>
<td>74.1 ± 22.3</td>
<td>8.7 ± 2.7</td>
<td>A322</td>
<td>9.0 ± 0.9</td>
<td>1410 ± 474</td>
</tr>
<tr>
<td>T325</td>
<td>A</td>
<td>1410 ± 474</td>
<td>165 ± 57</td>
<td>Q326</td>
<td>8.6 ± 0.7</td>
<td>988 ± 78</td>
</tr>
<tr>
<td>Q326</td>
<td>A</td>
<td>6.6 ± 1.3</td>
<td>0.8 ± 0.2</td>
<td>N327</td>
<td>23 ± 7.2</td>
<td>536 ± 109</td>
</tr>
<tr>
<td>N327</td>
<td>A</td>
<td>198 ± 60</td>
<td>23 ± 7.2</td>
<td>V328</td>
<td>77 ± 23</td>
<td>9.0 ± 0.9</td>
</tr>
<tr>
<td>V328</td>
<td>A</td>
<td>661 ± 185</td>
<td>77 ± 23</td>
<td>K329</td>
<td>1.0 ± 0.1</td>
<td>9.0 ± 0.9</td>
</tr>
<tr>
<td>K329</td>
<td>A</td>
<td>9.0 ± 0.9</td>
<td>1.0 ± 0.1</td>
<td>F330</td>
<td>0.6 ± 0.1</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>F330</td>
<td>A</td>
<td>5.3 ± 1.2</td>
<td>0.6 ± 0.1</td>
<td>V331</td>
<td>1.1 ± 0.3</td>
<td>1300 ± 256</td>
</tr>
<tr>
<td>V331</td>
<td>A</td>
<td>9.6 ± 2.0</td>
<td>1.1 ± 0.3</td>
<td>F332</td>
<td>151 ± 32</td>
<td>1300 ± 256</td>
</tr>
<tr>
<td>F332</td>
<td>A</td>
<td>1300 ± 256</td>
<td>151 ± 32</td>
<td>D333</td>
<td>0.7 ± 0.1</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>D333</td>
<td>A</td>
<td>6.4 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>A334</td>
<td>5.1 ± 0.9</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>A334</td>
<td>P</td>
<td>4.3 ± 6.5</td>
<td>5.1 ± 0.9</td>
<td>V335</td>
<td>2.1 ± 0.3</td>
<td>17.7 ± 2.2</td>
</tr>
<tr>
<td>V335</td>
<td>A</td>
<td>17.7 ± 2.2</td>
<td>2.1 ± 0.3</td>
<td>T336</td>
<td>3.8 ± 0.8</td>
<td>7.4 ± 2.0</td>
</tr>
<tr>
<td>T336</td>
<td>A</td>
<td>7.4 ± 2.0</td>
<td>0.9 ± 0.2</td>
<td>D337</td>
<td>5.1 ± 1.1</td>
<td>17.3 ± 4.2</td>
</tr>
<tr>
<td>D337</td>
<td>A</td>
<td>17.3 ± 4.2</td>
<td>2.0 ± 0.5</td>
<td>I338</td>
<td>4.3 ± 1.6</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>I338</td>
<td>A</td>
<td>9.3 ± 1.1</td>
<td>1.1 ± 0.2</td>
<td>I339</td>
<td>1.2 ± 0.3</td>
<td>35.4 ± 23</td>
</tr>
<tr>
<td>I339</td>
<td>A</td>
<td>35.4 ± 23</td>
<td>4.1 ± 2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The apparent rate constants were derived from fits of each data set to the exponential rise equation, \( y = c + 100(1-exp(-kt)) \). Each mutant was assayed at least 3 times (WT Go<sub>t</sub> was assayed 26 times), and an independent fit was made to each data set. The values reported are the mean \( k_{app} \times 10^4 \text{ min}^{-1} \pm 2*SEM.\)

<sup>b</sup>The catalyzed assay of wild-type Go<sub>t</sub> was conducted in the presence of 30nM photoactivated rhodopsin, 30nM Gβγ<sub>t</sub> and 14μM GTPγS.

<sup>c</sup>The fold increase over uncatalyzed wild-type is calculated at the \( k_{app}(mutant)/k_{app}(wild-type) \).

<sup>d</sup>The amino acid used to replace the wild-type residue.
FIG. 6-1. Structure of the \( \alpha5 \) helix of Go. A. Overall view of the structure of Go bound to GDP. The nucleotide is purple, the \( \beta6/\alpha5 \) loop is orange, and the \( \alpha5 \) helix (which is part of the Ras-like domain) is yellow. The nucleotide lies in a cleft between the Ras-like and helical domains of Go, adjacent to the \( \beta6/\alpha5 \) loop at the N-terminal end of the \( \alpha5 \) helix. An NMR structure of a peptide corresponding to the extreme carboxyl terminus of Go, residues 340-350, in the rhodopsin-bound conformation is shown in green, docked onto the end of the \( \alpha5 \) helix (Kisselev, et al., 1998). (This region was not ordered in the crystal structures, and it is not shown in the other panel). B. The view in panel A has been rotated 90 degrees about the vertical axis. The \( \alpha5 \) helix is viewed end-on. The helix packs into a groove formed by the central curved beta sheet of the Ras-like domain and the \( \alpha1 \) helix.
FIG. 6-2. Nucleotide exchange time courses of proline mutants in α5. Gαt and mutants were translated in vitro and combined with 100 μM GTPγS (panel A) or 30 nM R*/10 nM Gβγt/14 μM GTPγS (panel B). Aliquots (8 μl) were removed at the indicated times and the percent activation was determined by analysis of trypsin digestion patterns, as described in Chapter 5. The time-zero data point is calculated from protein mixed with 100 μM GDP for 10 min. Each data point is the average of 3-5 independent experiments. Error bars depict ± 2*SEM. The solid lines represent fits to an exponential rise function. A. Uncatalyzed nucleotide exchange time courses. B. R*/Gβγt-catalyzed nucleotide exchange time courses.
Chapter 6: The α5 helix of Gα₄

A

<table>
<thead>
<tr>
<th></th>
<th>330</th>
<th>340</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gα₄</strong></td>
<td>TDTQNVKFVFDAVTDIIIKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CT del 5</strong></td>
<td>TDTQNVKFVFDAVTDIIIKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CT del 10</strong></td>
<td>TDTQNVKFVFDAVTDIIIKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CT del 25</strong></td>
<td>TDTQNVKFVFDAVTDIIIKENLKDGLF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>330</th>
<th>340</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gα₄</strong></td>
<td>TDTQNVKFVFDAVTDIIIKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CT ala4</strong></td>
<td>TDTQNVKFVFDAVTDIIIKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α5 ala3</strong></td>
<td>TDTQNVKFVFDAVTDIIKAAAIAKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α5 ala4</strong></td>
<td>TDTQNVKFVFDAVTDIIKAAAIAKENLKDGLF</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α5 ile4</strong></td>
<td>TDTQNVKFVFDAVTDIIKAAAAIAKENLKDGLF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

**FIG. 6-3. Truncation and insertion mutants of Gα₄.**

A. Sequence alignment of truncation mutants of the carboxyl terminus of Gα₄.

B. Sequence alignment of insertion mutants of Gα₄.

C. Trypsin proteolysis of wild-type (WT), CT ala4, and α5 ala4 following treatment with GDP, GDP/AIF₄⁺, or R⁺/βγ/ΓTPγS (20 min incubation). The ala4 insertion mutants were resistant to activation by rhodopsin and ΓTPγS.
FIG. 6-4. Uncatalyzed nucleotide exchange time courses of alanine mutants in α5. Experiments were performed as described in Fig. 6-2 A.
FIG. 6-5. Trypsin digest analysis of wild-type, A322S, and the rapidly activating alanine mutants (T325A, V328A, and F332A). The arrows indicate the position of the ~34 and ~23 kDa bands. All the mutants displayed patterns of trypsin digestion identical to wild-type in the presence of GDP, GTP\textsubscript{yS}, and GDP/AlF\textsubscript{4}–, which suggests that the tertiary structure of the proteins is not grossly altered.
FIG. 6-6. R*Gβγt-catalyzed nucleotide exchange time courses of alanine mutants in α5. Experiments were performed as described in Fig. 6-2 B.
FIG. 6-7. Nucleotide exchange time courses of mutants of residues near α5 and in the β6/α5 loop. A. Mutations were prepared in several residues that interact with the α5 helix or which are part of the β6/α5 loop. Assays were conducted as in Fig. 6-2. B. Mutation of residues F185 and F192 led to disruption of the normal ~23 kDa band produced by GDP-bound Goα. Representative gels visualized by phosphorimaging from experiments with F185A, F192A, and WT suggest that the rate of GTPγS binding, as indicated by the change in intensity of the ~34 kDa band, is much faster than WT.
FIG. 6-8. Schematic representation of the phenotypes of proline mutants in α5. Top, helical wheel representation of α5, as viewed in Fig. 6-1B; bottom, close-up of the structure of α5. Residues are color-coded by phenotype: mutants similar to WT are yellow, those exhibiting rates faster than WT are green, and those slower than WT are red. Residues colored white did not yield functional proteins when mutated to proline. A. Uncatalyzed rates. B. Rhodopsin/Gβγt-catalyzed rates.
FIG. 6-9. Schematic representation of the phenotypes of alanine mutants in α5: a model for R*-catalyzed Gt activation. A, Close-up view of the α5 region. Most of the residues mutated in this study are shown in ball-and-stick representation. Within the α5 helix, residues that led to increases in both basal and R*-catalyzed nucleotide exchange rates when substituted with alanine are colored green; residues that led to decreases in R*-catalyzed exchange rate when substituted with alanine are colored red; and residues that were indistinguishable from wild-type when substituted with alanine are yellow. Also shown are residues which interact with α5 (blue). Mutation of these residues lead to increases in the basal nucleotide exchange rate. B, Helical wheel representation of the α5 helix, as viewed in Fig. 6-1B. The coloring is the same as in panel A. The sites of mutations that caused increases in both basal and R*-catalyzed nucleotide exchange map predominantly to a buried surface of the helix. The sites of mutations that caused impairment of R*-catalyzed nucleotide exchange map to a solvent exposed hydrophobic patch (including V331, I338, A334 and F330) adjacent to the buried surface of α5. Hydrophilic residues that map to the "bottom" of the helix did not affect either basal or R*-catalyzed nucleotide exchange when replaced with alanine. Together, the data provide evidence that R* catalyzes nucleotide exchange by perturbing the conformation of residues on the buried surface of α5 (green line). These residues communicate with the nucleotide-binding pocket. The perturbation may result from direct binding of R* to the exposed hydrophobic surface of α5 (red line).
Chapter 6: The α5 helix of Gαt

Discussion

The α5 helix has been proposed to mediate the effects of R* on Gαt (Onrust et al., 1997). This role was suggested by its location as a link between the carboxyl-terminal tail of Gαt, which binds to R*, and the nucleotide-contacting β6/α5 loop. To test this hypothesis, we prepared a large set of Gαt mutants in the α5 helix and surrounding regions. The mutants were expressed \textit{in vitro}, and the kinetics of nucleotide exchange, both uncatalyzed and R*-catalyzed, were determined directly by quantitative analysis of trypsin digestions of Gαt.

Alterations of the Structure of the α5 Helix by Site-Directed Mutagenesis Disrupted R*-Catalyzed Activation

One set of mutations in Gαt was designed to introduce structural perturbations into α5. If α5 were indeed involved in coupling R* to the nucleotide binding pocket, then one would predict that structural alteration on α5 would lead to reduced R*-catalyzed activation rates. Additionally, one might predict that structural alterations in α5 would lead to increases in basal nucleotide exchange since the natural structure of α5 has evolved for low nucleotide exchange in the absence of R*.

Prolines frequently introduce kinks into α helices, which bend away from the side of the helix containing the proline (Woolfson and Williams, 1990). The kinks arise because the side chain of proline bonds covalently to the backbone nitrogen, and disrupts the typical pattern of hydrogen bonding in α helices between the backbone oxygen of position n and the backbone nitrogen of position n+4. Thus prolines in positions 325-328 would not be expected to induce kinks per se since the n-4 residue does not exist; however, several of these mutations do appear to alter the structure of Gαt by other means. Proline substitutions at a variety of positions on the α5 helix, predominantly on the buried surface, severely diminished nucleotide binding in general (Fig. 6-8).
Possibly, prolines in some of these positions caused bending of the helix away from the rest of Gαt so as to disrupt a large number of intramolecular contacts and to interfere with protein folding and/or stability.

Among the proline mutants that were capable of binding GDP, increases in uncatalyzed nucleotide exchange rates were generally observed (Fig. 6-2A). This is consistent with the involvement of the α5 helix in interacting with the nucleotide binding pocket. In many cases, these effects were likely due to structural perturbation of α5, as opposed to disruption of contacts involving the substituted amino acid. For example, placement of alanines at the same positions generally did not cause the same phenotype as the prolines (with the exception of T325). Furthermore, in contrast to the alanine mutants (see below), the sites on α5 where substitutions with proline increased basal nucleotide exchange rates were not restricted to a particular surface of the helix (Fig. 6-8A). In addition, the effect on basal activation rates was most pronounced for replacement with proline of residues in the middle of the helix (Fig. 6-8). Kinks in the middle of the helix would be predicted to be most disruptive of interactions between α5 and the rest of Gαt. Proline mutants at the carboxyl terminus of the helix had a milder phenotype, plausibly because kinks at this sites were less disruptive to Gαt structure.

The proline mutants, with the exception of T325P, were generally partially defective in their response to R*-catalyzed activation (Fig. 6-2B, 6-8B). These mutants were not likely to be defective in binding to Gβγt, since the site of the mutation was on the opposite side of Gαt from the known Gβγt binding surface. The substitution of α5 residues with prolines seem to induce conformations in the α5 helix that disrupt communication between R* and the nucleotide; this result is further evidence for the involvement of α5 in mediating R*-catalyzed activation.
As in the uncatalyzed assay, the effects of the prolines in the catalyzed assay seem to result from structural perturbations of α5 and not merely from disruption of contacts involving the substituted amino acid. For example, the proline mutants of a given position were generally more defective in R*-catalyzed activation than were the corresponding alanine mutants. In addition, the sites on α5 where proline substitution led to disruption of R*-catalyzed activation were not restricted to a particular surface of α5 (Fig. 6-8B). The defect in R*-catalyzed activation of the proline mutants increased as the proline was placed at positions progressively closer to the carboxyl terminus of the α5 helix. For example, the Q326P mutant was only mildly defective, whereas the I338P mutant was nearly unresponsive to activation by R* (Fig. 6-2B). This phenomenon might relate to the fact that the alteration in the relative alignment of α5 and the R* binding site on the 340-350 region would be expected to be larger as the site of the kink moved closer to the 340-350 region-- i.e., closer to the carboxyl terminus of α5.

Additional alterations of the α5 helix, in the form of insertion and deletion mutants, were tested (Fig. 6-3). In general, such manipulations interfered with the folding and/or nucleotide binding of the resulting mutant proteins. Previously, it was reported that deletion of just the carboxyl terminal amino acid (F350) from Goαt eliminated nucleotide binding (Osawa and Weiss, 1995). In contrast, a related G protein, GoαO, was reported to tolerate a variety of deletions (Denker et al., 1992). It is not clear why Goαt is more sensitive than GoαO to deletions, but it might relate to the general difficulties in heterologously expressing functional Goαt.

For the Goαt insertion constructs that did yield interpretable data, α5 ala4 and CT ala4, trypsin digests indicated that the proteins could bind GDP and become activated in the presence of GDP/AlF4-. However, R*/Gβγt could not induce GTPγS uptake in these mutants (Fig. 6-3). It is possible that these mutants could not bind GTPγS (uncatalyzed GTPγS uptake was not tested), but it seems more likely that the mutations caused a
specific defect in R* interactions. The CT Ala4 mutant would be expected to have disruptions in the R*-binding site at positions 340-350 of G\(\alpha_t\), and thus might not bind to R*. However, the 340-350 region in the mutant \(\alpha5\) Ala4 would not be expected to be altered by the mutation. The phenotype of the \(\alpha5\) Ala4 mutant suggests that R* might need to interact not only with the 340-350 region of G\(\alpha_t\), but also with additional sites amino-terminal to I440. The binding of these latter sites might be disrupted by the insertion of four alanines following I339. Binding between R* and residues in the 330-338 region are also suggested by alanine substitution mutants (see below).

**Mutations of the \(\beta6/\alpha5\) Loop Increased Basal Nucleotide Exchange Rates**

Several mutations of the nucleotide-contacting \(\beta6/\alpha5\) loop have been described in other G protein subtypes as accelerating GDP release. A322 is highly conserved in both monomeric and heterotrimer G proteins. The mutation of this alanine to serine was first described (Iiri et al., 1994) as a naturally occurring mutant of G\(\alpha_S\) (A366S) found in patients with a combination of both gain and loss of endocrine function, in the form of testotoxicosis (gain) and pseudohypoparathyroidism type Ia (loss). Characterization in vitro of this mutant, as well as analogous mutants in G\(\alpha_i\) (Posner et al., 1998) and G\(\tau\) (Garcia et al., 1995) showed in all cases that the mutation accelerated uncatalyzed nucleotide exchange rates. This mutant has therefore been described as an approximation of the receptor-bound state (Posner et al., 1998). In the case of G\(\alpha_S\), this mutant was also found to be temperature sensitive. Hence the paradoxical disease phenotype was caused by high levels of receptor-independent activation of G\(\alpha_S\) in the relatively cool environment of the testis, and loss of G\(\alpha_S\) signaling due thermolability in the higher-temperature environment of the body.

The C321A mutant was previously reported to decrease the affinity for GDP in an equivalent mutant of G\(\alpha_O\) (Thomas et al., 1993). Our results confirm the finding of rapid GDP release rates in A322S and C321A mutants of G\(\alpha_t\), as well as demonstrate an
increased GDP dissociation rate in A322G. Together, these mutants suggest that a variety of perturbations of conserved regions in the β6/α5 loop can induce increased GDP release in Gαt. Additionally, we demonstrate that receptor-catalyzed activation of Gαt is not impaired by mutation of the β6/α5 loop (Fig. 6-7). These results are consistent with the previously proposed role of the β6/α5 loop and of the α5 helix in R*-catalyzed nucleotide exchange.

**Identification of Nucleotide-Release Control Microdomain on the α5 Helix**

Replacement of several residues in the α5 helix to alanine resulted in a gain-of-function phenotype, namely a marked (up to 165-fold) increase in the rate of uncatalyzed nucleotide exchange. These rates are comparable to or in excess of the basal exchange rate of mutant A322S (63-fold increase) (Table 6-1). These results suggest that alteration of the α5 helix, which is distant from the nucleotide, can accelerate GDP release to even a greater degree than replacement of amino acids known to be in direct contact with the nucleotide. Together the residues T325, V328 and F332, which cluster on an inward facing quadrant of α5 (Fig. 6-9), constitute a potent nucleotide-release control microdomain.

Significantly, mutations in the α5 nucleotide-release control region do not destroy the basic nucleotide-binding and conformational properties of Gαt (Fig. 6-5). Preservation of the patterns of trypsin digestion indicates that the overall folding patterns of the mutants are similar to that of wild-type Gαt. Each of the mutants is capable of binding GDP and GTPγS, and of being activated by GDP/AlF4⁻. The mutants also are responsive to rapid activation by R* (Fig. 6-6). Together, these data suggest that the mutations do not merely disrupt folding of the protein, but that they induce conformations that anticipate, or at least are compatible with, the R*-bound conformation. This contrasts with several of the proline mutants, which displayed increased basal activation rates, but reduced R*-catalyzed rates.
Chapter 6: The α5 helix of Gαt

T325 is located in the first turn of the α5 helix immediately adjacent to the β6/α5 loop (Fig. 6-9A). An extensive network of hydrogen bonds maintains the arrangement of this first helical turn. Mutation of the sidechains in this region would be expected to disrupt some of these hydrogen bonds, and alter the structure of the first turn and possibly of the β6/α5 loop. The T325P mutant also displayed accelerated basal activation rates. Mutation of N327, which is also located in the first turn of α5, resulted in an increase in the non-catalyzed activation rate (Fig. 6-2).

Two other residues, F332 and V328, point in towards the center of the molecule and contribute to the hydrophobic core of the protein (Fig. 6-9A). F332 and V328 make extensive hydrophobic contacts with residues from the α1 helix, as well as the β2 and β3 sheets. Loops emanating from these structural elements contribute to the canonical nucleotide-binding surfaces of Gαt (Sprang, 1997). Replacement of F332 or V328 by alanine may alter the packing of the hydrophobic core of the protein, including the β2 and β3 sheets and the α1 helix, such that a conformational change is communicated to the nucleotide-binding pocket.

The F332A mutant was previously studied in microsomes prepared from transiently-transfected COS cells (Onrust et al., 1997). In this assay system, it was described as being impaired in a rhodopsin activation assay. However, in the same study this mutant was found to bind to rhodopsin normally in an assay based on in vitro translated material. The rate of noncatalyzed activation was not assessed and the origins of these apparent inconsistencies were not clear.

Amino acids V328 and F332 are extremely conserved, not only in heterotrimeric G protein α subunits, but also among monomeric G proteins of the Ras superfamily (Valencia et al., 1991). A mutant of the analog of F332 in Ras, F156L, was found to have transforming properties in vivo and to greatly accelerate the rate of nucleotide
Chapter 6: The α5 helix of Gαt

release in vitro (Quilliam et al., 1995). NMR studies of this mutant revealed that the structures of α1, α5 and β1-β3 were altered. The mutation did not appear to reduce the stability of the protein, apparently due to induction of new intramolecular contacts not present in the wild-type version (Quilliam et al., 1995).

Interactions Between the α5 Helix and the Rest of Gαt

Since the α5 helix contains elements that, when perturbed, dramatically increase the GDP release rate, one would expect that α5 must be held in a very specific conformation to avoid inappropriate release of GDP. Thus, there should exist specific contacts between the α5 helix and the rest of the protein necessary to maintain such a conformation. In fact, mutation of a variety of residues located at different positions of Gαt that interact with the α5 helix do moderately increase the uncatalyzed nucleotide exchange rate (Fig. 6-7, 6-9). In particular, alanine substitution at positions Q168, F185, K188 or F192 lead to increases in nucleotide-exchange rate. Q168, which is located on αF of the helical domain, hydrogen bonds with the main chain carbonyl of T323. K188, which extends from a loop between β2 and β3 appears to form ionic interactions with two aspartic acid residues on the α5 helix, D333 and D337. These interactions likely serve to maintain the proper register of the α5 helix with respect to the rest of the protein. F185 and F192, which are located on β2 and β3 respectively, are part of a phenylalanine cluster which lies adjacent to F332. These contacts may serve both to stabilize the local tertiary structure near the α5 helix, to sense alterations in the position of F332 and to communicate structural changes on to the nucleotide-binding pocket. This latter role is suggested by the structural changes observed in β2 and β3 in NMR studies of the F156L mutant in Ras (Quilliam et al., 1995).

It has been previously suggested that interactions between the carboxyl terminus of the α5 helix and the rest of the protein are important for mediating the basal rate of nucleotide exchange (Denker et al., 1992; Denker et al., 1995; Natochin et al., 2000).
We suggest that these contacts, like those discussed above, contribute to the stability of amino acid side chains at the amino terminus of α5. Weakening of these contacts, as in the I339A mutant, moderately accelerated GDP release (Table 6-1), in agreement with previous results. However, the magnitude of the increase in GDP release resulting from these mutations is very small (~4-fold) compared with mutations closer to the amino terminus of α5.

A Mechanistic Model of R*-Catalyzed Nucleotide Exchange

Previously, it has been suggested that rhodopsin might induce GDP dissociation by deforming the β6/α5 loop via α5 (Onrust et al., 1997). The data in this study are consistent with this hypothesis. Mutations predicted to alter the structure of α5 disrupted R*-catalyzed nucleotide exchange, and a variety of mutants in the β6/α5 loop were found to increase basal activation rates in Goq, as had been previously reported in related G proteins.

The identification of a potent nucleotide-release control region in the α5 helix of Goq allows refinement of this hypothesis. The replacement of T325, V328 and F332 with alanine mimics the effects of R* binding on Goq in several key respects. The rate of GDP dissociation is tremendously increased, the primary perturbation is at a distance from the nucleotide-binding site, and the overall structure and function of the protein is preserved. Thus the data in this chapter suggest that α5 may not merely serve as a conduit between R* and the β6/α5 loop, but that perturbation of the nucleotide-release control region (T325, V328 and F332) on α5 itself by R* would be sufficient to induce rapid nucleotide exchange "at-a-distance."

Evidence that R* does in fact use this mechanism comes from alanine mutations in a F330, V331, D337 and I338 (Fig 6-9). These mutations did not affect the rate of basal activation, but they all decreased R*-catalyzed activation kinetics ~3-4 fold (Fig. 6-4B).
Chapter 6: The α5 helix of Gαₐ

Since the mutations did not completely abolish activation by R*, the functions of these residues may be partially redundant. The overall structures of these mutant proteins were not significantly disturbed as indicated by trypsin proteolysis patterns and basal nucleotide exchange rates that were similar to wild-type Gαₐ (Table 6-I and not shown). The mutations were unlikely to affect interactions with Gβγ, which is known to bind to the opposite face of Gαₐ from α5 (Lambright et al., 1996). The most direct interpretation of these loss-of-function results is that F330, V331, D337 and I338 make important contacts in the R*-Gαₐ complex, and that these contacts are disrupted by the substitutions with alanine.

In the absence of R*, these residues do not appear to be involved in any contacts that are relevant to nucleotide exchange rates, as indicated by the crystal structure and the basal nucleotide exchange rates of the corresponding mutants (Fig. 6-4, Table 6-I). In particular, F330 is more than 4 Å from any other residue. Thus, the formation of important contacts involving F330, V331, D337 and I338 in the R*-bound conformation necessitates a R*-induced conformational change in α5. Consistently, a site-directed spin labeling study with Gαₐ demonstrated that R*-induced conformational changes occur in a similar set of residues at the amino terminus of α5 corresponding to Q326, N327 and F330 of Gαₐ (W. Hubbell, personal communication).

Since R* appears to induce conformational changes at V331 and F330 and other residues of α5, it is plausible that R* also alters the T325/V328/F332 region on the adjacent face of the α5 helix (Fig. 6-9). This is evidence that R* does in fact catalyze nucleotide exchange at-a-distance by perturbing the T325/V328/F332 region and by exploiting the built-in structural connection between these residues and the nucleotide-binding pocket of Gαₐ. Subsequent alteration of the β6/α5 loop as previously hypothesized could also occur (Onrust et al., 1997).
Chapter 6: The α5 helix of Go

The proposed conformational changes of T325, V328 and F332 induced by binding of R* need be only subtle to induce nucleotide exchange. Since mutation of each residue by itself led to a dramatic increase in nucleotide exchange rate, even a minor structural perturbation of more than one residue simultaneously by R* would be expected to produce a potent effect on the nucleotide exchange rate. As a precedent, the 2.6 Å-resolution crystal structure of the GoA326S mutant heterotrimer (analogous to A322S in Go) did not reveal any significant structural alterations although the nucleotide exchange rate was dramatically increased (Posner et al., 1998).

Some or all of the contacts involving F330, V331, D337 and I338 that are inferred to exist in the R*-bound conformation may result from direct binding of R* to α5. The amino acid residues F330, V331, D337 and I338 map to a conspicuous solvent-exposed, yet predominantly hydrophobic surface of the α5 helix (Fig. 3). This surface is contiguous with the carboxyl-terminal region (amino acids 340-350), a well-documented R*-binding domain. Furthermore, the placement of either prolines in α5 or a four alanine insertion following I339 diminished R*-catalyzed activation (see above). These results are consistent with a R* binding surface that extends to include parts of α5. Thus, the exposed surface of α5 may be part of the R*-binding region on Go, as has been previously suggested (Lichtarge et al., 1996; Onrust et al., 1997).

The data are also consistent with the possibility that R* indirectly induces the formation of intramolecular contacts involving F330, V331, D337 and I338 or a subset thereof. In this scenario, R* would induce changes in the amino terminus of α5 by binding to other sites on the protein, such as the carboxyl terminal region. As a speculative example, R* might induce a counterclockwise rotation of α5 such that the exposed hydrophobic surface of α5 would become buried, and contacts between F330, V331, D337 and I338 would form with the mostly hydrophobic residues in the core of Go. Such a rotation would of course also displace T325, V328, and F332 on the inside
Chapter 6: The α5 helix of Gα₅

surface of α5. Additional experiments are needed to conclusively determine whether the interactions between R* and α5 are direct or indirect.

Conclusions

The data in this report support a significant role for the α5 helix in the mechanism of R*-catalyzed Gα₅ activation. Mutations predicted to disrupt the structure of α5 reduced R*-catalyzed nucleotide exchange rates. Furthermore, the α5 helix itself was found to contain residues that when mutated tremendously increased the GDP dissociation rate. These mutations, in residues F332, V328, and T325, mimic the receptor bound conformation in that the nucleotide exchange rate is very high, the primary perturbation is at a distance from the nucleotide binding pocket, and the overall integrity of the protein is maintained. Thus, R* needs only to distort the local structure of the α5 helix to achieve catalysis of nucleotide exchange. R* is known to bind to the adjacent carboxyl terminus of Gα₅. R* may also bind directly to α5, as suggested by the results of substitution of exposed residues in α5 with alanines or prolines, or the insertion of alanines after I339. Together, these results strongly suggest that R* achieves GDP-nucleotide release "at-a-distance" by perturbing the α5 helix.
Chapter 7: Perspectives

The fourth loop of rhodopsin in context of the rhodopsin crystal structure

The experiments described in Chapters 3 and 4 were performed, analyzed, and published (Marin et al., 2000; Ernst et al., 2000) before the crystal structure of rhodopsin became available (Palczewski et al., 2000). The structure does not alter any of the central conclusions from those studies. However, the new structure allows for a re-evaluation of the results in Chapters 3 and 4.

Helix 8

One of the more surprising features of the crystal structure of rhodopsin was the presence of an additional helix, the non-transmembrane Helix 8 (H8) (Fig. 7-1). H8 was not accurately predicted by earlier structural studies of C4 based on site directed spin labeling (Altenbach et al., 1999) and NMR analysis of peptides (Yeagle et al., 1996). This helix involves residues 312-321 in the C4 region. It is separated from TM 7 by a short linker, consisting of M309 to K311. The helix runs approximately parallel to the hypothetical intracellular membrane surface, and roughly perpendicular to TM 7. H8 points out from the helix bundle and increases the size of the cytoplasmic surface of rhodopsin. The helix is amphipathic and cationic; hydrophobic residues F313, M317, and L321 point in toward the lipid bilayer, parallel to the TM helices. Additionally, there is a highly conserved arginine in H8, which extends outward such that it could interact with negatively charged phospholipids. C322 and C323 are positioned such that attached palmitoyl groups, which are not included in current crystal structure model, could insert into the bilayer as predicted (Moench et al., 1994). Sequence analysis of related GPCRs reveals that hydrophobic residues are frequently conserved in positions 313, 317, and 321, suggesting that the presence of an amphipathic, membrane associated H8 may be a conserved feature of related receptors.
Chapter 7: Perspectives

The structure of H8 is redundant: 3 separate hydrophobic residues plus the 2 palmitoyl groups contribute to the formation and orientation of the amphipathic helix. Therefore, neither point mutations, chimeras with other related receptors, nor removal of the palmitoyl groups is guaranteed to effectively disrupt the H8 structure. For example, none of our the chimeric rhodopsin constructs CTr1, CTr2, CTr3, or CTr4 are likely to disrupt H8, since in all cases the amphipathic pattern is preserved.

The role of the helix itself in Gt activation is unclear. Truncation of rhodopsin following position 315 was reported to activate Gt normally (Weiss et al., 1994). This mutation would destroy H8, but it did not affect signaling. Additionally, the studies conducted in Chapter 3, and well as those from another group (Cai et al., 1999), suggest that the region of C4 that is important in activation of Gt is the amino-terminal region, which involves the linker between TM7 and H8, and only the first two amino acids of H8. Thus the majority of H8, despite its conservation, might not function in Gt activation. Additional studies of combinations of mutants designed to foil the redundancy in the stability of H8 structure, e.g., F313A/M317A/L321A, would clarify this point. Alternatively, mutations of two highly conserved residues, F313 and R314, which contribute directly to the cationic amphipathic nature of the helix should be explored. Neither of these residues was examined in our chimeras (Chapter 3) since they are conserved.

A likely possibility is that H8 serves to down-regulate the activity of rhodopsin in the dark state, perhaps by constraining the conformation of residues in the amino terminus of C4. The crystal structure of rhodopsin represents a conformation that does not interact with Gt. A conformational change in H8 upon MII formation might be required for efficient Gt activation. Light dependent changes in the structure of H8 have been suggested (Resek et al., 1993; Yang et al., 1996). Also supporting this hypothesis is the observation the depalmitoylation of rhodopsin leads to moderate hyperactivity (Chapter
3; (Morrison et al., 1991)), as does truncation of C4 (Weiss et al., 1994). Additionally, H8 is near the conserved NPXXY motif on TM7. Hydrogen bond interactions between this motif and residues on TM6 are hypothesized to be important in maintaining low dark and opsin activity (Palczewski et al., 2000; Han et al., 1998).

**Structural predictions regarding the amino terminus of C4**

We suggested (Chapter 3) that the amino terminus of C4 (residues 310-312) might interact with other parts of rhodopsin to contribute the Gt binding surface. The structure indicates that both N310 and Q312 are close to residues on TM 6 (E249) and the C1 loop (L68). Additional experiments with point mutations at these locations would be informative; perhaps it is disruption of these interactions involving 310 and 312 that are responsible for the reduction in Gt activation observed in two mutants of the 310-312 region, CTr2 and CTr4.

Also, we had speculated that 311 might be in the midst of a helical extension of TM 7, and that the proline placed there in CTr2 and CTr4 might disrupt this helix and cause the observed phenotype. This hypothesis proved inconsistent with the finding that the K311P point mutant activated Gt normally (Chapter 3). The structure reveals that 311 lies not in a helical extension of TM 7, but rather in a turn region between TM 7 and H8.

In Chapter 4, we hypothesized that replacement of the amino-terminal third of C4 with β2-AR sequence, as in CTr4, might disrupt interactions between the amino and carboxyl ends of C4. We speculated that these interactions could be restored by substitution of the entire C4 loop with amino acid sequence of the β2 AR, as in CTr2. This possibility was raised to explain the fact that the γ(50-71)-far peptide bound to CTr2 but not CTr4. However, the structure does not reveal any obvious reasons to support that hypothesis. The structural basis of the difference between the two mutants in binding to γ(50-71)-far is not clear.
FIG. 7-1. Close-up of Helix 8 (H8) of rhodopsin. The amino terminus of TM7 and the C4 loop, which includes H8, are shown based on the crystal structure of rhodopsin (PDB code 1f88). The orientation is similar to Fig. 1-2. N302 of the conserved NPXXY motif is labeled as are certain residues in C4.
Rhodopsin—$G_t$ interactions: the big picture

A Path of Interaction Between the Chromophore and the Nucleotide Binding Pocket

The data in this thesis partially delineate a path between the chromophore binding pocket of rhodopsin and the nucleotide binding pocket of $G\alpha_t$. This pathway represents a specific example of how information is transferred across distances within and between proteins. In Chapter 3, experiments with peptides and site-directed mutagenesis supported a specific role for the C4 loop of rhodopsin in interactions with $G_t$. In Chapter 4, a kinetic spectroscopic assay was used to demonstrate that the binding of both $G_t$ and of a peptide derived from the extreme C terminus of $G\alpha_t$ were sensitive to mutation of the C4 loop. In Chapter 5, we found that mutations predicted to disrupt interdomain interactions did not alter either basal or $R^*$-catalyzed nucleotide exchange rates. In Chapter 6, we found that mutation of key residues on $\alpha_5$, which are located at a distance from the nucleotide, but adjacent to the carboxyl terminus and other probable rhodopsin binding sites, can dramatically increase the nucleotide exchange rate.

Together, the data support the following scenario. Information regarding the isomerization of the chromophore moves from the chromophore binding pocket to the cytoplasmic surface of rhodopsin, where conformational and electrostatic changes occur. The cytoplasmic surface of $R^*$ then interacts with $G_t$, and in particular with the carboxyl-terminus of $G\alpha_t$, in a manner modulated by the amino terminus of C4. Interactions may also occur with the exposed surface of $\alpha_5$. As a result, the conformation of key residues at the amino terminus of $\alpha_5$ is altered. The altered structure of these residues (T325, V328, and F332) leads to structural changes in adjacent regions, such as the $\beta 6/\alpha 5$ loop and the $\beta 2$ and $\beta 3$ sheets. Ultimately, the nucleotide binding pocket is perturbed, culminating in the rapid release of GDP. Thus, the chromophore binding pocket of rhodopsin is functionally connected to the nucleotide binding pocket of $G\alpha_t$. 
Additional structures of R* and Gt are likely to participate in establishing the connection between the chromophore and the nucleotide. For example, interactions between the cytoplasmic surface of R* and the carboxyl terminus of Goαt certainly involve structures besides C4, such as C2 and C3 (Acharya et al., 1997). Other structures of Goαt, such as the α4/β6 loop, are also likely to participate in the complex (see Chapter 1). The interdomain cleft may open to facilitate the egress of GDP, although the opening per se does not appear to be an energetic barrier to nucleotide release as indicated by mutational analysis (Chapter 5).

**The Possible Role of Gβγt**

A second path connecting the chromophore to the nucleotide may involve Gβγt. Several authors have suggested that Gβγt does not merely facilitate R*-Goαt binding, but rather is mechanistically involved in nucleotide exchange (Bohm et al., 1997; Iiri et al., 1998). Several key observations support this idea. First, Gβγt is known to be required for efficient rhodopsin-catalyzed activation of Goαt (Fung, 1983). Second, the site at which the guanine nucleotide exchange factors Ef-Ts and Sos bind their cognate monomeric G proteins, Ef-Tu and Ras, overlaps with the equivalent region on Goαt where Gβγt binds (Iiri et al., 1998). In contrast, rhodopsin binds structures on the other side of the molecule. Third, several mutants have been identified in Gβt which do not disturb Goαt-Gβγt interactions, or rhodopsin-Gt interactions, but which do interfere with rhodopsin catalyzed nucleotide exchange (Ford et al., 1998). Possibly these mutants disrupt contacts necessary for the transmission of information from rhodopsin to the nucleotide binding pocket.

One attractive feature of the Gβγt dependent pathway is that the Switch I and Switch II regions, which mediate interactions with the Gβt subunit, are flexible and adopt different conformations in the free GDP-bound Goαt, the heterotrimeric GDP-bound, and the GTPγS-bound conformations of Goαt (Bohm et al., 1997). Therefore, in the empty
pocket conformation, it seems likely that a new conformation of the Switch I/II region could be induced. In contrast, the α5 region appears very rigid. However, only very small perturbations of the α5 helix (which might not be revealed in a crystal structure) could underlie the conformational shift necessary to induce GDP release.

Additional Questions

More detailed questions remain. For example it is not clear whether R* induces the proposed conformational changes in α5 of Gαt via direct or indirect mechanisms. A complete answer to these questions will eventually require high resolution structural data for the complex. As discussed, the crystal structure of the complex between R* and Gt is not known. A variety of studies, using mutational (Acharya et al., 1997), genetic (Liu et al., 1995) and crosslinking (Cai et al., 2001; Itoh et al., 2001) approaches, have sought to define specific point-to-point contacts between the R* and Gt. For example, the results in this thesis suggest a specific interaction between C4 and the carboxyl termini of Gαt and Gγt. These types of data serve as a starting point for the construction of models of the complex (Bourne, 1997). One vexing question that arises from the construction of these models, besides the aforementioned fact that rhodopsin needs to act "at a distance", is the apparent size mismatch between the cytoplasmic surface of rhodopsin and the R*-interacting surface of Gt (Hamm, 2001; Kisselev et al., 1999). The cytoplasmic surface of rhodopsin appears to be at best just barely large enough to simultaneously contact all the regions of Gt that are thought to interact with R*. Thus several possibilities must be considered. First, there may be large conformational changes in rhodopsin or Gt (Kisselev et al., 1995b; Kisselev et al., 1999) or both upon complex formation. Second, it may be possible that some interactions between R* and Gt occur sequentially, and not simultaneously (Kisselev et al., 1999). Finally, it may be the case that the stoichiometry of the reaction is not 1:1, but rather 2 R*: 1 Gt. A variety of recent experiments in related GPCRs (but not rhodopsin) have provided evidence that GPCRs can exist as dimers in
vivo (Bouvier, 2001). However, the functional importance of dimerization in G protein activation is unknown.
Reference List


References


References

313:310-313.


References


References


References


References


Skiba, N. P., Bae, H., and Hamm, H. E. (1996) Mapping of effector binding sites of
transducin α-subunit using G α i/G α i1 chimeras. J. Biol. Chem. 271:413-424.


References


