

Investigating Annexin A2/A2t-Membrane Interactions

By

Andrew Hadad

July 2026

Director of Thesis: Dr. Yumin Li

Major Department: Chemistry

Abstract

Annexin A2 (A2) and its heterotetrameric form, annexin A2t (A2t), participate in membrane organization, anionic phospholipid clustering, exocytosis, and other membrane-associated cellular processes. Both proteins selectively associate with membranes containing negatively charged phospholipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP₂). Cholesterol (CHOL) has also been proposed to influence these interactions, although its role in regulating A2- and A2t-mediated membrane binding and lipid-domain formation has not been fully characterized. Not only are these interactions important for cellular processes, but also they are implicated in various pathologies and cancers. The goal of this study was to investigate A2 and A2t–membrane interactions over extended simulation timescales and to determine how variations in membrane CHOL content affected protein binding, PIP₂ clustering, lipid mobility, preferred binding orientations, and interaction energetics.

Coarse-grained conventional and umbrella sampling molecular dynamics simulations were conducted using PIP₂-containing membranes with varying CHOL %. The A2 simulations demonstrated that increasing membrane CHOL content strengthened protein–membrane

association. Lipid-specific distance and linear interaction-energy analyses confirmed that PIP₂ was the membrane component most directly involved in A2 binding, as expected from previous studies. The A2–PIP₂ interaction also became increasingly favorable as CHOL concentration increased. CHOL itself was located deeper within the membrane and did not form extensive direct contacts with A2, suggesting that it enhanced protein binding indirectly by modifying membrane structure, lipid organization, and lipid mobility.

A2 also promoted the aggregation of PIP₂ lipids beneath the protein, as seen in PyMOL visualizations, PIP₂ self-distance heat maps, and self-contact analyses, and this action was increased with increased CHOL %. Membrane-only simulations provided insight into the mechanism underlying this effect. Lipid diffusion coefficients decreased for all lipid species as CHOL concentration increased, indicating that CHOL reduced membrane fluidity and lipid mobility. This reduction in diffusion limited the ability of PIP₂ lipids to move away from A2, thereby stabilizing the PIP₂-rich lipid microdomains formed beneath the protein. Consistent with these observations, umbrella-sampling simulations showed that the CHOL 21% membrane produced a shorter optimal binding distance and a more energetically favorable A2–membrane interaction than the CHOL 0% and 12% membranes.

The A2t simulations revealed a similar CHOL-dependent enhancement of membrane binding and PIP₂ clustering. Across all starting orientations, A2t tended to adopt a membrane-bound configuration in which both A2 subunits interacted with the membrane. This indicated that simultaneous membrane association of both subunits was the preferred overall binding mode. Increasing CHOL content generally increased interaction between A2t and the membrane.

The results supported a model in which A2 and A2t selectively bound PIP₂-containing membranes through the convex surfaces of their A2 subunits and promoted the formation of

PIP₂-rich lipid microdomains beneath the proteins. Increasing CHOL % in the membrane reduced lipid diffusion, stabilized PIP₂ clustering, increased protein–PIP₂ contacts, decreased protein–membrane distances, and produced more energetically favorable interactions. These and further systems that look into the more complex roles of A2 and A2t in exocytosis, membrane reorganization, and as an actin-binding-protein can provide a more in-depth understanding of the interactions throughout the body that our cells rely on and that can cause the metastasis of various cancers.

Investigating Membrane and Actin Interactions of Annexin A2/A2t

A Master's Thesis

Presented to the Faculty in the Department of Chemistry

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master's of Science Degree in Chemistry

By

Andrew Christopher Hadad

July 2026

Director of Thesis: Yumin Li, PhD

Thesis Committee Members:

Shouquan Huo, PhD

Adam Offenbacher, PhD

Xiaoping Pan, PhD

©Andrew Christopher Hadad, 2026

Acknowledgements

My years at ECU have shaped me in ways I could not have anticipated when I first began. During this time, I developed both academically and personally, while also forming relationships with my classmates, colleagues, and mentor. Many of them became people I can talk to not just about research and Chemistry, but about personal things as well. I am deeply grateful to everyone who contributed to this experience.

I would first like to express my appreciation to my thesis advisor, Dr. Yumin Li. Throughout my years in her laboratory, Dr. Li consistently provided encouragement, guidance, perspective, and innovative ideas. Her mentorship helped me strengthen my abilities as a researcher while also teaching me valuable lessons. Her influence has played an important role in the person I have become, and I will always be thankful for her support.

I am also grateful to my committee members, Dr. Adam Offenbacher, Dr. Shouquan Huo, and Dr. Xiaoping Pan. Their insight, encouragement, thoughtful feedback, and patience helped me view my research more critically, develop a deeper understanding of my work, and make it to this point.

My gratitude also extends to the ECU chemistry faculty. The knowledge and experience I gained within the department were invaluable, and I learned a great deal from the commitment and work ethic demonstrated by the faculty members with whom I studied and worked.

Joining Dr. Li's laboratory also gave me the opportunity to meet Sam, Wesley, Austin, and Jacob, who became friends and valued colleagues. Sam offered an incredible amount of assistance and guidance throughout my research, and my work would not have developed in the same way without his support. His willingness to share his knowledge, along with his humor,

made the laboratory a much more enjoyable place. Wesley was the one to introduce me to the computational work done in the Li lab while he helped my team in Instrumental Chemistry Lab to learn ICPMS. Without his support and recruitment of me there, I wouldn't be where I am today, even though he graduated just before I fully entered the lab. Austin graduated last year and was someone who I could talk to extensively about our similar plans for after graduation, as well as someone who was in a similar boat to me in regard to research progression. He was invaluable as a sounding board for my ideas and helping him with his research as well was both motivating and enlightening. Jacob will be joining our lab officially in the upcoming semester, and I am very excited for the work we will be doing in drug development and molecular dynamics simulations. He has already impressed me with his work ethic and ability to pick up new methods quickly, and it has inspired me to work with even more urgency.

I would also like to thank the many friends and family who supported me outside the laboratory, including my mother Cathy, my recently departed father Chris, my sister Megan and her husband Matt, my grandmother Shiela, and my best friend Conor. They have all been vital to my development and my mental wellbeing. I could have never gotten to this point without all of their support and inspiration.

To everyone who offered their time, knowledge, friendship, patience, and encouragement throughout this journey, thank you. Your support made this achievement possible, and I will carry your influence with me long after leaving ECU.

Table of Contents

Title Page	iv
Copyright Page.....	v
Acknowledgements.....	vi
List of Tables.....	x
List of Figures	xi
List of Equations.....	xiii
CHAPTER 1: BACKGROUND AND SIGNIFICANCE.....	1
1.1 Annexin	1
1.1.1 Annexin Family.....	1
1.1.2 Annexin A2	2
1.1.3 Annexin A2t.....	4
1.2 S100 Proteins and S100A10	7
1.3 Actin.....	8
1.4 A2-Actin Interactions.....	10
CHAPTER 2: COMPUTATIONAL METHODS	14
2.1 Molecular Dynamics	14
2.2 Coarse-grained Molecular Dynamics.....	15
2.3 Umbrella Sampling Molecular Dynamics.....	16
2.4 Simulation Details.....	17
2.4.1 System Preparation	17
2.4.2 Minimization	18
2.4.3 Equilibration	18
2.4.4 Production.....	18
2.4.5 Data Analysis.....	19
CHAPTER 3: EXPLORATION OF THE BINDING AFFINITY OF ANNEXIN A2 TO A MEMBRANE AND THE IMPACT OF THE CONCENTRATION OF CHOL IN THAT MEMBRANE	22
3.1 Background	22
3.2 Computational Methods	23
3.2.1 CGMD A2 System Details	23
3.2.2 CGMD Membrane-only System Details	28

3.2.3 USMD System Details.....	29
3.3 Results	31
3.3.1 A2-Membrane Distances	31
3.3.2 A2 Membrane Contacts	39
3.3.3 Lipid Contacts and PIP ₂ Clustering.....	42
3.3.4 Linear Interaction Energy by % CHOL.....	46
3.3.5 Lipid Diffusion Coefficients for Membrane-only Simulations	47
3.3.6 USMD Stable Interaction Distances	50
3.4 Conclusions	51
CHAPTER 4: EXPLORATION OF THE BINDING AFFINITY OF ANNEXIN A2T TO A MEMBRANE	54
4.1 Bckground	54
4.2 Computational Methods	55
4.2.1 CGMD A2t System Details	55
4.2.2 USMD System Details.....	60
4.3 Results	62
4.3.1 A2t-Membrane Distances by Subunit.....	62
4.3.2 A2t-Membrane Contacts.....	66
4.3.3 Lipid Contacts and Clustering	67
4.3.4 USMD Stable Interaction Distances	72
4.4 Conclusions	76
CHAPTER 5: EXPLORATION OF THE ACTIN AND MEMBRANE BINDING ACTIVITIES OF ANNEXIN A2	78
5.1 Background	78
5.2 Computational Methods	80
5.2.1 A2-Actin System Details.....	80
5.3 Conclusions and Further Study	84
REFERENCES	87

List of Tables

Table 3.1	25
Table 3.2	28
Table 4.1	56

List of Figures

Figure 1.1	3
Figure 1.2	3
Figure 1.3	4
Figure 1.4	5
Figure 1.5	6
Figure 1.6	9
Figure 1.7	12
Figure 1.8	13
Figure 3.1	25
Figure 3.2	27
Figure 3.3	32
Figure 3.4	34
Figure 3.5	35
Figure 3.6	37
Figure 3.7	38
Figure 3.8	38
Figure 3.9	39
Figure 3.10	40
Figure 3.11	41
Figure 3.12	43
Figure 3.13	44
Figure 3.14	45
Figure 3.15	46
Figure 3.16	48
Figure 3.17	49
Figure 3.18	50
Figure 4.1	57
Figure 4.2	59
Figure 4.3	63

Figure 4.4	64
Figure 4.5	65
Figure 4.6	66
Figure 4.7	68
Figure 4.8	70
Figure 4.9	71
Figure 4.10	73
Figure 4.11	75
Figure 5.1	81
Figure 5.2	82
Figure 5.3	83
Figure 5.4	84
Figure 5.5	86

List of Equations

Equation 2.1	14
Equation 2.2	16
Equation 2.3	21

Chapter 1: Background and Significance

1.1 Annexin

1.1.1 *Annexin Family*

Annexins are a calcium binding protein family that are distinct but closely related in mammals. These proteins are found in a wide range of tissues and cell types including near calcium sequestering intracellular organelles, in the nucleus, or in lung lavage fluid, prostate fluid, and matrix, and they are known for binding to anionic phospholipids upon calcium binding.¹ Annexins are comprised of a carboxyl core and an N terminal. The carboxyl core is highly conserved in this family and has four homologous domains with 70 residues each. These domains have five alpha helices (A-E), and helices AB and DE are connected by loops. There are pockets located in the convex surface between the domains that are potential targets for drug screening to inhibit the interaction between these proteins and membranes. The carboxyl core loops contain calcium binding sites, and these are located on the convex surface of the protein. This core is also where the binding sites for anionic phospholipids and actin are located. The N terminal in this family of proteins faces the cytosol and is variable and results in each protein's individual function.^{1,2} The N terminal contains post translational modification sites as well as protein-protein interaction sites. The N terminal also contains a nuclear exportation sequence that indicates a role in the nucleus for these proteins. One such role is through the reactivity of cysteine 8 to reactive oxygen species. Binding to the annexins' common binding partner, S100 proteins, which are small, dimeric, acidic, calcium-binding EF hand proteins, also occurs at the N terminal and leads to the formation of a heterotetramer consisting of two annexin proteins and two S100 proteins. These proteins in both monomer and heterotetramer states have many

functions including endocytosis, chromaffin granule exocytosis, plasminogen receptor, and redox sensing, among other important processes. One important process for this study is the actin-mediated remodeling of membranes, which when improperly regulated by various cancers, including breast cancer, renal cell carcinoma, and colorectal cancer, can lead to metastasis, so studies into this interaction could be of therapeutic significance.³

1.1.2 *Annexin A2*

Annexin A2, shown in **Figure 1.1**, is a 36 kDa protein that consists of 339 residues.³ The monomer has two sides, a concave and a convex side. A2's N terminal, shown in **Figure 1.2**, is different than the other annexin proteins, leading to its specific functions. It is where ligand and protein interactions occur, and its hydrophobic concave surface binds to S100A10 in order to form the heterotetramer (A2t) seen in **Figure 1.3**. The residues V3, I6, L7, and L10 form points of contact with the helix HI, HIV, and the hinge region of S100A10 after N-acetylation. The carboxyl core is made of four repeat domains of 70 residues each with four antiparallel and one perpendicular alpha helices wound in a right-handed superhelix. The convex side of the C terminal contains the binding site for calcium and anionic phospholipids. Prior studies have shown the potential of A2 and A2t to form protein clusters with anionic phospholipids like phosphatidylinositol (4,5) biphosphate (PIP₂) clustering beneath. This membrane binding action involves R273 and R284 binding to PIP₂, and it has been suggested that cholesterol (CHOL) likely enhances the binding affinity of A2 and A2t to the membrane. The C terminal also contains an actin binding site that binds to free barbed ends of actin filaments, leading to the proteins' role in exocytotic and endocytotic events as well as F-actin bundling.⁴ Simulations showing these various interactions have been completed and analyzed, though ones exploring the

effects of varying levels of cholesterol on the actin-A2/A2t-membrane interactions are yet to be studied in depth.

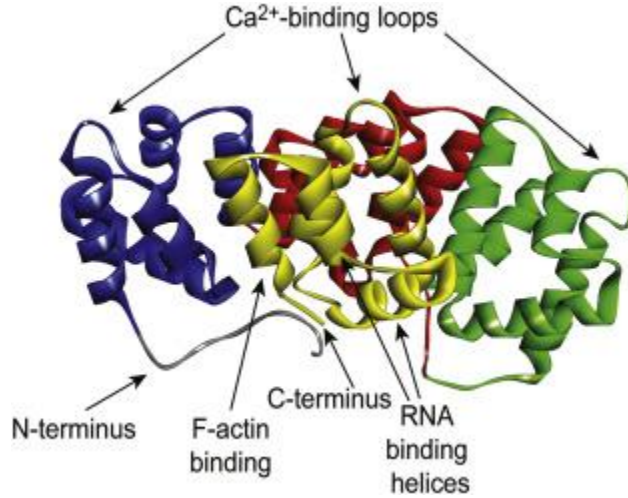
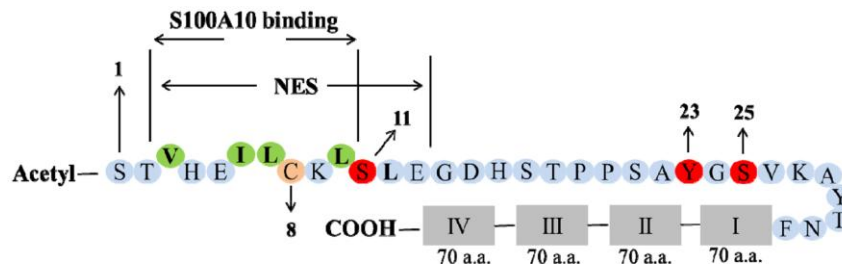


Figure 1.1: Cartoon representation of annexin A2 with important binding sites noted. The four domains of the carboxyl core are blue, yellow, green, and red. The convex (upper) side also contains R273 and R284, which are the binding sites of PIP₂. The N-terminus contains the binding sites for S100A10.⁵



Val 3, Ile 6, Leu 7 and Leu 10 – S100A10 binding hydrophobic residues

Ser 11 – PKC phosphorylation site

Tyr 23 – pp60Src phosphorylation site

Ser 25 – PKC phosphorylation site

Cys 8 – Redox active cysteine

Carboxyl domain – calcium, phospholipid, membrane, F-actin and heparin binding site

Figure 1.2: N terminal of annexin A2 along with the four carboxyl core domains. The N terminal is the site of many protein interactions, specifically with S100A10. The N terminal is also the site of posttranslational modifications and the reactive cysteine residue used for its antioxidant properties in the nucleus.⁵

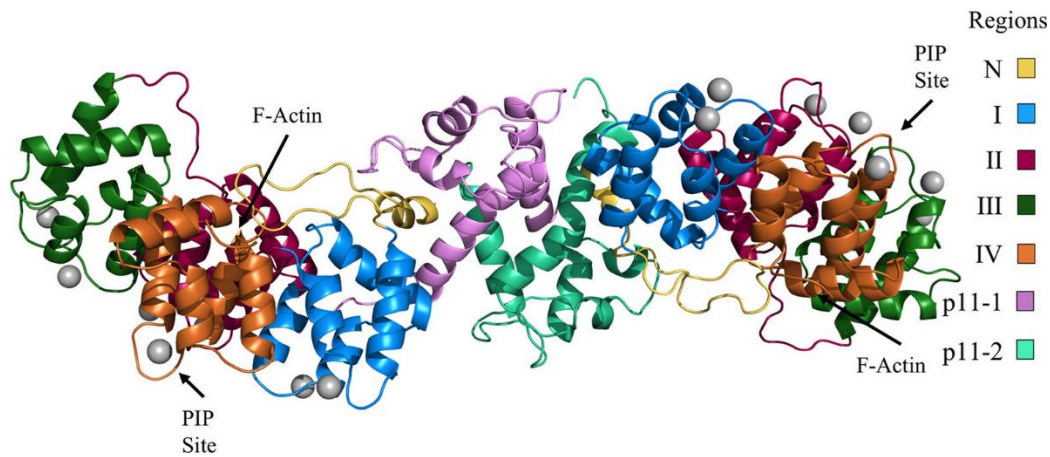


Figure 1.3: Cartoon representation of annexin A2 S100A10 heterotetramer with important regions and binding sites noted. The annexin A2 monomers are bound to p11-1 and p11-2, which are both S100A10 proteins, by their N terminals.⁶

1.1.3 Annexin A2t

Annexin A2t is the heterotetramer form of annexin A2, made of two annexin A2 monomers attached to a S100A10 dimer by their N terminals. S100A10 is a calcium binding EF hand protein that is small, dimeric, and acidic. EF hand proteins are characterized by a helix-loop-helix motif as seen in **Figure 1.4**.⁷ This heterotetramer has various roles in membrane organization events, as noted above, and one of these roles has been associated with the increase in metastasis for various cancers. While A2 itself is not a plasminogen receptor, its partner protein S100A10, whose association with the membrane A2 regulates, is through a C terminal lysine. S100A10 also is able to bind tissue plasminogen activator (tPa), which is necessary for this interaction. When plasminogen and tPa are bound to S100A10, plasminogen is converted to plasmin, as seen in **Figure 1.5**.³ Plasmin is a serine protease that is able to break down fibrin as well as membranes and tissues. This interaction has been implicated as increasing the metastasis of breast cancer, pancreatic ductal adenocarcinoma, renal cell carcinoma, colorectal cancer, hepatocellular carcinoma, and acute promyelocytic leukemia. Because of A2's regulation of

S100A10's association to the membrane, A2 has been viewed as an oncogene. However, it is of note that in other cancers, such as head and neck cancers, A2 had conflicting results, sometimes acting as a tumor suppressor. Studies have already shown that anti-A2 antibodies can be effective in reducing metastasis of various cancers, so further studies into inhibitors of the plasminogen and tPa binding action of S100A10 with A2 as a regulator or inhibitors of A2t heterotetramer formation could provide ways of reducing the impact of these proteins on the metastasis of various cancers.³

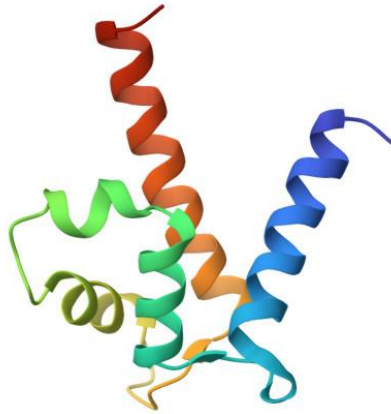


Figure 1.4: Structure of the calcium binding EF hand protein S100A10. S100A10 is a binding partner for annexin A2 and forms a dimer that binds to two A2's at their N terminals.⁷

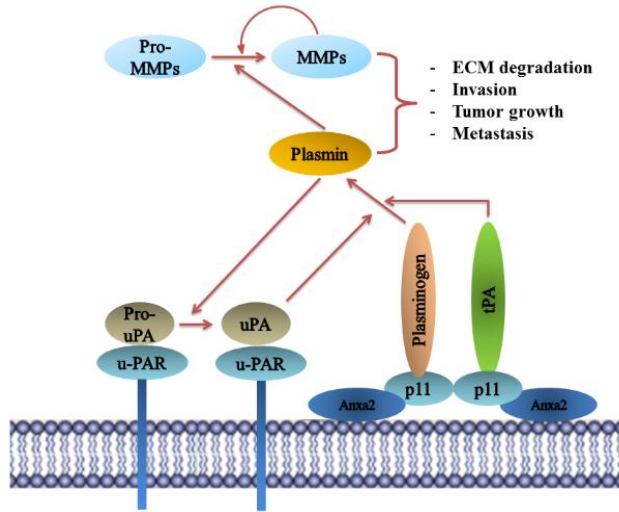


Figure 1.5: A model showing the binding of tPA and plasminogen to the C terminal lysine of S100A10. The A2s act as receptors for the cell membrane, and the urokinase-plasminogen activator (uPA) and receptor (u-PAR) complex is colocalized with the A2t. The binding of tPA and plasminogen to S100A10 promotes cleavage of plasminogen into plasmin, which is used to activate pro-matrix metalloproteases (MMPs), which break down proteins and tissues, to help spread some cancers. The plasmin also activates pro-uPA, which accelerates the cleavage of plasminogen.³

A2 also has other roles in cell polarity, mRNA binding, redox sensing, inflammation, and antiphospholipid syndrome. A2 is able to regulate actin dynamics through its binding to F-actin and PIP₂, both of which are heavily involved in cell-cell adhesion. A2 is found in ribonucleoprotein complexes and in the nucleus, which suggests a role in RNA translation, transport, and calcium dependent binding between negatively charged phosphates in mRNA and a lysine in A2. Localization of A2 with mRNA in the actin cytoskeleton also occurs, so A2 is considered to be a scaffolding protein that stabilizes mRNA in the cytoskeleton. A2 is able to act as an antioxidant in the nucleus, protecting DNA from reactive oxygen species. This action is done by cysteine 8 in the N terminal. A2 is also involved in the production of proinflammatory signals as part of the plasminogen and tPa interaction. Upon receiving a variety of stimuli,

macrophage infiltration and migration through the use of plasmin to break down tissues is promoted by extracellular A2t.

1.2 S100 Proteins and S100A10

S100 proteins, especially S100A10, are vital to this study because of S100A10's interactions with A2 and other proteins. S100 proteins were first discovered by B.W. Moore in 1965. They were named S100 because of their solubility in 100% saturated ammonium sulfate. There are a multitude of these calcium binding S100 proteins in a variety of tissues throughout the body, but the one of most interest to this study is S100A10. S100A10 is, as stated prior, a small, acidic, dimeric, EF hand protein. EF hand refers to a common motif seen in many proteins which describes a helix-loop-helix structure. The loop part of the structure is the one that engages in calcium binding in most S100 proteins. S100A10 is seen to have 2 of these EF hand motifs, which include most of the protein itself, but S100A10 specifically can not bind with calcium, making it distinct from the other members of its family.

This lack of calcium binding does not impede it from its main roles and interactions with other proteins. S100A10 has roles in membrane binding and reorganization events, commonly with a partner protein like A2. In this A2 interaction, S100A10 forms a homodimer, stabilized by non-covalent bonds, and then a complex with two A2 proteins to form A2t. A2t are commonly seen localized around the plasma membrane, allowing S100A10 to participate in membrane reorganization events utilizing other protein interactions, such as A2's interaction with the F-actin cytoskeleton or S100A10's own interactions with plasminogen and tPa.

1.3 Actin

Actin is one of the most abundant proteins in eukaryotic organisms. There are three main isoforms of actin named alpha, beta, and gamma. They are structurally similar and differ only slightly in their N terminals. The alpha isoforms are split into skeletal, cardiac, and smooth muscle to reference their locations and functions in muscle contractions. The beta and gamma actins are found in most cells and function in cell motility and structure.⁸

The general structure of an actin protein, seen in **Figure 1.6**,⁹ is highly conserved between species with 375 residues that fold into outer and inner domains, with the outer being subdomain one and its large insert subdomain two and the inner being subdomain three and its large insert subdomain four in **Figure 1.6A**. The protein is flat with a pointed (-) end and a barbed (+) end. There are two clefts between the domains. The upper cleft between subdomains two and four hold binding sites for nucleotides and divalent cations. The lower cleft between subdomains 1 and 3 at the pointed end has hydrophobic residues that contain the major binding sites for actin binding proteins (ABPs).

There are only slight conformational differences between ADP and ATP bound G actin, though the differences do affect the binding affinities to ABPs. The first change occurs in the subdomain one serine 14 beta hairpin loop, which engulfs the phosphates on the bound nucleotide. In the ATP bound state, there is a hydrogen bonding contact with the gamma phosphate of the nucleotide and the main chain of the methylated histidine 73 sensor loop. After hydrolysis, the serine 14 loop reorients to form a hydrogen bonding contact with the beta phosphate, and the methylated histidine 73 sensor loop moves into the gamma phosphate's previous location, seemingly "sensing" the nature of the nucleotide. These changes during

hydrolysis lead to a weakening of the link between the outer and inner domains, which leads to less stability for the ADP bound actin fibers, a necessary part of the depolymerization of F-actin.

Beta and gamma actins can either be in monomeric G actin form or filamentous F-actin form when polymerized. These forms can be transitioned between with the use of ATP. This process, known as treadmilling, involves actin monomers binding to the barbed end of another growing actin fiber in the more stable ATP bound F-actin state. The binding occurs by the D-loop of an F-actin subunit binding and inserting an amphiphilic helix into the lower target binding cleft of the subunit above it. As F-actin is a slow ATPase, hydrolysis can occur in the filament, and ADP state F-actins will dissociate from the pointed end, leading to a recycling of actin monomers. This process is key to actin proteins in their diverse roles of maintaining cell shape and polarity and cell motility.

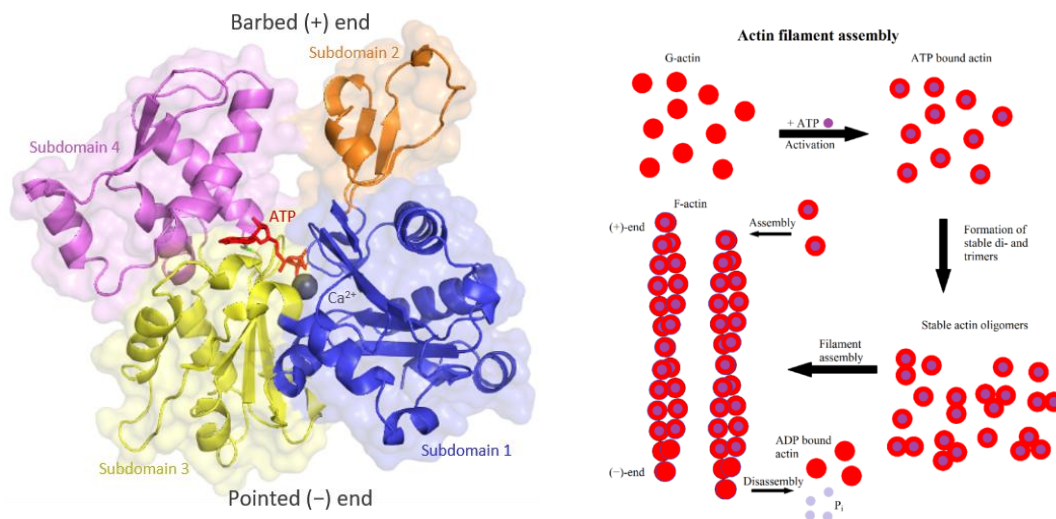


Figure 1.6: (A) Structure of an actin subunit subdomains 1 and 2 are considered the first domain, while subdomains 3 and 4 are considered the second domain. (B) The ATP dependent actin polymerization and depolymerization treadmilling process. ATP-bound actin monomers are added at the barbed (+) end of the fiber, and hydrolyzed ADP-bound subunits are dissociated at the pointed (-) end.⁹

1.4 A2-Actin Interactions

ABPs are directly involved with actin protein functions. Annexin A2 and A2t are two such proteins that bring more dynamic actin filaments closer to anionic phospholipid rich membranes in order to engage in exocytotic events and membrane organization. The K_d for F-actin binding to A2t is ~ 0.2 mM with two potential binding sites. The interaction between A2 and A2t and actin have been noted to occur when already relatively close to a membrane. It is also noted that A2 and A2t have not been found to associate with microvilli, stress fibers, and other more rigid actin structures, just the more dynamic F-actin fibril structures. The second major role for A2 and A2t is the bundling of actin filaments, which has the effect of controlling the number of exocytotic events. This has been explained in A2t when both annexin subunits bind to different actin fibers, and in A2 by the presence of two binding sites for actin and the likelihood of oligomerization of the actin-A2 complex.⁸⁻¹¹ An important example of an exocytotic process involving the A2-actin interaction is with neuroendocrine chromaffin granules, which synthesize, store, and release catecholamines, peptides, and ATP. These granules originate in the endoplasmic reticulum and mature in the Golgi system. Movement of the chromaffin granules to the membrane is mediated by dynamic F-actin structures with the use of myosin, as well as microtubule networks. Once stimulated by the cell, A2 in the cytosol moves closer to the plasma membrane. A2 binding to the membrane results in the aggregation of anionic phospholipids underneath it, mediating the formation of lipid microdomains. These actin bundles also bind to chromaffin granules and connect them to the plasma membrane, preparing the exocytotic event. In this way, the interaction of A2 and actin can control the number of exocytotic events, the number of lipid microdomains, and the number of membrane-attached granules. F-actin and A2 also provide the granules with the means for both fusion pore expansion and secretion, though the mechanics behind these can be further explored. A2 is also of interest

due to its PIP₂/CHOL/GM1 aggregation being necessary for binding with SNARE proteins, which are known to mediate the granule fusion process.

Misregulation of this interaction can thus lead to elevated numbers of exocytotic events, which can be harnessed by various cancers to aid in metastasis. Simulations and studies further investigating these roles would be important to understanding more about these interactions' roles in exocytotic events and the metastasis of various cancers.³

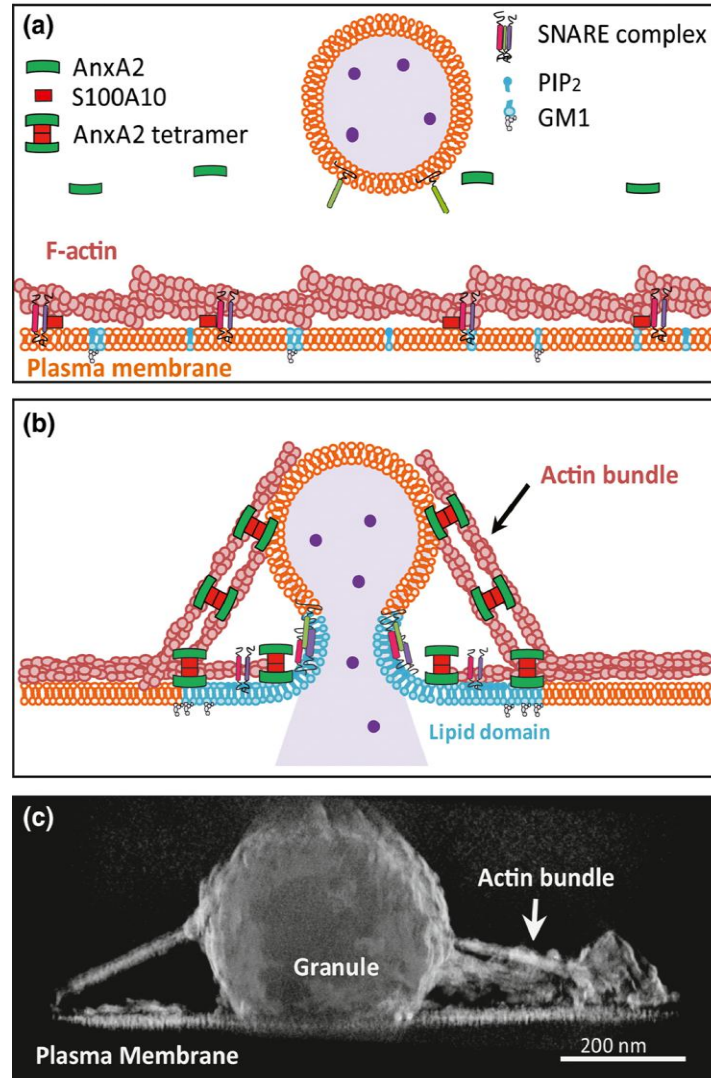


Figure 1.7: In an unstimulated chromaffin granule (A), the granules are not coated in F-actin and brought closer to the membrane. In a stimulated chromaffin granule during stress or danger (B) annexin A2t is able to bind to the membrane and to F-actin fibers near the membrane and coating the granule. This brings the granule to the prepared exocytotic site for fusion and secretion to occur. This is seen in the electron tomography analysis (C) with the annexin A2t's being located on the right side bound to the membrane and the fibrils.¹⁰

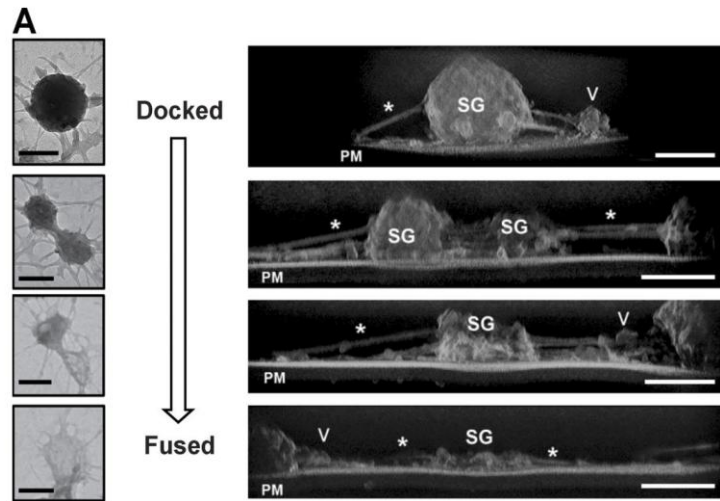


Figure 1.8: Electron tomography analysis of a chromaffin granule (SG) as it is docked to and fused to an exocytotic site on the membrane with the help of F-actin fibers and annexin A2t.¹⁰

Chapter 2: Computational Methods

2.1 Molecular Dynamics

A protein's function is closely tied to its structure. Viewing changes in a protein's structure and interactions with other proteins at the atomic level is difficult to do. As a result, computational approaches are used to simulate and visualize these proteins at the atomic scale. Among these approaches, molecular dynamics (MD) is one of the most widely used techniques, and it uses structure information, usually submitted to the Protein Databank, and classical mechanics to describe the motion of the atoms.

In MD simulations, atoms or groups of atoms are modeled as particles connected by springs. The motion of these particles is achieved using force fields, with an example given in **Equation 2.1**.

$$U = \sum_{bonds} K_b(b - b_0)^2 + \sum_{angles} K_\theta(\theta - \theta_0)^2 + \sum_{dihedrals} \frac{V_n}{2}[1 + \cos(n\psi - \varphi)] + \sum_{electrostatic} \frac{q_i q_j}{\epsilon_d r_{ij}} + \sum_{vdW} \left\{ \epsilon_{ij} \left[\left(\frac{R_{min,ij}}{r_{ij}} \right)^{12} - \left(\frac{R_{min,ij}}{r_{ij}} \right)^6 \right] \right\} \quad \text{Equation 2.1}$$

This potential energy function provides a general description for modeling proteins and other systems like membranes. The total potential energy includes both bonded and non-bonded terms. The bonded terms are bond stretching, angle bending, and dihedral rotations. The bonds term accounts for energy changes with deviations in bond length, where K_b represents the force constant and $b - b_0$ represents the deviation from the bond length b_0 . The angles term describes deviations from the bond angle θ_0 , with K_θ representing the angular force constant. In the

dihedrals term, V_n is barrier height and n is barrier periodicity. The dihedral angle between planes is ψ , and φ is the phase shift.

Non-bonded terms include electrostatic and van der Waals interactions. Coulomb's law is used for electrostatic interactions, in which charges q_i and q_j are divided by their distance from each other r_{ij} the dielectric constant ϵ_{ij} . Van der Waals interactions are modeled using the Lennard-Jones potential. This potential depends on the depth of the energy well ϵ_{ij} , the distance at which the potential energy equals zero, $R_{min,ij}$, and the distance between atoms r_{ij} .

The forces acting on the protein must be continuously recalculated as atomic positions change to create a simulation that explores the change in a protein over time. In order to do this, a series of steps must be completed. First is the creation of the system, which includes defining the positions of the atoms and their initial velocities. This allows calculations to include interactions with nearby atoms, which will affect the forces imparted on the atoms. These calculations are iterated upon according to the time-step set.

It is of note that these short timesteps can limit the ability of simulations to capture larger interactions for long periods of time due to computational load.

2.2 Coarse-grained Molecular Dynamics (CGMD)

Coarse-graining is used to simplify a system to lessen the computational load. Coarse-grained modelling involves representing four heavy atoms as a singular bead, created using the Martini force field.^{13,14} The bonded terms are taken from reference all-atomic simulations, while the non-bonded terms are taken from experimental free energies from many chemical compounds. This does reduce the resolution for the system, though some groups like rings are

represented with more resolution. There are also numerous subtypes for the beads to better describe their chemical nature. This method is used for the creation of the system, after which the typical steps for MD simulations are completed.

2.3 Umbrella Sampling Molecular Dynamics (USMD)

Umbrella sampling is an enhanced sampling method that can be used to calculate the relative free energy of different states along a reaction coordinate, which is a continuous parameter that is used to describe the system.¹⁵ These calculations involve the introduction of a harmonic bias potential, seen in **Equation 2.2**, to overcome any potential energy barriers.

$$w_i(s) = \frac{1}{2}k_i(s - s_{0,i})^2 \quad \text{Equation 2.2}$$

In the equation, k_i is the force constant, $s_{0,i}$ is the reference point on one window/bin i , and s is the point along the reaction coordinate at the current time in the simulation. Simulations are run and free energy is calculated for each of the bins in the reaction coordinate, and the output files can be analyzed by methods like weighted histogram analysis to obtain information according to the reaction coordinate. For example, a typical reaction coordinate is the distance between a protein and a membrane. For this reaction coordinate, the bins would be set stepwise at distances between the minimum and maximum distances. Within these bins, the protein's initial distance from the membrane is set, but the protein's distance is allowed to fluctuate slightly. This allows for overlaps with neighboring bins. The steps for these bins must be small enough for this overlap to occur to simulate a continuous parameter. The energy between the protein and membrane are then able to be represented and correlated with the distance of the protein from the membrane to determine the most stable binding distances.

2.4 Simulation Details

2.4.1 System Preparation

For each system in these studies, the proteins (A2, A2t, actin) were first obtained from the Protein Databank in a PDB structure file. This gives the positions of the atoms and their bonding interactions. Any mutations or alterations to the protein structure are made at this point. For the A2 and A2t, a mutation of calcium binding residues to alanine was required due to the incompatibility of coarse-graining and calcium. These files were then coarse-grained using the Martini forcefield to simplify each structure to reduce computational load and allow for longer term simulations. The lipids needed for the membranes were collected from the Martini Lipidome and were thus already coarse-grained. The lipids used in these studies were phosphatidylcholine (POPC), phosphatidylserine (POPS), phosphatidylinositol (4,5) biphosphate (PIP₂), and cholesterol (CHOL). These lipids were formed into a membrane using mMAEVE, a program for constructing more complex membrane and protein systems. The size of the membrane, the number of lipids, and the percent composition of the lipids can be provided, and the lipids will be evenly distributed across the structure selected. The protein can then be placed in reference to the membrane to create the full coarse-grained system.

In order to simulate cellular conditions, the system must be solvated. This was done using the GROMACS software suite. Water, as well as sodium and chloride ions, were distributed throughout a set simulation box with the same length and width as the membrane and a specialized height that depends on the system. The box uses a periodic boundary to ensure that the atoms of the system do not leave the box while also solving any temperature or pressure-related issues that may arise from using a stricter box. The periodic boundary allows for atoms

that would leave the box to cross over to the other side, thus simulating a membrane with infinite size.

2.4.2 *Minimization*

Each system was then minimized to reduce strain and remove contacts that presented problems. The minimization was run in two steps. The first was a softcore minimization with 5,000 steps and was followed by the second step of 5,000 steps. These steps generated output files for use in equilibration.

2.4.3 *Equilibration*

Equilibration was run on the minimized system for 250,000 steps with a timestep of 20 femtoseconds. The system was heated to 310K using v-rescale temperature coupling. The system also used semiisotropic Berendsen pressure coupling at a reference pressure of 1.0. The output files were used in the production step.

2.4.4 *Production*

Production was done using the GROMACS software suite. The equilibrated output files were used and run for a specified number of steps depending on the required simulation time, but with a timestep of 20 femtoseconds. A velocity-rescale thermostat set to 310 K and a coupling time constant of 1.0 ps was used separately for the proteins, lipids, and solvent. Pressure coupling was done using a semiisotropic Parrinello-Rahman (PR) barostat with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$ and a time constant of 12.0 ps to maintain a one bar pressure. PR is more accurate

and adaptable, so it is better for use when large changes for a system are expected. The result of this step are output files that contain the trajectories of the atoms in the system over the full simulation time. These trajectories, along with the structure files generated from the equilibration step, can be reimaged to obtain a viewable simulation for the set timeframe.

2.4.5 Data Analysis

Distance analysis was performed for the CGMD simulations. The distances between the protein and the membrane, the distance between protein residues and individual lipids, and the distance between individual lipid heads can be calculated over the entire simulation or during specific periods, such as the end of the simulation, or frames where the protein is most closely associated with the membrane. The scripts for these analyses was performed in Jupyter Lab analysis notebooks and will rely on Pandas, NumPy, SciPy, and MDAnalysis packages.²³⁻²⁵

Contact analysis was performed on the CGMD simulations to determine the total number of times that lipids and proteins come into contact with each other. This can be used to determine the number of close contacts between a protein and membrane or between a specific group of lipids to each other. A cutoff of 10 Å was used. This analysis was performed using the methods set out in the MDAnalysis project.

Linear interaction energy analysis was performed on the CGMD simulations to calculate the energy of the interaction between the A2 protein and the membrane. The van der Waals and electrostatic interactions were calculated for non-bonded atomic interactions between the protein and the membrane and averaged over the simulation. This analysis was performed using cpptraj.

Lipid diffusion coefficient analysis was performed on the CGMD simulations specifically with the protein absent. This analysis is used to determine how quickly the lipids in a membrane can diffuse, which can help in determining if there are interactions reducing the rate of diffusion and keeping lipids together. This can be calculated for individual lipid groups.

Variance analysis was performed for the CGMD simulations. This can determine an average of how far the lipids in a group are from the center of mass of that group over time. This can be correlated to association with the protein to gain more insight into any lipid clustering due to protein association.

Weighted histogram analysis was used to analyze the USMD simulations. This analysis can determine the distance between a protein and a membrane correlating to an energy minimum. This method analyzes the output files from USMD simulations and creates bins out of discrete states. The discrete states in these studies are the distances between the center of mass of the protein and the center of mass of the membrane. Histograms are then created with those distance bins that show the probability of observing that distance. The bins will have sufficient overlap in distances to create a continuous parameter. This method can then calculate the potential of mean force when analyzing these bins. The equations used can be seen in **Equation 2.3**.

$$P(\xi) = \frac{\sum_{i=1}^{N_w} g_i^{-1} h_i(\xi)}{\sum_{j=1}^{N_w} n_j g_j^{-1} \exp[-\beta(w_j(\xi) - f_j)]} \quad \text{Equation 2.3}$$

$$\exp(-\beta f_j) = \int d\xi \exp[-\beta w_j(\xi)] P(\xi)$$

β is inverse temperature $1/k_B T$ with k_B being Boltzmann's constant and T being temperature. n_j is the number of data points in the histogram h_j , and g_i is the statistical inefficiency $(1+2\tau_i)$ and τ_i is the integrated autocorrelation time of the bin. ξ is the reaction coordinate, w_j is the bias potential function, and f_j is the dimensionless free energy of the system.²⁷

Chapter 3: Exploration of the binding affinity of Annexin A2 to a membrane and the impact of the concentration of CHOL in that membrane

3.1 Background

Annexin A2 is found abundantly in the human body and is seen in a variety of tissue and cell types.³ They are commonly found in the cytosol and especially near the plasma membrane. A2 is known to interact with the membrane in a variety of functions. These include exocytotic events, such as the release of neurotransmitters from chromaffin granules, and membrane reorganization events using the actin cytoskeleton. A2 has been implicated as contributor to different pathologies, most importantly to this study, a variety of cancers. A2 has been seen to increase the metastasis of cancers like breast and colorectal cancer through its membrane binding and disrupting activities. One such activity is the ability for A2's binding partner S100A10 to bind both tPa and plasminogen, after which plasminogen is cleaved to plasmin, resulting in the breaking apart of nearby cell membranes. A2 allows for the close association of S100A10 to the membrane, so A2 is seen as a regulator of this interaction. Misregulation of interactions like these are what leads to increased cancer metastasis and worsened prognosis for patients. If a way to reduce A2's association to the membrane in cancer cells is discovered, this method of metastasis would be reduced and could aid the patient.

To begin this study, a look at A2-membrane interactions over a long-term period is required. A2 binds to membranes that include anionic phospholipids, such as PIP₂. These lipids bind to residues on the convex side of the protein. Studies have been done to investigate this interaction with both PIP₂ and POPS, and not only did A2 bind selectively to these lipids, but it also assisted in the formation of anionic lipid rafts underneath itself. It has also been noted that A2-membrane association is influenced by CHOL presence in the membrane, and that a

deficiency of CHOL may even inhibit A2-membrane binding all together. Another aspect is that CHOL is known to stabilize the formation of anionic lipid rafts as well. In this study, the influence of membranes with compositions that vary in CHOL amount on A2 binding and lipid raft formation was further explored on a larger time scale than previous studies. This was done using CGMD to simplify the large system to allow for longer simulations and USMD to overcome any potential energy barriers and provide optimal binding distances. The primary goal was to determine optimal binding distances in varying cellular environments and quantify contributions of A2 and CHOL to lipid raft formation and stability. The secondary goal was to provide a scaffold for A2-membrane interactions such as vesicle fusion and exocytosis.

3.2 Computational Methods

3.2.1 CGMD A2 System Details

The first system that was explored is a single A2 protein located over a lipid bilayer with varying CHOL amounts. The A2 protein was taken from the Protein Databank (2HYW.pdb). This file contains the structure of calcium-bound monomeric annexin A2 with no N terminal present. The N terminal's absence has been seen in previous studies to not affect the binding of A2 to membranes.²⁸ All non-A2 molecules were removed from the file to leave just the A2 protein in the file. This file was then coarse-grained using the Martini2 coarse-grained force field elnodyn22, which will use an elastic network with an upper cutoff of 0.9 nm, a lower cutoff of 0.5 nm, and a force constant of $700 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ as the basis for maintaining the protein's overall shape.¹⁷ This reduces four heavy atoms into one bead with specific designations to specify its properties. This reduces the resolution of the system and allows for longer-term simulations with less computational time. The lower resolution is reasonable for this study

because of the focus on overall protein and membrane interaction rather than smaller conformational changes, and it makes these longer-term simulations possible in a reasonable amount of time. The four coarse-grained lipids, POPC, POPS, PIP₂, and CHOL, were collected from the Martini Lipidome. From here, different starting structures were made using the mMAEVE program developed by Dr. Samuel Lindsay. The program allows the user to create various protein and membrane structures, like vesicles and bilayers, with varying compositions and positions. For the first system, one A2 protein was centered 50 Å over a 125x125 Å bilayer with a 7 Å separation between the layers and 250 lipids per layer. The A2 protein was oriented so that the convex side of the protein was facing the membrane. This system was split into eight different membrane compositions, 0%, 3%, 6%, 9%, 12%, 15%, 18%, and 21% CHOL (see **Table 3.1**). The POPS and PIP₂ percentages remained constant at 20% and 8% respectively. The POPC percentages were 72%, 69%, 66%, 63%, 60%, 57%, 54%, and 51% to accommodate the rising CHOL percentage. POPC was chosen to decrease because it is a neutral lipid and thus shouldn't have a noticeable effect on A2 binding to the membrane, as it selectively binds to anionic phospholipids. A representative starting structure can be seen in **Figure 3.1**.

Table 3.1: Compositions of the membranes for each of the eight CHOL compositions. The main lipids changing in percentage are CHOL and POPC.

Trial	% POPC	% POPS	% PIP ₂	% CHOL
CHOL 0	72	20	8	0
CHOL 3	69	20	8	3
CHOL 6	66	20	8	6
CHOL 9	63	20	8	9
CHOL 12	60	20	8	12
CHOL 15	57	20	8	15
CHOL 18	54	20	8	18
CHOL 21	51	20	8	21

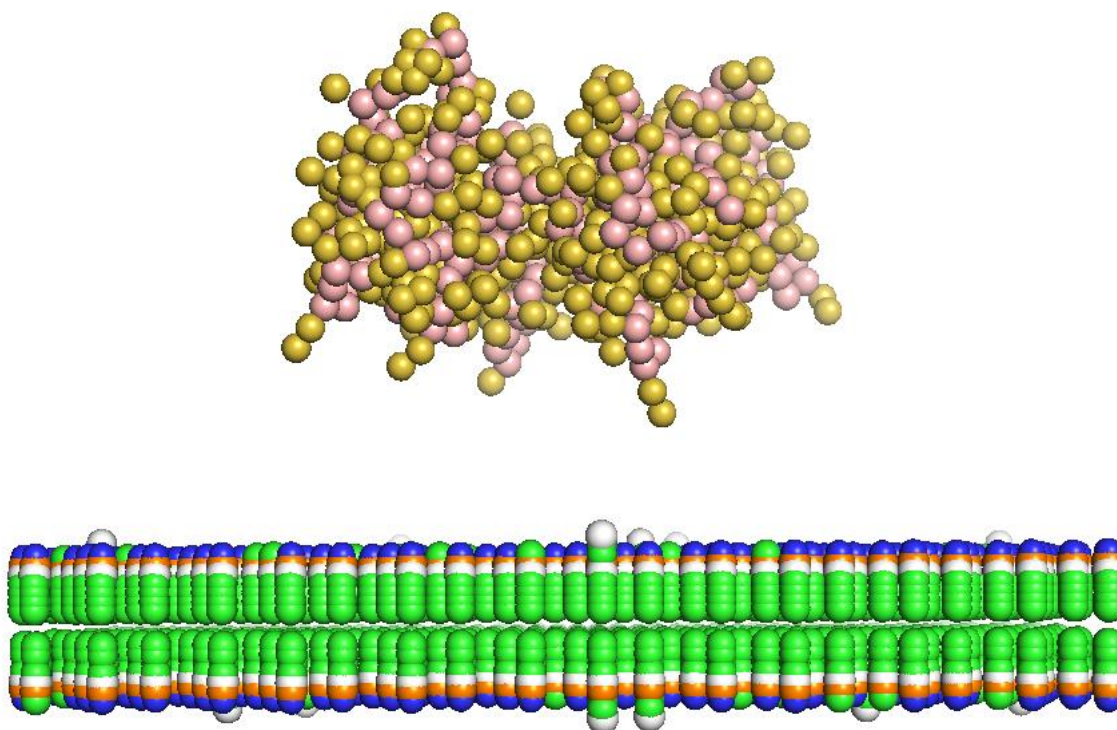


Figure 3.1: Starting structure for A2 coarse-grained simulations. For the conventional molecular dynamics simulations, the A2 was placed 50 Å above the membrane. For umbrella sampling, the protein ranged from 20-80 Å above the membrane. The distance above the membrane is the reaction coordinate and its reference point changed for each bin.

These eight starting structures were then solvated with water, sodium, and chloride ions to simulate a cellular environment. The GROMACS software suite was used for all of the following simulation steps. A box with periodic boundaries was created to contain the atoms of the system with dimensions of 125x125x450 Å. The height of the box was lengthened to avoid the A2 potentially moving towards the top of the box and remaining there for the duration of the simulation. A steep integrator was used with 5,000 steps to solvate the box with 0.15 M water and ions.

The output of the solvation step was then minimized to reduce strain and clear up problematic contacts. This occurred in two steps with 5,000 steps each with a steep integrator. These steps also generated starting velocities for 310K.

The output of the minimization step was then equilibrated to gradually raise the temperature to close to body temperature. This was done in 250,000 steps of 20 femtoseconds timesteps to raise the temperature to 310K using a v-rescale thermostat while maintaining 1.0 bar of pressure by using a semiisotropic Berendsen barostat with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$.

The output of the equilibration step was then used for the production step. The md integrator was used to simulate 10 μs through 500,000,000 steps with a timestep of 20 femtoseconds. A v-rescale thermostat was used to simulate the system at 310K, and a semiisotropic Parrinello-Rahman barostat was used to maintain 1.0 bar with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$. The output trajectories were then reimaged converting the trajectories using -pbc mol to enforce the periodic boundary for the system box. Each membrane composition was simulated three additional times to obtain a robust data set. The first and final frames of three of the CHOL compositions can be seen in **Figure 3.2**.

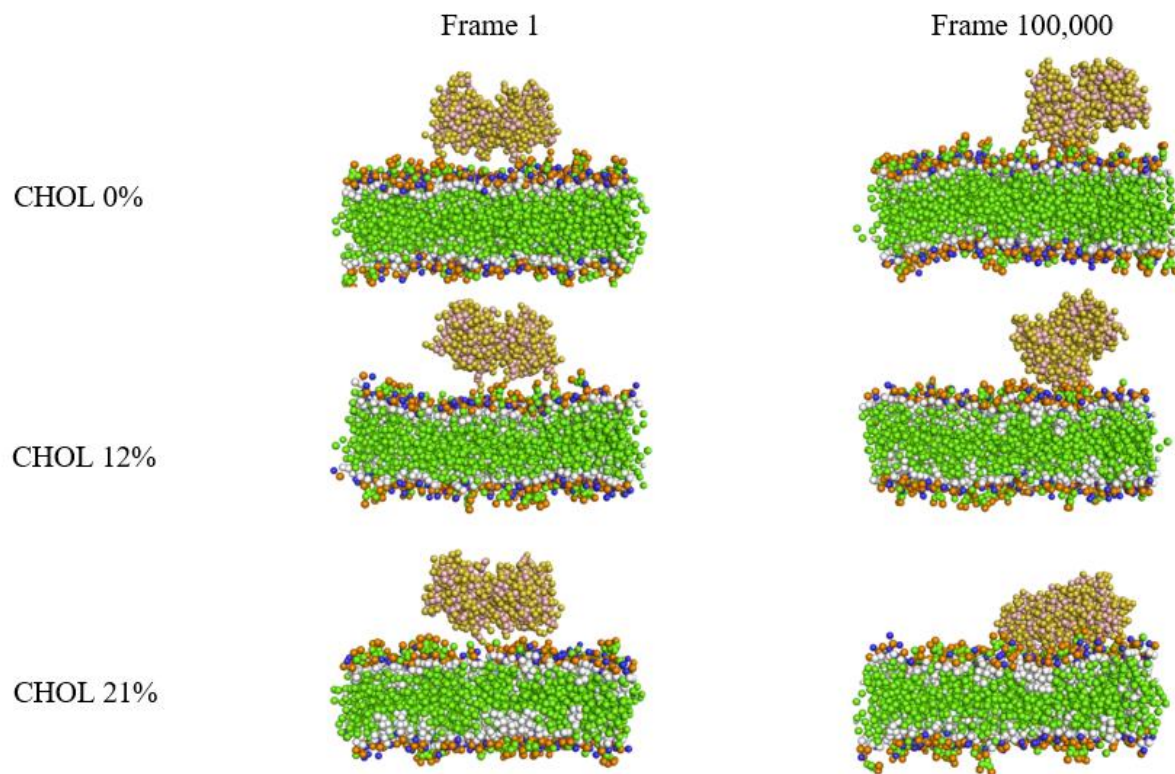


Figure 3.2: First and final frames of the CHOL 0, 12, and 21% simulations. The A2 is shown in yellow, and the membrane is shown below the proteins. The protein associates with the membrane more as the simulation progresses.

3.2.2 CGMD Membrane-only System Details

The second system tested excluded the A2 and had only the membrane included. This membrane was created using the Martini Lipidome's POPC, POPS, PIP₂, and CHOL lipids. These lipids were distributed equally in the 125x125 Å bilayer with 7 Å in between each layer. The composition of the bilayer followed the same specifications as trials CHOL 0, CHOL 12, and CHOL 21, as seen in **Table 3.2**.

Table 3.2: Compositions of the membranes for each of the three CHOL compositions used in the membrane-only simulations. The main lipids changing in percentage are CHOL and POPC.

Trial	% POPC	% POPS	% PIP ₂	% CHOL
CHOL 0	72	20	8	0
CHOL 12	60	20	8	12
CHOL 21	51	20	8	21

These three systems were then solvated with water and ions (sodium and chloride). The box size was reduced to 125x125x100 Å, as the protein is not included in the system and thus there is no chance of a biologically important molecule crossing the top boundary. The output of this solvation step was minimized with a steep integrator in 2 sets of 5,000 steps. The systems were then equilibrated using a v-rescale thermostat to 310K with a semiisotropic Berendsen barostat set to 1.0 bar with a compressibility of 3.4e-4 bar⁻¹. The systems were then run for 10 μs with 500,000,000 steps with a timestep of 20 femtoseconds. A v-rescale thermostat at 310K and a semiisotropic Parrinello-Rahman barostat set to 1.0 bar with a compressibility of 3.4e-4 bar⁻¹ were used for this production step. The resulting trajectories were then converted once with -pbc mol to enforce the periodic boundary condition for variance, contact, and distance analyses and converted a second time with -pbc nojump to remove the periodic boundary condition, allowing

full diffusion of the lipids to calculate lipid diffusion coefficients. Each membrane composition was simulated three times for a robust data set.

3.2.3 USMD System Details

The USMD system has a similar setup to that seen in the CGMD A2-membrane simulations, with A2 centered over a membrane. The A2 was collected from the Protein Databank (2HYW.pdb). This file contains the structure of calcium-bound monomeric annexin A2. All non-A2 molecules were removed from the file to leave just the A2 protein in the file. This file was then coarse-grained using the Martini2 coarse-grained force field elnedyn22, which will use an elastic network with an upper cutoff of 0.9 nm, a lower cutoff of 0.5 nm, and a force constant of $700 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ as the basis for maintaining the protein's overall shape.¹⁷ The four coarse-grained lipids, POPC, POPS, PIP₂, and CHOL, were collected from the Martini Lipidome. One A2 protein was centered over a 125x125 Å bilayer with a 7 Å separation between the layers and 250 lipids per layer, though in this study, the height varied from 20 Å to 80 Å with 2 Å steps. The A2 protein was oriented so that the convex side of the protein was facing the membrane, the same orientation seen in the CGMD A2 study. Three different compositions were used for the membrane, and they mirrored those used in the CGMD Membrane-only study, which can be seen in **Table 3.2**.

The system was then solvated with water, sodium ions, and chloride ions to a concentration of 0.15 M in a box with dimensions of 125x125x180 Å using a steep integrator with a maximum of 50,000 steps. The height of the box is noticeably less than the height seen in the CGMD A2 study due to the A2 in the USMD study being more restricted in the heights above the membrane between which a single bin's starting structure can fluctuate.

These systems were then minimized in two steps, both using a steep integrator with 5,000 steps, a Berendsen thermostat set to 310 K, and a semiisotropic Berendsen barostat set to 1.0 bar with a compressibility of $3.4\text{e-}4\text{ bar}^{-1}$. Next, the pull groups were established as the A2 center of mass and the membrane center of mass and a harmonic biasing potential was used along the z axis with a width of 1,000 to pull the A2 back towards its starting location if it diffuses too far. Each system was then equilibrated using a sd integrator in 50,000 steps with a Berendsen thermostat at 310 K and a semiisotropic berendsen barostat set to 1.0 bar with a compressibility of $3.4\text{e-}4\text{ bar}^{-1}$.

The outputs were then sent to the production step, where a sd integrator was used to simulate 10 μs simulations with 500,000,000 steps and a timescale of 20 femtoseconds. A v-rescale thermostat set to 310 K and a semiisotropic Berendsen barostat set to 1.0 bar with a compressibility of $3.4\text{e-}4\text{ bar}^{-1}$ were used for each of the systems. The outputs of this step were analyzed using the weighted histogram analysis method (WHAM).

3.3 Results

3.3.1 *A2-Membrane Distances*

The first goal of the study was to find optimal binding distances for A2 and a membrane containing anionic phospholipids and varied amounts of CHOL. The membranes in this study all contain PIP₂, to which the convex side of A2 selectively binds. There are eight membrane compositions tested in this study that range from 0% CHOL to 21% CHOL, and each of these membranes were simulated five times. A simple way to determine A2 associated binding distances is through distance analysis, where the distance between two groups selected by the user is calculated for specified times during the simulation. The simulations that were explored for this analysis are the CGMD A2-membrane systems. The groups selected for the distance analysis were the center of mass of A2 and the center of mass of the membrane, including all lipids in both leaflets. The distance between these groups was only calculated for the z coordinate, representing just the height of A2 over the membrane. Instead of selecting the entire simulations, or even the last half of the simulations, specific groups of frames representing the closest A2-membrane association were selected for each simulation to represent A2 being bound to the membrane. These groups of frames were between 10,000 and 20,000 frames in length, equaling from 1 to 2 μ s of the 10 μ s simulations. Those distances were collected for each trial's repeats and averaged to return **Figure 3.3**, shown below. These calculations involved the use and editing of code from the MDAnalysis project.

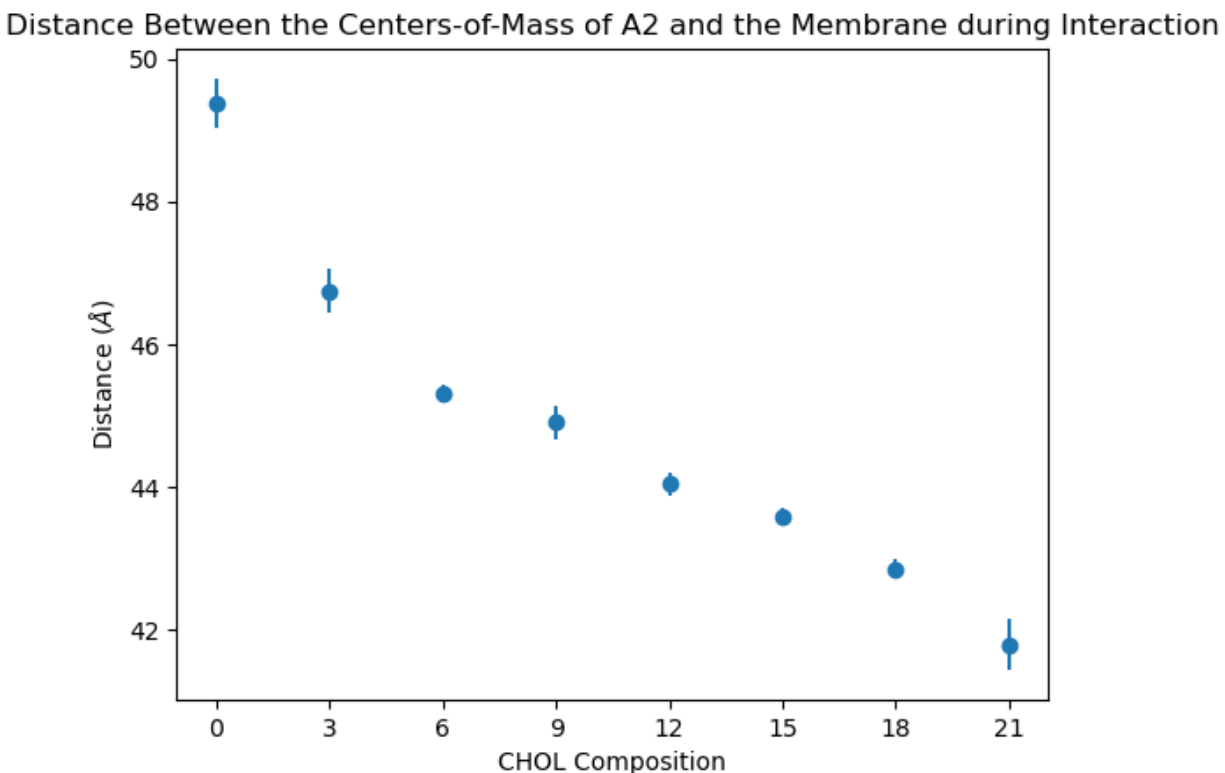


Figure 3.3: Plot showing the distance between the center-of-mass of A2 and center-of-mass of the bilayer for the different CHOL compositions. The frames selected for this analysis were during the times of most A2-membrane interactions during the simulations. Four simulations per CHOL % were averaged, and the standard deviations are shown.

As the amount of CHOL in the membrane increases, the height of A2 above the membrane during closest interaction decreases. Previous studies have posited that CHOL does have some impact on A2-membrane binding, and this distance analysis suggests the same.²² From the 0% CHOL average to the 21% CHOL average, the smallest and largest CHOL trials respectively, the binding distance decreased by 7.58 Å, illustrating that CHOL amount in a membrane does have an effect on at least the distance of the A2-membrane interaction. It is of note that a lack of CHOL has been correlated with potentially inhibiting A2-membrane interaction as well as anionic lipid clustering, and the binding distance average of 49.38 Å for the

CHOL 0% trials is only 0.62 Å less than the initial starting structure, so while there is still interaction occurring, it is weaker than with CHOL present.

Before focusing on specific lipids, a linear interaction energy analysis was completed to determine the interaction energy between each individual lipid group and A2. This was done to ensure that the PIP₂ lipids were the ones binding to A2 like suggested in previous studies. This was done by collecting the energy data for these specified groups and combining the van der Waals and electrostatic interaction terms for each of the CHOL composition simulations. The result of this analysis is shown in **Figure 3.4** below. In this plot, the PIP₂ lipid group has the most favorable interaction with A2 out of all the lipids present by a wide margin for every CHOL %, showing that PIP₂ is the lipid primarily responsible for A2 binding to the membrane. Also, PIP₂ is the lipid shown to be most affected by an increase in CHOL %, as the energy of the interaction becomes significantly more negative as the CHOL % increased. This analysis confirmed that PIP₂ is the lipid binding to A2, and lipid-based analysis methods used in the rest of this study will focus on PIP₂.

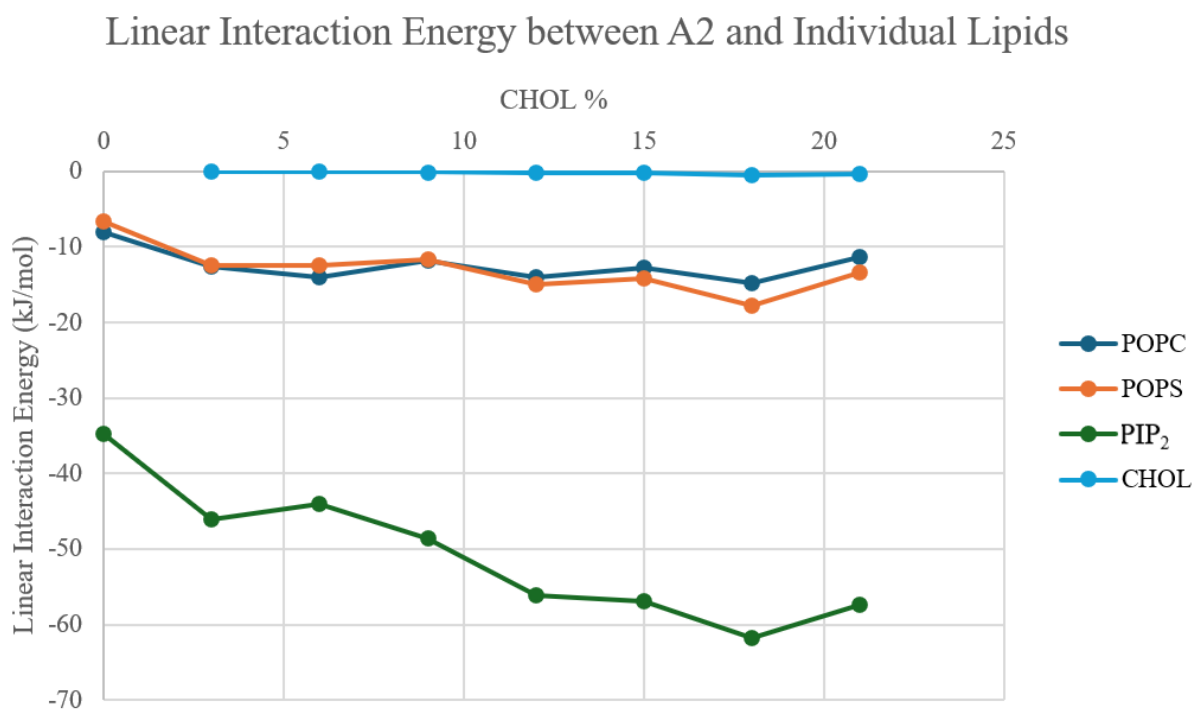


Figure 3.4: Linear interaction energy analysis plot for each individual and the energy associated with its interaction with A2. This analysis was performed for each CHOL %. The PIP₂ lipid both associates more with A2 in every CHOL % by a large amount and is affected most by the increase in CHOL %

A distance analysis was run to determine binding distances when A2 is associated closest with the membrane to PIP₂ in the upper leaflet of the membrane. Since A2 is only interacting with the upper leaflet of the membrane throughout all of the simulations, the headgroups of the can be selected for just the upper leaflet in order to determine the optimal binding distances to each one. These distances are the z coordinate height differences between the center of mass of A2 and the center of mass of lipid headgroups on the upper leaflet. The same groups of frames as used in **Figure 3.3** are represented in this lipid specific distance calculation. The results of this analysis are shown in **Figure 3.5**, shown below.

Distance Between the Centers-of-Mass of A2 and PIP₂ Headgroups during Interaction

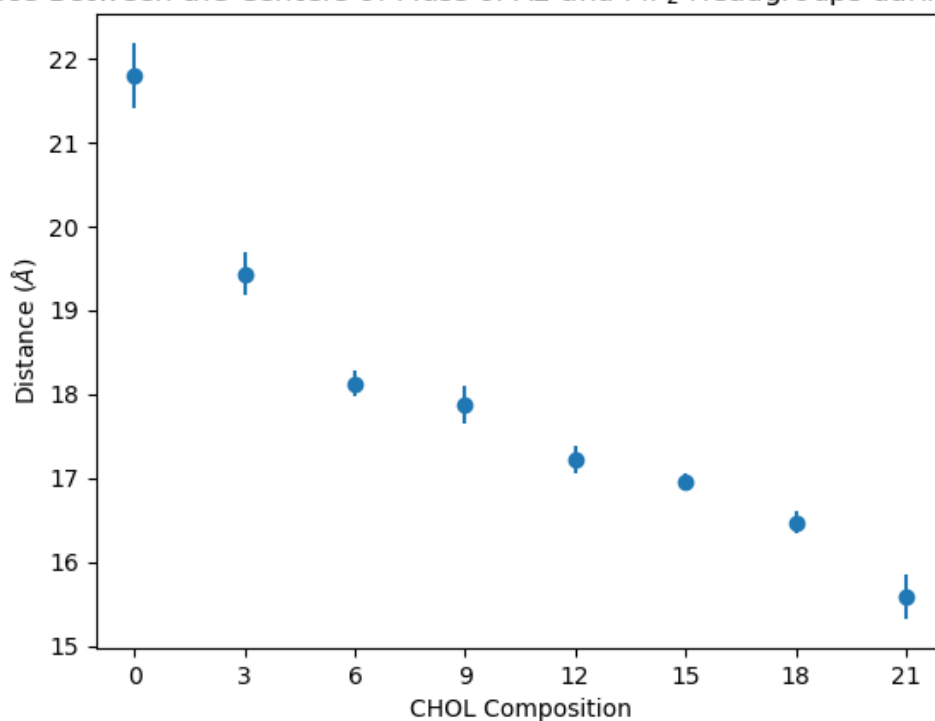


Figure 3.5: Plot showing the distance between the center-of-mass of A2 and center-of-mass of the top layer PIP₂ headgroups for the different CHOL compositions. The frames selected for this analysis were the same as the previous analysis. Four simulations per CHOL % were averaged, and the standard deviations are shown.

The distances shown give a better picture of the interaction between A2 and the surface of the membrane. The A2 has closest association with PIP₂. This matches what was expected, as A2 binds selectively to anionic lipids, in which PIP₂ is included. Of note, this analysis refers to the center of mass of A2, which is 14 Å higher than the surface of the convex side of the protein, leading to even closer distances between A2 and the PIP₂ headgroups. In further analysis, the A2-CHOL distances were noticeably larger than the other lipids, likely due to CHOL's location deeper in the membrane. The same trend as the previous one exists, where increased CHOL in

the membrane leads to closer binding distances between A2 and the PIP₂ in the upper layer of the membrane.

The headgroups of the PIP₂ lipids can be seen in close contact with A2 and are seen to be located higher in the z direction than any other lipid. This indicates that A2 has selective binding for PIP₂ as an anionic lipid. The CHOL lipids are localized more on the interior of the upper and lower leaflets and thus do not bind to A2 as much as the other lipids that extend outwards. This suggests a different role for CHOL in the increased A2-membrane interaction than strictly binding to the protein, and this different role will be explored further with other analyses.

The last application of this distance-based analysis was to visualize the distances between PIP₂ lipids and A2 residues. The MDAnalysis project code was used and altered to fit this goal. For three trials, CHOL 0, 12, and 21, the distance between each PIP₂ and each residue of A2 was calculated for the frames of closest association that are used for the previous figures. The results are displayed in a heatmap format to determine which residues are closer to the PIP₂ lipids. The results are shown in **Figure 3.6**.

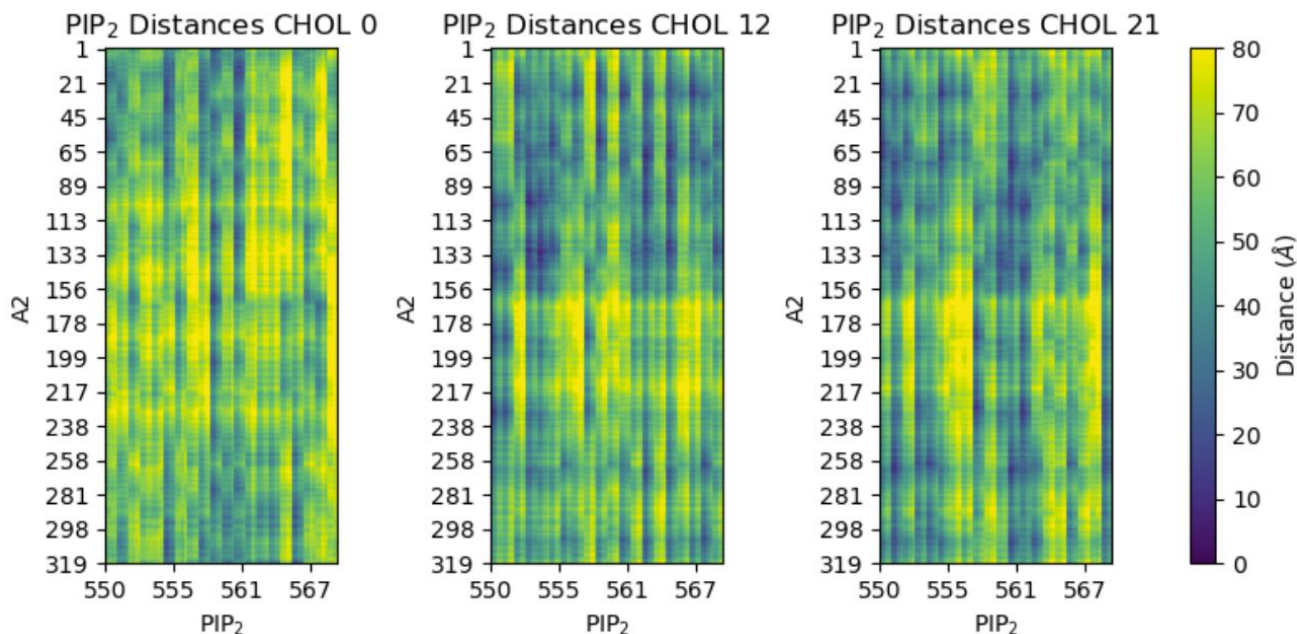


Figure 3.6: Heat maps showing the distances between A2 and PIP₂ headgroups in the upper leaflet of the membrane for CHOL 0, 12, and 21 trials. The yellow indicates larger distances and the blue indicates smaller distances.

The darker blue sections indicate the residues that are closest in distance to the specified PIP₂ lipid. First, as the CHOL increases, the closer distances that are observed become, which matches the previous distance analyses. In order to more clearly visualize the residues that are closest to the PIP₂ lipids, the CHOL 21 figure was enhanced and the cutoff for distance was reduced from 80 Å to 20 Å to create the following **Figure 3.7**. With this figure, it is possible to see that residues 26-30, 59-76, 183, 218-230, and 260-267 are in close proximity to the PIP₂ lipids, with residues K27, K60, S72, A76, R225, K261, R264, and D265 being the closest of those residues. K261 and, to a lesser extent, R264, as they are considered as part of the PIP site of A2, where it normally binds anionic lipids. The location of those lipids can be seen in blue in **Figure 3.8**. The rest of the close residues are all located on the convex side of A2 as expected.

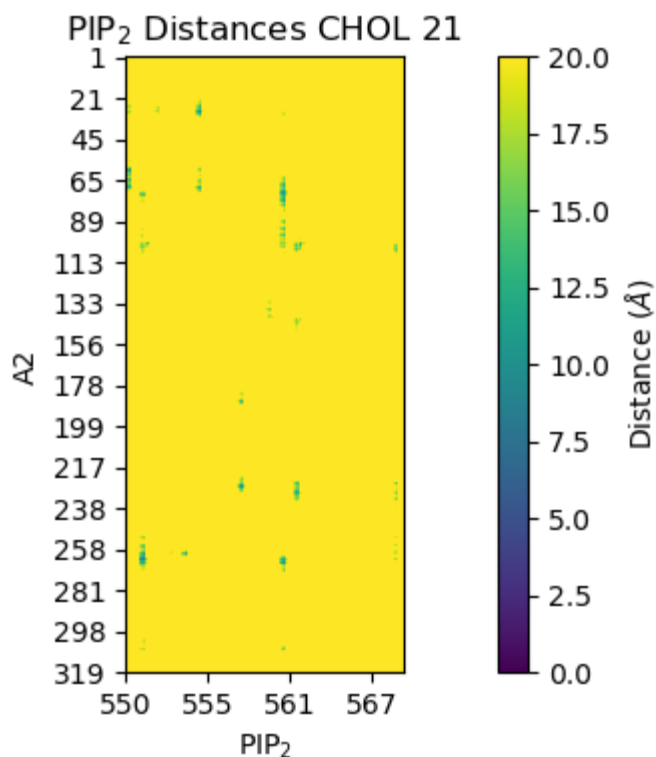


Figure 3.7: Heat map showing A2 residues in close proximity to PIP₂ headgroups. The scale has been adjusted to 0 to 20 Å to only include dark blue coloration for residues that are very close to the PIP₂ headgroups to identify PIP₂ binding sites on A2. Residues of closest contact are K27, K60, S72, A76, R225, K261, R264, and D265.

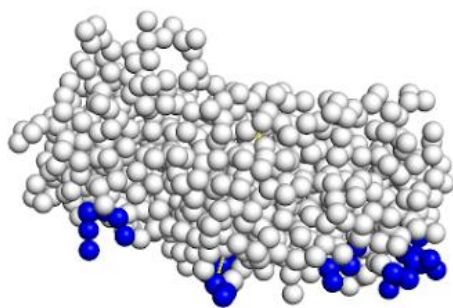


Figure 3.8: A2 residues in close proximity to PIP₂ headgroups. The close residues are colored in blue, while the farther away residues are in white. These residues correspond to those seen in **Figure 3.7**.

3.3.2 A2-Membrane Contacts

Another method used to quantify the effect of CHOL on A2-membrane association was contact analysis. This method utilized updated code from the MDAnalysis project. In this method, the number of close contacts between any A2 atoms and any membrane atoms throughout the second half of the simulations for the CHOL 0, 12, and 21% trials were calculated. The cutoff for close contact was selected as 10 Å. The results of this first analysis can be seen in **Figure 3.9**, shown below.

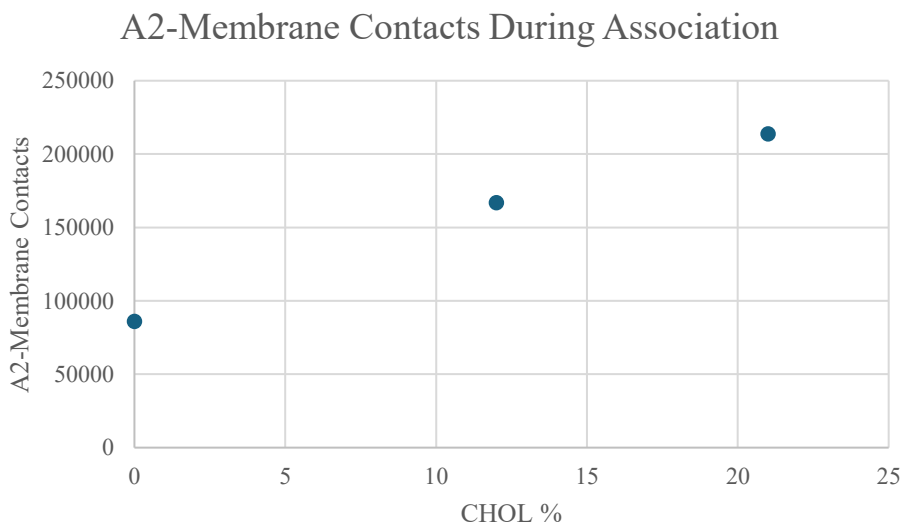


Figure 3.9: Plot showing the number of contacts between the A2 and the membrane during the entire simulation for CHOL 0, 12, and 21% compositions. The cutoff for consideration as a contact was 10 Å.

The number of close contacts between A2 and the membrane in the second half of the simulation increases as the amount of CHOL in the membrane increases, which works well alongside the previous distance analysis that concludes that A2 is more closely associated with the membrane. The frame-by-frame analyses for each of these data points can be seen in **Figure 3.10**, shown below.

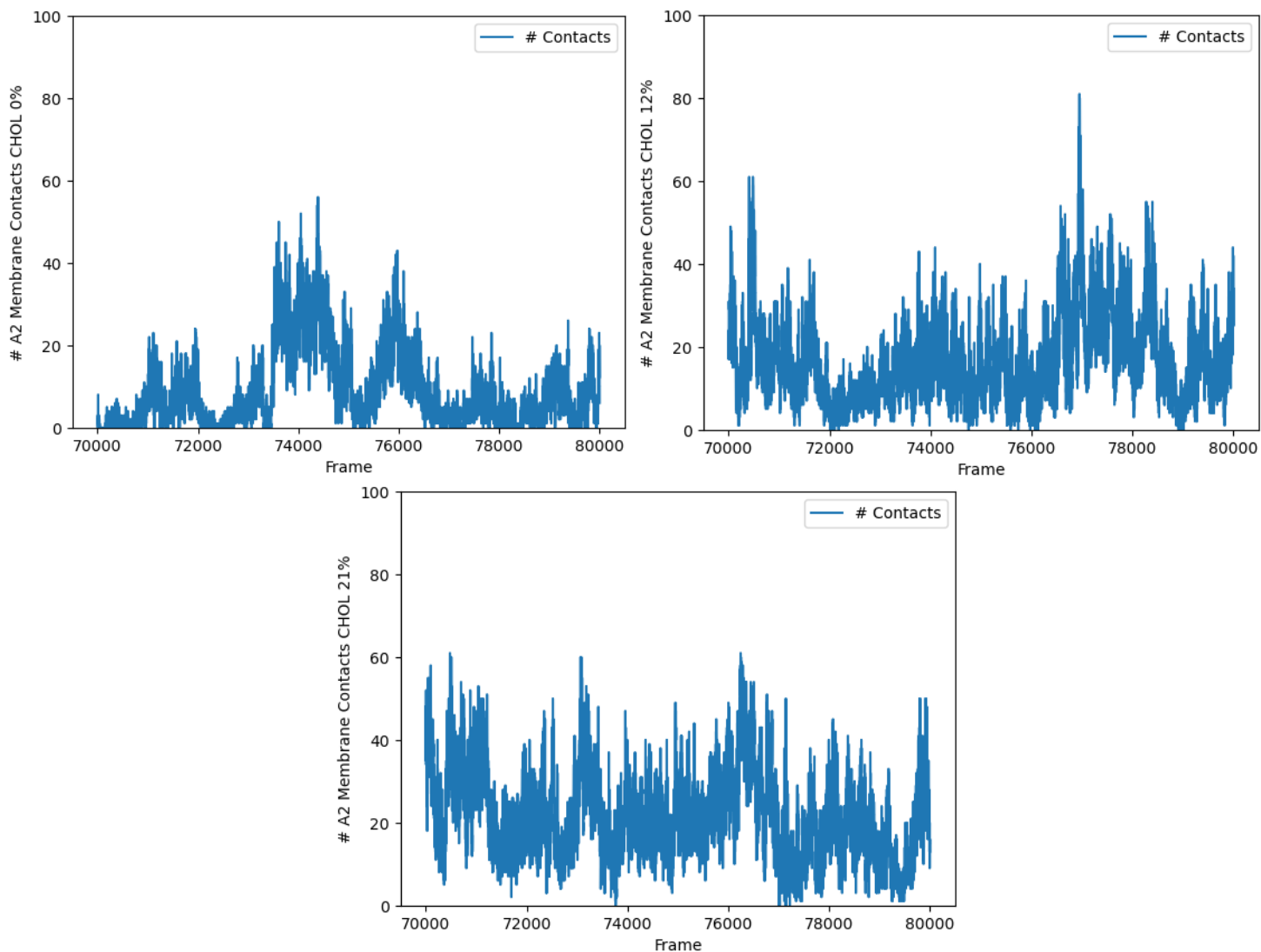


Figure 3.10: Plots showing the number of contacts on each frame of the CHOL 0, 12, and 21% simulations. The frames used are the ones where the protein and membrane are in closest contact, which were frames 70,000 to 80,000 for these simulations. These contact numbers were then totaled over these frames to give the total contact numbers for each CHOL composition.

Because A2 selectively binds to PIP₂ and other anionic lipids, the number of A2-PIP₂ contacts was of interest. A2 and the PIP₂ lipids in the upper leaflet were selected as the groups for contact analysis, and the same cutoff of 10 Å was used. The results for this are seen in **Figure 3.11**, shown below.

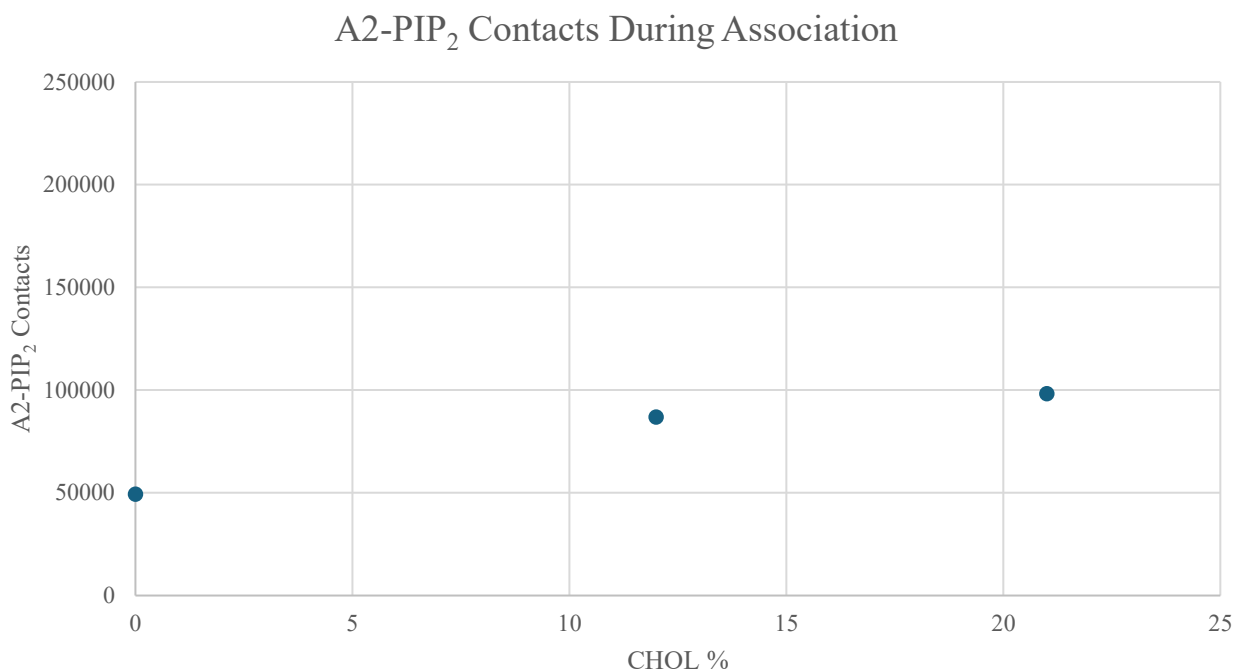


Figure 3.11: Plot showing the number of contacts between the A2 and the upper layer PIP₂ headgroups during the entire simulation for CHOL 0, 12, and 21% compositions. The cutoff for consideration as a contact was 10 Å.

As the CHOL % increases, the amount of A2-PIP₂ contacts increases as well, matching what was seen in the other contact and distance analyses. Also of note is that nearly 50% of all contacts with A2 during these three trials were with PIP₂, while PIP₂ is only 20% of the total lipids in the membrane, meaning PIP₂ has a larger impact on contacts with A2 than the other lipids.

3.3.3 Lipid Contacts and PIP₂ Clustering

A major role for A2 is the formation and stabilization of anionic phospholipid rafts. This function has been suggested to be enhanced by increased CHOL in the interacting plasma membrane. In order to study this interaction, analysis was completed to determine the extent of PIP₂ lipid clustering through the use of PyMOL visualization and lipid contacts.

Figure 3.12, shown below, depicts the top-down PyMOL visualization of the first and final frames of the simulations for CHOL 0, 12, and 21%. The A2 are yellow and slightly transparent, the PIP₂ lipids are blue, and the remaining lipids are white. As the simulations progress to their final frames, the A2 proteins can be seen aggregating more PIP₂ lipids underneath themselves as they interact more closely with the membrane. This aggregating can be seen to greater extents in the higher CHOL trials like CHOL 21%.

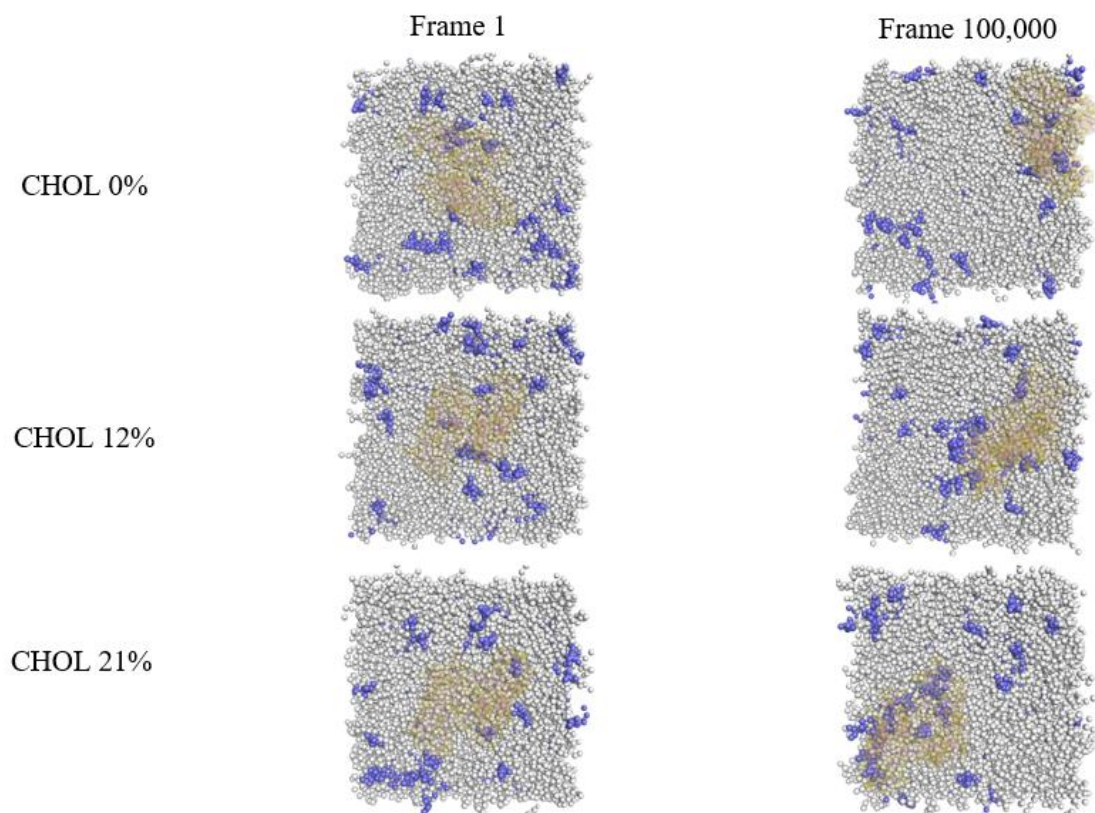


Figure 3.12: Top-down view of first and final frames of the CHOL 0, 12, and 21% simulations. The semi-transparent yellow object is the A2 above the membrane, the blue objects are PIP₂, and the white objects are the remaining lipids in the membrane.

The methods used to quantify this effect were PIP₂ self-distances and self-contacts. For the self-distances analysis, the distances between each PIP₂ headgroup during a frame of close association between the protein and the membrane were calculated and represented in a heat map for both the CHOL 0 and 21% trials, shown in **Figure 3.13** below. The distance range is reduced to 0 to 20 Å for a better view of which PIP₂ lipids are in close contact. The amount of darker blue sections in the CHOL 21% trial (bottom plot) is greater than the CHOL 0% trial (top plot). Thus,

the CHOL 21% had a greater number PIP₂ lipids in closer contact with 21 close contacts compared to the 10 seen in CHOL 0%.

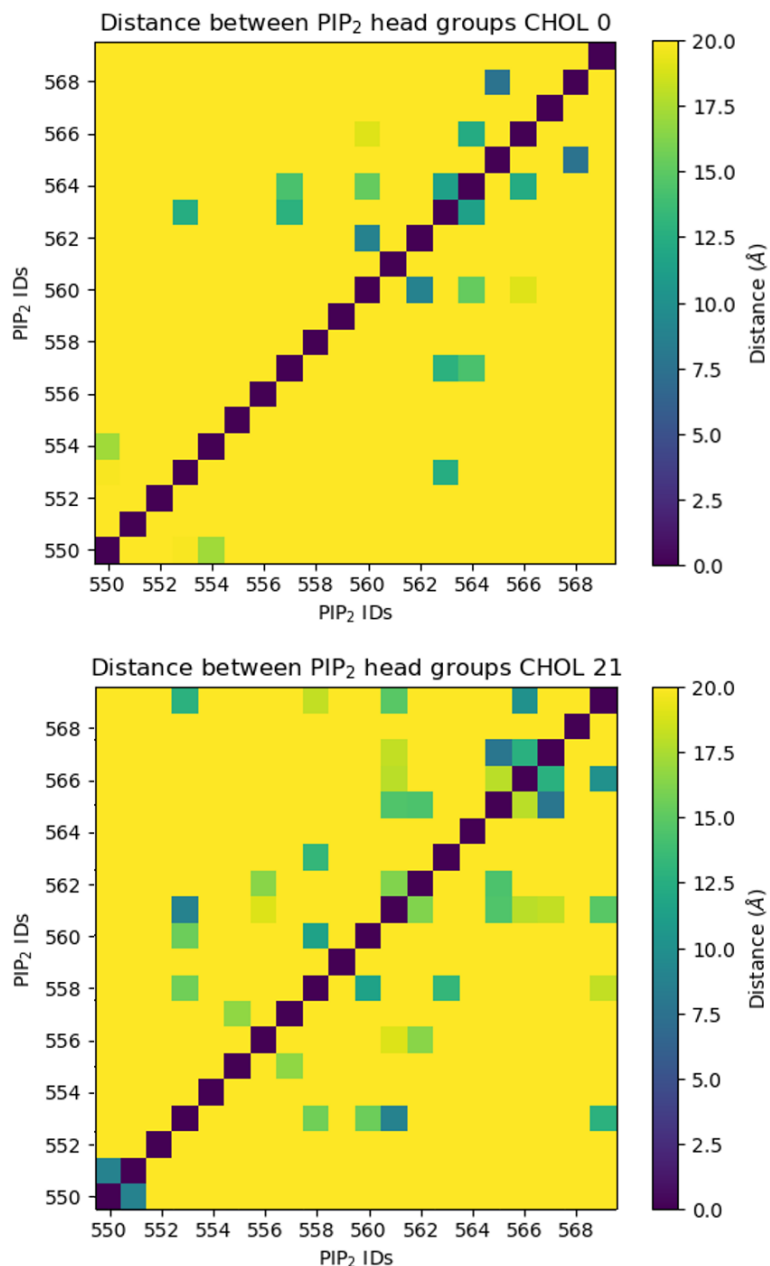


Figure 3.13: Heatmap visualization of the distance between each individual PIP₂ headgroup in CHOL 0% (top) and CHOL 21% (bottom) simulations during frames of close A2-membrane association. The plots show distances ranging from 0 to 20 Å to visualize PIP₂ headgroups that are clustering.

Figure 3.13 shows that higher CHOL% in a membrane results in closer distances between PIP₂ headgroups for frames of close protein association, but it was important to include an analysis method that showcases this over a larger amount of simulation time. To do this, self-contact analysis was used with a distance cutoff of 10 Å, and the group chosen for analysis was the PIP₂ lipids on the upper leaf of the membrane over the entire second half of the simulation for CHOL 0, 12, and 21% trials. The total number of self-contacts for each trial are represented in **Figure 3.14**, shown below. The trend of increased PIP₂ self-contacts with increased CHOL % is clear and supports the previous conclusion seen in just a single frame in **Figure 3.13** above.

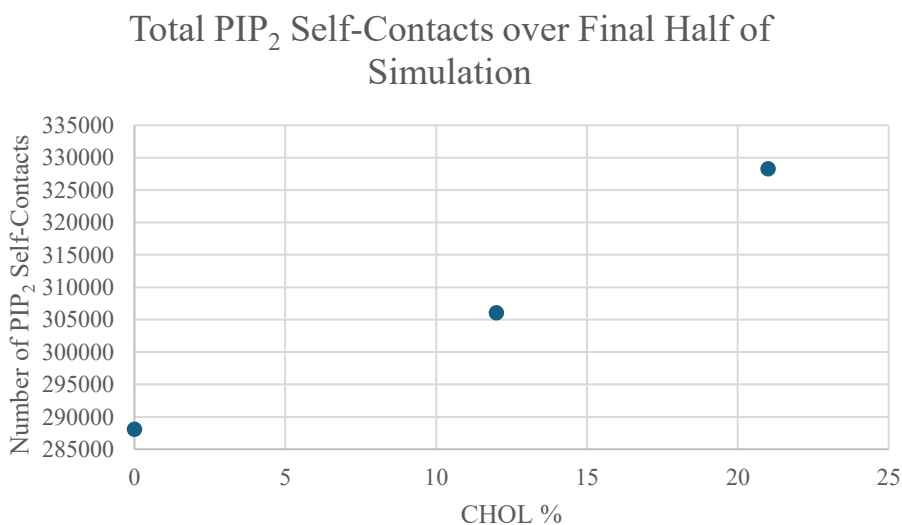


Figure 3.14: Plot showing number of self-contacts individual PIP₂ headgroups in CHOL 0, 12, and 21% simulations. The distance cutoff for contacts was set to 10 Å.

3.3.4 Linear Interaction Energy by %CHOL

In order to determine the energetic interactions between the A2 and the membrane, the linear interaction energy between A2 and the membrane was calculated over the final half of the simulations for all CHOL % trials. This was done by collecting the energy data for the van der Waals and electrostatic interactions between the protein and the membrane, combining them into a single linear interaction energy term. The results of this analysis can be seen in **Figure 3.15**, shown below.

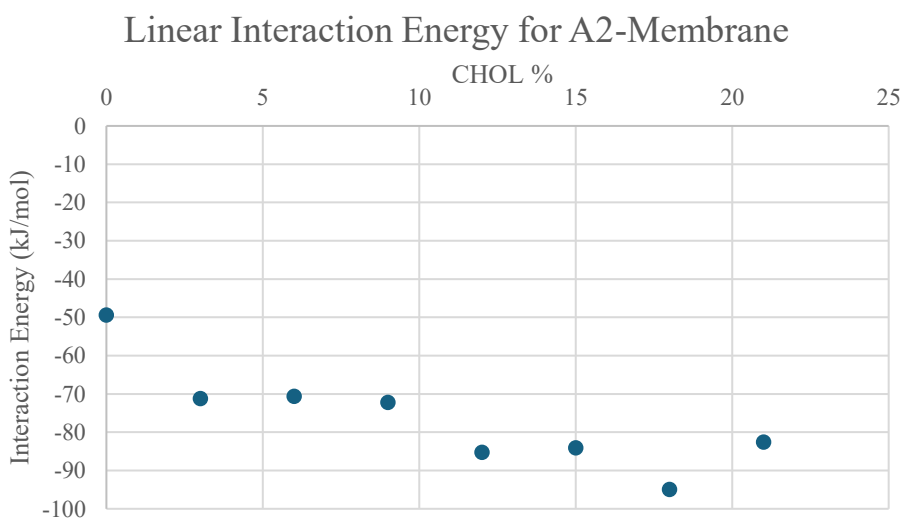


Figure 3.15: Plot showing the linear interaction energy over the final half of the simulations for the eight different CHOL compositions. The van der Waals and electrostatic interactions between the protein and the membrane were gathered from the production output files using the GROMACS energy tool.

This figure shows a similar trend to the distance and contact plots, showing more negative energies and stabler interactions between A2 and the membrane as the CHOL in the membrane is increased. This energy calculation is also supported by further energy calculations seen in the umbrella sampling simulations seen later.

3.3.5 Lipid Diffusion Coefficients for Membrane-only Simulations

The previous analyses showed that CHOL in the membrane increases A2 interaction and PIP₂ clustering, but insight into how CHOL does this is vital to this study. In order to elucidate this, simulations with only the membrane present in the absence of A2 were conducted for CHOL 0, 12, and 21% trials. The main form of analysis completed on these simulations was lipid diffusion coefficient analysis in order to determine how the fluidity of the membrane is affected by increased CHOL %. The mean squared displacement for each lipid was first determined over the trials, and a log-log plot was used to determine the most linear parts of the graphs, which were all located near the beginning of the simulations. The mean squared displacement graphs were reduced to just those straight-line sections, and the slope of each line was calculated to find the lipid diffusion coefficient. The lipid diffusion coefficients can be seen in **Figure 3.16**, shown below, and the method of achieving those lipid diffusion coefficients can be seen in **Figure 3.17**.

The lipid diffusion coefficients for all lipids decrease as the CHOL % increases. This indicates that increased CHOL leads to decreased diffusion speeds for the lipids, leading to stabler lipid rafts as the individual lipids cannot diffuse away as quickly as when lower CHOL % is present.

Lipid Diffusion Coefficients: Membrane Only

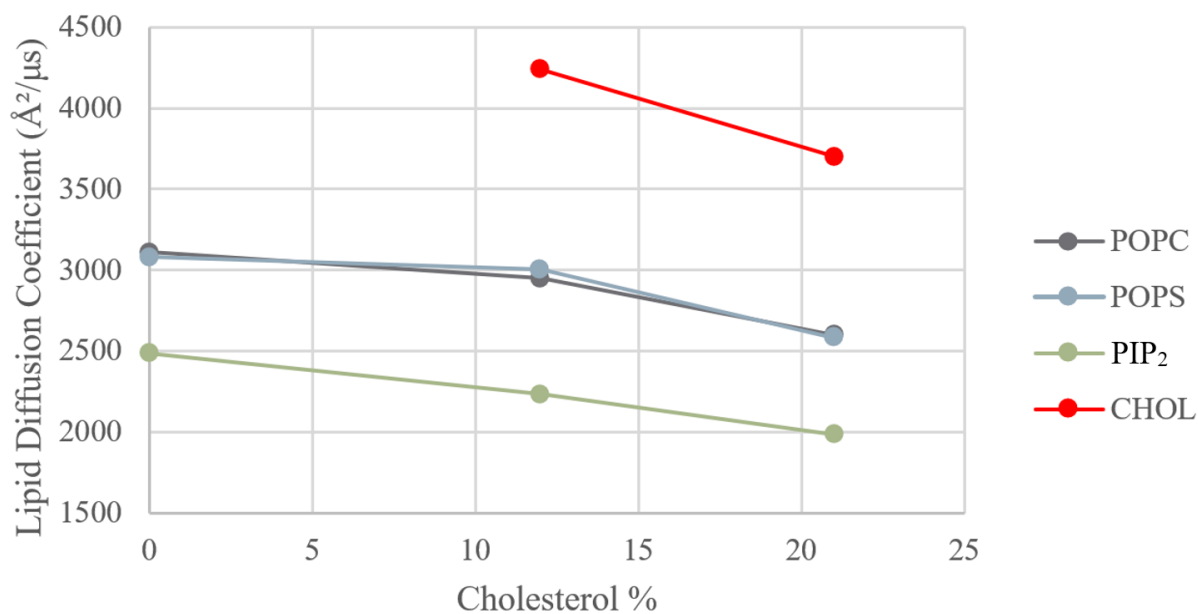


Figure 3.16: Plot showing the lipid diffusion coefficients for the four lipids in the membrane for CHOL 0, 12, and 21% membrane-only simulations. These simulations were reimaged in no-jump mode to allow free diffusion of the lipids, and the speed of diffusion for the lipids was collected for this calculation.

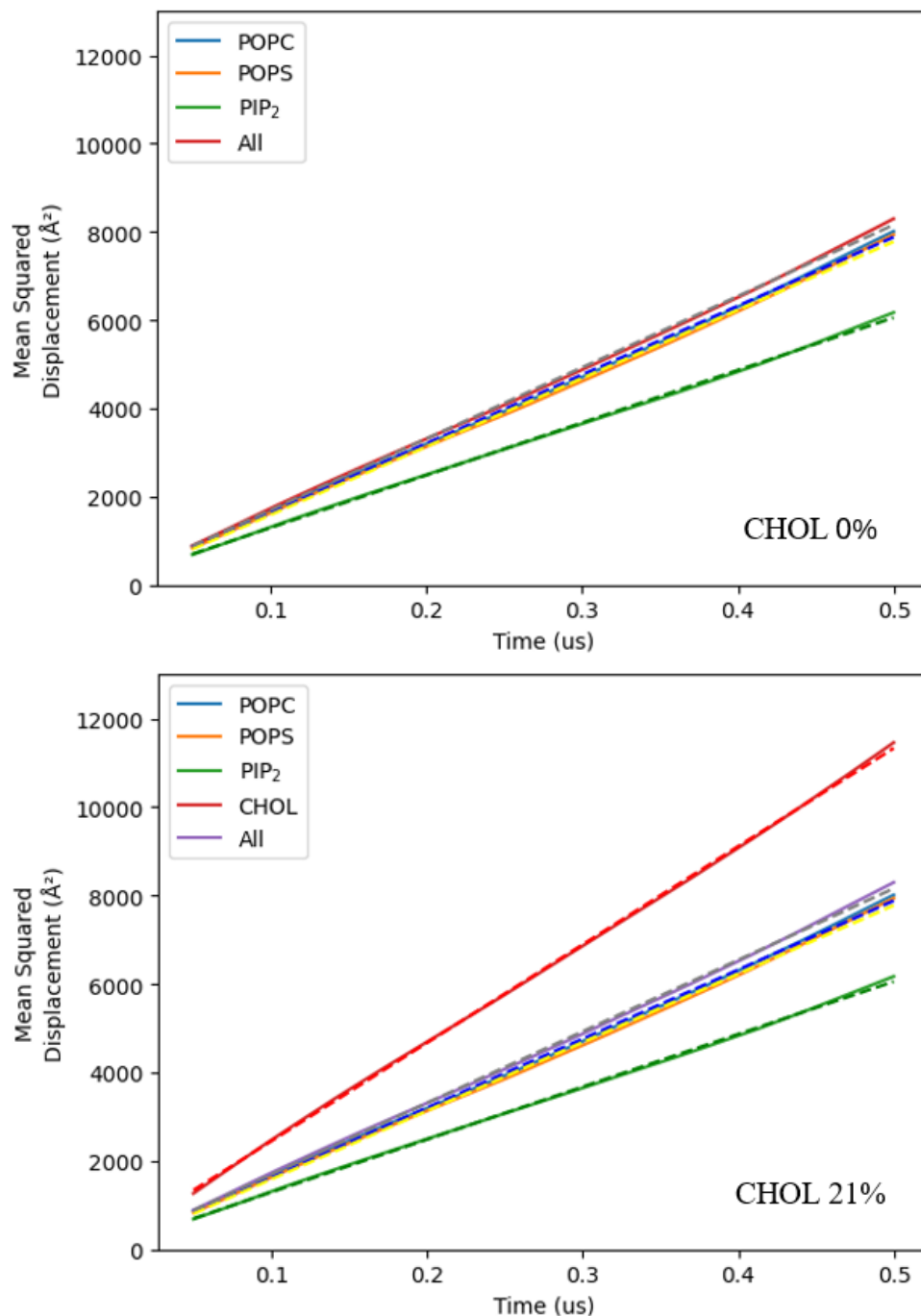


Figure 3.17: Plots showing the mean squared displacement for the four lipids in the membrane for CHOL 0% and 21% membrane-only simulations. These simulations were reimaged in no-jump mode to allow free diffusion of the lipids, and the displacement from the starting frame for each lipid was calculated over time. Log-log plots were used to determine the most straight-line portion of the plots. Only those straight-line portions are shown and they were used to calculate the lipid diffusion coefficients for each lipid by the slope of each line.

3.3.6 USMD Stable Interaction Distances

The final simulation type in this study was umbrella sampling, which was run on CHOL 0, 12, and 21% trials. From these simulations, the distance between the center of mass of A2 and the membrane and the energy of interaction can be correlated to find optimal binding distances, using the weighted histogram analysis method. The results of this analysis are shown in **Figure 3.18**, shown below.

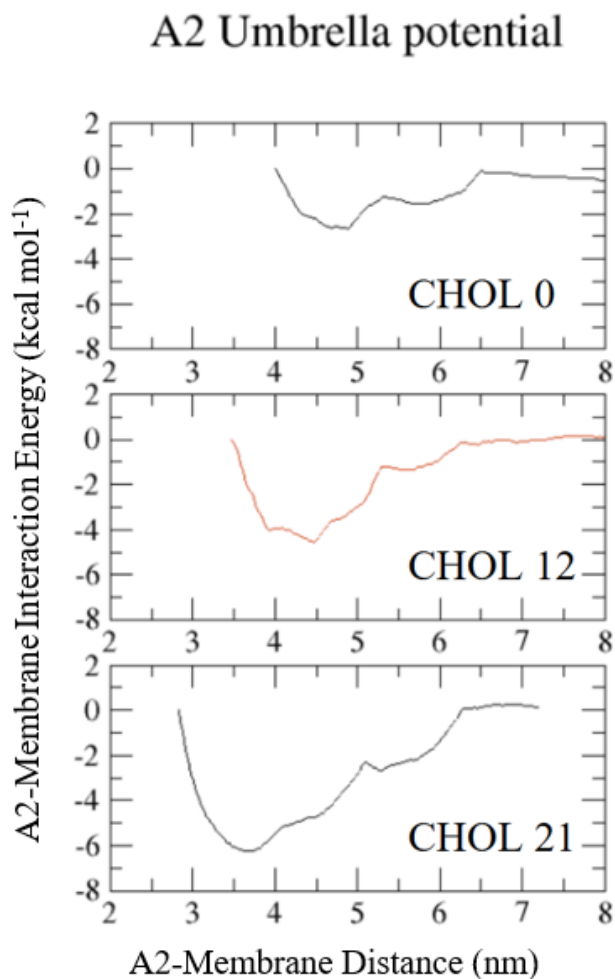


Figure 3.18: Plot showing the umbrella potential for the umbrella sampling molecular dynamics simulations of CHOL 0, 12, and 21% compositions. The energy associated with A2-membrane interaction is compared with the distance between the centers-of-mass of the protein and membrane, giving the energy associated with optimal binding distances.

The CHOL 21% trial shows an interaction between A2 and the membrane that is both at a closer interaction distance and that is more energetically favorable than the CHOL 0 and 12% trials. This indicates that increased CHOL % leads to closer and more favorable A2-membrane interactions, which supports the results seen in the coarse-grained simulations.

3.4 Conclusions

The goal of this study was to further investigate A2-membrane interactions with longer term simulations and determine the effects of cholesterol on these interactions. The results of this study demonstrated that membrane CHOL % played an important role in strengthening the association between annexin A2 and PIP₂-containing membranes. Across the eight membrane compositions examined, increasing CHOL % generally decreased the distance between A2 and the membrane during periods of close association.

Lipid-specific distance and interaction-energy analyses confirmed that PIP₂ was the primary lipid involved in A2 binding, matching what has been seen in previous studies.²² Among all membrane components, PIP₂ displayed the most favorable interaction energy with A2 and the closest association with the protein. Furthermore, the A2–PIP₂ interaction became increasingly favorable as CHOL concentration increased. Although CHOL itself was located deeper within the membrane and did not interact closely with A2, its presence appeared to indirectly promote A2 binding to PIP₂. These findings indicated that CHOL enhanced A2–membrane association by altering the organization and physical properties of the membrane rather than by binding directly to the protein.

Residue-level distance analysis identified several regions on the convex surface of A2 that were positioned close to PIP₂ lipids. Residues 26–30, 59–76, 183, 218–230, and 260–267

showed close proximity to PIP₂, with K27, K60, S72, A76, R225, K261, R264, and D265 exhibiting particularly short distances. The close association of K261 and R264 was consistent with their location within the previously identified PIP-binding region of A2. The remaining residues were also located along the convex membrane-binding surface, supporting the expected orientation of A2 during interaction with the membrane.

Contact analyses further supported the distance-based findings. The total number of A2–membrane contacts increased with increasing CHOL %, as did the number of contacts specifically formed between A2 and PIP₂. The simulations also showed that A2 promoted the aggregation of PIP₂ lipids beneath itself. PyMOL visualizations, PIP₂ self-distance heat maps, and self-contact analyses all indicated that PIP₂ clustering increased as CHOL % increased. These findings suggested that CHOL enhanced the ability of A2 to form and stabilize PIP₂ lipid microdomains.

The linear interaction-energy analysis showed that A2–membrane interactions became increasingly favorable as membrane CHOL % increased. This result was consistent with the observed reductions in binding distance and increases in protein–membrane contacts. Membrane-only simulations provided additional insight into the mechanism underlying this effect. Lipid diffusion coefficients decreased for all lipid species as CHOL concentration increased, indicating that CHOL reduced membrane fluidity. This reduced lipid mobility likely limited the ability of PIP₂ lipids to diffuse away from A2, thereby increasing the stability of the PIP₂ lipid microdomains formed beneath the protein.

Umbrella-sampling simulations supported the conclusions obtained from the coarse-grained molecular dynamics analyses. The CHOL 21% membrane produced a more energetically favorable A2–membrane interaction and an energy minimum at a shorter protein–membrane

distance than the CHOL 0 and 12% systems. These results confirmed that increased CHOL content promoted both closer and more stable A2 binding.

Overall, this study supported a model in which A2 selectively bound PIP₂ through residues located on its convex surface and promoted the formation of PIP₂ lipid microdomains beneath the protein. CHOL did not appear to strengthen this interaction through direct binding to A2. Instead, increasing CHOL content reduced lipid diffusion, stabilized PIP₂ clustering, increased the number of A2–PIP₂ contacts, and produced a closer and more energetically favorable A2–membrane interaction.

Chapter 4: Exploration of the binding affinity of Annexin A2t to a membrane and the impact of the concentration of CHOL in that membrane

4.1 Background

Annexin A2t is also found abundantly in the human body and is seen in a variety of tissue and cell types, found in the cytosol and near the plasma membrane.³ A2t is the heterotetrameric form of A2 that consists of two A2 monomers connected by a S100A10 dimer. A2t also interacts with the membrane in a variety of functions including exocytotic and membrane reorganization events. A2 has been implicated as contributor to different pathologies as well, including a variety of cancers. The ability for the S100A10 portion of the tetramer to bind both tPa and plasminogen, after which plasminogen is cleaved to plasmin, resulting in the breaking apart of nearby cell membranes is of keen interest when discussing and modelling A2t. A2t also acts as a regulator for S100A10 association with the membrane, due to S100A10 being a part of the heterotetramer. Misregulation of interactions like these are what leads to increased cancer metastasis and worsened prognosis for patients.

This study investigated the long-term interactions and binding modes seen when A2t binds to a membrane containing the anionic lipid PIP₂ and CHOL. A2t is also known to selectively bind to membranes that include anionic phospholipids, such as PIP₂. These lipids bind to residues on the convex side of both of the A2 subunits on A2t. Studies have been done to investigate this interaction with both PIP₂ and POPS, and A2t did bind selectively to these lipids, and it also formed anionic lipid rafts underneath itself. The previous study noted a role for CHOL in increasing A2 binding to a membrane containing PIP₂, and the same relationship is expected for the heterotetramer as well. CHOL was seen to increase the formation of anionic lipid rafts, and the reduced lipid diffusion speed suggests that CHOL stabilizes these lipid rafts as

well. In this study, the influence of membranes with compositions that vary in CHOL amount on A2t binding and lipid raft formation was simulated on a larger time scale than previous studies. This was done using CGMD to simplify the large system to allow for longer simulations and USMD to overcome any potential energy barriers and provide optimal binding distances. The primary goal for this study was to determine optimal binding modes for A2t and a membrane based off of three starting poses for the heterotetramer, determining whether it is more preferable for both A2 subunits to be bound or just a single subunit. The secondary goal was to provide a scaffold for A2t-membrane interactions such as vesicle fusion and exocytosis. A third goal was to ensure a similar CHOL role for A2t as seen in the A2 study.

4.2 Computational Methods

4.2.1 CGMD A2t System Details

The first system that was explored is a single A2t protein located over a lipid bilayer with varying CHOL amounts and three different starting poses. The A2t protein was taken from the Protein Databank (4HRE.pdb). All non-A2t molecules were removed from the file to leave just the A2t protein in the file. This file was then coarse-grained using the Martini2 coarse-grained force field elnedyn22, which used an elastic network with an upper cutoff of 0.9 nm, a lower cutoff of 0.5 nm, and a force constant of $700 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ as the basis for maintaining the protein's overall shape.¹⁷ This reduced resolution allowed for longer-term simulations with less computational time, which was especially important for the much larger system due to A2t being more than double the size of A2. The lower resolution is reasonable for this study because of the focus on overall protein and membrane interaction rather than smaller conformational changes, and it makes these longer-term simulations possible in a reasonable amount of time. The four

coarse-grained lipids, POPC, POPS, PIP₂, and CHOL, were collected from the Martini Lipidome. From here, different starting structures were made using the mMAEVE program. For the first system, one A2t protein was centered 80 Å over a 250x250 Å bilayer with a 7 Å separation between the layers and 1,000 lipids per layer. The A2t protein was oriented so that the convex side of the first A2 subunit was facing the membrane. This system was split into eight different membrane compositions, 0%, 3%, 6%, 9%, 12%, 15%, 18%, and 21% CHOL (see **Table 4.1**). The POPS and PIP₂ percentages remained constant at 20% and 8% respectively. The POPC percentages were 72%, 69%, 66%, 63%, 60%, 57%, 54%, and 51% to accommodate the rising CHOL percentage. POPC was chosen to decrease because it is a neutral lipid and thus shouldn't have a noticeable effect on A2 binding to the membrane, as it selectively binds to anionic phospholipids.

Table 4.1: Compositions of the membranes for each of the eight CHOL compositions used in the A2t simulations. The main lipids changing in percentage are CHOL and POPC.

Trial	% POPC	% POPS	% PIP ₂	% CHOL
CHOL 0	72	20	8	0
CHOL 3	69	20	8	3
CHOL 6	66	20	8	6
CHOL 9	63	20	8	9
CHOL 12	60	20	8	12
CHOL 15	57	20	8	15
CHOL 18	54	20	8	18
CHOL 21	51	20	8	21

Two other starting poses for A2t were also selected and simulated with the same membrane compositions. The second pose centered the S100A10 over the membrane, with both A2 subunits at equal heights above the membrane with the convex side facing down. The third pose had the second A2 subunit centered over the membrane with its convex side facing down. Each of the three poses had eight total simulations, each with a different CHOL amount. The starting structures for the three poses can be seen in **Figure 4.1**.

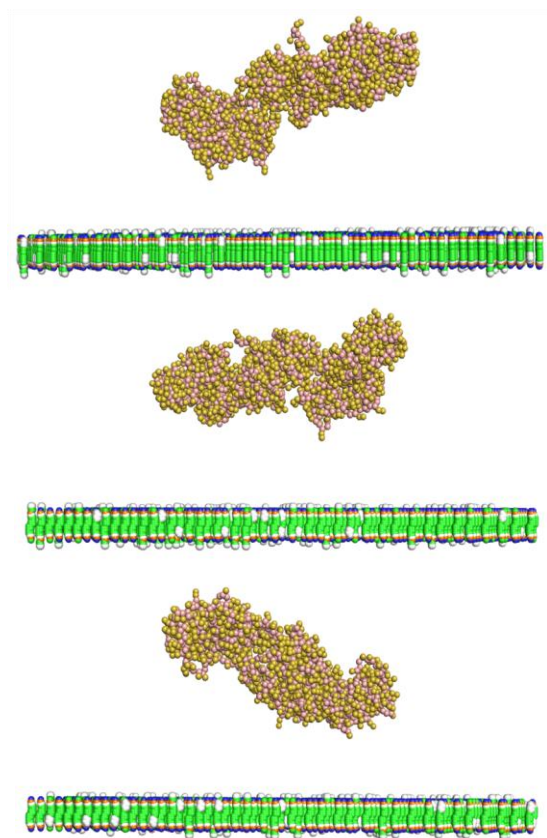


Figure 4.1: Starting structures for coarse-grained conventional and umbrella sampling molecular dynamics simulations. Poses 1 through 3 are shown top to bottom, and each pose is simulated with varying cholesterol percentages in the membrane.

These 24 total starting structures were then solvated with water, sodium, chloride ions to simulate a cellular environment. The GROMACS software suite was used for all of the following simulation steps. A box with periodic boundaries was created to contain the atoms of the system with dimensions of 250x250x550 Å. The height of the box was lengthened to avoid the A2t potentially moving towards the top of the box and remaining there for the duration of the simulation. A steep integrator was used with 5,000 steps to solvate the box with 0.15 M water and ions.

The output of the solvation step was then minimized to reduce strain and clear up problematic contacts. This occurred in two steps with 5,000 steps each with a steep integrator. These steps also generated starting velocities for 310K.

The output of the minimization step was then equilibrated to gradually raise the temperature to close to body temperature. This was done in 250,000 steps of 20 femtoseconds timesteps to raise the temperature to 310K using a v-rescale thermostat while maintaining 1.0 bar of pressure by using a semiisotropic Berendsen barostat with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$.

The output of the equilibration step was then used for the production step. The md integrator was used to simulate 10 μs through 500,000,000 steps with a timestep of 20 femtoseconds. A v-rescale thermostat was used to simulate the system at 310K, and a semiisotropic Parrinello-Rahman barostat was used to maintain 1.0 bar with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$. The output trajectories were then reimaged converting the trajectories using -pbc mol to enforce the periodic boundary for the system box. The first and final frames for these three poses can be seen in **Figure 4.2**.

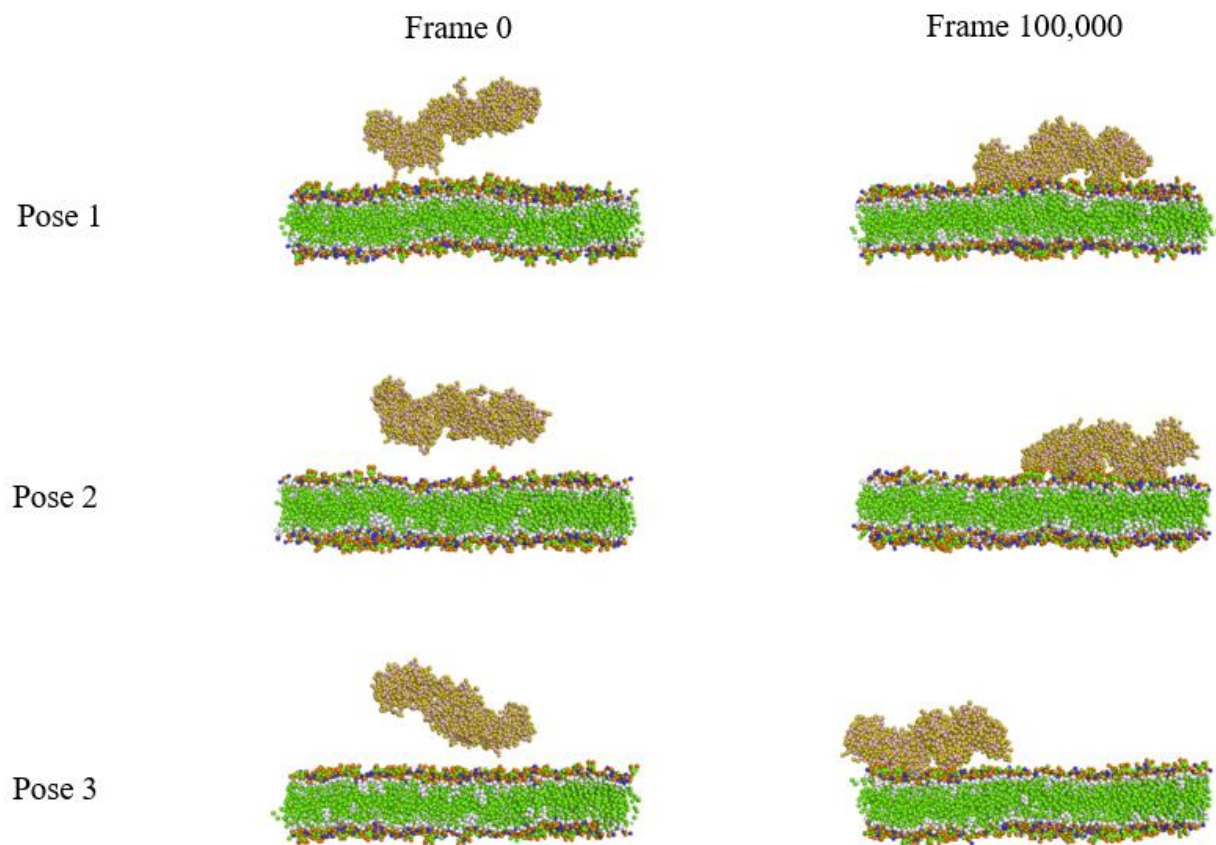


Figure 4.2: First and final frames of the Pose 1, 2, and 3 simulations. The A2t is shown in yellow, and the membrane is shown below the proteins. The protein associates with the membrane more as the simulation progresses.

4.2.2 USMD System Details

The USMD system has a similar setup to that seen in the CGMD A2t-membrane simulations, with different poses of A2t centered over a membrane. The A2t was collected from the Protein Databank (4HRE.pdb). All non-A2 molecules were removed from the file to leave just the A2 protein in the file. This file was then coarse-grained using the Martini2 coarse-grained force field elnedyn22, which will use an elastic network with an upper cutoff of 0.9 nm, a lower cutoff of 0.5 nm, and a force constant of $700 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ as the basis for maintaining the protein's overall shape.¹⁷ The four coarse-grained lipids, POPC, POPS, PIP₂, and CHOL, were collected from the Martini Lipidome. One A2 protein was centered over a 250x250 Å bilayer with a 7 Å separation between the layers and 1,000 lipids per layer, though in this study, the height varied from 20 Å to 80 Å with 2 Å steps. The first pose had the A2t protein oriented so that the convex side of the first A2 subunit was facing the membrane, the same orientation seen in the CGMD A2t study for pose 1. The second pose had both A2 subunits set to even heights with the convex sides of the subunits facing the membrane. These two poses both had only a single membrane composition, that being the CHOL 21 membrane for maximized binding to the membrane based on the previous study.

The systems were then solvated with water, sodium ions, and chloride ions to a concentration of 0.15 M in a box with dimensions of 250x250x300 Å using a steep integrator with a maximum of 50,000 steps. The height of the box is noticeably less than the height seen in the CGMD A2t study due to the A2t in the USMD study being more restricted in the heights above the membrane between which a single bin's starting structure can fluctuate.

These systems were then minimized in two steps, both using a steep integrator with 5,000 steps, a Berendsen thermostat set to 310 K, and a semiisotropic Berendsen barostat set to

1.0 bar with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$. Next, the pull groups were established as the A2t center of mass and the membrane center of mass and a harmonic biasing potential was used along the z axis with a width of 1,000 to pull the A2t back towards its starting location if it diffuses too far. Each system was then equilibrated using a sd integrator in 50,000 steps with a Berendsen thermostat at 310 K and a semiisotropic berendsen barostat set to 1.0 bar with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$.

The outputs were then sent to the production step, where a sd integrator was used to simulate 10 μs simulations with 500,000,000 steps and a timescale of 20 femtoseconds. A v-rescale thermostat set to 310 K and a semiisotropic Berendsen barostat set to 1.0 bar with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$ were used for each of the systems. The outputs of this step were analyzed using the weighted histogram analysis method (WHAM).

4.3 Results

4.3.1 *A2t-Membrane Distances*

The first method of analysis was a distance-based analysis to determine both if the effects of CHOL were similar to what was seen in the A2 study and the binding activities of each A2 subunit in A2t for the different poses. **Figure 4.3** shows the result of the distance between the center of mass of each A2 subunit of A2t and the bilayer for poses 1, 2, and 3: with poses 1 and 3 having an A2 subunit centered over the membrane and with pose 2 having both subunits evenly spaced above the membrane. This figure shows that no matter the starting pose, the A2t tends to bind to the membrane in a similar manner to the starting structure of pose 2. The same correlation between CHOL and distance between the protein and membrane exists to the one seen in the monomer study. As CHOL % in the membrane increases, the distance between the A2 subunits of A2t and the bilayer decreases, indicating a role for CHOL in increased A2t binding. As A2t is composed of two A2 monomers, the role of CHOL is likely the same as that seen in the A2 binding study, as the membrane compositions are similar. It is of note that when only one subunit was bound to the membrane closely and the other was farther away, that subunit was able to bind more closely to the membrane than when both subunits were associated with the membrane. This is likely due to a small twist in the A2t protein. When both subunits are associated with the membrane, they are slightly tilted to the side, and they are not able to bind to the membrane with the full convex side of the A2 subunits. This leaves the center of mass of each subunit slightly higher than when only one A2 subunit is allowed to bind to the membrane with its full convex side.

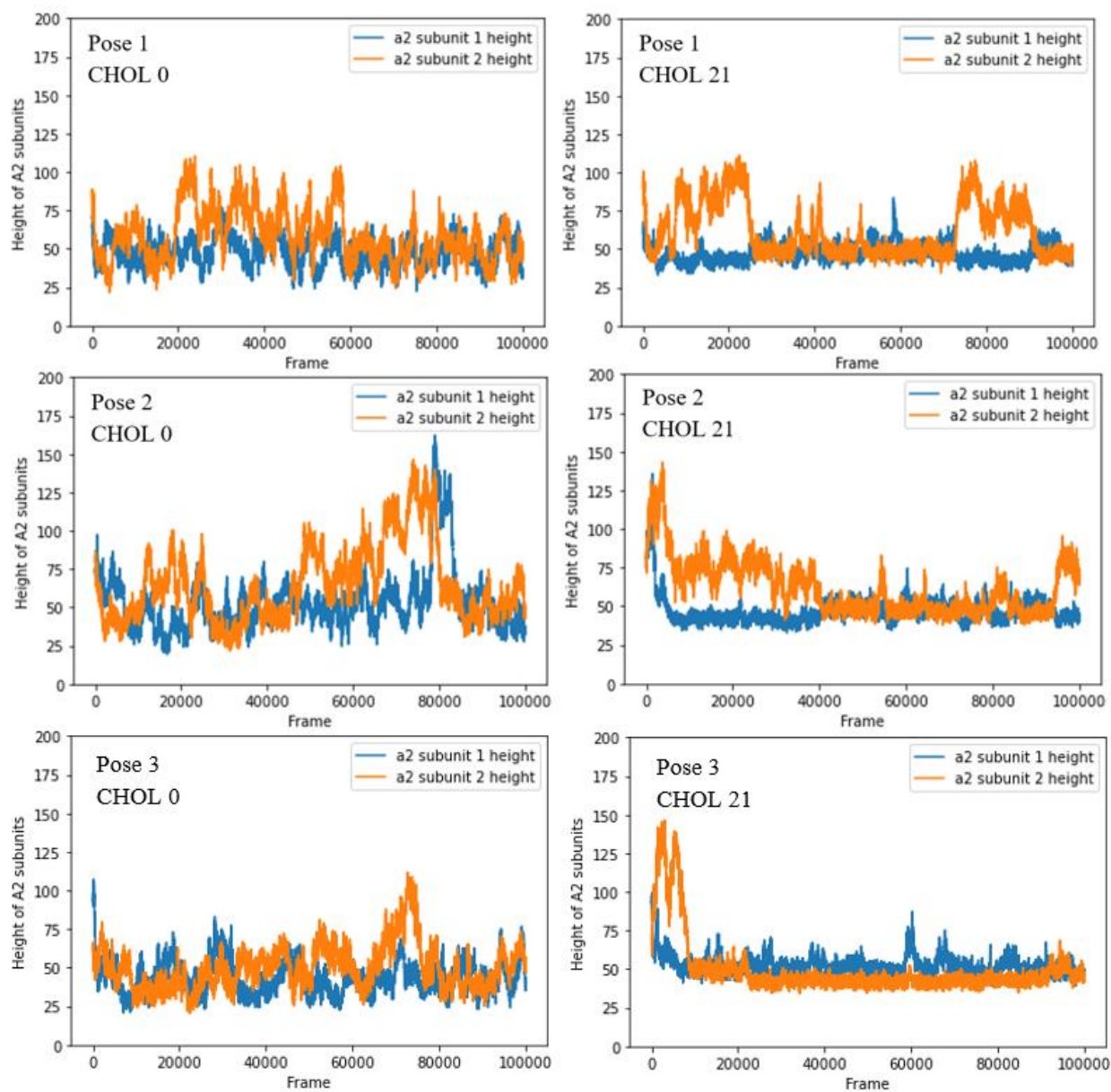


Figure 4.3: Distances between the center of mass of the A2 subunits of A2t for poses 1, 2 and 3 by frame for CHOL 0 and 21%. For each simulation, the CHOL 21% ones especially, the A2t tends towards binding to the membrane with both subunits, as seen in pose 2's starting structure.

Figure 4.4 limited the pose selection to just pose 2, as that is the starting pose most similar to the preferred binding mode of A2t as seen in the previous figure. In this figure, the distance between the center of mass of the entire A2t protein and the membrane was found for the eight different CHOL % trials. The trend of increased CHOL % leading to a decreased protein-membrane distance is even more prevalent and is very similar to the one seen in the A2 study.

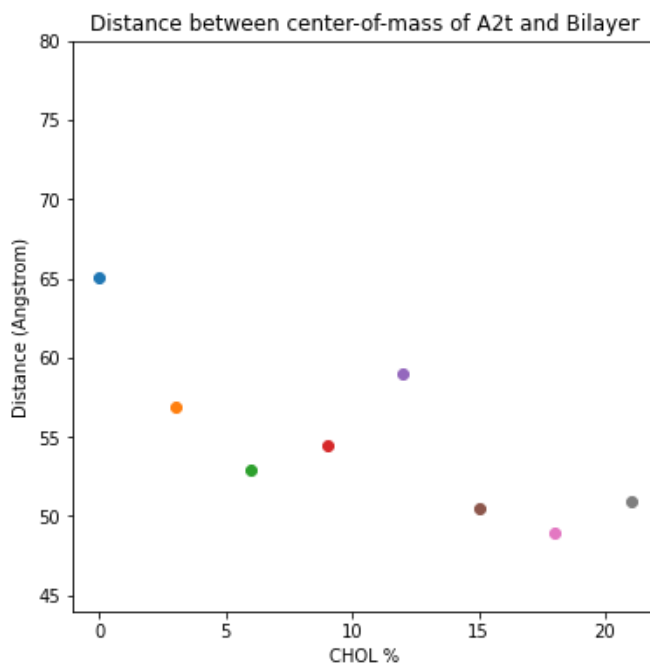


Figure 4.4: Distances between the center of mass of A2t and bilayer for pose 2 for the various CHOL % trials. The trend of increased CHOL % leading to closer protein-membrane interaction is present in this study as well.

Figure 4.5 expanded the selection of the protein to the center of mass of the A2 subunits of A2t to search for the same trend seen earlier, but also to determine any differences between the subunits. Both subunits followed the general trend shown earlier, where an increased CHOL % led to closer protein-membrane distances and interactions. This was very prevalent for subunit 1, but there was some variability in subunit 2 that led to larger protein-membrane distances. This is likely due to the slight twist that exists that does not allow both subunits to bind to the membrane using the full convex side of the A2.

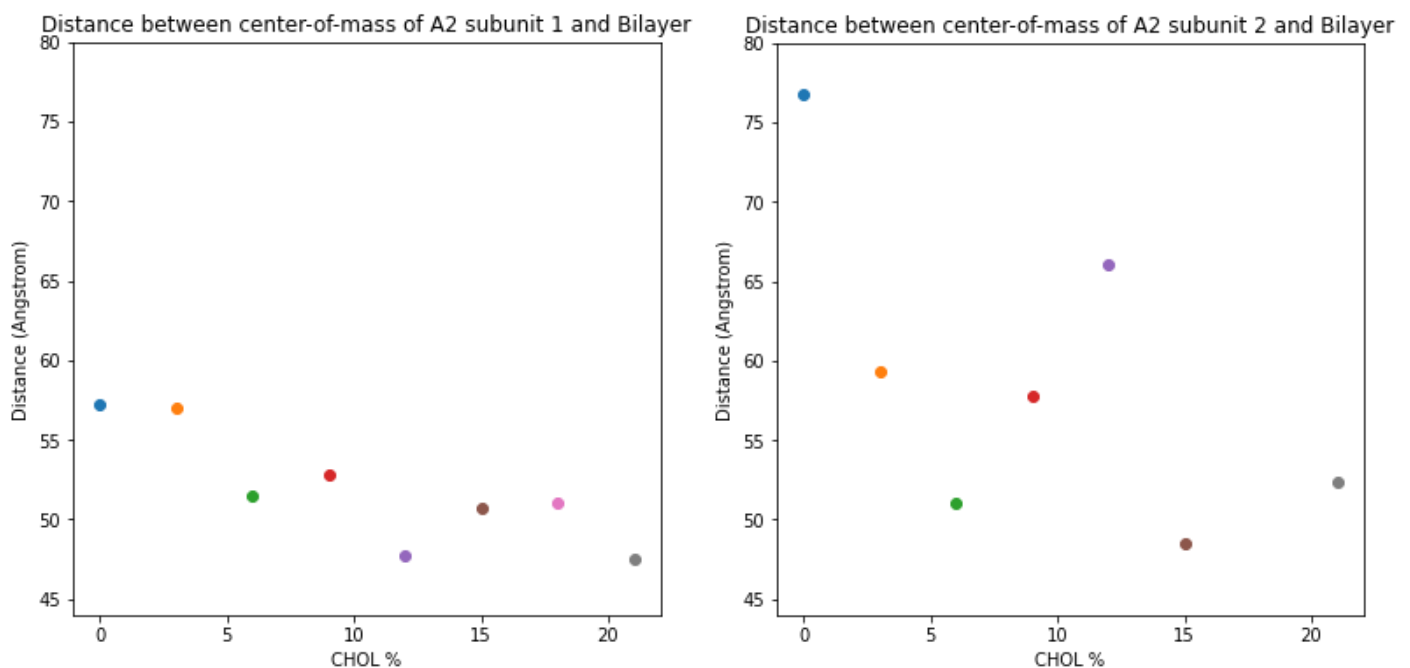


Figure 4.5: Distances between the center of mass of each subunit of A2t and bilayer for pose 2 at various CHOL % trials. Subunit 1 shows a clear trend of increased CHOL % leading to closer protein-membrane interactions. Subunit 2 shows more variability, but the general trend can be seen as well.

4.3.2 A2t-Membrane Contacts

Another method used to quantify the effect of CHOL on A2t–membrane association was contact analysis. This approach used updated code from the MDAnalysis project to calculate the number of close contacts between any A2t atoms and membrane atoms throughout the CHOL 0, 12, and 21% simulations. A cutoff distance of 10 Å was used to define a close contact. PIP₂ was highlighted in this analysis due to the monomer study showing that A2 was primarily binding with this lipid. Previous studies have also showcased this finding.²² The results of this initial analysis are presented in **Figure 4.6** below. The total number of close contacts between A2t and the PIP₂ lipids in the membrane increased as the amount of CHOL in the membrane increased. This finding is consistent with the previous distance analysis, which indicated that A2t becomes more closely associated with the membrane as CHOL concentration increases.

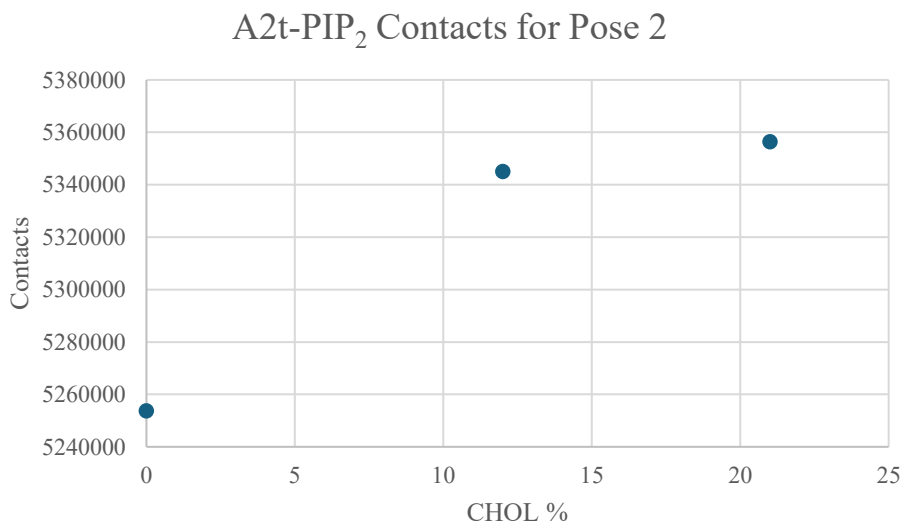


Figure 4.6: Plot showing the number of contacts between the A2t and the PIP₂ headgroups during the second half of the simulation for CHOL 0, 12, and 21% compositions for starting pose 2. The cutoff for consideration as a contact was 10 Å.

4.3.3 Lipid Contacts and Clustering

Like A2, a major role of A2t is the formation and stabilization of anionic phospholipid rafts. Previous studies, including the monomer study detailed earlier, have suggested that this function may be enhanced by increased CHOL content in the interacting plasma membrane. To investigate this relationship, analyses were performed to determine the extent of PIP₂ lipid clustering using PyMOL visualization and lipid contact measurements for starting pose 2.

Figure 4.7, shown below, presents top-down PyMOL visualizations of the initial and final simulation frames for the CHOL 0 and 21% trials. A2t is shown in yellow with slight transparency, PIP₂ lipids are shown in blue, and the remaining lipids are shown in white. As the simulations progress, the A2t proteins appear to aggregate increasing numbers of PIP₂ lipids beneath them while interacting more closely with the membrane. This aggregation is more pronounced in simulations with higher CHOL content, particularly the CHOL 21% trial.

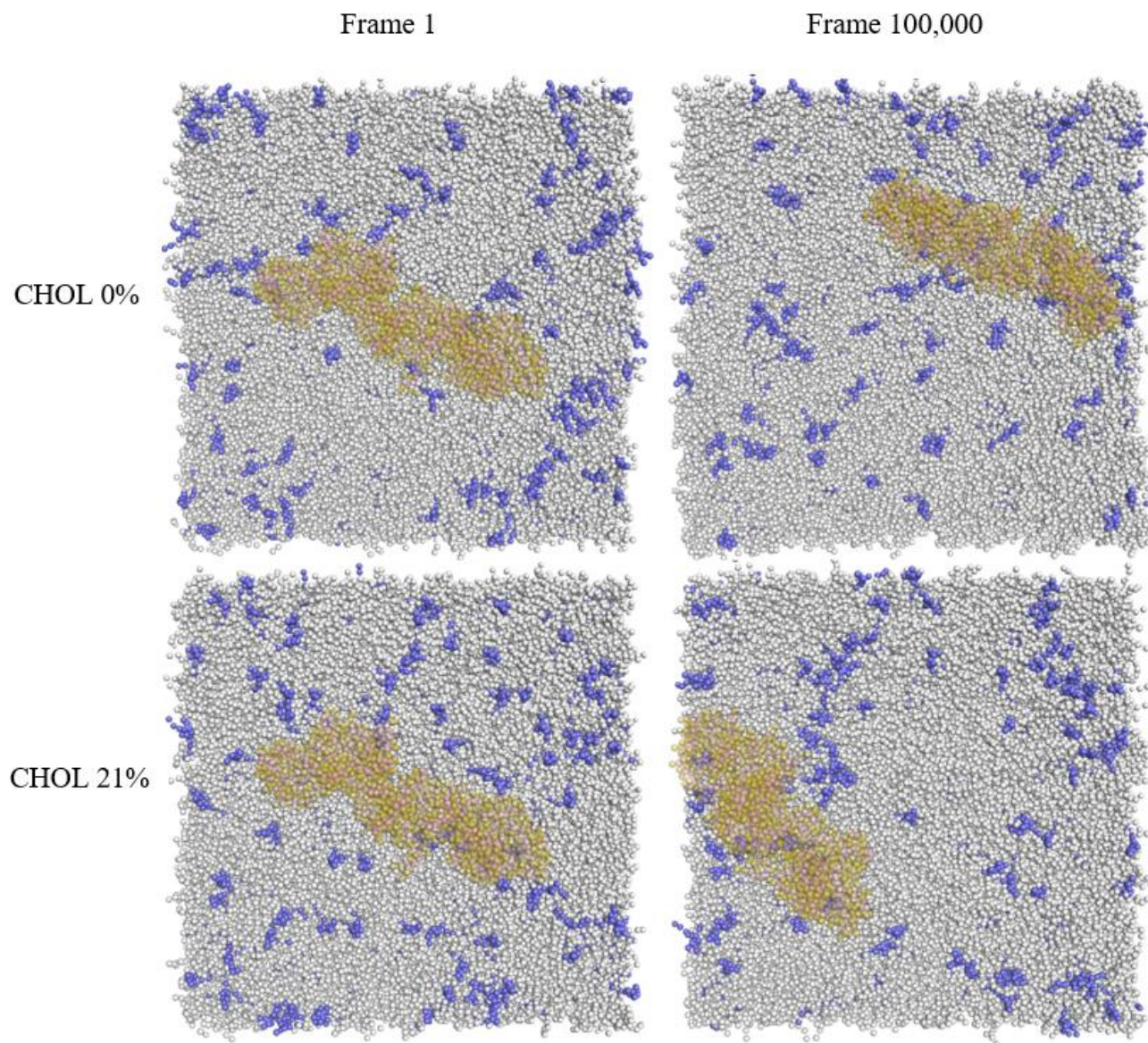


Figure 4.7: First and final frames for A2t simulations for pose 2 for CHOL 0 and 21%. The semi-transparent yellow is A2t, the blue is PIP₂, and the white is all the other lipids in the bilayer

PIP₂ self-distance and self-contact analyses were used to quantify this effect. For the self-distance analysis, the distances between each pair of PIP₂ headgroups were calculated during a frame in which the protein was closely associated with the membrane. These distances were represented as heat maps for the CHOL 0 and 21% trials, as shown in **Figure 4.8** below. The graphs in the right column restrict the range to 0–20 Å to provide a clearer view of the PIP₂ lipids in close proximity. The graphs show that the CHOL 21% trial contained a greater number of closely contacting PIP₂ lipids, with 83 close contacts compared with 65 in the CHOL 0% trial.

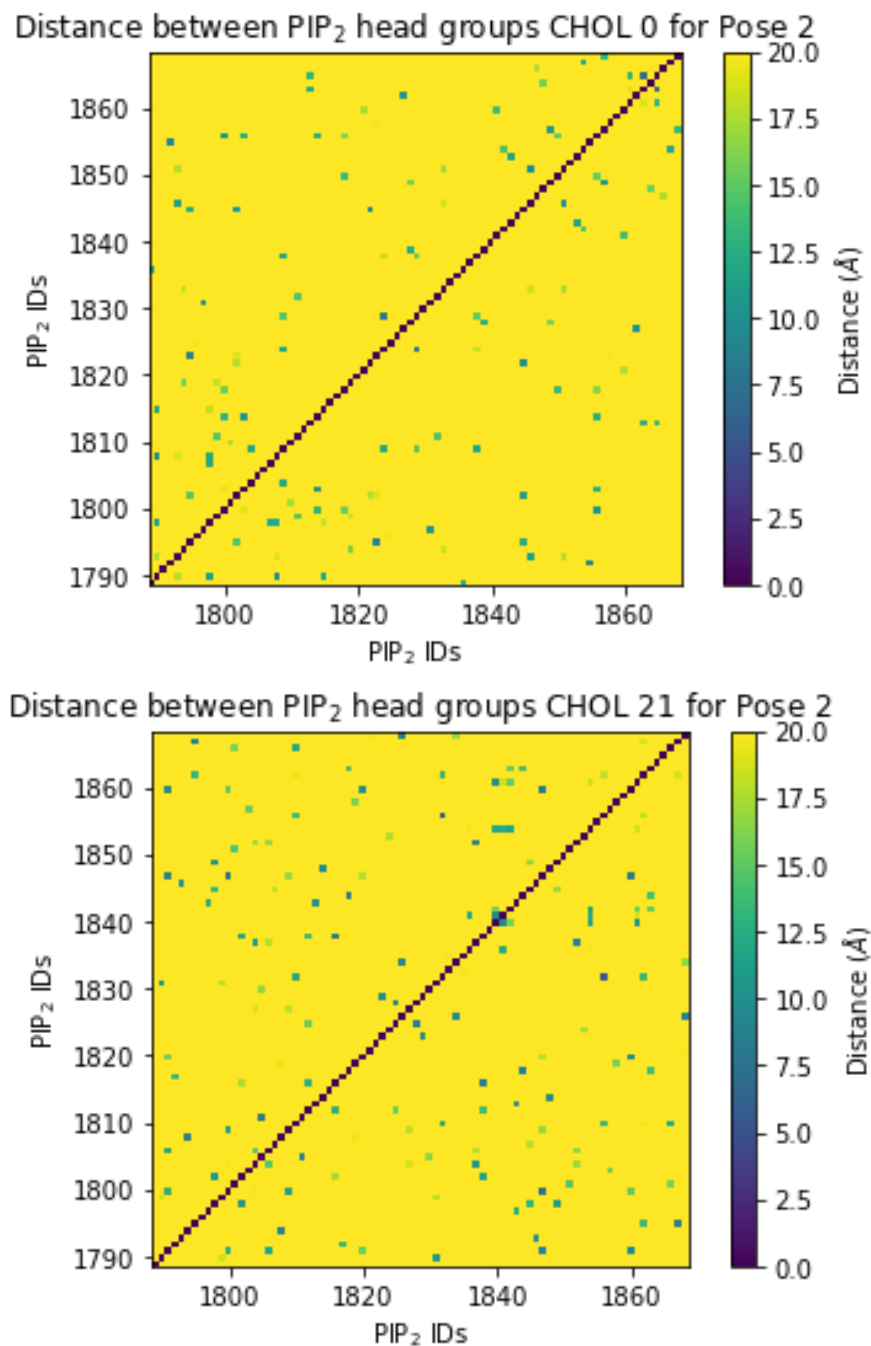


Figure 4.8: Heatmap visualization of the distance between each individual PIP₂ headgroup in CHOL 0% (top) and CHOL 21% (bottom) simulations during frames of close A2t-membrane association for pose 2. The plots show distances ranging from 0 to 20 Å to visualize PIP₂ headgroups that are clustering. The number of close associations is 65 for the top plot and 83 for the bottom plot.

Figure 4.8 therefore indicates that higher membrane CHOL content results in shorter distances between PIP₂ headgroups during periods of close A2t–membrane association. However, an additional analysis was needed to evaluate this effect over a longer portion of the simulations. For this purpose, a self-contact analysis was performed using a distance cutoff of 10 Å. The analysis included the PIP₂ lipids in the upper leaflet of the membrane throughout the second half of the CHOL 0, 12, and 21% simulations. The total number of PIP₂ self-contacts for each trial is presented in **Figure 4.9** below. The observed increase in PIP₂ self-contacts with increasing CHOL percentage is clear and supports the conclusion drawn from the single-frame analysis presented in **Figure 4.8**.

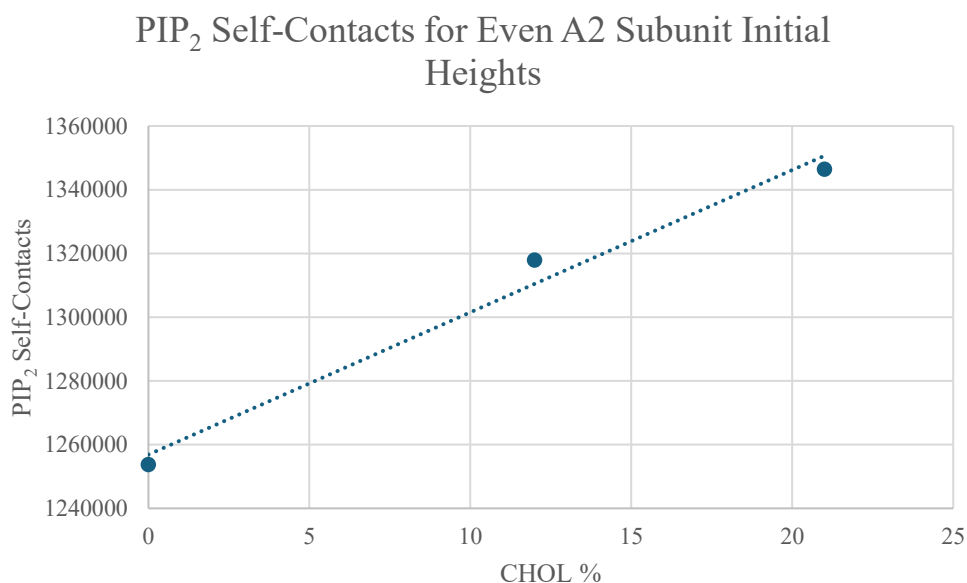


Figure 4.9: Plot showing the number of contacts between the PIP₂ headgroups during the second half of the simulation for CHOL 0% and 21% compositions. The cutoff for consideration as a contact was 10 Å.

4.3.4 USMD Stable Interaction Distances

Umbrella sampling simulations were run and analyzed by the weighted histogram analysis method for A2t over a membrane with 21% CHOL in order to determine optimal binding distance and the energy of the interaction. These simulations were run on both poses 1 and 2 first to compare with the results seen in the conventional molecular dynamics simulations. The results of this analysis are shown in **Figure 4.10**, below. This analysis shows a much more favorable interaction for the entire A2t protein when both subunits are positioned close to the membrane. This can be seen through the much more negative energy in the pose 2 plot compared to pose 1, with the center of mass of the protein in pose 2 also being at a smaller distance from the membrane at the energy minimum. This result was similar to what was seen in the conventional molecular dynamics simulations, where regardless of the starting pose, the binding of A2t to the membrane tended towards both subunits associating with the membrane, similar to pose 2's starting pose.

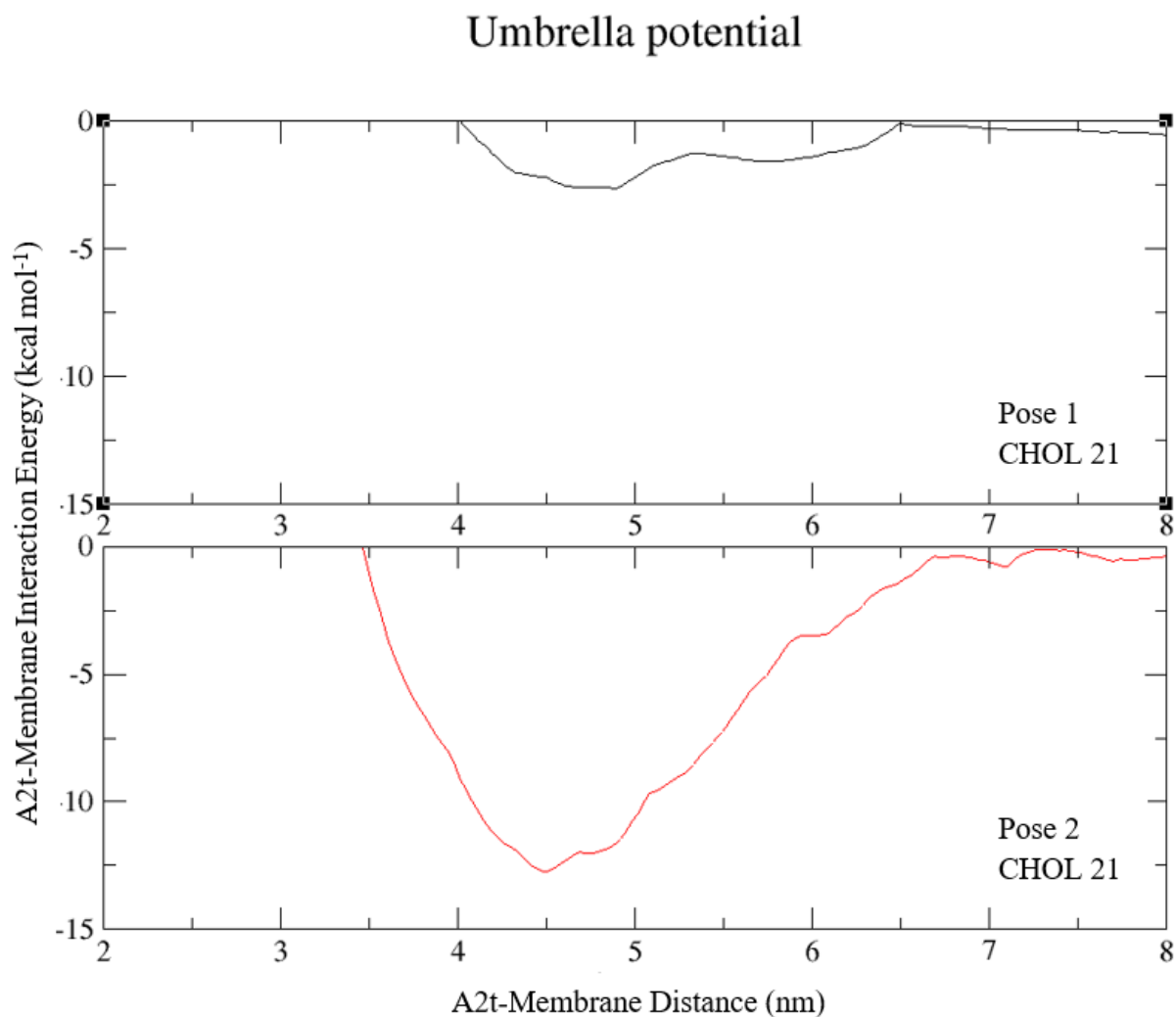


Figure 4.10: Plot showing the umbrella potential for the umbrella sampling molecular dynamics simulations of poses 1 and 2 for CHOL 21%. The energy associated with A2t-membrane interaction is compared to determine that pose 2, where both A2 subunits are evenly spaced above the membrane, has a more stable binding interaction at a closer distance.

In order to compare the umbrella sampling results to those seen in the monomer study, the results of pose 2 for A2t (in red) and the results of A2 CHOL 21% (in black) were overlaid in **Figure 4.11**, shown below. The A2 did have an energy minimum at a smaller distance to the membrane than A2t when comparing centers of mass. This does coincide with the result of the frame-by-frame distance analysis seen above in **Figure 4.3**, where it was noted that a twist in the A2t protein resulted in the inability of both A2 subunits to use their full convex sides to bind to the membrane. It is notable that A2t does bind with a more negative energy minimum however, likely due to it including two separate A2 monomers to bind to the membrane. The energy is slightly more than two times more negative for A2t compared to A2, suggesting a cooperative relationship between the two A2 subunits. More simulations are being run with the protein pull groups being specified for each A2 subunit instead of the whole protein to confirm this.

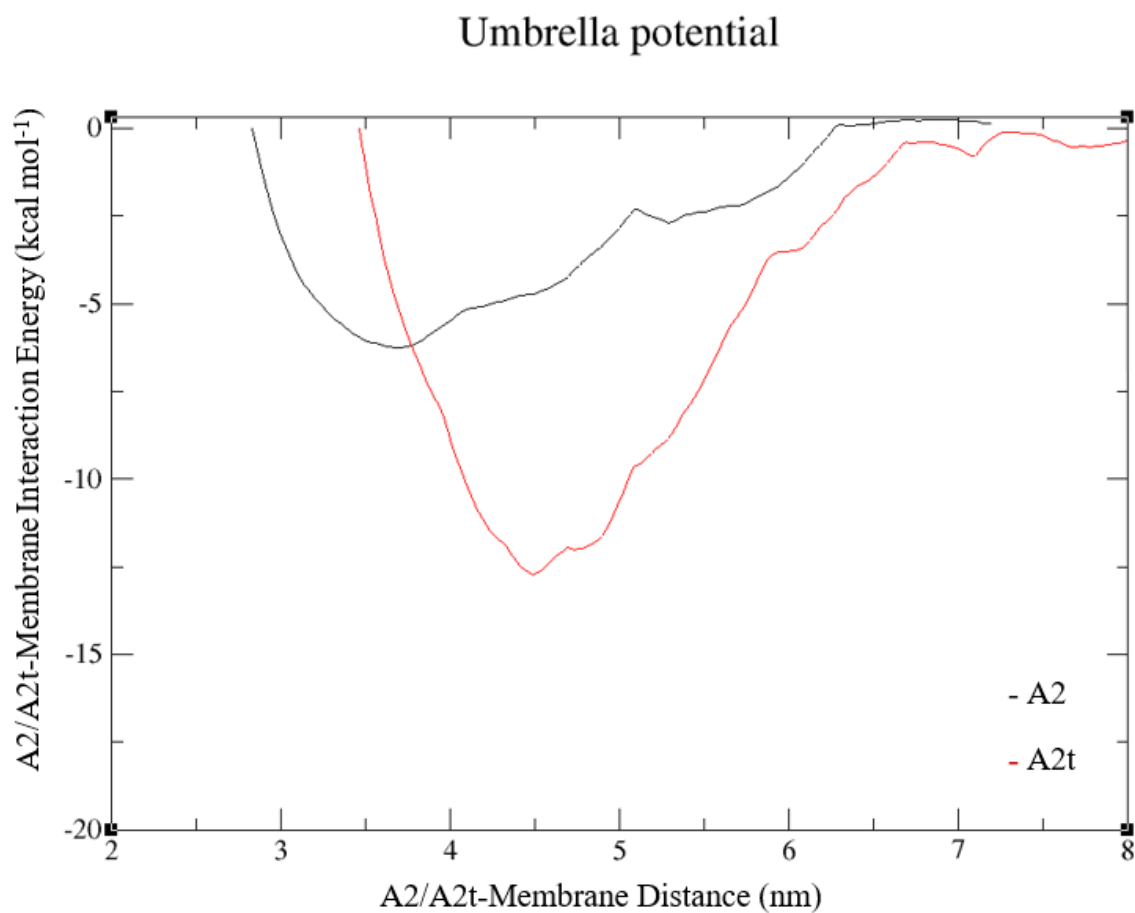


Figure 4.11: Plot showing the umbrella potential for the umbrella sampling molecular dynamics simulations of A2 (black) and A2t (red) for CHOL 21%.

4.4 Conclusions

The results of this study demonstrated that A2t associated favorably with PIP₂-containing membranes and that this association was strengthened by increasing membrane CHOL content. Across all three starting poses, the conventional molecular dynamics simulations showed that A2t tended to adopt a binding orientation resembling the starting orientation of pose 2, in which both A2 subunits interacted with the membrane. This indicated that simultaneous membrane association of both subunits was the preferred overall binding mode, regardless of the initial protein orientation. Distance analyses further showed that increasing CHOL percentage generally decreased the distance between A2t and the membrane, consistent with what was observed in the previous A2 study.

Although both A2 subunits followed the general trend of stronger membrane association at higher CHOL concentrations, some differences were observed between the subunits. These differences appeared to result from a slight twist in the A2t structure that prevented both subunits from placing their full convex surfaces against the membrane simultaneously. When both subunits were membrane-associated, the structural orientation of the A2t produced a slightly larger distance than when only one subunit was associated.

The contact and lipid-clustering analyses supported the conclusions obtained from the distance measurements. Increasing CHOL % resulted in a greater number of contacts between A2t and PIP₂ lipids and the membrane as a whole, indicating that CHOL promoted closer and more extensive interactions between the protein and PIP₂ lipids. PyMOL visualization, PIP₂ self-distance calculations, and self-contact analysis also showed that A2t aggregated PIP₂ lipids beneath the protein and that this clustering became more pronounced as CHOL % increased. Together, these findings suggested that CHOL not only strengthened A2t–membrane association

but also enhanced the formation and stabilization of PIP₂-rich anionic lipid domains, similar to what was seen in the A2 study.

Umbrella-sampling simulations provided additional support for the preferred binding orientation identified in the conventional simulations. Pose 2, in which both A2 subunits were associated with the membrane, produced a substantially more favorable energy minimum than pose 1, in which only one subunit was positioned for close membrane interaction. Comparison with A2 showed that A2 reached its energy minimum at a shorter center-of-mass distance from the membrane, likely because the monomer could fully use its convex side to bind to the membrane without the twist present in A2t. A2t exhibited a considerably more favorable energy minimum than A2, most likely because both A2 subunits contributed to the overall membrane interaction.

Overall, these results supported a model in which A2t preferentially bound with both A2 subunits associated with the membrane, while increasing CHOL % strengthened this interaction and led to increased PIP₂ lipid microdomain formation beneath the protein. These findings provided a structural and energetic basis for understanding how A2t binds to a membrane and organizes PIP₂ lipid microdomains over longer timeframes.

Chapter 5: Exploration of the Actin and membrane binding activities of Annexin A2

5.1 Background

Annexin A2 is widely expressed throughout the human body and is present in numerous tissues and cell types, where it is found in the cytosol and near the plasma membrane.³ A2t is the heterotetrameric form of annexin A2 and consists of two A2 monomers linked by an S100A10 dimer. Through its interactions with membranes, anionic phospholipids, and the actin cytoskeleton, A2 participates in processes such as exocytosis, membrane organization, and cytoskeletal remodeling. In particular, A2 and A2t function as actin-binding proteins that recruit dynamic F-actin filaments to membranes enriched in anionic phospholipids, like the PIP₂ focused on in the previous two studies.

A2 and A2t can also bundle actin filaments, thereby influencing the frequency and organization of exocytotic events. For A2t, this bundling may occur when the two A2 subunits bind separate actin filaments. A notable example is the exocytosis of neuroendocrine chromaffin granules, which store and release catecholamines, peptides, and ATP. Dynamic F-actin structures and myosin help transport these granules toward the plasma membrane. Following cellular stimulation, cytosolic A2 moves toward and binds the membrane, where it promotes the aggregation of anionic phospholipids and the formation of lipid microdomains. Actin bundles can then connect chromaffin granules to the plasma membrane, preparing them for fusion and secretion. A2 and A2t may regulate lipid microdomain formation, the number of membrane-associated granules, and the overall frequency of exocytotic events. These events have been implicated in various pathologies, so insight into their inner workings could prove valuable.

This study investigated the long-term interactions and binding modes of A2 and actin with membranes containing the anionic lipid PIP₂. A2t selectively binds membranes containing anionic phospholipids such as PIP₂, which interact with residues on the convex surface of the protein. The previous studies showed that A2 and A2t exhibited selective binding for PIP₂ and formed anionic lipid rafts beneath the protein. These lipid domains may contribute to the recruitment and organization of dynamic F-actin near the membrane, providing a connection between A2/A2t-mediated lipid clustering, cytoskeletal organization, and exocytosis.

The previous studies also indicated that CHOL strengthens A2/A2t association with PIP₂-containing membranes, increases the formation of anionic lipid rafts, and reduces lipid diffusion speeds, suggesting that CHOL stabilizes these lipid domains. In the present study, membranes with only 21% CHOL concentrations were simulated over long timescales to follow the results of the previous studies and optimize membrane-protein association. Coarse-grained molecular dynamics simulations were used to simplify the large system and permit longer simulation times.

The primary objective of this study was to begin to explore larger and more complex A2-membrane systems with molecular dynamics simulations. This particular system involved multiple A2 and actin proteins, so how they bound together and to the membrane was of interest as well. This study is in progress and will be expanded upon in the coming years. Understanding the interaction of these proteins may provide further insight into the normal regulation of exocytosis and membrane organization, as well as how their misregulation may contribute to various pathologies.

5.2 Computational Methods

5.2.1 CGMD A2-Actin System Details

Two systems are being explored. One is five A2 (2HYW.pdb) and four actin proteins (3HBT.pdb) located over a bilayer. The other is two actin proteins and two A2 proteins located over a lipid bilayer. The A2 and actin proteins were taken from the Protein Databank. All non-protein molecules were removed from the file to leave just the proteins in the file. This file was then coarse-grained using the Martini2 coarse-grained force field elnedyn22, which used an elastic network with an upper cutoff of 0.9 nm, a lower cutoff of 0.5 nm, and a force constant of $700 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ as the basis for maintaining the protein's overall shape.¹⁷ This reduced resolution allowed for longer-term simulations with less computational time, which was especially important for the much larger system due to these systems being very large in comparison to previous systems. The lower resolution is reasonable for this study because of the focus on overall protein-protein and protein-membrane interactions rather than smaller conformational changes, and it makes these longer-term simulations possible in a reasonable amount of time. The four coarse-grained lipids, POPC, POPS, PIP₂, and CHOL, were collected from the Martini Lipidome. From here, different starting structures were made using the mMAEVE program. For the first system, five A2 and four actin proteins were placed in a grid 80 Å over a 400x400 Å bilayer with a 7 Å separation between the layers and 2500 lipids per layer. The A2 proteins were oriented so that the convex sides were facing the membrane. The membrane was made up of 51% POPC, 20% POPS, 8% PIP₂, and 21% CHOL. The second system contained two A2 and two actin proteins located 80 Å over a 250x250 Å with a 7 Å separation between the layers and 1,000 lipids per layer. The starting structures for these systems can be seen in **Figure 5.1** and **Figure 5.2**.

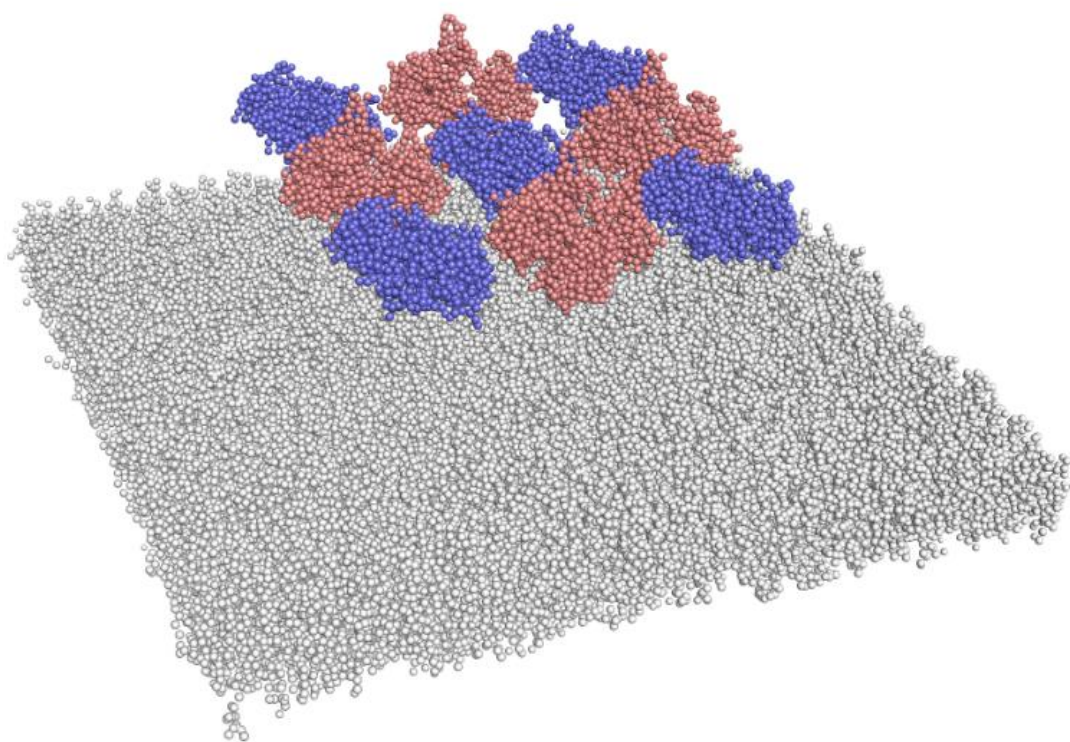


Figure 5.1: System setup of five Annexin A2 proteins (blue) and four actin proteins (salmon).

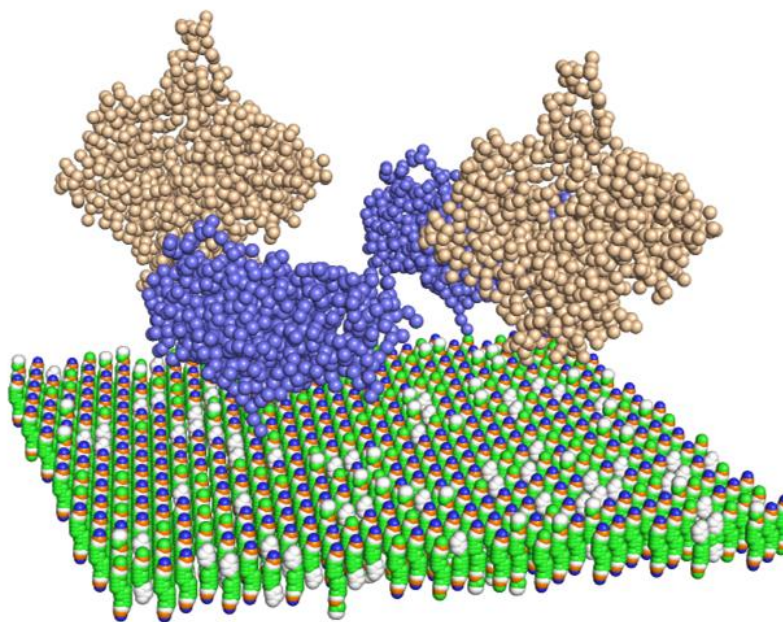


Figure 5.2: System setup of two Annexin A2 proteins (blue) and two actin proteins (tan).

These starting structures were then solvated with water, sodium ions , and chloride ions to simulate a cellular environment. The GROMACS software suite was used for all of the following simulation steps. A box with periodic boundaries was created to contain the atoms of the system with dimensions of 400x400x550 Å for system one and 250x250x550 Å for system two. The height of the box was lengthened to prevent the proteins potentially moving towards the top of the box and remaining there for the duration of the simulation. A steep integrator was used with 5,000 steps to solvate the box with 0.15 M water and ions.

The output of the solvation step was then minimized to reduce strain and clear up problematic contacts. This occurred in two steps with 5,000 steps each with a steep integrator. These steps also generated starting velocities for 310K.

The output of the minimization step was then equilibrated to gradually raise the temperature to close to body temperature. This was done in 250,000 steps of 20 femtoseconds

timesteps to raise the temperature to 310K using a v-rescale thermostat while maintaining 1.0 bar of pressure by using a semiisotropic Berendsen barostat with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$.

The output of the equilibration step was then used for the production step. The md integrator was used to simulate 10 μs through 500,000,000 steps with a timestep of 20 femtoseconds. A v-rescale thermostat was used to simulate the system at 310K, and a semiisotropic Parrinello-Rahman barostat was used to maintain 1.0 bar with a compressibility of $3.4\text{e-}4 \text{ bar}^{-1}$. The output trajectories were then reimaged converting the trajectories using -pbc mol to enforce the periodic boundary for the system box. The first and final frames for the first system can be seen in **Figure 5.3**.

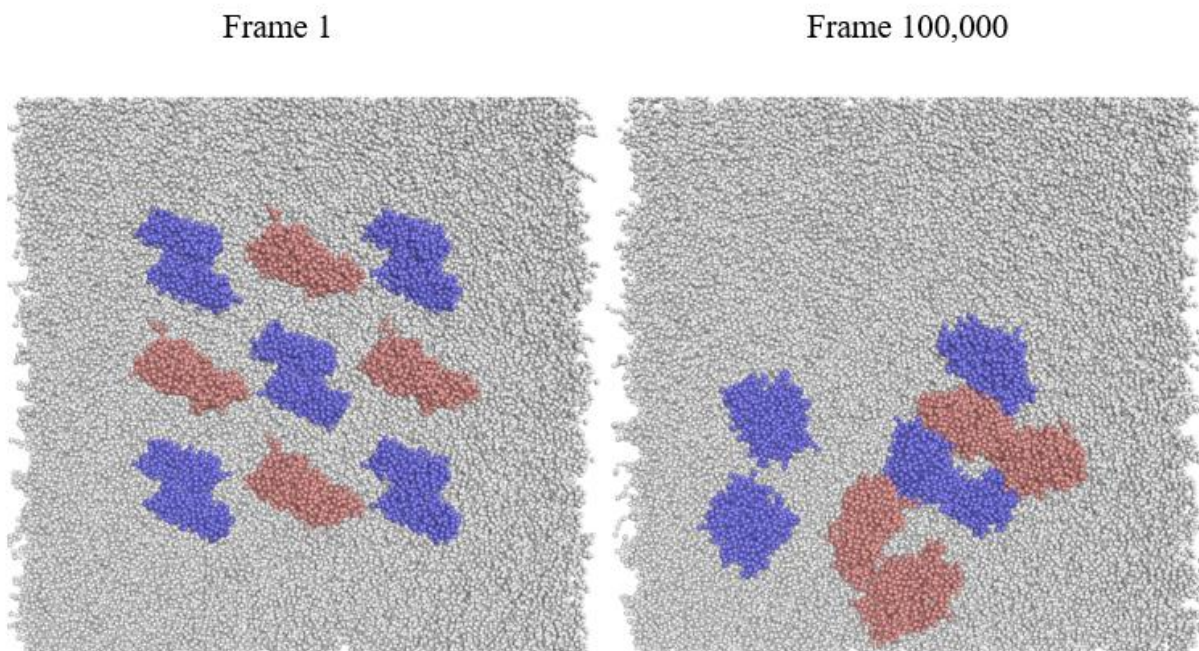


Figure 5.3: Top-down view of the first and final frames of the five A2 and four actin simulation. The A2s are shown in blue, the actins are shown in salmon and the membrane is shown in white behind the proteins. The proteins cluster together as the simulation progresses.

5.3 Conclusions and Further Study

The simulations for these actin systems are still ongoing, and the clustering of both PIP₂ lipids and A2 and actin proteins are expected. Other systems and models depicting the more complex roles of A2 are also being made and considered. One such model is one that includes two A2ts (4HRE.pdb), two actin chains (8DNI.pdb), and a vesicle over a membrane. This model is shown in **Figure 5.4**, below.

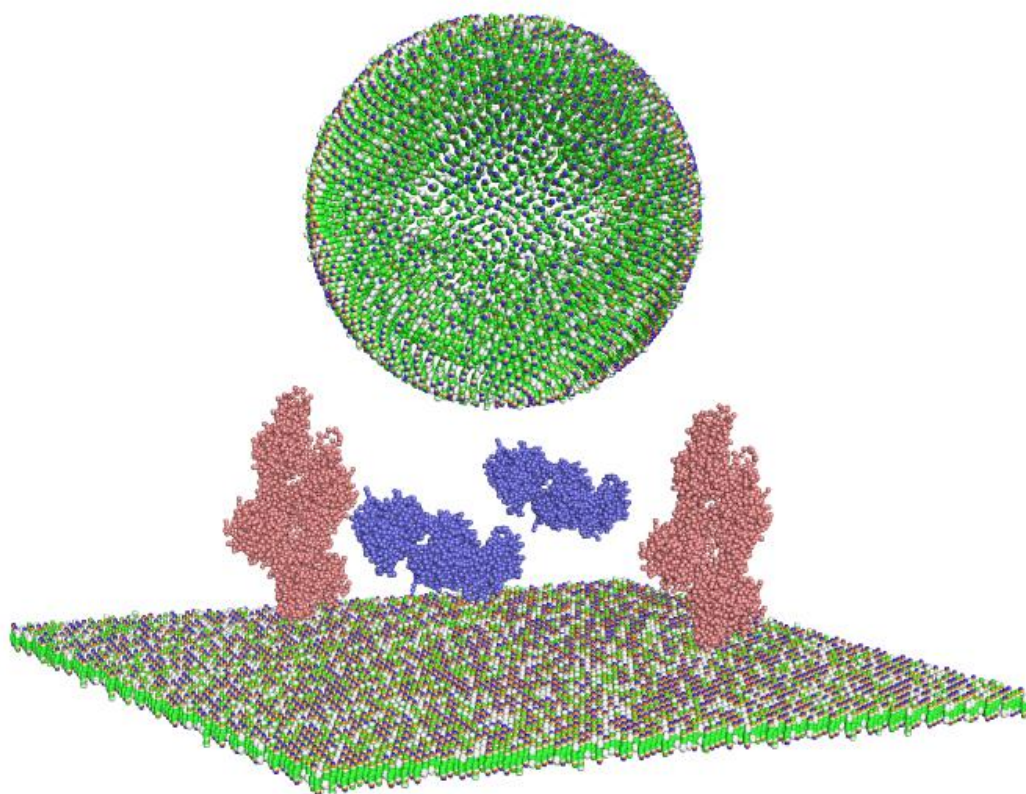


Figure 5.4: Model showing a membrane with two A2ts (blue) and two actin chains (salmon) with a vesicle above.

This model and other similar ones will be used to explore other roles of A2 that include exocytosis, membrane repair, and membrane reorganization through coarse-grained molecular dynamics simulations, umbrella sampling molecular dynamics simulations, and other powerful techniques. These further studies will elucidate more into A2 and A2t's roles in various cell processes and pathologies and the cellular environments that aid or inhibit the necessary protein and membrane interactions. A main goal of future systems will be to model the interactions seen in **Figure 5.5**, below.

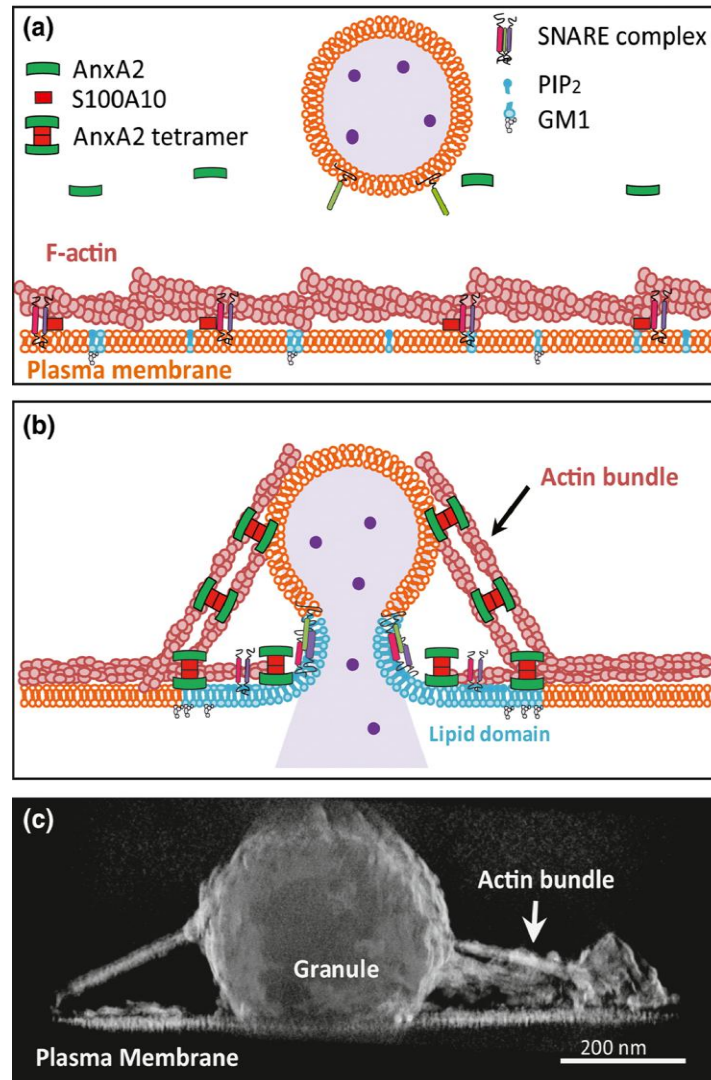


Figure 5.5: In an unstimulated chromaffin granule (A), the granules are not coated in F-actin and brought closer to the membrane. In a stimulated chromaffin granule during stress or danger (B) annexin A2t is able to bind to the membrane and to F-actin fibers near the membrane and coating the granule. This brings the granule to the prepared exocytotic site for fusion and secretion to occur. This is seen in the electron tomography analysis (C) with the annexin A2t's being located on the right side bound to the membrane and the fibrils.¹⁰

References

1. Burgoyne, R. D. & Geisow, M. J. The annexin family of calcium-binding proteins. *Cell Calcium* 10, (1989), 1–10.
2. Swairjo, M. A.; Seaton, B. A. Annexin Structure and Membrane Interactions: A Molecular Perspective. *Annual Review of Biophysics and Biomolecular Structure* (1994), 23 (1), 193–213.
3. Bharadwaj, A.; Bydoun, M.; Holloway, R.; Waisman, D. Annexin A2 Heterotetramer: Structure and Function. *International Journal of Molecular Sciences* (2013), 14 (3), 6259–6305.
4. Grieve, A. G.; Moss, S. E.; Hayes, M. J. Annexin A2 at the Interface of Actin and Membrane Dynamics: A Focus on Its Roles in Endocytosis and Cell Polarization. *International Journal of Cell Biology* 2012, (2012), 1–11.
5. Grindheim, A. K.; Vedeler, A. Extracellular Vesicles Released from Cells Exposed to Reactive Oxygen Species Increase Annexin A2 Expression and Survival of Target Cells Exposed to the Same Conditions. *Communicative & Integrative Biology*, (2016), 9 (4).
6. Lindsay S, Bartolotti L, Li Y. Interactions and conformational changes of annexin A2/p11 heterotetramer models on a membrane: a molecular dynamics study. *J Biomol Struct Dyn*. 2024;42(19):10342-10351.
7. Rety, S.; Sopkova, J.; Renouard, M.; Osterloh, D.; Gerke, V.; Russo-Marie, F.; Lewit-Bentley, A. *P11 (S100A10), ligand of Annexin II in complex with annexin II N-terminus* **1999**. DOI:10.2210/pdb1bt6/pdb.
8. Dominguez R, Holmes KC. Actin structure and function. *Annu Rev Biophys*. (2011), 40:169-86.

9. Hohmann, T.; Dehghani, F. The Cytoskeleton—a Complex Interacting Meshwork. *Cells* **2019**, *8* (4), 362. DOI:10.3390/cells8040362.
10. Gabel M, Delavoie F, Demais V, Royer C, Bailly Y, Vitale N, Bader MF, Chasserot-Golaz S. Annexin A2-dependent actin bundling promotes secretory granule docking to the plasma membrane and exocytosis. *J Cell Biol.* (2015), 210(5):785-800.
11. Hayes MJ, Rescher U, Gerke V, Moss SE. Annexin-actin interactions. *Traffic.* (2004), 5(8):571-6.
12. Gabel, M.; Chasserot-Golaz, S. Annexin A2, an Essential Partner of the Exocytotic Process in Chromaffin Cells. *Journal of Neurochemistry* (2016), 137 (6), 890–896.
13. D.H. de Jong, G. Singh, S.J. Marrink, *et al.* Improved Parameters for the Martini Coarse-Grained Protein Force Field. *J. Chem. Theor. Comput.*, 9 (2013), 687-697.
14. P.C. Kroon, F. Grünewald, S.-J. Marrink, *et al.* Martinize2 and Vermouth: Unified Framework for Topology Generation. Preprint at arXiv (2022).
15. S Gnanakaran, Hugh Nymeyer, John Portman, Kevin Y Sanbonmatsu, Angel E García. Peptide folding simulations. *Current Opinion in Structural Biology*, Volume 13, Issue 2, (2003), 168-174.
16. C. Shao, F. Zhang, B.A. Seaton, *et al.* Crystallographic Analysis of Calcium-dependent Heparin Binding to Annexin A2. *J. Biol. Chem.*, 281 (2006), 31689-31695.
17. X. Periole, M. Cavalli, M.A. Ceruso, *et al.* Combining an Elastic Network With a Coarse-Grained Molecular Force Field: Structure, Dynamics, and Intermolecular Recognition. *J. Chem. Theor. Comput.*, 5 (2009), 2531-2543.

18. Wang, H., Robinson, R.C. and Burtnick, L.D. (2010), The structure of native G-actin[‡]. Cytoskeleton, 67: 456-465.
19. T.A. Wassenaar, H.I. Ingólfsson, S.J. Marrink, *et al.* Computational Lipidomics with *insane* : A Versatile Tool for Generating Custom Membranes for Molecular Simulations. J. Chem. Theor. Comput., 11 (2015), 2144-2155.
20. 12. P. Drücker, V. Gerke., M. Pejic, *et al.* Lipid Segregation and Membrane Budding Induced by the Peripheral Membrane Binding Protein Annexin A2. J. Biol. Chem., 288 (2013), pp. 24764-24776.
21. M.J. Abraham, E. Lindahl., T. Murtola, *et al.* GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX, 1–2 (2015), 19-25.
22. S. Lindsay, Y. Li. Coarse-grained modeling of annexin A2-induced microdomain formation on a vesicle. Biophysical Journal, Volume 123, Issue 16, (2024), Pages 2431-2442.
23. T. pandas development team. Pandas-Dev/Pandas: Pandas (2020).
24. C.R. Harris, K.J. Millman, T.E. Oliphant, *et al.* Array programming with NumPy. Nature, 585 (2020), 357-362.
25. P. Virtanen, R. Gommers P., van Mulbregt, *et al.* SciPy 10 Contributors. SciPy 1.0: Fundamental Algorithms for Scientific Computing in Python. Nat. Methods, 17 (2020), 261-272.
26. N. Michaud-Agrawal, E.J. Denning, O. Beckstein, *et al.* MDAnalysis: A toolkit for the analysis of molecular dynamics simulations. J. Comput. Chem., 32 (2011), 2319-2327.

27. Kumar, S.; Bouzida, D.; Swendsen, R. H.; Kollman, P. A.; Rosenberg, J. M. J. Comput. Chem. 1992, 13, 1011–1021.
28. Grill, D., A. L. L. Matos, ., V. Gerke. 2018. Bridging of membrane surfaces by annexin A2. Sci. Rep. 8:14662.