

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**INVESTIGATION OF DIMERIZATION IN ANGIOTENSIN
RECEPTORS BY COMPUTATIONAL METHODS**

İSMAİL EROL
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF CHEMISTRY

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ANJİYOTENSİN RESEPTÖRLERİNDE
DİMERLEŞMENİN HESAPLAMALI
METODLAR İLE İNCELENMESİ

İSMAİL EROL
DOKTORA TEZİ
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GEBZE
2023



DOKTORA JÜRİ ONAY FORMU

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SUMMARY

Angiotensin receptors can interact with each other or with other receptors to form GPCR homo- or heterodimers. As a result of dimerization, their signaling pathways or the types of G proteins with which they couple and signal that they generate may change, and their signaling may increase or decrease. In order to elucidate this allosteric mechanism, it is important to determine the interaction interfaces. In this thesis, firstly, the possible homodimer forming interfaces of angiotensin II type 1 receptor (AT1R) were determined. Then, the interaction interfaces of AT1R-AT2R heterodimer formed by AT1R with angiotensin II type 2 (AT2R) were investigated. Interfaces were investigated using molecular dynamics (MD) simulations.

Four possible interfaces are proposed for the AT1R homodimer. These are TM1,2,8, TM5, TM4, and TM4,5 tight interfaces, respectively. Another important finding is that ILE238 plays an important role in maintaining the inactive state of the receptor. Two interaction interfaces were identified for the AT1R-AT2R heterodimer, a symmetric (TM3,5,6) and an asymmetric (TM1,2,8-TM3,4) one.

Key Words: GPCR, Angiotensin receptor, Homodimerization, Heterodimerization, Allosteric Mechanism.

ÖZET

Anjiyotensin reseptörleri birbirleriyle veya diğer reseptörlerle etkileşime girerek GPCR homo- veya heterodimerleri oluşturabilir. Dimerizasyonun bir sonucu olarak, sinyal yolları veya çiftleştikleri ve sinyal verdikleri G proteinlerinin türleri değişebilir ve sinyalleri artabilir veya azalabilir. Bu allosterik mekanizmanın aydınlatılabilmesi için etkileşim arayüzlerinin belirlenmesi önemlidir. Bu tezde ilk olarak anjiyotensin II tip 1 reseptörünün (AT1R) olası homodimer oluşturma ara yüzleri belirlenmiştir. Daha sonra, AT1R'nin anjiyotensin II tip 2 (AT2R) ile oluşturduğu AT1R-AT2R heterodimerinin etkileşim arayüzleri araştırılmıştır. Arayüzler moleküler dinamik (MD) simülasyonları kullanılarak araştırılmıştır.

AT1R homodimer için dört olası arayüz önerilmiştir. Bunlar sırasıyla TM1,2,8, TM5, TM4 ve TM4,5 sıkı arayüzleridir. Bir diğer önemli bulgu ise ILE238'in reseptörün inaktif durumunun korunmasında önemli bir rol oynamasıdır. AT1R-AT2R heterodimer için, biri simetrik diğeri asimetrik olmak üzere iki etkileşim arayüzü tanımlanmıştır. Bu arayüzler sırasıyla simetrik TM3,5,6 ve asimetrik TM1,2,8-TM3,4'tür.

Anahtar Kelimeler: GPKR, Anjiyotensin reseptörü, Homodimerizasyon, Heterodimerizasyon, Allosterik Mekanizma.

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations and</u>	<u>Explanations</u>
<u>Acronyms</u>	
α 1AR :	α 1 adrenergic receptor
α 1dAR :	α 1d adrenergic receptor
α 2AR :	α 2 adrenergic receptor
β 1AR :	β 1 adrenergic receptor
β 2AR :	Beta 2 adrenergic receptor
δ OR :	Δ opioid receptor
κ OR :	κ opioid receptor
μ OR :	μ opioid receptor
5HT2AR :	Serotonin (5HT) 2A receptor
A1AR :	Adenosine 1A receptor
A2AR :	Adenosine 2A receptor
A3AR :	A3 adenosine receptor
AA :	All atom
ACE :	Angiotensin converting enzyme
ACE2 :	Angiotensin converting enzyme 2
AFM :	Atomic force microscopy
ANGII :	Angiotensin II
ARB :	Angiotensin receptor blockers
AT1R :	Angiotensin II type 1 receptor
AT2R :	Angiotensin II type 2 receptor
B2R :	Bradykinin 2 receptor
BRET :	Bioluminescence resonance energy transfer
cAMP :	Cyclic adenosine monophosphate
CB1R :	Cannabinoid CB1 receptor
CCR2 :	CC type chemokine receptor 2
CCR5 :	CC type chemokine receptor 5
CG :	Coarse grained
D1R :	Dopamine D1 receptor
D2R :	Dopamine D2 receptor

D3R	:	Dopamine D3 receptor
D5R	:	Dopamine D5 receptor
DEER	:	Double electron-electron resonance
ECL	:	Extracellular loop
ER	:	Endoplasmic reticulum
ERK	:	Extracellular signal regulated kinase
FDA	:	U.S. Food and Drug Administration
FRET	:	Förster resonance energy transfer
FZD6	:	Frizzled class receptor 6
GDP	:	Guanosine Monophosphate
GIRK	:	G protein coupled inwardly rectifying potassium channel
GnRHR	:	Gonadotropin releasing hormone receptor
GPCR	:	G protein coupled receptor
GRAFS	:	Glutamate Rhodopsin Adhesion Frizzled Secretin
GRK	:	GPCR kinase
GTP	:	Guanosine Triphosphate
H8	:	Helix-8
ICL	:	Intracellular loop
M3R	:	Muscarinic acetylcholine M3 receptor
MAPK	:	Mitogen activated protein kinase
MD	:	Molecular dynamics
MEK5	:	Mitogen/extracellular signal regulated kinase 5
mGluR1	:	Metabotropic glutamate receptor type 1
mGluR5	:	Metabotropic glutamate receptor type 5
MM1R	:	Muscarinic m1 receptor
MPK1	:	Mitogen activated protein kinase 1
MT1R	:	Melatonin MT1 receptor
NAM	:	Negative allosteric modulator
O1R	:	Orexin 1 receptor
PAM	:	Positive allosteric modulator
PDB	:	Protein Data Bank
PI3K	:	Phosphoinositide 3 kinase
PKA	:	Protein kinase A

PKC	:	Protein kinase C
PLC	:	Phospholipase C
PP2	:	Protein phosphatase 2
PSN	:	Protein structure network
PYK	:	Proline rich tyrosine kinase
RAAS	:	Renin angiotensin aldesteron system
RAS	:	Renin angiotensin system
ROS	:	Reactive oxygen species
RXFP1	:	Relaxin family peptide receptor 1
S1I8	:	Saralasin-1 isoleucine-8
SDS	:	Sodium dodecyl sulfate
SSTR5	:	Somatostatin receptor type 5
TM	:	Transmembrane
VSD	:	Venus flytrap domain
WT	:	Wild type

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1. INTRODUCTION

G protein-coupled receptors (GPCRs) are cell membrane-bound proteins that regulate most of the cellular function by transmitting extracellular signals into intracellular processes. The extracellular vestibule, also known as the primary binding pocket targeted by various molecules, such as hormones, small molecules, neurotransmitters, peptides, and cytokines. The topology of these membrane-bound receptors consists of seven membrane-spanning helices, and three intra- and extracellular loops that connect heptahelical domains together [1]. General topology of a class A GPCR is given in Figure 1.1.

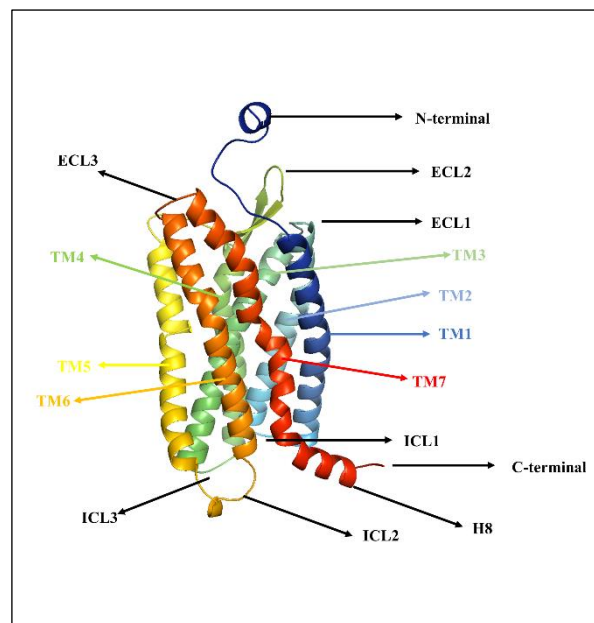


Figure 1.1: General topology of GPCRs.

They are one of the largest protein families in the mammalian genome and are mainly classified phylogenetically into five main groups; rhodopsin, glutamate, adhesion, frizzled, and secretin families. This classification is also known as the GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled, and Secretin) system [2]. The other classification divides GPCRs into six classes, class A (rhodopsin), class B (secretin), class C (glutamate), class D (pheromone), class E (cAMP receptors), and class F (frizzled and smoothened). The latter classification takes into account functional and sequential similarities. GPCRs can signal through three main interacting partners from the intracellular site; G proteins, GPCR kinases (GRKs), and

β -arrestins. G proteins are heterotrimeric entities and composed of three subunits, α , β , and γ , and divided into four families according to alpha subunits. G_s (G_s and G_{olf}), $G_{q/11}$ (G_q , G_{11} , G_{14} , G_{15}), $G_{12/13}$ (G_{12} and G_{13}), and $G_{i/o}$ (G_{i1} , G_{i2} , G_{i3} , G_o , G_z , G_{t1} , G_{t2} , G_{gust}). α subunit responsible for the conversion of guanosine triphosphate (GTP) into guanosine diphosphate (GDP). Upon activation α subunit and β - γ heterodimer are dissociated to initiate downstream signaling pathways. $G\alpha$ downstream effectors are mainly PKA (protein kinase A), PYK (proline-rich tyrosine kinase), proto-oncogene tyrosine-protein kinase Src, MEK5 (mitogen/extracellular signal regulated kinase-5), however $G\beta$ - γ effectors are PI3K (phosphatidylinositol-3 kinase), GRK, GIRK (G protein-activated inward rectifying K^+ channel). Signaling via heterotrimeric G proteins known as classical activation (canonical) pathway. However, in recent years, in contrast to the classical pathway, signaling through beta arrestins are well established. Desensitization of GPCRs starts with the GRK phosphorylation. Mammalian GRKs are investigated in seven classes GRK1-7, and on the basis of tissue expression and sequence homology they divided into three classes; rhodopsin kinases (GRK1 and GRK7), β adrenergic receptor kinases (GRK2 and GRK3), and the GRK4 subfamily (GRK4, GRK5, and GRK6). GRKs bind to the cytoplasmic C-terminal end of the GPCR to phosphorylate the active receptor. The phosphorylated GPCR then becomes capable to bind β arrestin through its phosphorylation sites. β arrestins are grouped into four, non-visual arrestins (arrestin2, and arrestin3, known as β -arrestin1, and β -arrestin2, respectively), and visual arrestins (arrestin1, and arrestin4). Most mammalian tissues and cell types ubiquitously express β -arrestin1, and β -arrestin2. The ability of GPCRs to signal through both G protein and β -arrestin has led to the term 'biased signaling'. To understand biased signaling or in other words, functional selectivity, it is necessary to define key pharmacological concepts in GPCR signaling. Endogenous ligand binding pocket is known as the orthosteric site (primary binding pocket). The ligands can bind and modify receptor function via pockets that are different from orthosteric sites. These are called allosteric sites. Receptor signaling without agonists is defined as constitutive signaling/activity. Ligands that produce the highest signal (maximum) at the pathway under investigation (G protein or β arrestin) named as full agonists (endogenous agonists are usually full agonists). Ligands that induce activity lower than its maximum are named as partial agonists. Constitutive activity of the receptors inhibited by inverse agonists. Molecules that increase receptor activity caused by ligands bound to orthosteric sites by binding to allosteric sites are

called positive allosteric modulators (PAM) and molecules that decrease receptor activity are called negative allosteric modulators (NAM). Additionally, molecules that simultaneously bind to both allosteric and orthosteric sites are known as bitopic molecules. The molecules that inhibit the agonist induced responses are called antagonists.

GPCRs, one of the most important therapeutic targets, have been the subject of active research for many years[3–5]. According to 2017 data, 475 U.S. Food and Drug Administration (FDA) approved drugs act on GPCRs [6]. According to data from the same year, 321 molecules in clinical trials (approximately 20% of all molecules in clinical trials) target GPCRs. Before the elucidation of the first experimental GPCR structures, the studies had been modeled on the basis of homology modeling principles. The first experimental GPCR structure of rhodopsin was determined by Palczewski et al. in 2000 at 2.80 Å resolution [7]. Deposited to Protein Data Bank (PDB) with the 1F88 ID. The first structures for the human non-rhodopsin GPCR, belonging to the β 2 adrenergic receptor (β 2AR), were solved in 2007 by the collaborating research groups of Stevens (with PDB ID 2RH1, at 2.40 Å resolution) and Kobilka (with PDB ID 2R4R, at 3.40 Å resolution) [8, 9]. After 4 years of long and painstaking work, the structure of the first GPCR- G_s protein complex has been elucidated by the research group led by Kobilka in 2011 [10]. The structure of the β 2AR, together with the G_s protein, was solved at 3.2 Å resolution and deposited in the PDB under the code 3SN6. The 2012 Nobel Prize in Chemistry was awarded to Brian Kobilka and Robert Lefkowitz for their enormous contributions to the field of GPCRs, both in terms of structure and function. In 2015, the first GPCR-arrestin complex structure was revealed by X-ray crystallography at 3.30 Å resolution and deposited to the PDB under the 4ZWJ PDB ID.

1.1. Renin-Angiotensin System (RAS)

RAS is an important regulatory system that is responsible for blood pressure and electrolyte-fluid balance. This endocrine system is also known as Renin-Angiotensin-Aldestoren System (RAAS), and is composed of hormones (aldosterone, renin, and angiotensin-II (ANGII)), proteases-enzymes (renin, angiotensin converting enzyme (ACE), and ACE2), GPCRs (ANGII type 1 (AT1), ANGI type 2 (AT2), and MAS receptors). In classical RAS, angiotensin-I (ANGI - ten amino acid length peptide) is

cleaved by ACE, resulting in eight amino acid length hormone ANGII. ANGII serves as an agonist of the AT1R, and AT2R. However, in the alternative RAS, ANGI is cleaved by ACE2, an ACE homolog to result in ANG1-9 (nine amino acids long peptide). ANG1-9, this time is cleaved by ACE to obtain ANG1-7 (seven amino acids long peptide). ANG1-7 is the endogenous agonist of the MAS receptor in the RAS. ANGII mediates almost all functions by binding to AT1R. Sympathetic facilitation, vasoconstriction, sodium and water retention, aldosterone, and vasopressin release were mediated by ANGII via AT1R. Through the activation of protein kinases, induction of remodeling and inflammation, and the production of reactive oxygen species (ROS), AT1R signaling eventually contributes to diabetic nephropathy, metabolic disorders, and various cardiovascular complications such as hypertension, cardiac arrhythmia, and stroke. The success of RAS blockade has been achieved with the use of ACE inhibitors (e.g. benazepril, enalapril, lisinopril, quinapril, and ramipril) and AT1R blockers (e.g. azilsartan, candesartan, irbesartan, losartan, olmesartan, telmisartan, and valsartan).

Class A GPCR family contains different receptor types that can accommodate aminergic (e.g. dopamine), peptide (e.g. angiotensin), protein (e.g. chemokine), lipid (e.g. leukotriene), melatonin, nucleotide (e.g. adenosine), steroid (e.g. estrogen), alicarboxylic acid (e.g. succinate), sensory (e.g. opsins) molecules. In addition to these receptors, there are several other class A GPCRs whose endogenous ligands are unknown or have not yet been identified, known as orphan receptors (e.g. GPR1, GPR75, and GPR84).

1.2. Angiotensin II Type 1 Receptor (AT1R)

AT1R is synthesized by a single gene, *AGTR1*, which is located on chromosome 3 (3q21-3q25). However, in rodents, two types of AT1R genes are available *Agtr1a*, and *Agtr1b*, but these two receptors share 95% sequence similarity. Human *AGTR1* encodes 359 amino acid long protein which has 41 kDa molecular weight. AT1R structure consists of two disulfide bridges, the first one connects N-terminal with transmembrane (TM) 7 (CYS18^{Nterm}-CYS274^{7.25}), and the second one bridges TM3 with ECL2 (CYS101^{3.25}-CYS180^{ECL2}). Human *AGTR1* is deposited to UNIPROT with the P30556 entry ID.

The AT1R can signal via either G proteins ($G_{q/1}$, G_{12} , G_{13} , and G_i) or β -arrestin. The G protein signaling is mediated via activation of phospholipase C (PLC), PLA, and PLD. G protein independent signaling (through β arrestins) occurs with the activation of ERK1/2.

The first structural model for the AT1Ra (rat) was built by Yamano et al in 1995 [11]. In this study, ARG167^{4.64}, LYS199^{5.42}, PHE259^{6.54}, and ASP263^{6.58} identified as important residues in ANGII binding. Another important result of this study was the identification of ARG167^{4.64} involvement in non-peptide antagonist binding, where previous studies were highlighting the importance of LYS199^{5.42} [11]. In 2001, Nikiforovich built the AT1Ra only considering steric and energetic complementary with ANGII [12]. The first 3D receptor model for the human AT1R came out in 2004 by Santos and colleagues [13]. They identified the interaction between ANGII's ASP1 and AT1R's ARG23^{Nterm} as a major finding. This helped to clarify the differences in activation between ANGII and [SAR1]-AngII (where ASP1 was mutated to sarcosine). Other homology modeling studies of AT1R were conducted mostly to find novel antagonist molecules for the blockade of the receptor [14–19]. In the literature cited above, the homology model of the AT1R has been used to predict the binding of proposed compounds at the binding site of the receptor and has provided a considerable amount of structural information where there is no experimental data. The first experimental structure for the AT1R was revealed in 2015, with the ZD7155 (an antagonist that is a precursor of candesartan, and structurally similar to tasosartan) at 2.9 Å resolution using X-ray laser serial femtosecond crystallography [20]. The structure was deposited to PDB with the 4YAY ID. Residues TYR35^{1.39}, PHE84^{2.60}, and ARG167^{4.64} were found to be important in the binding of small molecules, whereas these residues had not previously been suggested to be important in binding. Moreover, in the crystal structure TYR35^{1.39}, PHE84^{2.60}, ARG167^{4.64}, and LYS199^{5.42} were found important both for the peptide and non-peptide molecule binding. PHE182^{ECL2} and ILE288^{7.39} were identified as important for [SAR1, ILE8 (S1I8)]-ANGII binding, but mutations of SER109^{3.33}, and TYR292^{7.42} somewhat affected non-peptide antagonist binding, showing importance for the candesartan. Almost five months later, the AT1R crystal structure with inverse agonist olmesartan was revealed at 2.8 Å and the revealed structure was deposited with the 4ZUD PDB ID [21]. The most important finding for the AT1R-olmesartan complex was the identification of ASN111^{3.35} as an important residue for endogenous ANGII binding. However, TRV120027 (a β -arrestin biased

peptide) binding was not affected by the ASN111ALA^{3,35} mutation. A comparison of these two structures (4YAY and 4ZUD) revealed very similar conformations for residues, however in the latter case, conventional X-ray crystallography was used. In 2019 the first activated structure of AT1R complexed with partial agonist S1I8-ANGII at 2.9 Å and deposited to PDB with 6DO1 ID [22]. At that time, only one active and one inactive structure of a peptide receptor belonging to the rhodopsin family has been reported in the literature (the μ opioid receptor). Interestingly, Wingler et al. observed a dimerization interface in the activated structure of the AT1R where TM1, TM2, and TM3 contributed to the interactions. The comparison of active and inactive structures of AT1R resulted in differences in TM5, TM6, and helix-8 (H8). From the extracellular side, TM5 moved toward the receptor core in the active structure (6DO1) compared to the inactive structure (4YAY). In the intracellular end, TM6 moved away 11 Å from the receptor core to make space for the intracellular transducer binding, TM5 accompanied TM6, as it moved in the same direction as 4 Å. Another important structural difference was observed in H8, where it adopted its usual conformation that is parallel to the membrane, which was distinctly bent away from the membrane axis in inactive structure. Other conformational differences were observed in the phenylalanine ratchet (composed of PHE208^{5,51}, PHE249^{6,44}, and PHE250^{6,45}), in two asparagines from TM7 (as an outward movement of ASN294^{7,45}, and ASN295^{7,46} from the receptor core (specifically from ASN111^{3,35}) in active structure), and in tyrosine switch (TYR302^{7,53} came closer toward TYR215^{5,58} to form a hydrogen bond via bridging water molecule). In the accompanying paper, the effects of the ligands from different functional classes were investigated using double electron-electron resonance (DEER) spectroscopy [23]. In this methodology, PHE55CYS^{1,59}, ARG139CYS^{ICL2}, LYS220CYS^{5,63}, ASP236CYS^{6,31}, PHE304CYS^{7,54}, ARG311CYS^{8,52} mutations were introduced at the cytoplasmic ends of respected residues to enable spin labeling. Ten pairwise interactions were defined, between TM1-Intracellular loop (ICL)2, TM1-TM6, TM1-TM7, TM1-H8, ICL2-TM5, ICL2-TM6, ICL2-TM7, ICL2-H8, H8-TM5, and H8-TM6. Following systems were investigated, apo-form of the receptor, inverse agonist bound forms (olmesartan and candesartan), endogenous agonist bound form (ANGII), β -arrestin biased agonist bound forms (TRV023, TRV026, TRV027, and TRV034), G_q biased agonist bound forms (TRV055 and TRV056). As a result of DEER experiments four main states were defined for the AT1R, closed, occluded1, occluded2, and open. Apo form of the AT1R

stabilized mainly the states between closed and occluded1, but in inverse agonist bound forms the stabilization of the states which were much closer to the closed state. B arrestin-biased agonists, which stabilized the states between occluded1 and occluded2, showed the greatest differences. The endogenous agonist bound form stabilized the states between occluded2 and open, mainly the states closer to occluded2. G_q biased agonist bound forms stabilized the states that reside mainly in the open conformation [23]. In 2020, the activated structure of AT1R obtained with the endogenous ligand (ANGII) at 2.9 Å resolution using X-ray diffraction method and deposited with 6OS0 PDB ID [24]. In the same publication β-arrestin biased agonists TRV023 (2.79 Å, 6OS1 PDB ID), and TRV026 (2.70 Å, 6OS2 PDB ID) bound forms of the AT1R also revealed. One of the major differences at the binding pocket was observed in LEU112^{3.36} in the case of endogenous ANGII compared to the ZD7155 and TRV026 bound forms. LEU112^{3.36} moves away to make room for the PHE8 of ANGII, further altering the conformation of TYR292^{7.43}. ASN111^{3.35}, LEU112^{3.36}, and TYR292^{7.43} conformations were distinctive in active (ANGII bound) compared to other two structures (TRV026 and ZD7155). In fact, the side chain density of TYR292^{7.43} was not observed in the ANGII-bound form of AT1R (6OS0). The authors introduced two mutations, LEU112ALA^{3.36} and TYR292ALA^{7.43} to explain the differences that observed in ANGII-bound, TRV026-bound, and ZD7155-bound structures. LEU112ALA^{3.36} mutation decreased the affinity for [SAR1, ILE8]-AngII, TRV023, and TRV026, and increased the affinity for ANGII. The TYR292ALA^{7.43} mutation increased the affinity for TRV055 and TRV056. These are G_q-biased agonists with PHE at position 8. TRV023 with ALA at position 8, TRV026 with a seven amino acid long peptide and [SAR1, ILE8]-AngII with ILE at position 8 were not significantly affected in this mutant.

1.3. Angiotensin II Type 2 Receptor (AT2R)

AT2R is synthesized by a single gene, *AGTR2*, which is located on chromosome X (Xq22-Xq23). Human *AGTR2* encodes 363 amino acid long protein which varies between 41 kDa molecular weight, however depending on the tissues different molecular weights can be observed. AT2R structure consists of two disulfide bridges, the first one connects N-terminal with TM7 (CYS35^{Nterm}-CYS290^{7.25}), and the second one bridges TM3 with ECL2 (CYS117^{3.25}-CYS195^{ECL2}). Human *AGTR2* is deposited

to UNIPROT with the P50052 entry ID. AT1R and AT2R share 34% sequence similarity.

Although the AT2R has all the structural features and motifs required for the coupling of G proteins, classical G protein signaling does not take place. Although the inability to signal through G proteins has been well explained, there are also reports in the literature that AT2R stimulates ion channels through its G_i coupling [25]. Since AT2R primarily uses non-canonical (G protein and β -arrestin independent) pathway, it signals by activating protein phosphatases. AT2R activation leads to stimulation of protein phosphatase 2 (PP2) and mitogen-activated protein kinase phosphatase 1 (MKP1), which in turn antagonize the intracellular kinases such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2).

AT2R activation by ANGII is thought to produce vasodilatory effects and lower blood pressure by directly opposing AT1R signaling. Activation of AT2R by the other two endogenous ligands; ANG1-7 and ANG1-9, leads to protective properties by reducing blood pressure, fibrosis, and inflammation. Although AT2R was discovered shortly after AT1R, it was initially thought not to be a GPCR because of its interesting signaling properties [26]. The idea that ANGII exerts all its biological functions through AT1R was another issue that overshadowed AT2R. Existing studies in the literature have generally focused on elucidating the interesting signaling behavior of AT2R. With the revelation that the pathological signaling machinery of the AT1R is inhibited by the AT2R, it has been suggested that the AT2R is a useful receptor and has therefore not been the subject of modeling or molecular docking simulation studies [26].

The first crystal structure of the AT2R together with two high-affinity ligands, one AT2R-selective (compound 1, 5UNF and 5UNG, at 2.8 Å) and the other an AT1R-AT2R dual ligand (compound 2, 5UNH at 2.9 Å), was revealed in 2017 [27]. The compound 1 and 2 were quinazolinone-biphenyltetrazole derivatives. This crystal was obtained after many years of waiting. It has provided answers to many questions. The most interesting contribution to the literature is the observed conformation of H8. Considering the transmembrane regions, the structure that would be classified as active (mainly due to the openness of TM6 and in relation to the conformations of other structural motifs) is actually inactive. This is because the amphiphilic H8 was observed to be oriented towards the binding site of the effector protein in such a way that it can interact with TM5 and TM6. All three crystal structures (AT2R selective compound 1

bound monoclinic and orthorhombic forms, and AT1R-AT2R dual compound 2 bound forms) resemble the active-like conformations of GPCRs when seven transmembrane bundles are compared. However, the fact that the amphiphilic H8 is in a conformation that prevents possible coupling by orienting exactly towards the intracellular effector binding site has allowed it to be called "self-blocking receptor" [28]. Moreover, MD simulations using the obtained AT2R crystal structure showed that even with small positional perturbations applied to detach H8 from the effector binding site, H8 returned to its initial position. Furthermore, the non-canonical H8 conformation observed in the AT2R is very consistent with the G protein conformation observed in the NTS1 neurotensin receptor crystal (PDB ID : 4GRV). Just over a year later, the crystal structure of the S1I8-ANGII bound form of AT2R was solved at 3.2 Å resolution by the Iwata Group, and deposited in the PDB under the code 5XJM [29]. The most important finding revealed by the crystal structure obtained is that S1I8-ANGII binds to both the core and the extended binding site. The extended binding site is the binding site of allosteric molecules in muscarinic acetylcholine receptors [30]. It was also observed that, compared to the ET-1 bound form of the endothelin receptor, the peptides for both the AT2R and the endothelin type b receptor were oriented in the C-terminal down conformation at the binding site. However, the major shortcoming of this crystal structure was that the atomic positions of H8 could not be determined due to poor/weak electron density. This may indicate that H8 is highly flexible even in the S1I8-ANGII bound form. In 2020, after many years of waiting, the complex of ANGII, the main peptide of the RAS, with AT2R was elucidated by X-ray crystallography at a resolution of 3.2 Å resolution and deposited in the PDB under the 6JOD ID [31]. Two very important insights were gained from this crystal. The first is that H8, which has been observed in various conformations in existing experimental structures (self-inhibitory or undeterminable due to weak/uncertain electron density), has returned to the canonical form as an ANGII-bound AT2R. The second is that the activation cascade is initiated by the interaction of PHE8 of ANGII with MET128^{3,36}. In 2022, Perryman et al. crystallized the AT2R in complex with EMA401, which is a selective antagonist of the AT2R, and identified the AT2R as a target in glioblastoma [32]. The elucidated structure at 3 Å resolution, uploaded to the PDB with the 7JNI ID, showed similarities to previous experimental structures. H8 was also observed to be oriented towards the effector protein binding site in this structure. In 2004, Wan et al. reported the first AT2R selective agonist; compound 21 (M024), which was developed using

L162313 as a parent molecule [33]. Subsequently, the same research group synthesized the triphenyl analog 45 of M024 [34], compound 22 (a derivative of L162313) [35], compound 2 [36], compound 20 [37], compound 21 (phenylthiazole scaffold) [38], and 2-Alkyl substituted benzimidazoles [39].

Recently, AT2R was found associated with gout [40], glioblastoma [32], peripheral neuropathic pain [41], irritable bowel syndrome [42], paclitaxel-associated acute pain syndrome [43], oxidative stress and inflammation [44], necroptosis [45], cardiac baroreflex responses [46], postherpetic neuralgia [47], oxidative damage and ischemia–reperfusion injury [48], pulmonary function [49], neurite outgrowth [50], and osteogenesis [51]). However, information on the discovery and development of AT2R antagonists is very limited in the literature. EMA200 (PD123319), EMA300 (PD121981), EMA400 (PD126055), and EMA401 (S-EMA400, olodanrigan) were the reported non-peptidic antagonists of AT2R. The earliest report on AT2R antagonists was published by Blankey et al. in 1991 [52] where they reported compound 36 (PD121981), compound 78 (PD123177), and compound 79 (PD123319) as promising ones from their compound set. Wu et al. synthesized 1,4-disubstituted-piperazine-2-carboxylic acids derivatives and found the L159686 as the most potent compound [53]. In 1993, Klutchko et al reported tetrahydroisoquinoline-3-carboxylic acid derivatives were reported [54]. The most promising compound in this study was PD126055. However, EMA401 which is the S-enantiomer of PD126055 was also obtained but in a racemic mixture (EMA400). Later, enantiopure synthesis of R (EMA402) and S (EMA401) forms was reported [55]. Phase 2 trials were conducted for EMA401 [56], however, discontinued in 2015 due to animal toxicity data, then reinitiated again for painful diabetic neuropathy and postherpetic neuralgia [57]. Currently, there are no ongoing trials on EMA401, all trials were terminated. One of the most interesting AT2R studies in the literature was reported by Murugaiah and in this study a highly potent antagonist (more potent than PD123319) with a meta position substitution in the C21 molecule (AT2R agonist) was obtained [58]. The latest efforts on the discovery and development of novel AT2R antagonists were conducted by Larhed [59] and Hu research groups [60]. In 2022, Cong et al. performed the most detailed study of the conformational landscape of AT2R using Markov state modeling and MD simulations [61]. Different macrostates were defined and the transitions between these states were explained, together with a novel definition of the allosteric pocket.

1.4. Dimerization In GPCRs

The first report on GPCR dimerization came out in 1975 by Limbird et al. where they investigated radioligand binding in β 2AR. The negative cooperativity observed in alprenolol binding was attributed to spare receptors and receptor dimers [62]. The reports by Fraser et al in 1982 [63] and by Avissar et al. in 1983 [64] showed that β 2AR and muscarinic receptors could dimerize taking into account receptor weights by immunoaffinity chromatography and photoaffinity labeling, respectively. Avissar's work also showed that muscarinic receptors can form tetramers, depending on the tissue in which the receptors are isolated. In the 1980s, studies were carried out to explain the fact that many of the receptors could function in a dimer form. These include α 2-adrenergic (α 2AR) [65], D2 dopamine (D2R) [66], α 1-adrenergic (α 1AR) [67], gonadotropin-releasing hormone (GnRH) receptor (GnRHR) [68], and A1 adenosine receptor (A1AR) [69]. Venter et al. performed a size analysis of radiation inactivation and found 160 kDa for the α 2AR in human platelets, indicating a possible dimeric state [65]. A similar methodology was applied to the D2R, and 123 kDa was found to be identical in dog and human [66]. Another target size analysis was performed for the α 1-adrenergic receptor in rat liver membranes by Venter et al [67]. The results showed a molecular weight of 160 kDa, which accounts for two 85 kDa monomers. The functional unit of GnRHR was studied by Conn and Venter [68] in 1985 and found to be $136,346 \pm 8,120$ Da, which was twice the value obtained by the photoaffinity labeling method, indicating possible dimerization. In 1986, Frame et al. [69] conducted another radiation inactivation method on the A1AR and found 63 kDa, which differed from the previous reports on this receptor [70], and [71]. The authors concluded that receptor dimerization could account for the higher molecular weight obtained in the radiation inactivation experiments. The high molecular weights obtained in the above experiments may indicate receptor dimerization or may have been observed due to the tight interaction of GPCRs with their coupled effectors. Although immunodetection of high molecular weight species indicates dimerization, they alone have not been accepted as evidence of GPCR dimerization.

In a wise approach, Maggio et al. constructed a chimeric receptor in which the first five TMs belong to the α 2AR, and the last two TMs belong to the m3 muscarinic receptor (M3R) (and vice versa) applying trans-complementation. When these

chimeric receptors were expressed alone, neither ligand binding nor signaling was observed. However, when expressed together, the binding of both muscarinic and adrenergic agonists and subsequent signaling were detected [72]. One of the most seminal works on GPCR dimerization was conducted by Hebert et al., where they investigated β 2AR dimerization by differential coimmunoprecipitation, and found that sodium dodecyl sulfate (SDS)-resistant homodimers were formed and that TM4 could be in the dimerization interface [73]. In the same year, Ng et al. showed that the D2R dimerizes in cells and human brain tissue samples [74]. Experiments with peptides not from the TM regions of D2R, or from the TM6 region of the β 2AR, or from the C-terminal region of the D1 dopamine receptor (D1R), showed that these tested regions did not cause dissociation of the D2R structures. As an explanation for this, it has been suggested that the dimerization interfaces are receptor specific. Furthermore, the D2R dimers were SDS-resistant, the same as Hebert's study [73], but unlike Hebert, the receptor dimers were reported to dissociate upon temperature increase. In the same year, it was shown that dimerization of the metabotropic glutamate receptor 5 (mGluR5), a class C receptor, occurs covalently via disulfide bonds formed between the large extracellular N-terminal domains [75]. The first dimer receptor simulation was performed by Gouldson in 1997 using a peptide from the C-terminal end of ICL3 of the α 2AR [76]. The main result of the simulations is the demonstration that the interaction interface is formed by the interaction of TM5,6s and not by the TM1,2 interface observed in rhodopsin [77], and [78]. As a milestone study, Overton and Blumer demonstrated the dimerization of the alpha factor receptor in intact cells and membranes using fluorescent proteins where they applied fluorescence resonance energy transfer (FRET) methodology [79]. This study also showed that dimerization was not affected by agonist, antagonist, or G protein binding. In another study, using the bioluminescence resonance energy transfer (BRET) method, functional homodimers of the β 2AR were identified in vivo [80]. These two biophysical methods (FRET and BRET) have been successfully used to identify receptor dimers. Photobleaching FRET is another biophysical method to study both receptor homo- and heterodimerization. Patel group applied this methodology to identify somatostatin type 5 receptor (SSTR5) homo- and SSTR5- D2R heterodimers [81] and [82].

The first reported complex of receptor heterodimerization is the κ -opioid (κ OR) and δ -opioid (δ OR) heterodimer [83]. This heterodimer exhibits ligand binding and functional properties that differ from the behavior of its protomers under normal

conditions. Selective κ OR and δ OR agonists bind to the heterodimer with lower affinities. However, two selective agonists can bind to the heterodimer simultaneously, and this cooperative effect can lead to synergistic responses. Negative cooperativity in CC motif chemokine type 2 receptor (CCR2b)- CC chemokine type 5 receptor (CCR5) heterodimers were reported by El-Asmar et al. [84]. The most striking observation obtained in this study was that either in homodimer or in heterodimer case (both receptors also homodimerize), the resulting dimer bind only one chemokine. The first hetero-oligomeric complex was reported by Sohy et al., where they revealed the negative cooperativity using BRET and luminescence complementation together in CCR2, CCR5, and CXC motif chemokine type 4 receptor (CXCR4) receptors [85]. In their study, they emphasized that the cross-inhibition they observed in chemokine receptor hetero-oligomers can have both beneficial and lethal side effects, and showed that studies conducted to find molecules that target this class of receptors should take oligomerization into account. Allosteric cross-inhibition has been observed in CCR2-CXCR4 heterodimers, where specific antagonists of one protomer prevent chemokine binding to the adjacent protomer [86]. Inactivation of the cannabinoid CB1 receptor (CB1R) with its antagonist led to reduced activation levels of the orexin-1 receptor (O1R), similarly when treatment with an O1R antagonist also reduced CB1R activation [87]. These results indicate that orexin-1 and the CB1R form heterodimers and that specific antagonists of each protomer can modulate the function of the other receptor. Another heterodimer was reported to form between the dopamine D2R and the CB1R [88]. As a result of this heterodimerization, coupling with G_{as} proteins was observed instead of pairing with $G_{ai/o}$ proteins. Rocheville reported agonist-induced heterodimerization, where both receptors bind to their respective agonists to form the most active signaling complex [82]. Between the adenosine A2 receptor (A2AR) and the D2R, another receptor heterodimer has been observed [89]. When the A2AR is stimulated by an agonist molecule, the agonist affinity of the D2R is reduced due to heterodimer formation. Heterodimerization of dopamine receptor subfamilies has been reported [90], [91]. The first heterodimerization was observed between D1R and dopamine D3 receptors (D3R), with D1R increasing agonist binding affinity when D3R was stimulated [90]. The other has been observed between D1R and D2R receptors [91]. This heterodimerization has been reported to result in signaling through $G_q/11$, requiring simultaneous activation of both receptors. Heterodimerization of the melatonin MT1 receptor (MT1R) with the orphan receptor GPR50 impaired agonist

binding and G protein coupling at the melatonin MT1R protomer [92]. In this study, an orphan receptor was shown to be involved in heterodimerization for the first time. In 2005, Urizar et al. reported the thyrotropin (TSHr) and lutropin (LH/CGr) receptor heterodimer [93]. In 2004, Gomes et al. demonstrated that μ -opioid (μ OR) and δ OR receptors heterodimerize in living cells and their native membranes [94]. In this heterodimer state, it was shown that the agonist binding and signaling capacity of the μ OR is increased by the binding of antagonists to the δ OR protomer. The α 1d adrenergic receptor (α 1dAR), which is exclusively intracellular when expressed alone, was observed at the cell surface when coexpressed with the α 1b adrenergic receptor (α 1bAR) [95]. This was suggested to be the result of heterodimerization of the α 1bAR and α 1dARs. One of the first heterodimerization between class C and class A GPCRs was reported in 2008 by González-Maeso between serotonin 2A (5HT2AR) and metabotropic glutamate receptor 2 (mGluR2) [96]. This novel heterocomplex was proposed as a new target for the treatment of psychosis.

1.5. Computational Modeling Of GPCR Homo- And Heterodimers

In order to study GPCR dimer or oligomer states by computational methods, the starting point is to obtain/generate the dimer/oligomer complex structure. This can be done in several ways. The first is based on sequence analysis to identify the most conserved amino acids. Interaction interfaces can be identified by conservation analysis [97–100]. Another way is through information obtained from atomic force microscopy (AFM). This information was used to generate a dimer model for rhodopsin in its native membrane [101]. In this model, which was uploaded to the PDB model archive with the code 1N3M, TM4,5 was observed as the primary interaction interface. Another option is to use experimental structures that have been uploaded to the PDB. Examples of experimentally solved structures in dimer form include rhodopsin [102], C5aR [103], β 1 adrenergic receptor (β 1AR) [104], CXCR4 [105], and μ OR [106]. In addition to these methods, it is also possible to obtain structures of GPCR dimer/oligomer complexes using molecular docking simulations. The lutropin receptor homodimer [107], neurotensin receptor homodimer [108], thromboxane A2 prostanoid receptor homodimer [109], A2AR-D2R heterodimer [110], μ OR- δ OR

heterodimer [111], and mGluR2/5HT2AR heterodimer [112] are some examples of studies to obtain dimers by molecular docking simulations. The dimer/oligomer structures obtained as a result of the above mentioned interface determination studies are placed in the model membrane bilayer and atomic resolution or coarse-grained (CG) MD simulations are performed to study the stability of the determined interfaces and the structural changes that occur as a result of dimerization/oligomerization. Rodríguez's work on the crystal structure of the chemokine receptor CXCR4 in 2012 is one example [113]. In this study, it was shown that the TM5,6 interface maximizes inter-protomer interactions and that no ligand is required to stabilize the CXCR4 homodimer (in agreement with experimental studies, where ligand independent homodimerization was observed). Straßer and Wittmann modeled symmetric and asymmetric β 2AR dimers (with G protein heterodimer) [114]. The symmetric TM1,7 interface turned out to be one of the lowest energy interfaces, and large asymmetric conformational changes were observed in 20 nanosecond simulations with this interface. In a seminal study conducted in 2014, Prasanna et al. investigated the role of cholesterol in β 2AR dimerization [115]. Using CG simulations with different cholesterol ratios, it was shown that cholesterol molecules accumulating around helix 5 limit its involvement in the interaction interface and stabilize the formation of an interface consisting of TM1,2. Kaczor et al. tested a series of piperidine/piperazine derived homobivalent ligands by molecular docking to the asymmetric TM4,5 - TM1,7 interface D2R dimer [116]. It was reported that the homobivalent antagonists stabilized the inactive dimer structure and by making some changes at the interface, a dimer structure was obtained that differed by 4.61 Å from the initial structure. The dimer of Frizzled 6 (FZD6), a class F GPCR, has been shown to form by interaction at the TM4,5-containing interface [117]. This study also describes the mechanism of agonist-induced dimer disruption/re-formation. It was suggested that the monomeric form of FZD6 is the signaling unit preferentially over the dimer form. The A2AR – D2R heterodimer, which constitutes an important target complex in the central nervous system, was found to dimerize at the TM4,5 interface [118]. The obtained heterodimer interface was experimentally mutated and the validity of the predicted interface was demonstrated using computational methods. Shen et al. performed virtual molecule screening to target the interaction interface using the CXCR4 homodimer [119]. A promising molecule was discovered that binds to an allosteric site near TM4. This molecule showed an enhancing effect on inter-protomer interactions with the help of

hydrophobic interactions at the interaction interface. Recently, Dobitz et al. aligned somatostatin dimers to the human-smoothened receptor dimer and the gastrin-secretory peptide receptor dimer to the endothelin receptor dimer [120]. These dimer structures provided information about the distance between the two molecules in homobivalent ligand design. The results showed that intracellular uptake was optimized when the distance between the two molecules was 20 Å. In 2016, Baltoumas et al. conducted one of the most comprehensive studies of GPCR dimers which includes class A, C, and F members [121]. In this study, CGMD simulations were performed using the TM1,2,8 interface for rhodopsin, opsin, κ OR, and μ ORs and the TM4,5 interface for rhodopsin, A2AR, β 1AR, and human smoothened receptors, the TM5,6 interface for human CXCR4 and mouse μ ORs; and TM4,5,6 interface for human CXCR4 receptor. These interfaces were closely packed. For the loosely packed dimers, two different interfaces were simulated; the TM5 interface for squid rhodopsin, and the TM1 interface for human β 2ARs. In addition to these simulations, the authors also conducted MD simulation with cholesterol in the TM1,2 interface for the human metabotropic glutamate receptor 1 (mGluR1), and in the TM1,8 interface for the human β 2AR. In this study, the change in the initial simulation interfaces was investigated throughout the MD simulations. While the tightly packed TM1,2,8; TM4,5 and TM5,6 interfaces were retained, the TM5,6 and TM4,5,6 interfaces observed in the human CXCR4 receptor both changed to the TM4,5 interface. Both interfaces where loose packing dimer was observed were altered, the TM5 interface in squid rhodopsin to TM5,6 and the TM1 interface in human β 2AR to TM1,2,8. Both initial interfaces were converted to TM1,2,8 interfaces in simulations performed in the presence of cholesterol. In another study, Filizola et al. compared the dimer and monomer forms of rhodopsin by simulating them in a model membrane [122]. The TM4,5 interface was used in the simulations, and it was suggested that the changes observed in the monomer-dimer comparison were more intense at the interaction interface, which plays an important role in receptor activation. In 2020, Lei et al. investigated the activation mechanism of the mGluR dimer, a class C GPCR [123]. Class C GPCRs must be in dimer form to function. According to the results of the study, glutamate bound to the A-protomer was found to signal to the G-protein binding site by causing changes in the TM regions. Interestingly, glutamate bound to the B protomer negatively affected glutamate binding to the A protomer, but again caused changes on the A protomer, providing the necessary regulation of the effector protein

binding site. The general idea is that in GPCR dimers, the binding stoichiometry is one G protein for two GPCRs. Contrary to this idea, the ste2 receptor dimer bound to two separate G proteins was structurally elucidated by the Tate group [124]. However, MD simulations of class D GPCR ste2 dimer showed that one G protein remained stable while the other changed its conformation. Chen et al. simulated the apo state and DAMGO (balanced agonist) bound forms of the μ OR and δ OR heterodimer, a well-studied dimer system in the literature [125]. According to the simulation results, DAMGO, a balanced agonist, was transformed into a β arrestin-biased agonist in the heterodimer state, increasing TM1,8 interactions and making the heterodimer more stable. For the CXR4 receptor, dimer systems consisting of two different symmetric interfaces, TM5,6 and TM1, and an asymmetric interface, TM1-TM5,7, were formed [126]. These dimer systems were embedded in membrane layers containing 10% and 30% cholesterol and without cholesterol, and CG simulations were performed. In the simulations, the symmetric TM1 interface and the asymmetric TM1-TM5 and TM1-TM5,7 interfaces were found to be stable. With the addition of cholesterol to the medium, a different story emerged and the formation of the TM1/TM5-7 dimer was prevented by cholesterol molecules clustering around TM1 and TM7, resulting in a new cholesterol-induced interface, the TM3,4 interface. Gahbauer and Böckmann studied neurotensin NTS1 receptor dimerization in model membrane compositions with different physicochemical properties [127]. According to this study, cholesterol molecules clustered around TM5,6 allowed the TM5,6 interface to exhibit a loose packing pattern, while cholesterol accumulated around the positively charged portion of TM4 facing the intracellular side allowed the formation of a symmetrical TM3,4 interface. In their study of CCR5 homodimerization, Zhang et al. found that the TM1,2,3,4 symmetrical interface stabilizes dimerization [128]. As a result of dimerization, maraviroc binding was altered in each protomer. This change was seen as a decrease in binding in one protomer and an increase in binding in the other protomer. This study also revealed the allosteric interaction pathway between the two binding sites. The adenosine A2A receptor is known to form dimeric and even oligomeric structures [129]. Song et al. investigated the effect of receptor conformational state and membrane composition on dimerization/oligomerization using CGMD simulations (~2.6 milliseconds). In simulations performed with PIP2, an increase in interface stability was observed at interfaces where PIP2 was tightly bound. The full-length TAS1R2/TAS1R3 heterodimer model (together with venus flytrap

domain (VSD)) with TM5,6 as the heterodimerization interface was modeled by Kim et al. in 2017 [130]. Agonist binding to TAS1R2 changed the heterodimerization interface to the symmetric TM6 interface. This is similar to another class C GPCR, the metabotropic glutamate receptor dimer. The most significant finding of this study is that it is the first time that the activation of a class C GPCR has been modeled using MD simulation. The snake venom MT7 is a specific allosteric modulator of the muscarinic m1 receptor (MM1R). However, the atomic details of how MT7 interacts with the MM1R are not known. To answer this question, Marquer et al. performed a series of molecular docking simulations to elucidate the structure of the MT7-MM1R complex [131]. They found that MT7 toxin interacts with the extracellular loops of the human MM1R. Furthermore, in these interactions, the MM1R is a homodimer and the interaction interface consists of TM6,7. Perez-Aguilar et al. performed a series of MD simulations to determine the interaction interface of the sweet taste receptor heterodimer [132]. Heterodimer systems were generated from the interaction interfaces of TM4,5; TM5,6; TM1; TM5,4; and TM1,2. In addition, amino acids were identified that would allow allosteric ligand binding from both T1R2 and T1R3. In their 2010 study, Maurice et al. demonstrated that the melatonin MT1 receptor homodimer is capable of binding both RGS20 and G_i protein simultaneously [133]. This study demonstrated that the prior assumption, stating that only a single effector protein could bind to a dimer, is not universally accurate. Li et al. studied the A1AR homodimer using several computational methods (e.g., conventional MD, Gaussian accelerated MD) [134]. They observed asymmetric activation in both homodimer structures with two NECA molecules bound, as well as in the single NECA bound form, with one protomer remaining active while no activation was observed in the other protomer. Another adenosine receptor homodimer study was conducted by Kim and Jacobson in 2006 [135]. In this study, the adenosine A3 (A3AR) homodimer was subjected to MD simulations by constructing different systems to interact through different interaction interfaces. As a result of simulations using different symmetrical homodimerization interfaces such as TM1,2; TM2,3; TM2,4; TM3,4; TM4,5; TM5,6; TM6,7 and TM1,7, it was observed that the TM4,5 interface has both a high degree of conformational complementarity and the highest interaction surface area. In addition to the TM4,5 interface, the TM1,2,8 interface was also found to be a suitable interface for homodimerization. Jiang et al. studied GPCR homodimerization using three different receptors [136]. The TM1,2,8 interface was used for β 1AR and κ ORs; the

TM5,6 interface was used for the chemokine CXCR4 receptor. In the analyses, when the protomers were compared in the monomer and dimer states, it was observed that the amino acids that were important for the intra-receptor information flow in the monomer state retained their importance in the dimer state. However, as a result of dimerization, the information flow was weakened compared to the monomer state. Dijkman et al. reported that NTS1 receptor homodimerization of neurotensin occurs dynamically as a function of receptor density [137]. They showed that instead of homodimerization at a specific interface, different receptor interfacial conformations coexist and transform into each other. This new phenomenon is called "rolling dimer". For dopamine D2R homodimers, it is known that dimer formation and lifetime are prolonged when an agonist is added to the medium. To study the effect of antagonists, Wouters et al. interacted the D2R homodimer formed at the symmetric TM5,6 interface with haloperidol, olanzapine, risperidone, clozapine, droperidol, and spiperone [138]. Spiperone decreased D2R dimer formation while olanzapine increased dimerization. To better understand this peculiar effect of spiperone, the authors generated an A2AR-D2R heterodimer and examined the effect of spiperone on this heterodimer. There was no effect of spiperone on heterodimer formation, suggesting that the observed effect was D2R dimer/oligomer specific. Gahbaur et al. investigated the ability of chemokine receptors to form dimers using CGMD simulations [139]. The formation of CXCR4, CCR2 and CCR5 homodimers, CXCR4/CCR2, CXCR4/CCR5 and CCR2/CCR5 heterodimers was investigated in both model membrane POPC and model membrane POPC containing 30% cholesterol. In the simulations with cholesterol it was observed that the association rates of homodimers increased compared to the simulations without cholesterol. For CC chemokine receptors, TM1 was predominantly observed at the interaction interfaces, whereas TM4 was more important for CXCR4 homodimerisation. Although CCR2 and CCR5 have high sequence similarity, different interaction interfaces were observed in their heterodimers with CXCR4. Niv and Filizola investigated the oligomerisation of rhodopsin by normal mode analysis [140]. The intra-dimer interface was determined as TM4,5 and the interaction interface between dimers as TM5,6. Tetramer findings were compared with rhodopsin monomer. As a result, an increase in positive correlation between transmembrane amino acids in the monomer and an increase in positive correlation between amino acids at the interface in the dimer were observed. The D2R - neurotensin nts1 receptor heterodimer is an important target in

the treatment of diseases such as Parkinson's and schizophrenia. In their study, Hübner et al. designed bivalent ligands to bind to this heterodimer [141]. Bivalent ligands synthesised with polyethylene glycol linkers showed picomolar affinity and strongly activated the neurotensin nts1-dependent β arrestin2 pathway when bound to the heterodimer. Wang et al. investigated μ OR and δ OR heterodimerization and created inactive-inactive and active-inactive systems using receptor protomers in different activation states [142]. As a result, it was observed that the TM1,2,8 interface was the most stable interface for the μ - δ heterodimer, while TM7 also contributed to this interface with some amino acids. Upon activation of a protomer, the interface changed and the contribution of TM2 decreased.

In summary, the experimental results for GPCR homo- or heterodimers in different conditions and environments have clearly demonstrated the existence of these complexes and the functional consequences of dimer/oligomerization. The modeling and simulation results performed to validate these experimental studies or to provide explanations at the atomic level have added to our knowledge. Again, the information and details provided by computational methods will allow the design of new molecules with better pharmacological effects and selectivity, the latter being one of the most important factors in avoiding side effects.

1.6. Dimerization In Angiotensin Receptors

Angiotensin receptors form homo/hetero dimer/oligomer complexes by interacting with themselves or with other GPCR species in the membranes of the cells in which they reside. The homo/heterooligomers they form have different functions from the cellular signals they generate in their monomeric form. For example, while the AT1R in its monomeric form causes vasoconstriction when activated by ANGII, when AT1R-AT2R heterodimers are formed in the cell membrane, the AT1R is inhibited and vasoconstriction is prevented. Such situations further expand and complicate the current signaling spectrum of GPCRs. In this context, a historical review of the homo-/heterooligomers formed by angiotensin receptors with themselves and other GPCRs reveals that the first reported system was the AT1R-bradykinin B2 receptor (B2R) heterodimer. AT1R and B2R are co-expressed in many different tissues. These include renal medulla, smooth muscle, and glomerular mesangium. In 2000, AbdAlla et al. showed that AT1R-B2R heterodimers form functional and stable

dimer structures [143]. As a result of heterodimerization, $G_{\alpha i}$ - and $G_{\alpha q}$ -induced signals, which are the main signaling pathways of the AT1R, were found to be enhanced. This study is also important in the literature because it is the first study to demonstrate signal enhancement as a result of heterodimerization of two vasoactive hormone receptors. AT1R-B2R heterodimerization was studied by the same group in 2005 and it was shown that this dimerization contributes to ANGII hyperresponsiveness [144]. For the first time in the literature, the receptor-specific antagonistic effect of a GPCR was demonstrated by the same group. AbdAlla et al. reported in 2001 that AT1R functions are inhibited as a result of direct interaction of AT2R with AT1R [145]. The authors suggested that this antagonistic effect occurs when AT2R binding causes AT1R to adopt a conformation in which it cannot bind G protein. The first study to report that autophagy is regulated by ANGII also shows that cardiomyocyte autophagy mediated by AT1R is antagonized by AT2R [146]. In their 2010 study, Miura et al. examined whether the antagonistic effect of AT2R on AT1R is the result of heterodimerization at the cell surface or negative cross-talk in the cytoplasm [147]. They found that this effect may be due to negative cross-talk in the cytoplasm rather than AT1R-AT2R heterodimerization at the membrane. Inuzuka et al. reported that AT1R-AT2R heterodimerization occurs both *in vitro* and *in vivo* [148]. They showed that AT2R via protein kinase C (PKC) causes a decrease in ligand-induced activation of AT1R. Zha et al. found that high levels of glucose inhibited AT1R-AT2R heterodimerization [149].

AT1R-D1R [150] and AT1R-D3R [151] heterodimers were obtained by coimmunoprecipitation, demonstrating a direct interaction between these receptors in 2003. In these studies, the authors sought to investigate the effect of heterodimerization on receptor expression levels. In the AT1R-D1R heterodimer, fenoldopam, a D1R agonist, increased DD1R expression while decreasing AT1R expression. In the AT1R-D3R heterodimer, AT1R negatively affected D3R expression. In their study published in 2003, Barki-Harrington et al. showed that AT1R forms heterodimers with β adrenergic receptors (subtypes 1 and 2) [152]. Selective blockade of β adrenergic receptors resulted in blockade of AT1R signaling pathways. Similarly, selective blockade of AT1R also blocked $G_{\alpha s}$ coupling of β adrenergic receptors. This study has shown that selective inhibition of one of the two protomers in the heterodimer prevents the signaling of both receptors. Some older studies in the literature mention that AT1R can form homodimers. Their conclusion is based on the observation of high molecular

weight species in gel electrophoresis studies. Examples of these studies include those of Rondeau et al. [153] and Carson et al. [154]. In 2004, AbdAlla et al found that AT1R homodimer structures were formed when agonist-induced AT1R homodimers cross-linked GLN315s at their c-terminal ends by factor XIIIa transglutaminase [155]. Hansen et al. investigated the functional consequences of AT1R homodimerization using two mutant AT1Rs [156]. The first mutant, AT1R-EEA(A), is a mutant that inhibits coupling to G_q, while the other mutant, AT1R-K199A, inhibits ligand binding. In AT1R homodimers generated with these two mutants, extracellular signal-regulated kinase (ERK) activation was preserved, while G_q signaling was negatively affected. This study also demonstrated that homodimerization occurs in the endoplasmic reticulum (ER) during biosynthesis, even before surface expression of receptor dimers. Similar to the study by Hansen et al, in 2007 Karip et al investigated the functional consequences of AT1R homodimerization by creating an AT1R-AAV mutant that inhibits G_q binding and an AT1R-S109Y mutant that inhibits candesartan angiotensin receptor blocker (ARB) binding [157]. As a result of this study, they found that candesartan binding to one protomer prevents the other protomer from coupling to G_q and inhibiting signaling. In 2008, Yamada et al. showed that AT1R homodimerization is enhanced by aldosterone, which increases transglutaminase activity in mesenteric arterioles, leading to the formation of cross-linked AT1R dimers [158]. In their 2012 study, Szalai et al. observed that the unstimulated protomer in the AT1R homodimer maps to β -arrestin2 [159]. In addition, a change in conformation and a change in ligand binding were also observed in the unstimulated protomer. These findings suggest that the unstimulated protomer in the dimer structure is influenced by the stimulated protomer as a result of allosteric interactions. In 2017, it was reported by Zha et al. that high levels of glucose have an increase in AT1R homodimerization [149]. In the study of the AT1R and dopamine D5 receptor (D5R) heterodimer, it was shown that agonist activation of one protomer negatively affects the expression of the other protomer [160]. In 2005, Zeng reported that endothelin ETB receptors directly interact with AT1R (through heterodimer formation) and negatively affect AT1R expression [161]. In the AT2R, an intra-receptor disulfide bond is formed between CYS35^{Nterm} at the N-terminus and CYS290^{TM7} at TM7. In 2005, Miura et al. showed that this intra-receptor disulfide bond may also occur in the inter-receptor and provide for AT2R homodimerization [162]. This is the first study in the literature to demonstrate AT2R homodimerization. In 2009, AbdAlla et al. found that AT2R homooligomer formation

is a two-step process [163]. In the first step, AT2R homodimers are formed by oxidative cross-linking, followed by AT2R homotetramer/homooligomer formation in the second step by second cross-linking with transglutaminase. Amyloid β formed in Alzheimer's disease induces AT2R homodimer formation and leads to oligomer formation. The heterodimer formed between the Mas receptor (MasR) and the AT1R is not affected by antagonists or agonists of either receptor [164]. The MasR acts on the AT1R to antagonize the effects of ANGII. Interestingly, Canals et al. also found that when MasR is co-expressed with AT1R, the [3H]ANGII binding capacity is increased. In an attempt to explain this, the authors found that the observed increase in [3H]ANGII binding is mediated by constitutive $G_{aq}/G_{\alpha11}$ activation by MasR followed by PKC dependent phosphorylation of AT1R. The CYS18PHE^{Nterm}-LYS20ALA^{Nterm}-AT1R mutant is unable to bind ANGII and cannot generate the signals that should be generated upon G-protein coupling. In their 2007 study, Santos et al. found that the MasR- CYS18PHE^{Nterm}-LYS20ALA^{Nterm}-AT1R heterodimer restored ANGII binding and functional response generation [165]. This is evidence for direct interaction (heterodimerization) between the two receptors. AT2R forms a heterodimer with B2R [166]. This heterodimer formation was observed by confocal Förster resonance energy transfer (C-FRET) and coimmunoprecipitation methods. In addition, administration of agonists and antagonists of both receptors had no effect on heterodimerization. This new dimer system suggests that heterodimerization by selective induction of AT2R and B2R may be used in the treatment of cardiovascular and renal disorders and hypertension. The first apelin receptor (APJ)-AT1R heterodimerization reported in the literature was by Chun et al. in 2008 [167]. According to this article, APJ-AT1R heterodimerization was observed to increase with ANGII stimulation. In 2011, Sun et al. investigated APJ-AT1R heterodimerization and showed that unactivated APJ inhibited ANGII-induced AT1R signaling [168]. Interestingly, this inhibition was reversed when APJ was stimulated with apelin. In contrast to the findings of Sun et al. and Chun et al., in 2012, Siddiquee et al. showed that APJ-AT1R heterodimerization is dependent on APJ agonists [169]. As an explanation for the differences between these three findings, Siddiquee et al. pointed out that previous studies did not take into account the surface density of the receptors and the experiments were not performed in membrane preparations. In their study of α_{2c} adrenergic (α_{2c} AR)-AT1R heterodimerization, Bellot et al. observed that when both protomers bind their specific agonists (α_{2c} AR-norepinephrine (NE), AT1R-ANGII), a signaling pathway is initiated

that is very different from the signal generated by the protomers in their monomeric state [170]. This new pathway signals via G_s -cAMP-PKA. Goupil et al. studied prostaglandin F $_{2\alpha}$ receptor (PF2AR)-AT1R heterodimerization and observed altered PGF $_{2\alpha}$ binding in the presence of AT1R [171]. In the presence of agonist or antagonist binding to PF2AR, an increase in the binding affinity of ANGII to AT1R was observed. In the case of antagonist binding to AT1R, the signaling capacity of PF2AR via ERK1/2 was increased. However, in the case of antagonist binding of PF2AR, no change in AT1R signaling through ERK1/2 was observed. In contrast to this asymmetry observed in the ERK1/2 pathway, a symmetric modulation was observed in the $G\alpha_q$ pathway. In 2019, the group that brought PF2AR-AT1R heterodimerization to the literature examined the same heterodimer system and found that PF2AR, which does not have the ability to signal through β -arrestin under normal conditions, can signal through this pathway as a result of heterodimerization with AT1R [172]. In experiments conducted by Leonhardt et al. to study MasR-AT2R heterodimerization, they observed that each receptor-specific antagonist, when bound to the target of interest, inhibited agonist binding to the other protomer [173]. In this heterodimer, in which cross-inhibition is observed, each of the protomers is functionally dependent on the other protomer. In a follow-up study in the same year, it was shown that this heterodimer formation occurs by disulfide bonding between protomers [174]. In 2021, it was observed that the AT2R selective agonist C21 molecule increased MasR-AT2R interactions [175]. Chow et al. showed that AT1R-AT2R-relaxin family peptide receptor 1 (RXFP1) heterotrimers can form in myofibroblasts [176]. The antifibrotic effect of the RXFP1 agonist cerelaxin was shown to be abolished by AT1R or AT2R antagonists. Wang et al. studied RXFP1-AT2R heterodimerization and found that when RXFP1 or AT2R antagonists bind to their respective receptors, they antagonize agonist binding to the other receptor [177]. Interestingly, the same study also showed that AT2R agonists contribute to non-direct activation of RXFP1. In addition to the AT1R-AT2R-RXFP1 heterotrimer, Rivas-Santisteban et al. 2021 showed that AT1R-AT2R-MasR heterotrimers can also form in microglia and neurons [178]. Rozenfeld et al. showed that AT1R-CB1R heterodimerize by functionally interacting with each other and observed potentiation of AT1R signaling as a result of heterodimerization [179]. AT1R-CB1R heterodimers caused an increase in ANGII-induced signals and antagonism of CB1R prevented this increase.

1.7. Computational Modeling Of Angiotensin Homo- And Heterodimers

It is very important to understand the dynamic properties of these important players in the cardiovascular system at the atomic level. The details and information obtained in this way will allow the discovery of more selective molecules with fewer side effects. In addition, the recent increase in the number of studies reporting on dimerization/oligomerization of GPCRs, including these receptors, will have a positive impact on a more detailed understanding of the cardiovascular system. Answering and elucidating at the atomic level how angiotensin receptor homo- and heterodimers can alter the receptor signaling machinery will allow therapeutic targeting of these GPCR dimerizations/oligomerizations with pathological consequences.

1.8. Aim Of The Study

The aim of this work is the atomic-level understanding of two different GPCR dimers. Both receptors bind ANGII, a hormone composed of eight amino acids. The first of these receptors is the AT1R, which controls blood volume and pressure in the cardiovascular system, and the other is the AT2R. The AT1R is a receptor that has been studied more extensively in the literature, while there appear to be fewer studies on the AT2R than on the other receptor. When ANGII binds to AT1R, it causes vasoconstriction, leading to hypertension, whereas when it binds to AT2R, the opposite effect, vasodilation, is observed, and it is known that the pathological effects of AT1R are neutralized by AT2R. In the first part of the thesis, a series of computational efforts were made to understand the interfaces through which AT1R homodimerization takes place. First, the binding of ANGII to AT1R was predicted by molecular docking simulations. The resulting ANGII-AT1R complex was then subjected to MD simulations. In the MD simulations, several different interaction interfaces were used to determine the most stable interface. For the second part of the work, the AT1R-AT2R heterodimer system was considered. In order to understand this heterodimerization at the atomic level, all available experimental GPCR structures were examined, all possible interfaces were constructed and then CGMD simulations

were performed. AA simulations were performed according to the changes observed in the CGMD simulations.

2. METHODS

Molecular docking is a computational method used in the field of structural biology and drug discovery to predict the preferred binding mode or conformation of a small molecule (ligand) when it interacts with a target protein or biomolecule (receptor). It helps in understanding the interaction between the two molecules and provides insights into their binding affinity and potential biological activity. Equations expressed as scoring functions are used to calculate the pose of the ligand in the complex predicted by molecular docking and the energy values corresponding to that pose. Scoring functions can be broadly classified into two classes: physics-based and empirical. Examples of physics-based and empirical scoring functions are given in Equations 2.1 and 2.2.

$$E_{\text{bind}} = \sum_{i=1}^L \sum_{j=1}^R \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} + \frac{q_i q_j}{\varepsilon(r_{ij}) r_{ij}} \right) \quad (2.1)$$

$$E_{\text{bind}} = w_0 + w_1 \Delta G_{vdW} + w_2 \Delta G_{Hbond} + w_3 \Delta G_{\text{rot}} + w_4 \Delta G_{\text{hydro}} \quad (2.2)$$

Of the scoring functions shown as examples, the physics-based scoring function is used by the DOCK docking code, while the empirical scoring function is known as X-Score and is included in the X-tool docking program [180]. Physics-based scoring functions can be roughly divided into three categories: force field-based scoring functions, those that include a solvent model in addition to the force field, and quantum mechanical scoring functions that attempt to take advantage of quantum mechanics. Force field based one is shown below [180];

$$E_{\text{bind}} = E_{vdW} + E_{elec} \quad (2.3)$$

Scoring function that incorporate solvent models shown in equation 2.4,

$$E_{\text{bind}} = E_{vdw} + E_{elec} + \Delta G_{\text{solv}} \quad (2.4)$$

The quantum scoring function, which has a much higher level of accuracy but the highest level of computational cost, is as follows,

$$E_{\text{bind}} = E_{QM/MM} + \Delta G_{\text{solv}} \quad (2.5)$$

Molecular simulations, such as MD simulations, utilize force fields to calculate the forces acting on atoms and molecules, allowing them to predict the behavior and properties of the system over time. By integrating Newton's equations of motion, the trajectories of the atoms can be simulated, providing insights into various properties, including thermodynamics, kinetics, conformational changes, and intermolecular interactions. An example of the force fields used in molecular simulations is shown below (2.6). There are two main components: bonded interactions and non-bonded interactions. The bonded interactions are three: bonds, angles and dihedrals. The non-bonded interactions are electrostatics and Lennard-Jones interactions.

$$\begin{aligned} U(\vec{R}) = & \underbrace{\sum_{\text{bonds}} k_i^{\text{bond}} (r_i - r_0)^2}_{U_{\text{bond}}} + \underbrace{\sum_{\text{angles}} k_i^{\text{angle}} (\theta_i - \theta_0)^2}_{U_{\text{angle}}} + \\ & \underbrace{\sum_{\text{dihedrals}} k_i^{\text{dihe}} [1 + \cos(n_i \phi_i + \delta_i)]}_{U_{\text{dihedral}}} + \\ & \underbrace{\sum_i \sum_{j \neq i} 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] + \sum_i \sum_{j \neq i} \frac{q_i q_j}{\epsilon r_{ij}}}_{U_{\text{nonbond}}} \end{aligned} \quad (2.6)$$

Some components in the example force field potential function shown above are obtained from the geometry file, while other components are obtained from the parameter files used in the simulations. The components obtained from the geometry file can be listed as follows; $r_i, \theta_i, \phi_i, r_{ij}$. The components obtained from the parameter file are as follows; $k_i^{\text{bond}}, r_0, k_i^{\text{angle}}, \theta_0, k_i^{\text{dihe}}, n_i, \delta_i, \epsilon_{ij}, \sigma_{ij}, q_i, q_j$.

Here bonds represent the oscillations of two atoms from the equilibrium bond length, angles represent the oscillations of three atoms from the equilibrium bond angle, dihedrals represent the torsional rotations of four atoms around a central bond, and non-bonded interactions represent van der Waals and electrostatic interactions.

The motion of macroscopic objects at low speeds obeys physical laws known as Newton's laws. These laws of physics consist of three laws of motion. The first law

states that an object remains at rest or continues to move in a straight and true line until acted upon by an external force. The second law is expressed by $F=ma$ (m =mass, and a =acceleration) and states that the force acting on an object and its acceleration are proportional to each other. The constant of proportionality is the mass of the object. When more than one force acts on an object, the vectorial sum of the forces determines the acceleration. The third law is known as the law of inertia and states that an object is balanced by a force in the opposite direction of the force acting on it. In systems with a large number of interacting molecules, Newton's three laws of motion give a set of equations that describe the time dependence of several quantities. These quantities can be listed as position, $r_i(t)$, velocity, $v_i(t)$ (or momentum, $p_i(t) = m_i v_i(t)$), and force, $F_i(t)$ (or $a_i(t)$) for all i atoms. From these fundamental mechanical quantities, other mechanical quantities such as energy and angular momentum can be calculated for each atom and molecule as needed. Therefore, to overcome this situation, Newton's second law of motion ($F=ma$) can be expressed as a set of partial differential equations. These partial differential equations are quadratic equations in time. Their solutions give the change in the cartesian coordinates x , y , and z .

$$\begin{aligned} m_i \frac{d^2 x_i}{dt^2} &= F_{x,i}(\{\mathbf{r}\}) = -\frac{\partial U_i(\{\mathbf{r}\})}{\partial x_i} \\ m_i \frac{d^2 y_i}{dt^2} &= F_{y,i}(\{\mathbf{r}\}) = -\frac{\partial U_i(\{\mathbf{r}\})}{\partial y_i} \\ m_i \frac{d^2 z_i}{dt^2} &= F_{z,i}(\{\mathbf{r}\}) = -\frac{\partial U_i(\{\mathbf{r}\})}{\partial z_i} \end{aligned} \quad (2.7)$$

Here $F_i(\{\mathbf{r}\})$ is the force acting on atom i (in each of the x , y , and z planes), and the positions of all the other atoms in the system determine this force. The velocities and positions of all the other atoms in the system are linked by the forces acting on them. In Equation 2.7, $U_i(\{\mathbf{r}\})$ (the scalar potential energy of atom i) is expressed in terms of partial derivatives with respect to three Cartesian coordinate systems (x , y , and z). In many cases, the potential energy is much easier to determine than the force acting on the atoms. The equation in this equation can be solved in two different ways, analytically and numerically. However, while the analytical solution is rarely used, the numerical solution is often preferred. The numerical solution is the focus of MD simulation methodology. Newton's equations of motion give $3N$ quadratic differentials for a system of N atoms. Euler's method and Verlet's algorithm can be given as

examples of numerical solution methods[181]. By expressing the positions and velocities of the atoms in Cartesian coordinates as a Taylor expansion, a more accurate estimate can be obtained by using a finite number of terms in the function. This method is known as Euler's method and is an application of the finite difference method. The velocities and positions of the atoms at each simulation frame can be computed much faster and with higher numerical accuracy by using a more efficient algorithm, the Verlet algorithm. Unlike the Euler method, the Verlet algorithm attempts to obtain a solution by performing ΔT forward and ΔT backward Taylor expansions of the particles in time.

$$\begin{aligned} \mathbf{r}(t_0 + \Delta t) = & \mathbf{r}(t_0) + \mathbf{v}(t_0)(\Delta t) + \frac{1}{2} \mathbf{a}(t_0)(\Delta t)^2 \\ & + \frac{1}{6} \mathbf{j}(t_0)(\Delta t)^3 + \dots \end{aligned} \quad (2.8)$$

$$\begin{aligned} \mathbf{r}(t_0 + \Delta t) = & \mathbf{r}(t_0) + \mathbf{v}(t_0)(\Delta t) + \frac{1}{2} \mathbf{a}(t_0)(\Delta t)^2 \\ & + \frac{1}{6} \mathbf{j}(t_0)(\Delta t)^3 + \dots \end{aligned} \quad (2.9)$$

$$\begin{aligned} \mathbf{r}(t_0 + \Delta t) = & 2\mathbf{r}(t_0) - \mathbf{r}(t_0 - \Delta t) + \mathbf{a}(t_0)(\Delta t)^2 + O(\Delta t)^4 \\ & + \dots \end{aligned} \quad (2.10)$$

$$\mathbf{v}(t_0) = \frac{\mathbf{r}(t_0 + \Delta t) - \mathbf{r}(t_0 - \Delta t)}{2\Delta t} \quad (2.11)$$

The Verlet algorithm is given by equations 2.8, 2.9, 2.10, and 2.11. While Euler includes a 3rd order truncation error, Verlet's algorithm uses a 4th order truncation error, which allows for a more accurate determination of positions. In the Verlet algorithm, however, $\mathbf{r}(t_0)$ and $\mathbf{r}(t_0 - \Delta t)$ must be known. While $\mathbf{r}(t_0)$ can be obtained from the initial position, the following equation is used to calculate $\mathbf{r}(t_0 - \Delta t)$;

$$\begin{aligned}\mathbf{r}(t_0 + \Delta t) &= \mathbf{r}(t_0) + \mathbf{v}(t_0)\Delta t + \frac{1}{2}\mathbf{a}(t_0)(\Delta t)^2 \\ \mathbf{v}(t_0 + \Delta t) &= \mathbf{v}(t_0) + \mathbf{a}(t_0)\Delta t\end{aligned}\tag{2.12}$$

The Velocity-Verlet and Leapfrog algorithms are the other numerical methods to solve Newton's equations of motion [181].

3. HOMODIMERIZATION OF AT1R

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In order to understand AT1R homodimerization, the binding mode of the ANGII octapeptide was predicted at the active and inactive structures of the AT1R in the active site using peptide docking methodology. Approximately 800 different ANGII conformers were generated for this purpose, but the Glide SP-Peptide Docking module predicted 237 binding modes in the inactive structure and 137 in the active structure. Considering the AT1R-ANGII interactions reported in the literature, these poses were evaluated. From the poses obtained in the active and inactive structure, the ones that best fit the criteria were selected (one pose for the inactive structure and one pose for the active structure). The interactions found in the literature and searched for in the binding modes are as follows (the first amino acid belongs to ANGII, and the second belongs to the AT1R);

- i) Interaction between ASP1-ARG23 [13, 182],
- ii) Interaction between ARG2-ASP261, and ARG2-ASP281 [183–185],
- iii) Interaction or close proximity, between PHE8-LYS199 [186–188],
- iv) Vertical orientation of ANGII at the binding site of AT1R [184].

Figure 3.1 shows the predicted binding modes of ANGII in active and inactive AT1R constructs.

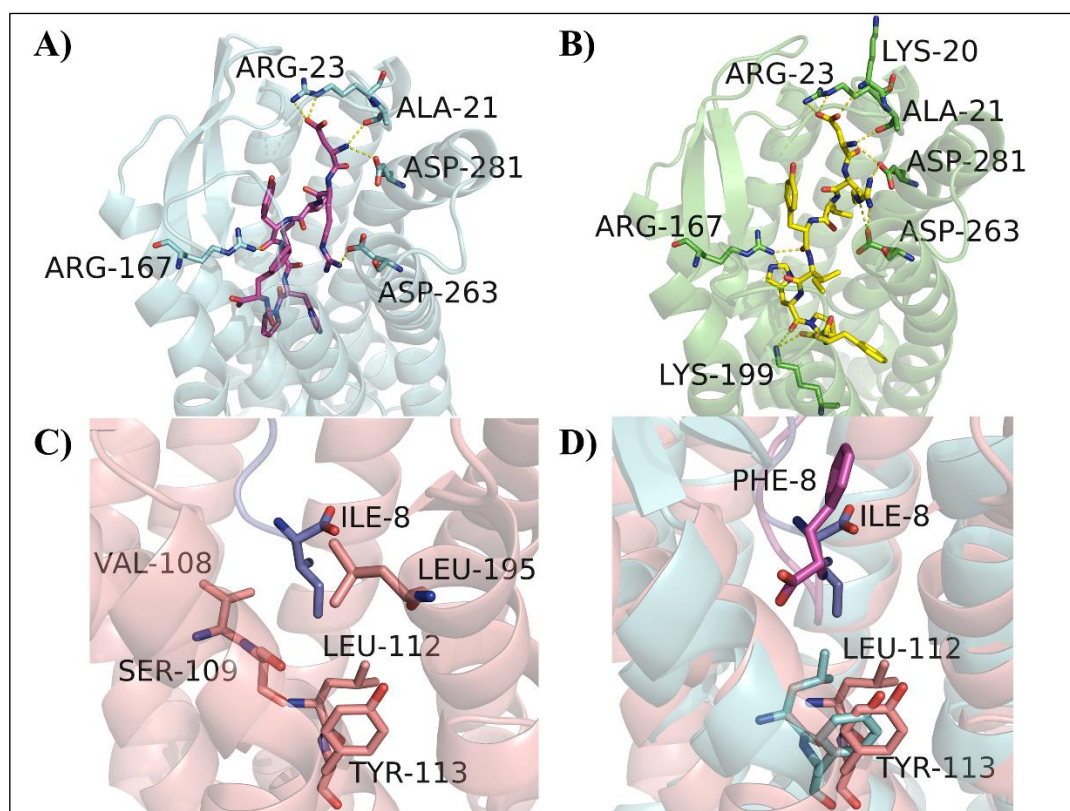


Figure 3.1: Binding modes of ANGII in active A) and inactive B) AT1R. Panel C shows the 4 Å around of ILE8 of ANGII. Panel D shows the alignment of ANGII and S1I8.

The binding modes of ANGII in active AT1R (A) and inactive AT1R (B) are shown in Figure 3.1. Careful examination of the binding mode of ANGII in active AT1R reveals that the amino acids in the N-terminal part of ANGII usually form polar interactions with AT1R. Amino acids ALA21^{Nterm}, ARG23^{Nterm} and ASP281^{7.32} of AT1R were observed to interact with ASP1, the first amino acid of ANGII. ASP263^{6.58} was found to interact with ARG2, the second amino acid of ANGII. ARG167^{4.64}, which is known to play an important role in ARB binding, was also observed to interact with ANGII in binding modes. TYR4, the fourth amino acid of ANGII, interacted with ARG167^{4.64}. However, this interaction occurred at the rear chain of TYR4. When the binding mode observed in the inactive structure was compared to the active structure, similarities were observed, but some differences were also reported. In the inactive structure, LYS20^{Nterm}, ALA21^{Nterm}, ARG23^{Nterm} and ASP281^{7.32} of AT1R strongly interacted with ASP1 of ANGII. ARG2 of ANGII was observed to interact with ASP263^{6.58} and ASP281^{7.32}, which were reported to be important in the literature. TYR4 and ILE5 of ANGII interacted with ARG167^{4.64} from their backbones. The

importance of LYS199^{5,42} of AT1R has been demonstrated by many studies in the literature. In the inactive ANGII binding mode, LYS199^{5,42} was observed to interact with HIS6 of ANGII and the C-terminal carboxyl group. It is well documented in the literature that ASP1 and ARG2 are important for the binding of ANGII to the AT1R [189]. It has been reported by Feng et al. that the ARG2 of ANGII has to interact with the ASP281^{7,32} of the AT1R in order to exhibit the full agonist properties [185]. This interaction reported by Feng has been observed in our molecular docking simulations as well. In high-resolution experimental structures of both AT1R and AT2R, ARG2 of ANGII was found to be in interaction with ASP263^{6,58} and ASP281^{7,32} of both receptors [183, 190]. Matsoukas et al. showed that the backbone of ANGII interacts with the ARG167^{4,64} of the AT1R by means of polar interactions [191]. This means that our peptide docking simulations were successful in predicting the key interactions between ANGII and AT1R that have been reported in the literature and in experimental angiotensin structures, and that the complex used as the starting structure in the MD simulations contains the correct binding modes and interactions. In addition, the ANGII binding modes obtained in the active and inactive structures are compared with the recently revealed AT1R-S1I8 crystal structure. As shown in panels C and D of Figure 3.1, it is observed that ILE112^{3,36} and TYR113^{3,37} ANGII occupy the space required for PHE8 in the crystal structure. In the crystal structure of the ligand, ILE is located at position 8 instead of PHE. Therefore, this amino acid occupies less space compared to PHE (Figure 3.1, C and D). In the predicted structure of ANGII in the active AT1R, PHE8 moved upwards due to steric hindrance and caused the formation of a U-shaped structure at the C-terminal end of ANGII. However, a different orientation was observed in the binding mode of ANGII in the inactive AT1R. In this binding conformation, PHE8 is positioned between TM5 and TM6 of the AT1R. This orientation confirmed the interaction reported in the literature between the carboxy-terminal part of ANGII and LYS199^{5,42} of the AT1R [188].

3.1. MD Simulations Of Dimer Systems With Different Interfaces

Each interaction interface used in this study (TM1,2,8; TM4,5 Loose; TM4; TM5; TM4,5 Tight; and TM6,7) was subjected to MD simulations at least twice with

different initial velocity distributions. At least 2.1 microseconds of all-atom (AA) simulations were performed for each dimer interface system. When all simulation trajectories are summed, a total of 12.5 microseconds of simulation data is obtained. Side views of all interfaces formed (the interacting TMs are colored in red) are shown in Figure 3.2.

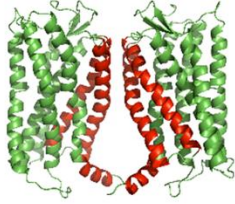
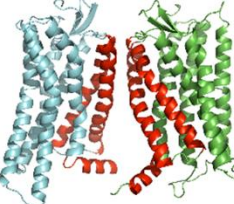
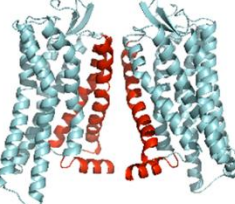
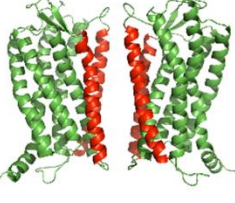
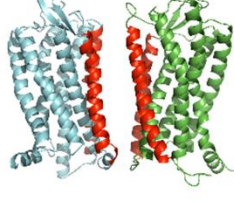
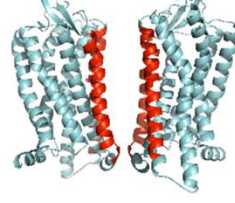
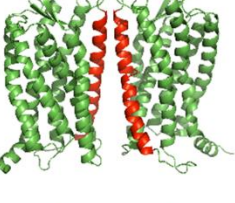
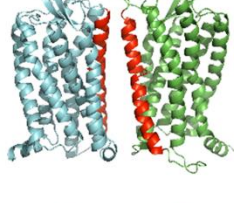
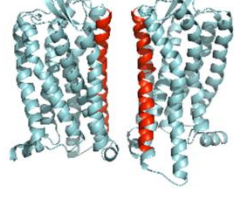
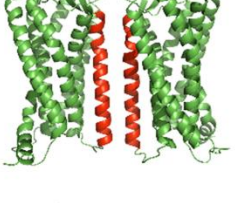
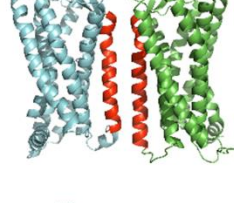
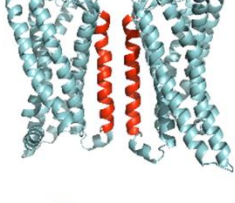
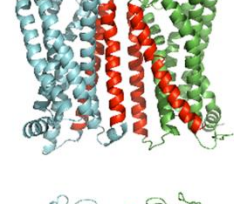
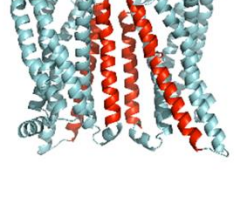
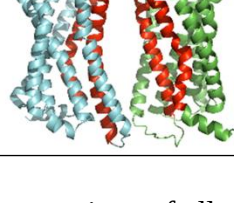
INACTIVE – INACTIVE	ACTIVE – INACTIVE	ACTIVE – ACTIVE	Interface
			TM1,2,8
			TM4,5 L
			TM5
			TM4
			TM4,5 T
			TM6,7

Figure 3.2: Side view representations of all studied dimer pairs and interfaces in this work. Inactive protomers are depicted in green and active protomers are in cyan color. Transmembrane domains that contributed to dimerization interface are depicted in red color.

We performed RMSD analysis on each of the extracellular, transmembrane and intracellular components of the AT1R, namely ECL1-3, TM1-7, H8 and ICL1-3. In our II (Inactive-Inactive), AI (Active-Inactive) and AA (Active-Active) dimer pairs, we compared all these structural changes to conclude how different dimer interfaces induce different stabilities or instabilities on all parts of the receptor. In the TM6,7 interface, to avoid steric clashes in TM6, which has an outward orientation in active forms of GPCRs, we did not generate AA dimer systems. In this section, we provide details only for II and AI dimer systems.

In TM1,2,8 interface, when we compared to TM1 backbone atom RMSDs, II dimer system stay stabilized around 1 Å, in AI and AA dimer pairs, active protomers have slightly higher value compared to inactive protomer. In the TM4,5 loose interface, TM1 was stabilized around 1.25 Å. Similar to TM4,5 loose interface, TM1 of TM5 interface was stabilized around 1.5 Å in all dimer pairs. At the TM4 interface, TM1 was stabilized between 1 and 1.5 Å in II, AI and AA dimer systems. In TM4,5 tight interface dimer systems, except for protomer B in the 500 ns run of II which deviated around 1.5 Å, all values were somewhat similar in TM1 RMSD values. In TM1 of all two systems studied (TM6,7 interface), similar trends were observed up to 200 ns of the trajectories, but sudden changes were observed in protomer A of II and in both protomers of AI. Protoner B of II dimer system was stabilized around 0.75 Å, and other 3 protomers were stabilized above 1 Å. Figure 3.3 shows the RMSD graph for the TM1 of each interface.

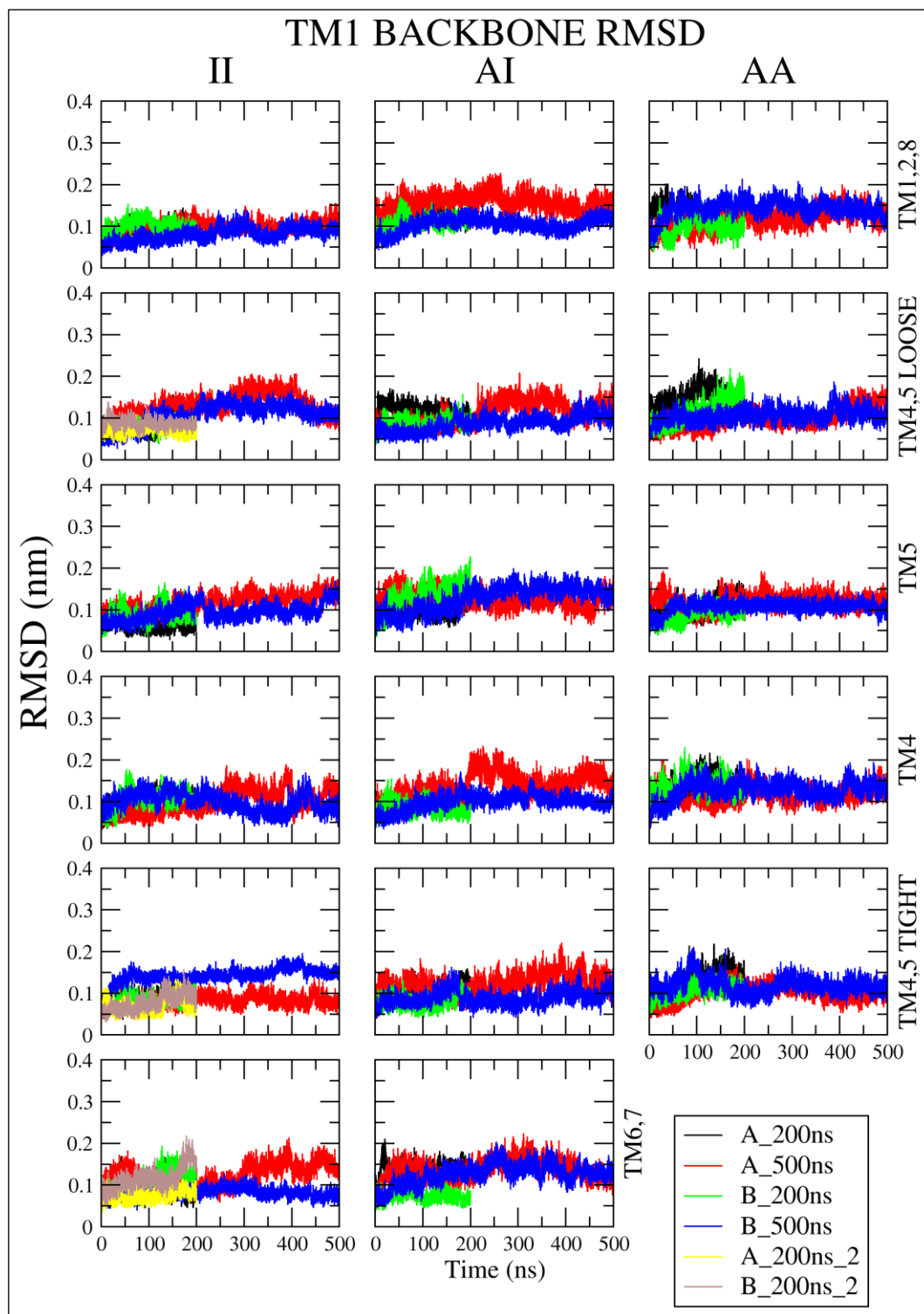


Figure 3.3: RMSD of TM1 over the course of MD simulations.

In TM2 of the TM1,2,8 interface, we have observed a slight difference in AA dimer pair, one of the active protomers fluctuated around 1 Å. In the TM4,5 loose interface RMSD values of TM2 was observed around 0.75 Å. In TM5 interface, TM2 of all pairs was observed around 0.75 Å. TM2 in II dimer pair of TM4 interface, stabilized around 0.5 Å, however increased deviations were observed in both AI and AA dimer pairs, especially in 200 ns run of protomer B and 500 ns run of protomer A. In TM2 RMSD values of TM4,5 Tight interface, all systems were below 1 Å. In TM6,7 interface, RMSD values of TM2 was around 1 Å in both II and AI dimer systems. Figure 3.4 shows the RMSD values for TM2 in each run.

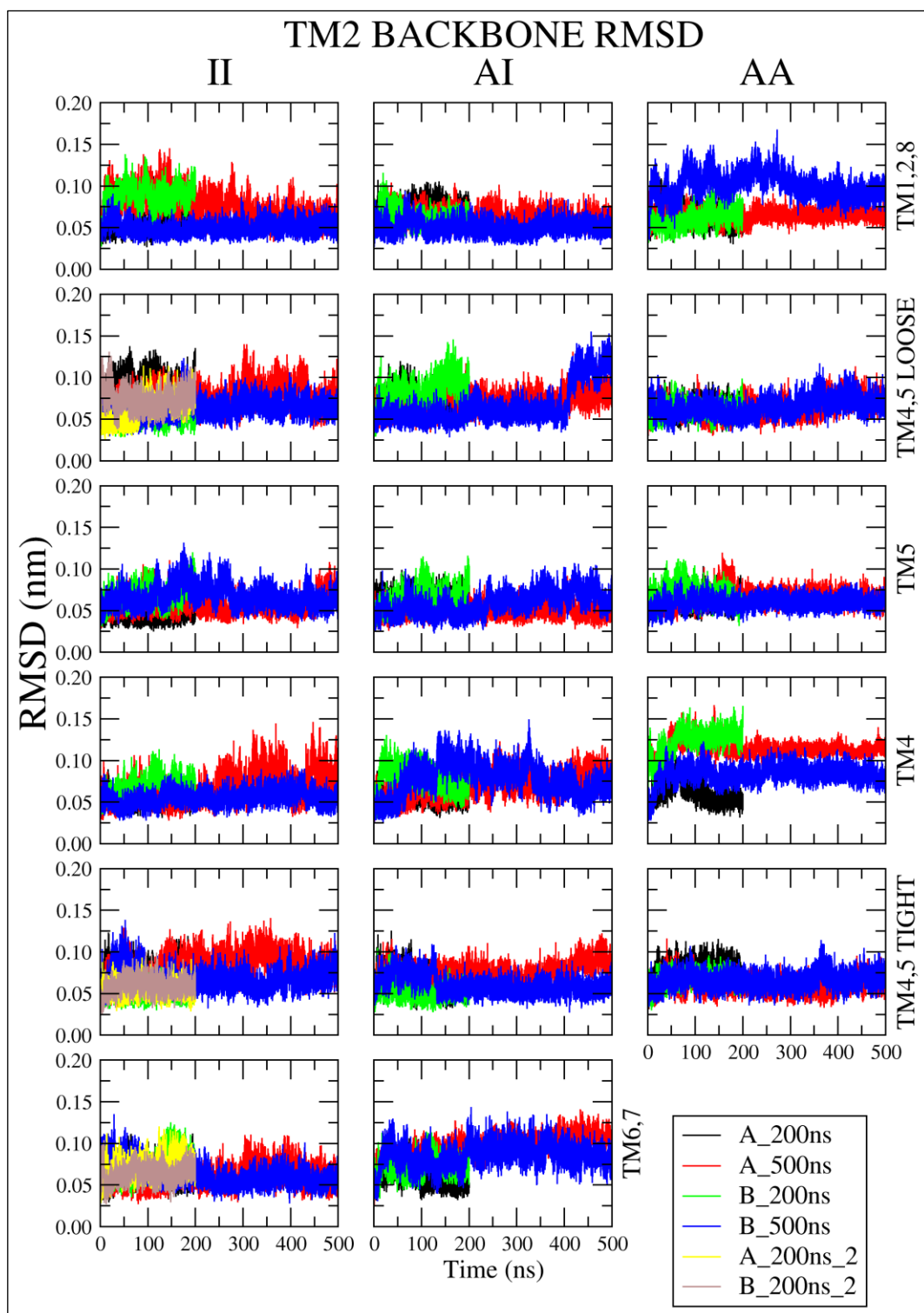


Figure 3.4: RMSD of TM2 over the course of MD simulations.

In TM3 of TM1,2,8 interface, the stabilities of transmembrane domain increasing values from II to the AA was observed. TM3, inactive protomers of the II and AI dimer system we observed smaller values compared to active protomers in TM4,5 loose interface. Interestingly, in AA dimer system, chain A first increased to 1.5 Å and then decreased to the value observed in inactive protomers (in II and AI), the other protomer, chain B, was stabilized around 1.5 Å. In TM3 RMSD values of TM5 interface, we observed higher deviations in AA dimer system compared to AI and II, which were less deviated. TM3 of TM4 interface, was found to be stabilized at similar values of each dimer system studied (II, AI, and AA, respectively). Compared to TM2 of TM4,5 tight interface, same situation was observed in TM3 RMSD values, we could not observe different behavior between protomers, and all systems were stabilized around 1.25 Å. In TM6,7 interface, we observed around 1 Å RMSD values for TM3. Figure 3.5 shows the RMSD graphs for the TM3 of each interface.

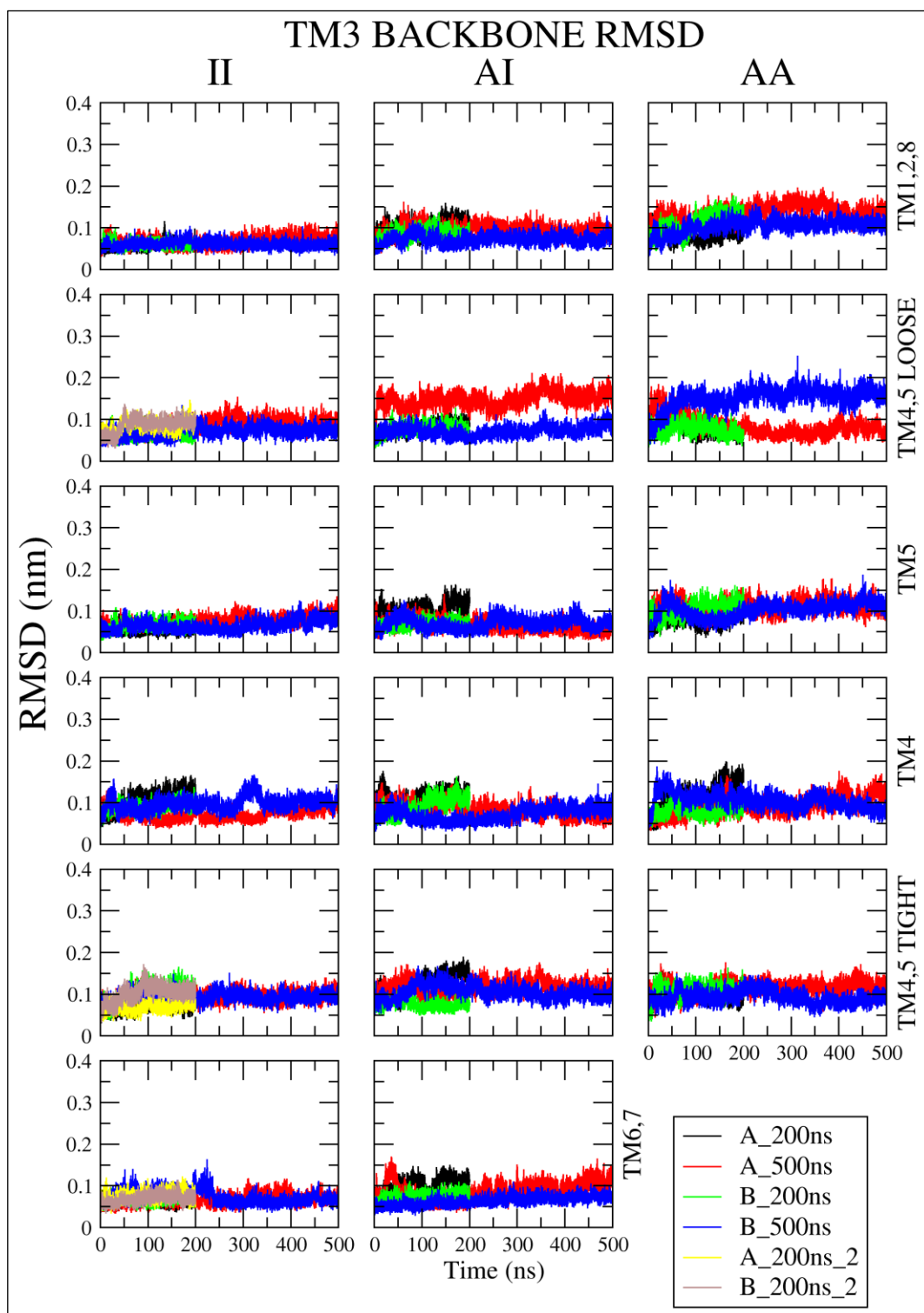


Figure 3.5: RMSD of TM3 over the course of MD simulations.

In the TM1,2,8 interface, the most dramatic changes were observed at TM4 of the active protomer of the AI dimer system, where the II system remained stable, also some higher fluctuations (around ~ 1.25 Å) were observed at both protomers in the AA dimer pair. In TM4 of TM4,5 loose interface, which is one of the interface TMs, in all dimer systems, one of the protomers fluctuated around 1 Å, the other protomer (chain B) showed higher fluctuations, such as around 1.5. In TM4 of TM5 interface, in II dimer system, except 200 ns run of protomer A, all other deviations were around 0.75 Å. In AI dimer system, when inactive protomer stay stabilized at around same values as observed in II dimer system, active protomer fluctuated around 2 Å. In AA dimer system, since both protomers were in active forms, they deviated around 3 Å. In the TM4 interface, the interface TM's RMSD values were found to be somewhat different, such as in II dimer system, protomer B deviated more than protomer A, and active protomer stabilized around 1.25 Å in AI dimer system, but toward the end of the simulation trajectory, it decreased to the same value that was observed in inactive protomer. In AA dimer system, TM4 was found to be the most fluctuating pair, deviating by about 2 Å compared to II and AI dimer pairs. However, in the TM4,5 tight interface, the TM4 values of inactive protomers were around 1 Å, active protomers except active protomer in AI, all active protomers had higher values. The active protomer of the AI dimer system had prominent values compared to other actives and especially to inactive protomers. In the TM6,7 interface we observed lower values in the inactive protomers compared to the active protomers in TM4, where the distinction between active and inactive pairs was so clear. Figure 3.6 shows the RMSD values for the TM4 of each studied systems.

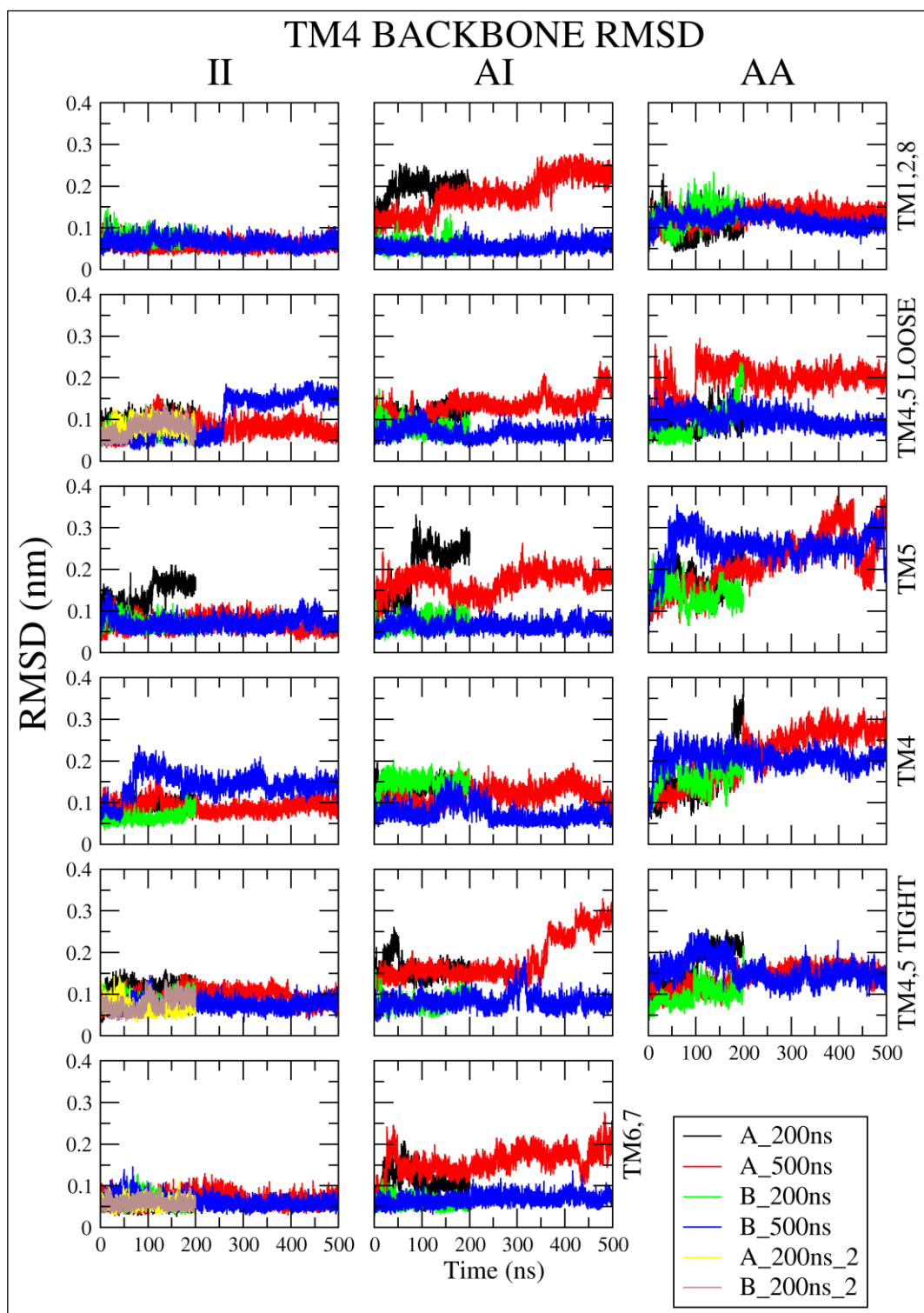


Figure 3.6: RMSD of TM4 over the course of MD simulations.

In TM5 of TM1,2,8 interface, almost all protomers in each dimer system deviated more or less similarly, but slightly higher values were observed in chain B of AA dimer system, which deviated somewhat higher than others. TM5 of TM4,5 loose interface was stabilized around 1 Å in the II dimer system. In AI and AA dimer systems, similar deviations observed in TM4 were detected in deviations of TM5, where one protomer (~1.5 Å) deviates slightly more than the other protomer (~1 Å). The interface TM of TM5 interface, we have observed similar fluctuations, only II dimer system had slightly higher values, all systems were deviated around 1.5 Å, showing stable TM5 interface in all studied systems. In TM5 of TM4 interface, in the RMSD values we have observed similar trends, whereas II stabilized around 1.5, AI stabilized 1.25 and AA stabilized around 1 Å. In TM4,5 tight interface, RMSD values of TM5 more stabilized RMSD values were observed in active protomers, compared to inactive ones, but the difference between RMSD values was not as pronounced as in TM4. In TM5 of TM6,7 interface, after 200 ns of simulations, inactive pairs of II dimer differed significantly from active protomer of AI dimer, where inactive protomer was found to be slightly different especially in the first 400 ns of simulation. Figure 3.7 shows the RMSD values for TM5 of each simulated interfaces.

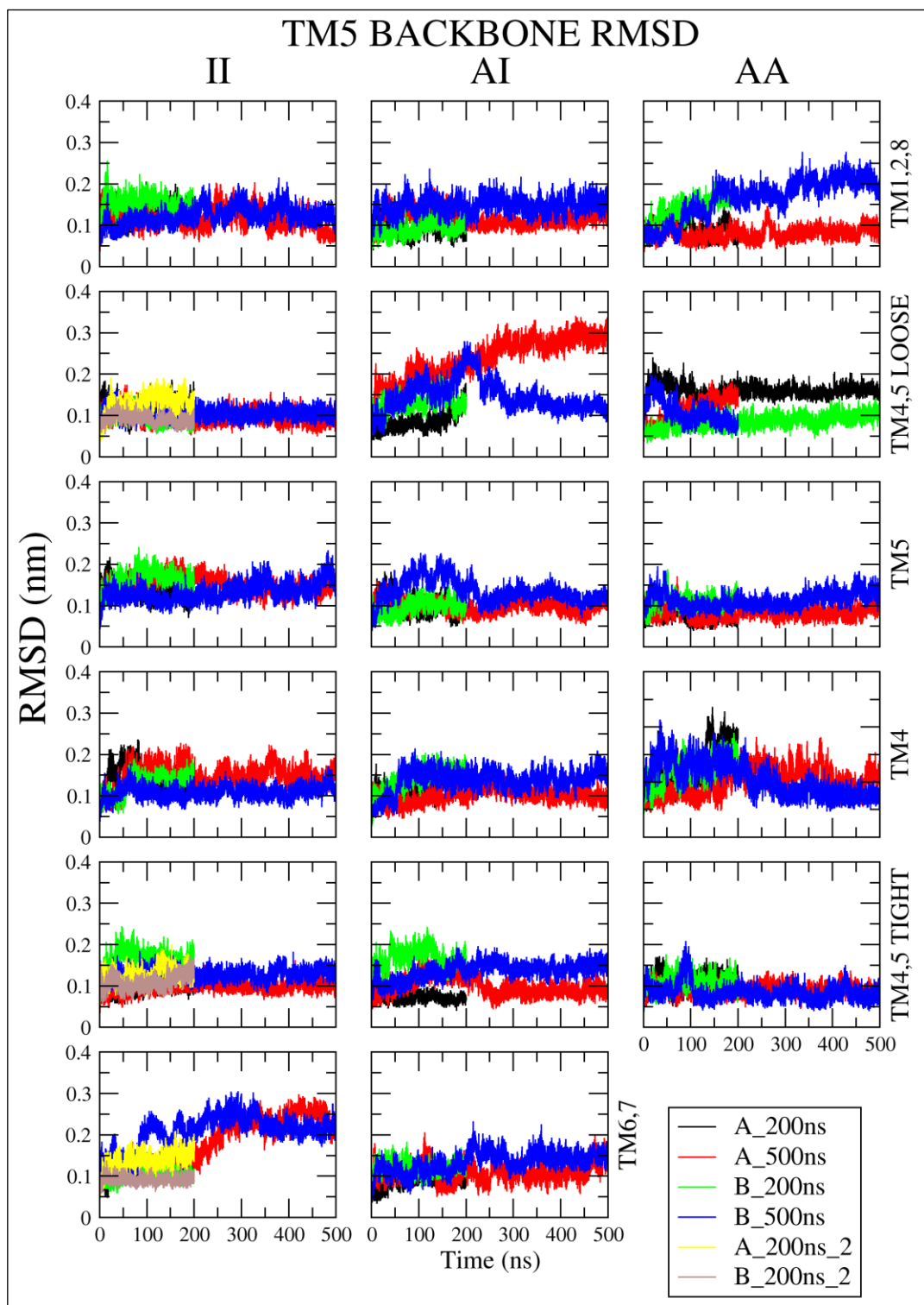


Figure 3.7: RMSD of TM5 over the course of MD simulations.

In all dimer systems of TM1,2,8 interface, TM6 stabilized around 1.5 Å. In TM6 of TM4,5 loose interface, we observed similar trends in the case of II and AA dimer systems, where after 150 ns of trajectories, the RMSD values stabilized around 1 Å, but a slight increase and stabilization around 1.5 Å was observed in the AI dimer system. In TM4,5 loose interface, chain A of II dimer system deviated significantly from the initial conformation in the first 200 ns run and stabilized around 2 Å, also in the second 500 ns run, until 200 ns deviation was observed and after 250 ns it started to decrease again to 1 Å, while chain B showed RMSD around 1 Å in all runs. Similar trend were observed in TM6 RMSD values compared to TM5 in TM5 interface. TM6 values of TM4 interface, were found to be in the same range in all dimer systems, around 1.5 Å. TM6 deviations were higher for both protomers in AI and AA dimer systems, and in protomer A of the II dimer pair we observed the similar trend observed for active protomers in all studied systems of TM4,5 tight interface. In TM6,7 interface, except for the 200 ns simulation of the AI dimer system, where the active protomer somehow fluctuated more, in all other trajectories we observed the same trend for both active and inactive protomers at around 1.25 Å in TM6 RMSD values. Figure 3.8 shows the RMSD changes throughout the simulation for the TM6 for all studied systems.

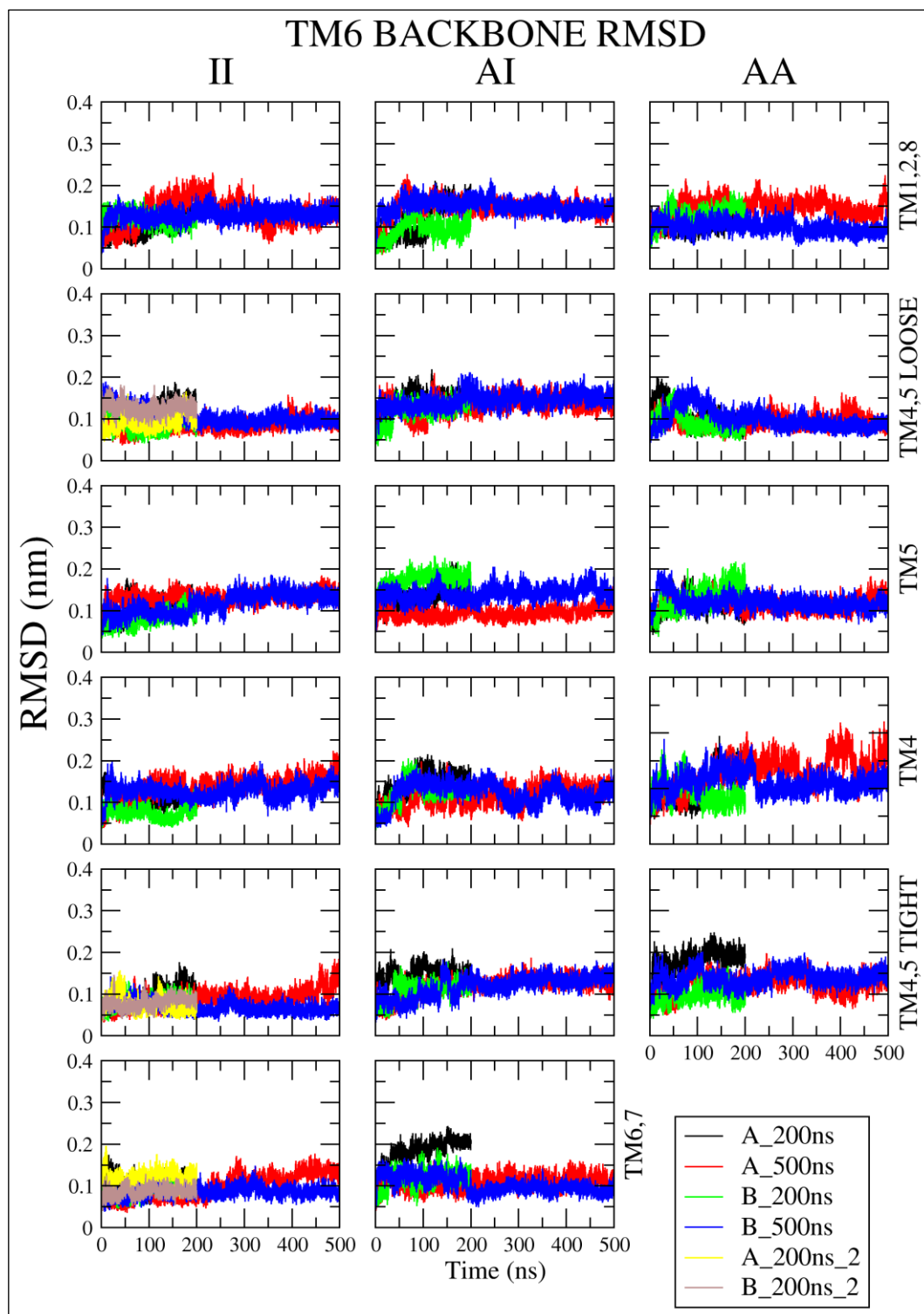


Figure 3.8: RMSD of TM6 over the course of MD simulations.

In all dimer systems of TM1,2,8 interface, TM7 stabilized around 1.25 Å. In AI and AA dimer systems, similar trends in TM7 RMSD values were observed in both chains, where they stabilized around 1.5 Å in TM4,5 loose interface. TM7 RMSD values of TM5 interface were somewhat different in each system, in II dimer system, protomer A and protomer B showed different deviations in both 200 ns and 500 ns runs, for example, in 200 ns run, protomer A fluctuated more than protomer B, but the story was changed in 500 ns, conversely, in this long run, protomer B tended to fluctuate more compared to protomer A, but in last 100 ns both protomers reached to same value. In the AI dimer system, the inactive protomer deviated more than the active protomer in both runs, but at different values. In AA dimer system, except for protomer B which showed higher RMSD values in 200 ns run, all other protomers stabilized around 1 Å in both 200 and 500 ns runs. In TM7 RMSD values of TM4 interface, inactive protomers of II and AI, have little differences, active protomers were found to be more stable in AI and AA dimer systems. Interestingly, in all studied systems, at least one protomer, whether active or inactive, deviated more in TM7 RMSD values compared to the other protomer in TM4,5 tight interface. Interestingly for the TM7 of TM6,7 interface; both protomers in II dimer system had same trend around 1.5 Å, but in AI dimer system, we have observed same trend in active protomer instead of inactive one. Inactive protomer fluctuated around 2 Å in AI dimer system. Figure 3.9 shows TM7's RMSD values for each system.

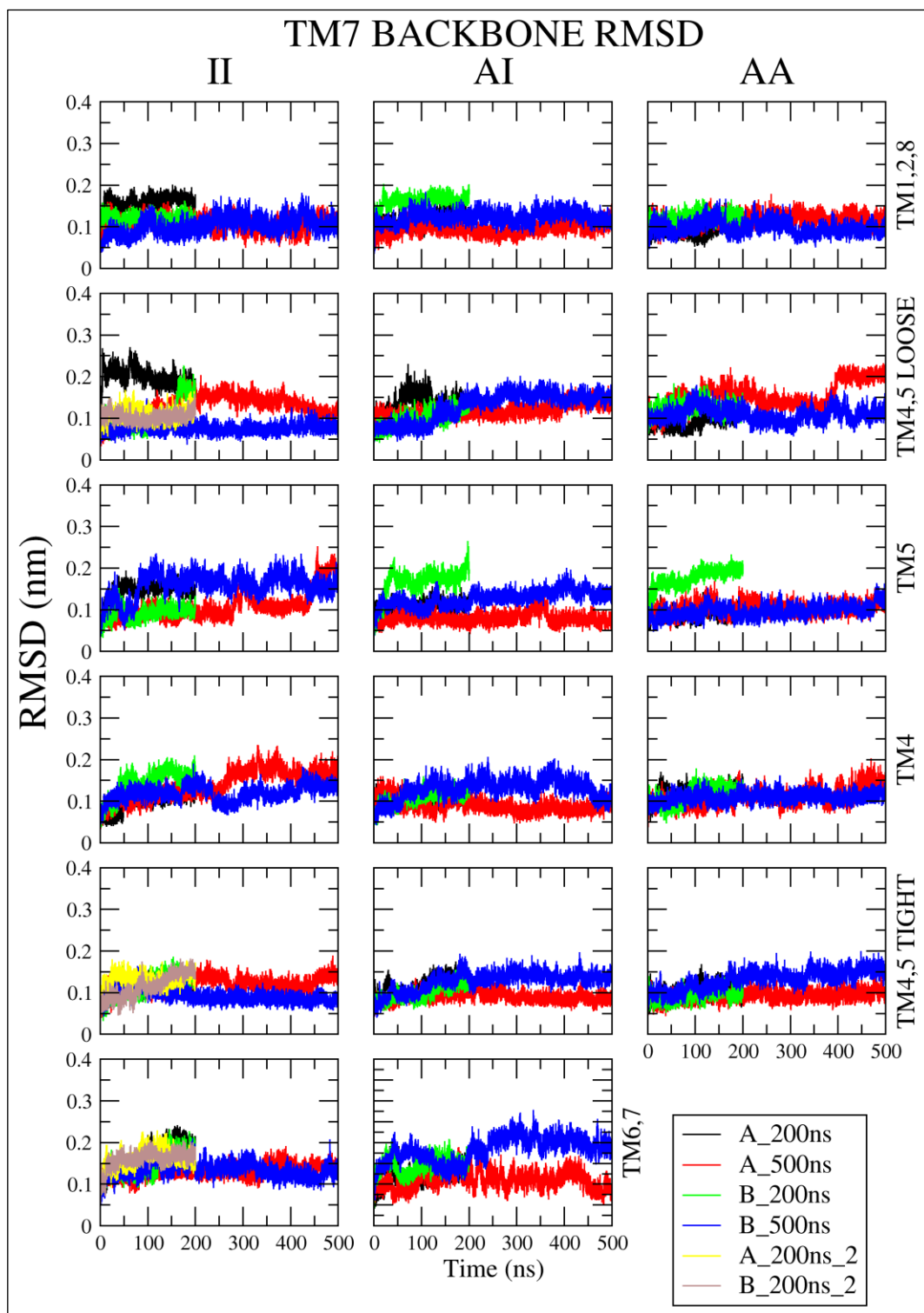


Figure 3.9: RMSD of TM7 over the course of MD simulations.

In H8 of TM1,2,8 interface dimer systems, we observed the same trend in inactive protomers of II and AI dimer systems, and in active protomer of AI and also chain B of AA dimer system deviated similarly, but in chain A of AA dimer system, H8 fluctuated between characteristic values of inactive protomers and active protomer of AI and chain B of AA dimer system. In H8 of TM4,5 loose interface, AA showed the lowest RMSD values compared to II and AI dimer systems, in the latter cases where higher deviations were observed, the stabilization of H8 was around 1.25 Å. In H8 of TM5 interface, inactive protomer of AI dimer system deviated dramatically around 3.5 Å, while active protomer and all protomers in other II and AA dimer systems stabilized around 1 or less values. In H8 of TM4 interface, active protomers of AI and AA dimer systems, we have observed distinct conformational changes compared to inactive protomers in II and AI dimer systems, although, in 500 ns run of protomer B of the AA dimer, some dramatic changes were observed in first 200 ns of the simulation, but after 200 ns, H8 of protomer B, converged to the same value as protomer A has. In the H8 of TM4,5 tight interface, RMSD values of the AI and AA dimer systems, where the inactive protomer stabilized at lower RMSD values compared to the active one in AI, and chain B stabilized below 1 Å in the AA dimer system. However, this was not possible in II dimer system, both protomers tended to deviate significantly between the first 300 ns part of the trajectories and somehow stabilized around 1 Å in the last 200 ns. In H8 of TM6,7 interface, we have observed slightly lower RMSD values for the active protomer compared to inactives. Figure 3.10 shows the RMSD values for H8.

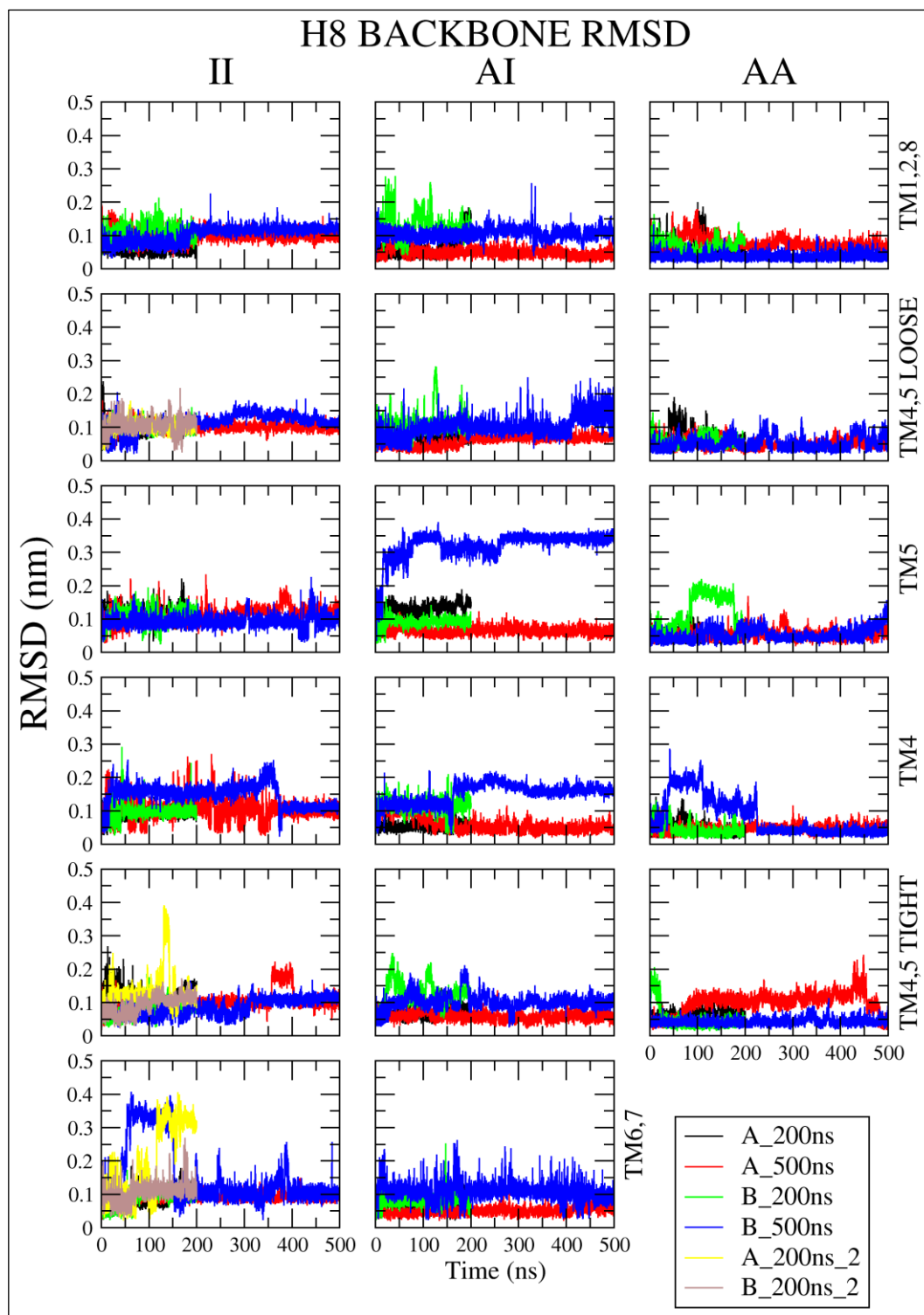


Figure 3.10: RMSD of H8 over the course of MD simulations.

In ICL1 of TM1,2,8 dimer interface, active protomers had slightly higher values compared to inactive protomers. In ICL1 of TM4,5 loose interface, II dimer system stabilized around 0.6 Å, but in the case of AI and AA dimer systems, higher deviations were detected, and ICL1 stabilized around 1 and 1.25 Å, respectively. On the intracellular side of TM5 interface, ICL1 had stabilized around similar values in all dimer pairs. In II and AI dimer systems of TM4 interface, ICL1 stabilized around 0.5 Å, however both protomers in AA dimer, had substantial deviations around 1 and 2.5 Å. In ICL1 backbone RMSD values of TM4,5 tight interface, we observed higher values for active protomers in AI and AA, and protomer B in II dimer systems. In ICL1 of TM6,7 interface, we have observed slightly lower RMSD values for the active protomer compared to inactives. Figure 3.11 shows the RMSD changes for the ICL1.

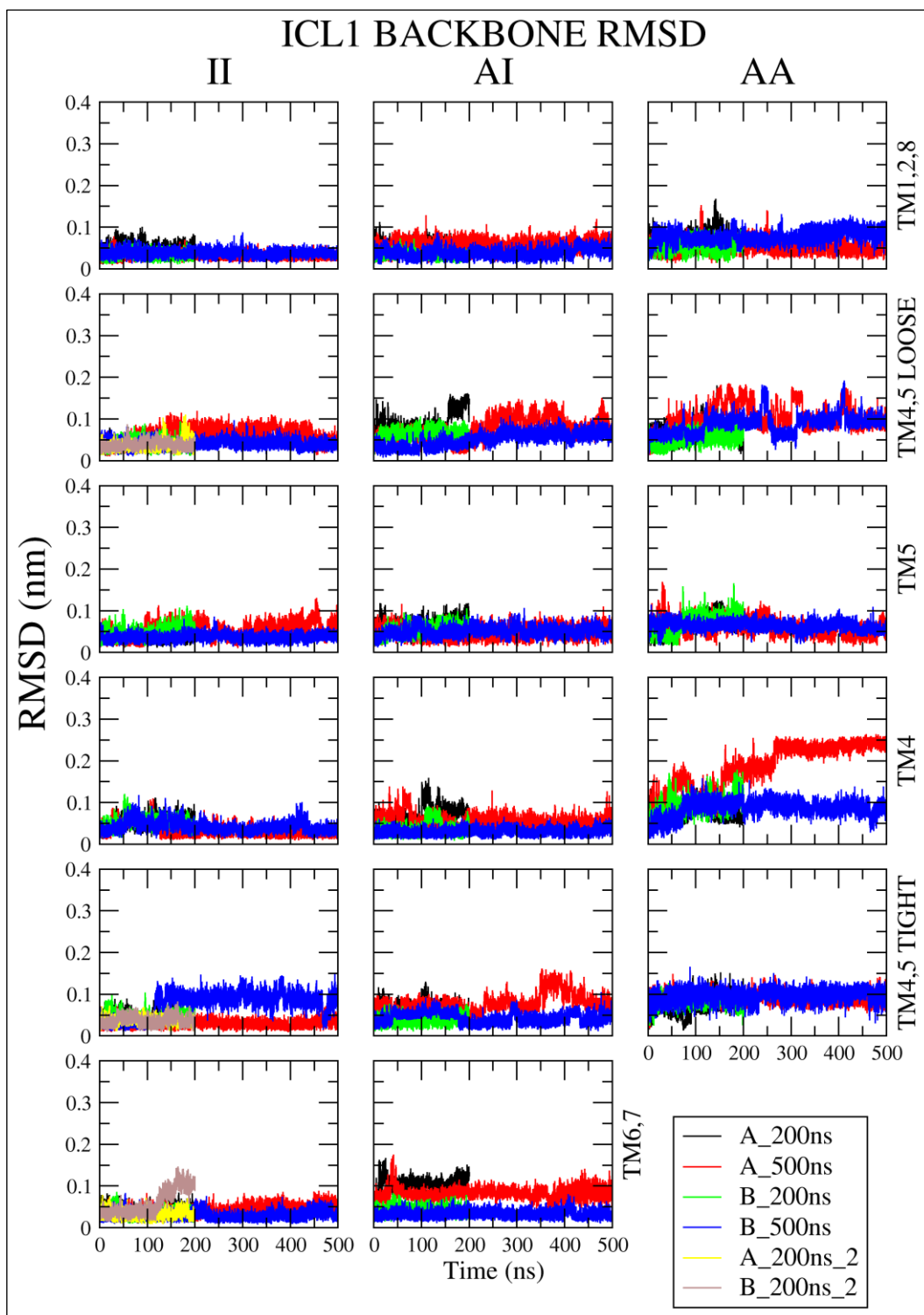


Figure 3.11: RMSD of ICL1 over the course of MD simulations.

In ICL2 of TM1,2,8 interface, we observed higher fluctuations in II and AI dimer system compared to AA dimer system. In all dimer systems, chain B had lower RMSD values than chain A, in ICL2 RMSD values of TM4,5 loose interface. In II dimer system of TM5 interface, after 250 ns of trajectories, both protomers deviated similarly, which was observed in AI dimer system protomers. In AA dimer system the deviation was found to be the highest after 200 ns of simulations when compared to II and AI systems, and protomers were fluctuated around 3 Å for the ICL2 of TM5 interface. In ICL2 of TM4, active protomer of AI dimer, stay stabilized around 0.5 Å, while inactive protomer and II dimer protomers fluctuated around 2 Å. After 100 ns of all trajectories in AA dimer system, both protomers behaved similarly as inactive protomers of II and AI dimer pairs. In ICL2 of TM4,5 tight interface, inactive protomers of II and AI and protomer B of AA dimer systems had higher values compared to protomer A of AI and protomer B of AA. We also observed this trend in protomer A of the AA dimer system after 200 ns simulation trajectories. In ICL2 of TM6,7 interface, we have observed slightly lower RMSD values for the active protomer compared to inactives. Figure 3.12 shows the RMSD values for ICL2.

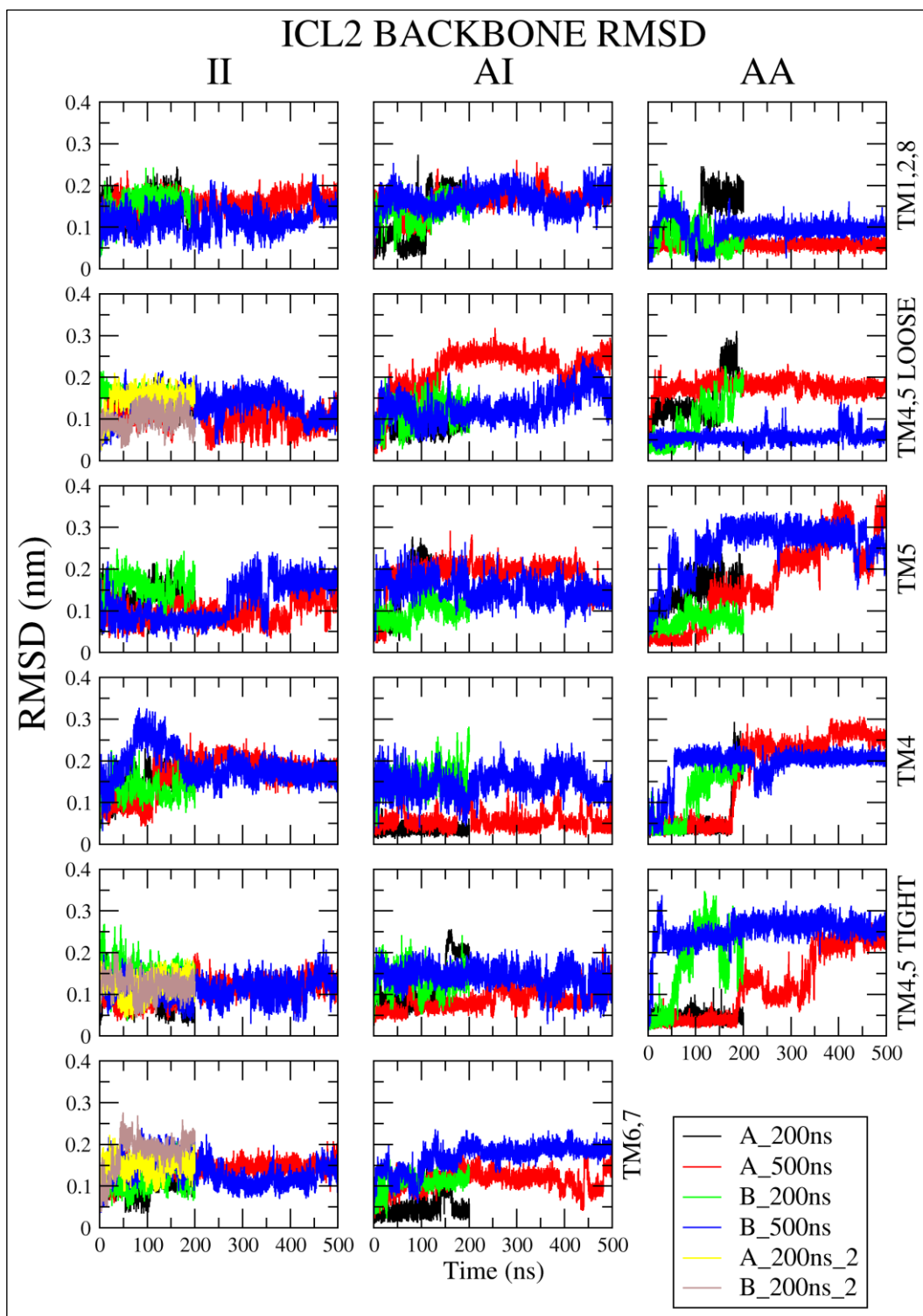


Figure 3.12: RMSD of ICL2 over the course of MD simulations.

ICL3 of inactive protomers of II and AI dimer system and chain B of AA dimer system tended to fluctuate more compared to active protomers of AI and chain A of AA dimer system in TM1,2,8 interface. In TM4,5 loose interface for the ICL3, except for II dimer system, where both chain A and B stabilized around 3 Å, chain A (1 and 1.5 Å) had lower RMSD values compared to chain B (3.5 and 3 Å) in AI and AA dimer systems, respectively. In TM5 interface, another distinct RMSD values were observed in ICL3 part where inactive protomers of II and AI system were found to be higher compared to active protomers. In ICL3 of TM4 interface, AA dimer system stabilized at lower RMSD values compared to AI and II dimer pairs. In ICL3 of TM4,5 tight interface, active protomers were found to be stabilized at lower RMSD values in all studied dimer pairs, except 200 ns run of protomer A of AI dimer system, where we observed higher trend. In ICL3 of TM6,7 interface, we have observed slightly lower RMSD values for the active protomer compared to inactives. Figure 3.13 shows the RMSD values for the ICL3.

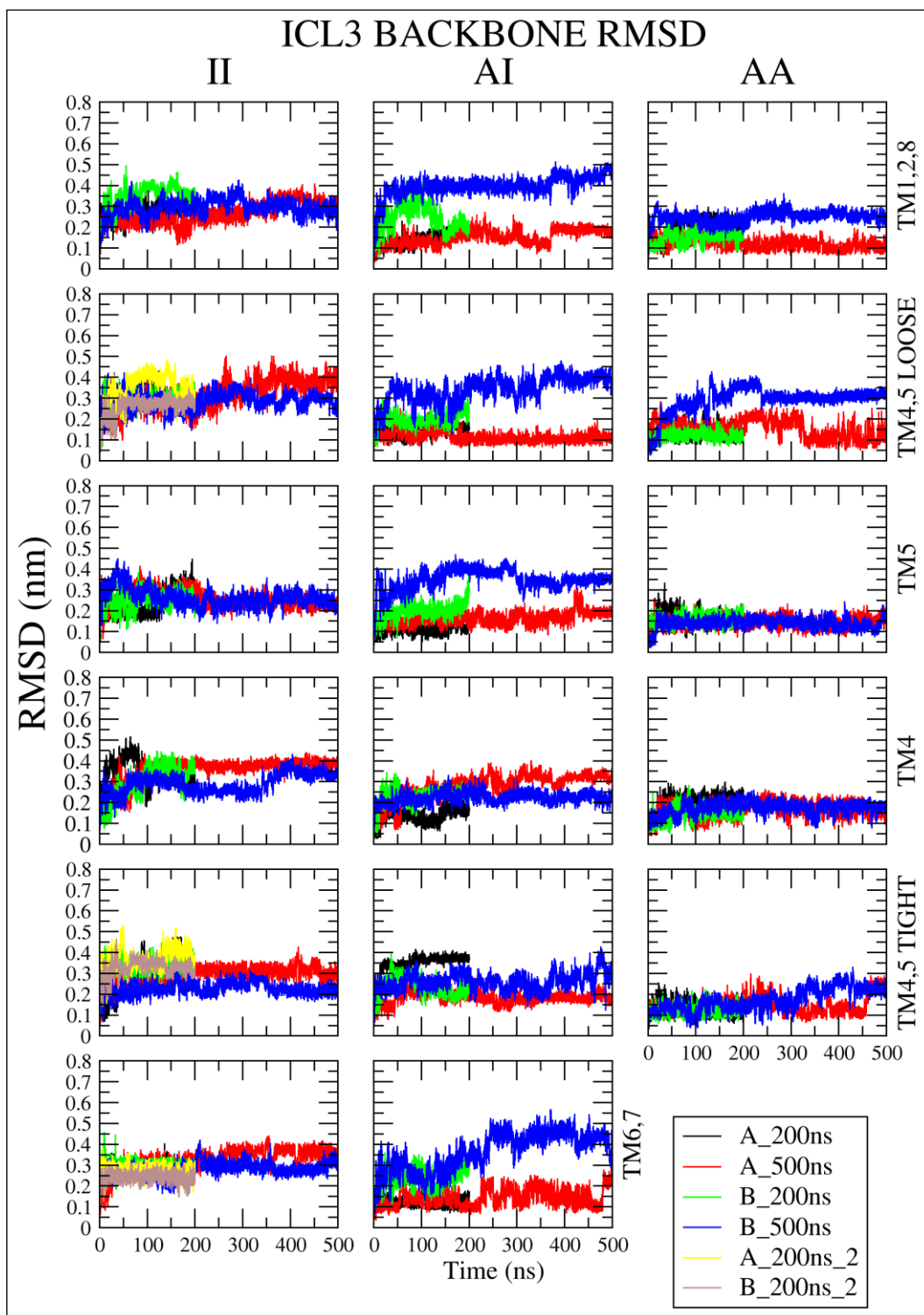


Figure 3.13: RMSD of ICL3 over the course of MD simulations.

RMSD values below 1 Å were observed in ECL1 of all dimer systems in the TM1,2,8 interface. ECL1 of TM4,5 loose interface, RMSD values were observed to deviate between 0.5 - 0.75 Å for all systems. ECL1 of TM5 interface stabilized with similar RMSD values for all protomer pairs in all studied systems. ECL1 of TM4 interface stabilized around 0.5 Å, expect the active protomer of AI system. ECL1 of TM4,5 tight interface stabilized around 0.5 Å, expect the active protomer of AI system. ECL1 of TM6,7 interface stabilized around 0.5 Å, expect the active protomer of AI system. Figure 3.14 shows the RMSD values of ECL1.

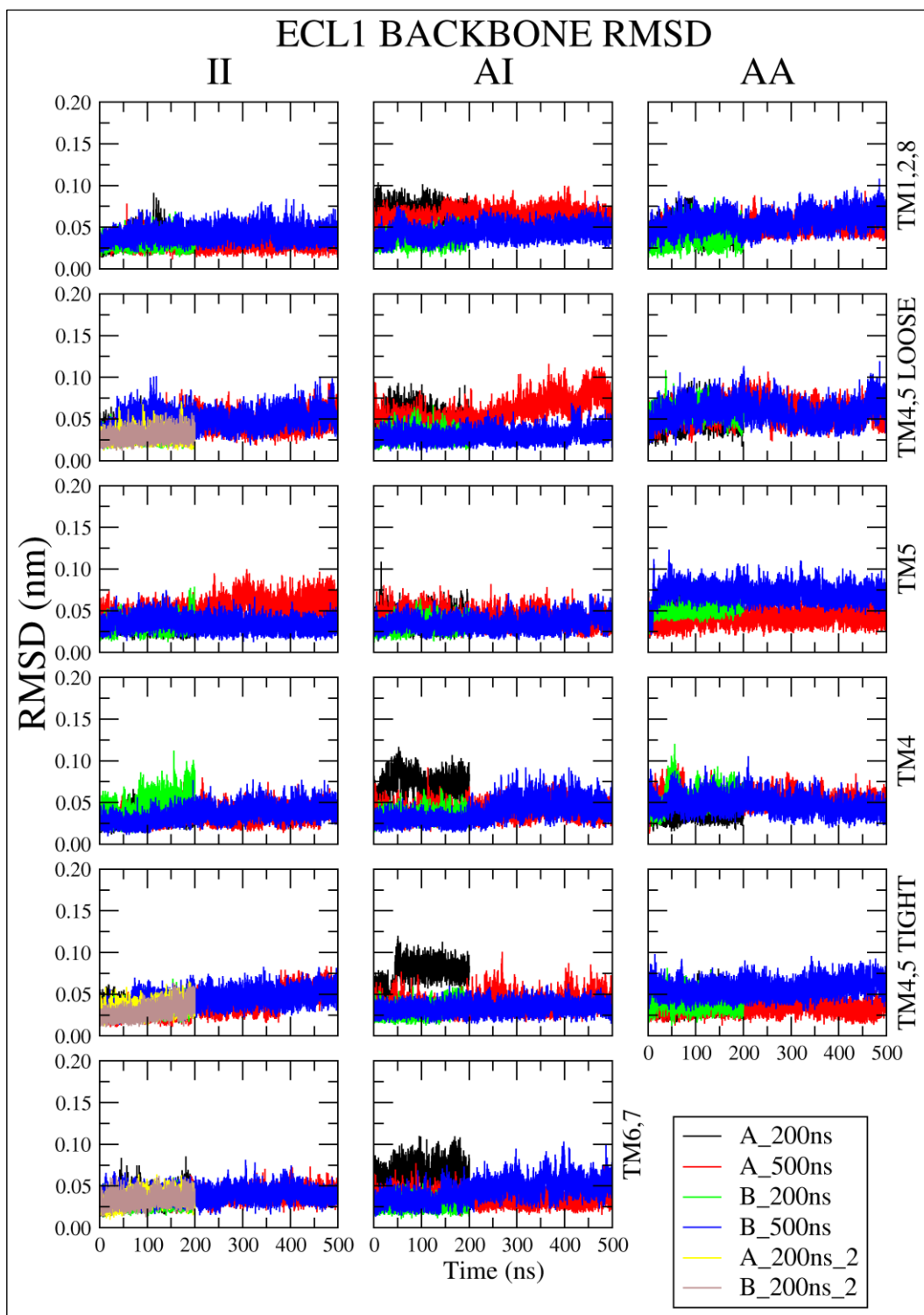


Figure 3.14: RMSD of ECL1 over the course of MD simulations.

The longest loop of GPCRs, ECL2, which has beta-hairpin motif, except for AA dimer system where both protomers fluctuated around 4 and 5 Å, in II dimer system, chain A and in AI the active protomer stabilized around 3 Å and other protomers both in II and AI remain stabilized around 1.5 and 2 Å respectively in the TM1,2,8 interface. In ECL2 of TM4,5 loose interface, for II dimer system, each protomer fluctuated more or less similarly, but in case of AI and AA, at least one protomer deviated more compared to other, we can conclude that, inactive protomer has more stabilized conformation compared to active protomer in AI dimer system, and in AA dimer system, chain A exhibited similar RMSD values which were observed for inactive protomers of II and AI systems. ECL2 of TM5 was found to be more flexible and stabilized around 2.5 Å in II and AI dimer pairs and around 3 Å in AA dimer system. ECL2 deviated differently at the beginning of the trajectories, but eventually all protomers were stabilized around 3 Å. In ECL2 of TM4,5 tight interface dimer systems, all protomers tended to deviate more after the first 200 ns of simulation trajectories. ECL2 of TM6,7 interface stabilized around 3 Å in all protomers. Figure 3.15 shows the RMSD graph for the ECL2 for all studied systems.

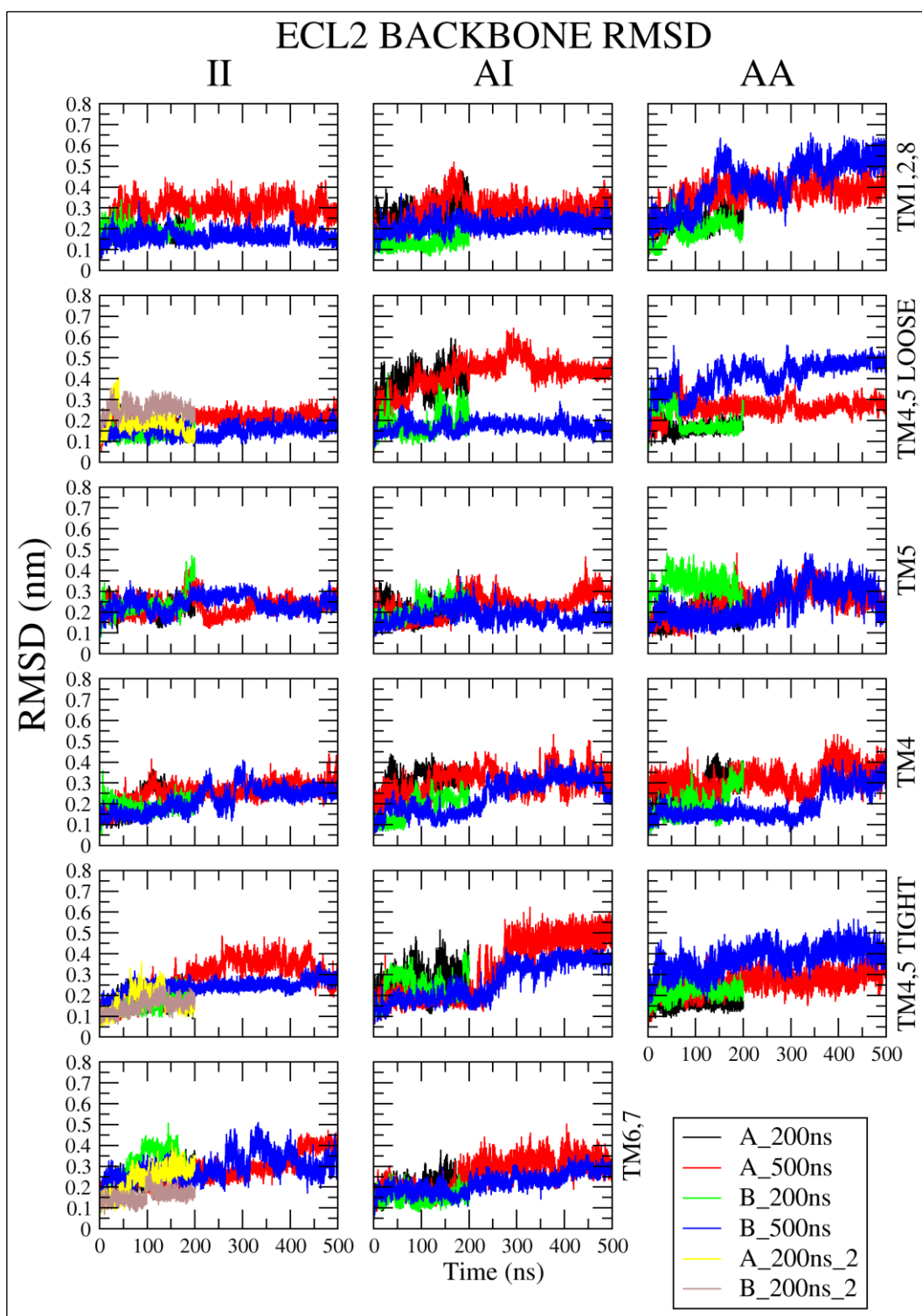


Figure 3.15: RMSD of ECL2 over the course of MD simulations.

In ECL3 of all dimer pairs we observed RMSD values lower than 0.5 Å. ECL3 of TM4,5 loose interface, RMSD values were stabilized around 0.5 Å. RMSD values of ECL3 of TM5 interface were very stable, similar to ECL1, all studied dimer pairs stabilized around 0.5 Å. ECL3 of TM4 interface stabilized around 0.5 Å. ECL3 of TM4,5 tight interface stabilized around 0.5 Å. However, we observed a slight difference in the RMSD values of the active protomer of the AI dimer system compared to the inactive protomers in both the II and AI dimer systems in the ECL3 RMSD values of TM6,7 interface. Figure 3.16 shows the RMSD values for the ECL3.

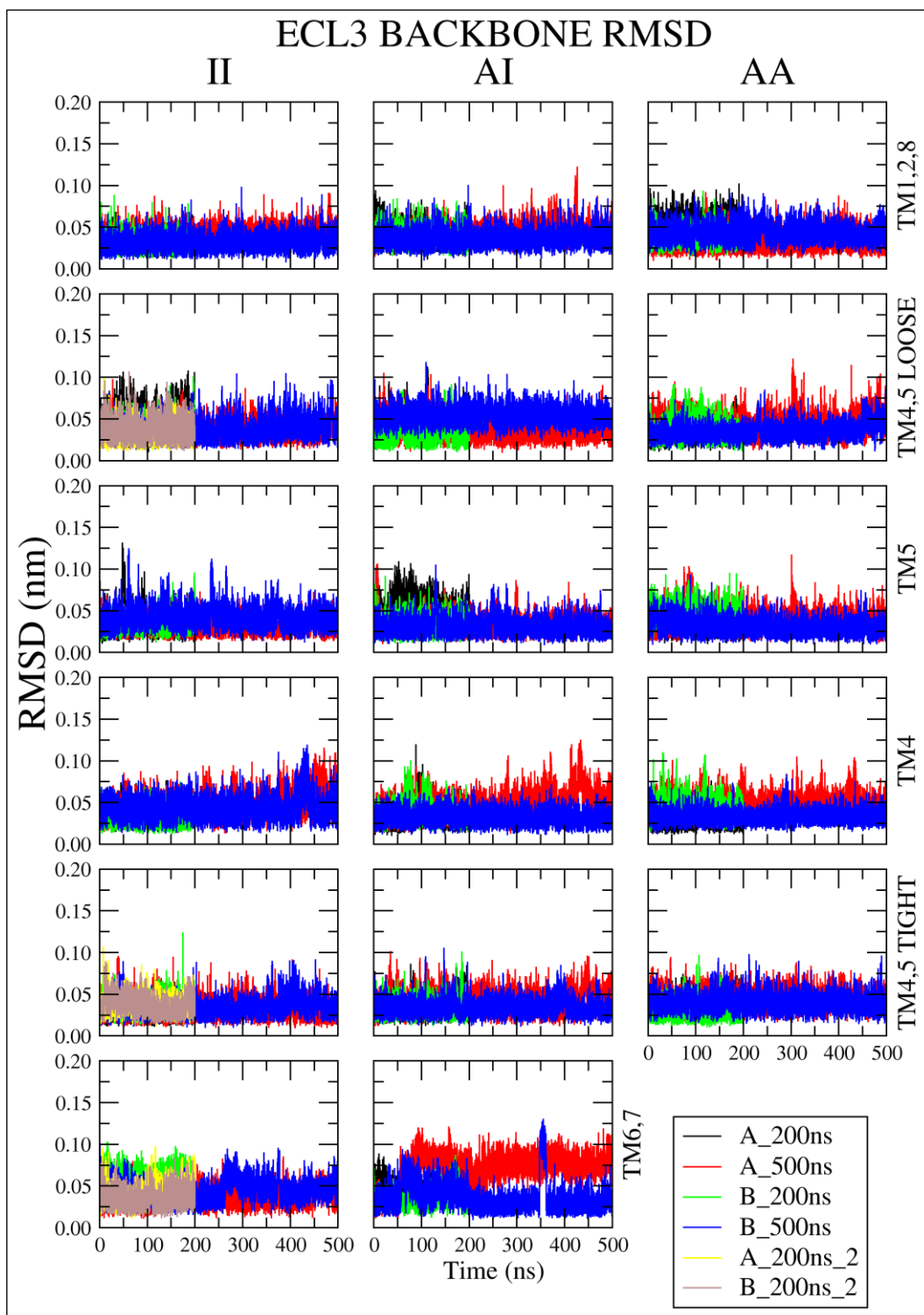


Figure 3.16: RMSD of ECL3 over the course of MD simulations.

When we examined the RMSD values of ANGII in dimer systems consisting of different interfaces, different observations were made. For example, in all systems of dimers with TM1,2,8 interfaces (II, AI, and AA), the RMSD of ANGII remained below 2 Å, indicating a very stable binding mode. With the exception of ANGII bound to protomer A of system II and protomer B of system AI, RMSD values below 2 Å were observed for TM4,5 loose dimers. In the dimer systems formed at the TM5 interface, RMSD values of about 2 Å or more were observed after the first 200 ns in both chains of the AI and AA dimers and in the B chain of the II system for ANGII. In dimers with TM4 interface, ANGII bound in the A protomer of the II system increased above 2 Å after the first 50 ns and maintained this trend until the end of the simulation. In the AI system, an increase in the RMSD values of ANGII was observed especially after the first 200 ns. Interestingly, in the AA system, the ANGII RMSD values remained quite stable in all simulations and values around 1 Å were observed. RMSD values of ANGII were generally low (around ~1.25 Å) in dimer systems with tight TM4.5 interfaces. In contrast, ANGII in the A protomer of the AI dimer system underwent a conformational change after the first 50 ns, with RMSD values averaging about 2.5 Å by the end of the simulation. In dimer systems consisting of the TM6,7 interface, the ANGIIs bound to each protomer had different average RMSD values. For example, the ANGII in the A protomer of the II system had RMSD values of about 1 Å, while the ANGII in the B protomer had higher average RMSD values. In the AI dimer system, the inactive protomer exhibited lower RMSD values, while the RMSD values of ANGII in the active protomer were observed to be 2 Å and above. Figure 3.17 shows the RMSD values of ANGII.

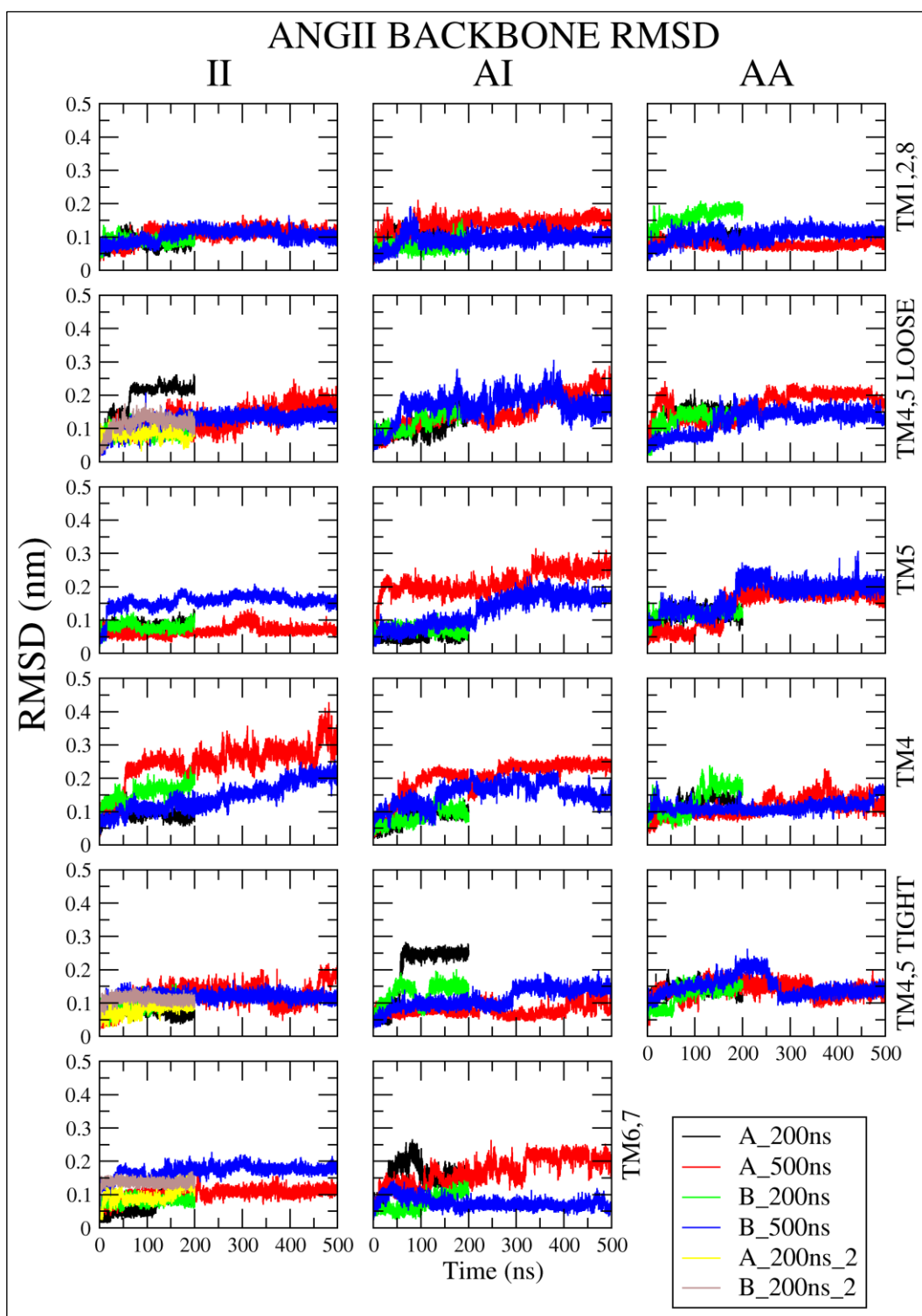


Figure 3.17: RMSD of ANGIO over the course of MD simulations.

Considering the RMSD values in detail, each topological subunit showed a different behavior during the simulation periods, some of them remained constant during the simulation, while others increased from the beginning of the simulation. It was not possible to draw any conclusions from the RMSD values of the TM regions and the intracellular and extracellular loops. Since it was not possible to determine which interface was the most stable by looking at the RMSD values, it was decided to perform further analyses. These analyses included principal component analysis (PCA), water pathway analysis, and distance analysis between specific amino acids. The results of these analyses are presented and interpreted in the following sections.

3.2. TM3-TM6 (ARG126^{3.50} - PHE239^{6.34}) Distance Analysis

In these analyses, the simulated trajectories were merged in order to make it easier to interpret the data obtained. The readers are reminded that before the merging, the first 25 ns of all simulations were considered as the time when the systems reached equilibrium and were excluded from the analysis. The remaining trajectories (175 ns for 200 ns simulations and 475 ns for 500 ns simulations) were concatenated to obtain a single 650 ns trajectory. When analysing the activation mechanism of class A GPCRs, it is observed that the most prominent change occurs in TM6. To reach this conclusion, it would be sufficient to align the inactive and active GPCR structures. The reason for this conformational change in TM6 is to make room for effector proteins (such as G-protein or beta-arrestin) to approach the receptor from the inside of the cell and bind to it. If there is enough space for coupling, the effector protein-GPCR complex forms and the required signalling pathway is initiated. In order to monitor this change observed in TM6, the distance between the carbon alpha atoms of ARG126^{3.50} in TM3 and PHE239^{6.34} in TM6 was calculated throughout the simulation. To correctly interpret the changes, the average values observed during the simulation were compared with the measured values between these two amino acids in the inactive AT1R crystal structure and in the active AT1R model. These values were measured to be 6.78 Å and 11.53 Å for the inactive and active AT1R structures, respectively. The distance between these two amino acids was therefore followed along the merged trajectories. The distance between TM3-TM6 was analysed at all interfaces and in all dimer pairs, and it was reported at which interfaces an active and

an inactive protomer were observed in concatenated MD trajectories. Figure 3.18 shows the change in TM3-TM6 distances throughout MD simulations.

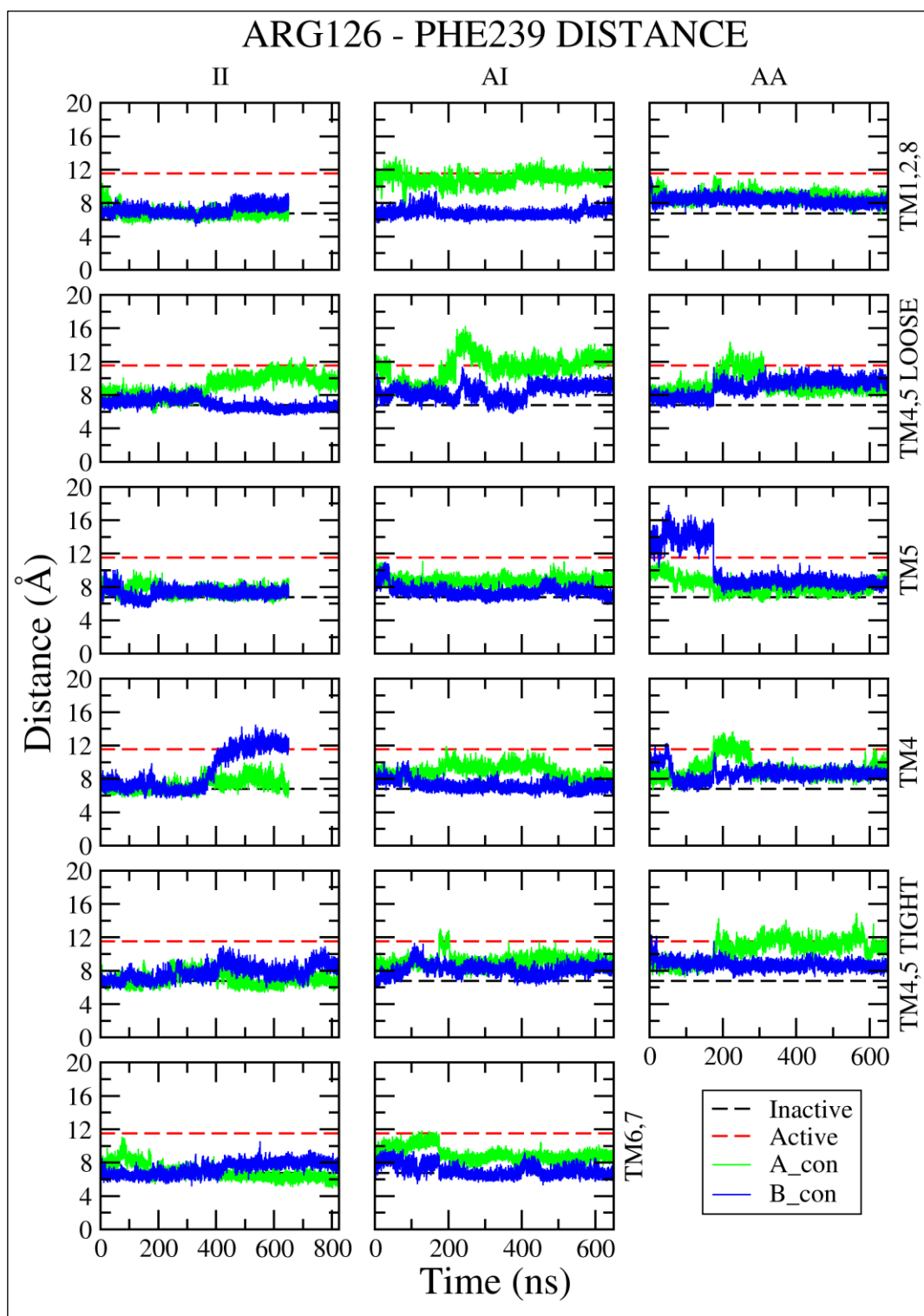


Figure 3.18: The distance between ARG126^{3.50}-PHE239^{6.34} in each dimer interface and pairs. The distance that observed in inactive crystal structure shown as black dashed lines. The distance that observed in active structure shown as red dashed lines.

When the distance between TM3-TM6 was analyzed in dimer systems with TM1,2,8 interface, it was observed that in the II dimer pair, both protomers maintained their initial state during the simulations. In the AI dimer pair, again as in II, the protomers maintained their initial state. However, when the AA dimer pair was analyzed, both protomers changed their conformation to have an inactive-like TM3-TM6 distance. However, it should be noted that in the AA system both protomers have higher averages than the TM3-TM6 distance observed in the inactive structure (Figure 3.18, first panel). As a result, in the dimer system consisting of two active protomers with TM1,2,8 interfaces, it was observed that both protomers shifted from their initial states to an inactive-like conformation.

In system II of the TM4,5 loose interface dimers, one protomer showed an active-like conformation, especially after the first 200 ns. The other protomer maintained its initial conformation throughout the simulations. Interestingly, in the AI dimer system, the initially active protomer exhibited an inactive-like conformation for the first 200 ns, but later reverted to the active conformation. However, the B-chain, which started the simulation in an inactive conformation, changed to an active-like conformation after the first 400 ns. In the AA system, where both protomers started in the active conformation, both chains switched to an inactive-like conformation and maintained this conformation for the first 200 ns. However, after 200 ns, they ended the simulation with a distance between TM3-TM6 between the active and inactive conformations (Figure 3.18, second panel).

In the II system of dimers with TM5 interface, both protomers maintained their inactive conformation from the beginning to the end of the simulation. In the AI system, the active protomer adopted an inactive-like conformation at the beginning of the simulation, while the inactive protomer did not change significantly during the simulation. Surprisingly, in the AA system, one of the protomers maintained the active conformation for the first 200 ns, but switched to the inactive-like conformation during the rest of the simulation. The other protomer in the AA system switched to the inactive-like conformation at the beginning of the simulation and maintained this conformation until the end of the simulation (Figure 3.18, third panel).

In the II system of TM4 interfaced dimers, quite unexpectedly, the protomer was observed to transition to the active structure after 400 ns in the B-chain, while the A-chain maintained the inactive structure, which started as the initial conformation, until the end of the simulation. Considering this result, we can conclude that the TM4

interface could be a possible interaction interface for the AT1R homodimer consisting of one active and one inactive protomer. In the AI system, while the protomer in the inactive conformation maintained its state, the protomer that started the simulation in the active conformation was observed to evolve into an inactive-like structure. In the AA system, the A-chain shows an active conformation only for a very short period of time (between 200-300 ns) of the simulation, while the rest of the time it shows an inactive-like structure characteristic of the other protomer (Figure 3.18, fourth panel).

In the TM4,5 tight interface dimer system II, although both protomers showed an inactive structure in the majority of simulations, the B-protomer moved slightly away from the inactive TM3-TM6 distance after the first 400 ns. In the AI system, both protomers moved away from the conformation in which they started and reached the end of the simulation with TM3-TM6 distances containing values between active and inactive structure properties. In the AA system, while both protomers switched to an inactive-like conformation at the start of the simulation, the A-protomer returned to the active structure after 200 ns. The B-protomer maintained its inactive-like conformation until the end of the simulation (Figure 3.18, fifth panel).

In system II of dimers with TM6,7 interface, protomer A deviated from the inactive structure at the beginning of the simulation (first 200 ns) and protomer B deviated from the inactive structure towards the end of the simulation (after the first 400 ns). In the AI system, the active protomer showed more active-like conformations during the first 150 ns and inactive-like conformations during the rest of the simulation. The protomer that started the simulation in the inactive conformation maintained this conformation throughout the simulation (Figure 3.18, sixth panel).

Determining the state of the systems by considering only the TM3-TM6 distance can lead to misinterpretations. To avoid this, the helix orientations of both TM3 and TM6 were also taken into account. Looking only at the distance between TM3-TM6 in the TM4,5 loose II and TM6,7 AI bound dimers, it can be seen that both systems consist of one active and one inactive protomer. However, upon closer examination, it was found that although this distance was measured to be 9.5 Å, this value was obtained by moving TM3 away from its proper position. Therefore, at this interface, TM3 moved in the direction of the interaction interface and caused the TM3-TM6 distance to be at active-like values. This situation is shown in Figure 3.19. As can be clearly seen in Figure T, the A protomer of the TM4.5 loose interface II system is

located in the G protein (or beta-arrestin) binding site, thus preventing binding of the effector protein. Therefore, the TM4,5 loose interface was not used in further analysis.

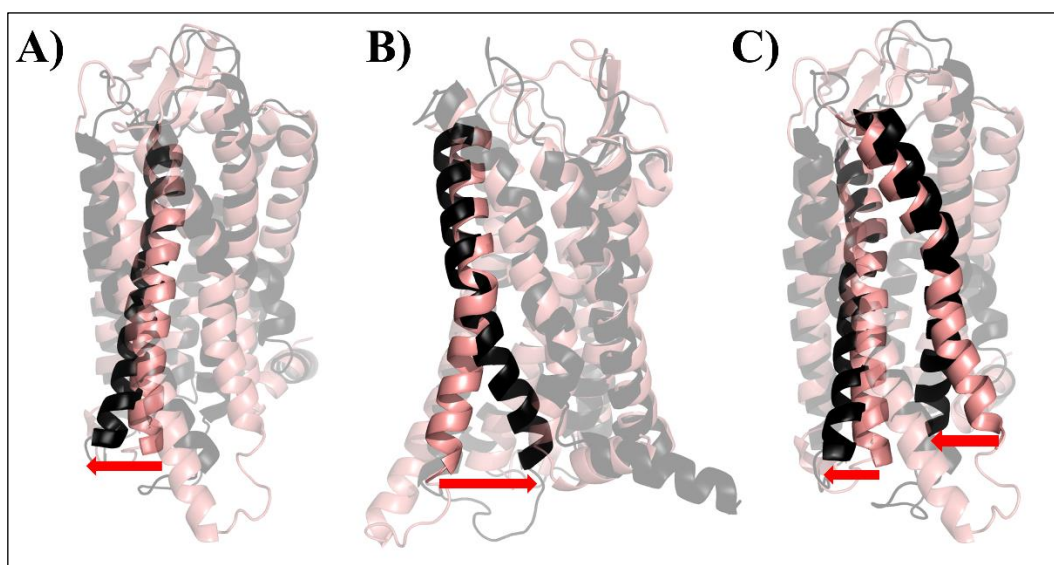


Figure 3.19: The most populated state of the protomer A of the TM4,5 loose interface aligned to AT1R crystal structure. Protomer A of the TM4,5 loose interface depicted in black, and AT1R crystal depicted in salmon color. A) Movement of TM3. B) Movement of TM6. C) TM3-TM6 movement.

Similarly, in the TM6,7 interface AI system, although the distance between TM3-TM6 contains active-like structural features, the inward movement of amino acids 229-242 towards the G-protein binding site prevented further analysis of this system. Figure 3.20 shows the inward movement of amino acids in the intracellular portion of TM6 in the TM6,7 interface AI system from side (A) and bottom (B) views.

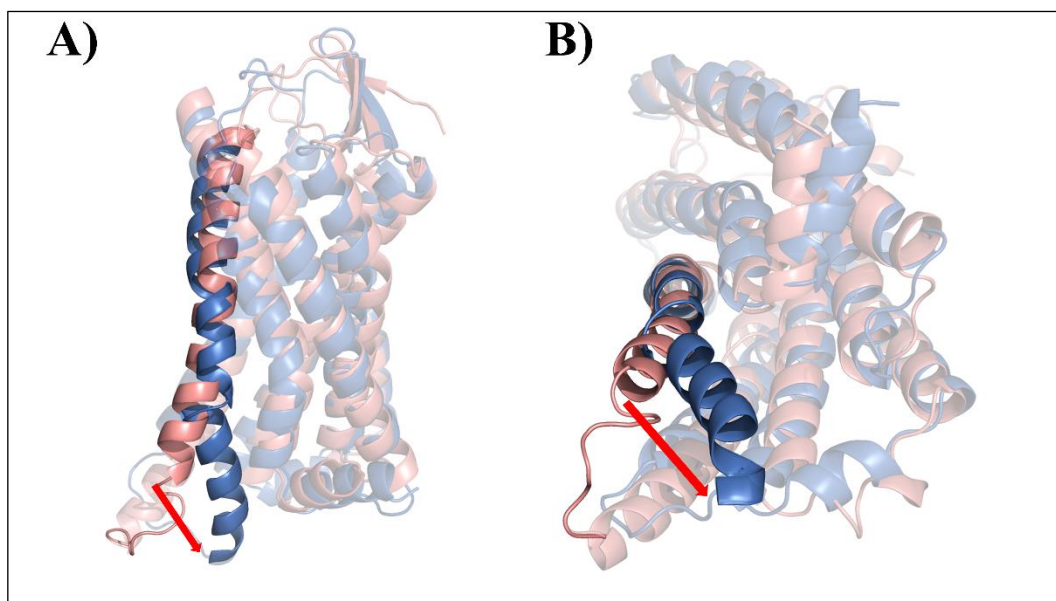


Figure 3.20: Inward movement of TM6 towards to the G protein coupling site.

Previous studies with GPCR dimers have reported that the interaction stoichiometry of dimers is 2:1. In this case, one protomer should be in the active form (able to interact with the intracellular effector) and the other in the inactive form [192–196]. In contrast to these studies, Parker et al. claimed that both protomers are capable of binding two G proteins simultaneously in the neuropeptide Y (NPY) Y2 receptor [197]. However, the 2:2 stoichiometry proposed by Parker et al. was not observed in any of our simulation systems. Therefore, in line with the literature, dimer systems consisting of one active and one inactive protomer were accepted as the most likely hypothesis in this study. Based on this hypothesis, seven systems, namely TM1,2,8 AI; TM4,5 loose AI and AA; TM5 AA; TM4 II and AA; and TM4,5 tight AA, were selected and subjected to further analysis.

3.3. Representative Structures From Clustering

The concatenated simulation trajectories were used in the clustering analysis. The gromos algorithm and a cut-off value of 2 Å were used in the clustering analysis. The results of the clustering analysis were evaluated together with the distance analysis between TM3-TM6. For this purpose, cluster members were selected that represent the opening of TM6 and the dimer consisting of one active and one inactive protomer according to the TM3-TM6 distance analysis. The cluster representatives selected for TM4.5 tight AA, TM1,2,8 AI, TM5 AA, TM4 II, TM4.5 loose AA, TM4.5 loose AI,

and TM4 AA represent 53.70, 51.57, 16.63, 15.35, 12.31, 11.90, and 9.46 percent of all trajectory frames, respectively. In other words, these cluster representatives fulfill all the characteristics such as the distance between TM3-TM6, the orientation of TM6, and the presence of an active and an inactive protomer in the mentioned percentages. However, it should be noted that the cluster representatives of the TM4.5 loose AA, TM4.5 loose AI, and TM4 AA systems represent less than 15% of all frames, so these systems were not used for further analysis. Therefore, four systems remained for further analysis (TM4.5 tight AA, TM1,2,8 AI, TM5 AA, and TM4 II).

The cluster representative structures were compared with the experimental structure of the AT1R-S1I8 complex and both the RMS value of the whole structure and the RMS values of the TM regions only were calculated. Since ANGII is also present in the cluster representative structures, the interactions between ANGII and AT1R were also evaluated. The interactions between ANGII-AT1R were compared between those observed at the beginning of the simulation (pose obtained by molecular docking) and those observed in the cluster representatives.

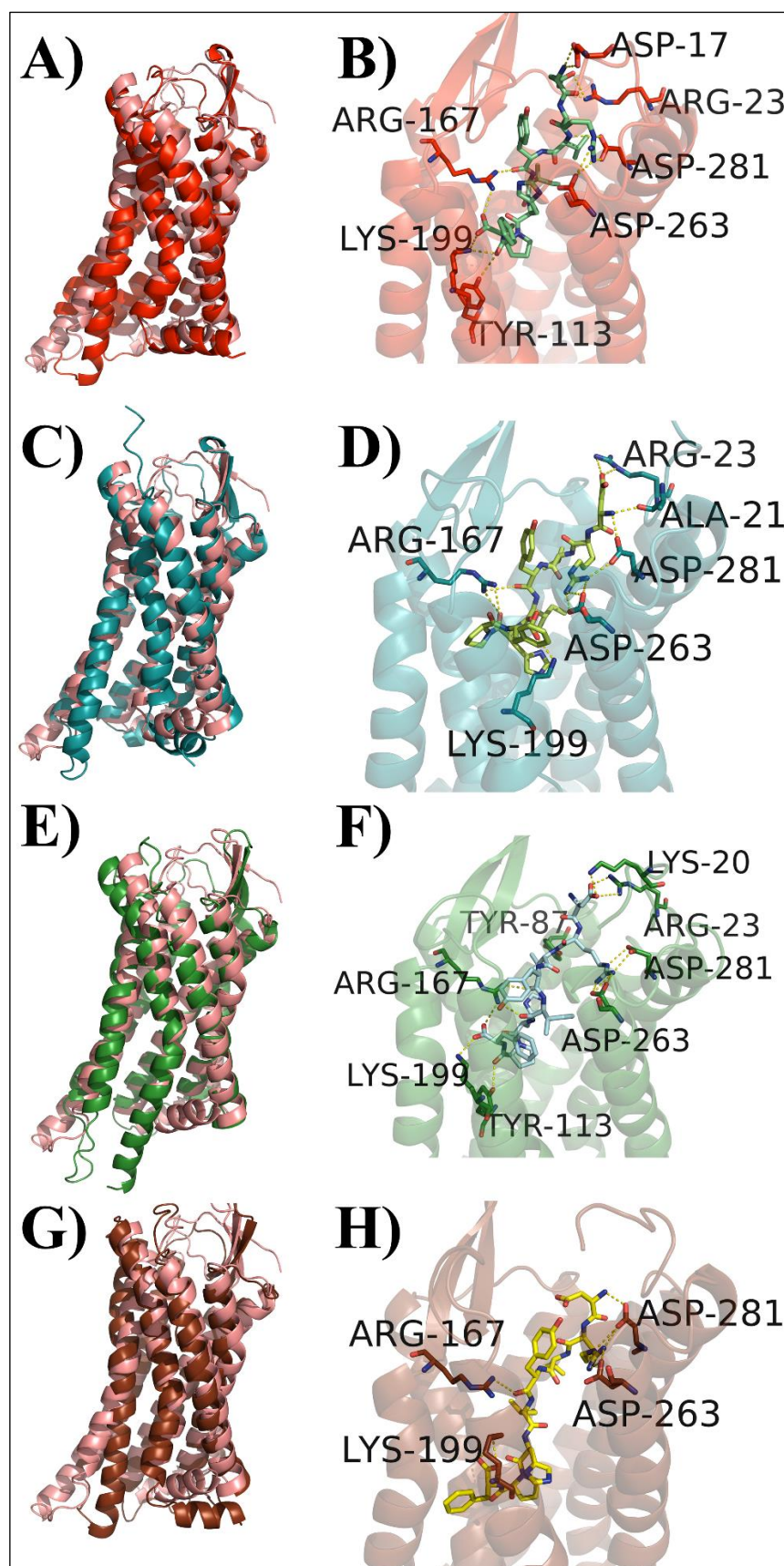


Figure 3.21: A), C), E) and G) Comparison of cluster representative structures with active AT1R crystal. B), D), F), and H) binding mode and interactions of ANGII at the binding pocket.

In the comparative analyses here, the active protomer of the dimer was used and a comparison was made with the experimental AT1R-S1I8 structure. It should be understood that the structure referred to as AT1R in this section is the experimental structure. When the cluster-representative structure of the TM1,2,8 AI dimer was aligned with the AT1R structure, an RMS value of 2.05 Å was obtained (an RMS value of 1.98 Å was observed when only the TM regions were considered). Figure 3.21, panel A shows the alignment of the cluster representative structure of TM1,2,8 AI and AT1R. The interaction between ASP17^{Nterm} of AT1R and ASP1 of ANGII, which was not observed in the starting pose, was observed in the cluster representative (Figure 3.21, panel B). The ARG23^{Nterm}-ASP1 and ASP263^{6.58}-ARG2 interactions observed in the initial pose were also observed in the cluster representative structure. Thus, this interaction is conserved. When Santos et al. mutated ARG23ALA^{Nterm}, they observed a decrease in ANGII binding and AT1R activation [13]. However, they found that this mutation did not alter SAR1-ANG binding and activation. Feng et al. showed that the ASP281^{7.32}-ARG2 interaction is essential for the full agonist effect [185]. Previous studies examining AT1R-ANGII interactions and the chemical nature of those interactions have reported the importance of ASP263^{6.58}-ARG2 and ASP281^{7.32}-ARG2 interactions. The work of Fillion et al. is one of these studies [184]. ASP281^{7.32} was also observed to interact with the backbone of VAL3 in the cluster representative structure. The importance of ARG167^{4.64} in ARB and ANGII binding has been reported in many studies in the literature [198–200]. When this amino acid is mutated, the binding of ARBs and ANGII is reduced. In the cluster representative structure, ARG167^{4.64} was observed to form two bonds with the backbone of TYR4 and the carboxy-terminal end of PHE8. This interaction is supported by another study. Matsoukas et al. also emphasized the importance of strong polar interactions between the C-terminal end and ARG167^{4.64} [191]. PRO7 has been reported as one of the key amino acids in the interaction of ANGII with AT1R. In the cluster representative structure, a bonding between the backbone of this amino acid and LYS199^{5.42} was observed. Mutation of PRO7 completely abolished the predisposition of ANGII to bind to AT1R [198]. Studies in the literature have highlighted the importance of the ionic interaction between LYS199^{5.42} and PHE8 [185–187]. This interaction was also observed in our cluster representative structure. Some other studies have highlighted the importance of the TYR113^{3.37}-PHE8 interaction. This interaction was also observed in the cluster representative structure [189, 201, 202].

In the TM5 AA dimer, it was protomer B that exhibited the active structural properties. The RMS value of 1,49 Å was obtained by superimposing protomer B on the AT1R crystal structure. When only the TM domain was considered, the RMS value obtained was 1,46 Å. The first two amino acids of ANGII, ASP1 and ARG2, interacted with ALA21^{Nterm}, ARG23^{Nterm}, ASP281^{7.32} and ASP263^{6.58}, respectively. Of the eight interactions, two are from the backbone (ALA21^{Nterm}-ASP1 and ASP281^{7.32}-ASP1) and the other six are from the side chains (ARG23^{Nterm}-ASP1 (2 bonds), ASP281^{7.32}-ARG2, and ASP263^{6.58}-ARG2 (3 bonds)). ARG167^{4.64} formed three interactions with the backbones of TYR4 and HIS6, one with TYR4 and two with HIS6. Another interaction that was observed in the cluster representative structure is the bond that is formed between LYS199^{5.42} and PHE8 through the carboxy terminal end of ANGII (Figure 3.21, panel D).

In the TM4 II dimer, it is protomer B that resembles the active structure features. Comparison of this protomer with the active AT1R structure yielded an RMS value of 1.982 Å. When only the TM regions were considered, the RMS value was calculated to be 1.952 Å. Amino acids at the N- and C-terminal ends of ANGII formed bonds with the AT1R through the interactions observed in the TM1,2,8 AI dimer. In contrast to these interactions, LYS20^{Nterm}-ASP1 and TYR87^{2.63}-VAL3 were observed to be specific for TM4 II (Figure 3.21, Panel F).

Of the four systems selected for detailed analysis, the highest RMS value was observed when the A protomer of the TM4.5 tight AA dimer was aligned with the AT1R. The RMS values obtained when comparing the whole structure and the TM domains only were 2.537 Å and 2.319 Å, respectively. The interactions observed in the cluster representative structure were ASP281^{7.32}-ASP1, ASP281^{7.32}-ARG2 (2 bonds), ASP263^{6.58}-ARG2 (2 bonds), ARG167^{4.64}-TYR4, and LYS199^{5.42}-PHE8 (2 bonds) (Figure 3.21, Panel H).

3.4. NPxxY Motif

One of the important structural motifs in GPCRs is the NPxxY motif. This motif is located in TM7 and consists of ASN298^{7.49}, PRO299^{7.50}, LEU300^{7.51}, PHE301^{7.52}, and TYR302^{7.53}. Of these amino acids, alanine mutants of ASN298^{7.49}, PRO299^{7.50}, and TYR302^{7.53} caused alterations in inositol phosphate responses and internalization kinetics [203, 204]. Upon receptor activation, the NPxxY motif undergoes

conformational changes in order to perform the structural rearrangements necessary for the intracellular binding partner to enable its binding to the receptor [205]. Therefore, active and inactive GPCR structures are expected to have different RMSD values of the NPxxY motif. A more accurate interpretation of the results would be a combined analysis of the TM3-TM6 distance and the NPxxY motif RMSD values. The graph of the RMSD values of the NPxxY motif plotted according to the distance change between TM3-TM6 is given in Figure 3.22. This figure has four panels. Each panel is divided into four sections. Of these areas, areas numbered II and IV show the conformations in which the active and inactive structures are found, respectively. Areas I and III represent the conformations in which inactive-like and active-like structures are found, respectively.

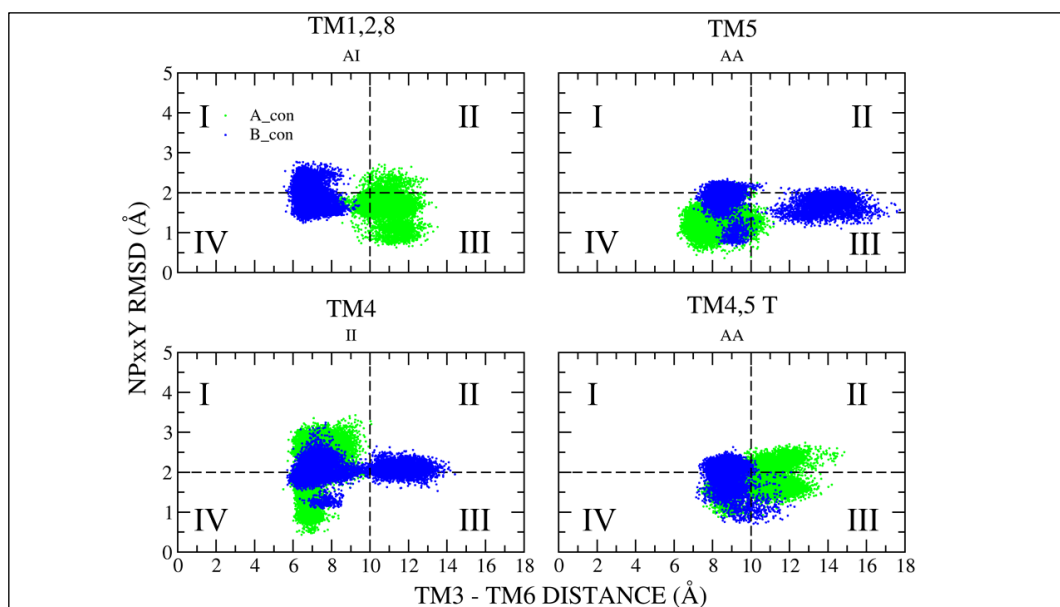


Figure 3.22: The NPxxY motif that reside in TM7 plotted together with TM3-TM6 distance in four selected dimer systems, TM1,2,8 AI, TM5 AA, TM4 II, and TM4,5 tight AA, right upper panel, left upper panel, right lower panel, and left lower panel, respectively.

The A chain of the TM1,2,8 AI dimer mostly visited active-like (III) and active (II) sites during the simulation. The B chain, on the other hand, did not leave the inactive (IV) and inactive-like (I) regions (Figure 3.22, upper left panel). Looking at the B-chain of the TM5 AA dimer, in terms of NPxxY RMSD and TM3-TM6 distance, the active-like region was the one in which this protomer spent the most time (i.e., had the most conformations), although it explored conformations in all regions. The majority of conformations of the A chain of the same dimer were observed in the

inactive region (Figure 3.22, upper right panel). The B chain of the TM4 II dimer showed active and active-like conformations. Some conformations of this chain were observed in the II and III regions in the lower left panel of Figure 3.22. Looking closely at the same panel, it can be seen that the A chain does not visit the II and III regions at all. In the case of the TM4,5 tight AA dimer, the A chain visits all regions of the NPxxY RMSD-TM3-TM6 distance landscape, but shows more conformations in the II region than in the other three regions (Figure 3.22, lower right panel). Taking all this information into account, the NPxxY RMSD-TM3-TM6 distance analysis supports the fact that the four selected systems consist of one active and one inactive protomer and that this is maintained for considerable periods of the simulation.

3.5. Intraprotomer Interactions That Stabilize Inactive States

Residue-residue distances were measured to determine which interactions between amino acids stabilize and maintain the active or inactive structures. These intra-protomeric residue-residue interactions were performed for all four systems selected for in-depth analysis. Three different residue-residue interactions were found to be significant for the TM1,2,8 AI dimer. These residue-residue distances are between MET57^{ICL1} CE - ILE238^{6.33} CD, ALA63^{2.39} CB - ILE238^{6.33} CG2, and LEU122^{3.46} CD2 - ILE238^{6.33} CG2 atoms. As shown in Figure 3.23, these interatomic distances are sufficiently different to distinguish between active and inactive systems. These interactions are thought to be much stronger in the inactive state and play an important role in stabilizing the inactive state. In the inactive state, these interatomic distances remain constant around 5 Å, stabilizing the inactive state. In the active state, these interatomic distances are stabilized at values of 15 Å and greater.

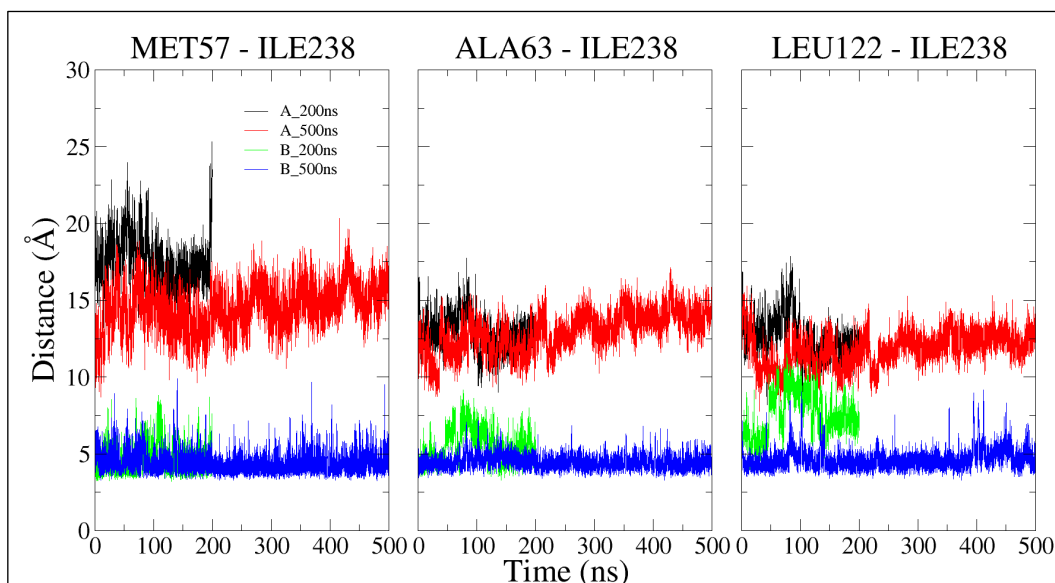


Figure 3.23: Crucial distances that stabilize inactive conformation in TM1,2,8 interface AI dimer.

In the TM5 AA dimer, two different interatomic distances were observed to be significantly different in the active and inactive structures in the simulations. These atom-to-atom distances were measured between ALA129^{3.53} CB - ILE238^{6.33} CD and ILE241^{6.36} CA - PHE301^{7.52} CZ atoms and are shown in Figure 3.24. In the protomer exhibiting inactive and inactive-like structures, these atom-to-atom distances were found to stabilize around 5 Å. The ALA129^{3.53} CB to ILE238^{6.33} CD distance showed values around 15 Å in the active structure, while the ILE241^{6.36} CA to PHE301^{7.52} CZ distance was around 7.5 Å in the active structure.

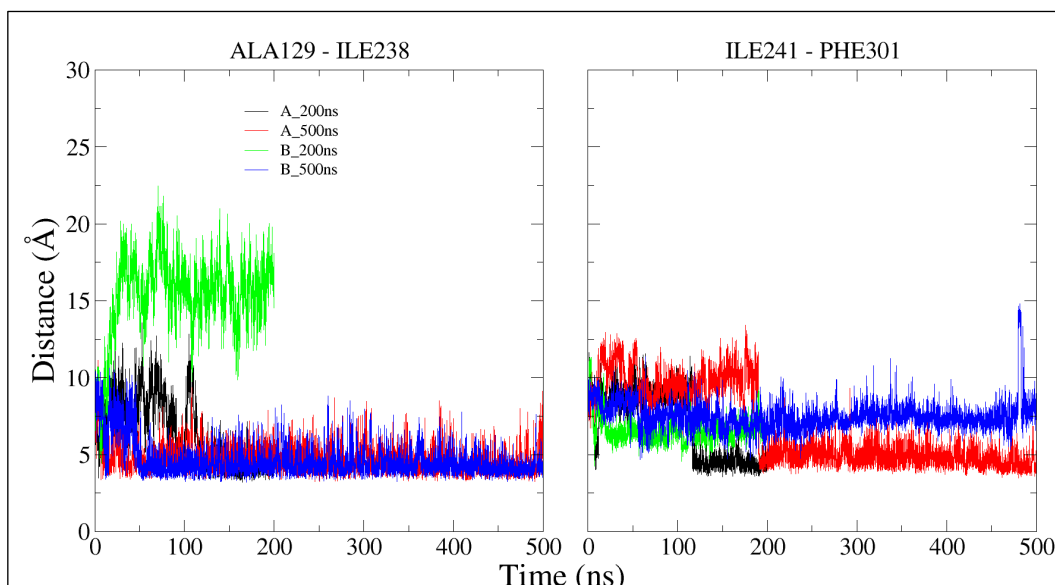


Figure 3.24: Crucial distances that stabilize inactive conformation in TM5 interface AA dimer.

The interatomic distances of MET57^{ICL1} CG - ILE238^{6.33} CD, ALA63^{2.39} CB - ILE238^{6.33} CG2, ALA63^{3.29} CB - ILE242^{6.37} CD, and LEU119^{3.43} CD1 - VAL246^{6.41} CG2 helped to separate the active and inactive protomers for the TM4 II interface. Figure 3.25 shows the variation of these interatomic distances throughout the simulation. These interatomic distances exhibited values around 5 Å for the inactive protomer, while values of 10 Å and above were observed for the active protomer.

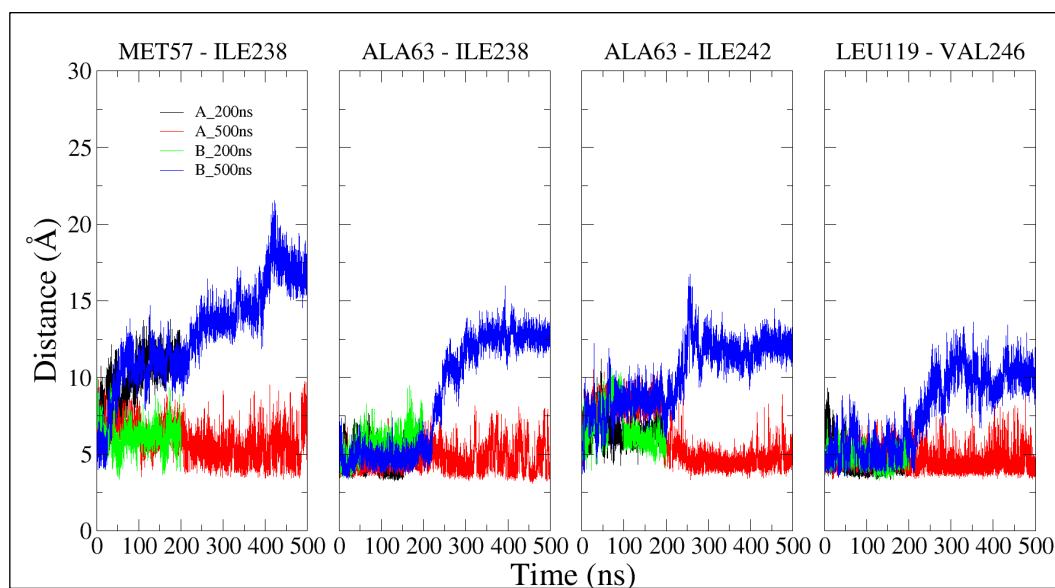


Figure 3.25: Crucial distances that stabilize inactive conformation in TM4 interface II dimer.

In the TM4.5 tight AA dimer, the differentiating interatomic distances separating the active and inactive protomers observed in the above three systems were not detected.

Considering the above atom-to-atom distances observed in the TM1,2,8 AI, TM5 AA, and TM4 II dimer systems, one residue stands out. This residue is ILE238 in TM6, and it was observed to stabilize the inactive structure by establishing an interaction network at the receptor core through interactions with non-polar amino acids from TM1, TM2, and TM3 in protomers where inactive structures were observed. These interactions were not conserved in the active structures, and when atom-to-atom distances were compared, higher values were observed in the active structures (Figure 3.23, Figure 3.24, and Figure 3.25). Mutation of ILE238^{6.33} was shown experimentally to prevent the production of inositol phosphate. Given this information, it can be suggested that ILE238^{6.33} is central to maintaining the inactive structure.

3.6. Phenylalanine Ratchet Rearrangement (FFF Motif) (PHE208^{5.51}, PHE249^{6.44}, and PHE250^{6.45})

For class A GPCRs, the PIF motif plays an important role in receptor activation. This motif consists of PRO^{5.50} from TM5, ILE^{3.40} from TM3 and PHE^{6.44} from TM6. These three amino acids are also referred to as the "core triad" and when the active and inactive structures of μ OR, β 2AR and M2Rs were compared, it was observed that the side chains of the amino acids in this motif were rearranged [206]. However, in the AT1R structure, instead of this motif, the FFF motif consisting of PHE^{5.51} from TM5 and PHE^{6.44} and PHE^{6.45} from TM6 plays an important role in activation, which was revealed by elucidating the active crystal structure of AT1R [183]. The conformation of the FFF motif in the inactive and active structures of AT1R is quite different. The importance of PHE^{5.51} [207, 208] and PHE^{6.44} [136, 209, 210] positions in this motif, called phenylalanine ratchet, has been reported in the literature. The stacking of the FFF motif in the active AT1R crystal compared to the cluster representative structures is shown in Figure 3.26.

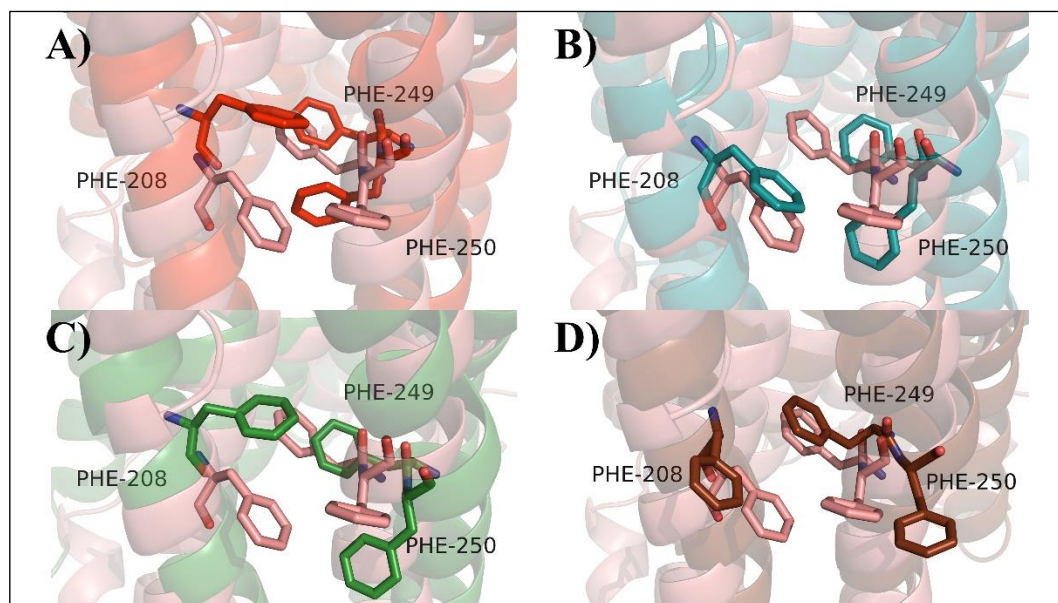


Figure 3.26: Phenylalanine ratchet in TM1,2,8 AI A), in TM5 AA B), TM4 II C), and TM4,5 tight AA D).

When we compared the FFF motif in the crystal structure with the cluster representative structures, the rearrangements in the FFF motif of the B chain of the TM5 AA dimer (Figure 3.26, panel B) and the A chain of the TM4,5 tight AA dimer (Figure 3.26, panel D) were similar, whereas the FFF motif in the TM1,2,8 AI A chain (Figure 3.26, panel A) and the TM4 II B chain (Figure 3.26, panel C) showed different packing properties.

3.7. TYR215^{5.58} – TYR302^{7.53} (YY Motif) Distance Analysis

Another motif that differs between the active and inactive structures of GPCRs is the YY motif, which consists of tyrosine residues from TM5 and TM7 [1, 208]. This motif consists of TYR^{5.58} in TM5 and TYR^{7.53} in TM7. These two tyrosine amino acids interact either directly or through a water molecule, forming a bond between TM5 and TM7 and allowing TM6 to move outward. When the crystal structures of the M2R [211], β 2AR [212] and rhodopsin [213] receptors are analyzed, it is clear that hydrogen bonding can occur through a water molecule. When the distance between TYR215^{5.58} and TYR302^{7.53} is measured in the active AT1R structure, a distance of 4.2 Å is observed [183]. Therefore, the formation of hydrogen bond network through the water molecule observed in M2R, β 2AR, and rhodopsin can also be expected for AT1R.

Figure 3.27 shows a comparison of the YY motif in cluster representative structures and the active AT1R crystal.

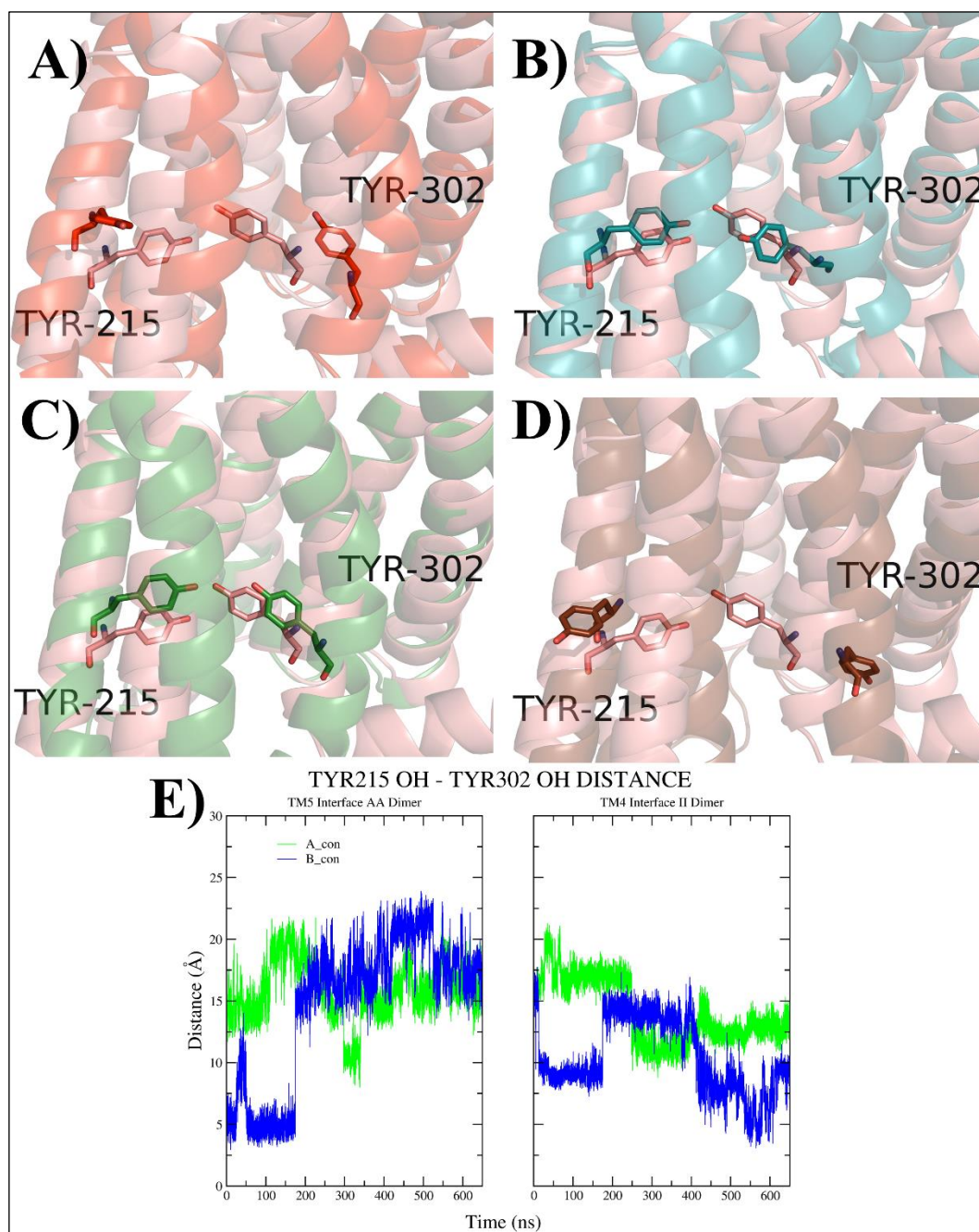


Figure 3.27: Tyrosine switch in TM1,2,8 AI A), TM5 AA B), TM4 II C), and TM4,5 tight D). Panel E shows the change of the distance in TM5 AA (left panel), and in TM4 II (right panel).

As can be seen in Figure 3.27, the A chains of the TM1, 2, 8 AI dimer (Figure 3.27, panel A) and the A chain of the TM4, 5 tight AA dimer (Figure 3.27, panel D) are far away from each other. There is also no interaction through the water molecule.

However, the YY motif is close enough to make a bond in the B chain of the TM5 AA dimer (Figure 3.27, panel B) and in the B chain of the TM4 II dimer (Figure 3.27, panel C). In Panel E, the B chain of the TM5 AA dimer is observed to be in the distance that allows contact in YY motif during the first 200 ns of simulation. When the distance between these two tyrosines was measured in the cluster representative structure, 4.2 Å was obtained. Similarly, in the B chain of the TM4 II dimer, the tyrosines moved closer to each other after 400 ns. The YY distance in the cluster representative of this structure was measured as 4.6 Å. In short, the YY motif in the B chains of TM5 AA and TM4 II dimers is at a distance that can bridge over a water molecule, which can be considered as an indication of the active structure [1]. The two distant tyrosine states observed in the A chains of TM1, 2, 8 AI and TM4, 5 tight AA dimers are similar to those observed in the active AT2R crystal structures [190, 214].

3.8. VAL108^{3.32} – TYR292^{7.43} and ASN111^{3.35} – ASN295^{7.46}

Distance Analysis

In their recent study, Singh et al. investigated the mechanism of AT1R activation by ANGII binding [198]. This study shows that the distance between TM3 and TM7 was very important. According to this study, the activation mechanism starts by breaking VAL108^{3.32}-TYR292^{7.43} and then ASN111^{3.35}-ASN295^{7.46} bonds. The TM3-TM7 interactions claimed to be important by Singh et al. were tracked throughout the simulation in the dimer systems used in this study. This was done by monitoring the changes in the VAL108^{3.32}-TYR292^{7.43} and ASN111^{3.35}-ASN295^{7.46} distances throughout the simulation.

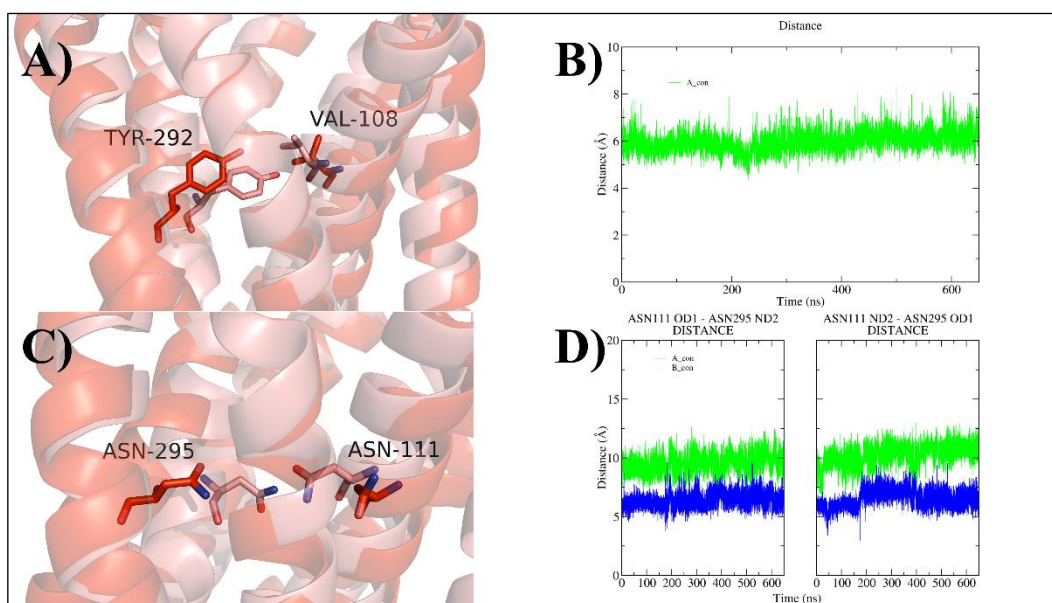


Figure 3.28: Comparison of VAL108^{3.32}-TYR292^{7.43} distance in cluster structure of TM1,2,8 AI and active AT1R crystal A), the change in the distance throughout MD simulation B). Comparison of ASN111^{3.35}-ASN295^{7.46} distance in cluster structure of TM1,2,8 AI and active AT1R crystal C), the change in the distance throughout MD simulation D).

Figure 3.28 Panel A compares the distance between VAL108^{3.32} and TYR292^{7.43} in the TM1,2,8 AI dimer A chain cluster representative structure and in the active AT1R crystal structure. Panel B plots the change in this distance during the simulation. As can be seen in panel B, the distance between the alpha carbon of VAL108^{3.32} and the OH atom of TYR292^{7.43} remained constant at around 6 Å, which is very similar to the distance in the active AT1R. A comparison of the ASN111^{3.35}-ASN295^{7.46} interaction in the cluster representative and the active AT1R crystal is shown in Figure 3.28, panel C. The interaction between these two amino acids can occur in two different ways, ASN111^{3.35} OD1 - ASN295^{7.46} ND2 or ASN111^{3.35} ND2 - ASN295^{7.46} OD1. These two distances were measured to be 2.9 and 3.0 Å in the active AT1R structure. In the cluster representative structure, these two distances were measured to be 7.5 and 8.8 Å, respectively. In panel D, it is observed that these atom-atom distances remain constant at 8 and 9 Å, respectively, throughout the simulation. The observed ASN111^{3.35}-ASN295^{7.46} distance in the simulations aligns with Singh et al.'s findings but deviates from the crystal AT1R structure. This discrepancy can be attributed to the crystal structure representing one of the low-energy states, leaving room for the potential presence of other similar low-energy states. Additionally, the variation might be influenced by the differences in temperature during the GPCR crystal formation

compared to physiological conditions, as well as the use of numerous detergent molecules to create a membrane-like environment surrounding the receptor.

3.9. Protein Coupling With The Active States Of The Selected Dimer Pairs

For a GPCR structure to be fully active (capable of generating a signal), it must be able to form complexes with its coupling proteins. In order to observe whether there is enough space in the cluster representative structures for the binding of intracellular effector proteins (G-protein or beta-arrestin), comparisons were made with μ OR-G_i-protein [215] and rhodopsin- β arrestin [216] complexes.

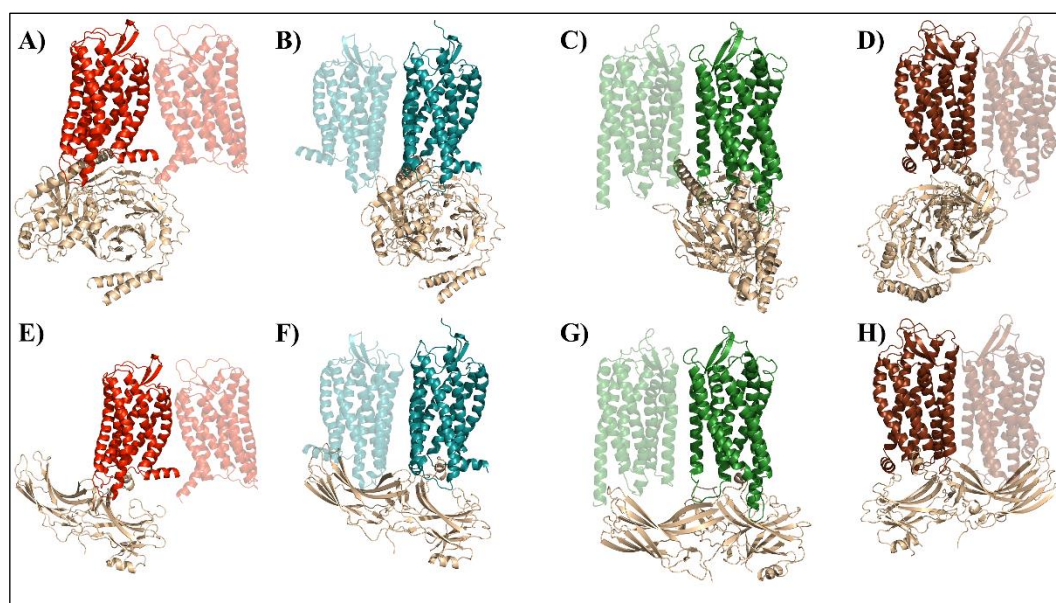


Figure 3.29: Cluster representatives with Gi protein from the recently crystallized GPCR-G_i protein complex, PDB: 6DDF **A)-D)**, and **(bottom)** with GPCR-arrestin complex, PDB: 5W0P **E)-H)**. G_i protein and arrestin colored in wheat, TM1,2,8 AI chain A, TM5 AA chain B, TM4 II chain B, and TM4,5 tight AA chain A, depicted as red, deep teal, forest, and chocolate, respectively.

For selected dimer systems, Figure 3.29 shows the complex structures of the cluster representative structures with G protein and arrestin. Gi protein was obtained from μ OR crystal (PDB ID : 6DDF) and arrestin from rhodopsin crystal (PDB ID : 5W0P). As shown in Figure 3.29, all dimer systems have enough space to bind both G protein and arrestin. Interestingly, however, in the G protein and arrestin complexes of the TM4 II dimer B chain, H8 appears to be in a different conformation. As shown in

more detail in Figure 3.30, the conformation of H8 is in the direction of the effector protein binding site. The H8s of the US28 [217] and AT2R [214] GPCRs are also similar to the H8 observed in the simulations performed in this study. This unusual conformation has led to the description of the AT2R as a "self-inhibitory receptor" [218]. Previous studies have emphasized the amphiphilic property of H8 and suggested that it could be oriented toward both the membrane and intracellular water [214, 217, 218].

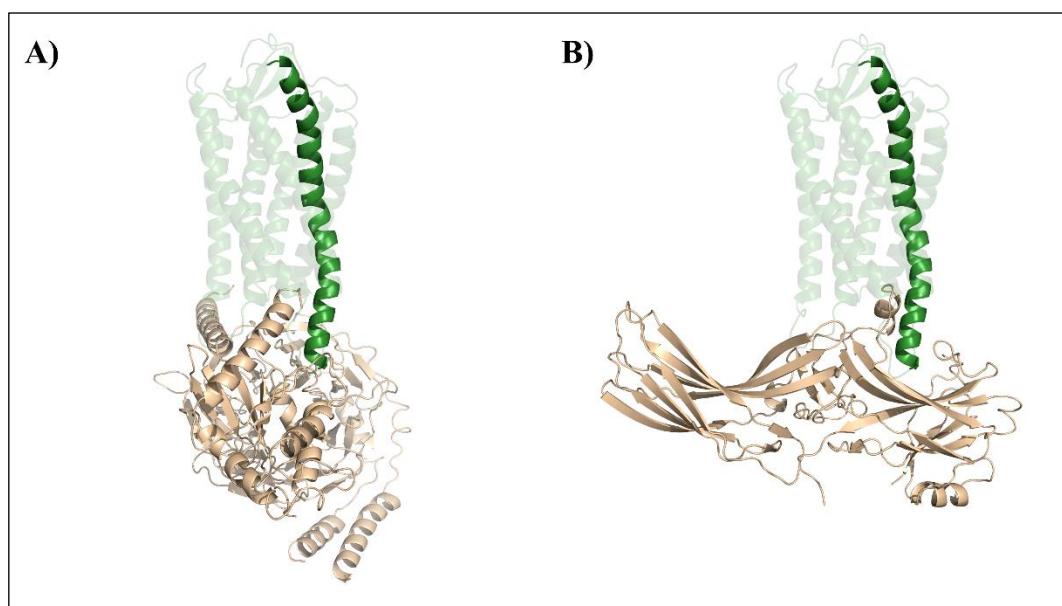


Figure 3.30: TM4 interface II dimers chain A with the Gi protein A) and arrestin B), for the sake of clarity except TM7-H8 part, the receptor depicted as 80% transparent.

Although the conformation clearly seen at H8 in Figure 3.30 is unexpected, no major steric overlap was observed in either the G protein or arrestin complex of the receptor.

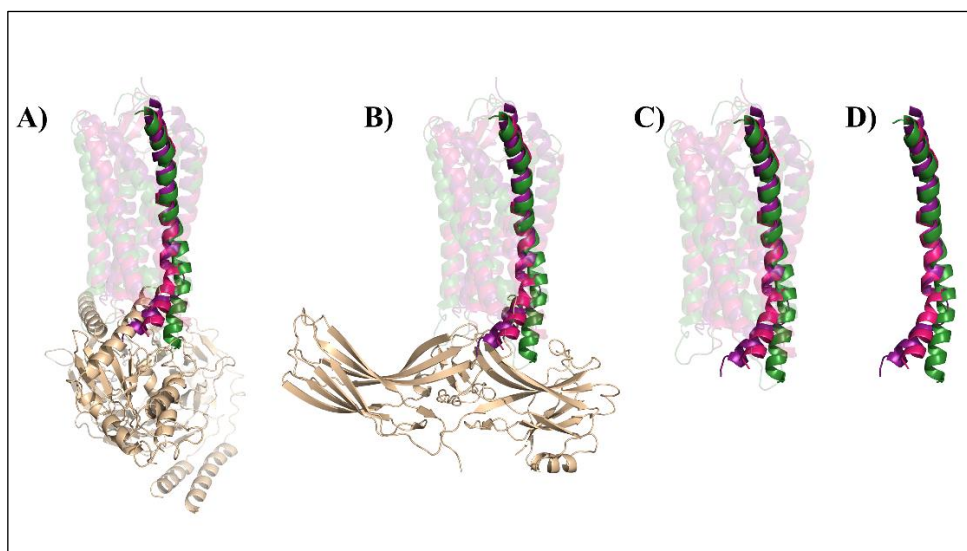


Figure 3.31: A) Gi protein – GPCR complexes (AT2R: hotpink, US28: deeppurple, and cluster structure of TM4 II chain B: forest). B) Arrestin – GPCR complexes, C) alignment of all three structures, D) TM7-H8 conformations of the three systems. For clarity, except TM7-H8 and intracellular binding partners, GPCR structures depicted as 80% transparent.

In Figure 3.31, the B chain of the TM4 II dimer, US28, and AT2R are compared. AT2R is shown in hotpink, US28 in shown deeppurple, and the B chain of the TM4 II dimer is shown in forest color. By comparing these three structures with similar H8s, it is clear that the conformation observed in US28 and AT2R is toward the inner cavity of the receptor. However, unlike the other two structures, the conformation observed in the B chain of the TM4 II dimer is slightly further away from the effector binding site.

3.10. Interface Analyses

The interactions observed at the interfaces and the conservation of these interactions significantly affect the stability of GPCR dimers. In order to evaluate the stability of interfacial interactions at the six different interfaces studied and the three dimer pairs (II, AI, and AA) used at each interface, analyses were performed using the PISA server. On the PISA server, simulation initial structures and cluster representative structures were used to perform the analysis and the results were compared. Three different colorings were used to examine the amino acids contributing to the interface. The amino acids colored in red were present at the interface in the simulation starting structure, but were not observed at the interface in

the cluster representative structure. Amino acids colored in yellow were present at the interaction interface in both the simulation starting structure and the cluster representative structure. Amino acids colored in green were used for those that were not involved in the interface in the initial structure but contributed to the interaction interface in the cluster representative structure.

Table 3.1: Changes in the interface residues in TM1,2,8 interface dimer system.

TM1,2,8						
4DKL_II		4DKL_AI		4DKL_AA		TM
A	B	A	B	A	B	
		LYS20				TM1
ASN25	ASN25		ASN25		ASN25	TM1
PHE28	PHE28	PHE28	PHE28	PHE28	PHE28	TM1
VAL29	VAL29	VAL29	VAL29	VAL29	VAL29	TM1
MET30						TM1
PRO32	PRO32	PRO32	PRO32	PRO32	PRO32	TM1
THR33	THR33	THR33	THR33	THR33	THR33	TM1
SER36	SER36	SER36	SER36	SER36	SER36	TM1
ILE37	ILE37	ILE37	ILE37	ILE37	ILE37	TM1
PHE39	PHE39	PHE39	PHE39	PHE39	PHE39	TM1
VAL40	VAL40	VAL40	VAL40	VAL40	VAL40	TM1
VAL41	VAL41	VAL41	VAL41	VAL41	VAL41	TM1
					ILE43	TM1
PHE44	PHE44	PHE44				TM1
PRO82	PRO82	PRO82	PRO82	PRO82	PRO82	TM2
					LEU83	TM2
ALA85	ALA85	ALA85	ALA85			TM2
VAL86	VAL86	VAL86	VAL86	VAL86	VAL86	TM2
ALA89	ALA89	ALA89	ALA89	ALA89	ALA89	TM2
MET90	MET90	MET90	MET90	MET90	MET90	TM2
GLU91	GLU91	GLU91	GLU91	GLU91	GLU91	ECL1
ARG93	ARG93	ARG93	ARG93	ARG93	ARG93	ECL1
PRO95	PRO95	PRO95	PRO95	PRO95		ECL1
PHE96	PHE96	PHE96	PHE96	PHE96		ECL1
			THR175			ECL2
			ILE177			ECL2
			LYS307			H8
LYS310	LYS310		LYS310			H8
PHE313	PHE313		PHE313			H8
LEU314	LEU314	LEU314	LEU314	LEU314		H8
LEU317	LEU317	LEU317	LEU317		LEU317	H8
LYS318	LYS318	LYS318				H8
TYR319	TYR319	TYR319				H8

In particular, amino acids from ECL 1 and ECL 2 (these amino acids were not present at the interface in the simulation starting structure) were contributed to the interaction interface in the cluster representative structure, and the number of amino acids contributing to the interface from H8 increased (Table 3.1).

Table 3.2: Changes in the interface residues in TM4,5 loose interface dimer system.

TM4,5 LOOSE						
4GPO_II		4GPO_AI		4GPO_AA		TM
A	B	A	B	A	B	
LYS102						TM3
TYR113	TYR113					TM3
			THR120			TM3
CYS121	CYS121					TM3
	ILE124	ILE124	ILE124			TM3
TYR127	TYR127		TYR127			TM3
LEU128	LEU128	LEU128	LEU128			TM3
VAL131	VAL131	VAL131				ICL2
HIS132	HIS132	HIS132	HIS132	HIS132	HIS132	ICL2
PRO133	PRO133		PRO133			ICL2
MET134	MET134	MET134	MET134	MET134	MET134	ICL2
LYS135	LYS135	LYS135	LYS135	LYS135	LYS135	ICL2
	SER136		SER136			ICL2
ARG137	ARG137	ARG137	ARG137	ARG137	ARG137	ICL2
	LEU138	LEU138		LEU138	LEU138	TM4
		ARG139		ARG139	ARG139	TM4
ARG140	ARG140	ARG140	ARG140			TM4
	THR141	THR141	THR141			TM4
		LEU143		LEU143	LEU143	TM4
VAL144	VAL144	VAL144	VAL144	VAL144	VAL144	TM4
		VAL147				TM4
	THR148					TM4
ILE151	ILE151	ILE151				TM4
ILE152	ILE152					TM4
		LEU154				TM4
LEU155	LEU155	LEU155	LEU155			TM4
LEU158	LEU158	LEU158			LEU158	TM4
ALA159	ALA159					TM4
LEU161	LEU161	LEU161			LEU161	TM4
PRO162	PRO162				PRO162	TM4
ILE165	ILE165				ILE165	TM4
HIS166	HIS166				HIS166	ECL2

Table 3.2: Continued.

TM4,5 LOOSE						
4GPO_II		4GPO_AI		4GPO_AA		TM
A	B	A	B	A	B	
PHE182	PHE182					ECL2
HIS183						ECL2
TYR184			TYR184			ECL2
GLU185	GLU185					ECL2
SER186	SER186					ECL2
GLN187	GLN187		GLN187			ECL2
	ASN188	ASN188	ASN188			ECL2
		SER189				ECL2
THR190	THR190					ECL2
LEU191	LEU191	LEU191	LEU191	LEU191		ECL2
PRO192	PRO192					ECL2
	ILE193					TM5
GLY194	GLY194					TM5
LEU195	LEU195		LEU195	LEU195		TM5
LEU197	LEU197					TM5
THR198	THR198		THR198	THR198		TM5
LYS199	LYS199					TM5
ILE201	ILE201		ILE201			TM5
LEU202	LEU202		LEU202	LEU202		TM5
GLY203	GLY203					TM5
	LEU205					TM5
PHE206	PHE206		PHE206	PHE206		TM5
PRO207						TM5
LEU209	LEU209					TM5
	ILE210					TM5
	THR213					TM5
LEU217	LEU217					TM5
	LYS220					TM5
		LYS224				ICL3
TYR226						ICL3
GLU227						ICL3
ILE228						ICL3

At the TM4,5 loose interface, TM3 contributed to the interface in the cluster representative structure II and AI dimer pairs. However, this was not observed in the AA dimer pair. In the II dimer system, unlike the other pairs, ICL3 was also observed to contribute to interactions at the dimerization interface. Table 3.2 shows the residues that are contributing to interface.

Table 3.3: Changes in the interface residues in TM5 interface dimer system.

TM5						
4RWS_II		4RWS_AI		4RWS_AA		TM
A	B	A	B	A	B	
		ILE124				TM3
TYR127	TYR127	TYR127	TYR127	TYR127	TYR127	TM3
LEU128	LEU128	LEU128	LEU128	LEU128	LEU128	TM3
VAL131	VAL131	VAL131	VAL131	VAL131	VAL131	ICL2
HIS132	HIS132	HIS132	HIS132	HIS132	HIS132	ICL2
	PRO133		PRO133	PRO133	PRO133	ICL2
MET134	MET134	MET134	MET134	MET134	MET134	ICL2
		LYS135	LYS135	LYS135	LYS135	ICL2
		SER136				ICL2
		LEU138		LEU138	LEU138	TM4
		ARG139		ARG139	RG139	TM4
		ARG140	ARG140	ARG140	ARG140	TM4
			VAL144	VAL144	VAL144	TM4
GLN187	GLN187		GLN187			ECL2
ASN188	ASN188		ASN188			ECL2
SER189	SER189		SER189			ECL2
THR190	THR190	THR190	THR190	THR190	THR190	ECL2
LEU191	LEU191	LEU191	LEU191		LEU191	ECL2
ILE193	ILE193	ILE193	ILE193			TM5
GLY194	GLY194	GLY194	GLY194			TM5
LEU195			LEU195			TM5
LEU197	LEU197	LEU197	LEU197	LEU197		TM5
THR198	THR198	THR198	THR198	THR198	THR198	TM5
ILE201	ILE201	ILE201	ILE201	ILE201	ILE201	TM5
LEU202	LEU202	LEU202	LEU202	LEU202	LEU202	TM5
	LEU205	LEU205	LEU205			TM5
PHE206	PHE206	PHE206	PHE206	PHE206	PHE206	TM5
LEU209	LEU209	LEU209	LEU209			TM5
ILE210	ILE210	ILE210	ILE210		ILE210	TM5
THR213		THR213	THR213			TM5
	LEU217	LEU217	LEU217	LEU217	LEU217	TM5
	LYS220	LYS220		LYS220	LYS220	TM5
	ALA221			ALA221		TM5
LYS224		LYS224		LYS224	LYS224	ICL3
		ALA225	ALA225			ICL3
		TYR226	TYR226			ICL3
			GLU227			ICL3
			ILE228			ICL3
		GLN267				TM6
LEU268	LEU268	LEU268		LEU268	LEU268	TM6
GLY269	GLY269	GLY269				ECL3
ILE270	ILE270					ECL3

In all dimer pairs with the TM5 interface, the TM5 interactions were conserved, although the amino acids contributing from TM5 to the interaction interface varied in the simulation starting structure and in the cluster representative structure. Again at this dimer interface, TM3 was observed to contribute to the interface in the cluster representative structures. The contribution of TM6 to the interface was only observed in the AI dimer pair, while in the other two systems (II, and AA) this interaction was lost in the cluster representative structure (Table 3.3).

Table 3.4: Changes in the interface residues in TM4 interface dimer system.

TM4						
5LWE_II		5LWE_AI		5LWE_AA		TM
A	B	A	B	A	B	
ASN98						TM3
TYR99	TYR99	TYR99				TM3
LYS102	LYS102	LYS102			LYS102	TM3
				PHE110	PHE110	TM3
LYS135	LYS135					ICL2
ARG137	ARG137	ARG137				ICL2
LEU138	LEU138	LEU138		LEU138		TM4
ARG139	ARG139	ARG139	ARG139			TM4
ARG140	ARG140		ARG140			TM4
		THR141		THR141		TM4
	MET142	MET142				TM4
LEU143	LEU143	LEU143	LEU143	LEU143	LEU143	TM4
VAL144	VAL144	VAL144	VAL144	VAL144	VAL144	TM4
		LYS146		LYS146	LYS146	TM4
VAL147	VAL147	VAL147	VAL147	VAL147	VAL147	TM4
THR148	THR148	THR148	THR148	THR148	THR148	TM4
ILE150	ILE150	ILE150	ILE150	ILE150	ILE150	TM4
ILE151	ILE151	ILE151	ILE151	ILE151	ILE151	TM4
				TRP153	TRP153	TM4
LEU154	LEU154	LEU154	LEU154	LEU154	LEU154	TM4
LEU155	LEU155	LEU155	LEU155	LEU155	LEU155	TM4
GLY157	GLY157	GLY157	GLY157	GLY157	GLY157	TM4
LEU158	LEU158	LEU158	LEU158	LEU158	LEU158	TM4
					SER160	TM4
LEU161	LEU161	LEU161	LEU161	LEU161	LEU161	TM4
PRO162	PRO162	PRO162		PRO162	PRO162	TM4
ILE164	ILE164	ILE164	ILE164	ILE164	ILE164	TM4
ILE165	ILE165	ILE165	ILE165	ILE165	ILE165	TM4
HIS166				HIS166	HIS166	ECL2
ARG167						ECL2
ASN168	ASN168					ECL2
GLU185	GLU185		GLU185			ECL2
	SER186					ECL2
	ASN188					ECL2
LEU191	LEU191		LEU191	LEU191		ECL2
LEU195						TM5

In AA, one of the dimer pairs in which the dimerization interface is TM4, TM3 interactions are present in the simulation starting structure, while these interactions are eliminated in the cluster representative structure. The contribution of ICL2 to interface formation observed in II and AI pairs was lost in the cluster representative structure in both systems. Table 3.4 shows the amino acids contributing to the interface for each dimer pair and their position in the structure.

Table 3.5: Changes in the interface residues in TM4,5 tight interface dimer system.

TM4,5 TIGHT						
5O9H_II		5O9H_AI		5O9H_AA		TM
A	B	A	B	A	B	
ASN98	ASN98	ASN98				ECL1
LYS102	LYS102	LYS102				TM3
TYR113			TYR113	TYR113		TM3
PHE117	PHE117	PHE117	PHE117	PHE117	PHE117	TM3
THR120		THR120	THR120	THR120		TM3
CYS121		CYS121		CYS121		TM3
ILE124	ILE124	ILE124	ILE124	ILE124		TM3
		ASP125				TM3
TYR127	TYR127	TYR127	TYR127	TYR127		TM3
LEU128	LEU128	LEU128	LEU128	LEU128		TM3
VAL131	VAL131	VAL131				ICL2
HIS132	HIS132	HIS132		HIS132		ICL2
PRO133	PRO133					ICL2
MET134	MET134		MET134	MET134		ICL2
LYS135	LYS135	LYS135		LYS135	LYS135	ICL2
	SER136		SER136			ICL2
ARG137			ARG137	ARG137		ICL2
		LEU138		LEU138	LEU138	TM4
		ARG139		ARG139	ARG139	TM4
ARG140	ARG140	ARG140	ARG140	ARG140	ARG140	TM4
		THR141	THR141	THR141	THR141	TM4
LEU143	LEU143	LEU143	LEU143		LEU143	TM4
VAL144	VAL144	VAL144	VAL144	VAL144	VAL144	TM4
VAL147	VAL147	VAL147	VAL147		VAL147	TM4
		THR148	THR148	THR148	THR148	TM4
ILE150	ILE150	ILE150	ILE150		ILE150	TM4
ILE151	ILE151	ILE151	ILE151	ILE151	ILE151	TM4
		ILE152	ILE152	ILE152	ILE152	TM4

Table 3.5: Continued.

TM4,5 TIGHT						
5O9H_II		5O9H_AI		5O9H_AA		TM
A	B	A	B	A	B	
	TRP153					TM4
LEU154	LEU154	LEU154	LEU154	LEU154	LEU154	TM4
LEU155	LEU155	LEU155	LEU155	LEU155	LEU155	TM4
GLY157	GLY157	GLY157	GLY157			TM4
LEU158	LEU158	LEU158	LEU158	LEU158	LEU158	TM4
ALA159	ALA159	ALA159	ALA159	ALA159	ALA159	TM4
LEU161	LEU161	LEU161	LEU161	LEU161	LEU161	TM4
PRO162	PRO162	PRO162	PRO162	PRO162	PRO162	TM4
ILE164	ILE164	ILE164			ILE164	TM4
ILE165	ILE165	ILE165	ILE165	ILE165	ILE165	TM4
HIS166	HIS166	HIS166	HIS166	HIS166	HIS166	ECL2
ASN168	ASN168	ASN168	ASN168		ASN168	ECL2
VAL169	VAL169					ECL2
PHE170	PHE170					ECL2
	PHE171					ECL2
	PHE182					ECL2
HIS183	HIS183	HIS183	HIS183	HIS183	HIS183	ECL2
TYR184		TYR184	TYR184	TYR184	TYR184	ECL2
GLU185	GLU185		GLU185		GLU185	ECL2
SER186	SER186		SER186		SER186	ECL2
GLN187	GLN187		GLN187	GLN187		ECL2
	ASN188			ASN188		ECL2
THR190	THR190		THR190	THR190	THR190	ECL2
LEU191	LEU191	LEU191	LEU191	LEU191	LEU191	ECL2
	PRO192					TM5
GLY194	GLY194	GLY194	GLY194	GLY194	GLY194	TM5
LEU195	LEU195	LEU195	LEU195	LEU195	LEU195	TM5
		LEU197				TM5
THR198	THR198	THR198	THR198	THR198	THR198	TM5
LYS199	LYS199		LYS199			TM5
ILE201	ILE201	ILE201	ILE201	ILE201	ILE201	TM5
LEU202	LEU202	LEU202	LEU202	LEU202	LEU202	TM5
GLY203			GLY203			TM5
PHE206	PHE206	PHE206	PHE206	PHE206	PHE206	TM5
PRO207		PRO207	PRO207			TM5
ILE210	ILE210	ILE210	ILE210			TM5

In all dimer pairs with TM4,5 tight dimer interfaces, the TM3 contribution to the cluster representative structure became more pronounced compared to the simulation starting structure. While TM4 and TM5 interactions were largely conserved, new amino acids from these two TMs were also observed to contribute to the dimerization interface. As shown in Table 3.5, in addition to TMs, ICL2 and ECL2 were also found to contribute to the interface in all dimer pairs.

Table 3.6: Changes in the interface residues in TM6,7 interface dimer system.

TM6,7				
5WIU_II		5WIU_AI		TM
A	B	A	B	
	ILE193		ILE193	TM5
	LEU205			TM5
	LEU209			TM5
TYR215				TM5
TRP219	TRP219	TRP219	TRP219	TM5
			LYS220	TM5
			ALA221	TM5
LEU222	LEU222			TM5
LYS223	LYS223		LYS223	TM5
LYS224	LYS224		LYS224	ICL3
ALA225	ALA225		ALA225	ICL3
TYR226	TYR226	TYR226	TYR226	ICL3
GLU227	GLU227	GLU227	GLU227	ICL3
ILE228	ILE228	ILE228	ILE228	ICL3
		GLN229		ICL3
		LYS232		ICL3
PRO233		PRO233		ICL3
ARG234				ICL3
ASP236		ASP236		TM6
		PHE239		TM6
LYS240	LYS240	LYS240		TM6
PHE251	PHE251			TM6
ILE254	ILE254			TM6
ILE258	ILE258		ILE258	TM6
PHE259	PHE259			TM6
PHE261	PHE261		PHE261	TM6
LEU262	LEU262		LEU262	TM6
LEU265	LEU265	LEU265	LEU265	TM6
LEU268	LEU268	LEU268	LEU268	TM6
GLY269	GLY269	GLY269	GLY269	ECL3
ILE270	ILE270	ILE270	ILE270	ECL3
ILE271	ILE271	ILE271	ILE271	ECL3
ARG272	ARG272	ARG272	ARG272	ECL3
ASP273	ASP273	ASP273	ASP273	ECL3
			CYS274	TM7
ARG275	ARG275	ARG275	ARG275	TM7
ILE276	ILE276	ILE276	ILE276	TM7
ILE279	ILE279	ILE279	ILE279	TM7

Table 3.6 shows the evolution of interfacial interactions at the TM6,7 interface in II and AI dimer pairs. While the contribution of TM6 and TM7 is preserved in both dimer pairs, especially in the AI dimer, the ICL3 interactions are completely lost in the cluster representative structure.

3.11. Center of Mass (COM) Distance Between Protomer Analysis

In all simulation systems, the inter-protomer distances were measured to investigate whether the chains in different dimer pairs moved closer or further apart during the simulation. This was done by determining the centers of mass of the two protomers and then plotting the change in the distance between the centers of mass. Figure 3.32 shows the change between the centers of mass for each interface and the dimer pairs (II, AI, and AA) with that interface.

around the distance observed in the simulation starting structure (Figure 3.32, panel I). The result of the interprotomer distances of TM_{4,5} loose interface is given in Figure AP, panel II. A large decrease in interprotomer distance was observed in II of the TM_{4,5} loose dimer pairs. The protomers were found to be moving closer together. In the AI protomer, the interprotomer distance in the first 250 ns of the simulations showed lower values compared to the initial simulation structure, while in the last 250 ns it showed higher values compared to the initial simulation structure. Although the interprotomer distances of the repeat simulations in the AA protomer were not similar, the 500 ns simulation showed the same distance values in the last 100 ns as the 200 ns simulation. In the II pair of the TM₅ interface dimer, the interprotomer distance was observed to be higher than the simulated initial structure. In the AI pair, lower values than the simulated starting structure were observed. In the AA protomer, the interprotomer distance remained largely unchanged and the distance observed in the simulation starting structure was maintained throughout the simulation (Figure 3.32, panel III). Considering the TM₄-interfaced dimers, the protomers in all dimer pairs (II, AI, and AA) moved away from each other during the simulation. As shown in Figure 3.32, panel IV, higher values of interprotomer distances were observed throughout the simulations compared to the initial simulation structure. In the II and AI systems of TM_{4,5} tight interface dimer pairs, a decrease in interprotomer distance was observed during the simulation, which can be interpreted as the chains moving closer together. However, this was not observed for the AA pair. In the AA pair, the distance between the protomers remained constant and did not differ from the distance observed in the initial structure of the simulation (Figure 3.32, panel V). Figure 3.32, panel VI shows the changes in interprotomer distance in II and AI dimer pairs with the TM_{6,7} interaction interface. Interestingly, in the 200 ns replicates of these dimer pairs, the inter-protomer distance was observed to decrease (II dimer pair) or remain constant (AI dimer pair) compared to the initial structure, whereas in the 500 ns simulation, the inter-protomer distance increased throughout the simulation. Overall, when the interprotomer distance was considered, different behaviors were observed in dimer systems where one protomer is active and the other protomer shows inactive properties. For example, in the AI dimer with TM_{1,2,8} interface, while the inter-protomer distance showed an increasing behavior until the middle of the simulation, values close to the initial values were observed especially in the last 200 ns of the simulation. In the AA dimer with TM₅ interface, the distance between the protomers was observed to be the

same as the initial values. In the TM4 II dimer, higher values than the initial distance were observed, indicating that the protomers are moving away from each other to some extent. In the AA dimer with TM4.5 tight interface, the distance between the protomers was observed to remain unchanged from the initial values.

3.12. Principal Component Analysis (PCA)

In molecular simulations, extracting functionally important motions from local and possibly unimportant motions is not an easy task. In order to extract functional motions from the simulations performed, Principal Component Analysis (PCA) was performed. When the local fast motions are eliminated from the global collective motions, it is seen that the meaningful motion of the protein is in the direction of a few collective coordinates. For this purpose, the covariance matrix of the atomic fluctuations was constructed taking into account the protein backbone atoms. This covariance matrix was diagonalized to obtain eigenvalues and eigenvectors [219, 220]. As shown in Figure 3.33, according to the PCA, a large motion was observed in H8 of the A chain of the AI dimer with the TM1,2,8 interface. While this movement of H8 in chain A was in the direction of TM1 and TM2 in the active protomer, it also caused a series of interrelated movements in chain B, which has inactive properties. According to this motion sequence, chain B H8 moved toward the effector protein binding site, and this movement caused TM7 to move toward TM1. Other significant movements in the inactive chain were observed in ECL2 and ECL3 in the extracellular portion and ICL3 in the intracellular portion.

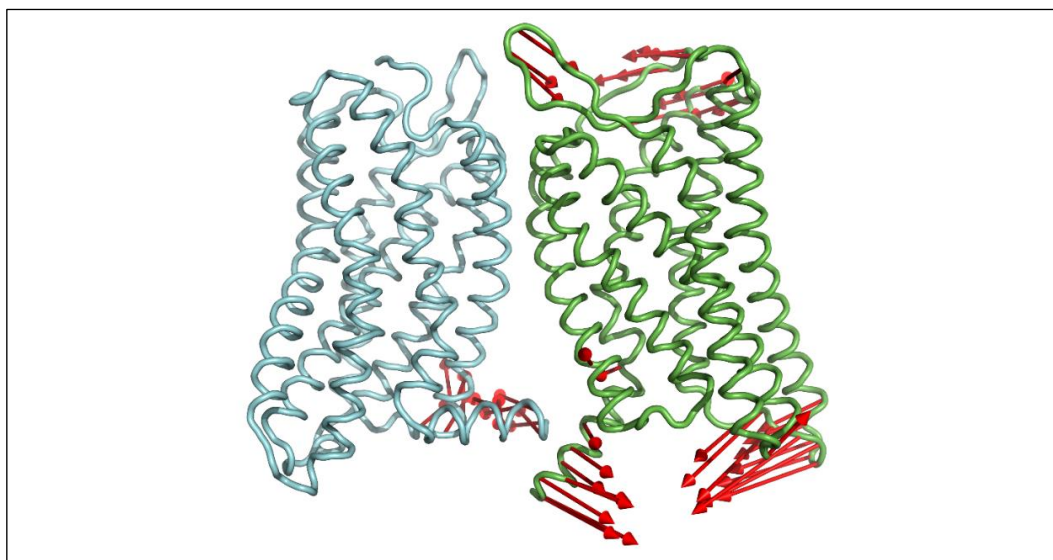


Figure 3.33: Principal component analysis fitted to initial structure from the trajectory of the MD simulations in TM1,2,8 interface AI dimer. Cyan color depicts, active protomer, and green color depicts inactive protomer.

In chain A of the TM5 interface AA dimer, the most pronounced movement was observed in the ICL2 region, while chain B was found to be more mobile. In chain B, the intracellular part of TM6, H8 and ICL3 have significant movements. In the same chain, the movement of ECL2 towards the N-terminal part caused the movement of the N-terminal part towards the extracellular side of TM7. These directions of movement are shown in Figure 3.34.

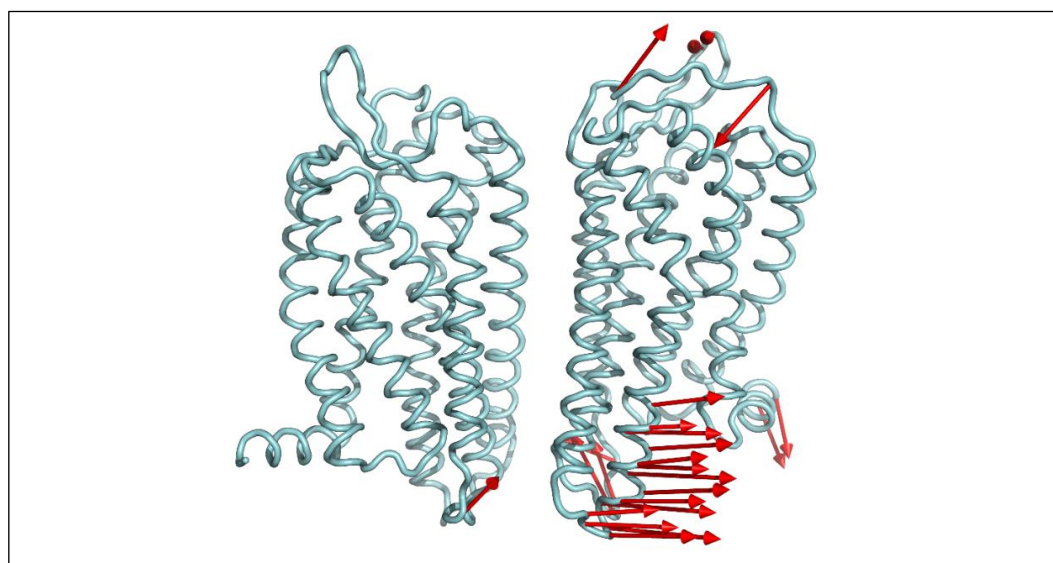


Figure 3.34: Principal component analysis fitted to initial structure from the trajectory of the MD simulations in TM5 interface AA dimer.

In the TM4 interface II dimer, movement of the N-terminal end of chain A toward ECL2 caused ECL2 to move toward the dimerization interface. The other mobile sites were TM5, ICL3, TM7 and H8. In the B chain, where activation was observed, the outward movement of TM6 and ICL3 helped to capture the movement required for activation. At the same time, the movement of H8 towards the effector protein binding site was also clearly observed. No directional movement was observed at TM4, which forms the interface. Figure 3.35 shows the important movements in TM4 interface II dimer.

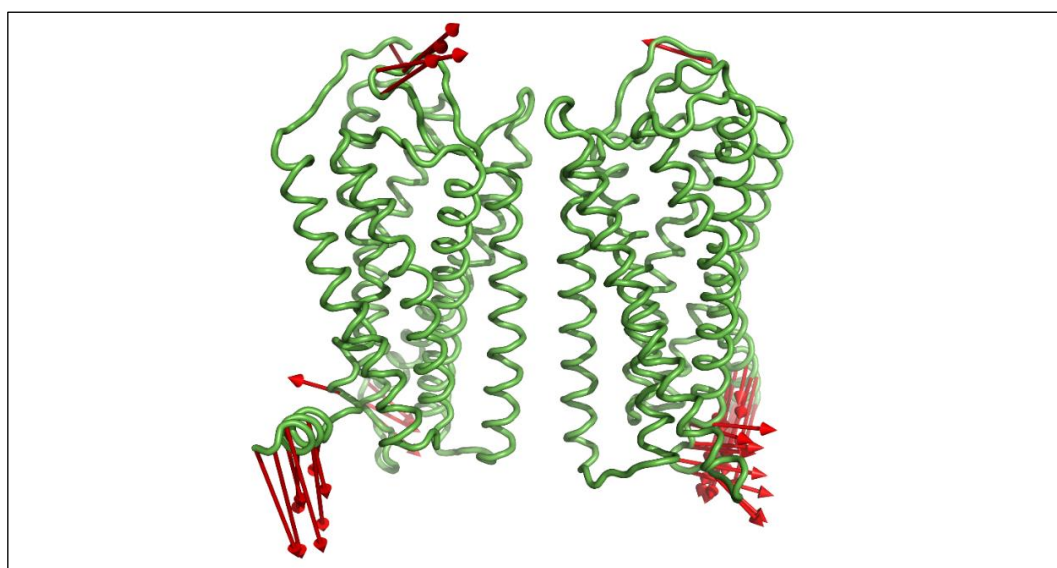


Figure 3.35: Principal component analysis fitted to initial structure from the trajectory of the MD simulations in TM4 interface II dimer.

Compared to the dimer pairs with three different interfaces mentioned above, the TM4,5 tight AA dimer pair was the system with the least observed movement (see Figure 3.36). In the A chain of this pair, movement of the N-terminus towards ECL2 was observed, while the small movement observed at ICL3 helped TM6 to transition to a slightly more open form. The only movement observed in the B chain was the movement of ECL1 towards ECL2.

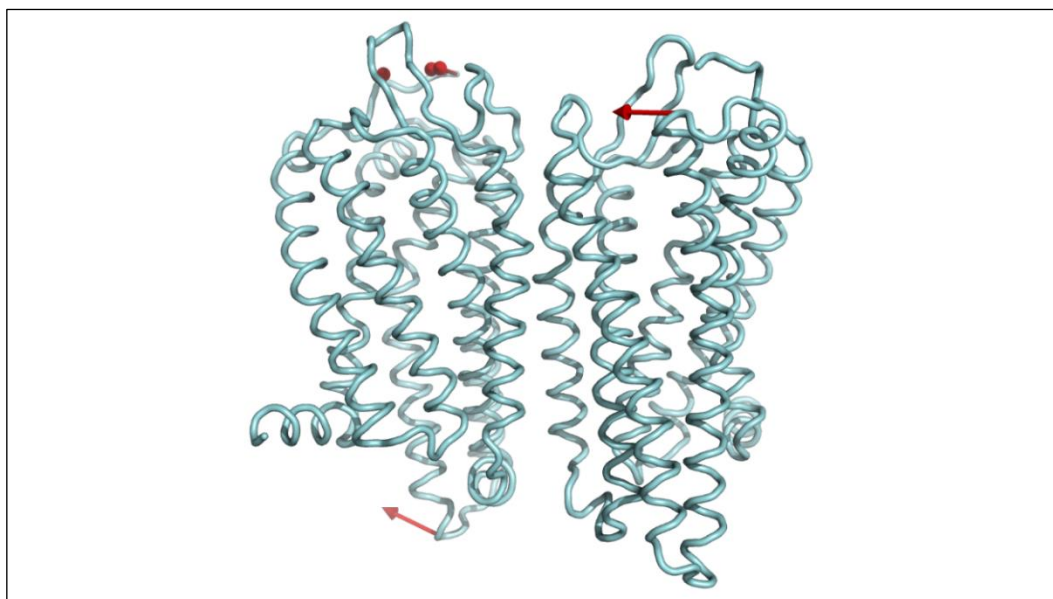


Figure 3.36: Principal component analysis fitted to initial structure from the trajectory of the MD simulations in TM4,5 tight interface AA dimer.

The PCA results are consistent with the other analyses presented above. For example, in the TM4 II dimer, where activation was observed as a result of TM3-TM6 analyses, PCA clearly showed movement of ICL3 in the TM6 direction. Therefore, it can be interpreted that the PCA results support the results of the other analyses.

3.13. Water Pathway Analysis

For GPCRs, whether the receptor is active or inactive can be understood by the formation of water channels in its inner cavity. However, there are conflicting results in the literature. For example, Bai et al. observed that water channels were formed in the inner cavity of the receptor in the inactive state of the inverse agonist-bound β 2AR [221]. In contrast, studies have been reported in which water channels were observed in the inner cavity of the receptor only in the active state [222–224].

All dimer pairs used in the study were analyzed to check for the formation of water channels during the simulation. In this context, simulation cluster representative structures were used. In chain A of the TM1,2,8 AI dimer, water molecules were observed to cluster on the side facing the intracellular side (Figure 3.37, panel A). In chain B, the denser water molecules are located on the extracellular side (Figure 3.37, panel B). As clearly observed in Figure 3.37, panel A, PHE77^{2.53}, VAL108^{3.32}, TRP253^{6.48}, and HIS256^{6.51}, located just below the orthosteric binding site, prevented

the formation of a continuous water channel. In the inactive protomer shown in panel B, VAL49^{1.53}, LEU67^{2.43}, LEU119^{3.43}, ILE241^{6.36}, ILE245^{6.40}, ASN298^{7.49}, and PHE301^{7.52} formed a cluster on the intracellular side, preventing water molecules from reaching the intrareceptor cavity. Also in the B chain, HIS256^{6.51}, THR260^{6.55}, MET284^{7.35}, and ILE288^{7.39} attenuated water channel formation through the interaction network they formed in the orthosteric region.

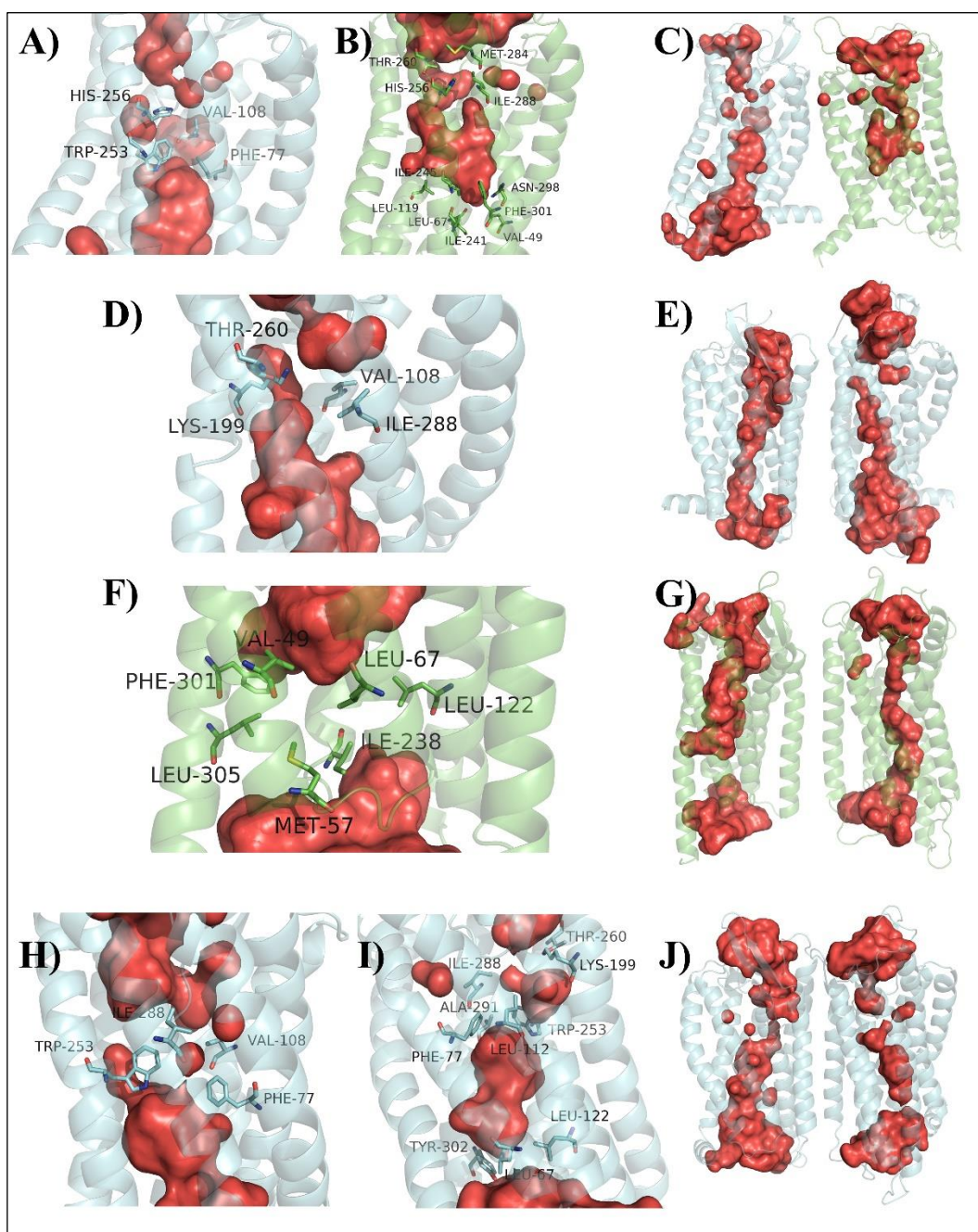


Figure 3.37: Water pathway formation in A) TM1,2,8 AI dimer chain A, B) chain B, D) TM5 AA dimer chain B, F) TM4 II dimer chain B, H) TM4,5 tight AA dimer chain A, I) chain B. Side view of selected dimer interfaces; C) TM1,2,8 AI, E) TM5 AA, G) TM4 II, and J) TM4,5 tight AA.

While continuous water channel formation was observed in the A chain of the TM5 AA dimer (Figure 3.37, panel E), the amino acids VAL108^{3,32}, LYS199^{5,42}, THR260^{6,55}, and ILE288^{7,39} were observed to prevent continuous water channel formation by clustering in the orthosteric binding site of the B chain (Figure 3.37,

panel D). In the TM4 II dimer, continuous water channel formation was observed in the B chain where active protomer properties were observed (Figure 3.37, panel G). In the A chain, where inactive protomer properties were observed, it was found that the formation of a continuous water channel was blocked by the interaction network established by the amino acids VAL49^{1.53}, MET57^{ICL1}, LEU67^{2.43}, LEU122^{3.46}, ILE238^{6.33}, PHE301^{7.52}, and LEU305^{7.56} located in the intracellular part (Figure 3.37, panel F). In the TM4.5 tight AA dimer, the formation of a continuous water channel in the A chain, where active receptor properties are observed, is attenuated by the interaction network formed by amino acids PHE77^{2.53}, VAL108^{3.32}, TRP253^{6.48}, and ILE288^{7.39} located just below the orthosteric binding site. Figure 3.37 panel H shows the amino acids that inhibit continuous channel formation. In chain B, where inactive receptor properties are observed, continuous channel formation is interrupted at two different sites. These interruptions are shown in Figure 3.37 panel I. The first interruption is due to the interaction network between PHE77^{2.53}, LEU112^{3.36}, LYS199^{5.42}, TRP253^{6.48}, THR260^{6.55}, ILE288^{7.39}, and ALA291^{7.42} around the orthosteric region, while the second interruption is due to the interaction network formed by amino acids LEU67^{2.43}, LEU122^{3.46}, and TYR302^{7.53} near the G protein coupling site. Figure 3.37 panel J shows the water channel formation in the TM4.5 tight AA dimer. The results of the water channel analyses performed on protomers of the four selected systems showing active receptor properties are interesting. Among the protomers exhibiting active receptor properties, continuous water channel formation was observed only in the B-chain of the TM4 II dimer (Figure 3.37, panel G). However, on closer inspection, the protomers of other interface dimer pairs (Figure 3.37; TM1,2,8 AI - panel C, TM5 AA - panel E, TM4,5 tight AA - panel J), which show active receptor properties in other analyses (e.g. TM3-TM6 distance measurement), although failing to achieve continuous water channel formation, appear to have their G-protein coupling sites filled with much more water molecules compared to other (inactive) protomers. In the case of the TM5 AA (Figure 3.37, panel E) and TM4.5 tight AA (Figure 3.37, panel J) systems, simulations initiated in the active state in both protomers were not sufficient to remove water molecules from the inner cavity of the protein in the inactive protomers, and continuous water channel formation was observed in these protomers. One could speculate that water molecules occupy the interior of the protomer as a result of the open conformation of TM6s in the initial simulation structure. It is also possible that the simulation duration was

inadequate, or the observed inactivation was insufficient to fully interrupt the formation of a continuous water channel.

4. ATOMIC DETAILS OF AT1R-AT2R HETERODIMERIZATION

One of the most intriguing ligand-independent functions of GPCRs is signaling through receptor dimers. It has been demonstrated that a number of GPCRs can modify the behavior of other receptors by heterodimerization [225–228]. GPCRs were thought to exist as monomers for a long time despite the fact that dimerization or oligomerization is a common theme in biology and can lead to the formation of functional protein complexes. This was due to the fact that the expected function and pharmacology could usually be achieved by the heterologous production of a single GPCR [229]. ANGII, which is an octapeptide, has an important role in the cardiovascular system for the regulation of blood pressure, maintaining electrolyte-fluid balance, and triggering sodium and water intake [230–232]. ANGII binds to two major receptors, namely angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). ANGII binding to AT1R initiates signaling through the canonical $G\alpha_q$ pathway. Activation of the AT1R can trigger multiple pathways leading to adverse outcomes such as; hypertension, arrhythmias, vascular and cardiac hypertrophy, remodeling, nephrosclerosis, and atherosclerosis [233]. However, AT2R activation countervails detrimental AT1R actions by tissue repair, reduction of cardiac and vascular hypertrophy, anti-atherosclerosis, lowering blood pressure, and organ protection [233]. Recent reviews were focused on class A GPCR dimerization [234, 235], specifically following reviews were concentrated on angiotensin II receptor dimers [236, 237]. Interestingly, although one study suggests that AT1R signalling is inhibited in the cytoplasm rather than blocked by heterodimerization with AT2R at the cell membrane [147], AT1R-AT2R heterodimerization was identified by various independent studies [148, 238–241]. AbdAlla et al. successfully identified one of the first GPCR heterodimer with their seminal work on AT1R-AT2R heterodimer, where they showed that AT2R directly binds to AT1R, and antagonize its function. Moreover, their study reveals that the AT1R specific antagonizing effect of AT2R is independent from its state, AT1R-AT2R heterodimers are neither induced nor inhibited by angiotensin II (ANGII - agonist) [241]. In another study, McMullen et al. conclude that AT1R induced uterine artery vasoconstriction and uterine blood flow are inhibited by AT2R [240]. Inuzuka et al. detected the AT1R-AT2R heterodimer in the cell membrane

using FRET analysis and showed that AT1R inhibition occurs via the PKC-dependent pathway [148]. Moreover, AT1R-AT2R heterodimers are present in central nervous system [238]. ANGII-AT1R/AT2R heterodimer formation and internalization was investigated by living cell imaging in LLC-PK1 cells, and as a consequence intracellular Ca^{2+} levels were increased [239]. Although many of the experimental findings mentioned above clearly demonstrate AT1R-AT2R heterodimerization, none of these studies provide structural insights and explain heterodimerization at the atomic level. Heterodimerization interfaces of following class A GPCRs; adenosine A1 receptor (A1R) / adenosine A2A receptor (A2AR) [242], AA2AR / dopamine D2 receptor (D2R) [243], melatonin MT1 receptor (MT1R) / melatonin MT2 receptor (MT2R), MT1R / orphan GPR50, MT2R / GPR50, MT2R / serotonin 2C receptor (5HT2CR) [244], κ -opioid receptor (KOR) / δ -opioid receptor (DOR) [142, 245], and serotonin 2A (5HT2AR) / D2R, cannabinoid B1 receptor (CB1R) / D2R, A2AR / CB1R [246] were identified using computational methodologies and provided substantial structural information.

4.1. Methodology

4.1.1. Interface Preparation

Firstly, we collected all available high-resolution GPCR structures from the protein data bank (PDB, rcsb.org)[247, 248]. As of 25.11.2019, we collected 352 GPCR structures. All possible dimerization interfaces are created by the symmetry mates option of the PyMol (The PyMOL Molecular Graphics System, Version 2.1 Schrödinger, LLC). PyMol reads the crystal symmetry information from the mmCIF or PDB files, and an asymmetric unit was used to derive complete biological assembly of the molecule. 17 symmetrical and 12 asymmetrical interfaces were obtained. Detailed information about the interfaces is provided in the Extended Methods section. All generated dimers used to align AT1R and AT2R structures on respective chains; A and B.

4.1.2. Protein Preparation

Active and inactive structures of AT1R retrieved from PDB with the PDB ID 6DO1 [183] and PDB ID 4YAY [20]. Nanobodies and co-crystallized ligands were deleted from the protein to obtain the apo form of the receptor. For the fixing the numbering of the sequence and for the filling the missing amino acids, GalaxyFill was used [249, 250] (<https://github.com/seoklab/GalaxyFill>). Full sequence model of 6DO1 and 4YAY were truncated from N-terminal and C-terminal. The AT1R monomer structure that was used in dimer formation starts with ASP17^{Nterm} and ends with LYS318^{H8}. The active form of AT2R was downloaded from PDB with the PDB ID: 5XJM [190]. This X-ray structure of AT2R does not include the helix-8 (H8), based on the sequence homology, H8 of the AT2R modeled using H8 of the 6DO1. Similarly, AT2R missing amino acids filled using GalaxyFill, and truncated from N and C-terminals. The AT2R structure that is used in dimer formation starts with ASN34^{Nterm}, and ends with ARG334^{H8}.

4.1.3. Coarse-grained (CG) Molecular Dynamics (MD) Simulations

Obtained dimers submitted to CHARMM-GUI web-server to generate CGMD inputs [251, 252]. Elnedyn (Elastic Network in Dynamics) force field (FF, an elastic network based) [253] is used for the protein, and Martini 2.0 FF [254] is used for the lipids and the non-polarizable water. All atom (AA) systems were converted to CG representation using The Martini-Maker module implemented in CHARMM-GUI [255]. The Martini model maps four atoms to one atom, i.e. on average four heavy atoms and all hydrogens are represented by a single particle. Each particle has its own subtypes to represent diverse atomistic natures. Only polar, non-polar, and charged interactions are taken into account, to make the model simple. CGMD simulations were performed using the MARTINI (v2.2) force field, using the ELNEDYN elastic network, and the GROMACS software (version 2018) [256, 257]. ELNEDYN elastic network applied with force constant of 500 kJ/mol/nm² and a cut off of 0.9 nm. Elastic networks were used along with Martini, which enables the system to remain close to the overall structure. The velocity rescaling method was used to set temperature to T = 310 K. The Berendsen barostat used to set pressure semi-isotropically to 1 bar using with a time coupling constant of 5 ps and a compressibility value of 3×10^{-4}

bar⁻¹ (for minimization and equilibration phases) and the Parrinello–Rahman barostat with a time coupling constant of 12 ps and a compressibility value of 3×10^{-4} bar⁻¹ (for production phase). Nonbonded interactions were used in their shifted form with electrostatic interactions shifted to zero in the range of 0–11 Å and Lennard–Jones interactions shifted to zero in the range of 9–11 Å. A timestep of 20 fs was used in the production run. Each interface was repeated at least 3 times, and each run was 10 μs long (3x10=30 μs for each).

4.1.4. All-Atom (AA) MD Simulations

15 systems out of 69 interfaces were selected to convert CG representations to all atom (AA) models (AT1R monomer system run directly from AA representation. For the monomer, CG simulations were not run). CHARMM36m forcefield was used for proteins, lipids, and ions, TIP3P was used for the water molecules. 6 steps minimization protocol applied with the following settings. In step 1 systems were equilibrated for 25 nanoseconds (ns), positional restraints were applied with the following constants; 4000 kcal/mol, 2000 kJ/mol/nm², and 1000 kJ/mol/nm² for the backbone (bb), side chain (sc), and lipid atoms. The dihedral restraints were also applied with 1000 kJ/mol/rad² force constant. In step 2, force constants (FCs) for the bb, sc, lipid, and dihedral angles decreased to 2000, 1000, 400 and 400 kJ/mol/rad², respectively. Step 2 equilibrated for 25 ns. Step 1 and 2 were in the NVT ensemble, the Berendsen thermostat was utilized. Step 3 was run for 50 ns, and 1000, 500, 400, and 200 kJ/mol/rad² FCs were used for the bb, sc, lipid, and dihedrals, respectively, in NPT ensemble. The berendsen barostat was used to set pressure to 1 bar, using semi-isotropic pressure coupling with 4.5×10^{-5} bar⁻¹ with a coupling constant of 5 ps. Step 4 simulated for 50 ns, with the same thermostat and barostat settings for the previous steps, however, gradually decreasing FCs were used as 500, 200, 200, and 200 kJ/mol/rad² for the bb, sc, lipid, and dihedrals. Step 5 was the last step with the FCs, where 200, 50, 40, and 100 kcal/mol/radian were applied for the bb, sc, lipid, and dihedrals, respectively. Step 6 ran for 50 ns the same as previous step (5), without any constraints. In the production run, systems were simulated for 1 μs, with Nose-Hoover thermostat and Parrinello-Rahman barostats. All interfaces were repeated ten times. In AA MD simulations Gromacs version 2020 was used. Cluster analysis were performed with gmx cluster, where gromos algorithm was used [258] with 3 Å cut-off.

4.1.5. Protein Ensemble Analysis

Ensemble analysis was performed using PENSEA (<http://doi.org/10.5281/zenodo.4362136>) [259]. Backbone dihedral angle distributions were compared using Jensen-Shannon Distance (JSD), and side-chain dihedral angle distributions were compared using state-specific information (SSI) to understand structural differences between active and inactive states of AT1R. In Pensea analysis, active-only, and inactive-only trajectories of the interfaces were used.

4.1.6. Protein Structure Network (PSN) Analysis

Protein structure network (PSN) analysis was performed using PSNtools [260]. Single trajectory PSNs were run locally, and results were analyzed using PSNwebserver [261]. Two types of PSN calculations were performed, in the first one global metapaths, which show the allosteric communication inside the protein were calculated. In the second one, the differences between PSNs were calculated (PSN difference). In PSN analysis, active-only, and inactive-only trajectories of the interfaces were used for the 2ZIY_1, 5WIV_2, 6AK3_1, and 6K1Q_1_1 systems.

4.1.7. Contact Analysis

Contact fingerprints for the selected interfaces were calculated using the GetContacts code (<https://getcontacts.github.io/>) to identify dimerization contributing residues and their changes under different states. The GetContact analysis was carried out only for the selected systems and their respective trajectories were used. Interaction networks at the dimerization interface were compared in different states (eg. for 2ZIY_1, the intermediate state was compared with the inactive state). All interaction types were considered (hydrogen bonding, salt bridge, π -cation, π -stacking, T-stacking, hydrophobic, and van der Waals).

In this study, 352 experimental structures were evaluated, and 71 systems were generated. These interfaces were embedded into model membrane bilayer and simulated in CG representation. For the CG simulations, 2.13 millisecond-long trajectories were accumulated in total ($71 \times 30 \mu\text{s} = 2130 \mu\text{s}$). Based on TM3-TM6 distance, 16 interfaces were selected for further investigations. The structure that was

closed to the inactive state was selected from CG simulation for these 16 interfaces and back-mapped to all-atom (AA) representation (named as CG2AA). In addition to 16 AA systems, for the comparison, AT1R in monomer form was also included into simulations. These 17 systems were simulated for 1 μ s and repeated 10 times. In AA simulations 170 μ s long trajectories were generated ($17 \times 10 \mu\text{s} = 170 \mu\text{s}$). Contact analysis at the heterodimerization interface, protein structure network (PSN), and protein ensemble analyses were carried out to extract information. Although CG2AA simulations were performed by extracting the structure closest to the inactive structure according to the TM3-TM6 distance; simulations at two interfaces (5WIV_2 and 6AK3_1) showed active state and simulations at two interfaces (2ZII_1 and 6K1Q_1_1_1) showed inactive state properties. Finally, the differences for these four interfaces were examined, and for AT2R-induced inactivation of AT1R was observed in symmetrical TM3,5,6 and asymmetrical TM1,2,H8/TM3,4 interfaces.

4.2. Interface Classification

Generated symmetry mates evaluated by PISA web server [262]. To assess whether any helix contributes to the interface, we hypothesized that at least three amino acids from that helix must be involved in the interfacial interactions in PISA analyses (For example, if one or two amino acids from a helix contribute to the interface formation, we did not use this helix in the interface naming. In more detail, when we name the TM1 / TM1 interface, we know that at least three TM1 amino acids from each protomer are involved in the interface formation.). According to PISA analyses, we obtained 17 symmetrical interfaces (where the same transmembrane helices from each protomer contributes to the interface formation). These 17 interfaces are distributed over 49 interfaces where some interfaces include slightly changing interface volumes, areas, angles, or number of interactions etc. First interface is TM1 / TM1 interface, which includes 8 structures from sphingosine 1-phosphate (3V2Y_2_2), chemokine CXCR4 receptor (3OE9_2_2), metabotropic glutamate receptor 1 (4OR2_1_2), jumping spider rhodopsin-1 (6I9K_1), neurotensin receptor NTSR1 (4XES_1 and 5T04_1_2), succinate receptor SUCNR1 (6RNK_1_2), and D4 Dopamine receptor (5WIV_2_2). μ -opioid (5C1M_1_2), adenosine A1 (5N2S_1_2), κ -opioid (6B73_1_2), and angiotensin II type 1 (6DO1_1) receptors are formed the TM1,2 / TM1,2 interface. The third interface is formed by the jumping spider

rhodopsin-1 (6I9K_2) structure and has TM1,2,3 / TM1,2,3 interactions. Fourth interface is TM1,2,8 / TM1,2,8 and consists of five different crystal structures; rhodopsin (2I37_1, and 6OFJ_1), β 1 Adrenergic (4GPO_1_2), κ -opioid (4DJH_1_2), μ -opioid (4DKL_1_3). Fifth interface observed in rhodopsin structure (3CAP_1) as TM1,6,8 / TM1,6,8. Prostaglandin E receptor EP3 (6AK3_1_2) and viral GPCR US28 (4XT3_1) formed the sixth interface with TM1,7 / TM1,7 interactions. Seventh interface TM1,8 / TM1,8 consist of β 2 Adrenergic (5D5B_1) and CC chemokine receptor type 9 (CCR9) (5LWE_2_2). The TM3 / TM3 interface is the eighth one, and contains endothelin type B (5GLH_1), and adenosine A1 receptor (5UEN_1_2). Ninth interface formed by histamine H1 receptor (3RZE_1_2) with TM3,4 / TM3,4 interactions. Tenth interface is TM3,5 / TM3,5 and observed in five different structures; β 1 Adrenergic (4BVN_1, 5A8E_1, and 4GPO_2_2), cysteinyl leukotriene receptor 1 (6RZ5_1_2), β 2 Adrenergic (5JQH_1_2). CC chemokine receptor type 9 (CCR9) (5LWE_1_2), Frizzled 4 receptor (FZD4) (6BD4_1_2), and sphingosine 1-phosphate (3V2Y_1_2) structures formed TM4 / TM4 as eleventh interface. TM4,5 / TM4,5 interface is the twelfth one with the structures from adenosine A2A (4EIY_1_2), rhodopsin (2Z73_1), smoothened (SMO) receptor (4JKV_1_2), and succinate receptor SUCNR1(6RNK_2_2). Thirteenth interface is TM5 / TM5, and rhodopsin (2Z1Y_1, and 4WW3_1), chemokine receptor CXCR4 (3OE9_1_2, and 3OE8_1_2), P2Y receptor P2Y12R (4NTJ_1_2) formed this interface. μ -opioid (4DKL_2_2) and Prostaglandin E receptor EP3 (6AK3_2_2) formed TM5,6 / TM5,6 interface. TM6 / TM6 interface observed in serotonin 2B receptor (5TUD_1_2) and metabotropic glutamate receptor subtype 5 (6N51_1_2) structures. Sixteenth interface formed by prostaglandin E receptor EP4 (5YWY_1_2) with TM8 / TM8 interactions. The last symmetrical interface observed in the D4 dopamine receptor (5WIV_1_2) differentiated from other interfaces by having ECL3 / ECL3 interactions. We observed 17 different interfaces in 49 clusters in symmetrical interfaces. Two of the interfaces; 1) 4XES_1 for the TM1/ TM1 interface, and 2) 4JKV_1_2 for the TM4,5 / TM4,5 were not suitable for the AT1-AT2 heterodimer system.

At asymmetrical interfaces, there are two possibilities for AT1 and AT2 for each asymmetric interface, as different transmembrane helices of each protomer contribute to interface formation. For example, for the TM1 / TM5,6,7 interface, two different interfaces occur when the AT1 receptor is located in the A chain and different when it is located in the B chain. 12 asymmetrical interfaces (where the different

transmembrane helices from each protomer contribute to the interface formation). Each interface has only one representative. TM1 / TM5,6,7 is the first asymmetrical interface that we observed in chemokine receptor CXCR4 (3OE8_2_2). Second asymmetrical interface observed in D4 dopamine receptor (6IQL_1_2) with TM1,2,3 / TM5,6 interactions. Endothelin receptor ETB (6K1Q_1_2) formed the TM1,2,8 / TM3,4 interface as a third cluster. The fourth interface has TM1,2,8 / TM7 interactions, and the adenosine A2A receptor (4UG2_1) is the only one member of this interface. The CCR5 chemokine receptor (4MBS_1_2) formed the TM1,7,8 / TM4 interface as the fifth cluster. The sixth interface TM1,8 / TM5 is observed in CC Chemokine Receptor 2A (6GPS_1_2). Lysophosphatidic Acid (LPA) receptor (4Z34_1_2) formed the TM3,4 / TM1,7,8 interface as the seventh interface. TM3,4 / TM1,7 interface is observed in corticotropin-releasing factor receptor 1 (4Z9G_1_2). The ninth interface is observed in the P2Y1 receptor (4XNV_1_2) as TM3,4,5 / TM5,6 interface. Same receptor is involved in another two interfaces as TM5 / TM1 (4XNW_1) and TM6 / TM1 (4XNV_2_2). The last asymmetrical interface is TM6,7 / TM3,4 and observed in Cannabinoid Receptor CB2 (5ZTY_1_2).

4.3. TM3-TM6 Distance Analysis In CG Simulations

The biggest conformational change is observed in TM6, where the end of the helix displaced in outward fashion (~10-14 Å) to provide adequate space for intracellular transducer binding. In addition, TM5 (inward movement) and TM7 also rearrange to accompany the TM6 displacement. In order to understand in detail how these changes occur, the trajectories of CG simulations were evaluated based on the difference of some important interatomic distances. In our previous publications, ARG126^{3.50}-PHE239^{6.34} distance successfully discriminated against the active and inactive conformations in the AT1R homodimer system [263]. Table 4.1 summarizes the simulation averages, simulation starting distances (Step5 distance) and the Δ distance. 75% threshold for the simulation repeats was used to select systems to proceed all-atom (AA) simulations. ARG126^{3.50}-PHE239^{6.34} distance threshold set to 2.75 Å. If a decrease from this threshold value was observed in not all three repeats (e.g. in two of the three repeats), the fourth simulation run was performed in these systems (2ZIIY_1, 3OE9_2_1, 4BVN_1, 4XNV_2_2, 4XT3_1, 5UEN_1_2, 5WIV_2, 6DO1_1_2, and 6K1Q_1_1). However, in the case of the 5GLH_1 interface, 4

independent runs were not enough to conclude whether system shifts to an inactive state or not, so 7 runs were performed. According to decrease in ARG126-PHE239 distance (the systems which have at least 2.75 Å decrease in all repeats were considered), we selected the following 11 systems to proceed AA simulations; 2ZIY_1, 4MBS_1_2, 4UG2_1_1, 4XNV_2_1, 4XT3_1, 5GLH_1, 5WIV_2, 6AK3_1, 6DO1_1_2, 6OFJ_1, and 6RNK_1.

Table 4.1: The distance between Backbone atoms of ARG126^{3.50} and PHE239^{6.34}, in CG simulations. Delta distance is the difference between the simulation starting distance and simulation average distance. The distances which has greater then 2.75 Å value, highlighted in green color.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
2I37_1_10ms_elndyn	12.13	13.96	1.84
2I37_1_10ms_elndyn_2	11.51	13.96	2.46
2I37_1_10ms_elndyn_3	11.44	13.96	2.53
2Z73_1_10ms_elndyn	12.33	14.06	1.74
2Z73_1_10ms_elndyn_2	11.74	14.06	2.33
2Z73_1_10ms_elndyn_3	12.72	14.06	1.34
2ZIY_1_10ms_elndyn	11.20	14.34	3.13
2ZIY_1_10ms_elndyn_2	12.93	14.34	1.41
2ZIY_1_10ms_elndyn_3	11.50	14.34	2.84
2ZIY_1_10ms_elndyn_4	11.47	14.34	2.87
3CAP_1_10ms_elndyn	12.55	14.33	1.77
3CAP_1_10ms_elndyn_2	12.40	14.33	1.93
3CAP_1_10ms_elndyn_3	12.52	14.33	1.80

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
3OE8_1_10ms_elndyn	12.17	14.05	1.88
3OE8_1_10ms_elndyn_2	13.42	14.05	0.63
3OE8_1_10ms_elndyn_3	12.95	14.05	1.09
3OE9_1_10ms_elndyn	12.93	14.30	1.37
3OE9_1_10ms_elndyn_2	11.53	14.30	2.77
3OE9_1_10ms_elndyn_3	13.46	14.30	0.83
3OE9_2_1_10ms_elndyn	11.66	14.03	2.37
3OE9_2_1_10ms_elndyn_2	11.02	14.03	3.01
3OE9_2_1_10ms_elndyn_3	11.04	14.03	2.98
3OE9_2_1_10ms_elndyn_4	11.78	14.03	2.24
3OE9_2_2_10ms_elndyn	12.20	14.01	1.81
3OE9_2_2_10ms_elndyn_2	11.81	14.01	2.20
3OE9_2_2_10ms_elndyn_3	12.02	14.01	2.00
3RZE_1_10ms_elndyn	13.61	14.16	0.55
3RZE_1_10ms_elndyn_2	13.55	14.16	0.60

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
3RZE_1_10ms_elnedyn_3	13.55	14.16	0.61
3V2Y_1_10ms_elnedyn	13.27	14.26	0.99
3V2Y_1_10ms_elnedyn_2	13.43	14.26	0.84
3V2Y_1_10ms_elnedyn_3	13.58	14.26	0.68
3V2Y_2_10ms_elnedyn	11.67	14.01	2.33
3V2Y_2_10ms_elnedyn_2	11.17	14.01	2.83
3V2Y_2_10ms_elnedyn_3	14.03	14.01	-0.02
4BVN_1_10ms_elnedyn	11.28	14.06	2.78
4BVN_1_10ms_elnedyn_2	12.19	14.06	1.87
4BVN_1_10ms_elnedyn_3	10.93	14.06	3.13
4BVN_1_10ms_elnedyn_4	12.40	14.06	1.66
4DJH_1_10ms_elnedyn	12.06	14.05	1.99
4DJH_1_10ms_elnedyn_2	12.42	14.05	1.63
4DJH_1_10ms_elnedyn_3	12.06	14.05	1.99
4DKL_1_10ms_elnedyn	11.33	13.69	2.36

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4DKL_1_10ms_elnedyn_2	11.20	13.69	2.50
4DKL_1_10ms_elnedyn_3	11.46	13.69	2.23
4EIY_1_10ms_elnedyn	13.13	14.01	0.89
4EIY_1_10ms_elnedyn_2	13.07	14.01	0.94
4EIY_1_10ms_elnedyn_3	13.00	14.01	1.02
4GPO_1_10ms_elnedyn	11.84	13.89	2.05
4GPO_1_10ms_elnedyn_2	13.24	13.89	0.66
4GPO_1_10ms_elnedyn_3	11.67	13.89	2.23
4GPO_2_10ms_elnedyn	12.11	13.79	1.68
4GPO_2_10ms_elnedyn_2	12.64	13.79	1.15
4GPO_2_10ms_elnedyn_3	13.60	13.79	0.19
4MBS_1_1_10ms_elnedyn	12.24	13.99	1.75
4MBS_1_1_10ms_elnedyn_2	11.74	13.99	2.26
4MBS_1_1_10ms_elnedyn_3	10.81	13.99	3.19
4MBS_1_2_10ms_elnedyn	10.97	13.96	2.99

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4MBS_1_2_10ms_elndyn_2	10.86	13.96	3.10
4MBS_1_2_10ms_elndyn_3	10.97	13.96	2.99
4NTJ_1_10ms_elndyn	11.33	13.78	2.45
4NTJ_1_10ms_elndyn_2	11.29	13.78	2.49
4NTJ_1_10ms_elndyn_3	11.56	13.78	2.22
4OR2_1_10ms_elndyn	12.18	13.95	1.76
4OR2_1_10ms_elndyn_2	12.19	13.95	1.76
4OR2_1_10ms_elndyn_3	11.82	13.95	2.13
4UG2_1_1_10ms_elndyn	10.74	13.85	3.11
4UG2_1_1_10ms_elndyn_2	10.83	13.85	3.02
4UG2_1_1_10ms_elndyn_3	10.90	13.85	2.95
4UG2_1_2_10ms_elndyn	11.38	13.86	2.48
4UG2_1_2_10ms_elndyn_2	11.97	13.86	1.88
4UG2_1_2_10ms_elndyn_3	13.06	13.86	0.80
4WW3_1_10ms_elndyn	11.62	14.06	2.44

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4WW3_1_10ms_elndyn_2	11.67	14.06	2.39
4WW3_1_10ms_elndyn_3	12.00	14.06	2.06
4XNV_1_1_10ms_elndyn	13.19	14.17	0.98
4XNV_1_1_10ms_elndyn_2	13.30	14.17	0.86
4XNV_1_1_10ms_elndyn_3	13.40	14.17	0.77
4XNV_1_2_10ms_elndyn	13.78	14.41	0.63
4XNV_1_2_10ms_elndyn_2	13.85	14.41	0.56
4XNV_1_2_10ms_elndyn_3	13.24	14.41	1.17
4XNV_2_1_10ms_elndyn	10.66	13.67	3.01
4XNV_2_1_10ms_elndyn_2	10.47	13.67	3.20
4XNV_2_1_10ms_elndyn_3	10.33	13.67	3.34
4XNV_2_2_10ms_elndyn	11.23	14.19	2.96
4XNV_2_2_10ms_elndyn_2	10.66	14.19	3.53
4XNV_2_2_10ms_elndyn_3	11.63	14.19	2.56
4XNV_2_2_10ms_elndyn_4	11.21	14.19	2.98

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4XNW_1_1_10ms_elndyn	11.52	14.04	2.52
4XNW_1_1_10ms_elndyn_2	13.73	14.04	0.31
4XNW_1_1_10ms_elndyn_3	12.88	14.04	1.16
4XNW_1_2_10ms_elndyn	12.13	13.70	1.57
4XNW_1_2_10ms_elndyn_2	12.22	13.70	1.48
4XNW_1_2_10ms_elndyn_3	11.79	13.70	1.92
4XT3_1_10ms_elndyn	10.98	13.93	2.95
4XT3_1_10ms_elndyn_2	10.38	13.93	3.54
4XT3_1_10ms_elndyn_3	11.15	13.93	2.77
4XT3_1_10ms_elndyn_4	10.77	13.93	3.16
4Z34_1_1_10ms_elndyn	12.61	14.05	1.44
4Z34_1_1_10ms_elndyn_2	13.48	14.05	0.57
4Z34_1_1_10ms_elndyn_3	12.72	14.05	1.33
4Z34_1_2_10ms_elndyn	13.20	14.20	1.00
4Z34_1_2_10ms_elndyn_2	13.37	14.20	0.83

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4Z34_1_2_10ms_elnedyn_3	10.88	14.20	3.32
5A8E_1_10ms_elnedyn	12.88	14.11	1.24
5A8E_1_10ms_elnedyn_2	11.15	14.11	2.97
5A8E_1_10ms_elnedyn_3	12.47	14.11	1.65
5C1M_1_10ms_elnedyn	12.72	13.94	1.22
5C1M_1_10ms_elnedyn_2	13.16	13.94	0.77
5C1M_1_10ms_elnedyn_3	11.88	13.94	2.06
5D5B_1_10ms_elnedyn	13.07	14.14	1.07
5D5B_1_10ms_elnedyn_2	12.71	14.14	1.43
5D5B_1_10ms_elnedyn_3	12.92	14.14	1.22
5GLH_1_10ms_elnedyn	10.62	13.77	3.15
5GLH_1_10ms_elnedyn_2	11.33	13.77	2.44
5GLH_1_10ms_elnedyn_3	10.41	13.77	3.36
5GLH_1_10ms_elnedyn_4	11.14	13.77	2.62
5GLH_1_10ms_elnedyn_5	11.14	13.77	2.63

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5GLH_1_10ms_elnedyn_6	10.36	13.77	3.41
5GLH_1_10ms_elnedyn_7	10.46	13.77	3.31
5JQH_1_10ms_elnedyn	12.06	13.87	1.80
5JQH_1_10ms_elnedyn_2	11.43	13.87	2.44
5JQH_1_10ms_elnedyn_3	11.27	13.87	2.59
5LWE_1_1_10ms_elnedyn	12.14	13.99	1.85
5LWE_1_1_10ms_elnedyn_2	11.56	13.99	2.42
5LWE_1_1_10ms_elnedyn_3	11.12	13.99	2.87
5LWE_1_2_10ms_elnedyn	12.96	14.06	1.11
5LWE_1_2_10ms_elnedyn_2	12.56	14.06	1.50
5LWE_1_2_10ms_elnedyn_3	12.86	14.06	1.20
5LWE_2_10ms_elnedyn	12.30	14.06	1.76
5LWE_2_10ms_elnedyn_2	12.15	14.06	1.91
5LWE_2_10ms_elnedyn_3	11.75	14.06	2.31
5N2S_1_10ms_elnedyn	13.38	14.08	0.70

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5N2S_1_10ms_elnedyn_2	13.53	14.08	0.55
5N2S_1_10ms_elnedyn_3	13.19	14.08	0.89
5T04_1_10ms_elnedyn	12.15	14.16	2.01
5T04_1_10ms_elnedyn_2	11.89	14.16	2.27
5T04_1_10ms_elnedyn_3	11.63	14.16	2.53
5TUD_1_10ms_elnedyn	12.60	13.72	1.12
5TUD_1_10ms_elnedyn_5	12.64	13.72	1.08
5TUD_1_10ms_elnedyn_6	13.31	13.72	0.40
5UEN_1_1_10ms_elnedyn	12.44	13.68	1.25
5UEN_1_1_10ms_elnedyn_2	11.67	13.68	2.01
5UEN_1_1_10ms_elnedyn_3	12.51	13.68	1.17
5UEN_1_2_10ms_elnedyn	11.30	14.13	2.83
5UEN_1_2_10ms_elnedyn_2	11.20	14.13	2.93
5UEN_1_2_10ms_elnedyn_3	11.76	14.13	2.36
5UEN_1_2_10ms_elnedyn_4	11.51	14.13	2.61

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5WIV_2_10ms_elndyn	10.68	14.01	3.33
5WIV_2_10ms_elndyn_2	11.25	14.01	2.76
5WIV_2_10ms_elndyn_3	10.86	14.01	3.15
5WIV_2_10ms_elndyn_4	10.60	14.01	3.42
5YWY_1_10ms_elndyn	12.58	13.97	1.39
5YWY_1_10ms_elndyn_2	12.46	13.97	1.51
5YWY_1_10ms_elndyn_6	12.71	13.97	1.26
5ZTY_1_1_10ms_elndyn	13.49	13.52	0.03
5ZTY_1_1_10ms_elndyn_2	13.40	13.52	0.12
5ZTY_1_1_10ms_elndyn_3	13.52	13.52	0.01
5ZTY_1_2_10ms_elndyn	11.64	13.84	2.19
5ZTY_1_2_10ms_elndyn_2	12.56	13.84	1.27
5ZTY_1_2_10ms_elndyn_3	11.76	13.84	2.08
6AK3_1_10ms_elndyn	11.00	13.88	2.88
6AK3_1_10ms_elndyn_2	10.63	13.88	3.25

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6AK3_1_10ms_elnedyn_3	10.93	13.88	2.95
6AK3_2_10ms_elnedyn	12.86	14.11	1.25
6AK3_2_10ms_elnedyn_2	11.89	14.11	2.22
6AK3_2_10ms_elnedyn_3	12.84	14.11	1.28
6AKX_1_1_10ms_elnedyn	12.74	14.21	1.47
6AKX_1_1_10ms_elnedyn_2	13.24	14.21	0.97
6AKX_1_1_10ms_elnedyn_3	11.91	14.21	2.30
6AKX_1_2_10ms_elnedyn	13.10	13.88	0.77
6AKX_1_2_10ms_elnedyn_2	11.14	13.88	2.74
6AKX_1_2_10ms_elnedyn_3	11.57	13.88	2.31
6B73_1_10ms_elnedyn	11.68	14.19	2.51
6B73_1_10ms_elnedyn_2	12.28	14.19	1.91
6B73_1_10ms_elnedyn_3	11.88	14.19	2.31
6DO1_1_1_10ms_elnedyn	11.55	13.95	2.40
6DO1_1_1_10ms_elnedyn_2	12.75	13.95	1.21

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6DO1_1_1_10ms_elndyn_3	12.69	13.95	1.27
6DO1_1_2_10ms_elndyn	10.78	14.15	3.37
6DO1_1_2_10ms_elndyn_2	11.15	14.15	2.99
6DO1_1_2_10ms_elndyn_3	10.78	14.15	3.37
6DO1_1_2_10ms_elndyn_4	10.86	14.15	3.28
6GPS_1_1_10ms_elndyn	12.85	14.01	1.17
6GPS_1_1_10ms_elndyn_2	13.31	14.01	0.70
6GPS_1_1_10ms_elndyn_3	13.04	14.01	0.98
6GPS_1_2_10ms_elndyn	11.60	13.81	2.22
6GPS_1_2_10ms_elndyn_2	12.50	13.81	1.32
6GPS_1_2_10ms_elndyn_3	12.79	13.81	1.03
6I9K_1_10ms_elndyn	12.94	14.00	1.07
6I9K_1_10ms_elndyn_2	12.95	14.00	1.05
6I9K_1_10ms_elndyn_3	12.92	14.00	1.09
6I9K_2_10ms_elndyn	11.20	13.83	2.63

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6I9K_2_10ms_elnedyn_2	11.19	13.83	2.64
6I9K_2_10ms_elnedyn_3	12.81	13.83	1.02
6IQL_1_1_10ms_elnedyn	11.79	14.09	2.30
6IQL_1_1_10ms_elnedyn_2	11.82	14.09	2.27
6IQL_1_1_10ms_elnedyn_3	11.96	14.09	2.13
6IQL_1_2_10ms_elnedyn	13.62	13.98	0.36
6IQL_1_2_10ms_elnedyn_2	13.41	13.98	0.57
6IQL_1_2_10ms_elnedyn_3	14.69	13.98	-0.71
6K1Q_1_1_10ms_elnedyn	10.96	13.61	2.65
6K1Q_1_1_10ms_elnedyn_2	10.68	13.61	2.93
6K1Q_1_1_10ms_elnedyn_3	11.20	13.61	2.41
6K1Q_1_1_10ms_elnedyn_4	11.31	13.61	2.30
6K1Q_1_1_10ms_elnedyn_5	10.65	13.61	2.96
6K1Q_1_2_10ms_elnedyn	12.79	14.07	1.28
6K1Q_1_2_10ms_elnedyn_2	13.27	14.07	0.79

Table 4.1: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}		
	254-514		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6K1Q_1_2_10ms_elndyn_3	12.49	14.07	1.58
6OFJ_1_10ms_elndyn	11.26	14.06	2.80
6OFJ_1_10ms_elndyn_2	11.26	14.06	2.80
6OFJ_1_10ms_elndyn_3	11.24	14.06	2.82
6RNK_1_10ms_elndyn	10.54	13.88	3.34
6RNK_1_10ms_elndyn_2	10.42	13.88	3.46
6RNK_1_10ms_elndyn_3	10.36	13.88	3.51
6RNK_2_10ms_elndyn	15.14	13.64	-1.50
6RNK_2_10ms_elndyn_2	12.88	13.64	0.76
6RNK_2_10ms_elndyn_3	12.97	13.64	0.67
6RZ5_1_10ms_elndyn	12.50	14.14	1.64
6RZ5_1_10ms_elndyn_2	11.01	14.14	3.14
6RZ5_1_10ms_elndyn_3	11.87	14.14	2.27

4.4. GPCRdb Distance Analysis In CG Simulations

Table 4.2 shows the change in the distance between the backbone atoms of VAL65^{2.41} and MET243^{6.38}. The cut-off distance was set to 1.50 Å. 2ZIY_1,

3OE9_2_1, 4MBS_1_2, 4UG2_1_1, 4WW3_1, 4XNV_2_1, 4XNV_2_2, 5GLH_1, 5WIV_2, 6AK3_1, 6DO1_1_2, 6K1Q_1_1, 6OFJ_1, and 6RNK_1 selected according to decrease in VAL65^{2.41}-MET243^{6.38} backbone atom distance.

Table 4.2: The distance between Backbone atoms of VAL65^{2.41} and MET243^{6.38}, in CG simulations. Delta distance is the difference between the simulation starting distance and simulation average distance. The distances which has greater then 1.50 Å value, highlighted in green color.

	VAL65 ^{2.41} - MET243 ^{6.38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	ΔDistance (Å)
2I37_1_10ms_elndyn	23.25	24.08	0.83
2I37_1_10ms_elndyn_2	22.73	24.08	1.35
2I37_1_10ms_elndyn_3	22.88	24.08	1.20
2Z73_1_10ms_elndyn	22.73	23.89	1.16
2Z73_1_10ms_elndyn_2	22.63	23.89	1.26
2Z73_1_10ms_elndyn_3	23.04	23.89	0.85
2ZIY_1_10ms_elndyn	22.05	24.37	2.32
2ZIY_1_10ms_elndyn_2	23.22	24.37	1.15
2ZIY_1_10ms_elndyn_3	22.39	24.37	1.98
2ZIY_1_10ms_elndyn_4	22.54	24.37	1.83
3CAP_1_10ms_elndyn	23.05	24.25	1.20
3CAP_1_10ms_elndyn_2	22.93	24.25	1.32

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
3CAP_1_10ms_elnedyn_3	23.03	24.25	1.22
3OE8_1_10ms_elnedyn	22.57	24.09	1.52
3OE8_1_10ms_elnedyn_2	23.44	24.09	0.65
3OE8_1_10ms_elnedyn_3	23.44	24.09	0.65
3OE9_1_10ms_elnedyn	23.27	23.95	0.68
3OE9_1_10ms_elnedyn_2	23.05	23.95	0.90
3OE9_1_10ms_elnedyn_3	23.19	23.95	0.76
3OE9_2_1_10ms_elnedyn	22.48	24.25	1.77
3OE9_2_1_10ms_elnedyn_2	21.97	24.25	2.29
3OE9_2_1_10ms_elnedyn_3	21.98	24.25	2.28
3OE9_2_1_10ms_elnedyn_4	22.44	24.25	1.82
3OE9_2_2_10ms_elnedyn	22.64	23.92	1.28
3OE9_2_2_10ms_elnedyn_2	22.56	23.92	1.36
3OE9_2_2_10ms_elnedyn_3	22.61	23.92	1.30
3RZE_1_10ms_elnedyn	22.84	23.94	1.11

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
3RZE_1_10ms_elnedyn_2	23.73	23.94	0.21
3RZE_1_10ms_elnedyn_3	23.48	23.94	0.46
3V2Y_1_10ms_elnedyn	23.98	24.23	0.25
3V2Y_1_10ms_elnedyn_2	23.65	24.23	0.57
3V2Y_1_10ms_elnedyn_3	23.39	24.23	0.84
3V2Y_2_10ms_elnedyn	22.33	24.19	1.86
3V2Y_2_10ms_elnedyn_2	21.73	24.19	2.46
3V2Y_2_10ms_elnedyn_3	23.63	24.19	0.56
4BVN_1_10ms_elnedyn	22.43	24.20	1.77
4BVN_1_10ms_elnedyn_2	23.05	24.20	1.15
4BVN_1_10ms_elnedyn_3	22.28	24.20	1.92
4BVN_1_10ms_elnedyn_4	22.70	24.20	1.49
4DJH_1_10ms_elnedyn	23.03	24.02	0.98
4DJH_1_10ms_elnedyn_2	23.00	24.02	1.02
4DJH_1_10ms_elnedyn_3	23.08	24.02	0.93

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4DKL_1_10ms_elnedyn	22.58	23.98	1.40
4DKL_1_10ms_elnedyn_2	22.42	23.98	1.56
4DKL_1_10ms_elnedyn_3	22.63	23.98	1.35
4EIY_1_10ms_elnedyn	23.59	24.19	0.60
4EIY_1_10ms_elnedyn_2	23.92	24.19	0.27
4EIY_1_10ms_elnedyn_3	23.53	24.19	0.66
4GPO_1_10ms_elnedyn	22.90	23.93	1.03
4GPO_1_10ms_elnedyn_2	23.48	23.93	0.45
4GPO_1_10ms_elnedyn_3	22.83	23.93	1.09
4GPO_2_10ms_elnedyn	22.81	24.11	1.30
4GPO_2_10ms_elnedyn_2	23.11	24.11	1.00
4GPO_2_10ms_elnedyn_3	23.46	24.11	0.65
4MBS_1_1_10ms_elnedyn	23.19	24.07	0.87
4MBS_1_1_10ms_elnedyn_2	22.47	24.07	1.60
4MBS_1_1_10ms_elnedyn_3	22.18	24.07	1.88

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4MBS_1_2_10ms_elnodyn	22.15	24.29	2.15
4MBS_1_2_10ms_elnodyn_2	22.22	24.29	2.07
4MBS_1_2_10ms_elnodyn_3	22.25	24.29	2.04
4NTJ_1_10ms_elnodyn	22.64	23.96	1.32
4NTJ_1_10ms_elnodyn_2	22.75	23.96	1.21
4NTJ_1_10ms_elnodyn_3	22.75	23.96	1.21
4OR2_1_10ms_elnodyn	23.02	23.96	0.93
4OR2_1_10ms_elnodyn_2	23.01	23.96	0.95
4OR2_1_10ms_elnodyn_3	22.73	23.96	1.23
4UG2_1_1_10ms_elnodyn	22.24	24.32	2.07
4UG2_1_1_10ms_elnodyn_2	22.23	24.32	2.09
4UG2_1_1_10ms_elnodyn_3	22.31	24.32	2.01
4UG2_1_2_10ms_elnodyn	23.39	24.12	0.74
4UG2_1_2_10ms_elnodyn_2	23.67	24.12	0.45
4UG2_1_2_10ms_elnodyn_3	24.29	24.12	-0.17

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4WW3_1_10ms_elndyn	22.26	24.10	1.84
4WW3_1_10ms_elndyn_2	22.39	24.10	1.71
4WW3_1_10ms_elndyn_3	22.53	24.10	1.57
4XNV_1_1_10ms_elndyn	23.72	24.26	0.55
4XNV_1_1_10ms_elndyn_2	23.62	24.26	0.65
4XNV_1_1_10ms_elndyn_3	23.53	24.26	0.73
4XNV_1_2_10ms_elndyn	24.27	24.43	0.16
4XNV_1_2_10ms_elndyn_2	24.46	24.43	-0.03
4XNV_1_2_10ms_elndyn_3	24.15	24.43	0.28
4XNV_2_1_10ms_elndyn	22.37	23.88	1.51
4XNV_2_1_10ms_elndyn_2	22.20	23.88	1.69
4XNV_2_1_10ms_elndyn_3	21.94	23.88	1.95
4XNV_2_2_10ms_elndyn	22.36	24.23	1.86
4XNV_2_2_10ms_elndyn_2	22.07	24.23	2.15
4XNV_2_2_10ms_elndyn_3	22.63	24.23	1.59

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4XNV_2_2_10ms_elnedyn_4	22.38	24.23	1.85
4XNW_1_1_10ms_elnedyn	22.32	24.31	1.99
4XNW_1_1_10ms_elnedyn_2	23.84	24.31	0.47
4XNW_1_1_10ms_elnedyn_3	23.22	24.31	1.09
4XNW_1_2_10ms_elnedyn	22.91	24.18	1.27
4XNW_1_2_10ms_elnedyn_2	23.07	24.18	1.11
4XNW_1_2_10ms_elnedyn_3	22.77	24.18	1.41
4XT3_1_10ms_elnedyn	22.31	23.75	1.44
4XT3_1_10ms_elnedyn_2	21.98	23.75	1.77
4XT3_1_10ms_elnedyn_3	22.01	23.75	1.74
4XT3_1_10ms_elnedyn_4	22.34	23.75	1.42
4Z34_1_1_10ms_elnedyn	22.87	24.23	1.36
4Z34_1_1_10ms_elnedyn_2	23.16	24.23	1.07
4Z34_1_1_10ms_elnedyn_3	23.23	24.23	0.99
4Z34_1_2_10ms_elnedyn	23.55	24.17	0.62

Table 4.2: Continued.

	VAL65 ^{2.41} - MET243 ^{6.38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4Z34_1_2_10ms_elnedyn_2	23.71	24.17	0.46
4Z34_1_2_10ms_elnedyn_3	22.36	24.17	1.81
5A8E_1_10ms_elnedyn	23.18	23.98	0.80
5A8E_1_10ms_elnedyn_2	22.71	23.98	1.27
5A8E_1_10ms_elnedyn_3	22.86	23.98	1.13
5C1M_1_10ms_elnedyn	23.19	24.25	1.05
5C1M_1_10ms_elnedyn_2	23.17	24.25	1.08
5C1M_1_10ms_elnedyn_3	22.56	24.25	1.68
5D5B_1_10ms_elnedyn	23.21	24.05	0.84
5D5B_1_10ms_elnedyn_2	22.99	24.05	1.06
5D5B_1_10ms_elnedyn_3	23.24	24.05	0.81
5GLH_1_10ms_elnedyn	22.32	24.14	1.82
5GLH_1_10ms_elnedyn_2	22.69	24.14	1.45
5GLH_1_10ms_elnedyn_3	22.36	24.14	1.78
5GLH_1_10ms_elnedyn_4	22.42	24.14	1.72

Table 4.2: Continued.

	VAL65 ^{2.41} - MET243 ^{6.38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5GLH_1_10ms_elnedyn_5	22.70	24.14	1.44
5GLH_1_10ms_elnedyn_6	22.12	24.14	2.01
5GLH_1_10ms_elnedyn_7	22.35	24.14	1.79
5JQH_1_10ms_elnedyn	22.77	24.24	1.47
5JQH_1_10ms_elnedyn_2	22.24	24.24	2.01
5JQH_1_10ms_elnedyn_3	22.30	24.24	1.94
5LWE_1_1_10ms_elnedyn	22.75	24.19	1.44
5LWE_1_1_10ms_elnedyn_2	22.79	24.19	1.40
5LWE_1_1_10ms_elnedyn_3	22.75	24.19	1.44
5LWE_1_2_10ms_elnedyn	23.46	24.23	0.77
5LWE_1_2_10ms_elnedyn_2	23.24	24.23	0.98
5LWE_1_2_10ms_elnedyn_3	23.63	24.23	0.60
5LWE_2_10ms_elnedyn	22.78	23.95	1.17
5LWE_2_10ms_elnedyn_2	22.59	23.95	1.36
5LWE_2_10ms_elnedyn_3	22.44	23.95	1.51

Table 4.2: Continued.

	VAL65 ^{2.41} - MET243 ^{6.38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5N2S_1_10ms_elnedyn	24.11	24.15	0.04
5N2S_1_10ms_elnedyn_2	24.43	24.15	-0.28
5N2S_1_10ms_elnedyn_3	23.93	24.15	0.21
5T04_1_10ms_elnedyn	22.98	24.33	1.35
5T04_1_10ms_elnedyn_2	22.91	24.33	1.42
5T04_1_10ms_elnedyn_3	22.76	24.33	1.57
5TUD_1_10ms_elnedyn	23.09	23.64	0.55
5TUD_1_10ms_elnedyn_5	22.97	23.64	0.67
5TUD_1_10ms_elnedyn_6	23.01	23.64	0.63
5UEN_1_1_10ms_elnedyn	23.26	24.07	0.81
5UEN_1_1_10ms_elnedyn_2	22.79	24.07	1.28
5UEN_1_1_10ms_elnedyn_3	23.66	24.07	0.41
5UEN_1_2_10ms_elnedyn	22.60	24.09	1.49
5UEN_1_2_10ms_elnedyn_2	22.65	24.09	1.44
5UEN_1_2_10ms_elnedyn_3	22.32	24.09	1.78

Table 4.2: Continued.

	VAL65 ^{2.41} - MET243 ^{6.38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5UEN_1_2_10ms_elnedyn_4	22.77	24.09	1.33
5WIV_2_10ms_elnedyn	22.18	24.06	1.87
5WIV_2_10ms_elnedyn_2	22.49	24.06	1.57
5WIV_2_10ms_elnedyn_3	22.23	24.06	1.82
5WIV_2_10ms_elnedyn_4	22.24	24.06	1.82
5YWY_1_10ms_elnedyn	23.32	24.58	1.26
5YWY_1_10ms_elnedyn_2	23.12	24.58	1.46
5YWY_1_10ms_elnedyn_6	23.08	24.58	1.50
5ZTY_1_1_10ms_elnedyn	22.91	23.45	0.54
5ZTY_1_1_10ms_elnedyn_2	22.89	23.45	0.56
5ZTY_1_1_10ms_elnedyn_3	23.39	23.45	0.07
5ZTY_1_2_10ms_elnedyn	22.56	23.78	1.22
5ZTY_1_2_10ms_elnedyn_2	22.73	23.78	1.05
5ZTY_1_2_10ms_elnedyn_3	22.58	23.78	1.20
6AK3_1_10ms_elnedyn	22.46	24.29	1.83

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6AK3_1_10ms_elnedyn_2	22.29	24.29	2.00
6AK3_1_10ms_elnedyn_3	22.54	24.29	1.75
6AK3_2_10ms_elnedyn	23.05	24.26	1.21
6AK3_2_10ms_elnedyn_2	22.58	24.26	1.68
6AK3_2_10ms_elnedyn_3	23.01	24.26	1.25
6AKX_1_1_10ms_elnedyn	23.45	24.29	0.84
6AKX_1_1_10ms_elnedyn_2	23.43	24.29	0.86
6AKX_1_1_10ms_elnedyn_3	22.64	24.29	1.65
6AKX_1_2_10ms_elnedyn	23.82	24.13	0.31
6AKX_1_2_10ms_elnedyn_2	22.02	24.13	2.11
6AKX_1_2_10ms_elnedyn_3	22.34	24.13	1.80
6B73_1_10ms_elnedyn	22.51	24.14	1.63
6B73_1_10ms_elnedyn_2	22.82	24.14	1.32
6B73_1_10ms_elnedyn_3	22.78	24.14	1.36
6DO1_1_1_10ms_elnedyn	22.92	23.86	0.95

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6DO1_1_1_10ms_elnodyn_2	23.57	23.86	0.30
6DO1_1_1_10ms_elnodyn_3	23.61	23.86	0.26
6DO1_1_2_10ms_elnodyn	22.16	24.10	1.94
6DO1_1_2_10ms_elnodyn_2	22.25	24.10	1.85
6DO1_1_2_10ms_elnodyn_3	22.09	24.10	2.01
6DO1_1_2_10ms_elnodyn_4	22.21	24.10	1.89
6GPS_1_1_10ms_elnodyn	22.88	24.13	1.25
6GPS_1_1_10ms_elnodyn_2	23.22	24.13	0.90
6GPS_1_1_10ms_elnodyn_3	22.81	24.13	1.32
6GPS_1_2_10ms_elnodyn	22.50	24.02	1.52
6GPS_1_2_10ms_elnodyn_2	23.17	24.02	0.85
6GPS_1_2_10ms_elnodyn_3	23.27	24.02	0.75
6I9K_1_10ms_elnodyn	23.26	23.85	0.59
6I9K_1_10ms_elnodyn_2	23.39	23.85	0.46
6I9K_1_10ms_elnodyn_3	23.44	23.85	0.41

Table 4.2: Continued.

	VAL65 ^{2,41} - MET243 ^{6,38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6I9K_2_10ms_elndyn	22.42	23.99	1.57
6I9K_2_10ms_elndyn_2	22.11	23.99	1.88
6I9K_2_10ms_elndyn_3	23.75	23.99	0.24
6IQL_1_1_10ms_elndyn	22.76	23.87	1.12
6IQL_1_1_10ms_elndyn_2	22.65	23.87	1.22
6IQL_1_1_10ms_elndyn_3	22.57	23.87	1.31
6IQL_1_2_10ms_elndyn	24.60	24.27	-0.33
6IQL_1_2_10ms_elndyn_2	24.66	24.27	-0.40
6IQL_1_2_10ms_elndyn_3	25.20	24.27	-0.93
6K1Q_1_1_10ms_elndyn	22.57	24.31	1.75
6K1Q_1_1_10ms_elndyn_2	22.37	24.31	1.95
6K1Q_1_1_10ms_elndyn_3	22.52	24.31	1.79
6K1Q_1_1_10ms_elndyn_4	22.80	24.31	1.51
6K1Q_1_1_10ms_elndyn_5	22.40	24.31	1.91
6K1Q_1_2_10ms_elndyn	23.76	23.83	0.07

Table 4.2: Continued.

	VAL65 ^{2.41} - MET243 ^{6.38}		
	114-525		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6K1Q_1_2_10ms_elndyn_2	23.66	23.83	0.17
6K1Q_1_2_10ms_elndyn_3	23.42	23.83	0.41
6OFJ_1_10ms_elndyn	22.62	24.21	1.59
6OFJ_1_10ms_elndyn_2	22.66	24.21	1.56
6OFJ_1_10ms_elndyn_3	22.59	24.21	1.63
6RNK_1_10ms_elndyn	22.40	24.08	1.68
6RNK_1_10ms_elndyn_2	22.20	24.08	1.88
6RNK_1_10ms_elndyn_3	22.25	24.08	1.83
6RNK_2_10ms_elndyn	24.08	24.35	0.27
6RNK_2_10ms_elndyn_2	23.65	24.35	0.69
6RNK_2_10ms_elndyn_3	23.85	24.35	0.49
6RZ5_1_10ms_elndyn	22.72	23.68	0.96
6RZ5_1_10ms_elndyn_2	21.96	23.68	1.72
6RZ5_1_10ms_elndyn_3	22.03	23.68	1.65

Table 4.3 shows the differences between the backbone atoms of THR120^{3,44} and PHE301^{7,52}. To detect the considerable change in studied systems we set a cut-off as 1.00 Å. 4DKL_1, 6K1Q_1_1, and 6K1Q_1_2 were the systems that fitted to this threshold. When we combined the systems that satisfied both the VAL65^{2,41}-MET243^{6,38}, and THR120^{3,44}-PHE301^{7,52} decrease, 6K1Q_1_1 was the only system that met both conditions.

Table 4.3: The distance between Backbone atoms of THR120^{3.44} and PHE301^{7.52}, in CG simulations. Delta distance is the difference between the simulation starting distance and simulation average distance. The distances which has greater then 1.00 Å value, highlighted in green color.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	ΔDistance (Å)
2I37_1_10ms_elndyn	14.81	15.95	1.14
2I37_1_10ms_elndyn_2	15.27	15.95	0.68
2I37_1_10ms_elndyn_3	15.17	15.95	0.78
2Z73_1_10ms_elndyn	15.40	16.01	0.60
2Z73_1_10ms_elndyn_2	15.01	16.01	0.99
2Z73_1_10ms_elndyn_3	15.33	16.01	0.67
2ZIY_1_10ms_elndyn	15.03	15.60	0.57
2ZIY_1_10ms_elndyn_2	15.26	15.60	0.34
2ZIY_1_10ms_elndyn_3	15.06	15.60	0.54
2ZIY_1_10ms_elndyn_4	14.99	15.60	0.61
3CAP_1_10ms_elndyn	15.42	16.01	0.59
3CAP_1_10ms_elndyn_2	15.57	16.01	0.44
3CAP_1_10ms_elndyn_3	15.46	16.01	0.55

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
3OE8_1_10ms_elnodyn	14.86	15.94	1.08
3OE8_1_10ms_elnodyn_2	15.06	15.94	0.88
3OE8_1_10ms_elnodyn_3	15.55	15.94	0.39
3OE9_1_10ms_elnodyn	14.93	15.77	0.84
3OE9_1_10ms_elnodyn_2	14.49	15.77	1.27
3OE9_1_10ms_elnodyn_3	14.20	15.77	1.57
3OE9_2_1_10ms_elnodyn	15.19	15.89	0.71
3OE9_2_1_10ms_elnodyn_2	15.30	15.89	0.59
3OE9_2_1_10ms_elnodyn_3	15.32	15.89	0.58
3OE9_2_1_10ms_elnodyn_4	15.25	15.89	0.64
3OE9_2_2_10ms_elnodyn	15.11	15.43	0.32
3OE9_2_2_10ms_elnodyn_2	15.23	15.43	0.20
3OE9_2_2_10ms_elnodyn_3	15.23	15.43	0.20
3RZE_1_10ms_elnodyn	15.26	15.65	0.39
3RZE_1_10ms_elnodyn_2	15.00	15.65	0.65

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
3RZE_1_10ms_elnedyn_3	14.90	15.65	0.75
3V2Y_1_10ms_elnedyn	15.22	15.92	0.70
3V2Y_1_10ms_elnedyn_2	15.61	15.92	0.31
3V2Y_1_10ms_elnedyn_3	15.27	15.92	0.65
3V2Y_2_10ms_elnedyn	14.98	15.81	0.83
3V2Y_2_10ms_elnedyn_2	14.96	15.81	0.85
3V2Y_2_10ms_elnedyn_3	15.24	15.81	0.57
4BVN_1_10ms_elnedyn	15.37	15.99	0.62
4BVN_1_10ms_elnedyn_2	15.45	15.99	0.54
4BVN_1_10ms_elnedyn_3	15.35	15.99	0.64
4BVN_1_10ms_elnedyn_4	15.47	15.99	0.53
4DJH_1_10ms_elnedyn	15.19	15.88	0.69
4DJH_1_10ms_elnedyn_2	15.21	15.88	0.66
4DJH_1_10ms_elnedyn_3	15.02	15.88	0.86
4DKL_1_10ms_elnedyn	14.68	16.10	1.42

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4DKL_1_10ms_elnedyn_2	15.08	16.10	1.02
4DKL_1_10ms_elnedyn_3	15.03	16.10	1.07
4EIY_1_10ms_elnedyn	15.16	15.77	0.61
4EIY_1_10ms_elnedyn_2	15.05	15.77	0.73
4EIY_1_10ms_elnedyn_3	15.28	15.77	0.50
4GPO_1_10ms_elnedyn	15.28	15.56	0.28
4GPO_1_10ms_elnedyn_2	15.17	15.56	0.40
4GPO_1_10ms_elnedyn_3	15.39	15.56	0.17
4GPO_2_10ms_elnedyn	14.97	15.57	0.60
4GPO_2_10ms_elnedyn_2	14.90	15.57	0.67
4GPO_2_10ms_elnedyn_3	15.19	15.57	0.37
4MBS_1_1_10ms_elnedyn	15.98	15.97	-0.02
4MBS_1_1_10ms_elnedyn_2	15.12	15.97	0.84
4MBS_1_1_10ms_elnedyn_3	15.52	15.97	0.45
4MBS_1_2_10ms_elnedyn	15.18	16.01	0.83

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4MBS_1_2_10ms_elnedyn_2	15.12	16.01	0.90
4MBS_1_2_10ms_elnedyn_3	15.15	16.01	0.86
4NTJ_1_10ms_elnedyn	14.95	15.51	0.56
4NTJ_1_10ms_elnedyn_2	14.79	15.51	0.72
4NTJ_1_10ms_elnedyn_3	15.01	15.51	0.49
4OR2_1_10ms_elnedyn	15.29	15.68	0.39
4OR2_1_10ms_elnedyn_2	15.26	15.68	0.42
4OR2_1_10ms_elnedyn_3	15.34	15.68	0.34
4UG2_1_1_10ms_elnedyn	14.81	15.38	0.57
4UG2_1_1_10ms_elnedyn_2	14.53	15.38	0.85
4UG2_1_1_10ms_elnedyn_3	14.56	15.38	0.81
4UG2_1_2_10ms_elnedyn	14.77	15.29	0.53
4UG2_1_2_10ms_elnedyn_2	14.72	15.29	0.57
4UG2_1_2_10ms_elnedyn_3	15.08	15.29	0.21
4WW3_1_10ms_elnedyn	14.82	15.52	0.70

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4WW3_1_10ms_elnedyn_2	14.67	15.52	0.85
4WW3_1_10ms_elnedyn_3	14.26	15.52	1.26
4XNV_1_1_10ms_elnedyn	15.17	15.57	0.41
4XNV_1_1_10ms_elnedyn_2	15.16	15.57	0.42
4XNV_1_1_10ms_elnedyn_3	15.15	15.57	0.42
4XNV_1_2_10ms_elnedyn	15.29	16.16	0.87
4XNV_1_2_10ms_elnedyn_2	15.45	16.16	0.71
4XNV_1_2_10ms_elnedyn_3	15.47	16.16	0.69
4XNV_2_1_10ms_elnedyn	15.46	15.33	-0.13
4XNV_2_1_10ms_elnedyn_2	15.25	15.33	0.08
4XNV_2_1_10ms_elnedyn_3	15.01	15.33	0.32
4XNV_2_2_10ms_elnedyn	15.36	16.12	0.77
4XNV_2_2_10ms_elnedyn_2	15.42	16.12	0.70
4XNV_2_2_10ms_elnedyn_3	15.46	16.12	0.66
4XNV_2_2_10ms_elnedyn_4	15.27	16.12	0.85

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4XNW_1_1_10ms_elnedyn	15.11	15.83	0.71
4XNW_1_1_10ms_elnedyn_2	15.31	15.83	0.52
4XNW_1_1_10ms_elnedyn_3	15.06	15.83	0.77
4XNW_1_2_10ms_elnedyn	15.50	16.07	0.57
4XNW_1_2_10ms_elnedyn_2	15.48	16.07	0.58
4XNW_1_2_10ms_elnedyn_3	15.58	16.07	0.49
4XT3_1_10ms_elnedyn	14.90	15.50	0.59
4XT3_1_10ms_elnedyn_2	15.18	15.50	0.32
4XT3_1_10ms_elnedyn_3	14.58	15.50	0.92
4XT3_1_10ms_elnedyn_4	15.13	15.50	0.37
4Z34_1_1_10ms_elnedyn	15.28	15.53	0.25
4Z34_1_1_10ms_elnedyn_2	15.08	15.53	0.45
4Z34_1_1_10ms_elnedyn_3	15.04	15.53	0.49
4Z34_1_2_10ms_elnedyn	15.19	15.71	0.52
4Z34_1_2_10ms_elnedyn_2	15.03	15.71	0.68

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
4Z34_1_2_10ms_elnedyn_3	15.14	15.71	0.57
5A8E_1_10ms_elnedyn	15.76	15.99	0.24
5A8E_1_10ms_elnedyn_2	14.69	15.99	1.30
5A8E_1_10ms_elnedyn_3	16.37	15.99	-0.38
5C1M_1_10ms_elnedyn	15.16	15.93	0.77
5C1M_1_10ms_elnedyn_2	15.23	15.93	0.71
5C1M_1_10ms_elnedyn_3	15.19	15.93	0.74
5D5B_1_10ms_elnedyn	15.08	15.92	0.84
5D5B_1_10ms_elnedyn_2	15.15	15.92	0.77
5D5B_1_10ms_elnedyn_3	15.19	15.92	0.73
5GLH_1_10ms_elnedyn	15.15	15.72	0.57
5GLH_1_10ms_elnedyn_2	14.97	15.72	0.75
5GLH_1_10ms_elnedyn_3	15.14	15.72	0.58
5GLH_1_10ms_elnedyn_4	15.37	15.72	0.35
5GLH_1_10ms_elnedyn_5	15.07	15.72	0.65

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5GLH_1_10ms_elnedyn_6	15.02	15.72	0.70
5GLH_1_10ms_elnedyn_7	15.14	15.72	0.58
5JQH_1_10ms_elnedyn	15.52	15.66	0.13
5JQH_1_10ms_elnedyn_2	15.29	15.66	0.37
5JQH_1_10ms_elnedyn_3	15.10	15.66	0.56
5LWE_1_1_10ms_elnedyn	15.39	15.72	0.33
5LWE_1_1_10ms_elnedyn_2	15.25	15.72	0.47
5LWE_1_1_10ms_elnedyn_3	15.26	15.72	0.46
5LWE_1_2_10ms_elnedyn	14.94	16.02	1.08
5LWE_1_2_10ms_elnedyn_2	15.12	16.02	0.89
5LWE_1_2_10ms_elnedyn_3	14.86	16.02	1.16
5LWE_2_10ms_elnedyn	15.12	15.62	0.50
5LWE_2_10ms_elnedyn_2	15.09	15.62	0.53
5LWE_2_10ms_elnedyn_3	15.06	15.62	0.56
5N2S_1_10ms_elnedyn	14.86	15.64	0.78

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5N2S_1_10ms_elnedyn_2	14.28	15.64	1.36
5N2S_1_10ms_elnedyn_3	14.99	15.64	0.65
5T04_1_10ms_elnedyn	15.46	15.77	0.31
5T04_1_10ms_elnedyn_2	15.46	15.77	0.31
5T04_1_10ms_elnedyn_3	15.50	15.77	0.27
5TUD_1_10ms_elnedyn	15.47	16.07	0.60
5TUD_1_10ms_elnedyn_5	15.48	16.07	0.60
5TUD_1_10ms_elnedyn_6	15.49	16.07	0.58
5UEN_1_1_10ms_elnedyn	14.65	15.34	0.69
5UEN_1_1_10ms_elnedyn_2	14.35	15.34	0.98
5UEN_1_1_10ms_elnedyn_3	14.62	15.34	0.72
5UEN_1_2_10ms_elnedyn	15.26	15.81	0.55
5UEN_1_2_10ms_elnedyn_2	15.11	15.81	0.70
5UEN_1_2_10ms_elnedyn_3	15.53	15.81	0.28
5UEN_1_2_10ms_elnedyn_4	15.35	15.81	0.46

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
5WIV_2_10ms_elnedyn	14.95	15.90	0.96
5WIV_2_10ms_elnedyn_2	15.05	15.90	0.85
5WIV_2_10ms_elnedyn_3	14.89	15.90	1.01
5WIV_2_10ms_elnedyn_4	14.99	15.90	0.91
5YWY_1_10ms_elnedyn	15.08	15.99	0.91
5YWY_1_10ms_elnedyn_2	15.07	15.99	0.92
5YWY_1_10ms_elnedyn_6	15.05	15.99	0.94
5ZTY_1_1_10ms_elnedyn	15.73	15.61	-0.11
5ZTY_1_1_10ms_elnedyn_2	15.48	15.61	0.13
5ZTY_1_1_10ms_elnedyn_3	15.28	15.61	0.33
5ZTY_1_2_10ms_elnedyn	15.16	15.57	0.41
5ZTY_1_2_10ms_elnedyn_2	15.48	15.57	0.09
5ZTY_1_2_10ms_elnedyn_3	15.13	15.57	0.44
6AK3_1_10ms_elnedyn	14.97	15.02	0.05
6AK3_1_10ms_elnedyn_2	14.90	15.02	0.12

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6AK3_1_10ms_elnedyn_3	14.93	15.02	0.09
6AK3_2_10ms_elnedyn	15.36	15.94	0.58
6AK3_2_10ms_elnedyn_2	15.76	15.94	0.17
6AK3_2_10ms_elnedyn_3	15.22	15.94	0.72
6AKX_1_1_10ms_elnedyn	15.59	15.93	0.33
6AKX_1_1_10ms_elnedyn_2	15.34	15.93	0.59
6AKX_1_1_10ms_elnedyn_3	15.52	15.93	0.40
6AKX_1_2_10ms_elnedyn	15.13	15.73	0.60
6AKX_1_2_10ms_elnedyn_2	14.91	15.73	0.82
6AKX_1_2_10ms_elnedyn_3	14.93	15.73	0.80
6B73_1_10ms_elnedyn	15.72	16.16	0.43
6B73_1_10ms_elnedyn_2	16.03	16.16	0.13
6B73_1_10ms_elnedyn_3	15.90	16.16	0.25
6DO1_1_1_10ms_elnedyn	14.91	15.59	0.69
6DO1_1_1_10ms_elnedyn_2	14.65	15.59	0.94

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6DO1_1_1_10ms_elnodyn_3	14.87	15.59	0.72
6DO1_1_2_10ms_elnodyn	14.70	15.88	1.18
6DO1_1_2_10ms_elnodyn_2	15.05	15.88	0.82
6DO1_1_2_10ms_elnodyn_3	14.86	15.88	1.02
6DO1_1_2_10ms_elnodyn_4	14.70	15.88	1.18
6GPS_1_1_10ms_elnodyn	15.43	15.94	0.50
6GPS_1_1_10ms_elnodyn_2	15.42	15.94	0.51
6GPS_1_1_10ms_elnodyn_3	15.46	15.94	0.48
6GPS_1_2_10ms_elnodyn	15.06	15.76	0.70
6GPS_1_2_10ms_elnodyn_2	15.17	15.76	0.60
6GPS_1_2_10ms_elnodyn_3	14.84	15.76	0.92
6I9K_1_10ms_elnodyn	15.26	15.68	0.41
6I9K_1_10ms_elnodyn_2	14.92	15.68	0.76
6I9K_1_10ms_elnodyn_3	14.77	15.68	0.91
6I9K_2_10ms_elnodyn	15.40	15.94	0.54

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6I9K_2_10ms_elnedyn_2	15.11	15.94	0.82
6I9K_2_10ms_elnedyn_3	14.69	15.94	1.25
6IQL_1_1_10ms_elnedyn	15.18	16.10	0.92
6IQL_1_1_10ms_elnedyn_2	15.69	16.10	0.41
6IQL_1_1_10ms_elnedyn_3	15.46	16.10	0.64
6IQL_1_2_10ms_elnedyn	15.33	15.63	0.31
6IQL_1_2_10ms_elnedyn_2	15.18	15.63	0.45
6IQL_1_2_10ms_elnedyn_3	14.44	15.63	1.19
6K1Q_1_1_10ms_elnedyn	14.03	15.41	1.38
6K1Q_1_1_10ms_elnedyn_2	14.31	15.41	1.10
6K1Q_1_1_10ms_elnedyn_3	14.05	15.41	1.37
6K1Q_1_1_10ms_elnedyn_4	14.13	15.41	1.29
6K1Q_1_1_10ms_elnedyn_5	14.23	15.41	1.18
6K1Q_1_2_10ms_elnedyn	14.33	16.02	1.70
6K1Q_1_2_10ms_elnedyn_2	14.66	16.02	1.36

Table 4.3: Continued.

	THR120 ^{3.44} - PHE301 ^{7.52}		
	242-659		
	Simulation Average (Å)	Step5 Distance (Å)	Δ Distance (Å)
6K1Q_1_2_10ms_elnedyn_3	14.67	16.02	1.35
6OFJ_1_10ms_elnedyn	15.78	15.54	-0.25
6OFJ_1_10ms_elnedyn_2	15.64	15.54	-0.10
6OFJ_1_10ms_elnedyn_3	15.68	15.54	-0.14
6RNK_1_10ms_elnedyn	15.44	15.57	0.13
6RNK_1_10ms_elnedyn_2	15.13	15.57	0.44
6RNK_1_10ms_elnedyn_3	15.15	15.57	0.42
6RNK_2_10ms_elnedyn	14.58	15.31	0.73
6RNK_2_10ms_elnedyn_2	14.32	15.31	0.99
6RNK_2_10ms_elnedyn_3	14.73	15.31	0.58
6RZ5_1_10ms_elnedyn	15.19	16.05	0.86
6RZ5_1_10ms_elnedyn_2	15.18	16.05	0.87
6RZ5_1_10ms_elnedyn_3	15.09	16.05	0.96

Table 4.4 shows the GPCRdb distance changes in all studied systems. If a decrease from the threshold value (set to 7.00 Å) was observed in at least two of the three independent simulations, these interfaces (3OE9_2_1, 4XNV_2_1, 6B73_1, 6OFJ_1 and 6RZ5_1) were selected to proceed AA simulations.

Table 4.4: The GPCRdb distance, in CG simulations. The distance between backbone atoms of THR120^{3.44} and PHE301^{7.52}, subtracted from the distance between VAL65^{2.41} and MET243^{6.38} to obtain GPCRdb distance. Delta distance is the difference between the simulation starting distance and simulation average distance. The distances which has less then 7.00 Å value, highlighted in green color.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
2I37_1_10ms_elnedyn	8.44	8.13	-0.31
2I37_1_10ms_elnedyn_2	7.46	8.13	0.67
2I37_1_10ms_elnedyn_3	7.71	8.13	0.42
2Z73_1_10ms_elnedyn	7.33	7.88	0.56
2Z73_1_10ms_elnedyn_2	7.61	7.88	0.27
2Z73_1_10ms_elnedyn_3	7.71	7.88	0.18
2Z1Y_1_10ms_elnedyn	7.02	8.77	1.75
2Z1Y_1_10ms_elnedyn_2	7.96	8.77	0.81
2Z1Y_1_10ms_elnedyn_3	7.32	8.77	1.45
2Z1Y_1_10ms_elnedyn_4	7.55	8.77	1.22
3CAP_1_10ms_elnedyn	7.63	8.24	0.61

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
3CAP_1_10ms_elndyn_2	7.36	8.24	0.88
3CAP_1_10ms_elndyn_3	7.57	8.24	0.67
3OE8_1_10ms_elndyn	7.71	8.15	0.44
3OE8_1_10ms_elndyn_2	8.39	8.15	-0.23
3OE8_1_10ms_elndyn_3	7.89	8.15	0.26
3OE9_1_10ms_elndyn	8.34	8.18	-0.16
3OE9_1_10ms_elndyn_2	8.55	8.18	-0.37
3OE9_1_10ms_elndyn_3	8.99	8.18	-0.80
3OE9_2_1_10ms_elndyn	7.30	8.36	1.06
3OE9_2_1_10ms_elndyn_2	6.66	8.36	1.70
3OE9_2_1_10ms_elndyn_3	6.66	8.36	1.70
3OE9_2_1_10ms_elndyn_4	7.19	8.36	1.17
3OE9_2_2_10ms_elndyn	7.53	8.48	0.96
3OE9_2_2_10ms_elndyn_2	7.33	8.48	1.16

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δ distance (Å)
3OE9_2_2_10ms_elndyn_3	7.39	8.48	1.10
3RZE_1_10ms_elndyn	7.58	8.29	0.71
3RZE_1_10ms_elndyn_2	8.73	8.29	-0.44
3RZE_1_10ms_elndyn_3	8.58	8.29	-0.29
3V2Y_1_10ms_elndyn	8.76	8.31	-0.45
3V2Y_1_10ms_elndyn_2	8.05	8.31	0.26
3V2Y_1_10ms_elndyn_3	8.12	8.31	0.19
3V2Y_2_10ms_elndyn	7.36	8.38	1.02
3V2Y_2_10ms_elndyn_2	6.77	8.38	1.60
3V2Y_2_10ms_elndyn_3	8.39	8.38	-0.01
4BVN_1_10ms_elndyn	7.06	8.21	1.15
4BVN_1_10ms_elndyn_2	7.60	8.21	0.60
4BVN_1_10ms_elndyn_3	6.93	8.21	1.27
4BVN_1_10ms_elndyn_4	7.24	8.21	0.97

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
4DJH_1_10ms_elnedyn	7.85	8.14	0.29
4DJH_1_10ms_elnedyn_2	7.79	8.14	0.35
4DJH_1_10ms_elnedyn_3	8.07	8.14	0.07
4DKL_1_10ms_elnedyn	7.89	7.88	-0.02
4DKL_1_10ms_elnedyn_2	7.35	7.88	0.53
4DKL_1_10ms_elnedyn_3	7.60	7.88	0.27
4EIY_1_10ms_elnedyn	8.43	8.42	-0.01
4EIY_1_10ms_elnedyn_2	8.87	8.42	-0.45
4EIY_1_10ms_elnedyn_3	8.26	8.42	0.16
4GPO_1_10ms_elnedyn	7.62	8.36	0.74
4GPO_1_10ms_elnedyn_2	8.31	8.36	0.05
4GPO_1_10ms_elnedyn_3	7.44	8.36	0.92
4GPO_2_10ms_elnedyn	7.84	8.55	0.70
4GPO_2_10ms_elnedyn_2	8.21	8.55	0.33

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
4GPO_2_10ms_elnedyn_3	8.26	8.55	0.28
4MBS_1_1_10ms_elnedyn	7.21	8.10	0.89
4MBS_1_1_10ms_elnedyn_2	7.34	8.10	0.76
4MBS_1_1_10ms_elnedyn_3	6.67	8.10	1.43
4MBS_1_2_10ms_elnedyn	6.96	8.28	1.32
4MBS_1_2_10ms_elnedyn_2	7.11	8.28	1.17
4MBS_1_2_10ms_elnedyn_3	7.10	8.28	1.18
4NTJ_1_10ms_elnedyn	7.70	8.45	0.76
4NTJ_1_10ms_elnedyn_2	7.96	8.45	0.49
4NTJ_1_10ms_elnedyn_3	7.74	8.45	0.72
4OR2_1_10ms_elnedyn	7.73	8.28	0.54
4OR2_1_10ms_elnedyn_2	7.74	8.28	0.53
4OR2_1_10ms_elnedyn_3	7.39	8.28	0.89
4UG2_1_1_10ms_elnedyn	7.44	8.94	1.51

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
4UG2_1_1_10ms_elnedyn_2	7.70	8.94	1.24
4UG2_1_1_10ms_elnedyn_3	7.75	8.94	1.19
4UG2_1_2_10ms_elnedyn	8.62	8.83	0.21
4UG2_1_2_10ms_elnedyn_2	8.95	8.83	-0.11
4UG2_1_2_10ms_elnedyn_3	9.21	8.83	-0.38
4WW3_1_10ms_elnedyn	7.44	8.58	1.14
4WW3_1_10ms_elnedyn_2	7.72	8.58	0.87
4WW3_1_10ms_elnedyn_3	8.27	8.58	0.31
4XNV_1_1_10ms_elnedyn	8.55	8.69	0.14
4XNV_1_1_10ms_elnedyn_2	8.46	8.69	0.23
4XNV_1_1_10ms_elnedyn_3	8.38	8.69	0.31
4XNV_1_2_10ms_elnedyn	8.98	8.27	-0.71
4XNV_1_2_10ms_elnedyn_2	9.01	8.27	-0.74
4XNV_1_2_10ms_elnedyn_3	8.69	8.27	-0.41

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
4XNV_2_1_10ms_elndyn	6.92	8.55	1.64
4XNV_2_1_10ms_elndyn_2	6.95	8.55	1.60
4XNV_2_1_10ms_elndyn_3	6.93	8.55	1.63
4XNV_2_2_10ms_elndyn	7.01	8.11	1.10
4XNV_2_2_10ms_elndyn_2	6.65	8.11	1.45
4XNV_2_2_10ms_elndyn_3	7.17	8.11	0.93
4XNV_2_2_10ms_elndyn_4	7.11	8.11	0.99
4XNW_1_1_10ms_elndyn	7.20	8.48	1.28
4XNW_1_1_10ms_elndyn_2	8.52	8.48	-0.04
4XNW_1_1_10ms_elndyn_3	8.16	8.48	0.32
4XNW_1_2_10ms_elndyn	7.41	8.11	0.69
4XNW_1_2_10ms_elndyn_2	7.58	8.11	0.52
4XNW_1_2_10ms_elndyn_3	7.19	8.11	0.92
4XT3_1_10ms_elndyn	7.41	8.26	0.85

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
4XT3_1_10ms_elnedyn_2	6.80	8.26	1.45
4XT3_1_10ms_elnedyn_3	7.43	8.26	0.82
4XT3_1_10ms_elnedyn_4	7.20	8.26	1.05
4Z34_1_1_10ms_elnedyn	7.59	8.70	1.11
4Z34_1_1_10ms_elnedyn_2	8.08	8.70	0.62
4Z34_1_1_10ms_elnedyn_3	8.19	8.70	0.51
4Z34_1_2_10ms_elnedyn	8.36	8.46	0.10
4Z34_1_2_10ms_elnedyn_2	8.68	8.46	-0.22
4Z34_1_2_10ms_elnedyn_3	7.22	8.46	1.24
5A8E_1_10ms_elnedyn	7.42	7.99	0.57
5A8E_1_10ms_elnedyn_2	8.02	7.99	-0.03
5A8E_1_10ms_elnedyn_3	6.49	7.99	1.51
5C1M_1_10ms_elnedyn	8.03	8.31	0.29
5C1M_1_10ms_elnedyn_2	7.94	8.31	0.37

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
5C1M_1_10ms_elndyn_3	7.37	8.31	0.95
5D5B_1_10ms_elndyn	8.13	8.13	0.00
5D5B_1_10ms_elndyn_2	7.85	8.13	0.29
5D5B_1_10ms_elndyn_3	8.05	8.13	0.08
5GLH_1_10ms_elndyn	7.16	8.41	1.25
5GLH_1_10ms_elndyn_2	7.72	8.41	0.70
5GLH_1_10ms_elndyn_3	7.22	8.41	1.20
5GLH_1_10ms_elndyn_4	7.05	8.41	1.37
5GLH_1_10ms_elndyn_5	7.63	8.41	0.78
5GLH_1_10ms_elndyn_6	7.10	8.41	1.31
5GLH_1_10ms_elndyn_7	7.21	8.41	1.21
5JQH_1_10ms_elndyn	7.25	8.59	1.34
5JQH_1_10ms_elndyn_2	6.95	8.59	1.64
5JQH_1_10ms_elndyn_3	7.21	8.59	1.38

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δ distance (Å)
5LWE_1_1_10ms_elnedyn	7.36	8.48	1.11
5LWE_1_1_10ms_elnedyn_2	7.55	8.48	0.93
5LWE_1_1_10ms_elnedyn_3	7.49	8.48	0.98
5LWE_1_2_10ms_elnedyn	8.52	8.21	-0.31
5LWE_1_2_10ms_elnedyn_2	8.12	8.21	0.09
5LWE_1_2_10ms_elnedyn_3	8.77	8.21	-0.56
5LWE_2_10ms_elnedyn	7.66	8.33	0.67
5LWE_2_10ms_elnedyn_2	7.50	8.33	0.83
5LWE_2_10ms_elnedyn_3	7.38	8.33	0.95
5N2S_1_10ms_elnedyn	9.25	8.51	-0.74
5N2S_1_10ms_elnedyn_2	10.15	8.51	-1.65
5N2S_1_10ms_elnedyn_3	8.95	8.51	-0.44
5T04_1_10ms_elnedyn	7.52	8.56	1.04
5T04_1_10ms_elnedyn_2	7.45	8.56	1.11

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
5T04_1_10ms_elnedyn_3	7.27	8.56	1.29
5TUD_1_10ms_elnedyn	7.62	7.57	-0.05
5TUD_1_10ms_elnedyn_5	7.49	7.57	0.07
5TUD_1_10ms_elnedyn_6	7.52	7.57	0.04
5UEN_1_1_10ms_elnedyn	8.61	8.73	0.12
5UEN_1_1_10ms_elnedyn_2	8.44	8.73	0.29
5UEN_1_1_10ms_elnedyn_3	9.04	8.73	-0.31
5UEN_1_2_10ms_elnedyn	7.34	8.28	0.94
5UEN_1_2_10ms_elnedyn_2	7.54	8.28	0.75
5UEN_1_2_10ms_elnedyn_3	6.79	8.28	1.49
5UEN_1_2_10ms_elnedyn_4	7.42	8.28	0.87
5WIV_2_10ms_elnedyn	7.24	8.15	0.91
5WIV_2_10ms_elnedyn_2	7.44	8.15	0.71
5WIV_2_10ms_elnedyn_3	7.34	8.15	0.81

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
5WIV_2_10ms_elndyn_4	7.25	8.15	0.91
5YWY_1_10ms_elndyn	8.24	8.59	0.35
5YWY_1_10ms_elndyn_2	8.05	8.59	0.54
5YWY_1_10ms_elndyn_6	8.03	8.59	0.56
5ZTY_1_1_10ms_elndyn	7.19	7.84	0.65
5ZTY_1_1_10ms_elndyn_2	7.41	7.84	0.43
5ZTY_1_1_10ms_elndyn_3	8.11	7.84	-0.27
5ZTY_1_2_10ms_elndyn	7.40	8.21	0.81
5ZTY_1_2_10ms_elndyn_2	7.25	8.21	0.96
5ZTY_1_2_10ms_elndyn_3	7.45	8.21	0.76
6AK3_1_10ms_elndyn	7.49	9.27	1.78
6AK3_1_10ms_elndyn_2	7.39	9.27	1.88
6AK3_1_10ms_elndyn_3	7.61	9.27	1.65
6AK3_2_10ms_elndyn	7.69	8.32	0.64

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
6AK3_2_10ms_elnedyn_2	6.82	8.32	1.50
6AK3_2_10ms_elnedyn_3	7.79	8.32	0.53
6AKX_1_1_10ms_elnedyn	7.86	8.37	0.51
6AKX_1_1_10ms_elnedyn_2	8.10	8.37	0.27
6AKX_1_1_10ms_elnedyn_3	7.12	8.37	1.25
6AKX_1_2_10ms_elnedyn	8.69	8.41	-0.29
6AKX_1_2_10ms_elnedyn_2	7.11	8.41	1.30
6AKX_1_2_10ms_elnedyn_3	7.41	8.41	1.00
6B73_1_10ms_elnedyn	6.79	7.99	1.20
6B73_1_10ms_elnedyn_2	6.80	7.99	1.19
6B73_1_10ms_elnedyn_3	6.88	7.99	1.11
6DO1_1_1_10ms_elnedyn	8.01	8.27	0.26
6DO1_1_1_10ms_elnedyn_2	8.91	8.27	-0.64
6DO1_1_1_10ms_elnedyn_3	8.74	8.27	-0.47

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
6DO1_1_2_10ms_elndyn	7.46	8.22	0.76
6DO1_1_2_10ms_elndyn_2	7.20	8.22	1.03
6DO1_1_2_10ms_elndyn_3	7.24	8.22	0.99
6DO1_1_2_10ms_elndyn_4	7.51	8.22	0.71
6GPS_1_1_10ms_elndyn	7.45	8.19	0.75
6GPS_1_1_10ms_elndyn_2	7.80	8.19	0.39
6GPS_1_1_10ms_elndyn_3	7.35	8.19	0.84
6GPS_1_2_10ms_elndyn	7.44	8.26	0.82
6GPS_1_2_10ms_elndyn_2	8.00	8.26	0.26
6GPS_1_2_10ms_elndyn_3	8.43	8.26	-0.17
6I9K_1_10ms_elndyn	8.00	8.17	0.17
6I9K_1_10ms_elndyn_2	8.47	8.17	-0.30
6I9K_1_10ms_elndyn_3	8.67	8.17	-0.50
6I9K_2_10ms_elndyn	7.02	8.05	1.03

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
6I9K_2_10ms_elnedyn_2	7.00	8.05	1.05
6I9K_2_10ms_elnedyn_3	9.06	8.05	-1.01
6IQL_1_1_10ms_elnedyn	7.58	7.77	0.20
6IQL_1_1_10ms_elnedyn_2	6.96	7.77	0.81
6IQL_1_1_10ms_elnedyn_3	7.11	7.77	0.67
6IQL_1_2_10ms_elnedyn	9.28	8.64	-0.64
6IQL_1_2_10ms_elnedyn_2	9.48	8.64	-0.85
6IQL_1_2_10ms_elnedyn_3	10.76	8.64	-2.12
6K1Q_1_1_10ms_elnedyn	8.53	8.90	0.37
6K1Q_1_1_10ms_elnedyn_2	8.05	8.90	0.84
6K1Q_1_1_10ms_elnedyn_3	8.47	8.90	0.43
6K1Q_1_1_10ms_elnedyn_4	8.67	8.90	0.22
6K1Q_1_1_10ms_elnedyn_5	8.17	8.90	0.73
6K1Q_1_2_10ms_elnedyn	9.43	7.81	-1.62

Table 4.4: Continued.

	(VAL65 ^{2.41} - MET243 ^{6.38})- (THR120 ^{3.44} - PHE301 ^{7.52})		
	[(114-525)- (242-659)] Å		
	Simulation Average (Å)	Step5 Distance (Å)	Δdistance (Å)
6K1Q_1_2_10ms_elndyn_2	9.00	7.81	-1.19
6K1Q_1_2_10ms_elndyn_3	8.74	7.81	-0.94
6OFJ_1_10ms_elndyn	6.84	8.68	1.84
6OFJ_1_10ms_elndyn_2	7.02	8.68	1.66
6OFJ_1_10ms_elndyn_3	6.91	8.68	1.77
6RNK_1_10ms_elndyn	6.96	8.51	1.55
6RNK_1_10ms_elndyn_2	7.07	8.51	1.44
6RNK_1_10ms_elndyn_3	7.10	8.51	1.41
6RNK_2_10ms_elndyn	9.49	9.04	-0.46
6RNK_2_10ms_elndyn_2	9.34	9.04	-0.30
6RNK_2_10ms_elndyn_3	9.12	9.04	-0.08
6RZ5_1_10ms_elndyn	7.53	7.63	0.10
6RZ5_1_10ms_elndyn_2	6.78	7.63	0.85
6RZ5_1_10ms_elndyn_3	6.94	7.63	0.69

4.5. Conversion Criteria For CG2AA Simulations

Table 4.5 shows the selection criteria for the conversion of the CG systems into AA representations. The ARG126^{3,50}-PHE239^{6,34} and the GPCRdb distances are assigned as 2 points, the other two (VAL65^{2,41}-MET243^{6,38} and THR120^{3,44}-PHE301^{7,52}) distances assigned as 1 point. The systems were selected if they scored at least 2 points and above. As a result of interatomic distance measurements, following 16 systems were converted to AA representation; 2ZIY_1, 3OE9_2_1, 4MBS_1_2, 4UG2_1_1, 4XNV_2_1, 4XNV_2_2, 4XT3_1, 5GLH_1, 5WIV_2, 6AK3_1, 6B73_1, 6DO1_1_2, 6K1Q_1_1, 6OFJ_1, 6RNK_1, and 6RZ5_1. Although the 4XNV_2_2 system does not meet the conditions, it has been added to the analysis (other interfaces were 4DKL_1, 4WW3_1, and 6K1Q_1_2). The aim was to see how the systems that get 1 point (see Table 4.5) will behave in AA simulations. For comparison, we also simulated the AT1R monomer system. In total we obtained 17 systems, and each system simulated for 1 μ s, and repeated 10 times (17x1 μ s = 170 μ s, in total).

Table 4.5: Selection criteria scoring for the systems that are selected to convert into AA representations and simulations. The ARG126^{3.50}-PHE239^{6.34} and the GPCRdb distances are assigned as 2 points, the other two (VAL65^{2.41}-MET243^{6.38} and THR120^{3.44}-PHE301^{7.52}) distances assigned as 1 point. The systems were selected if they scored at least 2 points and above. Selected 15 systems were highlighted as green. 4DKL_1, 4WW3_1, 4XNV_2_2, and 6K1Q_1_2 systems highlighted as red, since their score is below 2 points, they were not included in AA simulations.

	ARG126 ^{3.50} - PHE239 ^{6.34}	VAL65 ^{2.41} - MET243 ^{6.38}	THR120 ^{3.44} - PHE301 ^{7.52}	GPCRdb Average	Total Score
2I37_1					0
2Z73_1					0
2Z1Y_1	2	1			3
3CAP_1					0
3OE8_1					0
3OE9_1					0
3OE9_2_1		1		2	3
3OE9_2_2					0
3RZE_1					0
3V2Y_1					0
3V2Y_2					0
4BVN_1					0
4DJH_1					0
4DKL_1			1		1
4E1Y_1					0

Table 4.5: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}	VAL65 ^{2.41} - MET243 ^{6.38}	THR120 ^{3.44} - PHE301 ^{7.52}	GPCRdb Average	Total Score
4GPO_1					0
4GPO_2					0
4MBS_1_1					0
4MBS_1_2	2	1			3
4NTJ_1					0
4OR2_1					0
4UG2_1_1	2	1			3
4UG2_1_2					0
4WW3_1		1			1
4XNV_1_1					0
4XNV_1_2					0
4XNV_2_1	2	1		2	5
4XNV_2_2		1			1
4XNW_1_1					0
4XNW_1_2					0
4XT3_1	2				2
4Z34_1_1					0

Table 4.5: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}	VAL65 ^{2.41} - MET243 ^{6.38}	THR120 ^{3.44} - PHE301 ^{7.52}	GPCRdb Average	Total Score
4Z34_1_2					0
5A8E_1					0
5CIM_1					0
5D5B_1					0
5GLH_1	2	1			3
5JQH_1					0
5LWE_1_1					0
5LWE_1_2					0
5LWE_2					0
5N2S_1					0
5T04_1					0
5TUD_1					0
5UEN_1_1					0
5UEN_1_2					0
5WIV_2	2	1			3
5YWY_1					0
5ZTY_1_1					0

Table 4.5: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}	VAL65 ^{2.41} - MET243 ^{6.38}	THR120 ^{3.44} - PHE301 ^{7.52}	GPCRdb Average	Total Score
5ZTY_1_2					0
6AK3_1	2	1			3
6AK3_2					0
6AKX_1_1					0
6AKX_1_2					0
6B73_1				2	2
6DO1_1_1					0
6DO1_1_2	2	1	1		4
6GPS_1_1					0
6GPS_1_2					0
6I9K_1					0
6I9K_2					0
6IQL_1_1					0
6IQL_1_2					0
6K1Q_1_1		1	1		2
6K1Q_1_2			1		1
6OFJ_1	2	1		2	5

Table 4.5: Continued.

	ARG126 ^{3.50} - PHE239 ^{6.34}	VAL65 ^{2.41} - MET243 ^{6.38}	THR120 ^{3.44} - PHE301 ^{7.52}	GPCRdb Average	Total Score
6RNK_1	2	1			3
6RNK_2					0
6RZ5_1				2	2

4.6. Backbone RMSD Evaluation Of The AA MD Simulations

Figure 4.1 shows the chain A (AT1R) backbone RMSD values. The lowest chain A bb RMSD values were observed for the 4XNV_2_2 interface, 2.65 ± 0.55 Å for all repeats. For the individual runs, following RMSD values were obtained, 3.16 ± 0.41 , 2.91 ± 0.51 , 2.18 ± 0.37 , 2.10 ± 0.23 , 2.48 ± 0.28 , 2.25 ± 0.28 , 2.77 ± 0.33 , 2.49 ± 0.35 , 2.77 ± 0.55 , and 3.43 ± 0.60 Å, for the runs, respectively. The highest RMSD values for the AT1R bb atoms were observed in 3OE9_2_1 interface, 3.10 ± 0.68 Å average RMSD value was calculated for 10 repeats. 3.46 ± 0.56 , 3.36 ± 0.78 , 3.02 ± 0.89 , 3.23 ± 0.54 , 3.42 ± 0.60 , 3.59 ± 0.44 , 2.74 ± 0.55 , 2.91 ± 0.38 , 2.80 ± 0.44 , and 2.47 ± 0.37 Å obtained for the 10 repeats, respectively. Overall RMSD analyses show that the used timescale for the simulations is adequate.

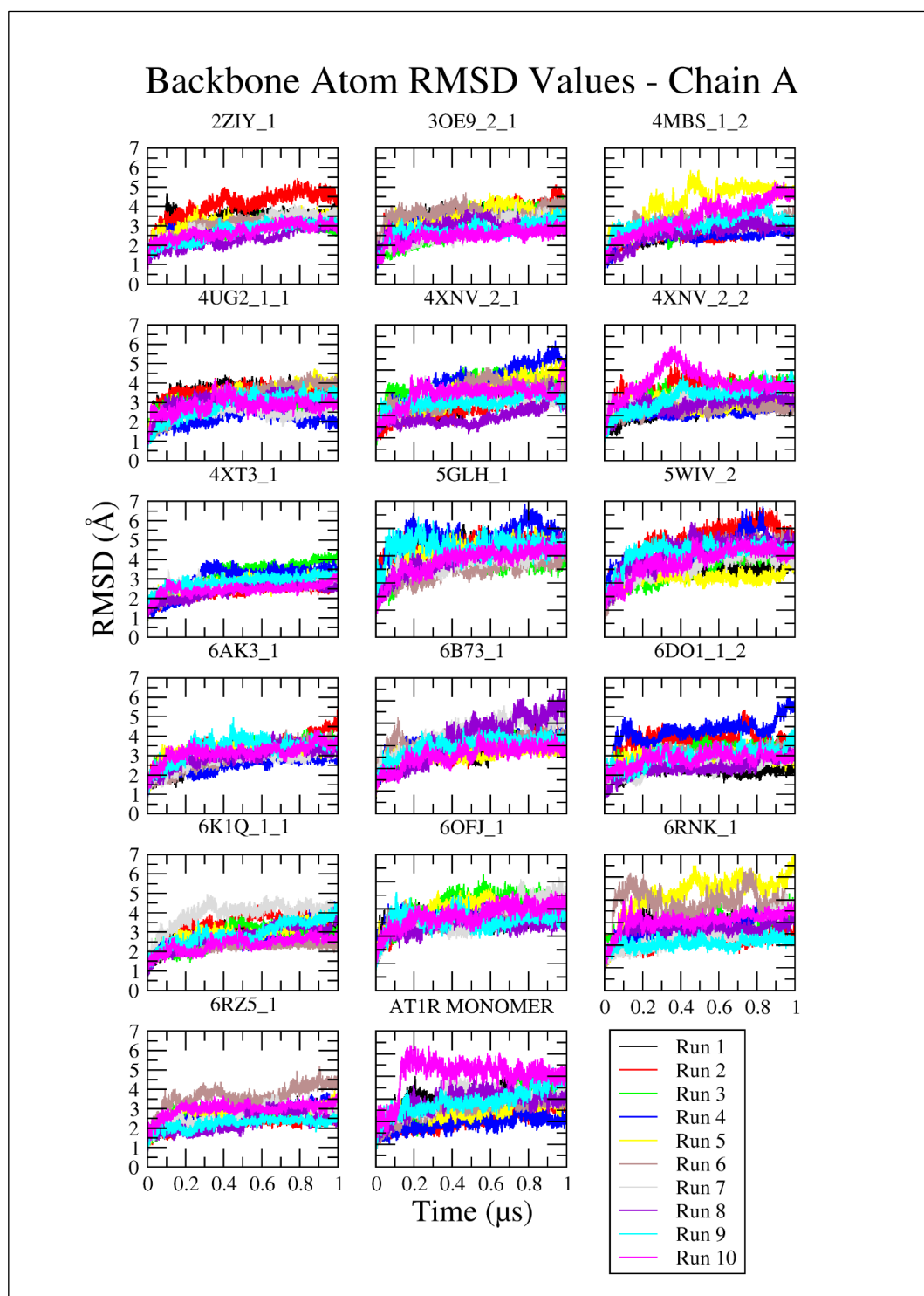


Figure 4.1: RMSD values for the backbone atoms of the chain A (AT1R). Each run is depicted in a different color (e.g. black, red, green, blue and yellow is used for the first, second, third, forth, and fifth runs, respectively).

4.7. GPCRdb Distance Analysis In AA MD Simulations

GPCRdb distance changes throughout the MD simulations for all repeats were given in Figure 4.2. The lowest average value for the GPCRdb distance was observed for the 4XNV_2_2 interface as 1.98 ± 2.79 Å, where the averages for each repeat obtained as 0.98 ± 1.71 , -1.86 ± 1.15 , 2.56 ± 3.35 , -0.07 ± 1.04 , 5.38 ± 0.96 , 3.19 ± 1.50 , 1.84 ± 2.73 , 1.74 ± 1.15 , 4.54 ± 2.49 , and 1.54 ± 1.69 Å, respectively. For the 4XNV_2_2 interface, according to GPCRdb distance criteria, first, second, forth, seventh, eighth, and tenth runs showed inactive structure properties. However, in the third, fifth, sixth, and ninth runs, intermediate structure characteristics were mostly observed. The 4UG2_1_1 interface has the highest average of the GPCRdb distance. The average was 7.83 ± 1.79 Å for this interface. The averages for the individual runs were, 7.96 ± 0.98 , 6.28 ± 0.81 , 6.51 ± 1.31 , 7.21 ± 1.21 , 7.23 ± 1.70 , 6.44 ± 1.39 , 9.03 ± 1.02 , 10.52 ± 1.35 , 8.96 ± 1.42 and 8.14 ± 0.88 Å. Except for the second, third and sixth runs (where intermediate structures were observed), all other repeats showed active structure features. Based on the all frame averages; the dimer systems with lowest GPCRdb distance averages were; 4XNV_2_2 (1.98 ± 2.79 Å), 2ZIY_1 (2.32 ± 2.96 Å), and 6K1Q_1_1 (2.46 ± 3.21 Å). The highest GPCRdb distance averages were observed in 4UG2_1_1 (7.83 ± 1.79 Å), 5GLH_1 (6.98 ± 2.26 Å), and 6RNK_1 (6.87 ± 1.95 Å).

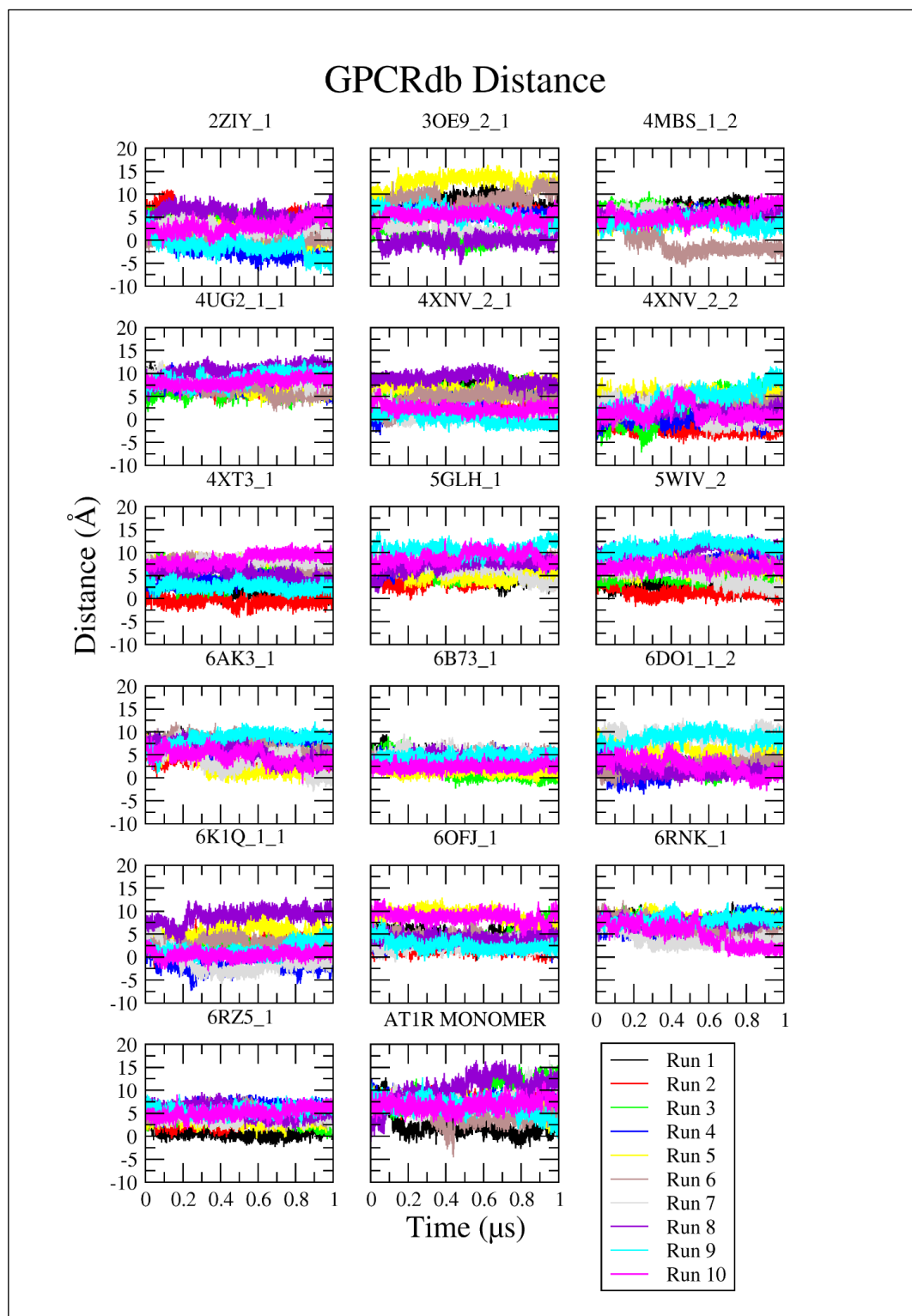


Figure 4.2: The deviation of GPCRdb distance throughout the MD simulations.

4.8. TM3-TM6 Distance Analysis In AA MD Simulations

TM3-TM6 distance changes throughout the MD simulations for all repeats were given in Figure 4.3. The lowest average value for the ARG126-PHE239 distance was observed for the 6K1Q_1_1 interface as 7.28 ± 1.74 Å, where the averages for each repeat obtained as 6.04 ± 0.69 , 6.40 ± 0.84 , 5.71 ± 0.42 , 8.49 ± 0.64 , 9.76 ± 0.96 , 6.29 ± 0.36 , 8.58 ± 0.44 , 11.52 ± 1.54 , 7.77 ± 0.87 , and 5.26 ± 0.30 Å, respectively. The ARG126^{3.50}-PHE239^{6.34} distance calculated as 6.70 Å, and 14.20 Å, in inactive (4YAY) and active (6DO1) crystal structures of AT1R, respectively. According to experimental structures, first, second, third, sixth, and tenth runs showed inactive structure features, however the forth, fifth, seventh, eighth, and ninth repeats were in intermediate structural characteristics. The highest average for the ARG126^{3.50}-PHE239^{6.34} distance was observed in 4UG2_1_1 interface with the average of 11.44 ± 1.90 Å. The averages for each repeat was as follows; 12.89 ± 0.55 , 9.09 ± 0.55 , 11.87 ± 1.06 , 11.03 ± 0.83 , 10.97 ± 1.70 , 8.78 ± 0.72 , 13.50 ± 1.08 , 14.10 ± 0.74 , 11.82 ± 0.51 , and 10.31 ± 0.61 Å. All repeats showed intermediate structural characteristics. Based on the all frame averages; the dimer systems with lowest ARG126^{3.50}-PHE239^{6.34} distance averages were 6K1Q_1_1 (7.58 ± 2.07 Å), 2ZIY_1 (8.36 ± 1.10 Å), 4XNV_2_2 (8.44 ± 1.00 Å). The highest ARG126^{3.50}-PHE239^{6.34} distance averages were observed in 4UG2_1_1 (11.44 ± 1.90 Å), 5WIV_2 (11.24 ± 2.81 Å), and 6RNK_1 (10.80 ± 1.74 Å).

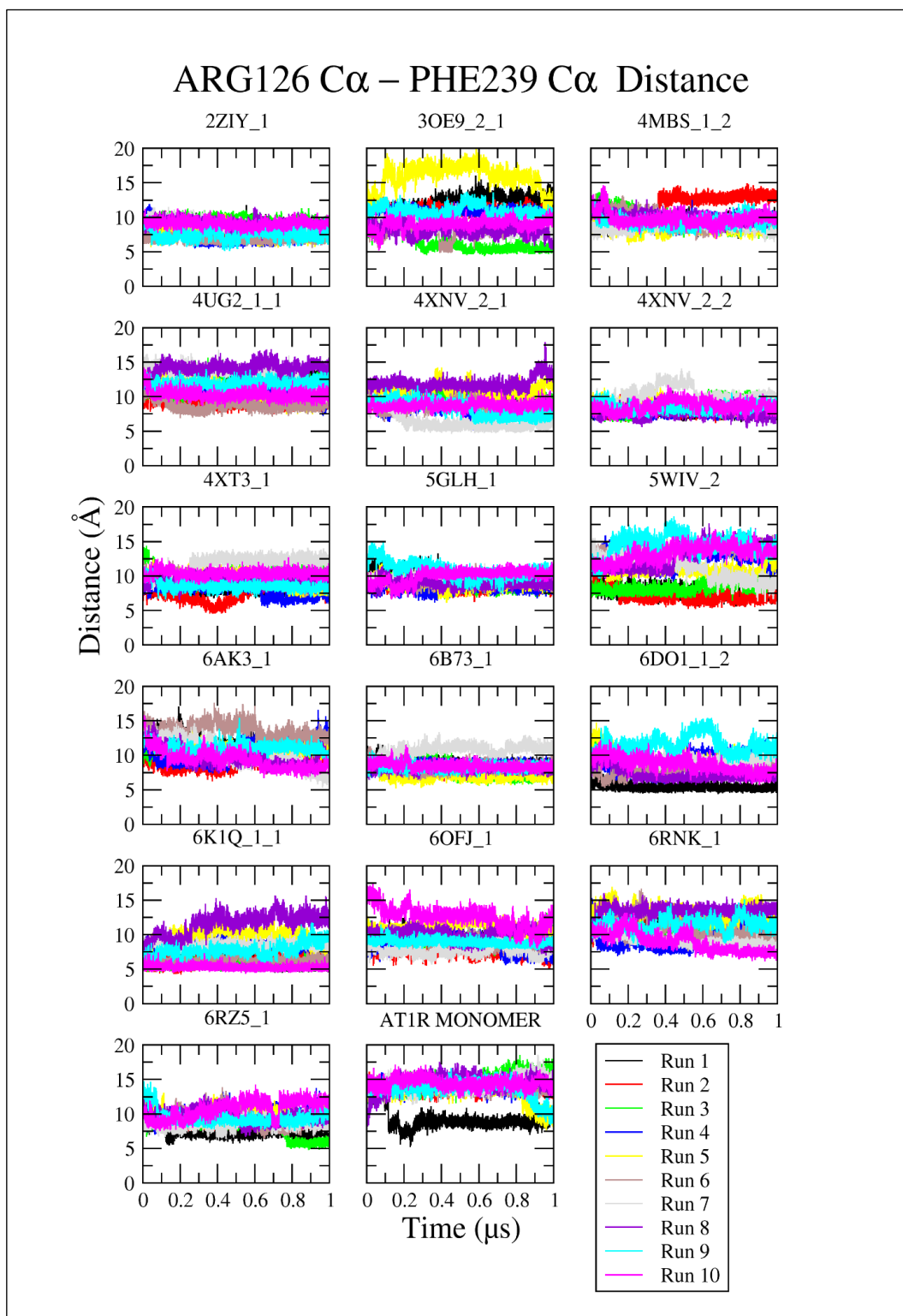


Figure 4.3: The distance between carbon α atoms of ARG126^{3,50}-PHE239^{6,34}.

4.9. AT1R Stabilized In Inactive State In 2ZIY_1 And 6K1Q_1_1 Interfaces

Figure 4.4 shows the conformational landscape of the AT1R, in different heterodimer interfaces. The conformational landscape is mapped with the projection of GPCRdb distance with the angle between VAL65^{2,41}, SER252^{6,47}, and PHE239^{6,34}. Table 4.6 summarizes the percentages of the frames for each system under investigation. In the 2ZIY_1, 4MBS_1_2, 4XNV_2_1, 4XNV_2_2, 4XT3_1, 6B73_1, 6DO1_1_2, 6K1Q_1_1, 6RNK_1, and 6RZ5_1 systems, the percentage of the active frames were lower than 1%. Similarly, in the 4MBS_1_2, 4UG2_1_1, 4XNV_2_2, 5GLH_1, 5WIV_2, 6AK3_1, 6B73_1, 6OFJ_1, 6RNK_1, 6RZ5_1 interfaces and in AT1R monomer system, the percentages of the inactive frames were lower than 1%. We considered the systems in which active or inactive structural features were observed at least 5% in simulation trajectories; 2ZIY_1, 3OE9_2_1, 4UG2_1_1, 4XT3_1, 5GLH_1, 5WIV_2, 6AK3_1, 6K1Q_1_1, and AT1R monomer. For these selected inactive interfaces, 2ZIY_1 was the second system based on the average GPCRdb distance, and 6K1Q was the third one (4XNV_2_2 was the lowest). When ARG126^{3,50}-PHE239^{6,34} distance is taken into account, 6K1Q_1_1, and 2ZIY_1 have the first and second lowest average values. For the active interfaces 5WIV_2, and 6AK3_1 were the fifth and sixth in terms of the highest GPCRdb distance averages (where 4UG2_1_1 has the highest average). 5WIV_2, and 6AK3_1 were the second, and fifth highest ARG126^{3,50}-PHE239^{6,34} distance average values (4UG2_1_1 has the highest average). The selected systems were either among the lowest (for inactive systems) or the highest (for active systems) in the specified interatomic distance comparisons and were in agreement with each other distances.

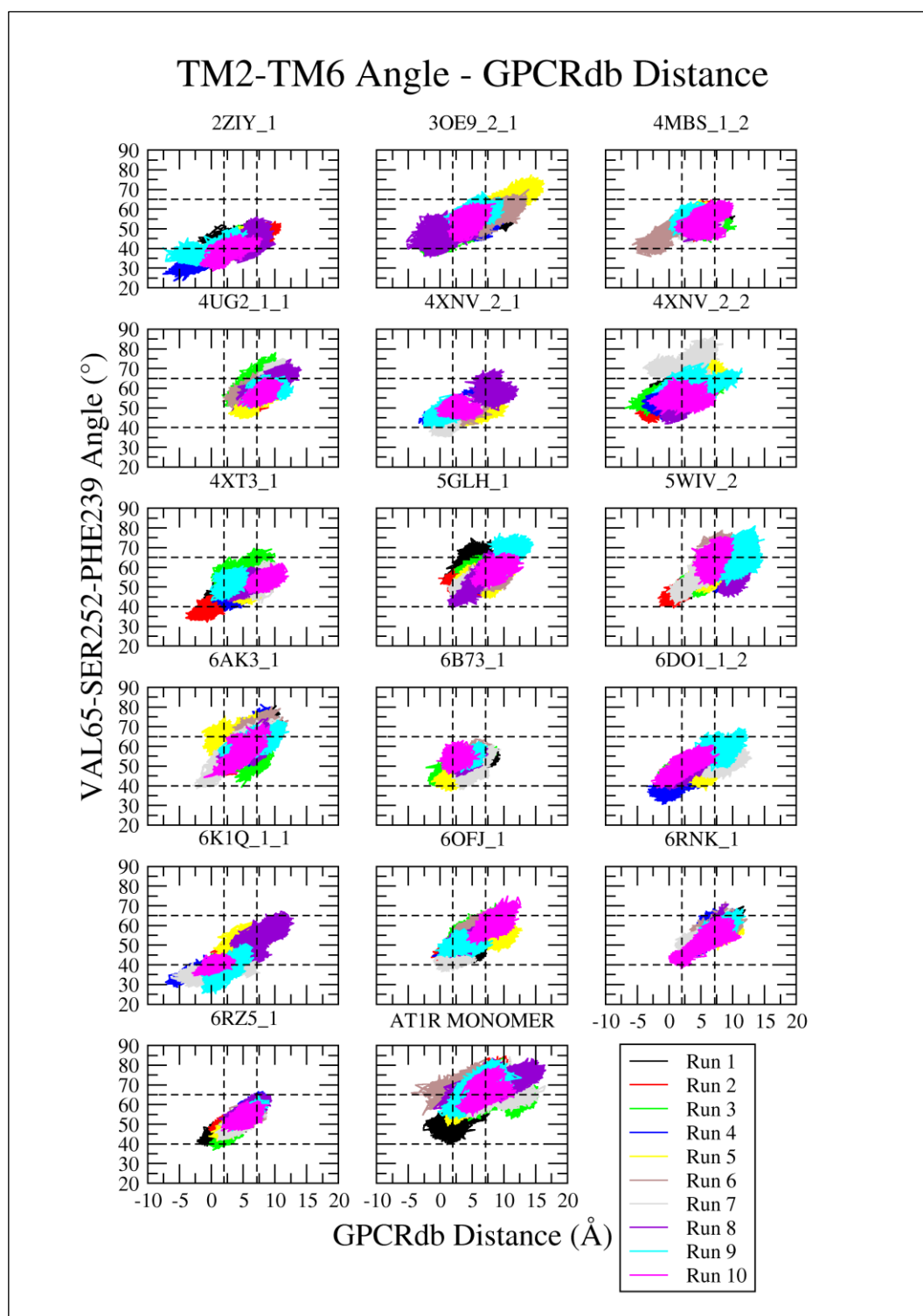


Figure 4.4: Conformational landscape of AT1R receptor in AT1R-AT2R heterodimer with different interfaces, generated using the GPCRdb distance, and the angle among the C α atoms of VAL65^{2.41}, SER252^{6.47}, and PHE239^{6.34}.

Table 4.6: The percentages for the state definitions. The angle between VAL65^{2,41}, SER252^{6,47}, and PHE239^{6,34} and GPCRdb distance were used to categorize the frames.

	Percentages			
	Active	Intermediate	Inactive	Not Satisfied
2ZIIY_1	0.00	43.25	36.82	19.93
3OE9_2_1	7.69	50.85	1.36	40.10
4MBS_1_2	0.00	80.82	0.70	18.48
4UG2_1_1	11.92	36.73	0.00	51.35
4XNV_2_1	0.18	50.51	2.06	47.25
4XNV_2_2	0.64	34.35	0.00	65.01
4XT3_1	0.09	50.07	6.04	43.81
5GLH_1	7.61	50.37	0.00	42.01
5WIV_2	20.25	37.45	0.00	42.29
6AK3_1	16.15	44.75	0.01	39.09
6B73_1	0.00	82.25	0.10	17.64
6DO1_1_2	0.21	48.25	4.07	47.47
6K1Q_1_1	0.02	33.56	34.90	31.52
6OFJ_1	1.85	66.42	0.35	31.38
6RNK_1	0.33	42.73	0.00	56.93
6RZ5_1	0.02	83.90	0.14	15.94
AT1R_Monomer	40.98	16.22	0.00	42.80

4.10. Cluster Analyses Corroborate The Similarities Of Most Populated States And Experimental Structures Of AT1R

First we compared the AT1R monomer most populated cluster with the experimental structure of AT1R (6DO1). In the AT1R monomer system, 26 clusters were obtained, where the first three clusters cover 74.39% of all active frames. The best cluster structure (most populated structure - from now on best cluster structure will be named as representative structure) cover 46.21% of all active frames, and when aligned to 6DO1 0.90 Å RMS value was obtained (see Figure 4.5, panel A).

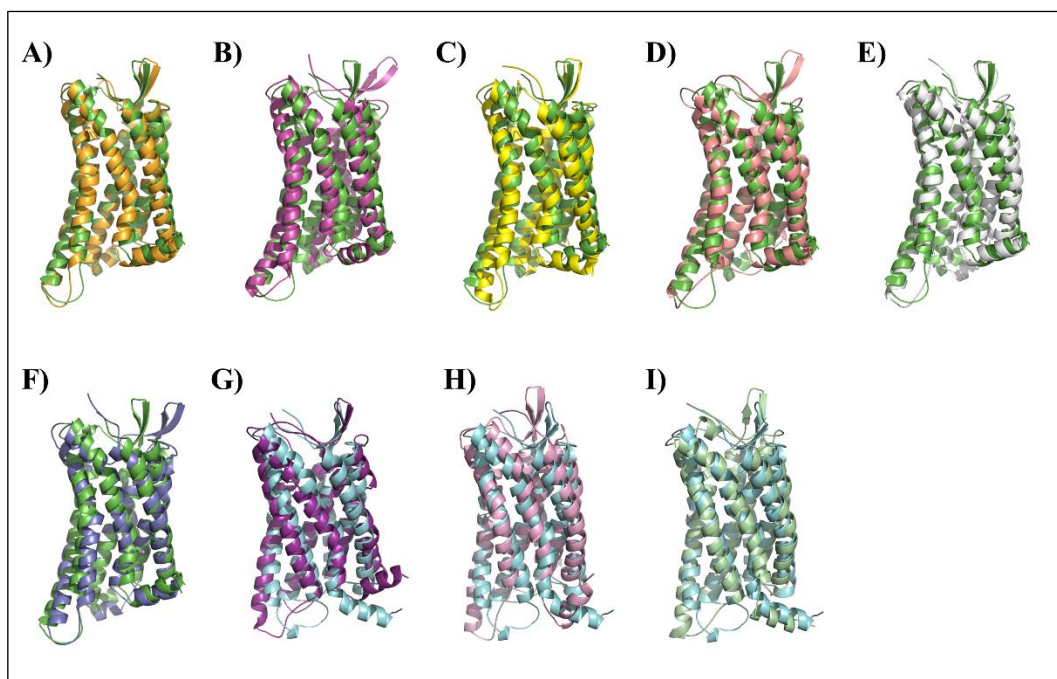


Figure 4.5: Alignment of the representative structures to the experimental active (6DO1) and inactive (4YAY) structures. 6DO1 colored in green, AT1R monomer colored in bright orange, 3OE9_2_1 colored in light magenta, 4UG2_1_1 colored in yellow, 5GLH_1 colored in salmon, 5WIV_2 colored in white-gray, 6AK3_1 colored in slate, 4YAY colored in cyan, 2ZIY_1 colored in purple, 4XT3_1 colored in pink, and 6K1Q_1_1 colored in palegreen. A) shows the alignment of the AT1R monomer representative to the 6DO1 crystal structure. B) 3OE9_2_1 aligned to the 6DO1 crystal structure. C) 4UG2_1_1 aligned to the 6DO1 crystal structure. D) 5GLH_1 aligned to the 6DO1 crystal structure. E) 5WIV_2 aligned to the 6DO1 crystal structure. F) 6AK3_1 aligned to the 6DO1 crystal structure. G) 2ZIY_1 aligned to the 4YAY crystal structure. H) 4XT3_1 aligned to the 4YAY crystal structure. I) 6K1Q_1_1 aligned to the 4YAY crystal structure.

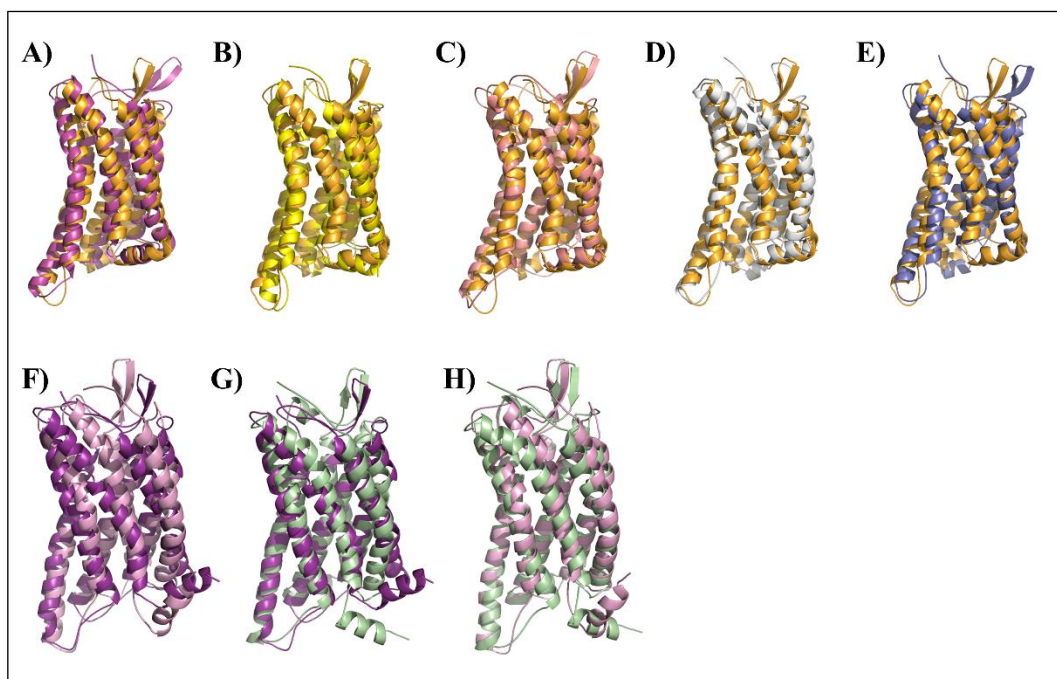


Figure 4.6: Alignment of the representative structures to the AT1R monomer representative structure. AT1R monomer colored in bright orange, 3OE9_2_1 colored in light magenta, 4UG2_1_1 colored in yellow, 5GLH_1 colored in salmon, 5WIV_2 colored in white-gray, 6AK3_1 colored in slate, 2ZIY_1 colored in purple, 4XT3_1 colored in pink, and 6K1Q_1_1 colored in palegreen. A) shows the alignment of the 3OE9_2_1 aligned to the AT1R monomer representative structure. B) 4UG2_1_1 aligned to the AT1R monomer representative structure. C) 5GLH_1 aligned to the AT1R monomer representative structure. D) 5WIV_2 aligned to the AT1R monomer representative structure. E) 6AK3_1 aligned to the AT1R monomer representative structure. F) 4XT3_1 aligned to the 2ZIY_1 representative structure. G) 6K1Q_1_1 aligned to the 2ZIY_1 representative structure. H) 6K1Q_1_1 aligned to the 4XT3_1 representative structure.

For the 3OE9_2_1 interface 11 clusters were obtained for the active frames. First two of the clusters cover 94.42% of all active frames. The representative structure which covers 85.86% of the all active frames were aligned to 6DO1 (Figure 4.5, panel B) and AT1R monomer representative structure (Figure 4.6, panel A), 3.01 and 2.81 Å RMS values were obtained.

For the 4UG2_1_1 interface, 19 clusters were obtained. First three clusters cover 82.14% of all active frames. The representative structure was aligned to 6DO1 (Figure 4.5, panel C), and to AT1R monomer representative structure (Figure 4.6, panel B); 2.03, and 1.87 Å RMS values were obtained.

For the 5GLH_1 interface 15 clusters were detected. First two clusters cover 73.19% of all active frames. The representative structure for this interface (covers 58.21% of all active frames) aligned to 6DO1 (Figure 4.5, panel D), and AT1R

monomer representative structure (Figure 4.6, panel C); 2.63, and 2.19 Å RMS values were obtained.

For the 5WIV_2 interface 18 clusters were obtained. First three clusters cover 73.01% of all active frames. The representative structure for the 5WIV_2 interface covers 24.79% of all active frames aligned to 6DO1 (Figure 4.5, panel E), and to AT1R monomer representative structure (Figure 4.6, panel D); 2.15, and 2.40 Å RMS values were obtained.

For the 6AK3_1 interface 21 clusters were obtained, where the first four of them cover 79.71% of all active frames. The representative structure which covers 29.43% of all active frames aligned to 6DO1 (Figure 4.5, panel F), and AT1R monomer representative structure (Figure 4.6, panel E); 1.97, and 2.14 Å RMS values were obtained.

In 2ZIIY_1, 32 clusters were detected, and the first five clusters cover 74.31% of all inactive frames. (which covers 21.85% of the all frames) compared with 4YAY and 2.21 Å RMS value was obtained (Figure 4.5, panel G). The representative structure for the 2ZIIY_1, was also compared with representative structures of 4XT3_1, and 6K1Q_1_1 interfaces. 2ZIIY_1 alignment to 4XT3_1 and 6K1Q_1_1 yielded 4.39, and 2.66 Å RMS values (Figure 4.6, panel F, and G, respectively).

For the 4XT3_1 interface, 8 clusters were obtained, where the representative covers 89.40% of all inactive frames. When the representative structure was aligned to 4YAY, 3.23 Å RMS value was obtained (Figure 4.5, panel H). The representative structure of 4XT3_1 interface was also compared with the representative structure of 6K1Q_1_1 interface, when aligned these two structure to each other, 2.31 Å RMS value was obtained (Figure 4.6, panel H).

In the 6K1Q_1_1 interface 33 clusters were obtained, the first five clusters cover 71.54% of all inactive frames. the representative structure for the 6K1Q_1_1 interface covers 18.77% of all frames, and when aligned to 4YAY, 2.48 Å RMS value was obtained (Figure 4.5, panel I).

4.11. Ensemble Analysis Reveal The Differences Of The Residues In Active And Inactive States

JSD and SSI analysis results were given in Figure 4.7. In these analyses, we only consider 5WIV_2, 6AK3_1, 2ZIIY_1, and 6K1Q_1_1 interfaces, where at least 15% of the trajectories were found as active or inactive. A, B, C, and D panels show the JSD comparison based on backbone atoms of 5WIV_2-2ZIIY_1, 5WIV_2-6K1Q_1_1, 6AK3_1-2ZIIY_1, and 6AK3_1-6K1Q_1_1, respectively. E, F, G, and H panels show the SSI comparison based on sidechain atoms of 5WIV_2-2ZIIY_1, 5WIV_2-6K1Q_1_1, 6AK3_1-2ZIIY_1, and 6AK3_1-6K1Q_1_1, respectively. JSD ranges between 0 and 1, where 0 shows identical distributions, 1 shows discrete distributions. Figure 4.7, Panel A shows the residues have JSD values higher than 0.6 when 5WIV_2 compared to 2ZIIY_1. PRO19^{Nterm}, PRO32^{1.36}, LEU34^{1.38}, LEU81^{2.57}, and PRO82^{2.58} backbone dihedral angle distributions were found discriminative in 5WIV_2 and 2ZIIY_1. These amino acids reside in N-terminal, TM1, and TM2, respectively. ARG93^{ECL1}, TRP94^{ECL1}, and PRO95^{ECL1} from ECL1, PHE171^{ECL2}, ILE172^{ECL2}, GLU173^{ECL2}, ASN176^{ECL2}, and THR178^{ECL2} from ECL2, TYR226^{ICL3}, ILE228^{ICL3}, LYS230^{ICL3}, ASN231^{ICL3}, and LYS232^{ICL3} from ICL3 were found with the maximum JSD of the backbone torsion angle distributions. ILE193^{5.36}, ARG234^{6.29}, ASP236^{6.31}, MET284^{7.35}, and PRO285^{7.36} (Figure 4.7, Panel A). It can clearly be seen that the JSD values differ in two different parts of the receptor; the upper part, where mainly ECL2 residues contributed, and the lower part, where residues from ICL3, and TM6 were the main contributors. In a recent study, Nicoli et al investigated the conformation and the flexibility of ECL2 in class A GPCR subfamily [263]. According to the results of that study, the highest C α RMSD was observed in Endothelin B receptor, which is a peptide binding receptor, similar to AT1R. Since one of the major differences between active and inactive receptor structures is the proximity and distance of TM6 to the receptor core region, the JSD values are consistent with this observation. The higher values for the upper part of the receptor were attributed to the simulated apo form of the receptor. This observation is in line with the previous studies [264, 265], where in the apo form ECL2 of the AT1R was found flaccid compared to agonist and antagonist bound forms.

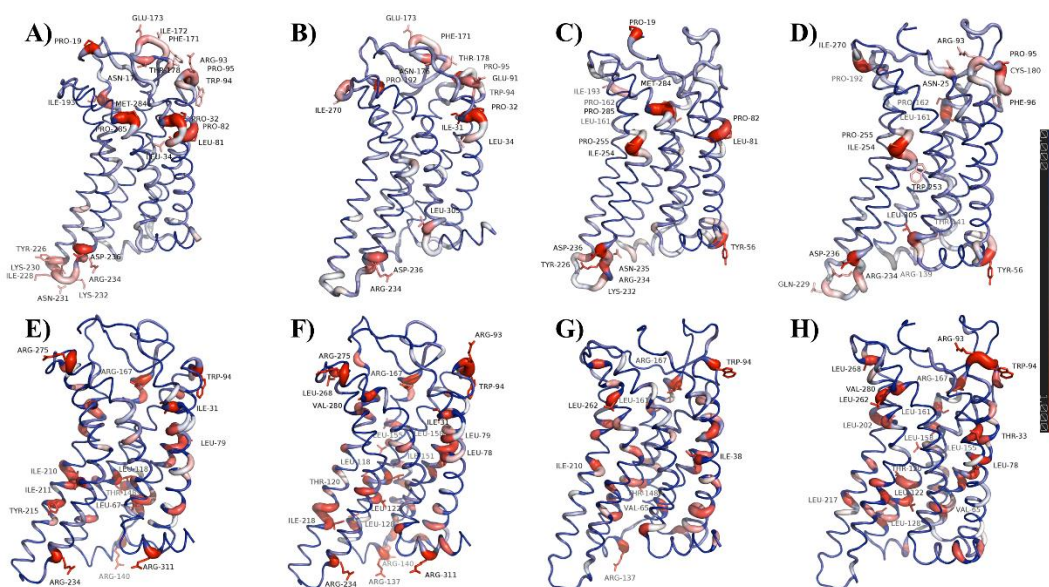


Figure 4.7: Comparison of backbone (Panels A, B, C, and D) and sidechain (Panels E, F, G, and H) torsion angles in active and inactive trajectories. Based on backbone JSD values of 5WIV_2 compared to 2ZIIY_1, and 6K1Q_1_1, 6AK3_1 compared to 2ZIIY_1, and 6K1Q_1_1 in panels A, B, C, and D, respectively (residues that have JSD values higher than 0.6, labeled). Based on sidechain SSI values of 5WIV_2 compared to 2ZIIY_1, and 6K1Q_1_1, 6AK3_1 compared to 2ZIIY_1, and 6K1Q_1_1 in panels E, F, G, and H, respectively (residues that have SSI values higher than 0.9, labeled).

Figure 4.7, panel B shows the comparison of 5WIV_2 and 6K1Q_1_1 interface JSD scores based on backbone atoms. ILE31^{1.35}, PRO32^{1.36}, and LEU34^{1.38} from TM1, and GLU91^{2.67} from TM2 were found different in both interfaces. TRP94^{ECL1}, PRO95^{ECL1} from ECL1, PHE171^{ECL2}, GLU173^{ECL2}, ASN176^{ECL2}, and THR178^{ECL2} from ECL2, and ILE270 from ECL3 had different JSD scores. PRO192^{5.35}, ARG234^{6.29}, ASP236^{6.31}, and LEU305^{7.56} differ in active (5WIV_2) and inactive (6K1Q_1_1) states. Similar pattern for the JSD values on the extracellular loops was observed in the case of 6K1Q_1_1 compared to 2ZIIY_1. However, LEU305^{7.56} differed in this case by interacting TM6 residues in the inactive state (6K1Q_1_1). The reason for the JSD score differences in the TRP94^{ECL1} residue (and surrounding residues) observed in Figure 4.7, panels A and B, is that this region is located at the 5WIV_2 interface.

Figure 4.7, panel C shows the residues with JSD values higher than 0.6 in 6AK3_1 compared to 2ZIIY_1. PRO19^{Nterm} from N-terminal, TYR56^{1.60}, LEU812.57, PRO82^{2.58}, LEU161^{4.58}, PRO162^{4.59}, ILE193^{5.36}, TYR226^{ICL3}, and LYS232^{ICL3} from ICL3, ARG234^{6.29}, ASN235^{6.30}, ASP236^{6.31}, ILE254^{6.49}, PRO255^{6.50}, MET284^{7.35}, and

PRO285^{7,36} differ in terms of JSD values in active (6AK3_1) and inactive (2ZIIY_1) ensembles. Figure 4.7, panel D shows the differences between active (6AK3_1) and inactive (6K1Q_1_1) based on backbone dihedral torsions. The JSD values for the following residues were differed in 6AK3_1 and 6K1Q_1_1 interfaces; ASN25^{1,29}, TYR56^{1,60}, ARG93^{ECL1}, PRO95^{ECL1}, and PHE96^{ECL1} from ECL1, ARG139^{ICL2} from ICL2, THR141^{4,38}, LEU161^{4,58}, and PRO162^{4,59}, CYS180^{ECL2} from ECL2, PRO192^{5,35}, GLN229^{ICL3} from ICL3, ARG234^{6,29}, ASP236^{6,31}, TRP253^{6,48}, ILE254^{6,49}, PRO255^{6,50}, ILE270^{ECL3} from ECL3, and LEU305^{7,56} (Figure 4, panel D). Comparison of 6AK3_1 with 2ZIIY_1, and 6K1Q_1_1 resulted in two similar patterns for the JSD values. However, TM6 proline kink (PRO255^{6,50}) played a significant role when 6AK3_1 compared to 5WIV_2. In both cases (2ZIIY_1, and 6K1Q_1_1) proline kink lead to the conformational change at the lower part of the TM6. The modulation of TM6 bending that initiated by PRO^{6,50}, was attributed to around residues (eg. TRP^{6,48}) [266]. In this context, the contribution of TRP^{6,48} is clearly seen when comparing the 6K1Q_1_1_1 and 6AK3_1 interfaces (Figure 4.7, panel D). Other differences were in ICL3, and the TM6, which pinpoints receptor activation/deactivation. The reason for the JSD score differences observed in Figure 4.7, panels C and D (compared to 2ZIIY_1 and 6K1Q_1_1_1) for the TYR56^{TM1} residue is that this region is located at the 6AK3_1 interface.

Figure 4.7 panels E, F, G, and H show the residues that have SSI values higher than 0.9 bits. The calculation of SSI values for each residue reveals the receptor segments that transmit information regarding the activation or deactivation process of the receptor. Figure 4.7, panel E shows the comparison of 5WIV_2 and 2ZIIY_1 interfaces based on SSI values of the sidechains. ILE31^{1,35}, LEU67^{2,43}, LEU79^{2,55}, TRP94^{ECL1} from ECL1, LEU118^{3,42}, ARG140^{ICL2} from ICL2, THR148^{4,45}, ARG167^{4,64}, ILE210^{5,53}, ILE211^{5,54}, TYR215^{5,58}, ARG234^{6,29}, ARG275^{7,26}, and ARG311^{8,52} show the changes in side chain torsion angles enables the activation/inactivation process of the AT1R. Figure 4.7, panel F shows the comparison of 5WIV_2 and 6K1Q_1_1 interfaces based on SSI values, following residues differ in each ensemble, ILE31^{1,35}, LEU78^{2,54}, LEU79^{2,55}, ARG93^{ECL1}, and TRP94^{ECL1} from ECL1, LEU118^{3,42}, THR120^{3,44}, LEU122^{3,46}, LEU128^{3,52}, ARG137^{ICL2}, and ARG140^{ICL2} from ICL2, ILE151^{4,48}, LEU155^{4,52}, LEU158^{4,55}, ARG167^{4,64}, ILE218^{5,61}, ARG234^{6,29}, LEU268^{6,63}, ARG275^{7,26}, VAL280^{7,31}, and ARG311^{8,52}. Figure 4.7, panel G shows the comparison of 6AK3_1 and 2ZIIY_1 ensembles. SSI

values of the sidechains of following residues differ in each ensemble, ILE38^{1.42}, VAL65^{2.41}, TRP94^{ECL1} from ECL1, ARG137^{ICL2} from ICL2, THR148^{4.45}, LEU161^{4.58}, ARG167^{4.64}, ILE210^{5.53}, and LEU262^{6.57}. Figure 4.7, panel H shows the ensemble differences between active (6AK3_1) and inactive (6K1Q_1_1) ensembles based on sidechain SSI values. THR33^{1.37}, VAL65^{2.41}, LEU78^{2.54}, ARG93^{ECL1}, and TRP94^{ECL1} from ECL1, THR120^{3.44}, LEU122^{3.46}, LEU128^{3.52}, LEU155^{4.52}, LEU158^{4.55}, LEU161^{4.58}, ARG167^{4.64}, LEU202^{5.45}, LEU217^{5.60}, LEU262^{6.57}, LEU268^{6.63}, and VAL280^{7.31} SSI values differ in each ensemble. Overall, based on SSI analyses of the selected interfaces, the differences were related with the receptor state (eg. the residues that reside at the interior of the receptor were highlighted in an inactive state that stabilizes the interactions (LEU118^{3.42}, THR148^{4.45}, etc)). In the active state, the interactions between TM5 and TM6 were more prominent, and this led to the differences in SSI values such as ILE210^{5.53}, LEU217^{5.60}, and ILE218^{5.61}.

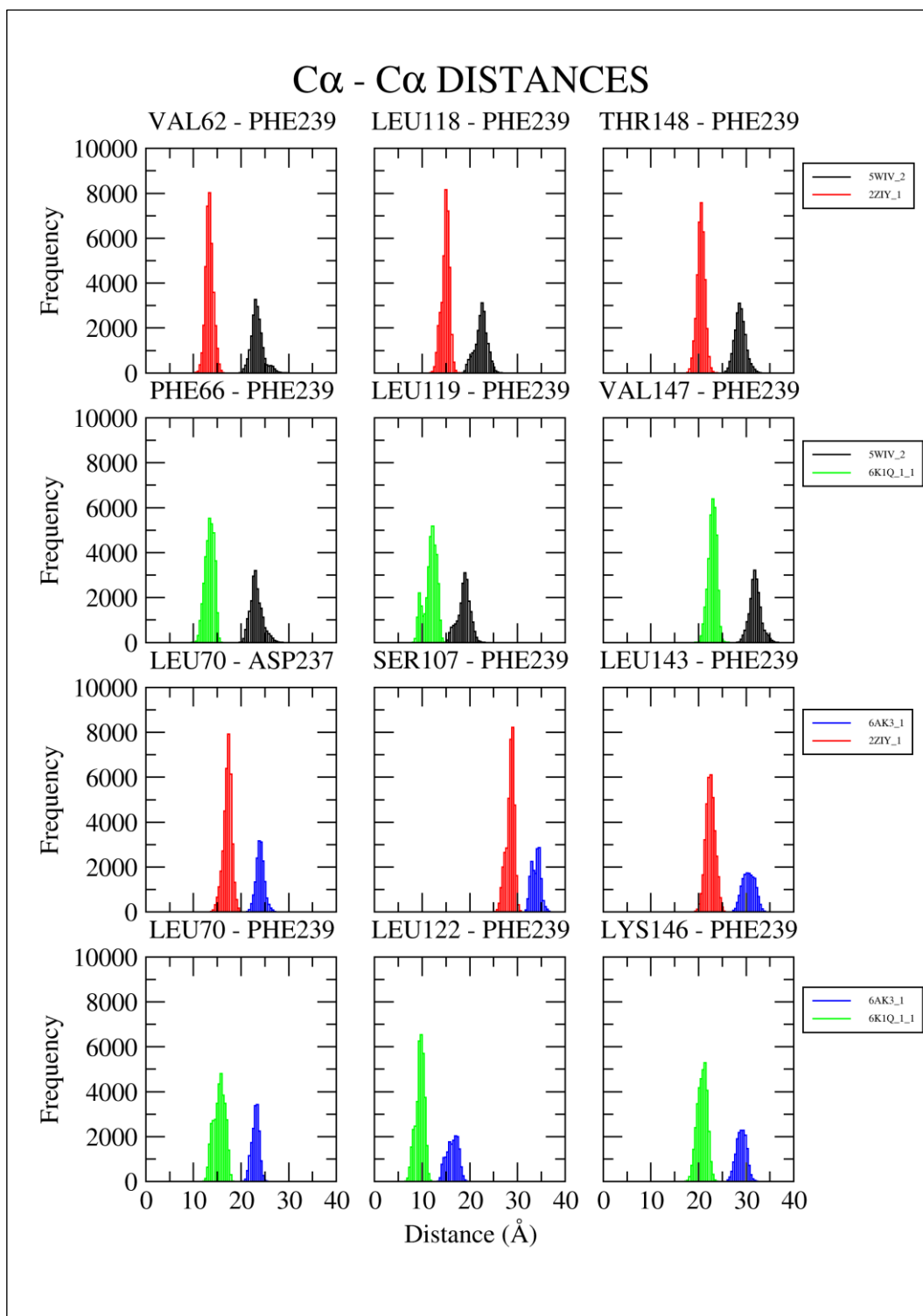


Figure 4.8: Histograms of C α -C α distance frequencies for the TM2-TM6, TM3-TM6, and TM4-TM6 distances. 5WIV_2 depicted in black, 2ZIY_1 depicted in red, 6K1Q_1_1 depicted in green, and 6AK3_1 depicted in blue. The interfaces where inactive frames (2ZIY_1, and 6K1Q_1_1) observed on the left, showing lower TMx-TM6 (x=2, 3, and 4) distance compared to active states (5WIV_2, and 6AK3_1).

Along with the ensemble analysis, the distance between all C α -C α pairs calculated in active and inactive ensembles. Three different C α -C α distances were selected from each comparison and plotted in Figure 4.8. The first panel in Figure 4.8 shows the VAL62^{2.38}-PHE239^{6.34}, LEU118^{3.42}-PHE239^{6.34}, and THR148^{4.45}-PHE239^{6.34} C α -C α distances in comparison of 5WIV_2 and 2ZIY_1 interfaces. The averages of VAL62^{2.38}-PHE239^{6.34} distances calculated as 23.40 $\pm$ 1.28, and 13.37 $\pm$ 0.79 Å in 5WIV_2, and 2ZIY_1 interfaces, respectively. LEU118^{3.42}-PHE239^{6.34} distance averages were 22.37 $\pm$ 1.28, and 14.89 $\pm$ 0.83 Å in 5WIV_2 and 2ZIY_1, respectively. The TM4-TM6 distance calculated by considering THR148^{4.45}-PHE239^{6.34} C α -C α , where the averages found as 28.75 $\pm$ 1.14, and 20.52 $\pm$ 0.81 Å in 5WIV_2 and 2ZIY_1, respectively. When 5WIV_2 and 6K1Q_1_1 interfaces were compared, three selected distances were plotted in Figure 4.8, second panel. The averages of PHE66^{2.42}-PHE239^{6.34}, LEU119^{3.43}-PHE239^{6.34}, and VAL147^{4.44}-PHE239^{6.34} C α -C α distances were found as 23.22 $\pm$ 1.28, 18.76 $\pm$ 1.29, and 31.91 $\pm$ 1.18 in 5WIV_2, and 13.38 $\pm$ 0.94, 11.75 $\pm$ 1.28, and 22.93 $\pm$ 0.86 in 6K1Q_1_1, respectively.

4.12. In Inactive Frames, The Receptor Is Stabilized By Interactions Between TM3-TM6, Whereas In Active Frames TM5-TM6 Interactions Become More Dominant

Next, protein structure network (PSN) analyses were conducted to reveal the signaling pathway inside the receptor. Figure 4.9 shows the PSNs for each system, respectively. In the 2ZIY_1 interface source residue for the allosteric communication was VAL108^{3.32}, and the sink residue selected as ILE238^{6.33}. The signal started from the receptor core and ended at the end of the TM6. The following residues were found in this communication path; VAL108^{3.32}, LEU195^{5.38}, LYS199^{5.42}, TRP253^{6.48}, PHE249^{6.44}, LEU119^{3.43}, LEU122^{3.46}, and ILE238^{6.33}. The path mainly consisted of residues from TM3, TM5, and TM6. The upper part of the TM6 was stabilized with the interactions from TM5. The receptor core contributed with residues from TM3 to stabilize the lower part of the TM6, which occupies the effector binding site and resembles the inactive conformation (see Figure 4.9, panel A). Figure 4.9, panel B shows the PSN for the 5WIV_2 interface. The sink residue selected was the same as

in the previous interface. In the 5WIV_2 interface, the source residue was selected as TYR184^{ECL2}. The path residues were TYR184^{ECL2}, GLN257^{6.52}, PHE204^{5.47}, PHE208^{5.51}, VAL246^{6.41}, TYR215^{5.58}, and ILE238^{6.33}. The residues from TM5, and TM6 were observed in the path. The interactions were between TM5 and TM6, that would allow TM6 to adopt open conformation. ASN188^{ECL2} selected as source residue in the 6AK3_1 interface. The allosteric path consisted of ASN188^{ECL2}, HSD256^{6.51}, PRO255^{6.50}, PHE251^{6.46}, LEU247^{6.42}, MET243^{6.38}, PHE239^{6.34}, and LEU222^{5.65}. Similar to the other active interface, in the case of 6AK3_1, the link that maintains the lower part of the TM6 close to the TM5 was also observed (see Figure 4.9, panel C). Figure 4.9, panel D shows the allosteric communication path for the 6K1Q_1_1 interface, where TYR92^{ECL1}, and ILE238^{6.33} were selected as source and sink residues, respectively. The path formed by TYR92^{ECL1}, LEU202^{5.45}, PHE206^{5.49}, PRO207^{5.50}, LEU119^{3.43}, ILE242^{6.37}, TYR302^{7.53}, and ILE238^{6.33}. The lower part of the TM6 was kept in the closed conformation by the interactions of LEU119^{3.43}-ILE242^{6.37}, and TYR302^{7.53}-ILE238^{6.33}.

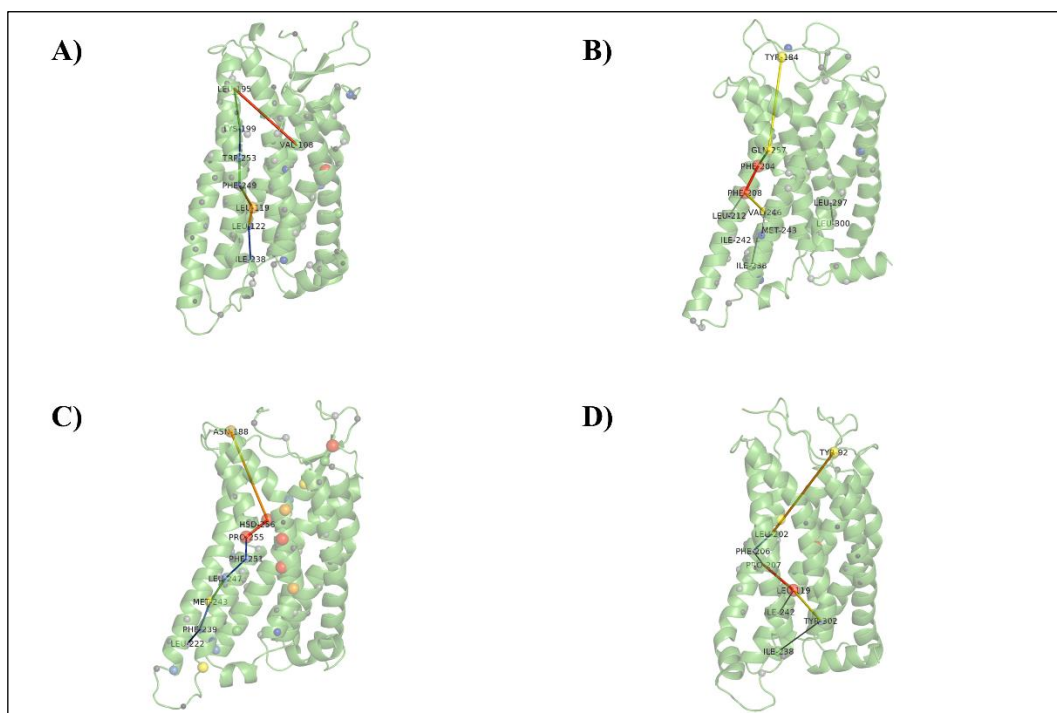


Figure 4.9: Highlighted metapaths for the 2ZIY_1, 5WIV_2, 6AK3_1, and 6K1Q_1_1, respectively. Dark blue, blue, green, yellow, orange, and red are used for relative recurrences, 40-50, 50-60, 60-70, 70-80, 80-90, and 90-100%, respectively.

PSN difference analyses were also conducted to highlight the structural differences between active and inactive states. Figure 4.10, panel A shows the most important interactions specific to the 5WIV_2 interface, compared to 2ZIIY_1. PHE208^{5.51}-VAL246^{6.41}, LEU212^{5.55}-ILE242^{6.37}, VAL246^{6.41}-TYR215^{5.58}, TYR215^{5.58}-ILE238^{6.33}, LEU222^{5.65}-PRO233^{6.28}, and PRO233^{6.28}-GLN229^{ICL3}. These links were formed between TM5 and TM6, to maintain the active form of the receptor. Panel B shows the interactions specific to the 2ZIIY_1 interface. LEU305^{7.56}-ILE238^{6.33}, ILE238^{6.33}-ARG126^{3.50}, ILE238^{6.33}-LEU122^{3.46}, ILE242^{6.37}-LEU119^{3.43}, and ILE245^{6.40}-LEU119^{3.43} interactions stabilized the inactive form of the receptor. These interactions are established mainly by TM3 located in the receptor core which keeps TM6 in closed conformation. Closed conformation of the TM6 toward the receptor interior, enables another interaction between TM6 and TM7. The PSN difference between 6AK3_1 and 2ZIIY_1 depicted in Figure 4.10, panel C. ILE211^{5.54}-ILE245^{6.40}, TRP219^{5.62}-MET243^{6.38}, LEU222^{5.65}-PHE239^{6.34}, and LEU222^{5.65}-ILE238^{6.33} were presented only 6AK3_1 (active state), where these interactions formed between TM5 and TM6, and kept TM6 in open conformation, eventually making space for effector binding from the intracellular site. However, the interactions which were observed between TM3-TM6, and TM6-TM7 were identical to 2ZIIY_1 (inactive state) (see Figure 4.10, panel D). LEU119^{3.43}-PHE249^{6.44}, LEU119^{3.43}-ILE245^{6.40}, LEU119^{3.43}-ILE242^{6.37}, LEU122^{3.46}-ILE238^{6.33}, LEU305^{7.46}-ILE238^{6.33}, and PHE304^{7.55}-ASP237^{6.32} interactions were only observed in 2ZIIY_1 interface. Among these links, LEU119^{3.43} served as a hub residue by forming three other interactions from the TM6, and kept the TM6 in the closed conformation. Figure 4.10, panel E shows the PSN difference for the 5WIV_2 and 6K1Q_1_1. The interactions that only observed in active state (5WIV_2) were, PHE208^{5.51}-VAL246^{6.41}, LEU212^{5.55}-ILE242^{6.37}, TYR215^{5.58}-ILE238^{6.33}, and LEU222^{5.65}-PRO233^{6.28}. Panel F shows the links specific to inactive state (6K1Q_1_1) were LEU112^{3.36}-PHE249^{6.44}, LEU119^{3.43}-PHE249^{6.28}, LEU119^{3.43}-ILE242^{6.37}, LEU122^{3.46}-ILE238^{6.33}, TYR302^{7.53}-ILE238^{6.33}, and TYR302^{7.53}-ASP237^{6.32} contributed to stabilize inactive conformation. The PSN difference between 6AK3_1 and 6K1Q_1_1 was depicted in Figure 4.10, panel G. The interactions that only observed in 6AK3_1 (active state) were ILE211^{5.54}-PHE249^{6.44}, ILE211^{5.54}-ILE245^{6.40}, TRP219^{5.62}-MET243^{6.38}, and LEU222^{5.65}-PHE239^{6.34}. LEU112^{3.36}-PHE249^{6.44}, LEU119^{3.43}-PHE249^{6.44}, LEU119^{3.43}-

ILE242^{6.37}, LEU122^{3.46}-ILE238^{6.33}, TYR302^{7.53}-ILE238^{6.33}, and TYR302^{7.53}-ASP237^{6.32} were specific to inactive state (6K1Q_1_1) in Figure 4.10, panel H.

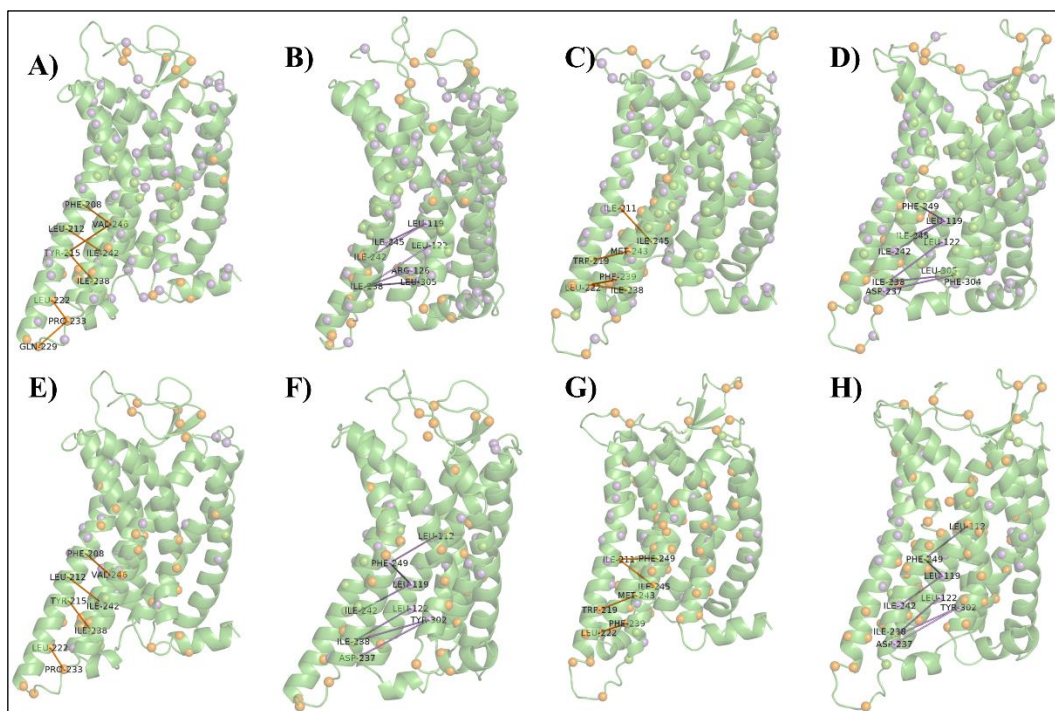


Figure 4.10: PSN difference graphs. Panels A, C, E, and G show the active structure differences for the 2ZIY_1-5WIV_2, 2ZIY_1-6AK3_1, 6K1Q_1_1-5WIV_2, and 6K1Q_1_1-6AK3_1 systems, respectively. Panels B, D, F, and H show the inactive structure differences for the 2ZIY_1-5WIV_2, 2ZIY_1-6AK3_1, 6K1Q_1_1-5WIV_2, and 6K1Q_1_1-6AK3_1 systems, respectively.

Consensus communities for the inactive and active states were given in Figure 4.11, Panel A, and B, respectively. For the inactive states (2ZIY_1, and 6K1Q_1_1) six communities were found.

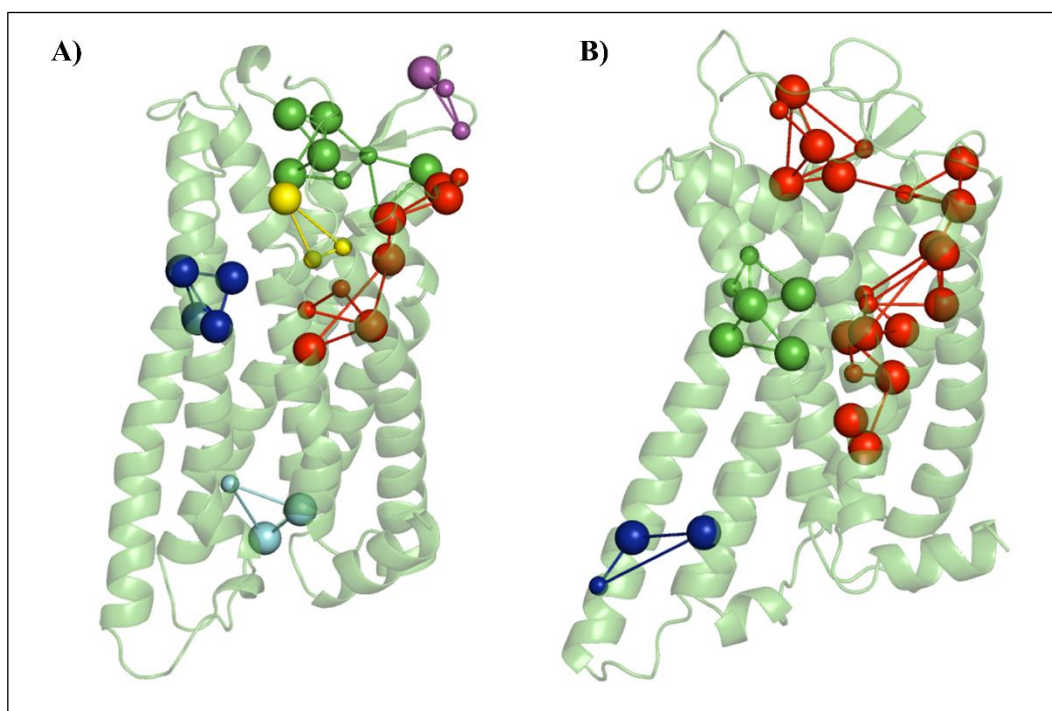


Figure 4.11: Consensus communities for the inactive states (2ZIY_1 and 6K1Q_1_1)(A), and active states (5WIV_2 and 6AK3_1)(B).

The first community has 9 nodes, 12 links, and 6 hubs, and consists of following residues; ILE31^{1.35}, TYR35^{1.39}, CYS289^{7.40}, TRP84^{2.60}, TYR92^{ECL1}, TYR87^{2.63}, TRP94^{ECL1}, PHE96^{ECL1}, and PRO95^{ECL1}. This community is shown in red color in Figure 4.11, Panel A, and mainly composed of ECL1, TM1, TM2, and TM7 residues. Second community has 8 nodes, 11 links, and 5 hubs, where LYS102^{3.26}, ASN98^{3.22}, ASN168^{ECL2}, HSD166^{4.63}, ARG167^{4.64}, PHE182^{ECL2}, HSD183^{ECL2}, and TYR184^{ECL2} involved. This community was formed mainly residues from ECL2, TM3, and TM4, and shown in green (Figure 4.11, panel A). The third community consists of PHE204^{5.47}, TRP253^{6.48}, GLN257^{6.52}, and HSD256^{6.51}, TM5 and TM6 contributed, and shown in blue color. Last three communities have three members in each. Yellow community (3 nodes, 3 links, and 1 hub) consists of TM1 residues; HSD24^{1.28}, ILE27^{1.31}, and PHE28^{1.32}. Cyan community (3 nodes, 3 links, and 2 hubs) formed by ASP125^{3.49}, VAL62^{2.38}, and ARG140^{ICL2}. Purple community (3 nodes, 3 links, and 1 hub) composed of ECL2 residues; ILE172^{ECL2}, THR175^{ECL2}, and ILE177^{ECL2}. For the active states (5WIV_2, and 6AK3_1) three communities were found. The first community depicted in red color in Figure 4.11, panel B. The first community formed by 22 nodes, 31 links, and 15 hubs, where residues contributed from TM1, TM2,

ECL1, TM3, TM4, ECL2, and TM7. ILE31^{1.35}, TYR35^{1.39}, CYS289^{7.40}, TRP84^{2.60}, ASN298^{7.49}, LEU70^{2.46}, PRO299^{7.50}, ASN111^{3.35}, ASP742.50, ASN295^{7.46}, VAL108^{3.32}, PHE77^{2.53}, TYR292^{7.43}, TYR87^{2.63}, PHE96^{ECL1}, CYS101^{3.25}, HSD166^{4.63}, ARG167^{4.64}, ASN168^{ECL2}, PHE182^{ECL2}, HSD183^{ECL2}, TYR184^{ECL2} were the contributing residues for the community 1. The second community was depicted in green, where mainly TM5, and TM6 residues contributed. ASN200^{5.43}, GLN257^{6.52}, THR260^{6.55}, PHE204^{5.47}, TRP253^{6.48}, HSD256^{6.51} were the nodes in community 2 (6 nodes, 8 links, and 4 hubs). The last community formed in the lower parts of TM5, and TM6, which consists of TRP219^{5.62}, LYS223^{5.66}, and PHE239^{6.34} colored in blue.

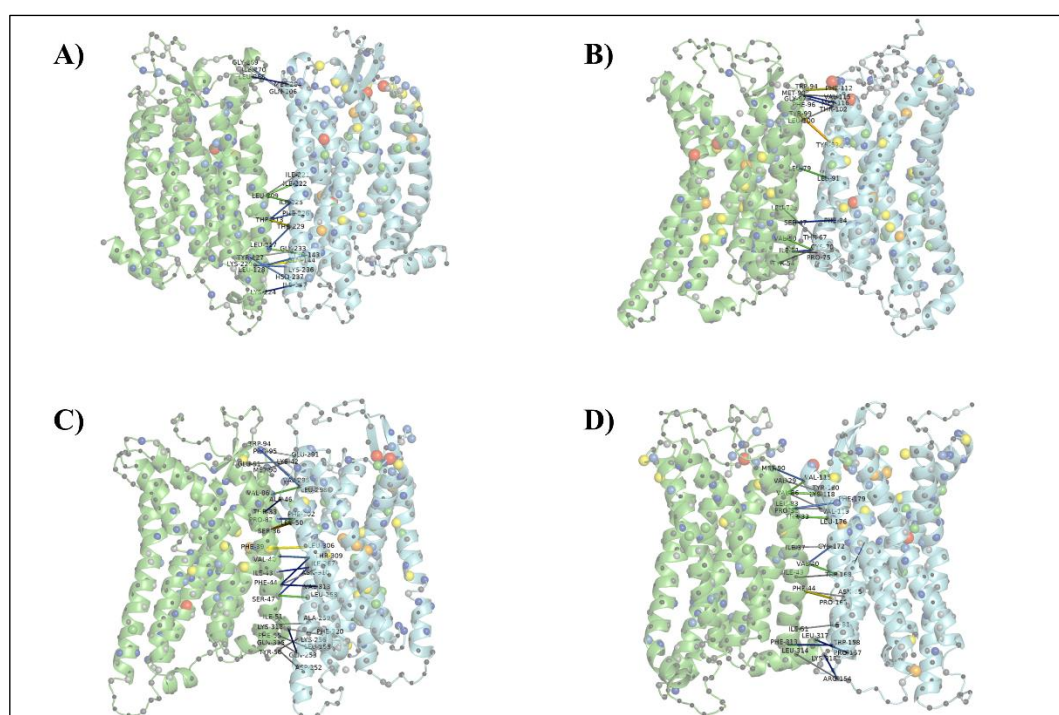


Figure 4.12: Links residues at the interface of 2ZIIY_1, 5WIV_2, 6AK3_1, and 6K1Q_1_1, respectively. These links were maintained at least 30% of the simulation trajectories.

When links at the interfaces were taken into account, hub residues at the interface were identified. For the 2ZIIY_1 interface, seventeen links were found. Figure 4.12, panel A shows the important links for the 2ZIIY_1 interface. These links have frequency higher than 30%, and given in the following; THR213^{5.56}-THR229^{5.56}, LYS220^{5.63}-GLN144^{3.52}, LEU209^{5.52}-ILE225^{5.52}, LEU217^{5.60}-TYR143^{3.51}, LEU209^{5.52}-ILE222^{5.49}, THR213^{5.56}-ILE225^{5.52}, LEU217^{5.60}-THR229^{5.56}, THR213^{5.56}-PHE226^{5.53}, LEU128^{3.52}-LYS236^{5.63}, LYS220^{5.63}-TYR143^{3.51},

ILE270^{ECL3}-GLN206^{5.33}, TYR127^{3.51}-HSD237^{5.64}, LEU268^{6.63}-MET284^{6.63},
 LYS224^{5.67}-ILE147^{3.55}, GLY269^{6.64}-GLN206^{5.33}, LEU209^{5.52}-ILE221^{5.48}, and
 TYR127^{3.51}-GLY233^{5.60}. Among these links, THR213^{5.56}, and LEU209^{5.52} were the
 hub residues at the interface by forming three interactions with the AT2R. In A2AR-
 D2R heterodimer LEU207ALA^{5.56} mutation was found to weaken the interaction at
 the interface [243]. In another study where bivalent ligands were docked into D2R
 homodimer, ILE^{5.52} was contributed to dimer formation [267]. More interestingly,
 SER^{5.52}ALA, and SER^{5.52}PRO when presented in rhodopsin were found to cause Blue-
 Yellow Color Blindness (disease-associated mutations) [268]. ^{5.52} was also found in
 smoothened, CXCR4 chemokine, mu-opioid and squid rhodopsin, and ^{5.56} was
 observed in smoothened, and squid rhodopsin dimer interfaces [269]. For the 5WIV_2
 dimer interface, following links (fourteen) were found with higher than 30%
 frequency; TRY99^{3.23}-TRY52^{1.40}, TRP94^{ECL1}-PHE112^{ECL1}, LEU79^{2.55}-LEU91^{2.51},
 VAL50^{1.54}-CYS70^{1.58}, GLY97^{3.21}-THR102^{2.62}, SER47^{1.51}-PHE84^{2.44}, ILE51^{1.55}-
 CYS70^{1.58}, PHE96^{ECL1}-THR102^{2.62}, MET90^{2.66}-MET116^{3.24}, LEU72^{2.48}-THR67^{1.55},
 ILE51^{1.55}-PRO75^{ICL1}, LEU100^{3.24}-THR102^{2.62}, MET90^{2.66}-VAL115^{3.23}, and
 TRY54^{1.58}-CYS70^{1.58}. Among these links, ILE51^{1.55}, and MET90^{2.66} were interacted
 with AT2R through two links. ^{1.55} was found in mu-opioid, adenosine A1, and
 metabotropic glutamate receptor dimer interfaces [269]. ^{2.66} was observed at the
 interfaces of mu-opioid, beta1-adrenergic, and bovine rhodopsin homodimers [269].
 Twenty five links were identified at the interface of 6AK3_1. SER36^{1.40}-LEU50^{1.38},
 PHE39^{1.43}-LEU306^{7.41}, SER36^{1.40}-PHE302^{7.37}, VAL86^{2.62}-LEU298^{7.33}, SER47^{1.51}-
 LEU263^{6.42}, VAL40^{1.44}-THR309^{7.44}, TRP94^{ECL1}-LEU298^{7.33}, PRO82^{2.58}-PHE302^{7.37},
 LYS318^{H8}-GLN253^{6.32}, PHE44^{1.48}-VAL313^{7.48}, THR33^{1.37}-ALA46^{1.34}, PHE44^{1.48}-
 THR309^{7.44}, SER47^{1.51}-ILE267^{6.46}, ILE43^{1.47}-ILE267^{6.46}, PHE55^{1.59}-LEU255^{6.34},
 MET90^{2.66}-LYS42^{Nterm}, PRO95^{ECL1}-GLU291^{7.26}, TRP94^{ECL1}-VAL295^{7.30}, LYS318^{H8}-
 PHE320^{7.55}, TYR56^{1.60}-LYS256^{6.35}, TYR56^{1.60}-ASP252^{6.31}, GLN315^{H8}-GLN253^{6.32},
 ILE51^{1.55}-ALA259^{6.38}, PHE44^{1.48}-ASN310^{H8}, and GLU91^{2.67}-LYS42^{TM1} had higher
 than 30% frequency throughout MD simulations. PHE44^{1.48} formed three interactions
 with AT2R. TYR56^{1.60}, TRP94^{ECL1}, SER47^{1.51}, SER36^{1.40}, and LYS318^{8.59} were
 contributed to the interface with two links from AT2R. ^{1.48} was found in the dimer
 interfaces of rhodopsin, adenosine A1, and metabotropic receptor [269] and predicted
 in the interface of D2R homodimer [267]. When PRO^{1.48}ARG mutation was introduced
 in rhodopsin, retinitis pigmentosa 4 (RP4) was detected [268]. Moreover, in a recent

study, Lu et al. identified a cryptic pocket in AT1R, where PHE44^{1.48} was contributed, when PHE^{1.48}ALA mutation together with other six sites impaired ANGII induced AT1R conformational change [270]. TYR56^{1.60} was found in the dimer interface of metabotropic glutamate receptor 1 [269]. ^{1.60} position is a generally conserved residue, and recent study revealed ^{1.60} resides in the Na⁺ ion exit pathway [271]. In another study, ^{1.60} found to stabilize the TM7-H8 interactions, and support the GPCR crystallization [272]. ^{1.51} was observed in Adenosine A1, and metabotropic glutamate receptor 1 interfaces [269]. ^{1.40} was observed as the one of the most presented residues at the dimerization interfaces; bovine opsin, CCR9 chemokine, μ -opioid, κ -opioid, β 1-adrenergic, and bovine rhodopsin receptors. LEU^{1.40}MET mutation in somatostatin 5 receptor related with prostate cancer risk, GLY^{1.40}ALA, and GLY^{1.40}ASP mutations in NK-3 receptor was found related with Hypogonadotropic hypogonadism 11 with or without anosmia (HH11), and PHE^{1.40}LEU mutation in rhodopsin associated with Retinitis pigmentosa 4 (RP4) [268]. ^{8.59} was found in the dimerization interfaces of bovine opsin, μ -opioid, κ -opioid, and bovine rhodopsin [269], when GLY^{8.59}GLU mutation introduced in to rhodopsin, as a result Colorblindness was observed [268]. For the 6K1Q_1_1 interface, nineteen links were found at the interface which have frequency values higher than 30%. These links were; PHE44^{1.48}-PRO165^{4.47}, VAL40^{1.44}-TRP168^{4.50}, THR33^{1.37}-LEU176^{4.58}, VAL86^{2.62}-LYS118^{3.26}, VAL86^{2.62}-VAL115^{3.23}, MET90^{2.66}-VAL115^{3.23}, PRO32^{1.36}-PHE179^{4.61}, VAL40^{1.44}-CYS172^{4.54}, PHE313^{8.54}-TRP158^{4.40}, LYS318^{8.59}-ARG154^{ICL2}, LEU317^{8.58}-PRO157^{4.39}, LEU314^{8.55}-ARG154^{ICL2}, LEU83^{2.59}-VAL119^{3.27}, ILE37^{1.41}-CYS172^{4.54}, VAL29^{1.33}-TYR180^{4.62}, ILE43^{1.47}-TRP168^{4.50}, ILE51^{1.55}-ILE81^{2.41}, VAL86^{2.62}-VAL119^{3.27}, and PHE44^{1.48}-ASN85^{2.45}. Among these links, VAL86^{2.62}, VAL40^{1.44}, and PHE44^{1.48} interacted with three, and latter two residues contributed to interface formation by interacting with two different residues from AT2R. VAL86^{2.62} was found in the dimer interfaces of μ -opioid, κ -opioid, β 1-adrenergic, and bovine rhodopsin [269]. ILE^{2.62}SER, and ILE^{2.62}THR mutations found to cause obesity when introduced in melanocortin receptor 4 [268]. LEU^{2.62}PRO mutation in vasopressin V2 receptor induced nephrogenic diabetes insipidus (NDI) [268]. Similarly, VAL40^{1.44} was observed in CCR9 chemokine, κ -opioid, β 1-adrenergic, bovine rhodopsin, and adenosine A1 dimer interfaces [269]. Recently, Tse et al. investigated MT2-5HT2C heterodimer interfaces, and ^{1.44} found in the heterodimer interface, and ALA^{1.44}THR

mutation impaired the heterodimer formation [244]. The last hub in the 6K1Q_1_1 interface was PHE44^{1.48}, which was also observed in the 6AK3_1 interface.

4.13. Structural Determinants For The Stabilization Of Active and Inactive States

Figure 4.13 show the heavy atom RMSD of the NPxxY and the ARG126^{3.50} C α -PHE239^{6.34} C α comparison in 2ZIY_1 (Panel A), 5WIV_2 (Panel B), 6AK3_1 (Panel C), and 6K1Q_1_1 (Panel D), respectively. For all systems RMSD values of the NPxxY motif were ranged between 0.5-3 Å. Which was expected to be small and concentrated, however it was not discriminative between inactive and active systems. The reason behind why NPxxY motif was not discriminative is since the transitions of this motif are local side chain rearrangements, they can occur very quickly [273].

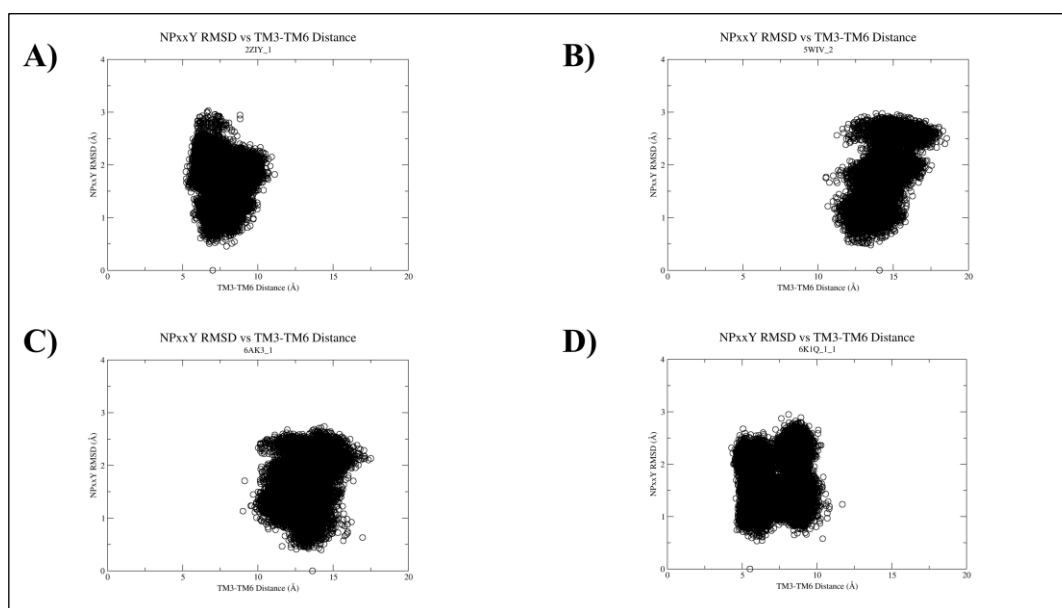


Figure 4.13: Comparison of the heavy atom RMSD of NPxxY and the distance between C α atoms of ARG126^{3.50} and PHE239^{6.34} for A) 2ZIY_1, B) 5WIV_2, C) 6AK3_1, and D) 6K1Q_1_1, respectively.

Figure 4.14 show the ARG126^{3.50} C α - TYR302^{7.53} C α distance distributions for the 2ZIY_1, 5WIV_2, 6AK3_1, and 6K1Q_1_1 interfaces, respectively. Upon activation TM3-TM7 contacts are formed [268]. In our analyses, we calculated the distance distributions for the selected systems, our simulation data was well aligned with the experimental structures of AT1 receptors, where the distance calculated as 16.5 Å, and 12.0 Å, in inactive (4YAY), and active (6DO1) structures.

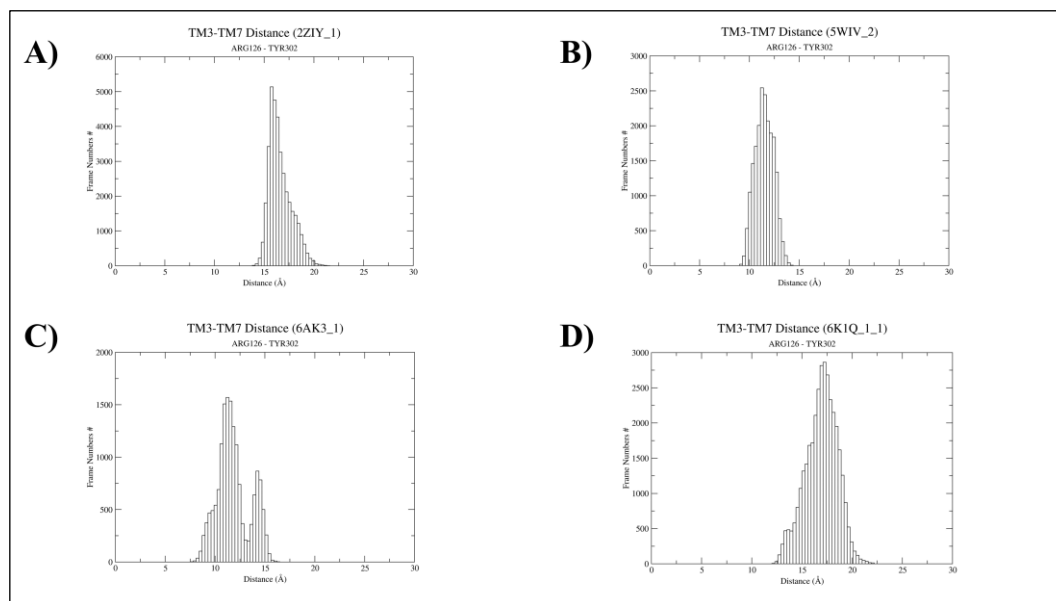


Figure 4.14: ARG126^{3.50} C α - TYR302^{7.53} C α distance distribution for the A) 2ZIY_1, B) 5WIV_2, C) 6AK3_1, and D) 6K1Q_1_1, respectively.

Another important microswitch is the TYR^{5.58}-TYR^{7.53}, where upon activation water bridged hydrogen bond was formed [183]. In inactive structure the distance between C α atoms of TYR215^{5.58}, and TYR302^{7.53} calculated as 18.7 Å, however in active structure these two tyrosine residues came closer, 14.0 Å. Our data is in accordance with experimental structures. Figure 4.15 show the TYR215^{5.58} C α - TYR302^{7.53} C α distance distributions for the 2ZIY_1, 5WIV_2, 6AK3_1, and 6K1Q_1_1 interfaces, respectively.

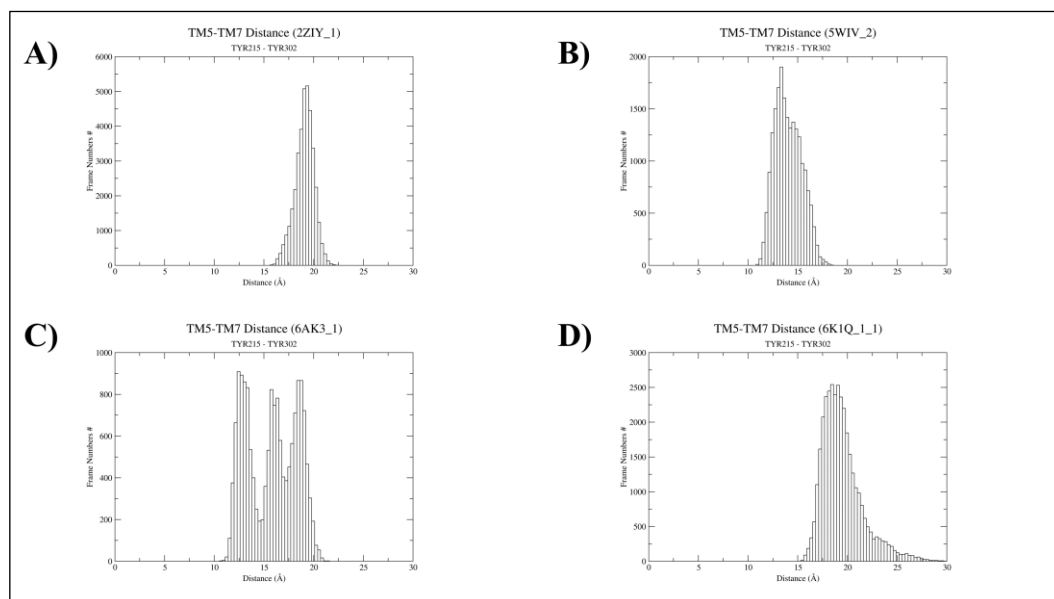


Figure 4.15: TYR215^{5.58} C α - TYR302^{7.53} C α distance distribution for the A) 2ZIY_1, B) 5WIV_2, C) 6AK3_1, and D) 6K1Q_1_1, respectively.

4.14. Inactivation Of AT1R Was Observed In TM3-TM5-TM6/TM3-TM5-TM6, And TM1-TM2-H8/TM3-TM4 Interfaces

Contact fingerprints for the selected interfaces were calculated and depicted in Figure 4.16. The 2ZIY_1 interface was identified as TM3-TM5-TM6/TM3-TM5-TM6. In the intermediate trajectory, GLY269^{6.64}-LYS203^{5.30} was abolished compared to the inactive trajectory (see Figure 4.16, panel A). TM6-TM5, and TM6-TM6 interactions have become stronger for the inactive trajectory. Panel B shows the interaction pattern for the 5WIV_2 interface. For this interface TM1-TM2-TM3/TM1-TM2-TM3 interactions were observed, also ECL1 of AT1R, and ECL1, and ICL1 of AT2R were also contributed to the interface. PHE39^{1.43}-LEU92^{2.52}, PHE55^{1.59}-LYS73^{ICL1}, LEU79^{2.55}-ALA95^{2.55}, LEU83^{2.59}-LEU99^{2.59}, and PRO95^{ECL1}-TYR106^{2.66} interactions were more weaker in active trajectory compared to intermediate trajectories. The residues that are participating in the dimerization interface of 6AK3_1 were depicted in panel C. TM1-TM2-H8/TM1-TM6-TM7 interface was observed. Interestingly, at the beginning of CG simulations, 6AK3_1 interface was classified as symmetrical where TM1-TM7/TM1-TM7 domains were contributed. However during MD simulations, AT1R was rotated and the TM7 contribution of this protomer totally

lost (see Figure 4.16, panel C). GLN315^{8.56}-GLN253^{6.32}, LYS318^{8.59}-PHE320^{7.55}, PRO82^{2.58}-PHE302^{7.37}, ALA89^{2.65}-LEU44^{Nterm}, and TRP94^{ECL1}-LEU298^{7.33} interactions were more stronger in active frames, compared to intermediate frames. Figure 4.16, panel D shows the interactions in the 6K1Q_1_1 interface, where TM1-TM2-H8/TM3-TM4 interactions were observed. LEU316^{8.57}-LYS76^{ICL1}, PHE551.59-GLN72^{1.60}, PHE55^{1.59}-GLY74^{ICL1}, and VAL86^{2.62}-VAL119^{3.27} interactions were more weaker in inactive frames compared to intermediate frames (see Figure 4.16, panel D).

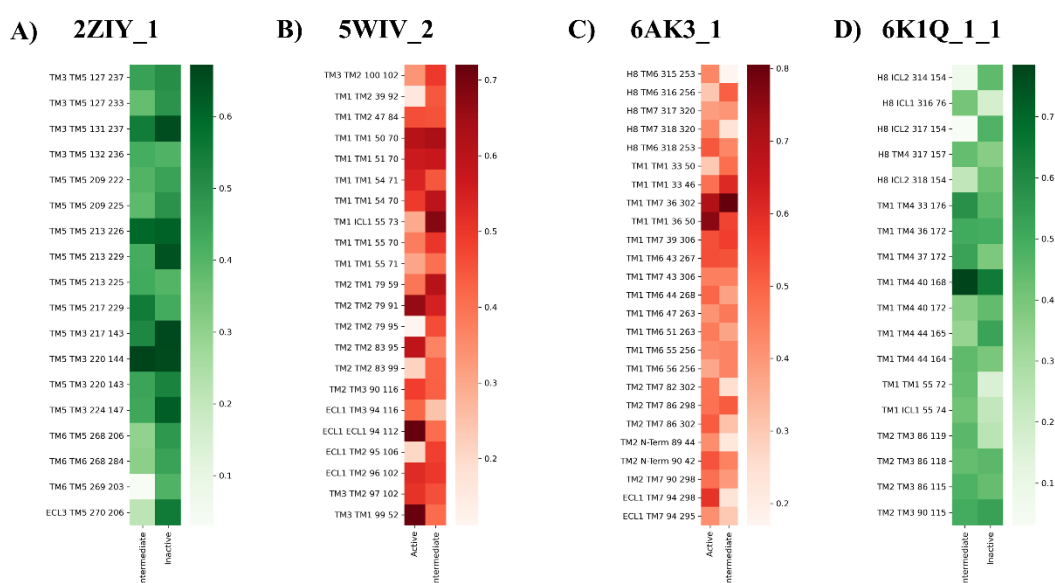


Figure 4.16: Contact analysis of selected interfaces compared in splitted trajectories. First label, and second label belong to AT1R, and AT2R, respectively. Similar labeling applied to residue numbers.

5. CONCLUSION

The first part of this thesis focused on investigating the possible dimerization interfaces for the AT1R. The study examined six different interfaces, each with three distinct dimer pairs (two for the TM6,7 interface) resulting in a total of 17 systems. Through classical MD simulations and post processing analyses, four interfaces emerged namely TM1,2,8 AI, TM5 AA, TM4 II and TM4,5 tight AA. These four interfaces consisted of one active and one inactive protomer as supported by the literature [274–276]. Interestingly, Lyngsø et al. based their study on Karip et al.'s findings and suggested that both protomers should be in the active form [277]. Karip et al. showed that the protomer in the inactive form would inhibit the signaling of the other protomer [278]. However, this study showed that dimer systems with one active and one inactive protomer are stable, particularly the TM1,2,8 AI system, which exhibited stable conformations in both independent MD simulations. While previous studies indicated the presence of H8 at the dimerization interface in one of the first reports of AT1R homodimerization [155], the TM1,2,8 AI system demonstrated positive distinctions from other AT1R dimerization interfaces, revealing that multiple interfaces might contribute to dimerization in recently reported GPCR structures [104, 106]. Additionally, this study highlighted the significance of ILE238^{6.33} in stabilizing the inactive structure, forming an interaction network with the amino acids in TM1, TM2 and TM3, resulting in a hydrophobic cluster just above the G-protein binding site, preventing water molecules from filling the receptor's inner cavity and impeding activation. The aim of this study was to understand AT1R dimerization at the atomic level using MD simulations and contribute to the development of molecules with desired efficacy and efficiency in the future. The methods employed here can be easily adapted to study other GPCRs and explore protein-protein interactions underlying various diseases and pathologies in greater detail.

In the second part of the thesis, several conclusions were drawn. GPCR dimers generate intracellular signals that significantly differ from those of their monomeric state. Dimerization allows the production of more selective intracellular signals than monomeric signaling, necessitating a comprehensive understanding of receptor interactions. To this end, MD simulations provided valuable dynamic characterization of interaction interfaces. Two interfaces where AT2R-induced inactivation of AT1R

was observed were identified: symmetrical TM3, 5, 6, and asymmetrical TM1, 2, H8-TM3, 4 interfaces. Protein structure network and ensemble analyses offered atomic insights, such as the importance of TM5-TM6 interactions for active state stabilization and TM2, 3, and 4 interactions with TM6 for maintaining the latter TM's proximity to the receptor core and stabilizing the inactive state.

The creation of precise dynamic molecular models of GPCR dimers is expected to enhance drug discovery, providing a comprehensive structural framework to understand the molecular mechanisms underlying GPCR function. Methodological advancements in studying GPCR oligomerization are predicted to lead to improved therapeutic options, given the significant role of GPCRs as contemporary pharmacological targets.

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BIOGRAPHY

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APPENDICES

Appendix A:

Erol I., Cosut B.,Durdagi S., (2019), "Toward Understanding the Impact of Dimerization Interfaces in Angiotensin II Type 1 Receptor", Journal of Chemical Information and Modeling, 59 (10), 4314-4327.