Ligand-Induced Conformations of Extracellular Loop 2 of AT1R

Hamiyet Unal  
*Cleveland State University*

Follow this and additional works at: [https://engagedscholarship.csuohio.edu/etdarchive](https://engagedscholarship.csuohio.edu/etdarchive)

Part of the *Biology Commons*

**How does access to this work benefit you? Let us know!**

**Recommended Citation**  
[https://engagedscholarship.csuohio.edu/etdarchive/294](https://engagedscholarship.csuohio.edu/etdarchive/294)

This Dissertation is brought to you for free and open access by EngagedScholarship@CSU. It has been accepted for inclusion in ETD Archive by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.
LIGAND-INDUCED CONFORMATIONS OF
EXTRACELLULAR LOOP 2 OF AT1R

HAMİYET UNAL

Bachelor of Science in Biology
Middle East Technical University, Turkey
June, 2003

Submitted in partial fulfillment of requirement for the degree
DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY WITH MOLECULAR
AND CELLULAR MEDICINE SPECIALIZATION
at the
CLEVELAND STATE UNIVERSITY
JUNE, 2010
This dissertation has been approved
for the Department of Biological, Geological, and Environmental Sciences
and the College of Graduate Studies by

________________________ Date: ________
Sadashiva S. Karnik, PhD, CCF-LRI
Major Advisor

________________________ Date: ________
Anton Komar, PhD, CSU-BGES
Advisory Committee Member

________________________ Date: ________
Crystal M. Weyman, PhD, CSU-BGES
Advisory Committee Member

________________________ Date: ________
Jun Qin, PhD, CCF-LRI
Advisory Committee Member

________________________ Date: ________
Barsanjit Mazumder, PhD, CSU-BGES
Internal Examiner

________________________ Date: ________
Saurav Misra, PhD, CCF-LRI
External Examiner
Dedicated to my Parents
ACKNOWLEDGEMENTS

This dissertation has been possible with the support of many people. I would like to express my sincerest gratitude to my advisor, Dr. Sadashiva Karnik for his invaluable support and guidance. He continually conveyed his deep knowledge, encouragement, enthusiasm and motivation that helped my growth as a scientist. I am grateful for his understanding, patience, and advices at all stages of my intellectual maturation. I feel honored to be supervised by such a distinguished scientist early in my career.

I would like to thank my committee members, Dr. Crystal Weyman, Dr. Anton Komar and Dr. Jun Qin for their time, encouraging support and constructive input during the progress of my research. I am thankful to Dr. Sathyamangala Naga Prasad for his critical suggestions and support during my training.

I gratefully acknowledge people who contributed my work, Dr. Robert Speth for providing me with $[^{125}I]$-[Sar1,Ile8] Ang II, Dr. Shin Miura and Dr. John Boros for constructing the CYS$^-$AT1R, Dr. Judith Drazba for assistance in confocal microscopy, Dr. Samuel Mazzei for assistance in binding assays, Dr. Manju Bhat for assistance in calcium measurement assays and Dr. Rajaganapathi Jagannathan for assistance in molecular dynamics simulation study.

I would like to give my special thanks to members of our lab, Russell Desnoyer, Dr. Anushree Bhatnagar, Dr. Hong Yue, Dr. Rajaganapathi Jagannathan and Jacqueline
Kemp for their enormous support, friendship and contribution to my training by helping me improve both experimental skills and knowledge by critical scientific discussions.

Financial support was provided by assistantship from Department of Biological, Geological and Environmental Sciences, Cleveland State University and NIH RO1 grant to Dr. Karnik.

I would like to thank my true friends Gulay and Aisulu for always being there to support, cheer and encourage me and making me feel better in difficult times.

I would like to express my love and gratitude to my beloved family for their constant support, prayers and endless love throughout my life. My parents deserve the greatest appreciation. I would like to thank them for raising me with the consciousness and responsibility to help the human being in all possible ways, with the strength to overcome difficulties and with the faith and confidence in myself. I would like to thank my sister, Mine, for being the role model for me to follow, the best friend and the best counselor relieving my worries.

I can not find words that express my great appreciation to my husband, Serdar, whose dedication, love and confidence in my success inspired me with the passion for my dreams. He always walked with me through the most difficult times and helped me with my struggles. Finally, I would like to thank my two-year old son, Sedat, for making me so happy with a huge smile and teaching me the meaning of life.
LIGAND-INDUCED CONFORMATIONS OF
EXTRACELLULAR LOOP 2 OF AT1R

HAMİYET UNAL

ABSTRACT

Angiotensin II type 1 receptor (AT1R) is a G-protein coupled receptor (GPCR) and an important regulator of blood pressure. It is a target for drug development, because abnormalities in its function are linked to hypertension, cardiac hypertrophy and heart failure. AT1R is composed of seven transmembrane helices connected by three extracellular loops and three intracellular loops. The extracellular loop 2 (ECL2) of AT1R directly interacts with the ligands. This loop is targeted by autoantibodies that activate AT1R in several pathologies such as preeclampsia, malignant hypertension and vascular allograft rejection. Therefore, we proposed that the conformation of ECL2 in AT1R is differentially regulated upon binding to agonists and antagonists.

We determined the conformation of the ECL2 of AT1R by reporter-cysteine accessibility mapping in different receptor states (i.e., empty, agonist-bound and antagonist-bound). We introduced cysteines at each position of ECL2 of a receptor surrogate lacking all non-essential cysteines and measured reaction of these with a cysteine-reactive biotin probe. The ability of biotinylated mutant receptors to react with a
streptavidin-HRP-conjugated antibody was used as the basis for examining differences in accessibility.

Two segments of ECL2 were accessible in the empty receptor, indicating an open conformation of ECL2. These segments were inaccessible in the ligand-bound states of the receptor. Using the accessibility constraint, we performed molecular dynamics simulation to predict ECL2 conformation in different states of the receptor. Analysis suggested that a ‘lid’ conformation of ECL2 was induced upon binding to both agonists and antagonists, but exposing different accessible segments delimited by a highly conserved disulfide bond between ECL2 and TMIII. We propose that the ligand-induced ECL2-lid is coupled to movements of transmembrane helices through the conserved disulfide bond to achieve the transition to the active state of the receptor.

Our study reveals the ability of ECL2 to interact with diffusible ligands and adopt a ligand-specific ‘lid’ conformation. Distinct conformations induced by the agonist and the antagonist around the conserved disulfide bond suggest an important role for the disulfide bond in producing different functional states of the receptor. Dynamics of ECL2 may regulate the fundamental process of receptor activation and pathophysiological processes involving GPCRs.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................... vi
NOMENCLATURE ................................................................................................. xi
LIST OF TABLES ................................................................................................. xiii
LIST OF FIGURES .............................................................................................. xiv

CHAPTER I: INTRODUCTION

1.1 G-protein Coupled Receptors ................................................................. 1
   1.1.1 General Features of GPCRs .............................................................. 1
   1.1.2 Classification and Evolution of GPCRs ......................................... 5
   1.1.3 Structural Features of GPCRs ......................................................... 9
   1.1.4 Activation of GPCRs ............................................................ ........12
   1.1.5 GPCRs and Disease ................................................................. 14
   1.1.6 Drug Discovery ............................................................ .......................... 17

1.2 Angiotensin II Type 1 Receptors ............................................................ 17
   1.2.1 Renin Angiotensin System ............................................................ 17
   1.2.2 Significance of AT1R ................................................................. 21
   1.2.3 Ang II binding and Activation of AT1R ....................................... 23

1.3 The Extracellular Loop 2 in Other GPCRs ........................................... 32

1.4 Significance of Current Work ................................................................. 36

CHAPTER II: MATERIALS AND METHODS

2.1 Construction of Plasmids ................................................................. 40

2.2 Cell Culture and Transfection ............................................................. 49

2.3 Western Blotting ................................................................. .......................... 52
CHAPTER III. HA-CYS−AT1R
3.1 Introduction.................................................................63
3.2 HA-CYS−AT1R Nucleotide Sequence Confirmation.................66
3.3 Characterization of HA-CYS−AT1R........................................69
3.4 Reaction of HA-AT1R and HA-CYS−AT1R with MTSEA-biotin.....74

CHAPTER IV. CYSTEINE SCANNING MUTAGENESIS
4.1 Introduction...........................................................................80
4.2 Expression Analysis of ECL2 Single Cysteine Mutants...............81
4.3 Effect of Cysteine Substitutions in ECL2.................................86

CHAPTER V. ACCESSIBILITY MAPPING ANALYSIS OF ECL2
5.1 Introduction...........................................................................89
5.2 Experimental Set Up for Accessibility Mapping.......................93
5.3 Accessibility of ECL2 in the Absence of Ligand.......................99
5.4 Accessibility of ECL2 Upon Ang II Binding............................102
5.5 Accessibility of ECL2 Upon Losartan Binding.........................102
5.6 Rate of Change in MTSEA-biotin Accessibility .......................... 103

CHAPTER VI. INSIGHTS FROM MOLECULAR DYNAMICS (MD) SIMULATIONS

6.1 Introduction ............................................................................. 106
6.2 Overall Approach ................................................................. 107
6.3 MD Simulation of ECL2 in the Absence of Ligand ................. 107
6.4 MD Simulation of ECL2 in the Presence of Ang II ............... 112
6.5 MD Simulation of ECL2 in the Presence of Losartan ............ 112
6.6 Analysis of Hydrogen Bonding Networks ............................. 112

CHAPTER VII. DISCUSSION

7.1 The Implications of Ligand Induced Conformational Change of ECL2 ................................................................. 126
7.2 The Potential Role of Conserved Disulfide Bond ............... 131
7.3 The Structural Basis for Binding Autoantibodies ............... 132
7.4 A Predicted Ang II-analogous Pharmacophore in ECL2 ....... 134
7.5 The ECL2 of AT1R in Context of GPCRs ............................ 136
7.6 Final Remarks ................................................................. 140

CHAPTER VIII. FUTURE DIRECTIONS

8.1 Introduction ............................................................................. 143
8.2 Experimental Design ............................................................. 147

BIBLIOGRAPHY ............................................................................ 158
# NOMENCLATURE

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ANG I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>ANG II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin Receptor Blockers</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin II Type 1 receptor</td>
</tr>
<tr>
<td>AT2R</td>
<td>Angiotensin II Type 2 receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>ECL</td>
<td>Extracellular Loop</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated Kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma Aminobutyric Acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein Coupled Receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol Trisphosphate</td>
</tr>
</tbody>
</table>
JAK, Janus Kinase

MAPK, Mitogen Activated Protein Kinase

MD, Molecular Dynamics

MPER, Mammalian Protein Extraction Reagent

MTS, Methanethiosulfonate

MTSEA, Methanethiosulfonate Ethylammonium

MTSEA-BIOTIN, N-biotinylaminoethylmethanethiosulfonate

MTSES, Methanethiosulfonate Ethylsulfonate

MTSET, Methanethiosulfonate Ethyltrimethylammonium

NMR, Nuclear Magnetic Resonance

PBS, Phosphate Buffered Saline

PCR, Polymerase Chain Reaction

PDB, Protein Data Bank

PLC, Phospholipase C

RAS, Renin Angiotensin System

RCAM, Reporter Cysteine Accessibility Mapping

SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEM, Standard Error of Mean

SNP, Single Nucleotide Polymorphism

SV40, Simian virus 40

TM, Transmembrane Domain
LIST OF TABLES

Table I.I. Examples of disease causing mutations in GPCRs…………………………….16
Table I.II. Differential effects of AT1R and AT2R stimulation by Ang II……………….20
Table II.I. The list of primers used for single cysteine substitutions…………………..48
Table III.I. Cysteine substitutions in HA-CYS−AT1R……………………………………67
Table IV.I. Cysteine scanning mutagenesis of ECL2………………………………………83
Table IV.II. Characterization of ECL2 single cysteine mutants………………………..87
Table V.I. Comparison of MTSEA-biotin reactivity of fully-glycosylated monomeric receptor and multimeric forms of the receptor………………………………………………..95
Table VI.I. Predicted interactions of inaccessible ECL2 residues with transmembrane residues in the absence of ligand…………………………………………………………118
Table VI.II.A. Predicted interactions of inaccessible ECL2 residues with transmembrane residues in the presence of Ang II………………………………………………………121
Table VI.II.B. Predicted interactions of Ang II with TM residues and ECL2……………121
Table VI.III.A. Predicted interactions of inaccessible ECL2 residues with transmembrane residues in the presence of losartan……………………………………………………………125
Table VI.III.B. Predicted interactions of losartan with transmembrane residues and ECL2……………………………………………………………………………………………………….125
Table VIII.I. The list of primers used to generate N111G, R23A and D281A substitutions………………………………………………………………………………………………….154
Table VIII.II. List of double mutants…………………………………………………………155
Table VIII.III. Binding analysis of ECL2 mutants with N111G mutation……………….157
LIST OF FIGURES

Figure 1.1. General configuration of GPCRs..............................................................3
Figure 1.2. Evolutionary tree of GPCRs in different species.................................6
Figure 1.3. The Renin-Angiotensin System (RAS)..................................................19
Figure 1.4. The major AT1R signal transduction pathway....................................22
Figure 1.5. Specific interactions of AT1R with Ang II and losartan.......................24
Figure 1.6. Ang II binding to AT1R.....................................................................25
Figure 1.7. Chemical structures of the angiotensin and AT1R non-peptide antagonists...28
Figure 1.8 Overlapping binding pockets of Ang II and losartan..........................30
Figure 1.9. Activation of AT1R..........................................................................31
Figure 1.10. The ECL2 structures in crystal structures of GPCRs..........................34
Figure 1.11. Schematic representation of ligand interference and protection assay....37
Figure 2.1. Cloning of HA-AT1R gene in pMT3 expression vector.......................41
Figure 2.2. Nucleotide and amino acid sequence of HA-AT1R.............................43
Figure 2.3. Schematic representation of construction of CYS¯AT1R......................45
Figure 2.4. Construction of ECL2 single cysteine mutants.....................................46
Figure 2.5. The agarose gel fractionation of 20 single cysteine mutants...............50
Figure 2.6. Transfection efficiency in COS1 cells..................................................51
Figure 2.7. Intracellular Ca2+ measurement by Fura2...........................................56
Figure 2.8. Assay standardization......................................................................58
Figure 2.9. Experimental strategy for MTSEA-biotin accessibility measurement.....59
Figure 2.10. Schematic explanation of MD simulation procedure.........................62
Figure 3.1. The secondary structure model of HA-CYS\textsuperscript{−}AT1R
Figure 3.2. Comparison of Chromatograms of HA-AT1R and HA\textsuperscript{−}CYS-AT1R
Figure 3.3. Expression analysis of HA-AT1R and HA-CYS\textsuperscript{−}AT1R
Figure 3.4. Binding analysis of HA-AT1R and HA-CYS\textsuperscript{−}AT1R
Figure 3.5. Activity analysis of HA-AT1R and HA-CYS\textsuperscript{−}AT1R
Figure 3.6. MTSEA-biotin accessibility of HA-AT1R and HA-CYS\textsuperscript{−}AT1R
Figure 3.7. Inhibition of ligand binding following reaction of MTSEA with AT1R and CYS\textsuperscript{−}AT1R
Figure 4.1. Expression of ECL2 single cysteine mutants
Figure 4.2. The expression analysis of Ile\textsuperscript{177} and Val\textsuperscript{179} mutants with cysteine, alanine and serine substitutions
Figure 5.1. MTSEA-biotin labeling of accessible cysteine reporters
Figure 5.2. Optimization of MTSEA-biotin reaction conditions
Figure 5.3. MTSEA-biotin accessibility of representative mutants
Figure 5.4. MTSEA-biotin accessibility maps of ECL2 single cysteine mutants
Figure 5.5. Rate of change in MTSEA-biotin accessibility for representative mutants
Figure 6.1. MD simulation of the ECL2 in the empty state of the receptor
Figure 6.2. The frame # 1 chosen for further analysis
Figure 6.3. The frame # 48 chosen for further analysis
Figure 6.4. The frame # 39 chosen for further analysis
Figure 6.5. Hydrogen bonding network of ECL2 in the absence of ligand
Figure 6.6 Hydrogen bonding network of ECL2 in the presence of Ang II
Figure 6.7 Hydrogen bonding network of ECL2 in the presence of losartan
Figure 7.1. Molecular dynamics simulation of ECL2........................................127
Figure 7.2. Predicted hydrogen bonding network formed by inaccessible residues of ECL2........................................................................................................130
Figure 7.3. Ang II analogous ECL2 Pharmacophore.............................................135
Figure 7.4. Alignment of ECL2 of AT1R with the ECL2 sequences of GPCRs for which crystal structures are available.............................................................................138
Figure 8.1. ECL2 interactions with NT, ECL3 and TM helices...............................148
Figure 8.2. The positions of substitutions to be analyzed........................................149
Figure 8.3. Characteristics of N111G-AT1R..............................................................150
Figure 8.4. Expression of ECL2 single cysteine mutants with N111G mutation.......156
CHAPTER I
INTRODUCTION

1.1 G-Protein Coupled Receptors (GPCRs)

1.1.1 General Features of GPCRs

G-protein-coupled receptors (GPCRs) are seven-transmembrane helix proteins that are coupled to guanine nucleotide-binding proteins (G-proteins) upon activation by a specific ligand. GPCRs are the largest family of cell surface receptors, having a major role in signal transduction. They are ubiquitously expressed in eukaryotes, from yeast to human, and represent 2% of the genes in the human genome with approximately 1000 members (Takeda et al, 2003). GPCRs convey extracellular signal across the cell membrane as a response to a diverse array of ligands including hormones, ions, neurotransmitters, proteins/peptides, fatty acids, small molecules and physical stimuli such as light, smell, taste and mechanical stretch (Lagerstrom and Schioth, 2008, Rosenbaum et al, 2009). GPCRs constitute a significant field of research due to their relevance to membrane biophysics, cell signaling, pharmacology, physiology, pathophysiology and drug discovery. 50% of all current human therapeutics is
targeted to GPCRs, which only constitutes a small fraction of known GPCRs. Therefore, there is an extensive search for new drugs that target GPCRs with high efficacy and specificity. Due to the relevance of GPCRs to drug discovery, understanding the roles of GPCR signaling in physiology and pathophysiology and delineating the structure-function relationships of GPCRs have been a major focus of studies in receptor biology.

GPCRs are transmembrane proteins with a common architecture (Fig 1.1 A) characterized by seven-transmembrane (TM) helices, connected by extracellular loops (ECL) and intracellular loops (ICL) (Lagerstrom and Schioth, 2008, Rosenbaum et al, 2009). The N-terminal tail faces the extracellular region while, the C-terminal region is intracellular. When the GPCRs fold to a three-dimensional structure within the membrane, the TM helices forms an aqueous channel open to the extracellular space. This channel is filled with water molecules forming a ligand binding pocket, 20-30 Å deep in the TM domain (Fig. 1.1 B&C). Ligands bind to the receptor on the extracellular side. The rigid body movements of TM helices, with a length of 25-30 residues, transmits the signal to the intracellular loops which bind and activate the heterotrimeric G-protein. The intracellular loops, particularly the second and third loops, are the critical sites for G-protein interaction. (Bockaert and Pin, 1999) The G-protein consists of an α-subunit, responsible for GTP/GDP binding and GTP hydrolysis, and a complex formed by β- and γ-subunits. The intracellular loops determine the specificity of a GPCR for a particular subtype of α-subunit of G-proteins such as Gaα, Gaι and Gaq. The intracellular loops of different GPCRs specifically interact with certain G-protein subtypes but, they also have a low affinity for other subtypes. Upon activation, the GDP of the G-protein is exchanged
Figure 1.1. *General configuration of GPCRs.* A) Depiction of the secondary structure of a GPCR. B) Depiction of the three dimensional structure of a GPCR. C) Depiction of the ligand binding pocket of a typical family A GPCR. (from Ji et al, 1998)
with GTP which results in dissociation of the $\alpha$ and $\beta\gamma$ subunits of the heterotrimeric protein. Dissociated $G\alpha$ and $G\beta\gamma$ subunits further activates intracellular downstream effectors such as adenylyl cyclases and phospholipases which in turn affect the levels of second messengers such as cAMP and calcium. In addition to the classical second-messenger mediated signaling pathways activated through G-protein coupling, recent studies have discovered a number of alternative signaling mechanisms that are related to diverse biological functions of these receptors (Pierce et al, 2002). GPCRs can also signal in a G-protein-independent manner, leading to activation of mitogen-activated protein kinase (MAPK) pathways (Miura et al, 2004). The cytoplasmic tail of GPCRs is target for agonist-induced posttranslational modifications such as phosphorylation and palmitoylation. The C-terminal tail of activated GPCRs is phosphorylated by G-protein-coupled receptor kinases (GRKs). The phosphorylated C-terminal tail of a GPCR recruits intracellular proteins such as $\beta$-arrestin and leads to internalization followed by recycling or lysozomal degradation (Tan et al, 2004). Palmitoylation of some GPCRs at the cysteine residues, found in the C-terminal tail, creates a fourth loop which may regulate the phosphorylation and internalization (Resh, 2006).

The assembly of GPCRs into dimers and oligomers adds an additional level of complexity to the mechanisms underlying the functioning of GPCRs. The ability of GPCRs to interact with various intracellular proteins that activate various signaling pathways provide a mechanism for physiological regulation of biological activity. Mechanisms for GPCR regulation occur during their biosynthesis by gene transcription, translation, and posttranslational processing or, during trafficking to the cell membrane.
Once the receptor is on the cell membrane, the regulatory mechanisms involve the process of GPCR desensitization and internalization, receptor recycling and lysosomal degradation.

1.1.2 Classification and Evolution of GPCRs

The gene repertoire of GPCRs extends from yeast to complex eukaryotic organisms including the nematodes, insects, plants, and mammals, indicating their evolutionary success (Fredriksson et al, 2003). GPCRs are not found in prokaryotes. Bacteriorhodopsin is a light-sensing seven transmembrane protein found in bacteria, which might have a common origin with GPCRs in eukaryotes. It is a proton pump involved in producing energy. However it is not a GPCR, does not couple to G-proteins, and does not have significant sequence homology to GPCRs (Okada and Palczewski, 2001).

Phylogenetic analysis classifies GPCRs into five families based on their sequence and structural similarities (Fredriksson et al, 2005). These families are Rhodopsin, Secretin, Adhesion, Frizzled/Taste2, and Glutamate families, known as the GRAFS classification system (Fig. 1.2). Surprisingly, there is minor sequence homology between different GPCRs families. In addition to lack of amino acid sequence conservation, there is variation in the length and function of their N-terminal and C-terminal domains and extracellular and intracellular loops. These types of variations give specific structural properties to different GPCRs. However, the sequence comparison within a particular receptor family shows some degree of sequence similarity. Sequence analysis within a
Figure 1.2. Evolutionary tree of GPCRs in different species. Adapted from Fredriksson et al, 2005.
particular family reveals conservation of amino acids with similar residue type, rather than the sequence identity. Compared to N-terminal (7–595 amino acids) and C-terminal regions (12–359 amino acids), the size and sequence of TM domains (20–27 amino acids) are highly conserved, as well as the size of the loops (5–230 amino acids).

Among GPCR families, the rhodopsin family is the largest and the most diverse, representing approximately 60% of the GPCR repertoire. The rhodopsin family is further divided into subgroups, based on ligand similarity, including opsins (rhodopsin etc.) amine receptors (muscarinic receptors, adrenoreceptors, dopamine receptor, serotonin receptor), peptide receptors (angiotensin receptor, bradykinin receptor, chemokine receptor, opioid receptor, etc.), glycoprotein binding receptors (follicle stimulating hormone receptor, thyrotropin receptor, etc.), olfactory receptors, and nucleotide like receptors (Fredriksson et al., 2003). The characteristic feature of the rhodopsin family is the presence of conserved amino acid sequences, such as the NPxxY motif at the end of TMVII, the DRY motif at the end of TMIII, and the conserved N residue in TMI. The presence of such motifs suggests common structural features and activation mechanisms. Most of the ligands for rhodopsin family GPCRs bind within a cavity formed by the TM helices (Baldwin, 1994). However, the ligand-binding domain in the glycoprotein binding receptor family is in the N-terminus.

The receptors in other GPCR families, particularly in the adhesion family, have long N-terminal regions. The long N-terminus (200-280 amino acids) of adhesion family receptors are suggested to interact with other membrane bound proteins which provides
cell-to-cell adhesion in a ligand independent manner. They have conserved sequence motifs within the N-terminus, such as epidermal growth factor (EGF)-like repeats, mucin-like regions, and conserved cysteine-rich motifs (Fredriksson et al, 2005).

The secretin family of GPCRs also contains the ligand binding domains in their N-terminal region. These binding domains interact with large peptides with paracrine actions that share sequence similarity. The N-terminus (60-80 amino acids) contains conserved cysteine bridges that are likely to participate in ligand binding. Some examples of secretin family GPCRs are the calcitonin receptor, glucagon receptor and parathyroid hormone receptor (Fredriksson et al, 2005).

The glutamate family GPCRs also has a long N-terminal region (280 to 580 amino acids) with the ligand binding domain that interacts with small molecules such as glutamate, gamma-aminobutyric acid (GABA), calcium ions, and taste molecules. The N-terminus forms a “Venus fly trap” structure with a cavity in which glutamate binds (Fredriksson et al, 2005).

The frizzled family of GPCRs signals through glycoproteins called Wnt and mediates cell fate, proliferation, and polarity during development. The N-terminus (200 amino acids) of the frizzled family of receptors contains conserved cysteines and participates in Wnt binding (Fredriksson et al, 2005).
1.1.3 Structural Features of GPCRs

The structural knowledge of GPCRs has been obtained by the three dimensional crystal structure of a prototypical GPCR, bovine rhodopsin covalently bound to the inverse agonist 11-cis retinal (Palczewski et al, 2000), sequence alignment and molecular models based on the rhodopsin structure, and site-directed mutagenesis studies. Complementary biophysical approaches have been performed to understand the dynamic features of GPCRs that enable transition of signals triggered by ligand binding to intracellular proteins. (Hamm, 2001) These approaches include site-directed spin labeling (Yang et al, 1996, Farrens et al, 1996, Altenbach et al, 1996) and electron paramagnetic resonance (EPR), fluorescence spectroscopy (Loewen et al, 2001), substituted cysteine accessibility mapping, and nuclear magnetic resonance (NMR) spectroscopy (Ahuja et al, 2009, Bokoch et al, 2010). All of these studies provided insight into the general configuration of GPCRs, positioning of the TM domains, and residues participating in ligand binding and interaction with intracellular signaling proteins. These studies improved our understanding of molecular mechanism of GPCR activation and signaling.

These studies have been followed by the high resolution inactive structures of several other GPCRs, including the human β2 adrenergic receptor bound to the inverse agonists carazolol and timolol (Cherezov et al, 2007, Rasmussen et al, 2007), the avian β1 adrenergic receptor bound to the antagonist cyanopindolol (Warne et al, 2008), and the human A2A-adenosine receptor bound to the antagonist ZM241385 (Jaakola et al, 2008). Furthermore, the structures of the active, unliganded opsin (Park et al, 2008) and opsin bound to the C-terminal sequence of the G protein, transducin (Scheerer et al, 2008) have
been solved and provided information about the conformational changes associated with active state of rhodopsin. There are both similarities and differences among the four known inactive structures of GPCRs. The extracellular domains, including the ends of the TM helices and the extracellular loops, show the greatest difference which may allow the recognition of a wide spectrum of ligands. (Rosenbaum et al, 2009)

The ligand binding domains of these rhodopsin family GPCRs share remarkable similarity. However, between GPCR families, the ligand binding domains show diversity. The ligand binding pocket for small molecular weight ligands is within the hydrophobic core formed by the TM helices whereas the ligand binding sites for larger peptides include the N terminus and the extracellular loops, as well (Perez and Karnik, 2005).

The conserved structural features of GPCRs suggest a common molecular mechanism for ligand-induced activation among different families of GPCRs (Gether, 2000). The previous studies of rhodopsin, a prototypical GPCR, provide useful knowledge on ligand-receptor interactions of TM and intracellular domains. However, the structure-function relationships of extracellular domains have not been well understood. Although the interactions with TM helices are recognized as the major factor for activation of GPCRs, accumulating evidence has revealed a new and unanticipated role for the extracellular loops (ECLs) in the functioning of GPCRs (Karnik et al, 2003). Compared to the variable structure of N-terminus, the lengths of all other ECLs are highly conserved among GPCRs, which implies that a conserved function is mediated by the extracellular domain. The significance of the extracellular domains for ligand binding
and receptor activation has been shown for the majority of GPCRs from different families. For the rhodopsin family GPCRs, the ligand binding site is found deep within the seven transmembrane bundle. However, small peptide ligands make contact with the ECLs while entering the TM core. In the secretin family of GPCRs which are characterized by longer N-terminal domains, ECLs contribute to receptor activation although the ligand binds to the N-terminal domain. The interaction of ligand with ECLs is crucial for the TM domains to activate the glutamate family of receptors. The ligand-induced activation of the other two GPCR families, adhesion and frizzled receptors has been shown to be impaired by the mutations in ECLs. These observations suggest a conserved role for the extracellular domains throughout the GPCR superfamily (Karnik et al, 2003).

In addition, the extracellular loop 2 (ECL2) is characterized by a highly conserved disulfide bond linking the loop to the TMIII in more than 90% of GPCRs. Disruption of this disulfide linkage directly affects receptor folding and function in most GPCRs, indicating an important role for the disulfide bond and ECL2 in receptor structure-function relationships (Karnik et al, 2003). The ECL2 attracted additional interest upon the elucidation of the high-resolution crystal structure of bovine rhodopsin, the first structure of a mammalian GPCR. In this structure, the loop makes extensive contacts with the other extracellular domains and adopts an anti-parallel β-hairpin structure that projects into the TM core, covering the retinal binding site (Palczewski et al, 2000). On the other hand, unlike the buried β-hairpin structure in rhodopsin, the ECL2 in the recently solved structures of β2 and β1 adrenergic receptors has an extra helix and an
intraloop disulfide bond in addition to the conserved disulfide bond between ECL2 and TMIII (Cherezov et al, 2007, Rasmussen et al, 2007, Warne et al, 2008). Despite this conformational difference, different GPCR structures predict that ECL2 is a part of the ligand binding pocket and has a vital role in receptor function.

1.1.4 Activation of GPCRs

Transmembrane signal transduction during the activation of GPCRs adheres to a common molecular mechanism (Karnik et al, 2003). Binding of activating ligands to a GPCR induces rigid-body movements of TM helices which facilitate G-protein activation, leading to cytoplasmic signal transduction. Elucidating the nature of the conformational changes in GPCRs that are induced by different types of ligands is critical to understand the molecular mechanism of GPCR signaling. GPCRs show some level of basal activity in the absence of an agonist and activate the G-protein in an agonist independent manner. This basal activity can be modulated by different types of ligands. Agonists are ligands that fully activate the receptor, whereas partial agonists induce submaximal activation of the G-protein, even at saturating concentrations. Inverse agonists reduce the basal activity and antagonists compete with the other ligands without affecting on basal activity (Kiya et al, 2010).

Different models have been proposed to explain GPCR activation upon agonist binding (Perez & Karnik, 2005). The “bimodal switch model” simply proposes that the receptor exists in equilibrium between the inactive state and the active state. The level of
basal receptor activity is determined by the equilibrium between these two states in the absence of agonists. The binding of ligand changes the equilibrium between active and inactive states.

A more complex model suggests that constraining interactions keep the receptor in an inactive conformation. Upon activation by agonists or activating mutations, these interactions are released, exposing the key residues for the G-protein interaction. The unbound receptor can assume several intermediate conformational states with different affinities for the G-protein. Each conformation can selectively interact with a ligand, leading to a specific intracellular signaling outcome. The inverse agonist stabilizes the inactive state by increasing the number of constraining interactions, whereas the agonist stabilizes the active state by releasing such interactions. Partial agonists may stabilize an intermediate state that has lower affinity for the G-protein. G-proteins may also interact with the empty receptor and stabilize the receptor in a different intermediate state that increases the affinity for agonist. The modulation of basal activity of GPCRs by different types of ligands indicates the dynamic nature and inherent flexibility of GPCRs and the existence of multiple conformational states in the absence of ligands (Perez & Karnik, 2005). Evidence suggests that during agonist binding and activation, the receptor undergoes a series of intermediate conformations through interactions formed between the receptor and the agonist, disruption of constraining intramolecular interactions that stabilize the basal state of the receptor and rearrangement of a new set of intramolecular interactions (Kobilka and Deupi, 2007, Ahuja and Smith, 2009). The flexibility of GPCRs is essential for their physiological functions (Kobilka and Deupi, 2007). A better
understanding of the dynamic nature of GPCRs might facilitate structure based drug discovery.

The fundamental question of the mechanism of GPCR activation is, what are the conformational changes induced by agonist which are then propagated to the cytoplasmic domain that couples to the G-protein. The recent crystal structures of active states of opsin (Park et al, 2008, Scheerer et al, 2008) and solid-state nuclear magnetic resonance (NMR) studies provide insight into the transmembrane helix rearrangements upon agonist binding. Ahuja et al (2009) has shown that conformational changes upon light activation of rhodopsin includes movement of TM helices that disrupts a hydrogen-bond network between ECL2 and the extracellular ends of TMIV, TMV and TMVI, displacing the ECL2 during rhodopsin activation. Using NMR spectroscopy, Bokoch et al (2010) reported that ECL2 of the β2-adrenergic receptor is in distinct conformations depending on the type of the ligand bound to the receptor, and identified ECL2 as a direct reporter of the receptor’s active state.

1.1.5 GPCRs and Disease

Coordination of signaling networks activated by GPCRs ensures proper functioning of biological systems. GPCRs have significant roles in the regulation of physiology. GPCR related defects have been shown to cause a wide range of genetic and somatic diseases including cardiovascular diseases and cancer (Paul et al, 2007). Mutations observed in GPCRs may cause deficiency in cell surface expression or ligand binding, generation of abnormal signals, constitutive activation, or uncontrolled
desensitization and internalization, all of which may lead to disease conditions. These mutations have been identified particularly in GPCRs that mediate the actions of sensory signals, hormones and neurotransmitters. Some examples are rhodopsin, gonadotrophin receptor, calcium sensing receptor, angiotensin receptors and vasopressin receptor. (Table I.1) Some beneficial mutations of GPCRs have also been reported, such as a mutation in a chemokine (CCR5) receptor. CCR5 receptor is a co-receptor for human immunodeficiency virus (HIV). A mutation in this receptor prevents the interaction of HIV with target cells, preventing HIV infection. (Galvani and Novembre, 2005) Diseases related to mutations in GPCRs are not common and observed in less than 1% of the human population. Some genetic variations in GPCRs, including single nucleotide polymorphisms (SNP), are also identified as contributors to diseases (Paul et al, 2007). Disease-related polymorphisms have been identified in angiotensin II type 1 and type 2 receptors (Baudin, 2005) and chemokine receptor (Insel et al, 2007). Many other diseases are caused by malfunctioning of GPCR signaling in cellular systems. Systemic disorders such as cardiovascular diseases (hypertension, heart failure), metabolic diseases (diabetes, obesity, kidney damage) respiratory diseases (asthma), and degenerative diseases (Parkinson’s disease) are related to loss of regulation of GPCR signaling. As a therapeutic approach, agonists and antagonists of GPCR have been proved useful for treatment of these diseases. Development of more specific and effective drugs is the major goal in the field of GPCR research.
Table I.I: Examples of disease causing mutations in GPCRs. Adapted from Paul et al, 2007.

<table>
<thead>
<tr>
<th>Receptor/Gene name</th>
<th>Mutation</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium-Sensing/CaSR</td>
<td>Multiple (e.g. Arg185Gln)</td>
<td>Autosomal Dominant Hypocalcemia, Sporadic and Familial Hypoparathyroidism</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Multiple (e.g. Ser338X)</td>
<td>WHIM syndrome</td>
</tr>
<tr>
<td>Endothelin receptor B/EDNORB</td>
<td>Multiple (e.g. Trp276Cys)</td>
<td>Hirschsprung’s disease</td>
</tr>
<tr>
<td>Follicle-stimulating hormone/FSHR</td>
<td>Multiple (e.g. Ala189Val)</td>
<td>Female infertility</td>
</tr>
<tr>
<td>N-formyl-peptide receptor/FPR1</td>
<td>Phe110Ser, Cys126Trp</td>
<td>Juvenile periodontitis</td>
</tr>
<tr>
<td>Frizzled/FZD4</td>
<td>Multiple (e.g. Arg417Gln)</td>
<td>Familial exudative vitreoretinopathy</td>
</tr>
<tr>
<td>Gonadotropin-releasing hormone/GNRHR</td>
<td>Multiple (e.g. Arg262Gln)</td>
<td>Hypogonadotropic hypogonadism</td>
</tr>
<tr>
<td>GPR54/GPR54</td>
<td>Multiple (e.g. Cys223Arg)</td>
<td>Hypogonadotropic hypogonadism</td>
</tr>
<tr>
<td>GPR56/GPR56</td>
<td>Multiple (e.g. Cys223Arg)</td>
<td>Bilateral frontoparietal polymicrogyria</td>
</tr>
<tr>
<td>vGPCR/KSHV-GPCR (constitutively active)</td>
<td></td>
<td>Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Relaxin family peptide receptor 2/LGR8</td>
<td>Multiple (e.g. Thr222Pro)</td>
<td>Cryptorchidism</td>
</tr>
<tr>
<td>MASS1</td>
<td>Multiple (e.g. Ser2652X)</td>
<td>Usher syndrome, Febrile seizures</td>
</tr>
<tr>
<td>Melanocortin/MC4R</td>
<td>Multiple (e.g. Pro78Leu)</td>
<td>Dominant and recessive obesity</td>
</tr>
<tr>
<td>Rhodopsin/RHO</td>
<td>Multiple (e.g. Pro23His)</td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td>Vasopressin receptor/AVPR2</td>
<td>Multiple (e.g. Arg113Trp)</td>
<td>Nephrogenic diabetes insipidus</td>
</tr>
<tr>
<td>Luteinizing hormone receptor/LHR</td>
<td>Gly578Asp</td>
<td>Familial male precocious puberty</td>
</tr>
<tr>
<td>Angiotensin II type 2 receptor/AT2R</td>
<td>Multiple (e.g. Arg324Gln)</td>
<td>X-linked mental retardation</td>
</tr>
</tbody>
</table>
1.1.6 Drug Discovery

The molecular models of GPCRs have been extensively used for in silico screening and identification of novel small-molecule ligands (Karnik et al, 2003). The availability of crystal structures of several GPCRs has enabled more precise screening for those GPCRs.

Three different types of therapeutic agents targeting GPCRs, including orphan GPCRs, have been identified by virtual screening of synthetic chemical libraries and natural compounds. First, novel agonists that activate GPCRs have been particularly useful for orphan GPCRs whose endogenous ligands are not known. Second, antagonists are also very useful for elucidating the cellular roles of orphan GPCRs. They are highly important for treatment of diseases related to GPCR overactivity for which we do not have an effective therapy. Third, inverse agonists can target the constitutively active mutants of GPCRs which can not be effectively inhibited by antagonists. The inverse agonists are also useful for characterization of orphan GPCRs. There are a few examples of other types of novel therapeutic agents that specifically target heterodimers of some GPCRs. An example is a drug specifically designed against the µ-opioid/δ-opioid receptor heterodimer (Daniels et al, 2005).

1.2 Angiotensin II Type 1 Receptor (AT1R)

1.2.1 Renin Angiotensin System

The renin angiotensin system (RAS) plays a critical role in maintaining the salt-water balance as well as blood pressure. The RAS is activated in response to decreased
plasma sodium and fluid volume, resulting in stimulation of the renin enzyme secretion from juxtaglomerular cells of the kidneys (Fig. 1.3). The enzyme renin cleaves circulating angiotensinogen to angiotensin I (Ang I), which is a decapeptide representing the N-terminus of angiotensinogen. The C-terminal dipeptide of Ang I is hydrolyzed by a carboxypeptidase, angiotensin converting enzyme (ACE), to produce Ang II. It is an extremely potent vasoconstrictor hormone and acts on the adrenal glands to stimulate the release of aldosterone, increasing blood pressure. Ang II interacts with two types of G-protein coupled receptors to elicit a cellular response; angiotensin II type 1 (AT1R) and angiotensin II type 2 (AT2R) receptors (Gasparo et al, 2000).

The major cardiovascular effects associated with Ang II are mediated through AT1R, such as vasoconstriction, aldosterone and vasopressin secretion, and activation of sympathetic nervous system (Dinh et al, 2001). The physiological effects of AT2R remain to be discovered. AT2R is known to antagonize the AT1R effects and possibly plays role in several AT1R-independent biological processes such as apoptosis and cellular differentiation (Table I.II). In contrast to global expression of AT1R in many tissues, AT2R is only expressed during fetal development. The vital functions of AT1R have also been indicated by the severe effect of complete ablation of AGT1R gene. However, AGTR2 gene depletion does not show defective phenotypes, except for a slight increase in the blood pressure (Oliverio et al, 1998). Moreover, AT1R mutations are very rare in populations, whereas AT2R mutations can be tolerated. Over activity of RAS is related to many diseases including hypertension, heart-failure, diabetes, renal and inflammatory diseases (Teerlink, 1996). The hypertrophic effects of RAS are elicited by
Figure 1.3. The Renin-Angiotensin system (RAS). Decreased blood pressure stimulates renin secretion from kidneys. Renin cleaves angiotensinogen into Ang I, which is in turn cleaved by ACE to produce the octapeptide Ang II. Ang II is the major active peptide of RAS that regulates blood pressure through vasoconstriction and stimulation of aldosterone and vasopressin (ADH) release. Ang II may also give rise to pathological conditions such as cardiac and vascular hypertrophy and end-organ damage.
Table I.II. Differential effects of AT1R and AT2R stimulation by Ang II. Adapted from Siverstein and Ram, 2005

<table>
<thead>
<tr>
<th>AT1R</th>
<th>AT2R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased vasoconstriction</td>
<td>Regulation of fetal tissue development</td>
</tr>
<tr>
<td>Increased aldosterone synthesis &amp; secretion</td>
<td>Regulation of cell growth and proliferation</td>
</tr>
<tr>
<td>Increased tubular sodium reabsorption</td>
<td>Regulation of extracellular matrix composition</td>
</tr>
<tr>
<td>Increased vasopressin secretion</td>
<td>Regulation of cell differentiation</td>
</tr>
<tr>
<td>Increased cardiac hypertrophy</td>
<td>Regulation of apoptosis</td>
</tr>
<tr>
<td>Increased cardiac contractility</td>
<td>Regulation of left ventricular remodeling</td>
</tr>
<tr>
<td>Increased vascular smooth muscle proliferation</td>
<td>Increased neuronal regeneration</td>
</tr>
<tr>
<td>Increased noradrenergic activity</td>
<td>Vasodilation</td>
</tr>
<tr>
<td>Cell growth and proliferation</td>
<td>Antiproliferation</td>
</tr>
</tbody>
</table>
the binding of Ang II to the AT1R. Genetic manipulations of the mouse \textit{AGTR1} gene significantly alter blood pressure as well as cardiac and renal functions (Paradis et al, 2000). Drugs targeting AT1R can effectively lower blood pressure, whereas drugs targeting AT2R do not have an effect on RAS induced pathologies.

### 1.2.2 Significance of AT1R

The AT1R is a 359 amino acid peptide hormone GPCR which belongs to the rhodopsin family. It is expressed in liver, kidney, aorta, lung, uterus, ovary, spleen, heart, adrenal, and vascular smooth muscles. Upon Ang II binding, AT1R productively couples to the G-protein, $\text{G}_a_{q/11}$, which leads to the activation of phospholipase C (PLC), producing inositol-1, 4, 5-triphosphate (IP3) and Diacylglycerol (DAG). Calcium mobilization results in smooth muscle cell contraction, which is associated with increased blood pressure (Fig. 1.4). The intracellular downstream signaling involves the phosphorylation of extracellular signal regulated kinases (ERK1/2) in cells (Fig. 1.4). In addition, the AT1R activates tyrosine kinases such as Janus Kinase (JAK) and epidermal growth factor receptor (EGFR) (Gasparo et al, 2000, Miura et al, 2004).

AT1R is an important target for drug development, because abnormal AT1R function causes hypertension, water-electrolyte imbalance, cardiac hypertrophy and heart failure, which are the main causes of death in the Western world. The importance of AT1R activation has been demonstrated in all of these diseases. ACE inhibitors and AT1R antagonists, also known as AT1R blockers (ARBs), are used effectively as therapeutic agents to lower blood pressure and to prevent cardiac and vascular hypertrophy.
Figure 1.4. The major AT1R signal transduction pathway. Ang II binding activates the Gq coupled signaling pathway leading to activation of PLC, which results in calcium mobilization and activation of MAPK.
ACE inhibitors compete with ACE to reduce Ang II production, whereas, ARBs compete with Ang II for binding to AT1R. The ARBs are well tolerated with fewer side effects than ACE inhibitors. ARBs completely block actions of Ang II on the AT1R, irrespective of whether Ang II is generated systemically or by non-ACE mediated pathways. Several ARBs available for the treatment of RAS associated disorders are losartan, for treatment of hypertension and hypertrophy, irbesartan, for treatment of diabetic nephropathy, and valsartan, for treatment of heart failure in people who cannot tolerate ACE inhibitors. Understanding the activation mechanism of AT1R is very important for development of more effective therapeutic agents.

1.2.3 Ang II binding and Activation of AT1R

Modification of Ang II, site-directed mutagenesis and crosslinking studies established the interactions between Ang II (DRVYIHPF) and AT1R (Fig. 1.5). The ligand binding pocket formed by the hydrophilic TM domain residues is shown in Fig. 1.6 A. There are two salt bridges, one between Arg^2 of Ang II and Asp^{281} of AT1R and second between the carboxyl group of Ang II and Lys^{199} of AT1R (Yamano et al, 1992, Noda et al, 1995, Feng et al, 1995, Lefkowitz et al, 1993). The Asp^1 of Ang II forms an ion pair with His^{183} in the ECL2 of AT1R (Feng et al, 1995). The aromatic stacking interactions between Phe^8 of Ang II and His^{256} of AT1R (Noda et al, 1995) and between Tyr^4 of Ang II and Asn^{111} of AT1R (Noda et al, 1996, Miura et al, 1998, Miura et al, 1999) were shown to be critical not only for ligand docking but also for receptor activation (Fig. 1.6 B). Crosslinking has shown two additional interactions, one between Phe^8 of Ang II and
Figure 1.5. Specific interactions of AT1R with Ang II and losartan. Secondary structure model of rat AT1R, showing the interactions of AT1R with Ang II, previously mapped by site directed mutagenesis (blue) and cross linking experiments (red). The AT1R residues interacting with losartan are shown in yellow.
Figure 1.6. Ang II binding to AT1R. A) Orientation of hydrophobic (magenta) and hydrophilic (cyan) side chains in the rat AT1R. Both membrane plane view and extracellular view is shown. (from Gogonea et al, 2006) B) The predicted hormone-binding site. Both membrane plane view and extracellular view is shown. Ang II in the binding site is shown in space filling model, with Phe$^8$ (magenta), Tyr$^4$ (orange) and other side chains (green) (from Gogonea et al, 2006). C) Replacement of water molecules in the ligand binding pocket in the empty state of the AT1R by Tyr$^4$ side chain of Ang II, interacting with Asn$^{111}$ in the active state of AT1R, as shown (from Husain and Graham, 2000).
Phe$^{293}$ and Asn$^{294}$ in TMVII of AT1R (Perodin et al, 2002), the other between Val$^3$ of Ang II and Ile$^{172}$ in the ECL2 region of the AT1R (Boucard et al, 2000). Thus, the hydrophobic C-terminal region of Ang II appears to enter the TM core of the receptor. The N-terminal region of Ang II is positioned towards the extracellular region of the AT1R.

Several Ang II analogs have been generated by modification of Ang II side chains to evaluate the effects on ligand binding and receptor activation (Miura and Karnik, 1999a). Ligand modification led to the identification of agonists, partial agonists and antagonists. For example, modification of either Tyr$^4$ or Phe$^8$ side chains of Ang II converts the agonist function into an antagonist function (Samanen et al, 1989). The effect of substitutions of Tyr$^4$ or Phe$^8$ side chains on receptor binding and activation has been further investigated by Miura et al (1999b). The aromatic side chains of these residues have been shown to be critical switches for G-protein activation (Fig. 1.6 C). Several other analogs retained partial agonist function. For example, [Ile4, Ile8] Ang II functions as a selective ligand, which does not lead to receptor internalization and stimulates β-arrestin dependent activation of ERK1/2 phosphorylation independent of Gq (Feng et al, 1998). The discovery of non-peptide Ang II antagonists and derivatives has significant therapeutic value. These antagonists have a structural pharmacophore similar to Ang II which enables them to compete with Ang II for binding to AT1R and to displace bound Ang II from the AT1R (Fig. 1.7 A). Losartan is a surmountable antagonist of AT1R which can be displaced by Ang II; however, carboxylated derivatives of losartan such as EXP3174, candesartan and irbesartan are insurmountable inhibitors. The
Figure 1.7. Chemical structures of the Ang II and AT1R non-peptide antagonists. A) Ang II and losartan pharmacophores (from Noda et al, 1995). B) Chemical structures of EXP3174, candesartan, and olmesartan. All compounds have biphenyltetrazole and imidazole groups. EXP3174, candesartan, and olmesartan, but not losartan, have a carboxyl group. Only olmesartan has a hydroxyl group (from Miura et al, 2006).
carboxylate group of these inhibitors provide an additional interaction with Gln$^{257}$ of AT1R (Fig. 1.7B) (Vanderheyden et al., 2000, Takezako et al., 2004). The structure of non-peptide antagonists mimics the contact points of Ang II side chains, and their binding sites overlap with the Ang II binding pocket in AT1R as shown by mutagenesis and crosslinking experiments (Yamano et al., 1992, Noda et al., 1995, Ji et al., 1995). For example, Lys$^{199}$ of AT1R forms a salt bridge interaction with both the carboxyl group of Ang II and the tetrazole group of losartan (Fig. 1.8).

Upon Ang II binding, activation of AT1R is shown to be associated with movements of TMII, TMIII, TMV, TMVI and TMVII (Fig 1.9) which is probably conserved in rhodopsin family GPCRs (Miura et al., 2002&2003, Boucard et al, 2003, Martin et al., 2004 & 2007, Domazet et al., 2009). The disulfide bond linking TMIII and ECL2 is highly conserved in more than 90% of GPCRs and has been suggested to have a role in coupling the movements of TM helices to the ECL2 during receptor activation (Karnik et al., 2003). Whether the ECLs play any role in the transition from inactive to active state is not known. The role of ECLs in regulating the actions of agonists and antagonists has not been studied in any GPCR.

We proposed a model of GPCR activation that involves the movement of ECL2, which results in the movements of transmembrane domains (Karnik et al., 2003). Our model is based on the findings that (i) the length of ECL2 in GPCRs is conserved; (ii) electrostatic and hydrophobic interactions between different TM helices and ECL2 are
Figure 1.8. Overlapping binding pockets of Ang II and losartan. A) Experimentally mapped Ang II binding residues. B) Overlap of Ang II (red) and losartan (yellow) interacting residues.
Figure 1.9. Activation of AT1R. The transmembrane domains, TMII, TMIII, TMV, TMVI and TMVII, that undergo conformational change upon AT1R activation are shown in box. The highly conserved disulfide bond between TMIII and ECL2 is shown in yellow.
predicted in almost all GPCR models; (iii) a highly conserved disulfide bond links the ECL2 to TM3 in 92% of GPCRs, and this disulfide bond is required for the structural integrity and function in GPCRs examined; and finally (iv) the available crystal structures of GPCRs confirmed the interaction of ECL2 with TM domains and the ligand.

Our proposal is further supported by observations reported by other investigators. These studies suggested significant interaction of ECL2 with Ang II and TM-helices. Two ECL2 residues, Ile\textsuperscript{172} and His\textsuperscript{183}, have been shown by ligand cross linking and mutagenesis to directly interact with Ang II (Feng et al, 1995, Boucard et al, 2000). Mutagenesis of Glu\textsuperscript{173} in ECL2 leads to increased sensitivity to a partial agonist, CGP42112A, implying that Glu\textsuperscript{173} chain imposes a constraint for activation in the absence of ligand (Parnot et al, 2000). In addition, mutational studies in our laboratory indicate that Phe\textsuperscript{182} in ECL2 is quite important for antagonist binding (unpublished observations). Recently, AT1R activating antibodies directed against ECL2 were found in preeclamptic patients (Wallukat et al, 1999, Zhou et al, 2008) and patients with refractory vascular allograft rejection (Dragun et al, 2005). This suggests that ECL2 plays a role in governing the active state of the AT1R. Therefore, interactions of the ECL2 with TM domains may be important to guide ligands to the binding pocket and to regulate the ligand dependent activation of the receptor.

1.3 The Extracellular Loop 2 in other GPCRs

Crystallographic structures and accumulating structure-function studies suggest an unanticipated canonical role for ECL2 in ligand-induced activation of GPCRs. ECL2
in the prototypical GPCR, bovine rhodopsin (Fig. 1.10 A), adopts a β-hairpin structure that projects into the site of covalently bound retinal as ‘a stable lid’ directly interacting with the ligand (Palczewski et al, 2000, Park et al, 2008). Upon light activation ECL2 moves away (Ahuja et al, 2009). Absence of a similar ‘lid’ in the recently solved structures of GPCRs suggests that a path for diffusible ligands to the binding pocket may be a specialization that evolved in other GPCRs. The ECL2 in β2 and β1 adrenergic receptors (Fig. 1.10 B & C) has an α-helical conformation stabilized by an intra-loop disulfide bond (Cherezov et al 2007, Warne et al, 2008). The ECL2 of A2A adenosine receptor (Fig. 1.10 D) with three disulfide bonds is unstructured (Jaakola et al, 2008). Despite these variations, all GPCR structures predict that ECL2 is a part of the ligand binding pocket. ECL2 makes direct contacts with bound inverse agonist, 11-cis-retinal in rhodopsin (Ser186 to Ile189, Glu181 and Tyr191), the antagonist cyanopindolol in β1 adrenergic receptor (Thr203 and Phe201), the inverse agonist carazolol in β2 adrenergic receptor (Phe193) and the antagonist ZM241385 in A2A adenosine receptor (Phe168 and Glu169). Positioned at the entrance to the binding cavity, ECL2 may regulate the access of ligands and receptor activation in rhodopsin-like and other GPCR families (Bourne and Meng, 2000).

The contribution of ECL2 in ligand binding and receptor activation has been shown for many GPCRs such as dopamine D2 receptor (Shi and Javitch, 2004), the adenosine A2A receptor (Jeongho et al, 1995), the M2 and M3 muscarinic acetylcholine receptor (Anu et al, 2004, Scarselli et al, 2007), adrenergic receptors (Zhao et al, 1996) and the histamine H1 receptor (Andrea et al, 2008). Importantly, some mutations on the ECL2
Figure 1.10. The ECL2 structures in crystal structures of GPCRs. A) ECL2 structure in Rhodopsin (from Cherezov et al, 2007). B) ECL2 structure in $\beta_2$ adrenergic receptor (from Cherezov et al, 2007). C) ECL2 structure in $\beta_1$ adrenergic receptor (from Warne et al, 2008). D) ECL2 structure in A2A adenosine receptor (from Jaakola et al, 2008). Disulfide bonds are shown in yellow.
led to constitutively active mutants in several GPCRs such as thyrotropin (TSH) receptor (Duprez et al, 1997) and complement C5A receptor (Klco et al, 2005), indicating that the ECL2 stabilizes the inactive conformation of these receptors. Importantly, receptor-activating auto-antibodies against the ECL2 regions have been discovered in several human pathologies related to muscarinic receptors (Borda et al, 2004), β1 and β2 adrenergic receptors (Magnusson et al, 1994, Lebesgue et al, 1998) and the bradykinin B2 receptor (Abdalla et al, 1995). Therefore, it is very important to study the structure of ECL2 in detail.

Accumulating evidence suggests that the extracellular domain of AT1R plays a critical role in guiding Ang II and ARBs to bind to TM helices, but the molecular details are unclear. Based on molecular modeling and dynamic simulation studies, the AT1R structure differs from both rhodopsin and adrenergic receptors in several key aspects. Unlike rhodopsin which has covalently attached ligand, the AT1R has a diffusible ligand similar to the ligand of adrenergic receptors. AT1R is different from the adrenergic receptors due to the absence of additional intraloop disulfide bond. Therefore, AT1R may have a different mechanism of structural stabilization. Molecular dynamic simulation studies performed on the AT1R model predicted significant movement of ECL2, which is seen in open and closed conformational states. Thus, ECL2 is likely a pivotal structural element in generating different conformational states of AT1R, but the role it plays in reversible binding of ligands is unknown.
1.4 Significance of Current Work

Evidence based on both biochemical studies and molecular modeling of AT1R suggests a critical role for ECL2 of AT1R in fundamental processes of ligand-mediated activation and inhibition of the receptor. Elucidating the conformational regulation of ECL2 by different types of ligands may help us dissect the pathophysiological processes mediated by AT1R. In this study, we examine the conformation of ECL2 of the AT1R specifically in basal, Ang II-bound and ARB-bound states by reporter-cysteine accessibility mapping (RCAM). RCAM analysis allows us to systematically map the surface accessibility of residues in a particular region of the receptor. RCAM involves site-directed mutagenesis to introduce cysteine residues at selected positions within the protein (Akabas et al, 1992). The reactivity of the introduced cysteines toward hydrophilic, thiol specific, alkylating reagents is the basis for determining their surface accessibility. The MTS reagents selectively and rapidly react with thiols (sulfhydryls) of water accessible cysteines. The reactivity of MTS reagents with a cysteine in the water accessible surface of a protein is much faster than one in the membrane or the protein interior. Thus, RCAM can give information on whether a cysteine at a particular position is buried or exposed (Fig. 1.11).

Our accessibility mapping analysis of ECL2 of AT1R in different states of the receptor has indicated a ligand-induced conformational change in the ECL2. The highly accessible ECL2 of AT1R is rendered inaccessible upon binding to agonist and antagonist. Molecular dynamic simulations based on the accessibility constraint reveal an open conformation of ECL2 in the empty receptor that may facilitate capture of Ang II,
Figure 1.11. Schematic representation of ligand interference and ligand protection assay.
losartan and autoantibodies. A lid conformation of ECL2 is shown in both Ang II- and losartan-bound states. The ligand-induced lid conformation of ECL2 may slow down the dissociation of ligands and prolong the bound conformation. Conformational dynamics of ECL2 may regulate ligand mediated processes in AT1R. These dynamic studies are essential to improve remedies targeting AT1R for treatment of hypertension, heart failure and end organ damage due to high blood pressure.

Our results have shown two different conformations of ECL2, open and closed conformations, where the open conformation seems ideal for facilitating autoantibody binding to AT1R. Autoantibodies have been shown to activate AT1R in several diseases, including preeclampsia and vascular allograft rejection (Liao et al, 2002, Dragun et al, 2005, Zhou et al, 2008). Haptens specific for the open conformation of ECL2 can be designed to prevent autoantibody interaction with the AT1R and block the immune response against these autoantibodies in patients with these diseases.

Our findings are significant in the context of the whole GPCR family of proteins. The ligand induced conformational changes of ECL2 shown in AT1R, which is a rhodopsin family GPCR, might have useful implications for other GPCR families, such as adhesion family GPCRs, for which there are no available antagonists. Novel agents or modifications could be developed to disrupt interaction of ECL2 with their long N-terminal tails as a mechanism to inactivate these GPCRs.
Specific conformational changes induced by agonist and antagonist binding provides an experimental basis for further analysis of the effects of various ARBs on AT1R dynamics and will aid in determining the high-resolution crystal structure of an ARB-bound AT1R.
CHAPTER II
MATERIALS AND METHODS

2.1 Construction of Plasmids

The hemagglutinin (HA)-tagged AT1R gene was cloned into the shuttle expression vector, pMT3, which contains unique EcoRI and NotI restriction sites (Fig. 2.1 A). The synthetic rat AT1R gene is flanked by EcoRI restriction site on the 5’ end and NotI restriction site on the 3’ end. N-terminal HA epitope tag was introduced at the 5’ end of the gene by polymerase chain reaction (PCR) amplification in the presence of FailSafe enzyme (Epicentre Biotechnologies, WI) using 10 pmol/µl of EcoRI-HA sense primer and 10 pmol/µl of NotI antisense primer (Fig. 2.1 B). The sequence was confirmed by automated DNA sequencing (Fig. 2.2).

CYS−AT1R was generated by PCR mutagenesis as described previously (Miura et al, 2002 & Miura et al, 2003). Briefly, single residue mutations were introduced by mismatched primer method and combined with PCR amplification. Primers were designed to change cysteine residues at positions 76, 121, 144, 289, 296 and 355 into...
Figure 2.1. Cloning of HA-AT1R gene in pMT3 expression vector. A) Schematic representation of pMT3 cloning vector. SV40, origin of replication; AdMLP, adenovirus major late promoter; TPL, tripartite leader sequence; IVN, intervening sequence; EcoR1-Not1, cloning sites for expression; DHFR, dihydrofolate reductase promoter; VA1, adenoviral VA1 gene; pBR-ori1, origin of replication; AmpR, ampicillin resistance. B) Schematic representation of cloning of PCR amplified HA-AT1R gene into pMT3 vector. Primers used for PCR amplification are EcoR1-HA sense primer “CGGGAATTCCGCAA
CCATGTACCCATACGACGTCCCAGACTACGCGGCCCTTTAICTCTCT” and Not1 antisense primer “CCCCCGCGCCGCGTATTAAGCTGGAGCAACTTG”. Template used was pcDNA3-AT1R. The parameters used for PCR reaction were an initial denaturing step of 5 min at 94 °C followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min and then a final extension at 72 °C for 10 min.
A

![Diagram of pMT3 vector](image)

B

1. **EcoR1-HA AT1R gene**
2. Cut with EcoR1 and Not1
3. Purify 1.2 kb fragment
4. PCR amplification of AT1R
   - Sense primer: EcoR1-HA-AT1
   - Antisense primer: Not1-AT1
5. Ligate
6. Transform into E.coli
7. pMT3 cut with EcoR1 and Not1
8. Vector
9. Insert
10. Nucleotide sequence of the insert is confirmed by DNA sequencing
**Figure 2.2. Nucleotide and amino acid sequence of HA-AT1R.** The introduction of HA epitope into AT1R gene is verified by sequencing. Red, EcoR1 restriction site; green, sequence encoding HA epitope; blue, stop codons; magenta, Not1 restriction site; yellow, cysteine codons and residues. Disulfide bonded cysteines are framed.

### HA-AT1R Nucleotide Sequence

```
GAATTCGCCACATGTA[ACATACGACGTCCCAGACTACGCGGCCCTTAACTCTTCTG
CTGAAGTGTCATCACCAAGATCCAGATCTGCGCTTGCCTGGCCACTGCGCCAGGGCTG
CTCTTCTTCTCAGGATCTGCTTCATGATTCCTACCCTCTACAGCATCATGTTTTGTTA
CAAACCACCTCTACCTATGCAATGCTGCCCGAAGGCTGGACGTCACAGTTACATATG
TTGTCATGATACCCTCTACAGCATGATTTGGAATACTCACTAGTGGTGATTGTCAT
TTACTTTTACTTTTACATGAAGCTGAAGACTGTGGCCAGCGTCTTTTCTTCTCAAT
CTCGCCTTGGCAGATTTATGCTTTTTGTTAACTTTGCCCCTGTGGGCAGTCTATAC
CGCCATGGAGTACCGCTGGCCCTTCGGTAACCACCTATGCTGAGCTTCAACCTCTA
CGCGTCTGTGTTCCTTCTCACTTGTCTCA
GCATCGACCGCTACTCCGGACATCGTGGACACTGCCATGCCCATCACGATATGCAT
AGTATTTTAACAACTGTTTAAACCCTCTGTTTTACGGCTTTCTGGGGAAGAAATT
CAAAAGTACTTCCTCCAGCTCTGAAATATATTCCCCCAAGGCCAAGTCCCACTCGA
GCTTTCTTACCGGCCTTCGGATAACATGAGCTCATCGGCCAAAAAGCCTGCGTCTT
GGTTTGAGGTGGAGTAATAGGCGGCCGC
```

### HA-AT1R Amino Acid Sequence

```
MYPYDVPSYATALNSAEDGIKRIQDPLPKAGRHYIFVMIPTLYSIIFVVGFNSLTV
IVIYFYMKLTAVASVFLNLALADLFLTLTPLAVYAMEYRPFWGNCVKISSASVS
FNLYASVFLTLTSLIDRYLAIVHPKRSRLRRTMLVAKTVCIWIMLGAASPPIVHRN
VYFIENNTIVCSAFHYESRNSTLPILGLTKNILGFLFPLIIITSYTLIKALKAYE
IQKNKPRNDIFRIIMAIVLFFIFWSVPHQTFLDVLQILGVHICSIDEVDTAMPI
TICIGIAYFNQCLNLPLFYGFLGKKKFKKYYFLQLLKYIPPKAKSHSSLSTKMSL
YRPSDMSNSSAKKPASCFEVE
```
alanine. Please note that different cysteine codons were used to avoid potential consequence of high abundance of a single codon during translation. The PCR products were fractionated on agarose gel, isolated by QIAquick Gel Extraction Kit (QIAGEN Sciences, Maryland), digested with appropriate restriction enzymes and purified with QIAquick PCR Purification Kit (QIAGEN Sciences, Maryland). The insert was subcloned into the synthetic gene which was digested with appropriate restriction enzymes at each step of mutagenesis. The schematic explanation of construction of CYS\(^{-}\)AT1R is given in Figure 2.3. The sequence of HA-CYS\(^{-}\)AT1R was confirmed by automated DNA sequencing.

The single cysteine mutants of ECL2 were generated using PCR mutagenesis as described in Figure 2.4 A. Briefly, sense and antisense mutagenic primers, complementary to the CYS\(^{-}\)AT1R sequence and carrying the nucleotide changes (TGC) to produce the desired amino acid (cysteine), were designed to substitute each residue on ECL2 one at a time. The list of primers is given in Table II.I. The mutagenic primers along with EcoR1 and Not1 primers were simultaneously annealed to the denatured pMT3-HA-CYS\(^{-}\)AT1R template in the first PCR reaction. The full-length construct was obtained in the second PCR reaction by amplification with EcoR1 and Not1 primers. The PCR products were fractionated on agarose gel, isolated by QIAquick Gel Extraction Kit (QIAGEN Sciences, Maryland), digested with EcoR1 and Not1 and purified with QIAquick PCR Purification Kit (QIAGEN Sciences, Maryland). The insert was ligated into pMT3, digested with EcoR1 and Not1, and then transformed into competent DH5\(\alpha\) cells (Fig. 2.4 B). The plasmids were isolated using QIAGEN Plasmid Maxi kit
Figure 2.3. Schematic representation of construction of CYS\textsuperscript{−}AT1R. Single cysteine substitutions, C76A, C121A, C144A, C289A, C296A, C355A were created by mismatched primer method and combined as shown by PCR amplification. Primers are shown as arrows. (Courtesy, John Boros, Shin-ichiro Miura & S. Karnik)
Figure 2.4. Construction of ECL2 single cysteine mutants. A) Schematic explains the introduction of I172C substitution as an example. pMT3-HA-CYS-AT1R was used as a template for PCR mutagenesis. The first PCR reaction involved either EcoR1 primer with I172C antisense primer or Not1 primer with I172C sense primer. The second PCR reaction annealed and amplified the purified fragments of the first PCR reaction in the presence of EcoR1 and Not1 primer. B) Left panel, PCR-1. Lane 1, EcoR1 & E173C antisense primers (product size: 571 kb); Lane 2, Not1 & E173C sense primers (product size: 613 kb); Lane 5, EcoR1 & N174C antisense primers (product size: 573 kb); Lane 6, Not1 & N174C sense primers (product size: 608 kb); Lane 9, EcoR1 & T175C antisense primers (product size: 577 kb); Lane 10, Not1 & T175C sense primers (product size: 605 kb). Lanes 3, 7 & 11, negative control without template. Right panel, PCR-2 with EcoR1 & Not1 primers. Lane 1, E173C PCR1 fragments of lanes 1 & 2 of left panel (product size: 1.2 kb); Lane 5, N174C PCR1 fragments of lanes 5 & 6 of left panel (product size: 1.2 kb); Lane 9, T175C PCR1 fragments of lanes 9 & 10 of left panel (product size: 1.2 kb). Lanes 2, 3, 6, 7, 10, 11, negative controls without either fragments.
A

- pMT3-HA-CYS\textsuperscript{-}AT1R
- EcoR1 Primer → I172C sense
- Target sequence
- I172C antisense → NotI Primer
- EcoR1 primer → I172C antisense
- NotI primer → I172C sense
- Calculate size and purify

- Remove I172C primers
- Denature, anneal
- 5' → 5'
- 3' → 3'
- 3' extension
- Confirm size & purify
- Cut w EcoR1 & NotI
- Purify & Clone

B

**PCR 1**

<table>
<thead>
<tr>
<th></th>
<th>E173C</th>
<th>N174C</th>
<th>T175C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

**PCR 2**

<table>
<thead>
<tr>
<th></th>
<th>E173C</th>
<th>N174C</th>
<th>T175C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Purify

Purify, Cut with EcoR1 & NotI
Purify & Ligate
### Table II.I. The list of primers used for single cysteine substitutions.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>bp</th>
<th>%GC</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I172C sense</td>
<td>GTATACCTTCGCGAGAACACC</td>
<td>21</td>
<td>47.62</td>
<td>75.74</td>
</tr>
<tr>
<td>I172C antisense</td>
<td>GGTGTTCGCGAGATAC</td>
<td>21</td>
<td>47.62</td>
<td>75.74</td>
</tr>
<tr>
<td>E173C sense</td>
<td>GTATACCTATCTGCAACACCAATATC</td>
<td>27</td>
<td>37.04</td>
<td>80.92</td>
</tr>
<tr>
<td>E173C antisense</td>
<td>GATTATGTTGTCAGATGAATATC</td>
<td>27</td>
<td>37.04</td>
<td>80.92</td>
</tr>
<tr>
<td>N174C sense</td>
<td>CTTCATCGAGTGCAACATATCAC</td>
<td>24</td>
<td>45.84</td>
<td>84.55</td>
</tr>
<tr>
<td>N174C antisense</td>
<td>GTGATATTGGTGTGCAGAGACTCAGTGAAG</td>
<td>24</td>
<td>54.84</td>
<td>84.55</td>
</tr>
<tr>
<td>T175C sense</td>
<td>CATCGAGAATCGAATATCACAGTGCAT</td>
<td>25</td>
<td>44.00</td>
<td>84.68</td>
</tr>
<tr>
<td>T175C antisense</td>
<td>CACACTGTGATGCGAGTGTATCTCG</td>
<td>25</td>
<td>54.17</td>
<td>88.97</td>
</tr>
<tr>
<td>N176C sense</td>
<td>CGAGAACACCATTATCAATATCACAGTC</td>
<td>24</td>
<td>45.84</td>
<td>88.23</td>
</tr>
<tr>
<td>N176C antisense</td>
<td>GATATGGTGTTGCGAGTCGATGGTCGAT</td>
<td>24</td>
<td>54.17</td>
<td>88.97</td>
</tr>
<tr>
<td>T178C sense</td>
<td>CATCGAGAATCTGCAATGTCGATTCGAG</td>
<td>25</td>
<td>44.00</td>
<td>84.68</td>
</tr>
<tr>
<td>T178C antisense</td>
<td>CAGTGTGCTGCGAGTCGATGGTCGAT</td>
<td>25</td>
<td>54.17</td>
<td>88.97</td>
</tr>
<tr>
<td>V179C sense</td>
<td>CCAATCACAATGCTGCGCATTTTCC</td>
<td>24</td>
<td>45.84</td>
<td>89.13</td>
</tr>
<tr>
<td>V179C antisense</td>
<td>GAAATGGCGACTGATCGGTATATGCG</td>
<td>25</td>
<td>54.17</td>
<td>92.10</td>
</tr>
<tr>
<td>A181C sense</td>
<td>CACAGTGTGCGCATTATGACGAG</td>
<td>25</td>
<td>54.17</td>
<td>92.10</td>
</tr>
<tr>
<td>A181C antisense</td>
<td>CTCATAATGAAAATGCAAGCCCAGTGTG</td>
<td>24</td>
<td>45.84</td>
<td>89.13</td>
</tr>
<tr>
<td>F182C sense</td>
<td>CAGTGTGCGCATTATGACGAG</td>
<td>25</td>
<td>52.00</td>
<td>91.31</td>
</tr>
<tr>
<td>F182C antisense</td>
<td>CACTGTGATGCGAGTCGATGGTCGAT</td>
<td>25</td>
<td>52.00</td>
<td>91.31</td>
</tr>
<tr>
<td>H183C sense</td>
<td>GTGCGCATTTTATGCAATTATGCTGAG</td>
<td>24</td>
<td>50.00</td>
<td>88.73</td>
</tr>
<tr>
<td>H183C antisense</td>
<td>CCGAGACTCATAATGCGACATGACGAGC</td>
<td>24</td>
<td>50.00</td>
<td>88.73</td>
</tr>
<tr>
<td>Y184C sense</td>
<td>GCATTTCATGCAACGCTCCCGATC</td>
<td>25</td>
<td>54.84</td>
<td>88.23</td>
</tr>
<tr>
<td>Y184C antisense</td>
<td>GAATTTCGAGACGCTCCCGATC</td>
<td>26</td>
<td>54.84</td>
<td>88.23</td>
</tr>
<tr>
<td>R187C sense</td>
<td>CATTATGAGTCTGCAATGACGAG</td>
<td>25</td>
<td>52.00</td>
<td>84.56</td>
</tr>
<tr>
<td>R187C antisense</td>
<td>GTCGAATTTCGAGACGCTCCCGATC</td>
<td>25</td>
<td>56.01</td>
<td>94.06</td>
</tr>
<tr>
<td>N188C sense</td>
<td>GAGTCGAGTCTGCAATGACGAG</td>
<td>25</td>
<td>50.00</td>
<td>95.83</td>
</tr>
<tr>
<td>N188C antisense</td>
<td>CAGCCGGACTGCGAGTCGACGACTC</td>
<td>25</td>
<td>50.00</td>
<td>95.83</td>
</tr>
<tr>
<td>S189C sense</td>
<td>GCTCAAATATTCGAGCTGCAATGCGAATGAAATGC</td>
<td>27</td>
<td>56.01</td>
<td>94.06</td>
</tr>
<tr>
<td>S189C antisense</td>
<td>GATCGGGCTGCAATGACGAGAG</td>
<td>26</td>
<td>57.70</td>
<td>94.83</td>
</tr>
<tr>
<td>T190C sense</td>
<td>CTCGAATATTCGAGCTGCAATGCGAATGAAATGC</td>
<td>26</td>
<td>57.70</td>
<td>94.83</td>
</tr>
<tr>
<td>T190C antisense</td>
<td>GATCGGGCTGCAATGACGAGAG</td>
<td>26</td>
<td>57.70</td>
<td>94.83</td>
</tr>
<tr>
<td>L191C sense</td>
<td>GAAATGGCACTGCAATGACGAG</td>
<td>25</td>
<td>54.84</td>
<td>88.23</td>
</tr>
<tr>
<td>L191C antisense</td>
<td>GCTCAAATATTCGAGCTGCAATGCGAATGAAATGC</td>
<td>25</td>
<td>56.01</td>
<td>94.06</td>
</tr>
<tr>
<td>P192C sense</td>
<td>GAAATGGGAAGCTGACGATGGGTCGAG</td>
<td>26</td>
<td>57.70</td>
<td>97.10</td>
</tr>
<tr>
<td>P192C antisense</td>
<td>GAGTCGAGTCTGCAATGACGAG</td>
<td>25</td>
<td>50.00</td>
<td>84.56</td>
</tr>
</tbody>
</table>

48
(QIAGEN Sciences, Maryland). The agarose gel fractionation of \textit{CYS}^\text{AT1R} \text{ and 20 single cysteine mutants} is shown in Figure 2.5. \text{All receptor mutants} were confirmed by automated DNA sequencing.

\section{2.2 Cell Culture and Transfection}

For expression in mammalian cells, we used COS1 cell line (American Type Culture Collection, Rockville, MD) which is a green monkey kidney fibroblast cell line. This cell line has been transformed with the large T antigen of SV40 which also immortalizes the cell line. High levels of expression can be achieved transiently by transfecting COS1 cells with plasmids that carry the SV40 origin of replication. COS1 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin/streptomycin and 10\% fetal bovine serum. Cell cultures were incubated at 37 °C in 5\% CO$_2$.

COS-1 cells were grown in 100-mm culture dishes to approximately 60-70\% confluence and transiently transfected by FuGENE 6 Transfection Reagent (Roche Applied Science, IN) according to manufacturer’s instructions. Briefly, 18 \text{μl} of FuGENE 6 reagent was added into 600 \text{μl} of OPTI-MEM reduced serum media. 6 \text{μg} plasmid DNA was then added and incubated for 15 min at room temperature. The mixture was then added into the culture medium. The transfection efficiency was 40-50 \% as determined by transfection of cells with a plasmid encoding both AT1R and green fluorescent protein (GFP) (Fig. 2.6).
Figure 2.5. The agarose gel fractionation of HA-CYS<sup>−</sup>AT1R and 20 single cysteine mutants. 2 µg plasmid DNA is dissolved in H2O in a total volume of 20 µl. 4 µl of 6X loading dye (Promega, WI) is added. M, 2 µl of 1 Kb Plus DNA ladder (Invitrogen) mixed with 14 µl of TE buffer and 4 µl of 6X loading dye. Samples were run on 1 % agarose gel at 90 Volts.
Figure 2.6. Transfection efficiency in COS1 cells. A) Phase picture of COS1 cells (Magnification X10). B) COS1 cells transfected with pEGFP-AT1R (Magnification X10). The transfection efficiency was estimated by a rough count of [number of GFP cells/number of DAPI stained cellsx100%].
2.3 Western Blotting

After 48 hours incubation, transfected cells were placed on ice and washed twice with ice-cold phosphate buffered saline (PBS). Cells were scraped and transferred into centrifuge tubes and spun at 3900 rpm for 5 min at 4°C. Cells were lysed in Mammalian Protein Extraction Reagent (MPER, 25mM bicine, CHAPS and N-tetradecyl-N, N-dimethyl-3-ammonia-1 propanesulfonate) (Pierce) containing protease (Sigma) and phosphatase inhibitors (Thermo Scientific) with continuous rotation at 4°C for 25 min. The lysate was centrifuged at 13200 rpm for 10 min at 4°C and the supernatant was analyzed for protein concentration by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a concentration standard. 50 μg of protein from each sample was incubated with 4X NuPAGE LDS sample buffer (Invitrogen) containing 10% 2-mercaptoethanol at room temperature for 5 min and resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) and electroblotted onto nitrocellulose membrane (0.45 µm) (Bio-Rad). The membrane was blocked in blocking buffer (PBS with 0.5 % Tween-20 and 5% nonfat dried milk) for 1 hour at room temperature. The membrane was then incubated with mouse anti-HA monoclonal antibody (Roche) (1:1000) in blocking buffer at 4°C for overnight. The membrane was washed three times for 10 min each with PBST (PBS containing 0.5 % Tween-20) and incubated with HRP-conjugated anti-mouse IgG (GE Healthcare) (1:5000) in blocking buffer for 1 hour at room temperature. The membrane was washed and the receptor expression was detected using ECL Plus western blotting detection system (Amersham).
2.4 Immunocytochemistry

24 hours post-transfection, cells were plated onto poly-L-lysine coated coverslips placed in 6-well culture dishes. Cells were cultured for 24 hours in DMEM supplemented with 10% fetal bovine serum and switched to serum-free DMEM 18 h before treatment. Cells were fixed with 4% paraformaldehyde in PBS for 30 min and washed three times with PBS for 10 min each. Cells were then permeabilized with 0.3% Triton X-100 in PBS for 20 min on ice and blocked with 5% nonfat dry milk in PBS for 1 hour at room temperature. Cells were incubated overnight with mouse anti-HA monoclonal antibody (1:1000) in blocking buffer at 4°C, washed and incubated with AlexaFlour 568 anti-mouse antibody (1:2000) for 1 hour at room temperature in the dark. Cells were then washed and mounted on coverslips on glass slides for confocal microscopy analysis.

2.5 Saturation Binding Assays

72 hours post-transfection, cells were placed on ice and washed twice with ice cold PBS. Cells were gently scraped and transferred into centrifuge tubes and spun at 1000 rpm for 5 min at 4 °C. Cells were gently resuspended in binding buffer (140 mM NaCl, 5.4 mM KCl, 1mM EDTA, 0.006% BSA, 25 mM HEPES, pH 7.4). Protein concentration was determined by Bradford method (Bradford, 1976) using BSA as a concentration standard. Binding assays were performed under equilibrium conditions, with \[^{125}\text{I}\]-[Sar1,Ile8] Ang II (Dr. Robert Speth, University of Mississippi) concentrations ranging between 0.125-12 nM (specific activity: 2176 Ci/mmol) for 1 hour at room temperature as previously described (Noda et al, 1996, Miura et al, 2002 & Miura et al, 2003). Nonspecific binding was measured in the presence of $10^{-5}$ M \[^{127}\text{I}\]-[Sar1,Ile8] Ang
II (Bachem). 25 μl of binding buffer or \(^{[127I]}\)-[Sar\(^1\)Ile\(^8\)] Ang II was mixed with 50 μl of increasing concentrations of \(^{[125I]}\)-[Sar\(^1\),Ile\(^8\)] and 50 μl of cell suspension in total volume of 125 μl. Each concentration was repeated in duplicates in 96-well plates. The cells were harvested with Combi cell harvester (Skatron Instruments, Norway) by filtering the binding mixture through Whatman GF/C glass fiber filters (102×256 mm), which were extensively washed with washing buffer (20 mM sodium phosphate, 100 mM NaCl, 10 mM MgCl\(_2\), 1 mM EGTA, pH 7.2). The bound ligand fraction was determined using 10/600 Apex gamma counter (Titertek Instruments) as the counts per minute (cpm) remaining on the membrane. The binding kinetics was analyzed by nonlinear curve fitting program Ligand\(^R\) which yields mean±S.D. for \(K_d\) and \(B_{max}\) values.

2.6 Analysis of ERK Phosphorylation

48 hours post-transfection, cells were serum starved for 18 h and treated with 1 μM Ang II (Bachem) for 10 min at room temperature. Cells were washed twice with ice-cold PBS. Cells were lysed and immunoblotted as described above and the membrane was blocked in blocking buffer (PBS with 0.5 % Tween-20 and 5% BSA) for 1 hour at room temperature. The membrane was incubated overnight with phospho-ERK 42/44\(^{MAPK}\) polyclonal antibody (Cell Signaling) (1:1000) in blocking buffer. The membrane was then washed and incubated with HRP-conjugated anti-rabbit IgG (1:5000, GE Healthcare) in blocking buffer for 1 hour at room temperature. Immunoreactivity was detected using ECL Plus western blotting detection system. The membranes were stripped and similarly analyzed for total ERK 42/44\(^{MAPK}\) (Cell Signaling) reactivity.
Results were used to correct pERK 42/44MAPK measurements for slight differences in sample protein content.

### 2.7 Intracellular Calcium Measurement

Changes in cytoplasmic Ca\(^{2+}\) were measured using fluorescent calcium indicator Fura-2 (excitation maximum 340 and 380 nm; emission maximum 510 nm) (Invitrogen) (29). The principle of Ca\(^{2+}\) measurement assay by Fura-2 is explained in Figure 2.7. Cells were grown on coverslips in 6-well culture dishes. 48 hours post transfection, cells were loaded with 1 µm Fura-2 acetoxy methyl ester 2 in BSS-Ca\(^{2+}\) buffer (140 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 5.5 mM Glucose, 10 mM Hepes, 0.1% BSA and 2 mM CaCl\(_2\), pH 7.4). After 30 min incubation at 37 °C cells were washed with BSS-Ca\(^{2+}\) to remove the extracellular dye. Calcium measurements were done in single cells using an inverted microscope (Zeiss Axiovert 135) connected to a CCD camera (Photon Technology International). The data were collected at 1.5 s intervals and analyzed using Image Master software (Photon Technology International). The release of intracellular Ca\(^{2+}\) in individual cells was measured after exposure to 1 µM Ang II in BSS-Ca\(^{2+}\) by rapid solution exchange. Results were presented as average changes in the ratio of Fura-2 fluorescence upon excitation at 340 nm and 380 nm and plotted using SigmaPlot.

### 2.8 MTSEA-biotin Labeling

MTSEA-biotin (N-biotinylaminoethylmethanethiosulfonate, Toronto Research Chemicals) in powder was stored as desiccated, dissolved in DMSO and aliquots of 0.1M
Figure 2.7. Intracellular Calcium measurement by Fura2. A) Fura-2 is a membrane impermeable UV-excitable fluorescent calcium indicator. Fura2-acetoxyethyl (Fura-2AM) is a membrane-permeable derivative of the Fura2. Once inside the cells, the acetoxyethyl groups are removed by cellular esterases. Removal of the acetoxyethyl esters traps Fura2 inside the cells. B) Fura2 is a ratiometric dye which is excited at 340 nm and 380 nm. The ratio of the emissions at 510 nm is directly correlated to the amount of intracellular calcium.
stock aqueous solutions were thawed just prior to use and kept on ice. Experiments were repeated at least three times as described below. 48 h post-transfection, cells were washed twice with PBS, harvested using non-enzymatic cell dissociation solution (Sigma) and suspended in PBS. 500 µl aliquots of cells were incubated with 10 mM MTSEA-biotin at 25 °C for 30 min. The incubation time and MTSEA-biotin concentration were standardized to obtain maximum signal strength (Fig. 2.8). The reaction was stopped by diluting with cold PBS. Cells were spun at 3900 rpm for 5 min at 4 °C and resuspended in NP40 lysis buffer [1% NP40, 20 mM Tris (pH 7.4), 137 mM NaCl, 20% glycerol, and protease and phosphatase inhibitors]. Protein concentration was determined by Bradford method (Bradford, 1976) using BSA as a concentration standard. 750 µg of protein was incubated overnight with 4 µl of mouse anti-HA monoclonal antibody in lysis buffer. The receptors were immunoprecipitated with Protein-G agarose beads (Millipore) by continuous rotation for 2 hours at 4 °C. Beads were then washed four times with cold PBS and proteins were extracted by heating at 70°C in NuPAGE LDS sample buffer for 10 min. Samples were immunoblotted as described above and probed with Streptavidin-HRP (Amersham) according to manufacturer’s instructions. Biotinylation level of receptors was determined using KODAK 1D 3.6. To control for the differences of receptors’ expression levels, the same blot was reprobed with anti-HA antibody as described above. MTSEA-biotin accessibility of each receptor was obtained by normalizing the biotinylation level of each receptor to its expression level. The same procedure was repeated following either 1 µM Ang II (Bachem) or 1 µM losartan (DuPont Merck Co, Wilmington, DE) treatment for 10 min at room temperature (Fig 2.9).
Figure 2.8. Assay standardization. A) Immunoprecipitation control experiment of HA-CYS\textsuperscript{−}AT1R (lane 1) and HA-AT1R (lane 3) with anti-HA antibody. Negative controls lack anti-HA antibody (lanes 2 and 4). Untransfected cells are also shown as negative controls. The fully glycosylated monomeric receptor band is shown by arrow. B) Standardization of MTSEA-biotin concentration was performed with 2 mM, 5 mM and 10 mM MTSEA-biotin concentrations. C) Standardization of incubation time was performed with 0-10 min time points using 10 mM MTSEA-biotin.
Figure 2.9. Experimental strategy for MTSEA-biotin accessibility measurement.

Intact COS1 cells transfected with HA-CYS\textsuperscript{AT1R} or mutants

- Buffer, 10 min
- 1\mu M Ang II, 10 min
- 1\mu M losartan, 10 min

10 mM MTSEA-biotin incubation, 30 min, 25°C

Stop reaction and solubilize with M-PER buffer

- IP: Anti-HA

- IB: Streptavidin

- Stripped

- IB: Anti-HA
2.9 Molecular Dynamics Simulations

Molecular Dynamics (MD) simulations were performed using the MD software package NAMD (Phillips et al, 2005) and the CHARMM 22. The modeled structure of AT1R (PDB ID: 2AC6) and AT1R with Ang II (PDB ID: 2AH3) (Gogonea and Karnik, 2006) was downloaded from the Protein Data Bank, and explicit hydrogen were added using the package VEGA ZZ 2.0.7 (Pedretti et al, 2004). This structure was subjected to 1000 steps of energy minimization. The energy-minimized system was heated from 0°C to 310°C in 31°C intervals over the course of 20,000 steps. Simulations were carried out for 14 ns. The root-mean-square deviation of the backbone of AT1R with Ang II was stabilized after 7 ns. MD simulations were carried out using the molecular modeling software VEGA ZZ 2.0.7. We simulated ECL2 residues (Ile\textsuperscript{165} - Pro\textsuperscript{192}) with selected loop constraints and other receptor regions were not simulated. The ECL2 simulations were carried out by imposing Cys\textsuperscript{101}-Cys\textsuperscript{180} disulphide bond as a constraint in different states. The disulphide bond constraint was relaxed for 1 ns at the end of 14 ns simulation. We used PyMOL (DeLano Scientific LLC) to visualize the output. A schematic explanation for steps of MD simulation is given in Figure 2.10.

2.10 Losartan Docking

Blind docking was carried out using AutoDock4 Vina (Trott and Olson, 2009). The starting conformation of losartan was an energy minimized form. The receptor was held rigid during the docking process while the ligand was allowed to be flexible to save computational time. The grid box size was 38.0Å -20.8Å-26.4Å in the x, y and z dimensions, with the center of the grid corresponding to the central axis of the pore at
Lys$^{199}$ and His$^{256}$ at the base of the selectivity filter. Exhaustiveness was set as default.

We used PyMOL to visualize the output.

2.11 Statistical Analysis

The results are expressed as the means ± Standard error of mean (SEM) and analyzed by unpaired t test for statistical significance to compare variables between mutants and wild type using Graphpad Prism$^R$. Significance is set at $p < 0.05$. 
Figure 2.10. Schematic explanation of MD simulation procedure.

The model of AT1R (PDB ID: 2AC6) and AT1R-Ang II (PDB ID: 2AH3)
14 ns simulation with backbone atoms and constrained disulfide bond
   ↓
1 ns simulation with relaxed disulfide bond
   ↓
1000 steps of energy minimization
   ↓
Select frames consistent with experimental results
   ↓
Add side chains
CHAPTER III

HA-CYS\textsuperscript{\textregistered}AT1R

3.1 Introduction

The structural analysis of transmembrane proteins such as AT1R through direct techniques, including x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy has been challenging. AT1R is expressed at very low levels (40-80 fmoles/mg) in native tissues. In addition, the structural flexibility of AT1R causes thermodynamic and proteolytic instability during detergent-solubilization and purification. Many of these experimental constrains have been overcome by higher level expression (>100-fold compared to native cells) of receptor genes in recombinant systems. Increasing the level of expression of a GPCR often requires alteration of some structural features, such as attachment of a signal sequence, introduction of stabilizing mutations, or deletion of some segments of the receptor. Recombinantly expressed β2 adrenergic, β1 adrenergic and the adenosine A1 receptors have been crystallized. Thus, protein engineering and recombinant expression of GPCRs has advanced the understanding of structure-function relationships of GPCRs.
Even when the high-resolution crystal structure is obtained, it represents a rigid, single conformation of a GPCR. We need other approaches to address questions concerning the dynamics and conformational changes associated with different states of activation, which allow the receptor to interact with a variety of diffusible ligands and generate diverse intracellular responses. Therefore we require additional indirect techniques to elucidate the dynamic properties of GPCRs such as AT1R. We planned to use a cysteine accessibility mapping approach to determine the dynamic changes in AT1R in different states including unbound, agonist-bound and antagonist-bound states. As described in Section 1.4, our accessibility mapping strategy requires introduction of cysteine residues into the region of interest one-at-a-time and measurement of the level of reactivity of engineered cysteines with thiol-specific alkylating reagents, which estimate the water accessibility of each residue.

To effectively use this technique for AT1R, it would be ideal to construct a receptor which lacks all non-disulfide bonded cysteine residues. AT1R has ten native cysteines, four of which are disulfide bonded and six of which are not disulfide bonded (Fig. 3.1). Two disulfide bonds in the AT1R are critical for maintaining the 7 TM structure and the conformational changes associated with receptor activation/inhibition (Ohyama et al, 1995, Feng et al, 2000). Disulfide bonded cysteines also do not react with thiol-specific alkylating reagents. Some of the free cysteines may be buried during folding of the receptor to the three dimensional structure and may not be accessible to the thiol-specific alkylating reagents. However, one can not be sure if the buried cysteines are exposed during receptor activation or inhibition. The cysteines that are partially
Figure 3.1. The secondary structure model of HA-CYS\textsuperscript{AT1R}. Native non-essential free cysteine residues, Cys\textsuperscript{76}, Cys\textsuperscript{121}, Cys\textsuperscript{144}, Cys\textsuperscript{289}, Cys\textsuperscript{296} and Cys\textsuperscript{355}, substituted with alanine are shown in green circles. Native cysteines involved in formation of essential disulfide bonds, Cys\textsuperscript{18}-Cys\textsuperscript{274} and Cys\textsuperscript{101}-Cys\textsuperscript{180}, are shown in gold circles.
accessible might interfere with the accessibility measurements of engineered cysteines. Therefore we created a receptor surrogate of HA-AT1R named as HA-CYS\textsuperscript{−}AT1R that lacks all non disulfide-bonded cysteines. It is very important to rigorously characterize the modified protein for functionality to be able to use it for mapping the conformation associated with different states of the receptor. In this chapter we describe the construction and characterization of HA-CYS\textsuperscript{−}AT1R. We show that pharmacological and signal transduction properties of HA-CYS\textsuperscript{−}AT1R are similar to those of HA-AT1R.

### 3.2 HA-CYS\textsuperscript{−}AT1R Nucleotide Sequence Confirmation

The mutagenesis steps used in the construction of HA-CYS\textsuperscript{−}AT1R are schematized in detail in Figure 2.1. In the HA-CYS\textsuperscript{−}AT1R, native cysteines involved in the formation of disulfide bonds, Cys\textsuperscript{18}-Cys\textsuperscript{274} and Cys\textsuperscript{101}-Cys\textsuperscript{180}, which are essential for receptor stability (Ohyama et al, 1995 & Feng et al, 2000) were not mutated (Fig. 3.1). The codons for these cysteine residues were unaltered (Table III.I). Codons for other native non-essential free cysteine residues (Cys\textsuperscript{76}, Cys\textsuperscript{121}, Cys\textsuperscript{144}, Cys\textsuperscript{289}, Cys\textsuperscript{296} and Cys\textsuperscript{355}) were substituted with alanine codons. The substituted alanine codons in the HA-CYS\textsuperscript{−}AT1R that were confirmed by DNA sequencing are listed in Table III.I. A comparison of nucleotide sequences of HA-AT1R and HA-CYS\textsuperscript{−}AT1R is shown in Figure 3.2 for three representative alanine substituted cysteine codons; Cys\textsuperscript{76}, Cys\textsuperscript{121} and Cys\textsuperscript{149}. The HA-CYS\textsuperscript{−}AT1R will serve as the background to introduce reporter cysteine residues in the ECL2.
Table III.I Cysteine substitutions in HA-CYS\textsuperscript{−}AT1R. HA-AT1R was subjected to site-directed mutagenesis as described in Section 2.1, creating HA-CYS\textsuperscript{−}AT1R in which 6 of the 10 cysteine residues of HA-AT1R was substituted with alanine. DNA sequence analysis confirmed C76A, C121A, C144A, C289A, C296A, C355A substitutions in HA\textsuperscript{−}CYS-AT1R as shown. The codons for Cys\textsuperscript{18}, Cys\textsuperscript{101}, Cys\textsuperscript{180} and Cys\textsuperscript{274} which are involved in disulfide bond formation were not modified as indicated in bold. The DNA sequencing chromatogram for corresponding residues of HA-CYS\textsuperscript{−}AT1R is also shown. Green, A; Red, T; Blue, T; Black, G.

<table>
<thead>
<tr>
<th>HA-AT1R</th>
<th>HA\textsuperscript{−}CYS-AT1R</th>
<th>Codon change</th>
<th>Chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys18</td>
<td>Cys18</td>
<td>TGC → TGC</td>
<td><img src="image1" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Cys76</td>
<td>Ala76</td>
<td>TGC → GCC</td>
<td><img src="image2" alt="Chromatogram" /></td>
</tr>
<tr>
<td><strong>Cys101</strong></td>
<td>Cys101</td>
<td>TGT → TGT</td>
<td><img src="image3" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Cys121</td>
<td>Ala121</td>
<td>TGT → GCT</td>
<td><img src="image4" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Cys149</td>
<td>Ala149</td>
<td>TGC → GCC</td>
<td><img src="image5" alt="Chromatogram" /></td>
</tr>
<tr>
<td><strong>Cys180</strong></td>
<td>Cys180</td>
<td>TGC → TGC</td>
<td><img src="image6" alt="Chromatogram" /></td>
</tr>
<tr>
<td><strong>Cys274</strong></td>
<td>Cys274</td>
<td>TGT → TGT</td>
<td><img src="image7" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Cys289</td>
<td>Ala289</td>
<td>TGC → GCC</td>
<td><img src="image8" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Cys296</td>
<td>Ala296</td>
<td>TGT → GCT</td>
<td><img src="image9" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Cys355</td>
<td>Ala355</td>
<td>TGT → GCA</td>
<td><img src="image10" alt="Chromatogram" /></td>
</tr>
</tbody>
</table>
Figure 3.2. Comparison of chromatograms of HA-AT1R and HA\textsuperscript{CYS}-AT1R. The sequences of Cys\textsuperscript{76}, Cys\textsuperscript{121} and Cys\textsuperscript{149} positions of HA-AT1R and the corresponding alanine substitutions in HA\textsuperscript{CYS}-AT1R are shown as an example. Green, A; Red, T; Blue, T; Black, G.
3.3 Characterization of HA-CYS\textsuperscript{−}AT1R

The expression of HA-AT1R and HA-CYS\textsuperscript{−}AT1R, compared by immunoblotting, showed similar levels in transiently transfected COS1 cells. Both receptors appear to be fully glycosylated (41.9 kDa). When analyzed by SDS-PAGE, unglycosylated (~37 kDa) and partially glycosylated (~40 kDa) monomeric forms of the receptors as well as small amounts of higher molecular weight oligomers (50-80 kDa) were present (Fig. 3.3 A) in both. Other properties in terms of cell surface expression, cellular localization, binding affinity for Ang II and agonist activated signaling were compared. Immunocytochemical anlaysis indicated that both HA-AT1R and HA-CYS\textsuperscript{−}AT1R were expressed on the plasma membrane at similar levels (Fig. 3.3 B). The $K_d$ values of HA-AT1R and HA-CYS\textsuperscript{−}AT1R for the peptide antagonist $^{125}$I-[Sar\textsuperscript{1}, Ile\textsuperscript{8}]Ang II, obtained by saturation binding analysis performed on intact cells, were 3.5 nM and 4.4 nM, respectively (Fig 3.4 A). The cell surface $B_{max}$ values measured from scatchered plots were 3.5 pmol/mg and 5.8 pmol/mg for HA-AT1R and HA-CYS\textsuperscript{−}AT1R respectively (Fig. 3.4 B).

To analyze the Ang II-induced signal transduction capacity of HA-CYS\textsuperscript{−}AT1R, we measured ERK1/2 activation and intracellular Ca\textsuperscript{2+} release upon 1 µM Ang II treatment (Fig. 3.5 A). The level of ERK1/2 activation of HA-CYS\textsuperscript{−}AT1R was comparable to HA-AT1R (Fig. 3.5 B). The Ang II-induced ERK1/2 activation was inhibited by the AT1R-selective non-peptide antagonist, losartan (Fig. 3.5 B). Ang II-induced mobilization of intracellular calcium, which is a measure of G protein activation, was similar in HA-CYS\textsuperscript{−}AT1R and HA-AT1R (Fig. 3.5 C). These results suggested that cell surface
Figure 3.3. Expression analysis of HA-AT1R and HA-CYS\textsuperscript{-}AT1R. A) Western blot analysis of HA-AT1R (lane 4) and HA-CYS\textsuperscript{-}AT1R (lane 5) expression in COS1 cells. Cells which are mock transfected (MT) (lane 1), transfected with pMT3 (lane 2) and CYS\textsuperscript{-}AT1R without HA tag (lane 3) are shown as negative controls. The 64 kDa band corresponds to a nonspecific, cross reacting protein. 3 differentially glycosylated monomeric bands of the receptor are identified as fully-, partial- and single-glycosylated bands. Higher molecular weight oligomeric forms of the receptor can be seen between 51-80 kDa. B) The localization of HA-AT1R (panel 2) and HA-CYS\textsuperscript{-}AT1R (panel 3) on the plasma membrane of COS1 cells. Untransfected cells are shown as negative control (panel 1). Cells were labeled with DAPI (blue) for nucleus and with mouse HA monoclonal primary antibody and Alexa flour 568 (red) anti-mouse secondary antibody for HA-tagged receptors. Plasma membrane is indicated by yellow arrow.
Figure 3.4. Binding analysis of HA-AT1R and HA-CYS\textsuperscript{-}AT1R. A) Saturation curves of HA-AT1R and HA-CYS\textsuperscript{-}AT1R determined using [125I]-[Sar\textsuperscript{1}, Ile\textsuperscript{8}] Ang II. Inset shows the corresponding Scatchard plot. B) Cell surface density of HA-AT1R and HA-CYS\textsuperscript{-}AT1R expressed as mean Bmax values derived from Scatchard plots. Error bars indicate the S.E.M., p=0.082 (n=3).
Figure 3.5. Activity analysis of HA-AT1R and HA-CYS−AT1R. A) The signal transduction pathway of AT1R activated by Ang II binding. The intracellular Ca2+ release and MAPK activation were analyzed as read-out of Ang II induced activity. B) ERK1/2 phosphorylation (p-ERK) upon treatment with 1 μM Ang II on untransfected cells (lanes 1-2), HA-AT1R (lanes 3-4) and HA-CYS−AT1R (lanes 7-8) transfected cells. ERK1/2 phosphorylation is inhibited by losartan treatment prior to Ang II treatment (lane 5 & 9) and together with Ang II treatment (lane 6 & 10). Total ERK (t-ERK) levels are shown as loading control. The plot corresponds to the p-ERK/t-ERK ratio of lanes 3,4,6,7,8,10. C) Measurement of intracellular Ca2+ mobilization upon 1 μM Ang II treatment in HA-AT1R and HA-CYS−AT1R transfected COS1 cells. The time point of Ang II treatment is indicated by the arrow.
expression, Ang II binding affinity, activation and inhibition properties of HA-CYS\(^{-}\)AT1R are comparable to those of wild type HA-AT1R.

### 3.4 Reaction of HA-AT1R and HA-CYS\(^{-}\)AT1R with Cysteine Reactive MTSEA-biotin

We anticipated that the HA-CYS\(^{-}\)AT1R would not react with thiol-specific modifying reagent, MTSEA-biotin (N-biotinylaminoethylmethanethiosulfonate). MTSEA-biotin consists of a biotin moiety attached to the highly reactive methanethiosulfanate. The MTSEA-biotin (Fig. 3.6 A) does not react with disulfide bonded or buried cysteine residues, but rapidly reacts with water-exposed reactive cysteines (Akabas et al, 1992).

![Chemical Reaction Diagram](image)

The resulting site-specific covalent attachment of biotin (red) to engineered cysteine was detected by streptavidin-HRP. Figure 2.9 explains the MTSEA-biotin labeling strategy. The receptor topology was preserved during MTSEA-biotin reaction as closely as possible to the native state in the plasma membrane by using adherent cells rather than fractionated membranes.

The anticipated reaction of HA-AT1R and HA-CYS\(^{-}\)AT1R with MTSEA-biotin is depicted in Figure 3.6 A. Theoretically; HA-AT1R carrying 6 free cysteines is
expected to be six times more reactive to MTSEA-biotin compared to HA-CYS\(^{-}\)AT1R, which lacks all the free cysteines. We compared the reactivity of HA-AT1R and HA-CYS\(^{-}\)AT1R with MTSEA-biotin (Fig. 3.6 B). Following MTSEA-biotin treatment, detergent solubilized HA-tagged receptors were immunoprecipitated using anti-HA antibody and probed with streptavidin-HRP. Band intensities observed by HA blot were similar, which controls for expression levels of two receptors. Fully glycosylated monomeric receptor band was monitored for the quantification (Fig. 3.6 C). The level of biotinylation was estimated by normalizing the streptavidin-HRP signal to the HA signal for each sample. MTSEA-biotin modification of HA-AT1R which has six native free cysteines is \(\approx 2\)-fold more than HA-CYS\(^{-}\)AT1R which lacks those cysteines (Fig. 3.6 D). This result is consistent with the previous study by Miura & Karnik (2002), which demonstrated that among the 6 native free cysteines in wild type AT1R, only Cys\(^{76}\) in TMII of AT1R is fully reactive to MTSEA (Fig. 3.7). The native Cys\(^{289}\) and Cys\(^{296}\) were partially reactive to MTSEA. The authors measured the MTSEA sensitivity of native cysteine residues of the AT1R and individual Cys\(\rightarrow\)Ala mutants. This data explains our observation of 2-fold more reactivity of HA-AT1R compared to HA-CYS\(^{-}\)AT1R.

The results discussed in this chapter suggest that HA-CYS\(^{-}\)AT1R is functionally similar to HA-AT1R. It appears that the disulfide bonds are formed appropriately and maintain the stability of the HA-CYS\(^{-}\)AT1R as anticipated. The four disulfide bonded cysteine residues in AT1R are sufficient for proper folding and maintenance of the tertiary structure of the receptor polypeptide. It is quite impressive to observe that replacing six free cysteines did not alter expression and affinity of the receptor for both
Figure 3.6. MTSEA-biotin accessibility of HA-AT1R and HA-CYS\textsuperscript{-}AT1R. A) The anticipated reaction of HA-AT1R which has 6 free cysteines with 6 MTSEA-biotin. B) HA and Streptavidin-HRP blots comparing levels of expression and MTSEA-biotin labeling, respectively. The band representing the fully-glycosylated, mature form of the receptor that has been used for quantification of MTSEA-biotin relative accessibility is shown in red box. C) The cropped image of panel A only indicates the receptor band that has been used for quantification of MTSEA-biotin relative accessibility. D) The corresponding plot shows the MTSEA-biotin relative accessibility which is the ratio of Streptavidin-HRP signal intensity to HA signal intensity for particular sample. The red line indicates the level of biotinylation of HA-CYS\textsuperscript{-}AT1R without any free cysteine which is determined as the cut-off below which the reactivity is considered inaccessible. The observed stoichiometry of the reaction is shown.
A

\[
\text{+ HA-AT1R (6 free cysteines) } \rightarrow \text{ HA-AT1R + 6 CH}_3\text{S-H + 6 S-biotin}
\]

\[
\text{+ HA-CYS AT1R (0 free cysteines) } \rightarrow \text{ HA-CYS AT1R + 6 MTSEA-biotin}
\]

B

IB: HA

IB: Streptavidin-HRP

C

D

MTSEA-biotin
relative intensity
Figure 3.7. Inhibition of ligand binding following reaction of MTSEA with AT1R and CYS\(^{-}\)AT1R. A) Time course inhibition of specific binding of \(^{125}\text{I-}[\text{Sar}^1, \text{Ile}^8]\text{Ang II}\) to the AT1R and mutant receptors, CYS\(^{-}\)AT1R, C76A, C289A and C296A after treatment with 2.5 mM MTSEA. B) Dose response inhibition of specific binding to the AT1R and mutant receptors, CYS\(^{-}\)AT1R, C76A and C289A after treatment with 2.5 mM MTSEA. Details are given in references 5 and 7. In the wild type receptor, MTSEA inhibited [Sar\(^1\),Ile\(^8\)]Ang II binding whereas binding in the CYS\(^{-}\)AT1R and C76A mutant was resistant to MTSEA reaction. Therefore, the inhibition of [Sar\(^1\), Ile\(^8\)]Ang II binding is due to reaction of MTS reagents with Cys\(^{76}\) in the TMII and the remaining free cysteine residues are not accessible (from Miura & Karnik, 2002).
agonist and antagonist. The Cys\textsuperscript{355} in AT1R is analogous to the cysteine residue that is palmitoylated in rhodopsin and adrenergic receptors. The palmitoylated cysteine may stabilize the C-terminal tail of the receptor by providing a pseudo cytoplasmic loop (Sachs et al, 2000). However, the substitution of Cys\textsuperscript{355} with alanine did not affect the stability and function of the AT1R in our study. In addition HA-CYS\textsuperscript{−}AT1R is less sensitive to MTSEA-biotin compared to HA-AT1R. Therefore, we used the HA-CYS\textsuperscript{−}AT1R as a template to create 20 single cysteine substituted ECL2 mutants used in our accessibility analysis.
4.1 Introduction

Chemical reactions that are rather specific for functional groups of each type of amino acid side chain have historically played a significant role in the development of foundational concepts of protein structure and function. Chemical modifications are possible for several residues carrying reactive groups on their side chains, including histidine, lysine, arginine, methionine, tryptophane, tyrosine and cysteine. Wider use of chemical modification is hampered because modification reactions use harsh conditions which may cause structural or conformational changes in the protein, affecting the biological function. The thiol group of cysteine residues is one of the most versatile functional groups, amenable to probing in terms of surface location or burial, location in static or dynamic regions of proteins. The cysteine thiol group is the most reactive among the functional groups of amino acids. Many reagents are available that specifically react with thiol groups. Thiol groups can be modified through metal binding, acylation, alkylation, cyanlation and oxidation under conditions that preserve the relevant biological functions of proteins (Chalker et al, 2009).
The advent of site-directed mutagenesis expanded the use of cysteine thiols as reporter groups in defining details of protein structure, function and dynamics that are not easily approachable by time-consuming methodologies including crystallography and NMR, which also require very expensive instrumentation. In many instances the reporter cysteine mapping is the only practical approach to study the protein in its native microenvironment. Cysteine substitutions can be directed to any region of a protein. The overwhelming experience is that the substituted cysteines are well tolerated in most biological systems. In the study of TM receptors, cysteine scanning mutagenesis is the method of choice because very few cysteine substitution mutants fail to yield functional receptors on the cell surface. Thus, cysteine substitutions are very well tolerated (Kaback et al, 1997).

In this chapter, we describe an extensive characterization of single cysteine mutants in ECL2, a 20 residue long segment of the extracellular domain of AT1R with poorly defined functions. It is necessary to ascertain that no gross conformational change occurred in the AT1R upon each modification. Only cysteine substitution mutants with near wild-type function can be used for further modification because we assume that the substituted cystiene side chain has wild-type-like orientation and rotational freedom.

4.2 Expression Analysis of ECL2 Single Cysteine Mutants

The mutagenesis strategy used in the construction of ECL2 mutants is schematized in detail in Figure 2.4. DNA sequencing confirmed that each native residue
in ECL2 was substituted with a cysteine, one at a time. The substituted cysteine codons in the ECL2 mutants are listed in Table IV.I.

The expression of each single cysteine substitution mutant upon transient transfection of COS1 cells was determined by western blotting. Western blot analysis showed that most of the mutant receptors were expressed at a similar level to that of HA-CYS-AT1R. Transient transfection of 18 single cysteine mutant plasmids yielded ≈ 42 kDa fully glycosylated mature form of the functional receptor protein in COS1 cells (Fig. 4.1). Variable expression seen due to variability in transient transfection was not statistically significant. Thus introduction of cysteine side chains at 18 different positions of ECL2 in the AT1R did not significantly interfere with the overall folding and transport to cell surface.

The expression level of mutant I177C was reduced and the V179C mutant receptor protein appeared to be degraded. To understand the reasons for instability of these two mutant receptors in COS1 cells, we carried out additional site directed mutagenesis. Serine substitution for Ile\textsuperscript{177} significantly reduced the expression whereas substitution of alanine was normal. This suggested that a hydrophobic residue is essential at position 177 for normal expression of the receptor (Fig. 4.2). Substitution of both serine and alanine for Val\textsuperscript{179} produced stable receptor protein, suggesting that the cysteine side chain introduced at position 179 causes instability. The reasons for instability of these mutant receptors are unclear. However, since Ile\textsuperscript{177} and Val\textsuperscript{179} are n-3 and n-1 positioned residues from conserved Cys\textsuperscript{180}, the side chains of these residues may
Table IV.I. Cysteine scanning mutagenesis of ECL2. HA-CYS^AT1R was subjected to oligonucleotide mediated site-directed mutagenesis, creating a series of 20 mutants in which each of the 20 residues within ECL2 was individually changed to cysteine. The native disulfide bonded cysteine is indicated in bold.

<table>
<thead>
<tr>
<th>Residue no</th>
<th>Amino acid change</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>172</td>
<td>Ile → Cys</td>
<td>ATC → TGC</td>
</tr>
<tr>
<td>173</td>
<td>Glu → Cys</td>
<td>GAG → TGC</td>
</tr>
<tr>
<td>174</td>
<td>Asn → Cys</td>
<td>AAC → TGC</td>
</tr>
<tr>
<td>175</td>
<td>Thr → Cys</td>
<td>ACC → TGC</td>
</tr>
<tr>
<td>176</td>
<td>Asn → Cys</td>
<td>AAT → TGC</td>
</tr>
<tr>
<td>177</td>
<td>Ile → Cys</td>
<td>ATC → TGC</td>
</tr>
<tr>
<td>178</td>
<td>Thr → Cys</td>
<td>ACA → TGC</td>
</tr>
<tr>
<td>179</td>
<td>Val → Cys</td>
<td>GTG → TGC</td>
</tr>
<tr>
<td>180</td>
<td>Cys → Cys</td>
<td>TGC → TGC</td>
</tr>
<tr>
<td>181</td>
<td>Ala → Cys</td>
<td>GCA → TGC</td>
</tr>
<tr>
<td>182</td>
<td>Phe → Cys</td>
<td>TTT → TGC</td>
</tr>
<tr>
<td>183</td>
<td>His → Cys</td>
<td>CAT → TGC</td>
</tr>
<tr>
<td>184</td>
<td>Tyr → Cys</td>
<td>TAT → TGC</td>
</tr>
<tr>
<td>185</td>
<td>Glu → Cys</td>
<td>GAG → TGC</td>
</tr>
<tr>
<td>186</td>
<td>Ser → Cys</td>
<td>TCT → TGC</td>
</tr>
<tr>
<td>187</td>
<td>Arg → Cys</td>
<td>CGA → TGC</td>
</tr>
<tr>
<td>188</td>
<td>Asn → Cys</td>
<td>AAT → TGC</td>
</tr>
<tr>
<td>189</td>
<td>Ser → Cys</td>
<td>TCG → TGC</td>
</tr>
<tr>
<td>190</td>
<td>Thr → Cys</td>
<td>ACG → TGC</td>
</tr>
<tr>
<td>191</td>
<td>Leu → Cys</td>
<td>CTC → TGC</td>
</tr>
<tr>
<td>192</td>
<td>Pro → Cys</td>
<td>CCG → TGC</td>
</tr>
</tbody>
</table>
Figure 4.1. Expression of ECL2 single cysteine mutants. Expression analysis of HA-CYS^−AT1R (lane 2) and HA-tagged single cysteine mutants (lanes 3-22) in transiently transfected COS1 cells. Untransfected cells served as negative control (lane 1). ~42 kDa band corresponds to the fully-glycosylated mature cell surface receptor. The multimeric receptor bands are observed between 50-80 kDa. Actin expression levels are shown as loading control.
Figure 4.2. The expression analysis of Ile\textsuperscript{177} and Val\textsuperscript{179} mutants with cysteine, alanine and serine substitutions.
be important to define the structural context for Cys^{181} to form the disulfide with Cys^{101}. Non-tolerated mutations at these positions may change the stability of this disulfide bond and the proper folding of the TM helices by increasing the energy of folding due to the nature and orientation of the substituted side chain. The introduced cysteine side chain at position 179 might directly interfere with the formation of the Cys^{101}-Cys^{180} disulfide bond or induce the formation of an abnormal disulfide bond resulting in an unstable receptor polypeptide.

### 4.3 Effect of Cysteine Substitutions in ECL2

The cell surface receptor expression (B_{max}) of twenty single cysteine mutants measured in intact COS1 cells is shown in Table IV.II. B_{max} values of mutants T178C, H183C, R187C, N188C and L191C was within 5-fold and <20-fold for the remaining mutants. The calculated cell surface receptor number shows that there are enough receptors on the cell surface to perform RCAM analysis in mutants with lower B_{max} values, I177C, V179C, E185C and P192C. The calculated fold changes in K_d values for mutants compared to HA-CYS-AT1R suggested that all mutants bound the ligand with high-affinity and specificity (Table IV.II). Binding of agonist Ang II elicited ERK1/2 activation response in all ECL2 mutants, demonstrating the capacity of mutants to assume active conformation (Table IV.II). Ang II-induced ERK1/2 activation capacity displayed by mutants E173C, T175C, E185C, S186C, S189C is reduced compared to HA-CYS^−AT1R, however the reduction is not statistically significant (Table IV.II). The antagonist losartan inhibited Ang II-dependent activation of all mutants.
Table IV.II. Characterization of ECL2 single cysteine mutants.

<table>
<thead>
<tr>
<th></th>
<th>Cell Surface Expression</th>
<th>ΔKd**</th>
<th>MAPK activation***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax (mean±SEM)</td>
<td>~Receptors/cell*</td>
<td></td>
</tr>
<tr>
<td>HA-CYS¬AT1R</td>
<td>5.80 ± 0.12</td>
<td>350,000</td>
<td>1.0</td>
</tr>
<tr>
<td>I172C</td>
<td>0.36 ± 0.07</td>
<td>21,500</td>
<td>1.3</td>
</tr>
<tr>
<td>E173C</td>
<td>0.34 ± 0.03</td>
<td>20,600</td>
<td>1.4</td>
</tr>
<tr>
<td>N174C</td>
<td>0.66 ± 0.05</td>
<td>40,000</td>
<td>1.4</td>
</tr>
<tr>
<td>T175C</td>
<td>0.72 ± 0.01</td>
<td>43,000</td>
<td>1.4</td>
</tr>
<tr>
<td>N176C</td>
<td>0.71 ± 0.12</td>
<td>43,000</td>
<td>1.3</td>
</tr>
<tr>
<td>I177C</td>
<td>0.26 ± 0.05</td>
<td>15,800</td>
<td>1.3</td>
</tr>
<tr>
<td>T178C</td>
<td>2.32 ± 0.32</td>
<td>139,600</td>
<td>1.6</td>
</tr>
<tr>
<td>V179C</td>
<td>0.12 ± 0.02</td>
<td>7,500</td>
<td>2.3</td>
</tr>
<tr>
<td>A181C</td>
<td>0.38 ± 0.03</td>
<td>22,700</td>
<td>2.1</td>
</tr>
<tr>
<td>F182C</td>
<td>0.71 ± 0.11</td>
<td>42,800</td>
<td>1.4</td>
</tr>
<tr>
<td>H183C</td>
<td>1.94 ± 0.47</td>
<td>116,700</td>
<td>1.4</td>
</tr>
<tr>
<td>Y184C</td>
<td>0.34 ± 0.04</td>
<td>20,600</td>
<td>1.8</td>
</tr>
<tr>
<td>E185C</td>
<td>0.11 ± 0.03</td>
<td>6,700</td>
<td>2.3</td>
</tr>
<tr>
<td>S186C</td>
<td>0.91 ± 0.32</td>
<td>55,100</td>
<td>1.9</td>
</tr>
<tr>
<td>R187C</td>
<td>6.50 ± 0.20</td>
<td>391,700</td>
<td>1.6</td>
</tr>
<tr>
<td>N188C</td>
<td>3.36 ± 0.21</td>
<td>202,600</td>
<td>1.4</td>
</tr>
<tr>
<td>S189C</td>
<td>0.78 ± 0.05</td>
<td>46,700</td>
<td>2.1</td>
</tr>
<tr>
<td>T190C</td>
<td>0.61 ± 0.05</td>
<td>36,800</td>
<td>1.9</td>
</tr>
<tr>
<td>L191C</td>
<td>1.59 ± 0.23</td>
<td>95,600</td>
<td>2.2</td>
</tr>
<tr>
<td>P192C</td>
<td>0.03 ± 0.01</td>
<td>1,700</td>
<td>3.0</td>
</tr>
<tr>
<td>HA-AT1R</td>
<td>3.5 ± 0.15</td>
<td>175,000</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Number of receptors on the cell surface calculated by the formula: [(Bmax*Avogadro’s number) / number of cells].

** Kd value for HA-CYS¬AT1R is 4.4 nM. Kd value for HA-AT1R is 3.5 nM.

*** MAPK activation indicates that ERK is phosphorylated upon treatment with 1µM Ang II, demonstrating the ability of mutants to assume active conformation upon binding Ang II. P-values are derived from two-tailed, unpaired t-test.
The results presented in this chapter suggest that, the single cysteine substitution in the ECL2 region did not cause gross defect in the conformation which indicates that substituted cysteine residue orientation is similar to that of the wild type residue at each position.
CHAPTER V
ACCESSIBILITY MAPPING ANALYSIS OF ECL2

5.1 Introduction

Variations of the site-directed cysteine labeling (modification) techniques have been a very powerful approach in structure-function studies of GPCRs. Site-directed cysteine residues are used as sites for attachment of spin-label reporter groups (Loewen et al, 2001), for binding metal ions or for inducing site-specific cross linking (Itoh et al, 2001, Cai et al, 2001) to unravel structural and dynamic features of functional motifs in rhodopsin and other neuroendocrine GPCRs. Site-directed spin labeling studies pioneered by Hubbell and Khorana (Farrens et al, 1996, Altenbach et al, 1996) employed site-directed cysteine substitutions to analyze the folding and conformational dynamics. Specific positions in receptors can be labeled with $^{13}$C-methyl to facilitate structural analysis by nuclear magnetic resonance (NMR) spectroscopy (Bokoch et al, 2010). Sulfhydryl groups can be modified with specific reagents such as 2-nitrobenzoic acid (DTNB) that enable spectrophotometric monitoring of the reaction (Yang et al, 1996).
Methanethiosulfanate (MTS) reagents are more recently described thiol-specific probes, which provide advantages due to their high selectivity and rapid reactivity with the thiolate anion (S$^-$) rather than the reduced -SH (Akabas et al, 1992). Only water accessible cysteines ionize to thiolate and thus react with hydrophilic MTS reagents a billion times faster than buried cysteines, which cannot ionize to a significant extent (Roberts et al, 1986). Thus, rapid MTS-reactivity indicates location of a cysteine in the water-accessible regions of a protein. A cysteine group facing the membrane or the protein interior would not react or react poorly. The influence of MTS-reacted cysteines on ligand binding is a measure of accessibility of a reporter cysteine in the ligand binding pocket. The criterion for identifying an accessible reporter cysteine is the inhibition of ligand binding due to the reaction with the MTS reagent. In addition, the residue is identified as a part of the ligand binding pocket if the MTS reaction is retarded in the presence of agonists or antagonists. If a position does not tolerate cysteine substitution due to its role in protein stability, folding or ligand binding, its accessibility can be inferred from neighboring reporters. Thus, exposure of site-directed cysteines, found in the water-exposed surface and in the aqueous pockets, or channels, within the TM proteins can be very rapidly and accurately measured by MTS reagents in situ, in the native membrane environment, without purifying the protein to a homogenous preparation.

Reporter cysteine accessibility mapping methodology was previously used to determine activation-induced conformational changes in the TM helices of AT1R (Miura et al, 2002, Miura et al, 2003, Boucard et al, 2003, Martin et al, 2007, Yasuda et al, 2008,
Domazet et al, 2009). In these studies, the reaction rate and the degree of modification of substituted cysteines by MTS reagents, measured by radioligand binding, was used to infer dynamic changes in specific TM helices. However, the indirect measurement of MTS-reactivity with a reporter cysteine has limitations. False negative results are possible, since a reporter may be accessible, but ligand binding may not be inhibited by its reaction with MTS reagent. If a residue is covered by ligand upon treatment, its reactivity can not be determined.

We developed a new probing method which overcomes some of these limitations in the current study. Using MTSEA-biotin (N-biotinylaminoethylmethanethiosulfonate) reaction, followed by streptavidin-HRP detection (Fig. 3.6), we could directly analyze the degree of modification of reporter cysteines. Figure 5.1 explains the principle behind the MTSEA-biotin labeling strategy. This procedure can be performed both in the absence and presence of the ligand. It allows us to get a direct readout for every position in different ligand-bound states of the receptor.

In this chapter, the MTSEA-biotin accessibility of single cysteine mutants of ECL2 was compared in different ligand-bound states; empty, agonist-bound and antagonist-bound states. The reactivity of HA-CYS−AT1R to MTSEA-biotin is considered as baseline to identify a residue as accessible or inaccessible (Fig. 3.6). Any significant measurement above the reactivity of HA-CYS−AT1R indicates accessibility of the particular reporter cysteine. The receptor topology was preserved during MTSEA-biotin reaction as closely as possible to the native state in the plasma membrane by using
Figure 5.1. MTSEA-biotin labeling of accessible cysteine reporters. Schematic explanation of principle behind MTSEA-biotin labeling strategy. MTSEA-biotin preferentially reacts with water-accessible cysteines and does not react with buried or disulfide bonded cysteines. The reaction of MTSEA-biotin with thiol group of an accessible cysteine can be detected by Streptavidin-HRP.
adherent cells rather than fractionated membranes. This analysis allowed us to deduce the conformational changes of ECL2 associated with AT1R activation/inhibition.

5.2 Experimental Set Up for Accessibility Mapping

Intact COS1 cells expressing each of the reporter cysteine mutants were exposed with 10 mM MTSEA-biotin for 30 min at room temperature (23 °C). We did not use >10 mM concentration for reasons of cost of the MTSEA-biotin reagent. Carrying out the reaction at room temperature was necessary to minimize spontaneous and ligand-induced receptor internalization. 20-30% of AT1R internalize spontaneously at 37 °C in 60 min. Ang II induces internalization of >80% of AT1R at 37°C in 60 min. At room temperature, Ang II-induced internalization of AT1R is <10% in 30 min. Under these conditions, we were able to obtain optimal cysteine-biotinylation in AT1R without affecting the integrity of cells (Fig. 5.2). As previously described in Section 3.4, the modification levels of single cysteine mutants of ECL2 were determined by normalizing the streptavidin-HRP signal of immunoprecipitated receptors to the HA signal. The streptavidin-HRP signal of the HA-CYS¯AT1R was used as the control. The relative MTSEA-biotin accessibility of a mutant was calculated as the ratio of streptavidin-HRP signal for that mutant to the same signal for HA-CYS¯AT1R in the same experiment. This control minimizes confounding factors which might influence the readout due to different cell surface expression levels. Fully glycosylated monomeric receptor band was monitored for the mapping studies. As seen in Table V.I, examination of monomeric band yields more consistent results with lower standard error compared to multimeric band. The overall accessibility pattern was same regardless of whether the monomeric or
Figure 5.2. Optimization of MTSEA-biotin reaction conditions. A) Concentration dependence of streptavidin-HRP signal. B) Time course analysis of Streptavidin-HRP signal upon treatment with 10 mM MTSEA-biotin.
Table V.I. Comparison of MTSEA-biotin reactivity of fully-glycosylated monomeric receptor and multimeric forms of the receptor.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>41.9 kDa Monomeric form Relative Accessibility</th>
<th>S.E.M.</th>
<th>50-80 kDa Multimeric Relative Accessibility</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-CYS&lt;sup&gt;−&lt;/sup&gt;AT1R</td>
<td>1.00</td>
<td>0.24</td>
<td>1.00</td>
<td>0.28</td>
</tr>
<tr>
<td>I172C</td>
<td>1.39</td>
<td>0.40</td>
<td>1.04</td>
<td>0.24</td>
</tr>
<tr>
<td>E173C</td>
<td>1.86</td>
<td>0.48</td>
<td>1.53</td>
<td>0.79</td>
</tr>
<tr>
<td>N174C</td>
<td>3.08</td>
<td>0.60</td>
<td>1.52</td>
<td>1.21</td>
</tr>
<tr>
<td>T175C</td>
<td>3.18</td>
<td>0.49</td>
<td>1.24</td>
<td>0.97</td>
</tr>
<tr>
<td>N176C</td>
<td>1.61</td>
<td>0.06</td>
<td>1.07</td>
<td>0.35</td>
</tr>
<tr>
<td>I177C</td>
<td>0.58</td>
<td>0.17</td>
<td>1.31</td>
<td>0.38</td>
</tr>
<tr>
<td>T178C</td>
<td>1.63</td>
<td>0.09</td>
<td>1.16</td>
<td>0.26</td>
</tr>
<tr>
<td>V179C</td>
<td>0.73</td>
<td>0.16</td>
<td>0.94</td>
<td>0.10</td>
</tr>
<tr>
<td>A181C</td>
<td>0.01</td>
<td>0.01</td>
<td>0.84</td>
<td>0.21</td>
</tr>
<tr>
<td>F182C</td>
<td>0.81</td>
<td>0.20</td>
<td>0.83</td>
<td>0.42</td>
</tr>
<tr>
<td>H183C</td>
<td>0.42</td>
<td>0.06</td>
<td>0.88</td>
<td>0.32</td>
</tr>
<tr>
<td>Y184C</td>
<td>1.04</td>
<td>0.17</td>
<td>1.56</td>
<td>1.04</td>
</tr>
<tr>
<td>E185C</td>
<td>2.03</td>
<td>0.56</td>
<td>1.67</td>
<td>0.62</td>
</tr>
<tr>
<td>S186C</td>
<td>2.38</td>
<td>0.47</td>
<td>1.91</td>
<td>1.46</td>
</tr>
<tr>
<td>R187C</td>
<td>3.52</td>
<td>0.23</td>
<td>1.29</td>
<td>0.92</td>
</tr>
<tr>
<td>N188C</td>
<td>0.57</td>
<td>0.15</td>
<td>0.55</td>
<td>0.13</td>
</tr>
<tr>
<td>S189C</td>
<td>2.08</td>
<td>0.28</td>
<td>1.56</td>
<td>1.43</td>
</tr>
<tr>
<td>T190C</td>
<td>1.57</td>
<td>0.34</td>
<td>0.90</td>
<td>0.17</td>
</tr>
<tr>
<td>L191C</td>
<td>0.92</td>
<td>0.20</td>
<td>0.97</td>
<td>0.75</td>
</tr>
<tr>
<td>P192C</td>
<td>1.21</td>
<td>0.23</td>
<td>0.99</td>
<td>0.96</td>
</tr>
</tbody>
</table>
the multimeric bands were analyzed.

The binding of agonist (Ang II) and antagonist (losartan) caused changes in the reactivity of two representative cysteine reporters, replacing His$^{183}$ and Glu$^{185}$. The H183C did not react in the absence of ligand (Fig. 5.3 A) but reacted very well in the Ang II-bound state (Fig. 5.3 B). In contrast, Ang II binding rendered E185C less reactive, although it was highly reactive in the absence of Ang II. In the presence of the antagonist losartan, the reactivity of both cysteine reporters was reduced. These results provided the first indication that the binding of agonists and antagonists induce distinct changes in the conformation or position of ECL2 in AT1R. Differential labeling of a particular mutant with and without agonist/antagonist suggests that the binding of ligand influences the position/conformation of ECL2 and alters accessibility of the reporter.

In the empty-state, the observed MTSEA-biotin reactivity for H183C was significantly lower compared to HA-CYS−AT1R (Fig. 5.3 B), which has no free cysteines. The reasons for lower reactivity of H183C and several other mutants are not clear at this time. Theoretically, the MTSEA-biotin reaction for substituted cysteine should be greater than or at least equal to HA-CYS−AT1R. The observed discrepancy is not correlated with the level of expression of H183C or other mutants and potential masking of other cysteines in the basal conformation. Since Ang II or losartan binding was able to change the H183C reactivity (Fig. 5.3 B), the substituted cysteine at this position was acceptable as a sensor of local conformational change. We apply the same
Figure 5.3. MTSEA-biotin accessibility of representative mutants. A) Immunoprecipitated receptors were probed for HA (left) and Streptavidin-HRP (right) to detect receptor expression and biotinylation levels respectively. The same blot was used for probing with HA and Streptavidin-HRP. The blots for representative mutants H183C and E185C are shown under three experimental conditions; in the absence of ligand (upper), in the presence of 1 μM Ang II (middle) and in the presence of μM losartan (lower). The fully glycosylated monomeric receptor band at 41.9 kDa was used for determination of MTSEA-biotin accessibility. HA signal intensity and streptavidin-HRP signal intensity of each sample was compared to the HA-CYS\textsuperscript{-}AT1R in the same gel as indicated by numbers below the bands. The corresponding plots show the MTSEA-biotin relative accessibility, which is the ratio of relative Streptavidin-HRP signal to relative HA signal for particular sample. Relative MTSEA-biotin accessibility of each mutant was compared to the HA-CYS\textsuperscript{-}AT1R in the same gel. Inset is the schematic representation of reporter cysteines which point up when inaccessible and point down when accessible, reacted with MTSEA-biotin (shown as -SR in red).
consideration for other mutants.

5.3 Accessibility of ECL2 in the Absence of Ligand

The accessibility level of each single cysteine mutant was determined in comparison to the HA-CYS\(^{-}\)AT1R control. The accessibility map of cysteine reporters in ECL2 without ligand is shown in Figure 5.4 A. The mean ± S.E.M. observed for HA-CYS\(^{-}\)AT1R in nine independent experiments represents the streptavidin-HRP signal for the receptor that lacks free cysteine residues. The red bar indicates the cut off value below which the measurement is considered inaccessible. Significantly higher streptavidin-HRP signal (mean ± S.E.M.) was observed for reporter cysteines residues in two distinct regions of ECL2. The accessible N-terminal (Glu\(^{173}\), Asn\(^{174}\), Thr\(^{175}\), Asn\(^{176}\) and Thr\(^{178}\)) and the C-terminal (Glu\(^{185}\), Ser\(^{186}\), Arg\(^{187}\), Ser\(^{189}\) and Thr\(^{190}\)) regions were separated by a region of low MTSEA-biotin reactivity. The inaccessible Ile\(^{177}\)-Tyr\(^{184}\) segment contains at its center the highly conserved disulfide bond linking ECL2 to TMIII. In addition, reporter cysteine residues at Ile\(^{172}\), Ile\(^{177}\), Asn\(^{188}\), Leu\(^{191}\) and Pro\(^{192}\) were also inaccessible. All of the mutants in which MTSEA-biotin labeling did not increase, were expressed on the cell surface (Table IV.II) and efficiently immunoprecipitated as shown by the HA blot, suggesting that the absence of signal was due to the inaccessibility of the particular cysteine reporter, and not due to the lack of cell surface expression. Unlabeled receptors with reduced cell surface expression levels (I177C, P192C) were still present at sufficient quantity to yield accessibility signal, as seen upon ligand exposure (see section 4.3).
Figure 5.4. MTSEA-biotin accessibility maps of ECL2 single cysteine mutants. The MTSEA-biotin relative accessibility of mutants are expressed as mean ± S.E.M., n=3. Red line shown on graph designates the significance cut off (S.E.M. of HA-CYS^-AT1R accessibility, n=9) that determines the accessibility of mutants. Mutants with significantly higher accessibility compared to HA-CYS^-AT1R are indicated with red asterisk. The conserved Cys180 residue is marked by gold star. A) MTSEA-biotin accessibility map of ECL2 mutants in the absence of ligand. The autoantibody binding epitope sequences are indicated in dark red. B) MTSEA-biotin accessibility map of ECL2 mutants in the presence of AngII. C) MTSEA-biotin accessibility map of ECL2 in the presence of losartan.
5.4 Accessibility of ECL2 Upon Ang II Binding

Surprisingly, Ang II treatment prior to MTSEA-biotin reaction significantly reduced the overall accessibility of ECL2 (Fig. 5.4 B). Residues Glu\textsuperscript{173}, Asn\textsuperscript{174}, Thr\textsuperscript{175}, Asn\textsuperscript{176} and Thr\textsuperscript{178}, Glu\textsuperscript{185}, Ser\textsuperscript{186}, Arg\textsuperscript{187} and Thr\textsuperscript{190} which were found to be highly reactive in the absence of ligand did not react in the presence of Ang II. The protection of residues by Ang II implies two possibilities, either that Ang II masked these residues directly or that ECL2 undergoes an Ang II-dependent conformational change. The pattern of accessibility throughout the ECL2 residues clearly indicates that blocks of residues show similar accessibility patterns rather than individual ones. Decreased accessibility of consecutive residues of ECL2 in two segments rather than individual residues supports the idea that Ang II induces conformational rearrangement of ECL2. Ang II binding increased the accessibility of Ala\textsuperscript{181}, Phe\textsuperscript{182} and His\textsuperscript{183}, which are immediately C-terminal to the disulfide bonded Cys\textsuperscript{180}. Thus, the Ang II-induced change is consistent with increase in local conformational entropy involving the disulfide bond.

5.5 Accessibility of ECL2 Upon Losartan Binding

The presence of AT1R-selective antagonist, losartan, during the MTSEA-biotin labeling (Fig. 5.4 C) also decreased the overall reactivity of ECL2 similar to Ang II-bound state. These accessibility changes in the presence of antagonist are similar to that observed in rhodopsin (Palczewski et al, 2000, Ahuja et al, 2009) and dopamine receptor (Shi et al, 2004) where ECL2 forms a cap over the inhibitor binding site.
However, losartan binding increased the reactivity of residues in the Asn\textsuperscript{176}-Val\textsuperscript{179} region, which are immediately N-terminal to the disulfide bonded Cys\textsuperscript{180}. Thus, losartan binding induced flexibility on the other side of the disulfide bond compared to Ang II bound-state. Thus, conformational changes induced near the disulfide bond upon binding of the agonist are different from those induced by binding of the antagonist. These results suggest that ECL2 adopts different conformations in Ang II- and losartan-bound states.

### 5.6 Rate of Change in MTSEA-biotin Accessibility

The observation that the accessibility of residues on either side of the disulfide bond-tether of ECL2 changes upon ligand binding raised an important question: Do the induced conformational changes indicate a flexible or rigid-body motion of ECL2? Does the conformation of ECL2 change as a single entity or do different segments (residues) of ECL2 have different rates of accessibility changes?

We determined the rate of accessibility change for two representative reporter cysteines in ECL2, on each side of the disulfide-tether. The reactivity of MTSEA-biotin was measured following 0 min, 2 min, 5 min and 10 min treatment with Ang II. The accessibility of N174C reporter decreased in the presence of Ang II, with a slope of 0.298 which suggested a gradual conformational change for this residue (Fig. 5.5 A). On the other hand, the accessibility of F182C reporter increased in the presence of Ang II, with a slope of 0.058. The slower rate observed for Phe\textsuperscript{182} residue compared to Asn\textsuperscript{174} might be due to the fact that Phe182 residue is closer to the disulfide bond which might have restricted its flexibility to some extent compared to a residue far from the disulfide bond.
restriction. The result indicates that the two segments in ECL2 have different rates of accessibility changes (Fig. 5.5 B). This observation suggested that ECL2 does not change its conformation as a single entity; instead, two regions undergo conformational changes independently from each other.

ECL2 is highly accessible in the absence of any ligand. Agonist and antagonist binding differentially alters the accessibility of ECL2 leading to distinct conformations in two different ligand-bound states. The data suggests that ECL2 is a flexible region as predicted by theoretical models. Tethering ECL2 to TMIII via the conserved disulfide bond is an important structural constraint, giving different flexibility to two segments of ECL2, which allows ECL2 to follow different folding depending upon the nature of the ligand. A ligand-specific conformational change of ECL2 may be associated with the activation/inhibition of AT1R.
Figure 5.5. Rate of change in MTSEA-biotin accessibility for representative mutants. A) Rate of change in MTSEA-biotin accessibility for N174C mutant. Experiment was performed by treating cells with 1µM Ang II for 0 min, 2 min, 5 min and 10 min. The relative accessibility to 20 mM MTSEA-biotin was measured as described before. Inset shows the linear trend line and the equation.  B) Rate of change in MTSEA-biotin accessibility for F182C mutant. Inset shows the trend line and the equation.
CHAPTER VI

INSIGHTS FROM MOLECULAR DYNAMICS (MD) SIMULATIONS

6.1 Introduction

As a computational approach, molecular dynamics (MD) simulations provide insights into the dynamic nature of biological molecules and their inherent motion as a function of time. It helps the interpretation of experimental data. We performed MD simulations using the accessibility constraints determined by MTSEA-biotin labeling (Fig. 5.3) to predict the conformation of the ECL2. MD simulations were performed using model structures of AT1R (PDB ID: 2AC6) and AT1R with Ang II (PDB ID: 2AH3) as explained in Figure 2.10. The ECL2 simulation in the presence of losartan was performed on a de novo AT1R model docked with losartan. Together, these studies provide a greater insight into the functional role of dynamic motions in ECL2 and improve our understanding of AT1R interaction with Ang II, losartan and auto-antibodies.
6.2 Overall Approach

MD simulation on empty, Ang II-bound and losartan-bound models was performed as explained in Figure 2.10. In each instance, 100 frames were obtained at the end of 14 ns simulation. To illustrate the strategy, all 100 frames for the empty state of the receptor are shown in Figure 6.1 as an example. The overall backbone conformation of ECL2 in each frame is examined for conformity with the experimental results obtained using MTSEA-biotin accessibility mapping of the ECL2 in the empty state of the receptor. Among several frames which displayed an ECL2 conformation concordant with the experimental data, we chose the frame number 1 (Figure 6.2 A) for the empty state of the receptor. When the side chains of the ECL2 region were added (Figure 6.2 B) and energy minimization was carried out, the final orientation of every side-chain matched the accessibility data for this frame. The same analysis was done for the Ang II- and losartan-bound states.

6.3 MD Simulation of ECL2 in the Absence of Ligand

The MD simulation in the absence of any ligand indicated that ECL2 conformation is open toward the extracellular space (Fig. 6.2). The accessible residues on the N-terminal (Glu$^{173}$, Asn$^{174}$, Thr$^{175}$, Asn$^{176}$ and Thr$^{178}$) and the C-terminal (Glu$^{185}$, Ser$^{186}$, Arg$^{187}$, Ser$^{189}$ and Thr$^{190}$) regions of the ECL2 are shown in red color. The Val$^{179}$ to Tyr$^{184}$ region harboring the conserved disulfide bond is deeper and more restricted than the N-terminal and C-terminal regions of the loop. Disulfide bonded cysteines, Cys$^{101}$ and Cys$^{180}$, are shown in yellow. The access to the ligand-binding pocket is wider near TMV-TMVII, providing an open aqueous channel (see Chapter VII for discussion).
Figure 6.1. MD simulation of the ECL2 in the empty state of the receptor. A) The 100 frames obtained from the MD simulation analysis of ECL2 in the empty state of the AT1R following 1000 steps of energy minimization using the backbone structure of AT1R model. (PDB ID: 2AC6) The ECL2 is shown in red. B) Superimposition of 100 frames from the MD simulation analysis of ECL2 in the empty state of the AT1R.
Figure 6.2. The frame #1 chosen for further analysis. A) Among the 100 frames, the frame #1 was chosen since the ECL2 conformation is consistent with the experimental results found by MTSEA-biotin accessibility mapping of ECL2 in the empty state of the AT1R. B) The side chains were then added to the ECL2 backbone. The disulfide bonded cysteines are shown in yellow.
6.4 MD Simulation of ECL2 in the Presence of Ang II

The MD simulation in the presence of Ang II depicts ECL2 forming a tightly packed lid against bound Ang II, which is shown in magenta in Figure 6.3. The accessible residues Ala$^{181}$, Phe$^{182}$ and His$^{183}$ which are immediately C-terminal to the disulfide bond are shown in red.

6.5 MD Simulation of ECL2 in the Presence of Losartan

The MD simulation in the presence of losartan which is shown in blue in Figure 6.4 also depicts a lid conformation of ECL2 similar to Ang II bound state of the receptor. The accessible residues, Asn$^{176}$-Val$^{179}$, which are immediately N-terminal to the highly conserved disulfide bond, are shown in red color. These results suggest that ECL2 adopts lid conformations in both Ang II- and losartan-bound states, but in a distinct orientation. Varying positions of the disulfide bond and the neighboring accessible residues in Ang II- and losartan-bound conformations of ECL2 indicates flexibility of the region that is normally thought to be constrained because of the disulfide bond.

6.6 Analysis of Hydrogen Bonding Networks

The overall decrease of ECL2 accessibility in the Ang II- and losartan-bound states compared to the empty state indicates that ECL2 conformation changes upon receptor activation. We used inaccessible residues as constraints to perform molecular dynamic simulation to gain insights on ECL2 conformational rearrangements. We analyzed the hydrogen bonding of the inaccessible residues in the empty, Ang II-bound, and losartan-bound states of the AT1R. To assign the rearrangement of hydrogen bonding
Figure 6.3. **The frame # 48 chosen for further analysis.** A) Among the 100 frames, the frame #48 was chosen since the ECL2 conformation is consistent with the experimental results found by MTSEA-biotin accessibility mapping of ECL2 in the Ang II-bound state of the AT1R. B) The side chains were then added to the ECL2 backbone. The disulfide bonded cysteines are shown in yellow. Ang II is shown in magenta.
Figure 6.4. The frame # 39 chosen for further analysis. A) Among the 100 frames, the frame # 39 was chosen since the ECL2 conformation is consistent with the experimental results found by MTSEA-biotin accessibility mapping of ECL2 in the losartan-bound state of the AT1R. B) The side chains were then added to the ECL2 backbone. The disulfide bonded cysteines are shown in yellow. Losartan is shown in blue.
network in each receptor state, we simulated 3.2 Å hydrogen bond contacts of inaccessible ECL2 residues. In the empty state, ten inaccessible residues (Ile$^{172}$, Ile$^{177}$, Val$^{179}$, Ala$^{181}$, Phe$^{182}$, His$^{183}$, Tyr$^{184}$, Asn$^{188}$, Leu$^{191}$ and Pro$^{192}$) are involved in extensive interaction with TMII, TMIII, TMIV and TMV and ECL3 (red) (Fig. 6.5). A list of interactions in the absence of ligand is given in Table VI.I.

In the Ang II-bound state, sixteen residues inaccessible to MTSEA-biotin (Ile$^{172}$, Glu$^{173}$, Asn$^{174}$, Thr$^{175}$, Asn$^{176}$ Ile$^{177}$, Thr$^{178}$, Val$^{179}$, Tyr$^{184}$, Glu$^{185}$, Ser$^{186}$, Arg$^{187}$, Asn$^{188}$, Thr$^{190}$, Leu$^{191}$ and Pro$^{192}$) increase intra-molecular contacts of ECL2 with TMI, TMII, TMIII, TMIV, TMV, TMVII and extra-cellular segments, N-terminal tail, ECL1 and ECL3 (red, Fig. 6.6). Two intermolecular hydrogen bonding networks; the first (green) between Ang II and TM-helices and the second (blue) between Ang II and ECL2 highlights the major difference between basal and active states of AT1R. A list of interactions in the presence of Ang II is given in Table VI.II A and B. The N-terminal residues of Ang II are involved in interactions with TMII and TMVII (green). The C-terminal residues of Ang II are involved in interactions with N-terminal residues of ECL2 (blue).

The intra-molecular hydrogen bonding network in the presence of losartan is even more extensive including interactions with all TM helices (except TMI), N-terminal tail, ECL1 and ECL3, however these contacts are distinct compared to Ang II-bound state. The rearrangement of hydrogen bonding network in the inhibited receptor state upon
Figure 6.5. Hydrogen bonding network of ECL2 in the absence of ligand. A) Predicted intramolecular interactions of inaccessible residues of ECL2 with the TM helices in the absence of ligand are shown in red. TM helices are depicted as helical wheel diagram generated using Helical Wheel Projections (http://rzlab.ucr.edu/scripts/wheel/). Loop and extracellular residues are highlighted in yellow. B) Cartoon depiction of ECL2 interactions with TMD.
**Table VI.I.** Predicted interactions of inaccessible ECL2 residues with transmembrane residues in the absence of ligand.

<table>
<thead>
<tr>
<th>ECL2</th>
<th>TM interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I172</td>
<td>Y170</td>
</tr>
<tr>
<td>I177</td>
<td>T80, T88, L100, L81</td>
</tr>
<tr>
<td>V179</td>
<td>F171, A104, C101, S105</td>
</tr>
<tr>
<td>C180</td>
<td>V169, F171, Y170, Disulfide bond with C101</td>
</tr>
<tr>
<td>A181</td>
<td>V169, N168</td>
</tr>
<tr>
<td>F182</td>
<td>R167, H272, I271</td>
</tr>
<tr>
<td>H183</td>
<td>L112, N168, V270, I271, H272, D273, C274</td>
</tr>
<tr>
<td>Y184</td>
<td>S109, V108, I271, C274</td>
</tr>
<tr>
<td>N188</td>
<td>S107, K199, H272, D273</td>
</tr>
<tr>
<td>L191</td>
<td>H166, I165, V164, I193, G196, L195</td>
</tr>
<tr>
<td>P192</td>
<td>G194, L195</td>
</tr>
</tbody>
</table>
Figure 6.6 Hydrogen bonding network of ECL2 in the presence of Ang II. A) Predicted intramolecular interactions of inaccessible residues of ECL2 with the TM helices in the presence of Ang II are shown in red. Two intermolecular hydrogen bonding network between Ang II and ECL2 (blue) and between Ang II and TM helices (green) are also shown. Loop and extracellular residues are highlighted in yellow. B) Cartoon depiction of ECL2 interactions with TMD (red), ECL2 interaction with Ang II (blue) and Ang II interactions with TMD (green). Ang II is shown in yellow. Three different hydrogen bonding networks stabilize the lid conformation.
Table VI.II

A. Predicted interactions of inaccessible ECL2 residues with transmembrane residues in the presence of Ang II.

<table>
<thead>
<tr>
<th>ECL2</th>
<th>TM interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I172</td>
<td>L100, Y170, W94, R93, C101</td>
</tr>
<tr>
<td>E173</td>
<td>A89, Y92, R93</td>
</tr>
<tr>
<td>N174</td>
<td>A104, Y170, F171, T88, C101</td>
</tr>
<tr>
<td>T175</td>
<td>A89, A85, P82, T88, L81</td>
</tr>
<tr>
<td>N176</td>
<td>I32, Y35, F31</td>
</tr>
<tr>
<td>I177</td>
<td>I288, Y292</td>
</tr>
<tr>
<td>T178</td>
<td></td>
</tr>
<tr>
<td>V179</td>
<td></td>
</tr>
<tr>
<td>C180</td>
<td>Y170, Disulfide bond with C101</td>
</tr>
<tr>
<td>Y184</td>
<td>C274, H272, I271</td>
</tr>
<tr>
<td>E185</td>
<td>Y170, F171, R13, I14</td>
</tr>
<tr>
<td>S186</td>
<td>V169, R13, I14</td>
</tr>
<tr>
<td>R187</td>
<td>F171, R13, K275, C274</td>
</tr>
<tr>
<td>N188</td>
<td>A7, K12</td>
</tr>
<tr>
<td>T190</td>
<td>I165, R167, V164, G196, L195, I271</td>
</tr>
<tr>
<td>L191</td>
<td></td>
</tr>
<tr>
<td>P192</td>
<td>G196, G194</td>
</tr>
</tbody>
</table>

B. Predicted interactions of Ang II with transmembrane residues and ECL2.

<table>
<thead>
<tr>
<th>Ang II</th>
<th>TM interactions</th>
<th>ECL2 interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>L112</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>N111,A114,S115</td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>N294,N295</td>
<td></td>
</tr>
<tr>
<td>Y4</td>
<td>I290,A291,Y292,F293,N294,N295</td>
<td></td>
</tr>
<tr>
<td>I5</td>
<td>S107,V108,S109</td>
<td>E173</td>
</tr>
<tr>
<td>H6</td>
<td>I177,T178,V179</td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>I172,T178</td>
<td></td>
</tr>
<tr>
<td>F8</td>
<td>S109,Y113</td>
<td></td>
</tr>
</tbody>
</table>
losartan binding is shown in detail in Figure 6.7. The intermolecular hydrogen bonding network between TM helices and losartan (green) is less extensive than that between losartan and ECL2 (blue). A list of interactions in the presence of losartan is given in Table VI.III A and B.

Together, molecular dynamic simulation studies predict a flaccid ECL2 conformation in the empty state. The ECL2 becomes more strongly hydrogen bonded in the ligand-bound state. Extensive intra-molecular hydrogen bonding network of ECL2 with the TM helices, ECL1, ECL3 and N-terminal tail stabilizes a compact ‘lid’ like structure in both Ang II- and losartan-bound states. Two intermolecular hydrogen bonding networks between Ang II and TM helices and Ang II and ECL2 stabilize the agonist bound to the receptor. Individual contacts for the same ECL2 residues differ in agonist- and antagonist-bound states. Cooperatively, these hydrogen bonding networks may account for the experimentally found inaccessibility of ECL2 in the ligand-bound state and govern the high-affinity of AT1R towards Ang II and losartan and their rapid binding followed by slow dissociation (see chapter VI for discussion). Leaning of Ang II-bound ECL2 towards one set of residues and losartan-bound ECL2 towards a different set appears to contribute respectively to receptor activation and inhibition.
**Figure 6.7 Hydrogen bonding network of ECL2 in the presence of losartan. A)**
Predicted intramolecular interactions of inaccessible residues of ECL2 with the TM helices in the presence of losartan are shown in red. Two intermolecular hydrogen bonding network between losartan and ECL2 (blue) and between losartan and TM helices (green) are also shown. Loop and extracellular residues are highlighted in yellow. 1; imidazole ring, 2; phenyl ring 1, 3; phenyl ring 2, 4; tetrazole ring. **B)** Cartoon depiction of ECL2 interactions with TMD (red), ECL2 interaction with losartan (blue) and losartan interactions with TMD (green). Losartan is shown in cyan. Three different hydrogen bonding networks stabilize the lid conformation.
Table VI.III

A. Predicted interactions of inaccessible ECL2 residues with transmembrane residues in the presence of losartan.

<table>
<thead>
<tr>
<th>ECL2</th>
<th>TM interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I172</td>
<td>R13, I14, D17, D16, W84, W94, F96, L100</td>
</tr>
<tr>
<td>E173</td>
<td>K12, R13, Q15, Y170, F171, A104, S107, V108, C101</td>
</tr>
<tr>
<td>N174</td>
<td>P95, L81, P82</td>
</tr>
<tr>
<td>T175</td>
<td>Y170, W94, A85</td>
</tr>
<tr>
<td>C180</td>
<td>Y170, F171, Disulfide bond with C101</td>
</tr>
<tr>
<td>A181</td>
<td>Y170</td>
</tr>
<tr>
<td>F182</td>
<td>P285, M284</td>
</tr>
<tr>
<td>H183</td>
<td>S107, I271, V270, T287, M284, S109</td>
</tr>
<tr>
<td>Y184</td>
<td>S160, A106, H272, F259</td>
</tr>
<tr>
<td>E185</td>
<td>H166, V164, V169</td>
</tr>
<tr>
<td>S186</td>
<td>Y170, F171, V169</td>
</tr>
<tr>
<td>R187</td>
<td>F171, V169, N168, H166, R167, Y170, F259</td>
</tr>
<tr>
<td>N188</td>
<td>S109, V108, S105</td>
</tr>
<tr>
<td>T190</td>
<td>V270, L195, G196</td>
</tr>
<tr>
<td>L191</td>
<td>H272, L197, G196</td>
</tr>
<tr>
<td>P192</td>
<td>L195, G196</td>
</tr>
</tbody>
</table>

B. Predicted interactions of losartan with transmembrane residues and ECL2. 1; imidazole ring, 2; phenyl ring-1, 3; phenyl ring-2, 4; tetrazole ring.

<table>
<thead>
<tr>
<th>Losartan</th>
<th>TMD interactions</th>
<th>ECL2 interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H256, I288</td>
<td>A181, F182, H183, Y184, S186, R187, N188</td>
</tr>
<tr>
<td>2</td>
<td>H256, T260</td>
<td>A181, H183, Y184, S186, N188, S189, T190</td>
</tr>
<tr>
<td>3</td>
<td>Y113, K199, N200</td>
<td>S189, T190</td>
</tr>
<tr>
<td>4</td>
<td>H256, Y113, Q257</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER VII

DISCUSSION

7.1 The Implications of Ligand Induced Conformational Change of ECL2

Results presented here shed new light on ligand-specific structural dynamics of ECL2 in AT1R. In many peptide hormone GPCRs including AT1R, ECL2 directly interacts with agonists and is also a target of auto-antibodies. Hence the ligand-specific conformational dynamics of ECL2 in AT1R we describe here are insightful.

An open conformation of ECL2 has been indicated by the accessibility measurements and molecular dynamics simulation of ECL2 in the empty state of the AT1R (Fig. 7.1 A). The open conformation of ECL2 may facilitate capture of ligands in AT1R by providing an aqueous channel to facilitate ligand entry and exit from the binding pocket in milliseconds (Bourne & Meng, 2000, Cherezov et al, 2007, Warne et al, 2008, Jaakola et al, 2008). As ligands enter the binding pocket through this aqueous channel, they may have transient interactions with the ECL2, an interpretation which is consistent with previous biochemical studies. Photoaffinity cross-linking studies have shown that Val$^3$ in Ang II is cross-linked with Ile$^{172}$ in ECL2 (Perodin et al, 2002).
Figure 7.1. Molecular dynamics simulation of ECL2. A) Molecular dynamics simulation of ECL2 in the absence of ligand. TMI, TMII, TMIII, TMVI and TMV are shown in cartoon model. The ECL2 residues and side chains are shown in stick and surface model. The accessible residues are shown in red. The disulfide bonded cysteines Cys101 (TMIII) and Cys180 (ECL2) are shaded in yellow. B) Molecular dynamics simulation of ECL2 in the presence of Ang II. Ang II is shown as magenta stick. The accessible residues are shown in red. C) Molecular dynamics simulation of ECL2 in the presence of losartan. Losartan is shown as blue stick. The accessible residues are shown in red. D) Zoomed view of ECL2 simulations in the empty, Ang II-bound and losartan-bound states of the receptor.
Site-directed mutagenesis studies have shown that Ile\textsuperscript{172} and His\textsuperscript{183} in ECL2 specifically interact with Val\textsuperscript{3} and Asp\textsuperscript{1} side chains in Ang II, respectively (Feng et al, 1995, Boucard et al, 2000).

The accessibility measurements and molecular dynamics simulation of ECL2 indicated a “lid” conformation of ECL2 in the Ang II-bound state of the AT1R (Fig. 7.1 B). Mutagenesis studies established direct interactions between TM helices II, III, V, VI & VII and Ang II. Photoaffinity cross-linking studies have shown that Phe\textsuperscript{293}/Asn\textsuperscript{294} in TMVII interact with Phe\textsuperscript{8} of Ang II (Perodin et al, 2002). A molecular model of AT1R predicted that ligands bind in the TM domain \(\approx 25\ \text{Å} \) away from the membrane border (Gogonea et al, 2006). The model suggests that the N-terminal part of ECL2 dips into the binding pocket reaching the bound Ang II (Gogonea et al, 2006). Binding of Ang II may induce folding of ECL2 to an elaborate ‘lid’ conformation similar to that seen in the case of bovine rhodopsin (Fig. 5.3 B). This lid may interact with additional elements of the receptor's extracellular domain, i.e. ECL1, ECL3 and the NT. Thus, the ligand-induced folding of ECL2 requires considerable rearrangement of the hydrogen bonding networks in AT1R (Fig. 7.2).

A similar “lid” conformation of ECL2 in the losartan-bound state of the AT1R is indicated by the accessibility measurements and molecular dynamics simulation of ECL2 (Fig. 7.1 C). Mutagenesis, photoaffinity cross-linking and modeling studies (Boucard et al, 2000, Yamano et al, 1992, Noda et al, 1995, Schambye et al, 1994, Ji et al, 1994,
Figure 7.2. Predicted hydrogen bonding network formed by inaccessible residues of ECL2. The ECL2 is highlighted in dark grey. A) Intramolecular interactions of ECL2 with the TM helices (red) in the absence of ligand. B) Intra-molecular interactions of ECL2 with the TM helices (red) in the presence of Ang II. Ang II is shown in yellow spheres. Two intermolecular hydrogen bonding network between Ang II and ECL2 (blue) and between Ang II and TM helices (green) are also shown. C) Intra-molecular interactions of ECL2 with the TM helices (red) in the presence of losartan. Losartan is shown in cyan spheres. Two intermolecular hydrogen bonding network between losartan and ECL2 (blue) and between losartan and TM helices (green) are also shown.
Nouet et al, 2000) show that losartan competitively occupies the Ang II binding site, implying that conformational rearrangement induced by losartan in another part of the receptor is not the cause of reporter Cys reactivity in ECL2. Molecular modeling studies show that the only mechanism for simultaneous ECL2 and TM domain interaction with losartan would be through folding of ECL2 into the pocket within the TM domain. Molecular dynamic simulation studies shown in Figure 5.3 C provide strong support for ECL2 forming a lid over losartan bound to the TM domain. Simulated interactions include direct contact between ECL2 and losartan and between ECL2 and TM-domain as well as ECL2 and other EC loops (Fig. 6.7).

**7.2 The Potential Role of Conserved Disulfide Bond**

Ligand-specific folding of ECL2 appears to induce specific perturbation around the disulfide bond, which may play a critical role in the inhibition and activation of the receptor. A distinct pattern of accessibility changes caused by losartan on the N-terminal side of the disulfide bond and by Ang II on the C-terminal side of the disulfide bond suggest that type of constraint experienced by the disulfide bond plays a critical role in the inhibition and activation of TM signaling, respectively.

The N-terminal residues in Ang II interact with ECL2 and the C-terminus with the receptor’s inter-helical crevice, leading Ang II to fold into a ‘C’ shape, a conformation predicted in many studies (Marshall et al, 1974, Samanen et al, 1994, Garcia et al, 1992, Nikiforovich et al, 1994). Dynamics of ECL2 might be critical in the Ang II binding process, since three N-terminal residues in Ang II sequentially interact with Ile$^{172}$, His$^{183}$
in ECL2 and Asp\textsuperscript{281} in ECL3/TMVII, while the Ang II C-terminus inserts into receptor’s inter-helical crevice. The Ang II docking process may be completed, upon folding of the peptide ligand to a ‘C’ shape. We propose that ligand binding in AT1R leads to specific reorganization of the hydrogen bond network formed primarily with residues on ECL2, which is then coupled to the movements of TM helices involving the Cys\textsuperscript{101}–Cys\textsuperscript{180} bond.

The ECL2 length (27±13) is conserved in the GPCR family (Karnik et al, 2003), but the frequency of activating mutations targeting this region is lower than in the TM helices. Individual mutations in a flexible region of receptors may not relax the constraint for activation as readily as single residue mutations in TM helices. What is the significance of ligand-specific conformation of ECL2 in the receptor activation process? One possibility is that in the ligand bound state, the ECL2 interacts with multiple TM helices, which enables ECL2 to integrate conformational cues originating at multiple contacts between ligand and the receptor. In fact, the hydrogen bonding network analysis of ECL2 (Fig. 6.6) has shown that there is a considerable rearrangement of hydrogen bonding, increasing the number of contacts between ECL2 and TM domain. The disulfide bond located in the middle of ECL2 may be required for transmitting the conformation to TMIII. Conservation of the disulfide bond in >90% GPCR may indicate a general role for this bond in efficiently coupling ligand binding to GPCR activation.

7.3 The Structural Basis for Binding of Autoantibodies

The open conformation of the ECL2 in the empty receptor is independently supported by autoantibody epitope mapping studies in human AT1R (Liao et al, 2002,
Dragun et al, 2005, Zhou et al, 2008). Autoantibodies directed towards ECL2 in many GPCRs mimic the action of agonists, but their mechanism of action is unclear. Circulating autoantibodies recognize the readily accessible Ile\textsuperscript{172}–Thr\textsuperscript{178} and Glu\textsuperscript{185}–Thr\textsuperscript{190} segments. For instance, agonistic autoantibodies from preeclampsia patients bind to the epitope ‘AFHYESQ’ (Zhou et al, 2008), and agonistic autoantibodies from patients with malignant hypertension and refractory, vascular allograft rejection bind to epitopes ‘ENTNIT’ and ‘AFHYESQ’ in ECL2 (Liao et al, 2002, Dragun et al, 2005). It is interesting to note that the antigenic epitopes (dark red in Fig. 5.3 A) are interrupted by the Val\textsuperscript{170}–Tyr\textsuperscript{184} region adjoining the disulfide bond that was found to be inaccessible by RCAM. Our analysis suggests that these autoantibodies can capture the accessible region and losartan could mask these epitopes to prevent such binding. Losartan binding, which masks epitopes, is known to protect patients harboring autoantibodies.

In many other GPCRs the auto-antibodies directed towards ECL2 may also modulate the conformation of the EC-domain (Lebesgue et al, 1998, Wang et al, 2000). Autoantibodies directed against ECL2 regions were discovered in several human pathologies involving the muscarinic receptors, \(\alpha\)1 and \(\beta\)1 adrenergic receptors and the bradykinin B2 receptor. In a more general sense, the conformational dynamics of ECL2 may regulate the fundamental process of autoantibody-mediated activation and pathophysiological process involving GPCRs. Conformational display of epitopes in ECL2 may be the basis for allostERIC activation of GPCRs by autoantibodies. Autoantibody binding to ECL2-epitopes may exert tension on the disulfide bond to
activate the AT1R directly or may open the entrance channel for circulating Ang II. Our findings might offer strategies for improved therapies.

7.4 A Predicted Ang II-analogous pharmacophore in ECL2

Of great interest, out of ten inaccessible residues in the empty state of the receptor, seven residues are similar to the residues in Ang II (Fig. 7.3), but their order is not the same as in Ang II. We speculate that the inaccessible residues of ECL2 may actually regulate low basal activity of ligand free receptor by presenting an Ang II-analogous pharmacophore hidden within ECL2. Our RCAM analysis uncovered existence of this hidden pharmacophore, a set of structural features in ECL2 that might have a pharmacological effect on the receptor. Effect of the hidden pharmacophore in the empty receptor may be inhibitory. The hydrogen bonding network analysis of ECL2 in the absence of ligand (Fig. 6.5 A) has shown that some of the ECL2 interacting residues in TM domain are within the 3.2 Å of Ang II interacting residues (e. g. Lys$^{199}$ is an Ang II binding residue). When the primary sequence of Ang II is scrambled, an inhibitory pharmacophore is generated as shown earlier (Marshall et al, 1974, Bumpus et al, 1977, Samanen et al, 1994, Miura et al, 1999). For instance exchanging residues at 4$^{th}$, 6$^{th}$ and 8$^{th}$ positions in Ang II converts the agonist to an antagonist, and lowers affinity for AT1R. Whether the predicted Ang II-analogous pharmacophore in ECL2 works like an extremely weak agonist or antagonist needs further study.
Figure 7.3. Ang II analogous ECL2 Pharmacophore. *The inaccessible residues of ECL2 in the empty-state which are similar to Ang II residues are shown. Three Ang II analogs with different properties are also shown where the scrambled residues are indicated in bold italic.

<table>
<thead>
<tr>
<th>ECL2*</th>
<th>Ile Ile Val Phe His Tyr Asn Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
</tr>
<tr>
<td>[Ile⁴] Ang II</td>
<td>Asp-Arg-Val-<strong>Ile</strong>-Ile-His-Pro-Phe</td>
</tr>
<tr>
<td>[Ile⁸] Ang II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-<strong>Ile</strong></td>
</tr>
<tr>
<td>[Ile⁴, Ile⁸] Ang II</td>
<td>Asp-Arg-Val-<strong>Ile</strong>-Ile-His-Pro-<strong>Ile</strong></td>
</tr>
</tbody>
</table>
7.5 The ECL2 of AT1R in context of GPCRs

Previous studies in different GPCRs suggested that ECL2 is important for inactive receptor conformation and participates in ligand binding. Therefore, we evaluated the dynamic role of ECL2 in AT1R as the basis for complex functions attributed to it. Our RCAM results indicated that the ECL2 reacts with MTSEA-biotin with exception of the conserved disulfide bonded region. Binding of an agonist or antagonist limited solvent accessibility of most residues in ECL2, but the accessible residues differ in the presence of agonist and antagonist. These findings can be reconciled assuming that the ECL2 undergoes specific ligand-induced conformational changes as a consequence of ligand-receptor interaction. The conformational changes associated with TM and cytoplasmic domains of GPCRs during activation are conserved. We propose that specific kinetic and thermodynamic prerequisite for ligand and autoantibody recognition are achieved through conformational dynamics of ECL2.

Structural changes in the ECL2 of GPCRs upon activation have been characterized for several GPCRs including rhodopsin and β-adrenergic receptors. In bovine rhodopsin, a compact ‘lid’ conformation of the extracellular domain which interacts with the inverse agonist form of the chromophore is documented (Bourne and Meng, 20005, Massotte et al, 2005, Palczewski et al, 2000, Park et al, 2008, Ahuja et al, 2009). A less compact ‘lid’ conformation is maintained in opsin with an aqueous channel opening near the extracellular ends of TMV-TMVII and ECL3 (Park et al, 2008). NMR studies in light activated rhodopsin have shown the movement of ECL2 away from the activating ligand, all-trans-retinal. The ECL2 sequence of AT1R and rhodopsin share 11
of 21 residues (Fig. 7.4) and the predicted structures of ECL2 are ideally positioned to form multiple interactions with the bound ligand and other segments of the receptor (Gogonea et al, 2006). The ECL2 sequence of AT1R shows high propensity to form a β-hairpin structure and a salt-bridge similar to rhodopsin. Direct interaction of Ang II possibly induces folding of ECL2 to a ‘lid’ conformation (Fig. 5.3 B), which is different when compared to the movement of ECL2 away from the activating ligand, all-trans-retinal in light-activated rhodopsin (Ahuja et al, 2009). Considering the conformational changes in the TM domain, movements of TMIII, TMV, TMVI and TMVII have been shown to be critical for activation of both rhodopsin and AT1R.

Similar extracellular lids blocking the exit of bound inhibitory ligands are not found in the subsequently solved GPCR structures of β-adrenergic and adenosine receptors. Although tethered to TMIII, the ECL2 in these GPCRs is stabilized outside the ligand pocket by additional disulfide bonds (Cherezov et al, 2007, Warne et al, 2008, Jaakola et al, 2008). In the crystal structure of the β-adrenergic and adenosine receptors, ECL2 is not as extensively associated with the ligand binding pocket, leading to the speculation that absence of extracellular lid facilitates ligands’ entry and exit through the binding pocket in milliseconds. In rhodopsin-like peptide hormone receptors, significantly greater involvement of the ECLs in agonist interaction is documented (Karnik et al, 2003). What is the effect of reversible binding of diffusible ligands on the ECL2 conformation of peptide GPCRs? A recent solid-state NMR study on β2-adrenergic receptor has shown that agonists induce a conformational change in ECL2 that differs from those induced by inverse agonists and an inward movement of ECL2 is
Figure 7.4. Alignment of ECL2 of AT1R with the ECL2 sequences of GPCRs for which crystal structures are available. The conserved cysteine is indicated by asterisk. The cysteines which are involved in disulfide bond formation in each GPCR is boxed in gold (1-7). The alignment was generated by using ClustalW2 (http://www.ebi.ac.uk/clustalw/).

<table>
<thead>
<tr>
<th>GPCR</th>
<th>ECL2 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGTRA_RAT</td>
<td>IENTN-^-ITVC</td>
</tr>
<tr>
<td>OPSD_BOVIN</td>
<td>PEMQ-^-CS</td>
</tr>
<tr>
<td>OPSD_TODPA</td>
<td>LECV-^-LC</td>
</tr>
<tr>
<td>ADRB2_HUMAN</td>
<td>THQE-^-AIN</td>
</tr>
<tr>
<td>ADRB1_MELGA</td>
<td>EDPQ-^-ALK</td>
</tr>
<tr>
<td>AA2AR_HUMAN</td>
<td>QPEGKNHS-^-قو</td>
</tr>
</tbody>
</table>
associated with the active state of the β2-adrenergic receptor (Bokoch et al, 2010).

In all five main human GPCR families (rhodopsin, glutamate, secretin, adhesion and frizzled/taste2), mutations targeting ECLs impair ligand dependent activation, also suggests a conserved function of the EC-domain throughout the GPCR superfamily (Karnik et al, 2003). Thus, the dynamic conformational changes in the ECL2 may be critical for the activation mechanism of the GPCRs. Mutations on the ECL2 led to constitutive activation in GPCRs such as TSH receptor and C5A receptor (Duprez et al, 1997, Klco et al, 2005). ECL2 is a negative regulator of C5a receptor activation as discovered by genetic analysis. A similar role in stabilizing the inactive state in other GPCRs is proposed. In our study, none of the mutations caused constitutive activation and a previous genetic analysis also did not find mutations in ECL2 that cause activation of AT1R. ECL2 possibly serves an essential function in protein folding and maintaining the folded-state of the AT1R as indicated by I177C and V179C mutations. The susceptibility of folded AT1R to reducing agents is due to breaking the Cys101–Cys180 disulfide linkage with TMIII (Ohyama et al, 1995, Feng et al, 2000).

A rhodopsin-like ‘lid’ formed by ECL2 in the losartan-bound state is quite similar to the proposed arrangement of ECL2 in the D2 dopamine receptor in the antagonist bound state (Shi & Javitch, 2004). In other GPCRs the conformation of ECL2 in the presence of agonist is not reported. Similar conformational changes are likely in other GPCRs, where a direct role of ECL2 in ligand specificity is documented (Zhao et al, 1996, Duprez et al, 1997, Banares et al, 2005).
Current ideas about how an extracellular lid may limit the access of ligands to cognate GPCRs are based on extrapolation from elegant dynamic and structural studies on bovine rhodopsin (Palczewski et al, 2000, Park et al, 2008, Ahuja et al, 2009). Our analysis allows us to reach a different conclusion, that a ‘lid’ conformation is induced in AT1R upon binding of both agonists and antagonists.

7.6 Final Remarks

The results of the current work provide explanation for several previous observations that remained unanswered. The open conformation of ECL2 provides an aqueous channel that facilitates access of ligands to the ligand binding pocket deep in the TM domain. As ligands enter the binding pocket through this aqueous channel, they may have primary interactions with the ECL2, an interpretation which is consistent with previous mutagenesis and cross-linking studies. The N-terminal residues in Ang II sequentially interact with Ile^{172} and His^{183} in ECL2, while the C-terminal residues in Ang II inserts into the binding crevice, interacting with TM residues (Gogonea et al, 2006). Photoaffinity cross-linking studies have shown that Val^{3} in Ang II is cross-linked with Ile^{172} in ECL2 (Perodin et al, 2002) and Phe^{293}/Asn^{294} in TMVII interact with Phe^{8} of Ang II (Perodin et al, 2002), leading Ang II to fold into a ‘C’ shape, a conformation predicted in NMR studies (Marshall et al, 1974, Samanen et al, 1994, Garcia et al, 1992, Nikiforovich et al, 1994). The mechanism for simultaneous interaction of Ang II with ECL2 and TM domain can only be explained through folding of ECL2 into the pocket within the TM domain, as we have shown here. The Ang II docking process may be
achieved by specific rearrangement of the hydrogen bond network of ECL2, which is then coupled to the movements of TM helices involving the conserved disulfide bond.

The open conformation of the ECL2 in the empty receptor is independently supported by autoantibody epitope mapping studies in human AT1R (Liao et al, 2002, Dragun et al, 2005, Zhou et al, 2008). Autoantibodies directed towards ECL2 of AT1R recognize the Ile<sup>172</sup>–Thr<sup>178</sup> and Glu<sup>185</sup>–Thr<sup>190</sup> segments, which are found to be readily accessible in our results. Our analysis suggests that these autoantibodies can capture the accessible region on the ECL2. It has been shown that, losartan binding could mask these epitopes to prevent autoantibody binding and protect patients harboring autoantibodies. The lid conformation of ECL2 induced by losartan suggests that the epitope regions are rendered inaccessible to autoantibodies, preventing their interaction with the AT1R.

Transient formation of an EC-lid is most likely very important in GPCRs for blocking rapid exit of the ligand from the pocket. Their ligands bind to the pocket rapidly and leave the binding pocket at a much slower rate. For example, the dissociation rate (k<sub>a</sub>) is five times larger than the association rate (k<sub>a</sub>) of both Ang II and losartan for AT1R (Timmermans et al, 1993, Ojima et al, 1997). A transiently formed ‘lid’ can prevent the ligands from leaving the pocket rapidly, thus sustaining the ligand-specific conformation longer. In the agonist-bound state, this would allow the receptor to amplify the signal by prolonged G protein activation. Unlike rhodopsin, the activation in most other GPCRs may require different dynamic parts of the receptor to achieve a sharp transition to active
state. In this way, the ‘lid’ may help to ensure that the strain energy gain by agonist and/or antagonist binding is efficiently transformed into helix rearrangement. In other words, the ‘lid’ allows the ligand to flip the GPCR switch more easily and perhaps faster, as suggested by Bourne and Meng (2000).
CHAPTER VIII
FUTURE WORK

8.1 Introduction

The results presented in this thesis have shown that binding of agonist and antagonist in AT1R induces formation of a distinct lid structure in ECL2, which closely interacts with the ligand. The specific conformations of the lid, particularly around the highly conserved disulfide bond that links ECL2 to TM III, were different in the Ang II-bound and losartan-bound states. Are specific ECL2-lid conformations necessary for receptor activation and inhibition? Could ECL2 form a lid without the agonist, during constitutive activation of AT1R? How are the dynamics of ECL2 conformation affected by gain-of-function mutations located on TM-domain and loss-of-function mutations in the N-terminal tail and ECL3? Is the ECL2 conformation in mutant receptors similar to that of the empty-state or the ligand-bound state?

Coordinated movement of different domains within a GPCR is critical for its biological function, transduction of signal across the membrane. Binding of a molecule to
the EC-domain initiates local conformational changes that involve several intermediate
tates of dynamic movements within the protein. These dynamic movements take place in
the nanosecond timescale, and lead to long-range rearrangements in the TM and
cytoplasmic domains of GPCRs. Conformational changes also occur in the reverse order.
For instance, G-protein binding to a GPCR initially changes the local conformation of the
cytoplasmic loops, which subsequently propagates to the TM helices and the extracellular
domains. Domain coupling has been shown to be critical for the biological activity of
many different protein families with signaling and structural functions such as enzymes
and receptors. However, the regulation of dynamic processes that facilitate domain
coupling in proteins are poorly understood.

The critical nature of domain coupling in transmembrane signaling by GPCRs is
well documented. GPCRs exist in equilibrium between active and inactive conformations
in the absence of any ligand. Different types of ligands stabilize distinct conformations.
The inactive state of the receptor is stabilized by constraining intramolecular interactions;
agonists release the constraints leading to coupling of the cytoplasmic domain to the G-
protein. Thus, agonist activation is associated with the initiation of the signal at the ligand
binding site and the transmission of the signal along the transmembrane helices. Ligand
induced rearrangement of helices consequently leads to structural changes on the
cytoplasmic domain. On the other hand, antagonists and inverse agonists stabilize a more
constrained inactive conformation that decreases the affinity towards the G-protein.
Ligand-free GPCRs can also be driven to a high affinity state upon binding of G-protein
to the cytoplasmic face of the receptor which suggests that the conformational changes in the cytoplasmic domain are coupled to the conformation of the agonist binding site (Karnik et al, 2003).

Gain-of-function, loss-of-function and misfolding mutants of GPCRs directly alter domain coupling between the extracellular, transmembrane and cytoplasmic domains of GPCRs (Karnik et al, 2003). Mutations that constitutively activate and increase the G-protein coupling efficiency of GPCRs also increases the affinity towards agonist by leading to a favorable reconfiguration at the agonist-binding site made up of extracellular and transmembrane domain residues. In contrast, mutations that impair agonist binding disrupt favorable interactions between the agonist and the binding pocket, thus keeping the cytoplasmic domain in a low-affinity state towards G-proteins. These observations suggest that a mutation in a particular domain of the receptor causes a local structural perturbation that can be transmitted to other domains leading to a global consequence in terms of affinity towards ligands and G-protein.

Misfolding mutations of rhodopsin, known as retinitis pigmentosa (RP) mutations, have been extensively studied. These studies suggested that the cooperativity between different domains originated from the tertiary folding of the receptor polypeptide. (Spiegel, 1996, Berson, 1996, Hwa et al, 1997) RP mutations can also be located in the extracellular, transmembrane and cytoplasmic domains of rhodopsin and results in partially or completely misfolded receptors, suggesting that the coupling of tertiary structures of different domains take place during folding of the receptor.
The misfolding of the receptor due to a RP mutation is usually associated with the loss of formation of the disulfide bond between TMIII and ECL3 of rhodopsin, which is conserved in more than 90% of GPCRs (Hwa et al, 1999 & 2001). This disulfide bond in rhodopsin is critical for generating the functionally active structure of the rhodopsin (Karnik et al, 1988).

We propose that domain coupling is a central phenomenon of the GPCRs including AT1R (Karnik et al, 2003). The major structural evidence of domain coupling in GPCRs is the interhelical contacts in the TM domain. Intramolecular contacts, hydrophobic interactions and salt bridges between the extracellular domain and the TM domain indicate that the activity of TM domain is regulated by the structural features of the extracellular domain in GPCRs.

Very little is known about the ligand-specific conformational changes in the extracellular domain and coupling of these movements to the TM domain. The results of this thesis project provide evidence for conformational coupling between the ECL2 and the ligand binding site, showing that different molecules targeting this region stabilize distinct conformations that would eventually affect the transmembrane and cytoplasmic domain conformation. In the AT1R model, ECL2 has been shown to have extensive contacts with the N-terminal tail, ECL3 and TM helices. (Fig. 8.1) Therefore we propose that the ECL2 conformation is influenced by mutations in the N-terminal tail, ECL3 and TM domain. Several residues on the N-terminal tail, ECL3 and TM domain of AT1R have been found to be important for both ligand binding and activation of the receptor.
What is the effect of specific residue mutations in these domains on the conformation of ECL2 in the absence and presence of different types of ligands?

8.2 Experimental Design

In order to determine the effects of structural changes in the other domains of the receptor on the ECL2 conformation, we will utilize the gain-of-function and the loss-of-function mutants of AT1R. The conformational effects of these substitutions on the ECL2 will be analyzed by measuring the accessibility of selected ECL2 residues in the presence of activating or inactivating substitutions, using the reporter cysteine accessibility mapping analysis with the same approach applied before.

First, we want to analyze the effect of a gain-of-function substitution of Asn\textsuperscript{111} residue, found on TMIII with glycine (Fig. 8.2). Asn\textsuperscript{111} residue interacts with the Tyr\textsuperscript{4} of Ang II (Feng et al., 1998) and is positioned close to the highly conserved disulfide bond that links TMIII to ECL2. Asn\textsuperscript{111} to Gly\textsuperscript{111} substitution leads to a constitutively active AT1R. The N111G-AT1R has been shown to have 50% more activity compared to wild type receptor (Feng et al., 1998). As the size of the substituted residue decreases, the basal IP3 production increases (Fig. 8.3). The N111G-AT1R showed higher affinity for Ang II and lower affinity for losartan (Fig. 8.3). Since N111G-AT1R mutants is constitutively activated, it is assumed to be in an active conformation. Is the conformation of ECL2 in N111G-CYS\textsuperscript{−}AT1R similar to that of the Ang II-bound state of CYS\textsuperscript{−}AT1R?
Figure 8.1. ECL2 interactions with NT, ECL3 and TM helices. A) Predicted interactions of NT (purple) with ECL1 (blue), ECL2 (green) and ECL (red). B) Predicted interaction of ECL2 (green) with ECL1 (blue) and ECL3 (red). C) Predicted interaction of ECL2 (green) with TM helices: TMI (purple), TMII (purple), TMIII (red), TMVII (blue). (Courtesy of Camelia Gogonea and S.Karnik)
Figure 8.2. The positions of substitutions to be analyzed. The substitutions selected to analyze are \( \text{Arg}^{23} \) on N-terminal tail, \( \text{Asn}^{111} \) on TMIII and \( \text{Asp}^{281} \) on ECL3, shown in red circles. These substitutions are combined with ECL2 reporter cysteines replacing \( \text{Asn}^{174} \), \( \text{Asn}^{176} \), \( \text{Phe}^{182} \) and \( \text{Arg}^{187} \), shown in pink circles. Cysteine residues substituted with alanine are shown in green circles. Cysteine residues involved in formation of disulfide bonds are shown in gold circles.
Figure 8.3. Characteristics of N111G-AT1R. A) IP production without agonist activation is measured as the percentage of IP produced by the same receptor when stimulated with 1 µM [Sar1] Ang II (100%) in parallel. Details are given by Miura et al, 2002. B) The affinities of wild type AT1R and N111G-AT1R for Ang II and losartan. Details are given by Feng et al, 1998.

**Table:**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Affinity (nM)</th>
<th>WT</th>
<th>N111G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>10.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Losartan</td>
<td>10</td>
<td>235</td>
<td></td>
</tr>
</tbody>
</table>
The other substitution that will be analyzed for its effects on ECL2 conformation is Arg\textsuperscript{23} to alanine substitution. Arg\textsuperscript{23} is located on the N-terminus of AT1R (Fig. 8.2). It interacts with Asp\textsuperscript{1} of Ang II. Its interaction with Asn\textsuperscript{176} of ECL2 has also been predicted (Gogonea & Karnik, 2006). The R23A-AT1R has been shown to have a reduced efficacy for Ang II (Santos et al, 2004). On the other hand it has a similar efficacy for losartan compared to wild type AT1R indicating that the mutation does not cause a gross defect in the receptor structure. Similarly, substitution of Asp\textsuperscript{281} to alanine also reduces the efficacy of the receptor towards Ang II, without changing the efficacy towards losartan (Hjorth et al, 1994, Feng et al, 1995, Costa-Neto et al, 2000, Santos et al, 2004). Asp\textsuperscript{281} residue is located on the ECL3 and interacts with the Arg\textsuperscript{2} of Ang II (Fig. 8.2). Its interaction with Asn\textsuperscript{174} of ECL2 is also predicted (Gogonea & Karnik, 2006). Since these two substitutions result in loss-of-function mutants, they are assumed to be in a conformation that disfavors efficient binding of Ang II and accounts for a low-affinity state. Is the conformation of ECL2 in R23A-CYS\textsuperscript{−}AT1R and D281A-CYS\textsuperscript{−}AT1R similar to that of empty state or losartan-bound state of CYS\textsuperscript{−}AT1R?

In order to determine the conformational effects of these substitutions on the ECL2, we will measure the accessibility of reporter cysteines replacing selected ECL2 residues generated on the background of N111G-CYS\textsuperscript{−}AT1R, R23A-CYS\textsuperscript{−}AT1R and D281A-CYS\textsuperscript{−}AT1R. The selected ECL2 single cysteine mutants are N174C, N176C, F182C and R187C. These residues are shown to be highly sensitive to changes in the conformational state of the receptor based on our previously generated accessibility maps. The reasons for choosing these ECL2 residues for this analysis are listed below.
N174C:

- close to Ile^{172} which is crosslinked to AngII
- accessible in the absence of ligand and inaccessible in the presence of both AngII and losartan
- in the autoantibody binding region

N176C:

- inaccessible in the absence of ligand, accessible in the presence of losartan
- close to the disulfide bond
- in the autoantibody binding region

F182C:

- inaccessible in the absence of ligand, accessible in the presence of AngII
- close to the disulfide bond
- in the autoantibody binding region

R187C:

- accessible in the absence of ligand, inaccessible in the presence of both AngII and losartan
- in the autoantibody binding region

The NIIIG mutation, R23A mutation and D281A mutation were simultaneously introduced into these single cysteine mutants by PCR mutagenesis as described in Figure
2.4. NIIIG-CYS\textsuperscript{\texttt{AT1R}} mutant, R23A-CYS\textsuperscript{\texttt{AT1R}} mutant and D281A-CYS\textsuperscript{\texttt{AT1R}} mutant were also created for use as a control. The list of primers used in the PCR mutagenesis is provided in the Table VIII.I. The double mutants constructed for MTSEA-biotin accessibility are listed in the Table VIII.II. All receptor mutants were confirmed by automated sequencing.

Following construction of mutants, we started characterization of mutants. The expression analysis of N111G single cysteine mutants was performed by western blotting (Fig. 8.4). The expression patterns of all mutants were similar to HA-CYS\textsuperscript{\texttt{AT1R}}, although there is a decrease in the expression. Further we analyzed the cell surface expression and binding affinities of N111G mutants by calculating $K_d$ and $B_{\text{max}}$ values from scatchard plots (Table VIII.III). As expected we observed a higher affinity for $[^{127}\text{I}]-[\text{Sar}^{1}\text{Ile}^{8}]$ Ang II with the N111G single cysteine mutants compared to HA-CYS\textsuperscript{\texttt{AT1R}}. We also calculated the number of receptors on the cell surface to make sure that we have enough number of receptors on the cell surface especially for mutants with low $B_{\text{max}}$ values to perform accessibility analysis. The characterization of R23A and D281A mutants will be completed in the same way. The mutants will also be analyzed for their abilities to activate Ang II induced signaling pathway by Ca\textsuperscript{2+} transient measurements. After completion of characterization of mutants, we will determine the accessibility of mutants using the RCAM analysis with the same approach applied before in order to determine the conformational effects of these substitutions on the ECL2.
Table VIII.I. The list of primers used to generate N111G, R23A and D281A substitutions.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>bp</th>
<th>%GC</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N111G sense</td>
<td>GCCAGCGTGAGCTTCGGCCTCTACGCG</td>
<td>27</td>
<td>70.38</td>
<td>102.06</td>
</tr>
<tr>
<td>N111G antisense</td>
<td>CGCGTAGAGGCCGAAGCTACGCTGGC</td>
<td>27</td>
<td>70.38</td>
<td>102.06</td>
</tr>
<tr>
<td>R23A sense</td>
<td>CCGAAGGGCTGGAGGCTCACAGTTAC</td>
<td>24</td>
<td>58.34</td>
<td>89.09</td>
</tr>
<tr>
<td>R23A antisense</td>
<td>GTAACTGTGAGGCTCCAGCTTCG</td>
<td>24</td>
<td>58.34</td>
<td>89.09</td>
</tr>
<tr>
<td>D281A sense</td>
<td>GACATCGTGGCCACTGCCCAGCCCTGCCC</td>
<td>24</td>
<td>66.67</td>
<td>98.76</td>
</tr>
<tr>
<td>D281A antisense</td>
<td>GGGCATGCCAGTGGCCACGATGTC</td>
<td>24</td>
<td>66.67</td>
<td>98.76</td>
</tr>
</tbody>
</table>
Table VIII.II. List of double mutants. N111G-CYS−AT1R, R23A-CYS−AT1R and D281A-CYS−AT1R are generated using HA-CYS−AT1R background. The respective single cysteine mutants of ECL2 (available from the previous study) were used as templates to incorporate N111G, R23A and D281A substitutions simultaneously. All mutants have the HA epitope to facilitate analysis.

<table>
<thead>
<tr>
<th>N111G single cysteine mutants</th>
<th>R23A single cysteine mutants</th>
<th>D281A single cysteine mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>N111G-CYS−AT1R</td>
<td>R23A-CYS−AT1R</td>
<td>D281A-CYS−AT1R</td>
</tr>
<tr>
<td>N111G-N174C-CYS−AT1R</td>
<td>R23A-N174C-CYS−AT1R</td>
<td>D281A-N174C-CYS−AT1R</td>
</tr>
<tr>
<td>N111G-N176C-CYS−AT1R</td>
<td>R23A-N176C-CYS−AT1R</td>
<td>D281A-N176C-CYS−AT1R</td>
</tr>
<tr>
<td>N111G-F182C-CYS−AT1R</td>
<td>R23A-F182C-CYS−AT1R</td>
<td>D281A-F182C-CYS−AT1R</td>
</tr>
<tr>
<td>N111G-R187C-CYS−AT1R</td>
<td>R23A-R187C-CYS−AT1R</td>
<td>D281A-R187C-CYS−AT1R</td>
</tr>
</tbody>
</table>
Figure 8.4. Expression of ECL2 single cysteine mutants with N111G mutation. Expression analysis of HA-CYS\textsuperscript{-}AT1R (lane 2) and HA-tagged single cysteine mutants with N111G substitution (lanes 3-7) in transiently transfected COS1 cells. Untransfected cells served as negative control (lane 1). Actin expression levels are shown as loading control.
Table VIII.III. Binding analysis of ECL2 single cysteine mutants with N111G mutation. The $K_d$ value of HA-CYS$^-$AT1R was 5.10 nm and $B_{\text{max}}$ value was 7.46 pmol/mg in the same experiment (n=3). *Number of receptors on the cell surface calculated by the formula: [(\(B_{\text{max}}\) \times Avogadro’s number) / number of cells].

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_d$ (nm) ± S.E.M</th>
<th>$B_{\text{max}}$ (pmol/mg) ± S.E.M</th>
<th>~Molecules/cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N111G-CYS$^-$AT1R</td>
<td>1.21 ± 0.43</td>
<td>2.76 ± 0.63</td>
<td>166,211</td>
</tr>
<tr>
<td>N111G-N174C</td>
<td>3.43 ± 0.20</td>
<td>0.78 ± 0.15</td>
<td>46,972</td>
</tr>
<tr>
<td>N111G-N176C</td>
<td>1.15 ± 0.49</td>
<td>0.03 ± 0.01</td>
<td>1,806</td>
</tr>
<tr>
<td>N111G-F182C</td>
<td>2.47 ± 0.38</td>
<td>0.39 ± 0.14</td>
<td>23,486</td>
</tr>
<tr>
<td>N111G-R187C</td>
<td>2.45 ± 0.37</td>
<td>0.79 ± 0.12</td>
<td>47,574</td>
</tr>
</tbody>
</table>


of the Cytoplasmic Domain of Mammalian Rhodopsin. Proc. Natl. Acad. Sci. 98; 4888-4892


73. Miura, S. I., Karnik, S. S. (1999a) Angiotensin II type 1 and type 2 receptors bind angiotensin II through different epitope recognition. J. Hypertension. 17; 397-404


293 and 294 are ligand contact points of the human angiotensin type 1 Receptor.

*Biochemistry* 41; 14348-14356


Hamster Ovary Cells expressing human angiotensin II type 1 receptors. *Biochem. Pharmacol.* 59, 927-935


