

Computational Investigation of the Annexin A2-Membrane Interaction and Annexin A2-Induced Microdomain Formation

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Computational methods including molecular dynamics and molecular docking were utilized to investigate multiple facets of the peripheral membrane binding protein, Annexin A2 (A2). The combined works are presented here as five independent studies. All atomic molecular dynamics simulations were used to conduct the first two studies on the heterotetrameric A2/p11 complex. The first investigated the impact of Ca^{2+} on the organization of membrane and F-Actin binding sites found within the heterotetramer. It was shown that Ca^{2+} modulates the availability of key membrane-binding residues and the stability of F-Actin-binding C-terminal residues of A2. The second study investigated the organization of the heterotetramer on the surface of a membrane model. A model of the heterotetramer consistent with measurements from the literature was generated. Additionally, previously unreported interactions between p11 and the membrane were observed.

A2 has been implicated in a variety of pathologies where its inhibition would be desirable. A study was undertaken to identify potential inhibitors of A2-membrane binding activity. The stability of A2-ligand binding and effects of ligand binding on A2-membrane

interactions was assessed via molecular dynamics simulation. Multiple ligands were identified that disrupt A2-Ca²⁺ binding and A2-membrane binding.

The final two studies involved modelling A2-induced PIP2 microdomain formation. A novel Python package, called MMAEVe, was developed that facilitates the creation of biological-scale membrane-protein models. For the final study, MMAEVe was used to generate a system of A2 surrounding a vesicle. Coarse-grained molecular dynamics simulations were run for the system and the process of A2-induced PIP2 microdomain formation was observed. It was shown that the process occurs in three steps, A2-membrane association, localized PIP2 clustering, and A2 aggregation causing local PIP2 clusters to merge into microdomains. A novel method was developed to track microdomain formation which utilizes the Fréchet mean to define a system-appropriate variance measurement.

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CHAPTER 1: Introduction

Annexin A2 (A2) is a well-studied peripheral membrane binding protein. It is key to a number of biological processes that include F-Actin organization, various forms of endocytosis, exocytosis, cellular polarity, cellular migration, plasminogen regulation, angiogenesis, and redox activity, among others.¹ See **Figure 1.1** for schematic representations of these activities. The upregulation of A2 is crucial for the survival and proliferation of many cancers. In fact, A2 has been demonstrated to contribute to the lifecycle of aggressive metastatic cancers in no less than three facets, its role in cellular migration via its interactions with structural proteins, its role in angiogenesis, and its protection of genetic information in the presence of reactive oxygen species

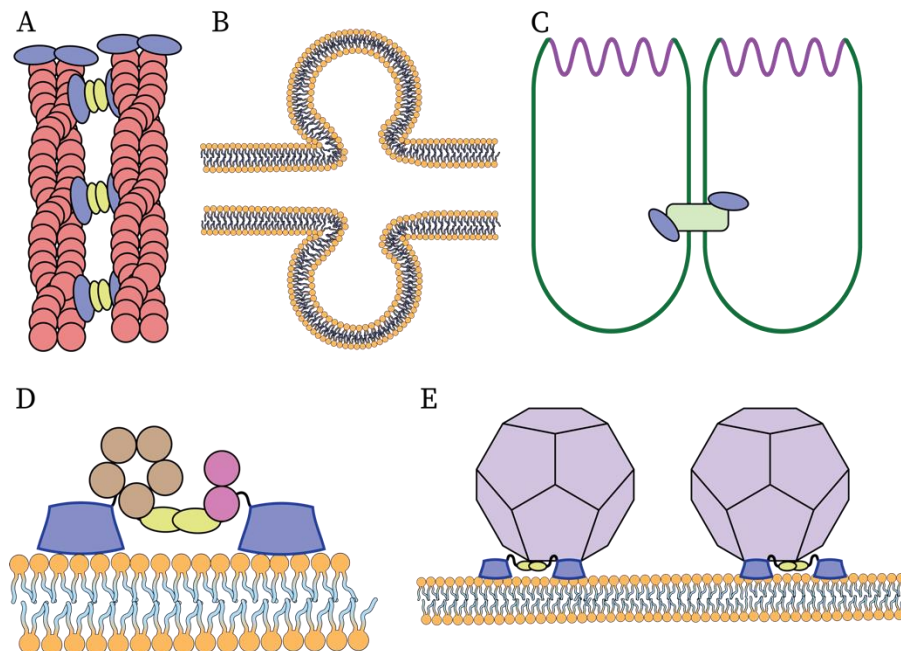


Figure 1.1. Schematic representation of the various functions of A2. (A) A2t induced bundling of F-Actin along with A2 serving as Actin fibril caps. (B) Exosome (top) and endosome (bottom) formation. (C) A2 colocalized with E-cadherin at an epithelial cell adheren junction. (D) Colocalization of plasminogen and tissue-plasminogen activator by A2t. (E) Docking of virion to plasma membrane by A2t. The color scheme is consistent across all images. A2, p11, actin, lipid heads, lipid tails, epithelial baseolateral domain, epithelial apical domain, E-cadherin, plasminogen, tissue-plasminogen activation, and virions are colored blue, yellow, salmon, orange, cyan, dark green, dark purple, light green, brown, pink, and light purple, respectively.

via its redox activity.² A2 has also been demonstrated as a primary or secondary cellular receptor for many viruses.³

To gain a better understanding of A2, its role in disease, and outstanding questions regarding its function, a comprehensive literature review was undertaken. This review synthesizes the content of many existing reviews as well as more recent work involving A2. It facilitated the creation of the primary goals of this dissertation which were derived from outstanding questions regarding A2. The review is presented in the second chapter of this dissertation. Following the review of A2, a brief overview of molecular dynamics, the applied force fields, and virtual screening is provided in the third chapter. This chapter serves to familiarize or refresh the reader with the topic.

In the literature, virtually all introductions of A2 or its heterotetrametric complex with p11 (A2t), seen in **Figure 1.2D**, mention their Ca^{2+} -dependent binding to phospholipid membranes. Given the ubiquity of this statement we were surprised to find little structural information regarding the specific role of Ca^{2+} in facilitating the A2-membrane interaction. Ca^{2+} -binding sites are seen in **Figure 1.2C**. In the first decades of A2 research, it was suggested that Ca^{2+} -bridged the membrane interaction directly. Work characterizing the Ca^{2+} -binding sites, conducted by Filipenko et al. demonstrated that mutation of acidic Ca^{2+} -binding residues to alanine restored membrane-binding capability of A2 in the absence of Ca^{2+} . This led them to suggest that in the absence of Ca^{2+} , acidic residues capture important, basic membrane-binding residues which are co-localized with the Ca^{2+} binding loops.⁴ K279 and K281, seen in **Figure 1.2A**, are two highly conserved residues within A2. K279A and K281A mutants have a K_d that is reduced by factors of ~20 and ~40, respectively.⁵ The effects of Ca^{2+} on A2 activity are not limited to membrane interactions. It has also been demonstrated that the bundling of F-Actin by

A2t is enhanced in the presence of Ca^{2+} .⁶ The F-Actin binding site is shown in **Figure 1.2B**. We sought to investigate the structural features of A2t pertinent to both binding sites that are altered by Ca^{2+} -binding. In Chapter 4 the results of an MD investigation of A2t in the presence and absence of Ca^{2+} are presented to answer these questions.

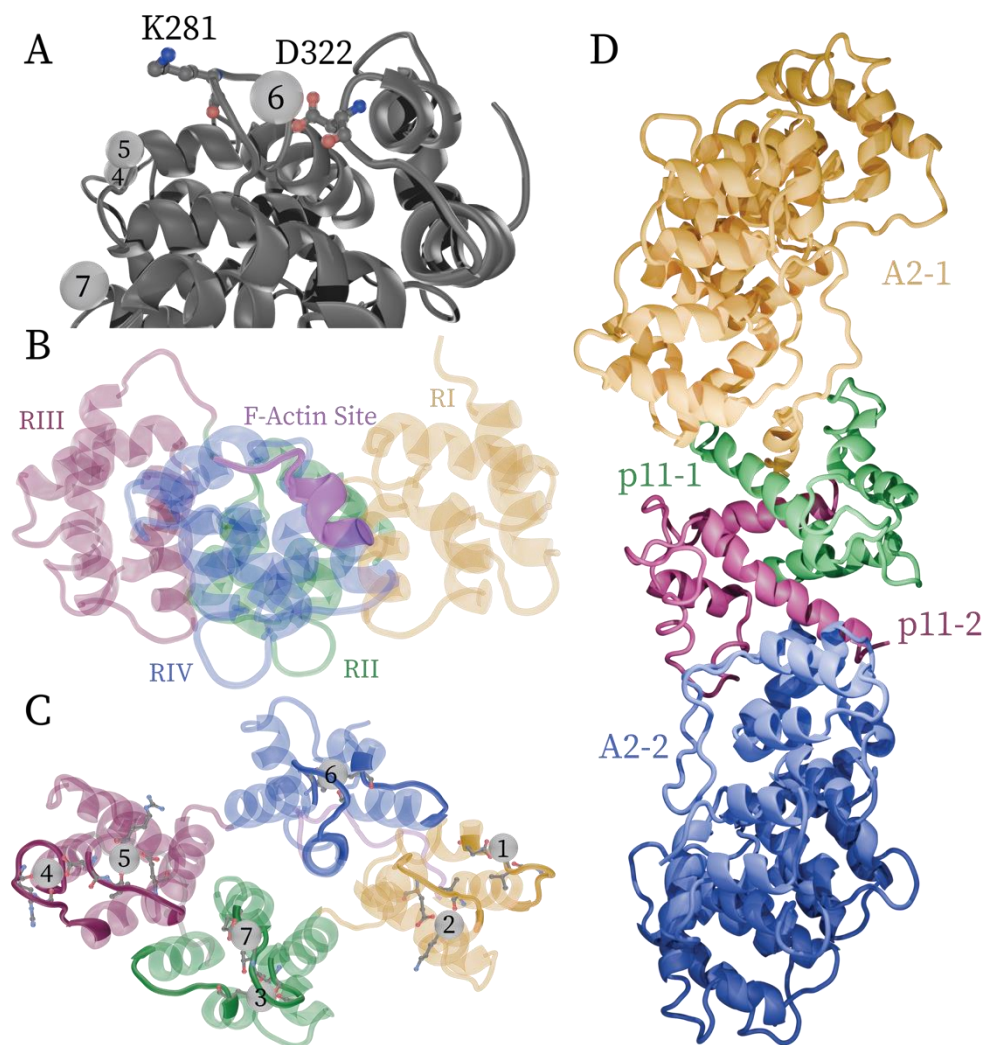


Figure 1.2. Cartoon representations of A2 or A2t to show various structural features. (A) PIP2-specificity site residue K281 and Ca^{2+} -binding residue D322 are shown using ball and stick models. Ca^{2+} are labeled, labels are consistent with those in (C). (B) The repeats of the A2-core domain with color-coded labels. The F-Actin binding site is also colored and has a corresponding label. (C) Ca^{2+} -binding loops of A2 with Ca^{2+} -coordinating residues shown using ball and stick models. Individual Ca^{2+} are labeled. (D) Structure of A2t with its subunits labeled. For ball and stick models found in (A, C), carbons are colored grey, oxygens red, and nitrogen blue; Ca^{2+} are shown using white spheres.

The orientation of A2t of the membrane surface has been the focal point of biostructural studies beginning in the 1990s. A cryogenic electron microscopy (cryo-EM) study conducted by Lambert et al. investigated the structure of A2t at GUV junctions. This study was motivated by previous observation of A2 and Annexins generally bridging chromaffin granules. Their results demonstrated that at the interface of two vesicles, A2t appears to be oriented such that the two A2 monomers are each associated with one of the vesicles with the p11 dimer located in-between, as shown in **Figure 1.3A**.⁷ Menke et al. conducted scanning force microscopy (SFM) studies on A2 and A2t on membrane models. It was observed that the dimensions of A2 are consistent with what would be expected if its convex face were associated with the membrane, as seen in **Figure 1.3B**. The dimensions of A2t were consistent with the whole complex being flush against the membrane surface.^{8,9} The A2t crystal structure was solved by Oh et al. in the absence of a membrane component.¹⁰ The structure did not resemble what has been observed in the literature regarding the orientation of membrane-associated A2t. Thus, a physical model of the A2t complex on the membrane surface is still unavailable. We built models consistent with the

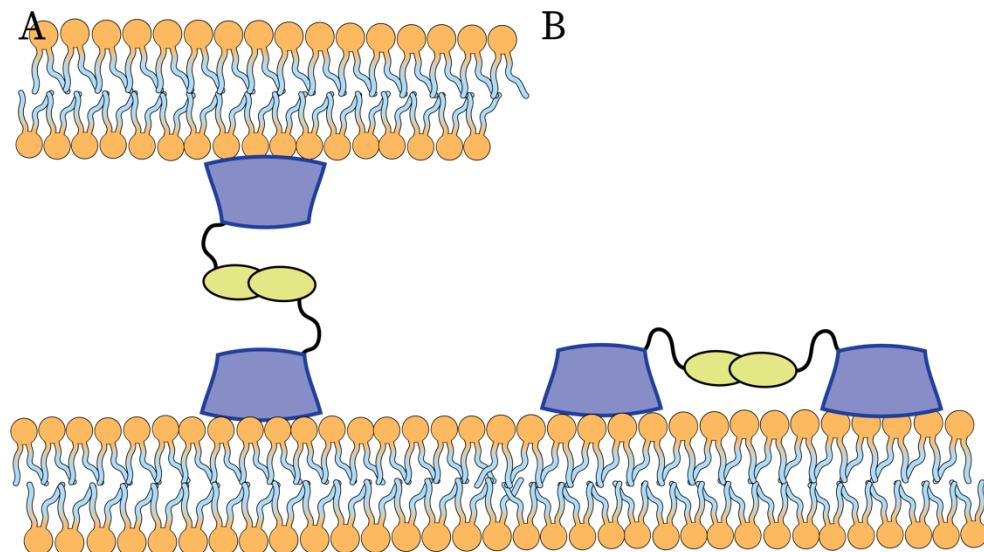


Figure 1.3. Schematic representation of A2t at (A) a membrane junction and (B) on a membrane monolayer.

reported literature and performed MD to observe their interactions in the presence of a single membrane. The results of this endeavor are reported in Chapter 5.

A2's role in cancer was briefly introduced earlier. Its role in angiogenesis makes it important for the development of new vasculature. This process sees A2t serve as a receptor for colocalizing plasminogen with tPA and uPA with p11 being the most important component as it is the subunit responsible for interaction with plasminogen and its activators.¹¹ Indeed, p11 knockout mice demonstrate enhanced clotting ability because the ability to activate plasminogen to the fibrin-targeting protease plasmin is reduced.¹² A2 knockout mice demonstrate defects in the process of both plasminogen activation and angiogenesis. Intriguingly, A2-knockout mice remain viable even though their capacity for angiogenesis is reduced.¹³ However, aggressive cancers which need a significant amount of novel vasculature to fuel their growth are highly susceptible to A2t inhibition.¹⁴ This makes A2t a desirable target for developing novel therapeutics. Unsurprisingly, efforts have been made to identify inhibitors of A2t formation. These efforts employed virtual screening of the p11 dimer. Substituted 1,2,4-triazoles were identified as inhibiting A2t complex formation.¹⁵ The A2 membrane interaction is another desirable target process for inhibition as the A2t complex rests on the surface of vascular endothelial cells. A natural site to focus ligand discovery efforts on is the PIP2-binding site which was introduced earlier.⁵ The sixth chapter details the process of applying computational drug discovery methods for identifying and assessing the capacity of candidate ligands.

We also developed an interest in computationally reproducing and investigating some of the more striking behaviors of A2 that are observed *in vitro*. Specifically, A2's ability to induce the formation of PI(4,5)P2 microdomains on membrane models, as seen in **Figure 1.4B**.¹⁶ We believed this to be an excellent use of molecular dynamics simulations but challenges for

accomplishing this task remained. This is largely because of the limited timescale that is accessible for all atomic molecular dynamics simulations; high-quality force-fields will provide excellent results for transport properties such as diffusion coefficients but the accessible timescales on consumer-grade workstations and small to medium-size clusters are not sufficient for observing membrane dynamics. Indeed, only truly massive supercomputers can do so at the required tens of μs to ms timescale.¹⁷ To circumvent this, coarse-grained molecular dynamics force fields have been developed. These accomplish massive speed-ups over all-atomic force fields by a variety of mechanisms. The first is a reduction in the number of overall particles that are needed to represent a system, see **Figure 1.4A** for a coarse-grained PI(4,5)P2. This is typically on the order of a 10x reduction in the number of required particles. The second is by a reduction in the highest frequency bonds observed in a system. This allows for the usage of a larger timestep, on the order of 10x or greater. Finally, the reduced complexity results in a smoother potential energy landscape which allows for processes to occur much more quickly. This allows for a roughly 100x speedup in observed times and up to a 1000x effective speedup depending on the process being observed. Diffusion coefficients of water molecules in Martini are approximately 4x larger than observed experimentally. The Martini Force Field is by and far the most successful of the coarse-grained force fields to-date and has been extended to lipids, proteins, carbohydrates, and small molecules. Because of its success and widespread application, we chose to utilize it for our system.¹⁸

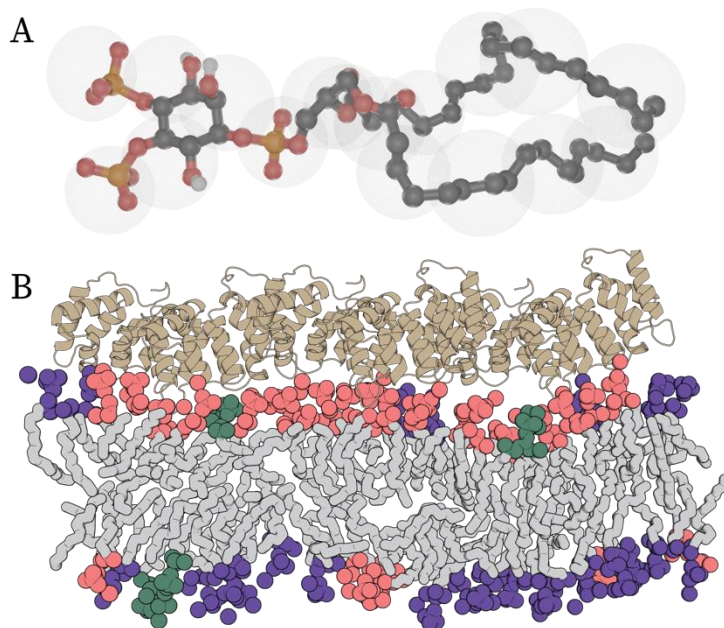


Figure 1.4. (A) Ball and stick model of PI(4,5)P2 with large spheres shown to demonstrate how atoms can be grouped during a coarse-graining procedure. Oxygen atoms are colored red, phosphorus orange, carbon grey, and hydrogen white. (B) Representation of A2-induced microdomain formation. Lipid tails are colored grey, PI(4,5)P2 heads red, POPC head purple, POPS heads green, and A2 tan.

Even with an appropriate force field readily available, setting up a vesicle model with multiple A2 present remained challenging. The existing tools for constructing membrane systems for MD simulations suffered from limitations regarding the incorporation of protein components in the systems. To overcome these limitations, we sought to develop a program for quickly and efficiently generating large, complex protein-membrane simulation systems of mixed composition. The project was titled MMAEVe, short for Membranes, Micelles, and Even Vesicles. MMAEVe is the topic of Chapter 7, including how it works, performance benchmarks, and example applications. With MMAEVe constructed we were able to construct a simulation system that allowed us to investigate the process of A2-induced PI(4,5)P2 aggregation. This investigation is the final chapter, Chapter 8, of this dissertation.

CHAPTER 2: Review of Literature Regarding Annexin A2

2.1. Annexins

2.1.1. *Discovery of Annexins*

Originally named Synexin and discovered in 1978 by Creutz et al., Annexin A7 was the first member of the Annexin superfamily to be identified. It was isolated from chromaffin granules, intracellular vesicles originating in chromaffin cells of the adrenal gland that are commonly utilized as a model for the study of exocytosis. Its ability to cooperatively aggregate chromaffin granules in a Ca^{2+} -dependent manner led Creutz et al. to suggest that it served as an intracellular Ca^{2+} -receptor involved in triggering exocytotic events in the adrenal medulla.¹⁹

2.1.2. *Structural Features of Annexins*

As previously alluded to, the name “Annexin” denotes a superfamily of proteins which contains five families, A, B, C, D, and E found in vertebrates, invertebrates, fungi, plants, and protists. The specific identity of an annexin protein is denoted by a numeric suffix to the family, i.e. Annexin A2. Eukaryotes have anywhere from 1 to 20 Annexin genes.²⁰

All annexins have similar structural organization; they contain two domains referred to as the N terminal domain (ND) and C terminal domain (CD). In animals and fungi, the former is unique to each annexin and can contain anywhere from a dozen to over one-hundred amino acids. This unique nature of Annexin NDs is seen in the upper panel of **Figure 2.1** where a pairwise alignment of all Human Annexin NDs is shown. The latter is highly conserved, composed of four homologous repeats, denoted by RI-RIV, each containing about 70 amino acids (see **Figure 2.2B**). Across vertebrate Annexins, the core domains share ~50% sequence identity. Secondary and tertiary structure are highly conserved. This is demonstrated by an MSA

of all Human Annexins, shown in the lower panel of **Figure 2.1**. The one observed exception to the highly conserved structure is Annexin A6 which is composed of eight repeats instead of four. This unique structure is the result of Annexin A6 being a compound-gene, likely created from the fusion of the genes for Annexins A5 and A10. Each repeat is in-turn composed of five individual α -helices, A, B, C, D, and E. Helices A, B, D, and E are wound into a right-handed superhelix while helix C is oriented atop the other, nearly perpendicular to the superhelix (see **Figure 2.2A**). Helices are joined by flexible loops. The overall structure of the CD has a slight curvature with distinct convex and concave surfaces. The ND is located at the concave surface. Ca^{2+} -binding sites are found at the convex surface (see **Figure 2.2C**). The loops between helices A and B, and D and E are found at the convex surface and commonly contain Ca^{2+} -binding sites.²⁰

Each Annexin repeat can contain up to two Ca^{2+} -binding sites. To date, three types of Ca^{2+} -binding sites have been identified in human annexins, type II, type III, and AB' binding sites. Type II sites are known as the endoexin-fold and have the characteristic sequence GXGT-[38X]-D/E. All three sites are shown in **Figure 2.2D**.¹

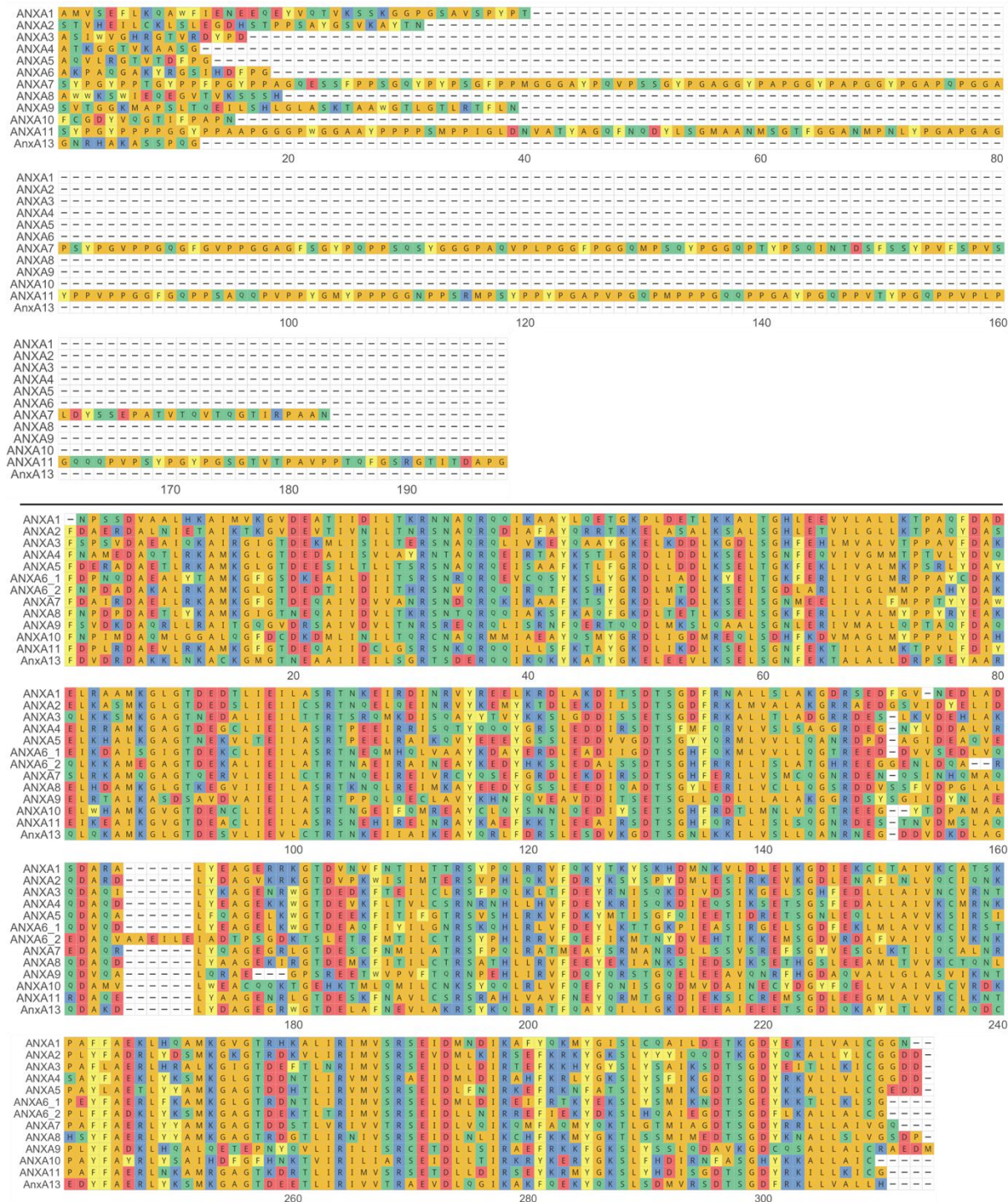


Figure 2.1. Sequence alignments of the ND and CD of Human Annexins. Pairwise sequence was performed for the ND (upper panel). Multiple sequence alignment was performed for the CD (lower panel). For the MSA, the two CD repeats of Annexin A6 were split into two. Amino acids are color-coded. Non-polar, polar, aromatic, acidic, and basic residues are colored orange, green, yellow, red, and blue, respectively.

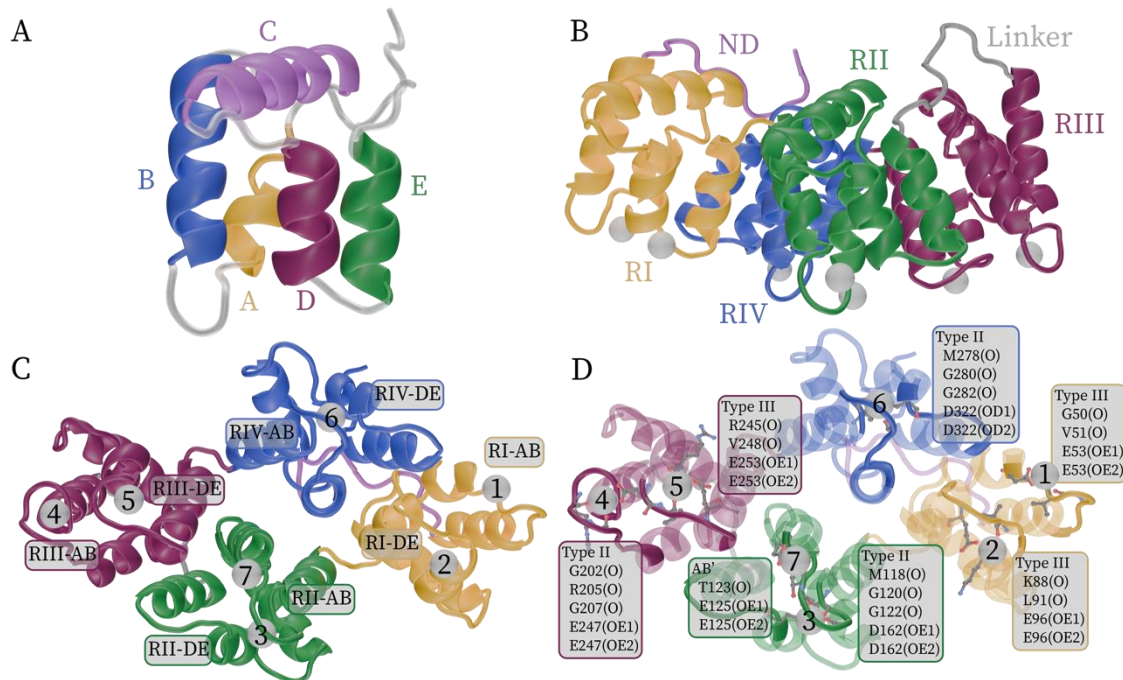


Figure 2.2. Ribbon diagrams of (A) RI of A2 with helices labeled using color coded letters. (B) Full A2 structure with the ND, RI-RIV, and Linker labeled using color-matched text. (C) Full view of the convex face of A2 with Ca²⁺ ions and loops labeled. (D) Full view of the convex face of A2 with Ca²⁺-binding sites shown using a ball-and-stick representation. Each Ca²⁺-binding site is labeled with its site type and a list of coordinations. Across images, Ca²⁺ ions are shown using white spheres.

At the biological level, vertebrate Annexins serve a variety of functions that have been elucidated using knockout mouse-model studies. The effects of Annexin knockouts range from lethal, in the case of Annexin A7, to enigmatic, in the case of Annexin A6.^{21,22} Annexin A1 knockouts demonstrate defects in glucocorticoid-induced inflammation responses.²³ Annexin A2 is involved in vascular tissue development and regulation of fibrin levels.¹³ Given the diversity of roles played by Annexins, their diversity of binding partners is unsurprising. Vertebrate Annexins have over 30 known binding-partners which include a diversity of receptors and structural proteins. See **Table 2.1** for a comprehensive table reproduced from a review article by Moss and Morgan.²⁰

Table 2.1. Known Vertebrate Annexin Binding Partners.

Annexin	Binding Partners
A1	Epithelial growth factor receptor, formyl peptide receptor, selectin, actin, integrin A4
A2	Tissue plasminogen activator, angiostatin, insulin receptors, tenascin C, caveolin 1, actin
A3	Unknown
A4	Lectins, glycoprotein 2
A5	Collagen type 2, vascular endothelial growth factor receptor2, integrin B5, protein kinase C, cellular modulator of immune recognition (MIR), G-actin, helicase, DNA (cytosine-5-)methyltransferase 1 (DNMT1)
A6	Calcium-responsive heat stable protein-28 (CRHSP-28), ras GTPase activating protein, chondroitin, actin
A7	Sorcin, galectin
A8	Unknown
A9	Unknown
A10	Unknown
A11	Programmed cell death 6 (PDCD6), sorcin
A13	Neural precursor cell expressed, developmentally down-regulated 4 (NEDD4)

2.2. Annexin A2

Annexin A2 (A2) is one of the most studied proteins of the Annexin superfamily. It was first detected in cell lines transformed by the *Rous sarcoma* virus by Cheng and Chen in 1981.²⁴ Its structure adheres to the previous discussion of Annexin structural features and characteristics with the highly conserved core domain composed of RI-RIV and each repeat composed of α -helices A-E (see **Figures 2.2A** and **2.2B**). It's ND is 32 residues in length and largely non-polar. It serves as the primary interface between A2 and S100A10 (p11) with which A2 forms the A2/p11 heterotetramer (A2t). A2 contains up to 7 Ca^{2+} -binding sites, three type II, three type III, and one AB' (see **Figures 2.2C** and **2.2D**).¹

2.2.1. Roles in Biology

Since, its original discovery, A2 has been implicated in numerous biological functions. These include F-Actin Organization, endocytosis and exocytosis, cellular polarity in endothelial and epithelial cells, gene expression, redox reactivity, and plasmin regulation.¹

2.2.1.1. F-Actin Interaction.

A2's ability to bind F-Actin was first discovered in 1984 by Gerke et. al. They isolated the protein from porcine intestinal tissue, specifically, the apical region of vesicles isolated from endothelial and epithelial tissue, along with p11. A2 was further demonstrated to bind F-Actin in a Ca^{2+} -dependent manner.²⁵ Subsequently, Ikebuchi and Waisman demonstrated that A2t cooperatively induces the formation of F-Actin bundles in a Ca^{2+} -dependent manner (see **Figure 2.3A**). They also showed that A2 bundles F-Actin to a lesser extent.⁶ Filipenko and Waisman demonstrated that the final nine residues of A2, LLYLCGGDD, are responsible for Actin binding.²⁶ Hayes et al. investigated the role of A2 in actin polymerization and showed that A2 prevents Actin fibril elongation at the barbed end (see **Figure 2.3B**).²⁷ Though, the behavior of A2 and A2t as Actin-bundling proteins has been thoroughly investigated, Hayes et al. suggest that rather than Actin bundling, the primary reason for A2-Actin association is to facilitate the Actin-membrane interaction (see **Figure 2.3C**). This suggestion is supported by their observation that *in vivo* A2 is not localized throughout large actin bundles, as other actin bundling proteins are, but specifically at the barbed-ends of Actin fibrils.²⁷

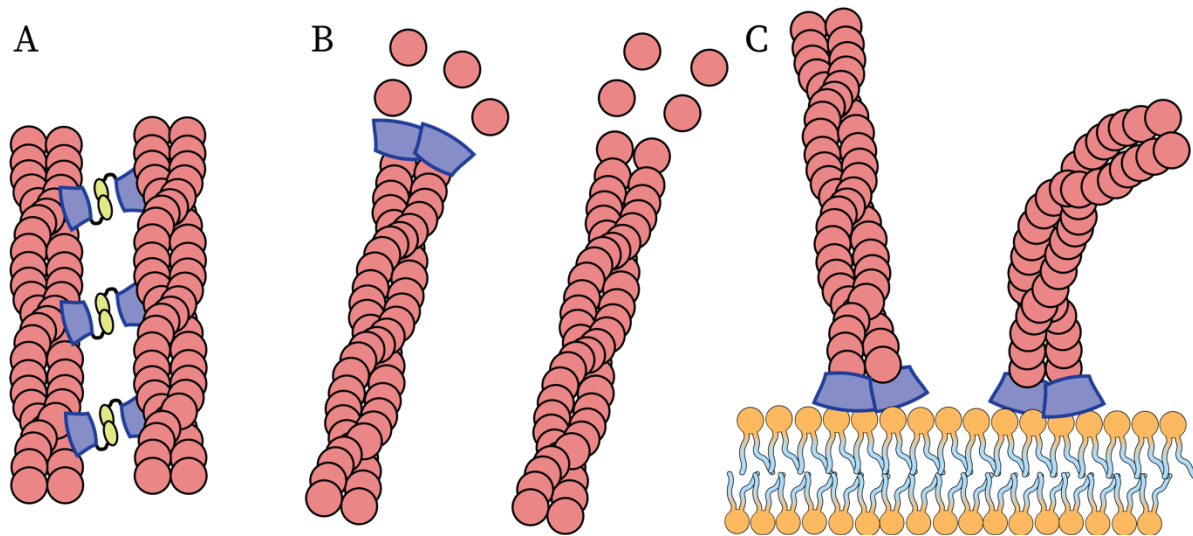


Figure 2.3. Schematics of A2's and A2t's proposed interactions with F-Actin. (A) Bundling of F-Actin by A2t. (B) Capping of F-Actin by A2 to prevent further elongation. (C) Bridging of F-Actin a phospholipid membrane by A2. A consistent color scheme is used across each image. Actin is colored salmon, A2 blue, p11 yellow, lipid heads orange, and lipid tails blue.

2.2.1.2. Macropinosome Formation, Phagocytosis, and Endocytosis.

Annexin's role in facilitating Actin-membrane interactions is related to its role in membrane-trafficking events, including macropinosome formation, phagocytosis, and endocytosis. This is known because of the identified co-localization of A2 and Actin during said events. However, the exact roles of A2 across the listed membrane-trafficking events, along with finer mechanistic details are still poorly resolved.

Macropinosomes are large endosomes used for uptake of solutes from the extracellular matrix.²⁸ See **Figure 2.4A** for a schematic representation. A key step of macropinosome formation is the rapid extrusion of oblong Actin structures, commonly referred to as “rocketing”. Merrifield et al. demonstrated that during rocketing, A2 was located at the tips of the actin fibrils. In rat leukemia cells with suppressed A2 expression, macropinosome formation was not observed.²⁹

Phagocytosis is a process by which a cell ingests large particles. Specifically, particles over 0.5 μm in diameter.³⁰ See **Figure 2.4B** for a schematic representation. Law et al. observed the accumulation of A2 at the early phagocyte of retinal epithelial pigment cells. These early phagocytes were rich in Actin. A2(-/-) cells were shown to have retarded phagocytotic trafficking in retinal epithelial cells as phagocytes aggregated near the apical domain of cells instead of being trafficked into the cell and processed.³¹

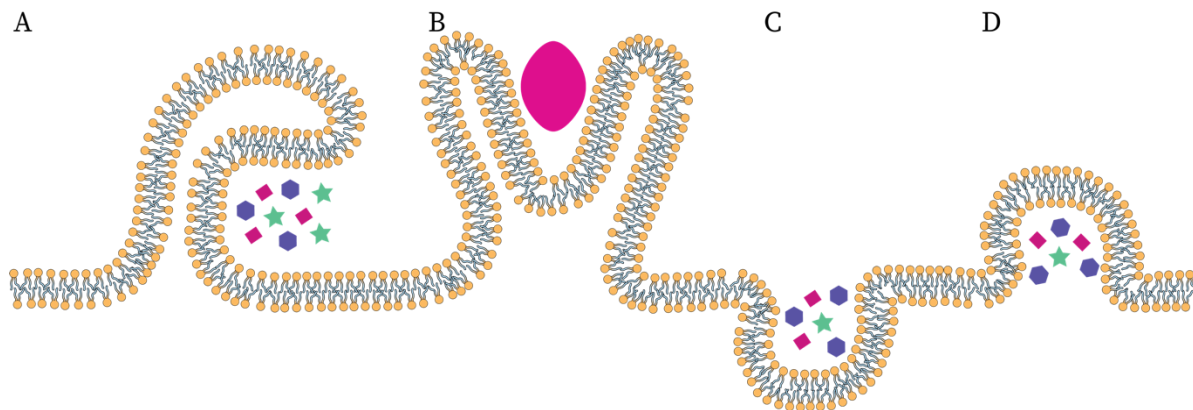


Figure 2.4. Schematics of (A) micropinocytosis, (B) phagocytosis, (C) endocytosis, and (D) exocytosis. Lipid heads are colored orange and tails cyan. Other elements do not have a specific identity associated with them and serve as conceptual placeholders.

Calthrin-mediated endocytosis is involved in processes such as the uptake of nutrients and intercellular communication.³² See **Figure 2.4C** for a schematic representation of non-specific endocytosis. Turpin et al. were the first to identify A2 on calthrin-coated vesicles. They found that A2 interacted with a protein on the CCVs and that the interaction was Ca^{2+} in dependent.³³ Later work by Creutz and Snyder demonstrated that A2 binds with the $\mu 2$ -subunit of the calthrin assembly-protein complex AP-2. This interaction was found to be dependent upon two YXX Φ copies, where Y is tyrosine, X is any residue, and Φ is a bulky non-polar residue, which are also present in transmembrane $\mu 2$ -receptors.³⁴ The $\mu 2$ interaction with other proteins has been shown to be PI(4,5)P2 mediated.³⁵ Given the previous it seems reasonable to extrapolate that the A2- $\mu 2$ interaction is also mediated by PI(4,5)P2 as was done by Grieve et al.

in their review of A2's role in Actin and membrane dynamics.³⁶ When A2 is downregulated, the process of endocytosis seems to be disrupted and large amalgams of CCVs are seen, further establishing A2's role in the process of Calthrin-mediated endocytosis.³⁷

2.2.1.3. Cellular Polarity.

Epithelial cell polarity is an important property of epithelial cells forming the lumen of vasculature or intestines. This polarity sees epithelial cells organized into two distinct domains, the basolateral and apical domains. The former composes the structural interface of the many cells which compose a lumen. The latter forms the inner face of the lumen.³⁸ The membrane composition of the basolateral and apical domains also differs. The basolateral domain contains a higher concentration of phosphatidylcholine and sphingomyelin while the basal membrane contains a higher concentration of glycosphingolipids.³⁹ Additionally, PIP2 localization is characteristic of the apical domain while PIP3 localization is characteristic of the basolateral domain.⁴⁰

The process of epithelial cell polarization is initiated in response to external conditions.³⁸ A2 has been demonstrated to be a key orchestrator of this process.^{41–43} One mechanism by which A2 is involved in orchestrating cellular polarity is in its role recruiting Rho GTPases. In A2-depleted cells cell-wound assays demonstrate that A2-depleted cells are unable to properly migrate owing to a decreased recruitment in required Rho GTPase.⁴¹ Proper cellular migration is dependent upon cellular polarization. Intriguingly, A2 also plays a role in the facilitation the early stages of cellular adhesion. It does so by recruiting the Rac1 GTPase to early junctions where Rac1 interacts with E-cadherin.⁴² In addition to Rac1, A2 plays a role in recruiting E-cadherin to nascent adheren junctions. Though, tight junctions will still form in the absence of A2. The migration of A2 itself to junctions has been hypothesized to be driven by interaction

with either Phosphoinositide 3-kinase or PI(3,4,5)P3.³⁶ The latter case seems somewhat unlikely as A2 has traditionally been understood as a PI(4,5)P2-binding protein and A2 has been demonstrated to have an ~2x larger K_d for membranes containing a PI(3,4,5)P3 component instead of PI(4,5)P2.⁵ However, PIP2 is typically located in the apical domain while PIP3 is determinant of the basolateral domain which appears to have motivated the suggestion.⁴⁰

In endothelial cells, A2 plays a role in the formation of vascular endothelial cadherin (VE-cadherin) junctions. Upon cellular confluence (the interaction of two cellular surfaces) it was observed by Heyraud et al. that A2 is recruited to the cell surface in the form of the A2t complex. At the cell surface, A2t was found to associate with both Actin and VE-cadherin, keeping the complexes docked to cholesterol rich rafts. Further, they demonstrated that interference with the A2t-cadherin interaction resulted in a transition from the non-proliferating quiescent state to the of cellular state from that of the immature state.⁴⁴ Cells in an immature state are pluripotent, that is they can differentiate into a number of different cell types.⁴⁵

2.2.1.4. Vascular Fibrinolysis and Angiogenesis.

Vasculature is lined by vascular endothelial cells. In response to environmental insult, vascular endothelial cells initiate clotting cascades. Fibrinogen is the primary component of fibrin clots that form as a results of clotting cascades.⁴⁶ Persistent or unnecessary clots are undesirable and can lead to serious acute pathologies such as ischemic strokes, pulmonary embolism, and deep vein thrombosis.^{47,48} Thus, anticoagulant pathways are also activated that help to limit the proliferation of clotting by dissolving blood clots and thereby maintaining blood fluidity. This dissolution of blood clots is known as fibrinolysis. Fibrinolysis is carried out by the protease plasmin. Prior to activation, plasmin exists as the zymogen plasminogen. To activate plasminogen, two other proteins are required, tissue-type plasminogen activator (tPA) and

urokinase-type plasminogen activator (uPA).⁴⁶ Cellular receptors facilitate the activation of plasminogen by colocalizing it with both tPA and uPA. Two key characteristics are common among many plasminogen receptors. The first is the low binding-affinity of receptors for plasminogen. This is understood as a method to help limit excessive fibrinolysis; a high-affinity receptor would likely more easily activate plasminogen. The second characteristic is a C-terminal lysine residue which interacts with the plasminogen kringle-domain.⁴⁹

The A2 monomer was first suggested as a plasminogen receptor by Cesarman et al. with the simultaneous suggestion by Hajjar et al. of a proteolytic event which would expose residue K307 of A2 whose terminal residue is D339.^{50,51} Later, Hajjar et al. suggested that the tPA-A2 interaction was driven by a disulfide bridge involving C9 of the A2 ND.⁵² However, within a year Kassam et al. demonstrated both the presence of the A2t complex on the endothelial surface and that the C-terminal lysine residue of the p11 was responsible for A2t interaction with both tPA and plasminogen and that the binding was independent of A2.^{53,54} Studies regarding the effects of A2 and p11 expression have further reinforced that p11 is the receptor and that A2 plays an important but indirect role in the complex.^{55,56} To this point, A2 phosphorylation has been shown to regulate the A2t translocation to the extracellular surface.⁵⁷ Studies conducted by He et al. showed that A2 prevents the rapid degradation of p11.⁵⁸ Both facts suggest that it may be most accurate to describe A2's role in plasminogen activation as a p11 receptor and/or carrier protein which in turn is the receptor for plasminogen.¹¹ A more direct demonstration of this was made via a surface plasmon resonance study investigating the binding of A2, p11, and A2t with tPA, plasminogen, and plasmin. In the study, no direct binding of A2 to either tPA or plasminogen was observed. Both p11 and A2t were demonstrated to bind with tPA, plasminogen, and plasmin, with A2t having a greater binding affinity for plasminogen ($K_d = 0.11 \mu\text{M}$) and

plasmin ($K_d = 75 \text{ nM}$) when compared to p11 ($1.81 \text{ }\mu\text{M}$ and $0.36 \text{ }\mu\text{M}$, respectively).⁵⁹ Finally, the same group demonstrated enhancement of tPA-dependent plasminogen activation by factors of 2, 46, and 77 for A2, p11, and A2t, respectively.⁵⁴ See **Figure 2.5A** for a schematic representation of plasminogen activation facilitated by A2t.

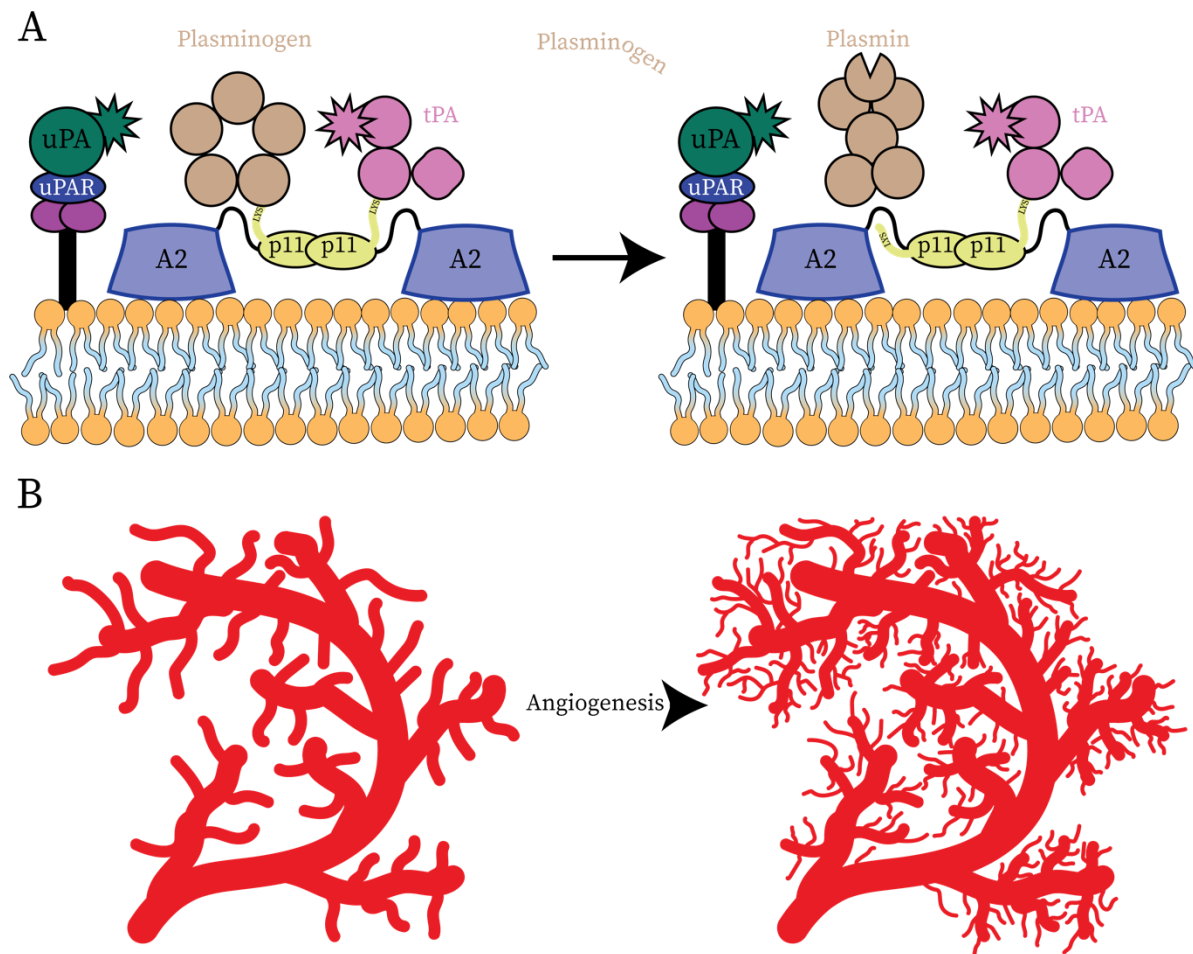


Figure 2.5. Schematics of (A) plasminogen activation to plasmin with A2t serving as the receptor for the necessary protein components. (B) Formation of vasculature by angiogenesis.

Angiogenesis is the process by which new vasculature is formed from pre-existing vasculature. See **Figure 2.5B** for a schematic representation. This process requires the degradation of surrounding extracellular matrix by metalloproteases. These metalloproteases are

activated by plasmin.⁶⁰ As a component of the plasmin receptor this indirectly implicates A2 in angiogenesis.¹ Indeed, in the absence of either component of A2t, angiogenesis is hindered.⁶¹

2.2.1.5. Redox Activity.

Cysteine residues are sensitive to several redox active signaling events. redox events can serve as a concerted mechanism to change cellular activity and metabolism.⁶² To this end, reactive oxygen species (ROS) serve as important signaling molecules in cell cycle arrest, autophagy, and cellular differentiation.^{63–65} However, excessive ROS can cause lasting damage to proteins, DNA, and the cellular membrane. The accumulation of such damage can lead to carcinogenesis. Thus, coping with excessive ROS is important for the overall maintenance of cellular health.⁶⁶ A2 contains a total of four cysteine residues, C8, C132, C261, and C334. Residues C8 and C334 are located at the N and C-termini, respectively.⁶⁷ The latter has been implicated in the activation of plasminogen to plasmin while the former has been suggested as a redox-sensitive cysteine residue.^{68,69} C132 and C261 are located near each other and have been shown to form a disulfide bridge.⁷⁰ Oxidative stress-induced glutathionylation of C8 and C132 have been demonstrated as modulators of A2 function. Glutathionylation of both residues results in the disruption of both phospholipid and F-Actin binding.

A2 null mice demonstrate elevated levels of ROS in both liver and lung cells. Elevated levels of ROS are also observed in A2-depleted cancer cells. These examples suggested some role for A2 in coping with oxidative stress. Subsequently, Caplan et al. demonstrated that the C8 residue is reversibly oxidized hydrogen peroxide and reduced by thioredoxin. Further, it was observed that A2-depleted tumor cells demonstrate increased levels of oxidation of redox sensitive proteins.⁷¹

Studies have demonstrated that A2 is primarily localized in the cytoplasm or at the cellular membrane with a small percentage of A2 present in the nucleus. The N-terminus of A2 contains a nuclear export sequence (NES) that prevents nuclear accumulation of A2.⁷² It was demonstrated by Madureira et al. that increase nuclear accumulation of A2 is observed in cases when a cell is exposed to a genotoxic insult such as ROS and radiation, as seen in **Figure 2.6**. The observed accumulation was blocked in response to the introduction of an antioxidant. Their work was the first to demonstrate the migration of A2 to the nucleus in response to genotoxins.⁶⁹

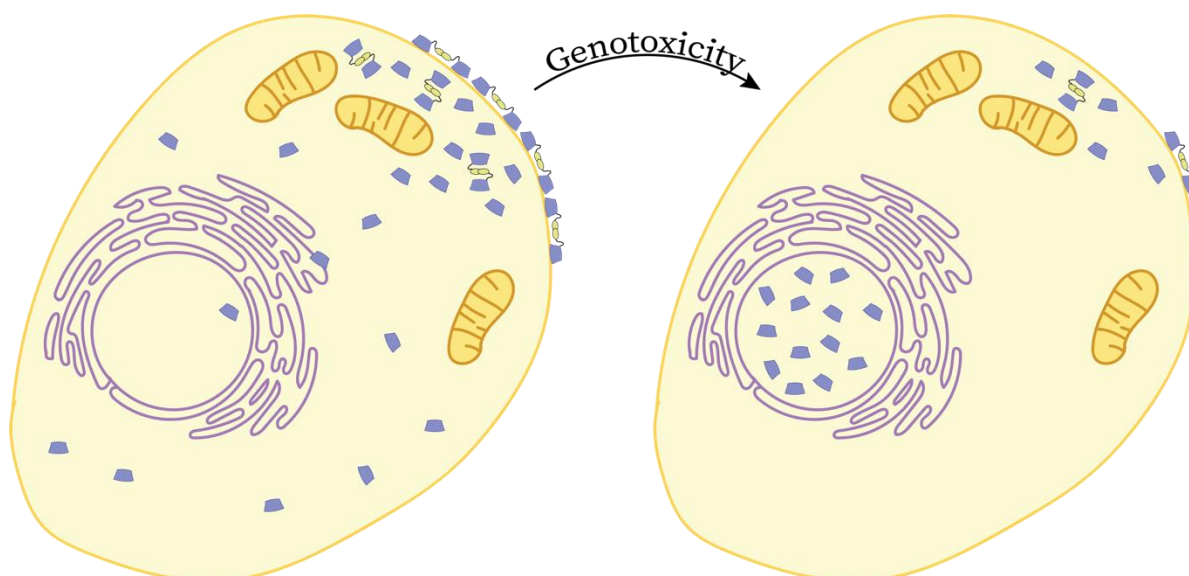


Figure 2.6. Schematic representation of A2 accumulation in the nucleus in response to genotoxic insult.

2.2.2. Roles in Disease

2.2.2.1. Cancer.

A2 is upregulated in a multitude of cancers that include acute lymphoblastic leukemia, acute promyelocytic leukemia, multiple myeloma, clear cell renal cell carcinoma, urothelial cancer, breast cancer, cervical cancer, endometrial cancer, ovarian cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma, oral squamous cell carcinoma, glioblastoma, and non-small cell lung cancer.^{73–87} It has also been observed to be downregulated in cancers that include

prostate cancer, esophageal squamous cell carcinoma, and oral squamous cell carcinoma.^{85,88,89}

In all cases a firm link has been established between A2 levels divergent from what is observed in healthy cells and negative outcomes in patients.^{73–89}

The specific mechanisms by which upregulation of A2 acts to enhance cancer cell activity are not yet fully resolved but, in many cases, have been directly linked to its role as either a plasminogen receptor or its redox activity.^{73,74,78,79,90–95} This enhancement has been linked to a multitude of negative consequence. Chief among these is the ability of cancer cell to metastasize.⁹⁶ Metastasis being the process by which cancer cells migrate from their original location to colonize other tissues.⁹⁷ A2-related plasmin activation preceded the activation of matrix metalloproteases (MMPs). MMPs are in-turn important for remodeling of the extracellular matrix and neovascularization. Both processes promote the invasion and metastasis of cancer cells as well as their proliferation. As one might expect, colonization of distant tissue is characteristic of advanced cancers. Correspondingly, A2 expression levels have been linked to the progression of several cancers with higher levels corresponding to a greater level of progression. Cancers in which A2 has been linked to metastasis or proliferation include all those in which it is upregulated. Higher expression of A2 in all these cancer types also correlates with lower survival rates.⁹⁶

A2t's role as a plasminogen receptor has not only been linked to metastasis. It has been demonstrated that in acute lymphoblastic leukemia (ALL), A2t confers glucocorticoid resistance. This resistance was not only linked to A2t but it was show that the resistance required the A2t complex be expressed on the extracellular surface. Extracellular migration of A2 is dependent upon Src-kinase dependent phosphorylation of A2. While the exact mechanism by which A2t confers glucocorticoid resistance, it seems likely to involve its role as a plasminogen receptor.⁷³

A2t also plays another role in ALL; it serves to facilitate the adhesion to osteoblasts.⁹⁸ This activity is also observed in multiple myeloma and prostate cancer.^{75,90}

A2's role in coping with genotoxic agents was previously discussed. This activity is highly desirable in healthy cells as it prevents lasting harm from occurring to genetic information because of environmental insult. This activity manifests negatively in certain cancer cells. Chemotherapies are commonly a part of cancer treatments and are typically genotoxic agents. In prostate cancer, cervical cancer, ovarian cancer, and pancreatic duct adenocarcinoma, chemoresistance, that is resistance to chemotherapies, has been linked to the upregulation of A2.^{79,90,92,93} This establishes yet another role of A2 upregulation in enhancing the survivability of cancer cells.

2.2.2.2. Viral Infection.

The viral infection cycle, across all viruses can be split into six separate phases which include attachment, penetration, uncoating, gene expression and replication, assembly, and release.⁹⁹ A2 has been implicated in at least one of these stages in over a dozen viruses. Some of the viruses in which A2 is involved include human papillomavirus (HPV), enterovirus 71 (EV71), respiratory syncytial virus (RSV), cytomegalovirus (CMV), influenza A virus (IAV), and measles virus (MV).³ In HPV, A2t colocalizes with the virion during entry though it is not required for HPV attachment. However, only A2 is required for entry while p11 appears to play a role in later infection stages involving intracellular trafficking.^{100,101} Yang et al. demonstrated that A2 and EV71 colocalize on the cellular surface during infection and the A2 C-terminus is important for the interaction.¹⁰² It was demonstrated by Malhotra et al. that A2 is an important receptor during RSV infection.¹⁰³ Neither study investigated the mechanism of action but rather they only identified A2 as the primary receptor. In CMV, A2 was demonstrated to enhance viral

entry but it was not found to be necessary for entry.^{104,105} Later stages of the viral infection cycle for CMV are dependent upon A2.¹⁰⁶ A2 and A2t have both been identified on the outside of IAV. Further, plasmin has been implicated in the replication process of IAV. This suggests that A2's role on this virus is that of a plasminogen receptor.¹⁰⁷ A2 knockout has been demonstrated to hinder the proliferation of MV while having no impact on entry. Further, Koga et al. demonstrated that A2 is involved in the MV M protein localization, which is aberrant in the absence of A2.¹⁰⁸

2.2.2.3. A2 as a Therapeutic Target

A2's role in both cancer and viral pathologies was highlighted in the two preceding sections. The involvement of A2, or any protein for that matter, in a given pathology does not establish it as a suitable mechanism of treatment. To this point, the cessation of certain cellular activities, say protein synthesis via a hypothetical ribosomal inhibitor, would result in cancer and viral death but would also likely have adverse negative impacts on the host. This is not the case in A2 deficient mice, they remain viable, capable of reproduction and progress through a normal life cycle. Ramifications from A2 inhibition or silencing are observed. Specifically, clotting increases and the capacity for angiogenesis is reduced.¹³ When combined with the information presented in the previous two sections, detailing A2's role in cancer and viral pathologies, the therapeutic potential of A2-specific inhibitors becomes apparent. Additionally, enhancement or inhibition of A2 activity could have therapeutic applications in pathologies beyond cancer and viral infection. As we have extensively discussed, A2's role as a plasminogen receptor implicates it in blood clot regulation. Inhibition or enhancement of this activity would both have therapeutic applications. The former in cases when clotting is desired such as excessive bleeding and congenital bleeding disorders. The latter could have applications for the treatment of clotting

disorders which A2 has recently been implicated in.¹⁰⁹ Additionally, inhibition or enhancement of A2's redox activity might have applications. Enhancement of A2's redox activity could help individuals who have been exposed to genotoxic agents such as radiation or chemical spills. It should be noted that these latter suggestions are highly speculative.

We hope to have thoroughly established the potential of A2 as a therapeutic. Efforts have already been made toward developing ligands that target A2. One such effort was undertaken by Reddy et al. and targeted the formation of the A2t complex via p11 inhibition.¹⁵ They were successful in identifying 1,2,4-triazoles as inhibitors of A2t formation. The identified compound disrupts A2t formation by directly binding at the location of A2-ND association within the p11 dimer. Additionally, the ligand has been tested and verified to prevent infection by HPV *in vitro*.¹¹⁰ This is promising but room still exists for development of novel inhibitors of both A2t and A2. Specifically, inhibitors which target the membrane interaction.

2.2.3. In vitro Studies of Annexin A2

A2 has been studied extensively *in vitro*. These studies include structural studies of A2 and its tetramer, investigations of A2/A2t conformation on membrane models, and observations of A2's behavior on membrane surfaces. Many high-quality structural investigations of A2 have been conducted. The first crystal structure of A2 was completed by Burger et al. in 1996.⁷⁰ This was not the first Annexin structure for which a crystal structure was generated. Huber et al. elucidated the Annexin A5 crystal structure in 1990. They described all the hallmarks of Annexin structures that were discussed at the start of this chapter.¹¹¹ Burger et al. not only elucidated the structure of A2 but described its character as an ion channel, an activity which Annexins A1, A2, A5, A6, and A7 have been shown to demonstrate.⁷⁰ Rosengarth and Leucke solved structures for A2 both in the presence and absence of Ca^{2+} . They observed that A2 crystallized as a dimer in

the presence of Ca^{2+} and as a monomer in the absence of Ca^{2+} . The orientation of the dimer led them to suggest that the A2 monomer bridged membrane surfaces by adopting a so-called “up-side-down” orientation similar to what is observed in their crystal structure.⁶⁷ The first crystal structure of the full A2t complex was solved by Oh et al. They showed that A2t targets the chromatin remodeling factor SMARCA3 and enhances its DNA binding. In the crystal structure they demonstrated that SMARCA3 interacts with a hydrophobic pocket of the A2t complex.¹⁰

Studies investigating the structure of A2 at the membrane interface have also been performed. Lambert et al. utilized cryo-electron microscopy to investigate A2t at membrane junctions. They identified seven distinct bands in density profiles of A2t junctions. They correspond to the inner leaf, outer leaf, A2, p11, A1, outer leaf, and inner leaf. They demonstrate that the arrangement of A2t at membrane junctions sees the two A2 opposite each other interacting with separate membranes with the p11 dimer in the center (see **Figure 2.7**). They also observed A2-induced membrane aggregation and produced density profiles for the junctions.⁷ A subsequent study conducted by Grill et al. demonstrated clearly that A2 will only bridge membrane models if two monomers are linked into a dimer involving the ND residue C8. Given that the conditions in the cytoplasm are reducing and that no A2 dimers have been isolated from cell lysate, it seems likely that direct membrane bridging by A2 is not observed *in vivo*.¹¹²

Menke et al. performed two studies investigating both A2 and A2t on membrane monolayers using scanning force microscopy (SFM).^{8,9} In the first study they demonstrated that in the absence of a second membrane, and in contrast to the membrane junction cryo-EM study conducted by Lambert et al., that both A2 subunits of A2t associate with the membrane in the monolayer. This initial study was performed using saturated lipids, DPPC and DPPS.⁸ In their second study they performed the same measurements using unsaturated lipids,

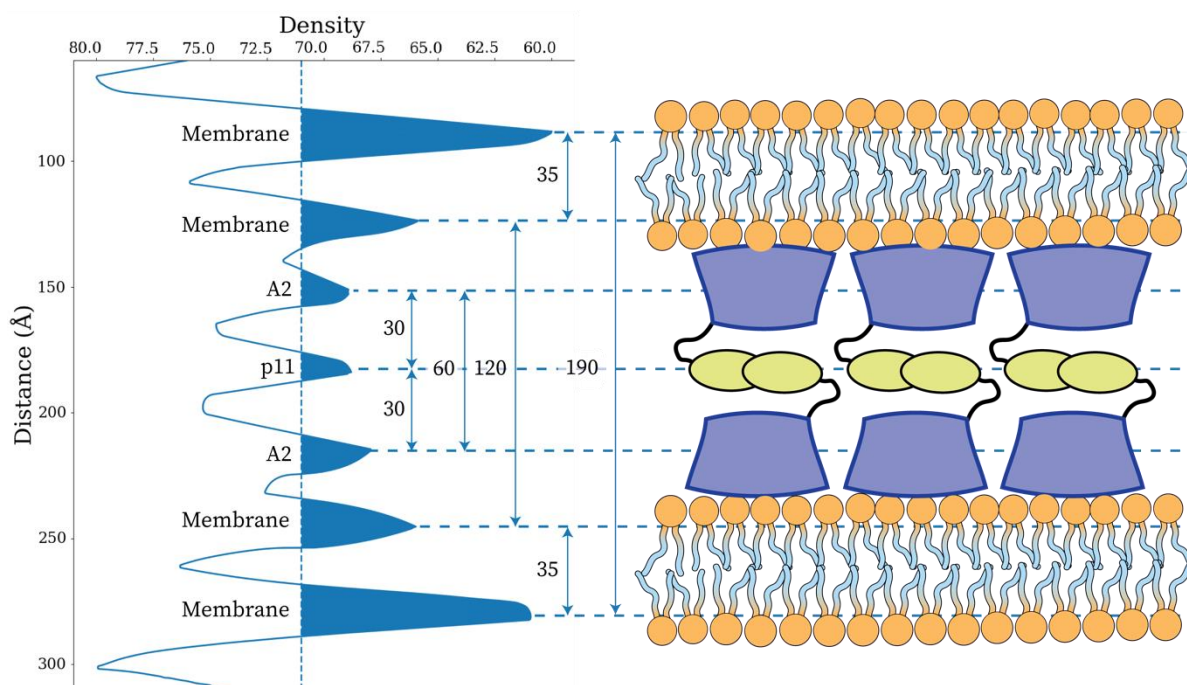


Figure 2.7. Reproduction of the pixel density graph generated by Lambert et al. via cryo-electron microscopy. Minima correspond to the centers of mass of junction bands. A schematic of the protein and membrane components was added to enhance visualization.

POPC and POPS. They observed slight differences in height with the complexes appearing to be shorter than in the original study. They postulated that A2 and A2t were able to partially insert into the membrane or that the SFM measurement itself had a greater impact because the lipids were in the gel phase. The latter suggestion was supported by their observation that the protein domain heights were larger when the measurements were taken in contact mode versus intermittent contact mode.⁹

While A2 and A2t induce POPS microdomains in POPC/POPS membrane models, A2 is more recognized for its specificity toward PI(4,5)P2 specificity and its ability to induce both CHOL and PI(4,5)P2 microdomains.¹ The specificity of A2 for PI(4,5)P2 has been attributed to residues K279 and K281 and A2 has been shown to preferentially bind PI(4,5)P2 containing membranes when compared to other types of phosphoinositide-containing membranes.⁵ The

ability of A2 to induce PI(4,5)P2 microdomains was clearly demonstrated in a study conducted by Drücker et al. They also demonstrated that this is a unique property of A2 and not

Annexins broadly; Annexin A1 did not exhibit this same behavior. A2t and A2 also demonstrated an ability to induce “budding” the formation of smaller vesicles within the vesicle models.¹⁶ Several of the discussed studies have shown that lateral aggregates of A2 appear to be key for the formation of A2-induced lipid microdomain formation. Matos et al. investigated the mechanism by which these lateral aggregates form. Crosslinking studies they performed found that membrane-binding promotes A2-aggregation and that the aggregation itself is largely driven by charged residues at the side of A2.¹¹³

2.3. Computational Studies of Annexins

Computational studies of Annexin family proteins have been performed as early as 1998. The first instance of such a study that could be found was performed by Cregut et al. They investigated the essential motions of both Annexin A5 (A5) and Annexin A1 (A1). Their primary interest was a suggested “hinge-bending” motion that had been postulated to control the ability of ions to pass through the central pore of A5, a known Ca^{2+} channel protein. Ca^{2+} -binding was found to limit the mobility of the hinge motion with more open conformations being favored in A5.¹¹⁴ A later study investigated the isolated RI of A1. They found that the maintenance of its structural integrity was sensitive to the supplied simulation parameters.¹¹⁵ Shesham et al. investigated the effects of Ca^{2+} -binding on A1. Ca^{2+} -coordination was found to increase the flexibility of A1 core-domain residues.¹¹⁶ Lewis et al. investigated the Ca^{2+} -induced ejection of the A1 ND from RIII of the CD using the nudged elastic band method. They found that the ejection of the helix slid out of the pocket, rather than swung out. The flexibility of the ND-linker residues S27-N42 was highlighted as an important feature of the ND expulsion from RIII. The

helix that takes the place of the ND in RIII was found to form in 2 steps. First residues M247–V250 form a helix followed by residues L251–E254.¹¹⁷

More recent studies have focused upon the interaction of Annexins with membrane models or the dynamics of Annexin tetramers. Chen et al. investigated the binding of A5 to a membrane model. They demonstrated direct coordination of lipids by Ca^{2+} . The carboxyl oxygens of POPS were the favored interactions for the membrane coordinating Ca^{2+} . The A5-membrane interactions were driven by H-bonds and non-polar contacts. A5 was found to have a large impact on membrane morphology, causing the membrane model to bend significantly.¹¹⁸ Donohue et al. investigated the N-terminal domain as a potential secondary membrane-binding site. This was suggested in a previously discussed cryo-EM study where it appeared that the A1 and A2 monomers were not required to dimerize at some membrane junctions. They found that K26 and K29 of the ND, strongly interacted with the membrane and suggested might serve as key contributors if the ND served as a secondary membrane-binding site.¹¹⁹ The same group performed a subsequent study investigating the effects of mutations that have been investigated experimentally to either promote or hinder membrane aggregation by A1, for S27A and S27E, respectively. The S27A mutation destabilized the ND of A1 and caused it to rapidly migrate toward the core domain. The S27E mutation stabilized the ND and prevented its migration toward the core domain. It was asserted by the authors that these observations are consistent with those observed experimentally.¹²⁰ Simulation of an A1/S100A11 heterotetramer model was performed by Sanchez et al. They identified three distinct conformations of the A1 ND. The different conformations were attributed to the W12-M63 interaction which caused the formation of a kink in the first 11 residues of the ND.¹²¹

Hakobyan et al. performed the first simulations of an A2-membrane system. Their primary focus was investigating orientations of A2 at the membrane surface. They found that the most stable A2-membrane interactions occurred in the conformation where the convex face of A2 was in direct contact with the membrane. Lysine residues at the convex face were shown to be important Ca^{2+} -independent contributors to the A2-membrane interaction. They also investigated the consequences of A2-binding for membrane properties. They found that A2 binding caused membrane deformations in the form of bending at the site of A2 binding. Strength of lipid-A2 interaction was shown to have a negative correlation with the lipid order parameter while the lipid- Ca^{2+} interaction had the inverse relationship. This suggests that A2-binding enhances local membrane fluidity and Ca^{2+} -decreases it. Intriguingly, lipid-A5 binding was found to have the opposite effect on lipid order parameter.¹¹⁸ This was noted by the authors, and it was suggested that the observed differences could have been related to structural differences between A2 and A5.¹²²

2.4. Recognition of Sources

The composition of this review of Annexin A2 while not entirely dependent upon was facilitated and greatly expedited by several high-quality reviews. These include reviews regarding Annexins broadly, A2 specifically, A2's role in Actin dynamics, A2's role in cancer, and A2's role in viral infection, which largely guided the discussion.^{1-3,20,36} No such reviews existed to guide the sections regarding *in vitro* studies of A2 at the membrane or the computational study of Annexins. We wanted to highlight their importance and provide direct citations with a mention of these reviews as well as thank their authors.

CHAPTER 3: Molecular Dynamics and Virtual Screening

3.1. Molecular Dynamics

The simulation of chemical systems has been an active area of research since before the development of silicon-based processor. In recent years, the most popular method for computational investigation of biomolecular systems has been all atomic molecular dynamics (aaMD). This method has aptly been referred to as: “Laplace’s vision of Newtonian physics on modern supercomputers.”¹²³ I would venture to replace “supercomputer” with graphical processing unit (GPU) as many modern MD codes, including GROMACS, LAMMPS, and AMBER among others, have been adapted to operate on GPUs.^{124–126} This has been because of the explosion in GPU availability and power.¹²⁷ This has essentially removed the monetary barrier to entry for MD which used to require the usage of supercomputers costing tens of thousands to millions of dollars and specialized support staff. Now, anyone can use widely available consumer-grade workstations to simulate sizeable biological systems, such as whole protein complexes in a membrane, albeit within a limited context. This has made MD an ever-growing presence in biochemical studies.¹²³

At the heart of MD is the desire to achieve two goals. The first is to “see” the time-evolution of biomolecules using MD as a “computational microscope”. The second, and more rigorous application is to generate a series of images referred to as a trajectory that faithfully represents the potential energy surface of the system (PES). This PES will contain a number of minima that corresponds to the accessible conformation of the system. Given an accurate probability distribution of molecular structures:

$$(3.1) \quad \rho(q) = \frac{e^{-V(q)/kT}}{\int e^{-V(q)/kT} dq}$$

where $\rho(q)$ is the probability distribution as a function of molecular coordinates, $V(q)$ is potential energy as a function of molecular coordinates, k is the Boltzmann constant and T is temperature. Then observable properties can be determined using the generated sampling:

$$(3.2) \quad \langle A \rangle = \rho_1 A_1 + \rho_2 A_2 + \rho_3 A_3 + \cdots + \rho_\infty A_\infty = \int A(q) \rho(q) dq$$

where A is some quantity such as the potential energy of a system. In reality, the distribution is an approximation of $\rho(q)$ and a high-quality distribution requires the generation of multiple, independent MD trajectories or the application of advanced sampling techniques.

3.1.1. Equations of Motion

The first step in understanding MD is understanding the underlying equations that govern the behavior of the system. There are more formal ways of arriving at the equations, but it is conceptually easier and most approachable to investigate the equations using a single atom and beginning from definitions commonly introduced in undergraduate physics courses. The first is the Newtonian definition of force:

$$(3.3) \quad F_i = m_i a_i = m_i \frac{dv_i}{dt} = \frac{dp_i}{dt}$$

where F is force, m is mass, a is acceleration, v is velocity, t is time, and p is momentum.

The second is the relationship between the potential energy of a system that includes only conservative forces. Conservative forces are those which only depend on position; they are independent of the momenta of the system's constituent particles. The relation is as follows:

$$(3.4) \quad F_i = -\frac{dV(q_i)}{dq_i}$$

where V is the potential energy of the particle and q is its position.

Combining these equations, we get a relationship between the momenta and the potential energy of a conservative system:

$$(3.5) \quad \frac{dp_i}{dt} = -\frac{dV}{dq_i}$$

We now define the kinetic energy of a system:

$$(3.6) \quad K_i = \frac{1}{2} m_i v_i^2 = \frac{m_i^2 v_i^2}{2m_i} = \frac{p_i^2}{2m_i}$$

where K is the kinetic energy. And show that its derivative with respect to momentum can be related to position:

$$(3.7) \quad \frac{dK_i}{dp_i} = \frac{2p_i}{2m_i} = \frac{p_i}{m_i} = \frac{m_i v_i}{m_i} = v_i = \frac{dq_i}{dt}$$

Yielding:

$$(3.8) \quad \frac{dq_i}{dt} = \frac{dK_i}{dp_i}$$

Taken together we can describe the propagation the system through phase space using equations (3.5) and (3.8). However, the systems explored using MD contain a large number of constituents making the derivation of an analytical solution impossible. Thus, we are forced to use a numerical scheme to solve the equations. The first step in formulating this scheme is to take a Taylor expansion of the position to the third term:

$$(3.9) \quad q(t + \Delta t) = q(t) + \Delta t \frac{dq(t)}{dt} + \frac{\Delta t^2}{2!} \frac{d^2 q(t)}{dt^2}$$

We can then substitute velocity into the second term and use a rearranged form of equation (3.4) to substitute into the third term. The rearranged form of (3.4) is:

$$(3.10) \quad a(t) = \frac{F(t)}{m} = -\frac{1}{m} \frac{dV(t)}{dq}$$

And with both substitutions performed, equation (3.9) becomes:

$$(3.11) \quad q(t + \Delta t) = q(t) + v(t)\Delta t - \frac{\Delta t^2}{2m} \frac{dV(t)}{dq}$$

which contains only components we have defined. Having arrived at an equation for describing the propagation of positions, we now need an equation describing the forward propagation of the velocities. As with positions, we begin with a Taylor expansion of the velocity and preserve the first three terms:

$$(3.12) \quad v(t + \Delta t) = v(t) + \Delta t \frac{dv(t)}{dt} + \frac{\Delta t^2}{2!} \frac{d^2v(t)}{dt^2}$$

The second term is easily simplified but the third term is complex and not easily simplified with the equations we have utilized thus far. So, to arrive at a suitable substitution for the third term we begin with a Taylor expansion of $dv(t)/dt$, truncating after the second term:

$$(3.13) \quad \frac{dv(t + \Delta t)}{dt} = \frac{dv(t)}{dt} + \Delta t \frac{d^2v(t)}{dt^2}$$

We seek to arrive at a suitable substitution for the third term of (3.12) so we multiply (3.13) by a factor of $\Delta t/2$ then isolate the term corresponding to the third term of (3.12):

$$(3.14) \quad \frac{\Delta t}{2} \frac{dv(t + \Delta t)}{dt} = \frac{\Delta t}{2} \frac{dv(t)}{dt} + \frac{\Delta t^2}{2} \frac{d^2v(t)}{dt^2}$$

$$(3.15) \quad \frac{\Delta t^2}{2} \frac{d^2v(t)}{dt^2} = \frac{\Delta t}{2} \frac{dv(t + \Delta t)}{dt} - \frac{\Delta t}{2} \frac{dv(t)}{dt}$$

Substituting (3.15) into (3.12) allows us to replace the problematic term:

$$(3.16) \quad v(t + \Delta t) = v(t) + \Delta t \frac{dv(t)}{dt} + \frac{\Delta t}{2} \frac{dv(t + \Delta t)}{dt} - \frac{\Delta t}{2} \frac{dv(t)}{dt}$$

which can then be rearranged:

$$(3.17) \quad v(t + \Delta t) = v(t) + \frac{\Delta t}{2} \left(\frac{dv(t + \Delta t)}{dt} + \frac{dv(t)}{dt} \right)$$

The final two terms can be rewritten in terms of the potential energy using (3.10)

$$(3.18) \quad v(t + \Delta t) = v(t) - \frac{\Delta t}{2m} \left(\frac{dV(t + \Delta t)}{dq} + \frac{dV(t)}{dq} \right)$$

Combining equations (3.11) and (3.18) we have everything we need to update positions and velocities from t to $t + \Delta t$. The Verlet integration scheme, commonly applied for molecular dynamics, operates in five steps, outlined below:

- 1) Calculate $\frac{dV(t)}{dq}$
- 2) Calculate $q(t + \Delta t)$ using (3.11)
- 3) Calculate $\frac{dV(t + \Delta t)}{dq}$ using the updated positions
- 4) Calculate $v(t + \Delta t)$ using (3.18)
- 5) Repeat steps 1-4 until the desired time is reached.

Though we now have a suitable system of equations and scheme for solving them numerically, questions remain. Chiefly, we have not discussed how to arrive at initial positions and velocities nor have we explicitly defined $V(q)$. The former we will discuss in section **3.1.2.**

System Initialization. $V(q)$ may take several forms: it may be defined using a forcefield, quantum mechanics, or a mixture of the two. The first method is by and far the most popular technique for large biomolecular systems because of its relative computational simplicity which makes it more suitable for sampling longer timescales. Classical MD is driven by force fields which will be discussed in more detail in section **3.1.3. Forcefields.**^{128,129}

3.1.2. System Initialization

Initial coordinates can be generated in a variety of ways. For systems with a protein component, experimentally derived models are typically utilized. Nuclear magnetic resonance, x-ray crystallography, and cryogenic electron microscopy are three experimental methods utilized to elucidate protein structure. Structures are commonly deposited in the Research Collaboratory for Structural Bioinformatics PDB, a database for sharing experimentally derived structural models of proteins.¹³⁰ Said structures are available to the public and serve as a valuable source for initial coordinates. Methods also exist for generating protein models without experiment. Homology models are generated using a protein sequence and a close analogue for which structural information is available.¹³¹ Many known proteins sequences that lack structures have homology models available on the SWISS-MODEL Repository, a database of homology models.¹³² Deep learning models have also demonstrated great utility for protein structural prediction. DeepMind's AlphaFold2 recently won the Critical Assessment of Structure Prediction, a competition for assessing models that predict protein structures without the need for a pre-existing structural scaffold.¹³³

Small molecule structures can be derived from experimental data, but it is often feasible to generate a structural model by optimizing an initial structural model using *ab initio* methods. The Automated Topology Builder maintains a collection of over one million small molecules that have been optimized for use.¹³⁴ Additionally, CHARMM-GUI maintains an archive of both lipid and ligand structures available for use and compatible with their forcefield.¹³⁵

While one may have a protein and/or small molecule model suitable for simulation it is often desired that they be assembled into a more complex system. There is no single way to achieve this. If one sought to model the interaction of a protein and a ligand, they might first use

a ligand docking software to generate a starting model. For a protein-protein system there are analogous docking models. Lipid systems are also complex and while lipid-only systems will self-organize during a simulation it might be desirable to have the membrane pre-formed. This is useful when modeling membrane-protein interactions. A number of software for generating DNA, RNA, and other polymer structures are also available such as 3D-DART and polyply.^{136,137}

While implicit solvation models do exist, most force-fields are developed to be coupled with explicit solvation. Solvation of a system is relatively straightforward. It typically involves the repetitive placement of a pre-equilibrated water box at non-overlapping intervals within a specified area.

Arriving at initial velocities is much simpler when compared to initial coordinates. Particle velocities are sampled from a Boltzmann distribution which describes the distribution of velocities according to:

$$(3.19) \quad P(v) = \left(\frac{m}{2\pi kT} \right)^{3/2} 4\pi v^2 e^{\frac{-mv^2}{2kT}}$$

3.1.3. Forcefields

The potential energy of a system is defined by a forcefield which is only a function of a system's internal coordinates. A common definition of a forcefield equation follows:

$$(3.20) \quad V_{total} = \sum_{i=1}^{N_{bond}} V_{bond} + \sum_{i=1}^{N_{angle}} V_{angle} + \sum_{i=1}^{N_{dihedral}} V_{dihedral} + \sum_{i=1}^{N_{nb}} V_{nb}$$

The first three terms are referred to as the bonded terms while the final term of the equation is referred to as the non-bonded terms. The bond, V_{bond} , and angle, V_{angle} , terms are defined using simple harmonic potentials:

$$(3.21) \quad V_{bond} = \frac{1}{2} k_b (r - r_0)^2$$

$$(3.22) \quad V_{angle} = \frac{1}{2} k_a (\theta - \theta_0)^2$$

where k_b is the bonded force constant, r is the bond length, r_0 is the equilibrium bond length, k_a is the angular force constant, θ is the angle formed by three bonded atoms, and θ_0 is the equilibrium angle. Harmonic potentials describe equilibrium geometry well, but they cannot be used to describe the breaking and forming of bonds. This would require the application of a different potential, such as the Morse potential, which describes bond dissociation. See **Figures 3.1A** and **3.1B** for examples of bonded and angular potentials.

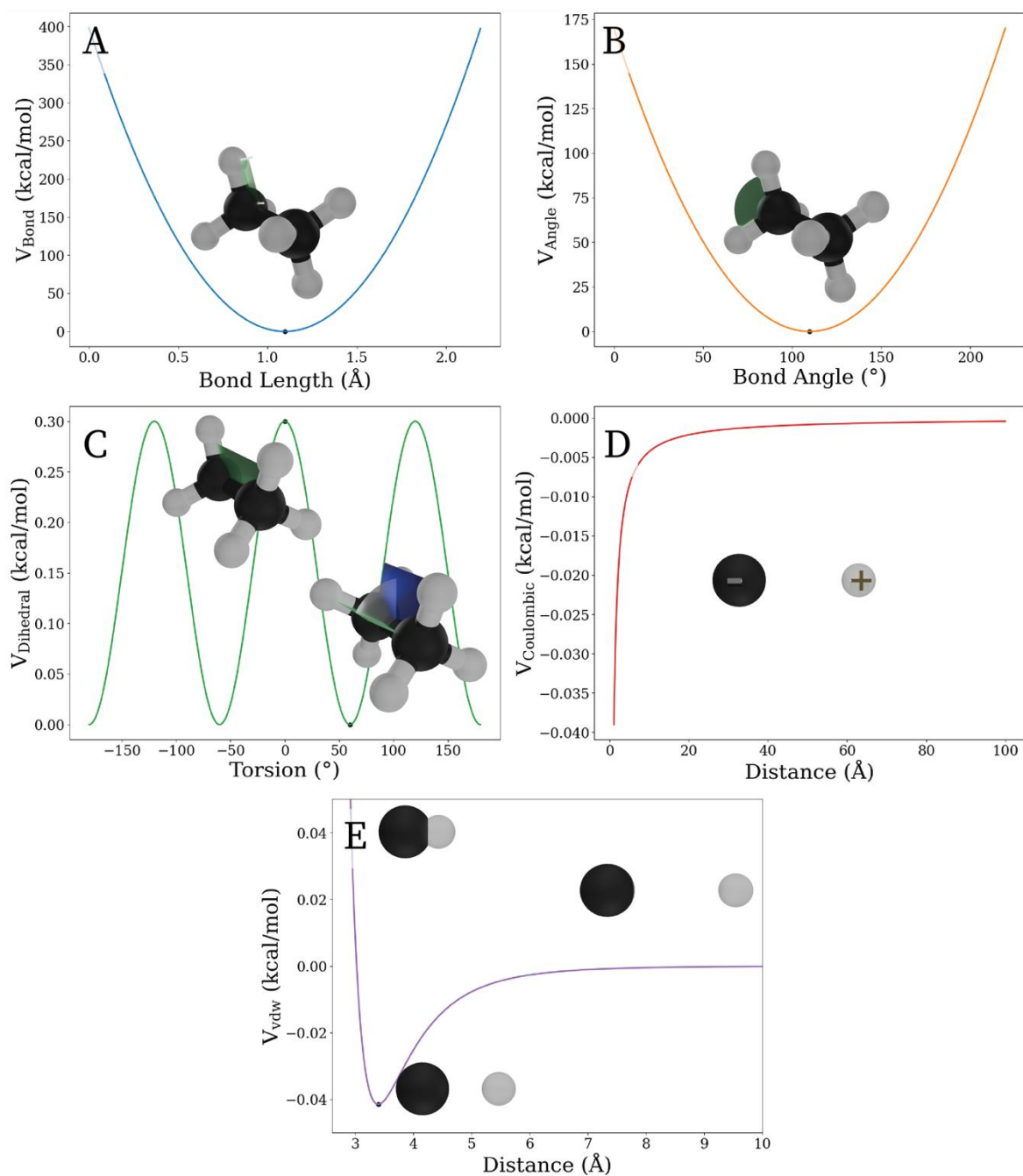


Figure 3.1. Examples of potential energy force-field terms for ethane. Carbon and hydrogen are colored black and grey respectively. All terms were created using GAFF parameters for c3 and hc. (A) c3-hc bond potential with bond length shown on a 3D model. (B) hc-c3-hc angle potential with angle shown on an ethane model. (C) hc-c3-c3-hc torsional potential with angle between hc-c3-c3 planes shown for energetic minimum and maximum. (D) hc-c3 coulombic potential with charge signs shown on 3D representations of each atom. (E) hc-c3 Lennard-Jones potential with different distances along the potential shown using spheres.

Dihedrals are described using a periodic potential:

$$(3.23) \quad V_{dihedral} = V_d(1 + \cos(\eta\omega - \gamma))$$

where V_d is the barrier height, η is the periodicity, ω is the angle between the two planes of the dihedral, and γ is the phase. See **Figure 3.1C** for an example of a dihedral potential.

The non-bonded term, unlike the previous potentials, is composed of three components: coulombic, van der Waals, and repulsive terms:

$$(3.24) \quad V_{coulombic} = \frac{q_i q_j}{4\pi\epsilon r_{ij}}$$

$$(3.25) \quad V_{vdw} \propto -\frac{1}{r_{ij}^6}$$

$$(3.26) \quad V_{repulsive} \propto \frac{1}{e^{r_{ij}}} \approx \frac{1}{r_{ij}^{12}}$$

where q is the atomic partial charge, ϵ is the permittivity of free space, and r is the distance between two atoms. See **Figure 3.1D** for an example of a coulombic potential.

Equations (3.25) and (3.26) are usually combined into a Lennard Jones potential taking the form:

$$(3.27) \quad V_{LJ} = 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right]$$

The ϵ_{ij} and σ_{ij} are parameters that determine the depth of the potential well and point where potential is zero, respectively. ϵ and σ are defined individually for all atom types. The terms used to describe the interaction between two specific atoms are generated using the following equations:

$$(3.28) \quad \epsilon_{ij} = \sqrt{\epsilon_i \epsilon_j}$$

$$(3.29) \quad \sigma_{ij} = \frac{\sigma_i \sigma_j}{2}$$

See **Figure 3.1E** for an example of a Lennard-Jones potential.

With all components of the potential introduced in (3.20) explicitly define we can rewrite (3.20) using those definitions:

$$(3.30) \quad \begin{aligned} V_{total} = & \sum_{i=1}^{N_{bond}} \frac{1}{2} k_b (r - r_0)^2 + \sum_{i=1}^{N_{angle}} \frac{1}{2} k_a (\theta - \theta_0)^2 \\ & + \sum_{i=1}^{N_{dihedral}} V_d [1 + \cos(n\omega - \gamma)] + \sum_{i>j}^{N_{nb}} \frac{q_i q_j}{4\pi\epsilon r_{ij}} \\ & + 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] \end{aligned}$$

With a complete definition of the force-field, we have now completed a full description of the equations of motion that are needed to describe our system. It should be noted that “force-field” refers both to the potential energy function that is used, whose exact form can vary, as well as the parameters that are associated with it, i.e. force constants. Several nuances remain to be discussed and are the topic of the proceeding section.^{128,129}

3.1.4. Periodic Bounds, Pressure and Temperature Regulation

3.1.4.1. Periodic Boundary Conditions.

Let us consider two cubic systems. The first has dimensions that allow for 50 atoms to be aligned in a row along one dimension of the cube. The second cube allow for 10^8 atoms. Next let us calculate roughly how many atoms would be in contact with the surface of our cube versus how many total atoms can sit inside of the cube. We can see that for a small system, the percentage of atoms that would be in contact with the surface of a container for the large system

is 2×10^6 times smaller (see **Table 3.1**). This discrepancy gives rise to undesirable artifacts and requires a solution.

Table 3.1. Comparison of System Size and Number of Atoms in Contact with Container.

System	Width	Surface Atoms	Volume Atoms	Surface Percent (%)
Small	50	15,000	125000	12
Large	10^8	6×10^6	10^{24}	0.000006

One solution is to simulate the system using what are known as periodic boundary conditions (PBC). A schematic representation of PBC can be seen in **Figure 3.2**. When using PBC we define the dimensions of the system to match those of a periodic unit cell. If a constituent of the system crosses a periodic boundary, then it is wrapped around to the other side. This scheme results in what is effectively an infinitely continuous system. Naturally, this method of handling boundary conditions also comes with challenges. The most pronounced being the calculation of nonbonded terms. From a naïve perspective, by making the system periodic, we have taken the already computationally intensive, $O(n^2)$, nonbonded calculation and made it infinitely more difficult. With PBC we need to calculate the V_{nb} term across periodic bounds. However, there are commonly employed methods to greatly simplify this calculation.

The first and most obvious applies to the Lennard-Jones potential. The attractive tail of the system quickly converges to 0 which allows for a cutoff to be applied as a reasonable approximation. This value of this cutoff for an explicitly solvated system can vary but is usually ~ 10 Å. It can extend beyond the unit cell and when doing so acts as if the interaction were within a replica cell, see **Figure 3.2** for an example of this. The cutoff can be implemented as a hard cutoff or coupled with a smoothing function that tapers to 0 at the desired distance.

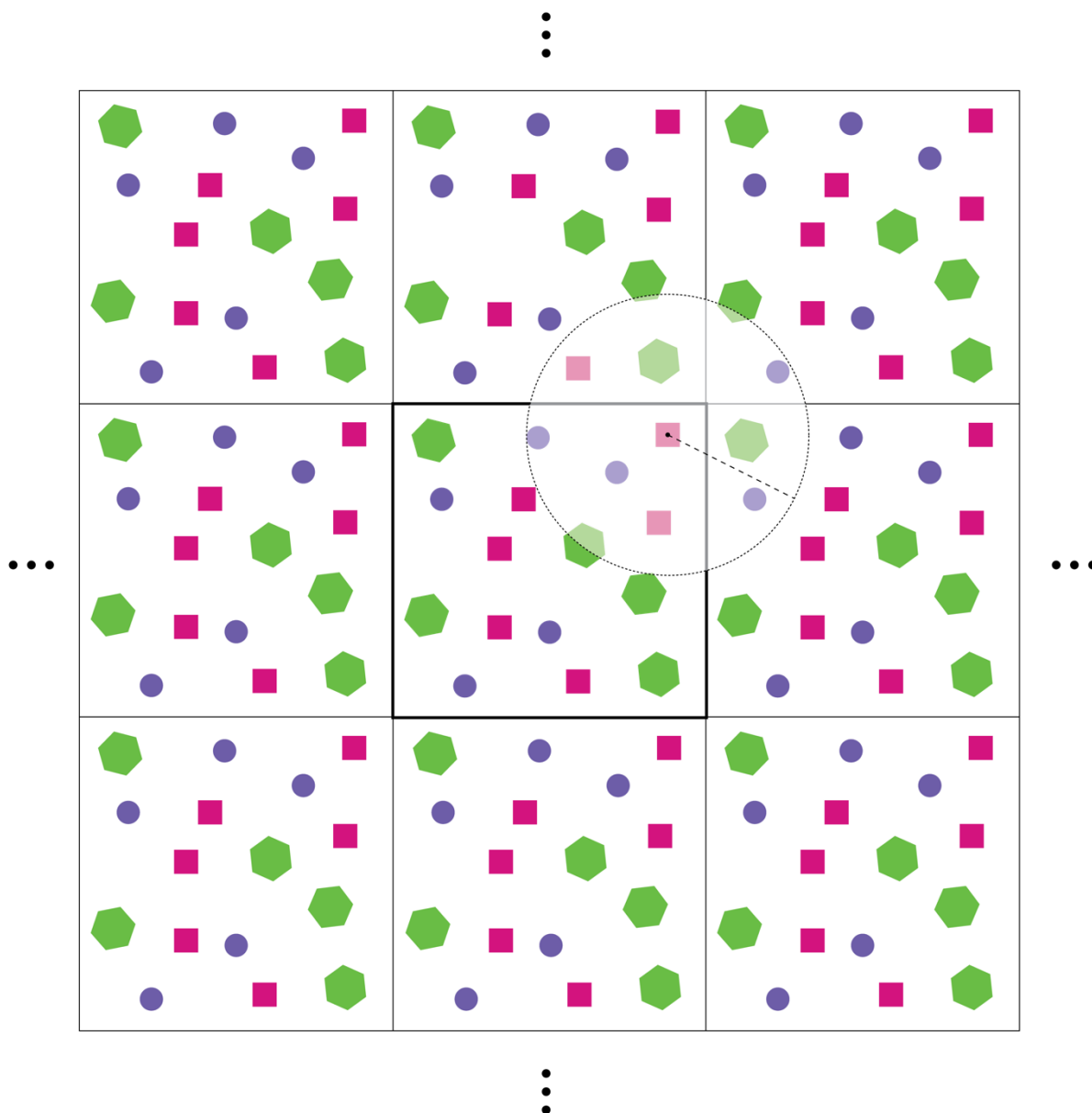


Figure 3.2. Example of 2D periodic boundary conditions. The primary cell is located at the center. A non-bonded cutoff radius is shown for one of the atoms in the primary cell.

The coulombic interactions are more difficult to handle. The most naïve form, where all coulombic interactions across boxes are summed converges slowly and is conditionally convergent. Conditional convergence means that convergence is dependent upon the order in which the individual terms of the summation across unit cells are added. The equation for this approach is shown below for a single atom:

$$(3.31) \quad V_{coulombic}(r_i) = \sum_n \sum_{j=1}^N \frac{q_j}{|r_{ij} + n|}$$

The n in this equation represents the surrounding unit cells. The $\sum_n$ indicates that the $i = j$ calculations are not performed for the central cell.

Ewald created an alternative method for calculating the electrostatic contribution to the energy. It takes the original interaction over all periodic boxes and splits into two rapidly converging series, one in real space and one in reciprocal space.

$$(3.32) \quad V_E(r_i) = \sum_n \sum_{j=1}^N q_j \frac{\text{erfc}(\alpha|r_{ij} + n|)}{|r_{ij} + n|} + \frac{4\pi}{L^3} \sum_{k \neq 0} \sum_j q_j \exp\left(\frac{-|k|^2}{4\alpha^2}\right) \exp\left(i\mathbf{k} \cdot (\mathbf{r}_j - \mathbf{r}_i)\right) - \frac{2\alpha}{\pi^{1/2}} q_i$$

While a full discussion of the Ewald summation and its derivation is beyond the scope of this section, its importance should be noted. It takes the scaling of the electrostatic calculation from $O(N^2)$ to $O(N^{3/2})$. Further it serves as the foundation for methods like Particle mesh Ewald method which further increases the efficiency of scaling from $O(N^{3/2})$ to $O(N \log(N))$. This greatly reduces the time that must be spent on the calculation of the coulombic term.^{128,129}

3.1.4.2. Temperature Regulation.

In equation (3.6) we defined the kinetic energy of a particle. We can define the total kinetic energy of a collection of particles as:

$$(3.33) \quad K = \frac{1}{2} \sum_i \frac{p_i^2}{m_i}$$

The relationship between total kinetic energy and the temperature of a system is directly proportional. It is given by:

$$(3.34) \quad K = \frac{3N}{2} kT$$

where N is the number of particles and T is the temperature.

Both (3.33) and (3.34) can be combined to yield a direct relationship between the momenta and temperature of a system:

$$(3.35) \quad T = \frac{1}{3Nk} \sum_i \frac{p_i^2}{m_i}$$

Regulation of temperature during MD simulations is achieved using a thermostat. Thermostats maintain temperature by scaling the velocities of the individual particles. The simplest algorithm is a direct scaling:

$$(3.36) \quad v_i^{new} = \lambda v_i^{old}$$

λ is defined as follows:

$$(3.37) \quad \lambda = \left(\frac{T_d}{T_{MD}(t)} \right)^{1/2}$$

Where T_d is the desired or target temperature and $T_{MD}(t)$ is the temperature at the current time.

The Berendsen thermostat follows a similar form to (3.36) but λ is redefined:

$$(3.38) \quad \lambda = \left[1 + \frac{\Delta t}{\tau} \left(\frac{T_d}{T_{MD}(t)} - 1 \right) \right]^{1/2}$$

In its redefined form λ is meant to represent the coupling of the system to a theoretical heat bath where the rise time, τ , controls the strength of the coupling with smaller values τ corresponding to a stronger coupling between the heat bath temperature and the simulation temperature. A value of $\tau = 1$ would result in $v_i^{new} = v_i^{old}$ meaning there is no coupling between the heat bath and the system.¹³⁸

The Andersen thermostat adopts a somewhat different approach when compared to the rescaling and Berendsen thermostats. Particles are selected at random and assigned velocities sampled from a Boltzmann distribution corresponding to the desired temperature. The coupling of the system is controlled by the frequency with which atoms are selected for rescaling. A higher frequency corresponds to a tighter coupling.¹³⁹

The Langevin thermostat operates by modifying one of the original equations of motion, (3.5). It becomes:

$$(3.39) \quad \frac{dp_i}{dt} = -\frac{dV}{dq_i} - \gamma_i p_i + f_i$$

These additional terms modify the behavior of the system. The second term corresponds to a “friction” term that acts against the motion of a particle in a manner proportional to its momentum. The friction coefficient, γ , controls how powerful this effect is. The third term corresponds to random forces meant to simulate the transfer of energy via collision. These forces are sampled from a Gaussian distribution with variance:

$$(3.40) \quad \sigma_i^2 = \frac{2m_i\gamma_i k_B T}{\Delta t}$$

As with the Andersen thermostat, the frequency with which the random forces are applied is also a parameter.¹²⁸

3.1.4.3. Pressure Regulation.

The final component of molecular dynamics simulations that will be explored is the regulation of pressure. However, pressure is not a parameter that we can controlled or scaled directly. Instead, we rely upon the relationship between pressure and volume as defined by the Virial equation:

$$(3.41) \quad \frac{PV_m}{RT} = 1 + \frac{B}{V_m} + \frac{C}{V_m^2} + \dots$$

Thus, the pressure can be controlled by modifying the volume of the system. The method of maintaining pressure is referred to as a barostat. One common barostat is the Berendsen Barostat. Its form should be familiar in that it resembles that of the Berendsen thermostat. Given a box defined by vectors of length, L , the lengths are scaled according to:

$$(3.42) \quad L_i^{new} = \mu L_i^{old}$$

$$(3.43) \quad \mu = \left[1 - \frac{\Delta t}{\tau} \gamma (P_d - P_{MD}(t)) \right]^{1/3}$$

where P_d is the target pressure and $P_{MD}(t)$ is the pressure at the current time.

As with the Berendsen thermostat, τ , corresponds to how tightly coupled to the desired temperature the system is. In this case γ corresponds to a term referred to as the thermal compressibility.¹³⁸

3.2. Molecular Docking

Molecular docking is a method by which the interaction between a ligand and a macromolecular receptor, usually a protein, can be modelled. Docking is generally divided into two different steps, conformational sampling and scoring. Each of these will be discussed in turn. This discussion will be largely guided by that of Meng et al. to survey methods that have historically been successful.¹⁴⁰ An additional section at the end will address some of the recent advances that have been made using deep learning techniques.

3.2.1. Conformational Sampling

Matching algorithms utilize information about the docking site to construct pharmacophores. A pharmacophore is an abstract representation of a molecule that can be used to describe or identify target binders. Matching algorithms seek to generate an alignment between both the protein and ligand where each is represented using a pharmacophore.

Incremental construction deconstructs molecules about rotatable bonds. The individual pieces are incrementally added to the docking site from largest to smallest and the ligand is reconstructed.

Stochastic methods utilize randomness for conformational sampling. Monte Carlo methods are one such approach. They apply rotation about ligand bond, translation, or ligand rotation at random. A selection criterion can then be applied to determine if the generated conformation will be kept or discarded. This procedure is not unique to protein-ligand interactions, it is a common method of conformational sampling that can be applied to many different categories of systems.

Genetic algorithms are another class of stochastic methods which can be applied to conformational sampling. They have found great success for a wide variety of optimization problems such as the notoriously difficult travelling salesman problem. They represent various aspects of the system state using individual “genes” to encode the smallest subunit of the problem. These are then combined to create a proposed solution known as a “chromosome”. Pools of chromosomes are generated as random initial guesses. These are then assessed, and the best chromosomes are preserved. These best chromosomes are then used as scaffolds to generate a second generation. This second generation is generated via two mechanisms, crossover and mutation. Crossover occurs when two of the chromosomes swap genes. Mutations are random

changes in the chromosome. The canonical definition of each iteration of chromosome generation and assessment is “epoch”. The number of iterations is controlled by either convergence or a limit to the number of epochs. A chromosome is considered converged when the quality between epochs does not increase by a specified threshold. Alternatively, a hard cutoff for the number of epochs can be provided.^{140,141}

3.2.2. Scoring

Forcefield based scoring functions operate in the same manner as the previously discussed MD forcefield potential. Docking forcefields usually differ from MD forcefields in that they will only include a non-bonded term. The atom typings tend to be less complex as well with fewer distinct categories of atoms explicitly defined. Additional modifications can be made to correct for entropic contributions, differing solvents, or contributions that are weighted differently such as hydrogen bonds.

Empirical scoring functions decompose the contributions of each interaction into categories such as hydrogen bonds, dipole interactions, ionic interactions, hydrophobic interactions, and binding entropy. Each of these terms is weighted against coefficients. Practically, empirical models generally take the form of a linear regression trained on a dataset of similar protein-ligand complexes.

Knowledge-based scoring functions use structural data to construct a scoring function. The favorability of a given interaction is assumed to be correlated with the frequency of the interactions in the training dataset. This is then used to weight the favorability of all the observed types of interactions.¹⁴⁰

3.2.3. Deep Learning for Docking Problems

Within the past decade, deep learning has been applied successfully to many notoriously difficult and important problems. Perhaps one of the most impactful is in the field of image recognition. High quality models now exist that are capable of human-level performance for tasks such as recognizing objects, faces, animal and plant species, diseases, etc. These models have applications in disparate fields such as ecology and medicine.¹⁴² More recently deep learning has been applied to generate the high-quality protein-structure prediction model AlphaFold2 which has outcompeted the outcompete any of the other available models.¹³³ AlphaFold2 also shows promise as a tool for drug discovery.

Even more recently, diffusion models such as stable diffusion 3 have become popular for generating images from user-provided prompts.¹⁴³ This same class of model was used to create RFDiffusion a model capable of generating protein structures designed to bind a specific target with high specificity.¹⁴⁴ Another diffusion model DiffDock has been published which generates docked structures of ligand-protein complexes.¹⁴⁵ This continues the trend of an increasing presence of deep-learning models in computational drug design and discovery.

CHAPTER 4: Ca²⁺ Ions Facilitate the Organization of the Annexin A2/S100A10

Heterotetramer

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Title: “Ca²⁺ ions facilitate the organization of the Annexin A2/S100A10 heterotetramer”

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Abstract

Annexin A2 (A2) is a member of the Annexin family which contains Ca²⁺-regulated phospholipid-binding proteins. Annexins associate with S100 proteins to form heterotetramers. The A2/S100A10 heterotetramer (A2t) is the most extensively studied of these heterotetramers. It induces membrane microdomain formation, causes membrane budding, and facilitates proliferation of some cancers. In this work, the first molecular dynamics (MD) study on the complete A2t of 868 amino acids was performed. MD trajectories of more than 600 ns each were generated for complete A2t complexes with and without Ca²⁺ ions. The outward extension of membrane-binding residues A2-K279 and A2-K281 was shown to be inhibited in the absence of Ca²⁺ as they were captured by Ca²⁺-binding residue D322. F-Actin binding residue A2-D339 was observed to occupy either an exposed or buried state in the absence of Ca²⁺ while it only occupied the buried state in the presence of Ca²⁺. The observed motions of the A2t subunits are highly organized with a strongly correlated central region which is negatively correlated with the periphery of the complex. The central region contains the S100A10 (p11) dimer, A2-N, and A2-I while the periphery contains A2-II, A2-III, and A2-IV. Novel interactions between A2 and p11 were identified. A2 residues outside of A2-N (K80, R77, E82, and R145) had strong interactions with p11. Residue R145 of A2 may have a significant effect on the dynamics of the system with its interaction resulting in asymmetric motions of A2. The presented results provide novel insights to inform future experimental studies.

4.1. Introduction

The Annexin A2/S100A10 heterotetramer (A2t) has important biological functions that have been demonstrated extensively in the literature.¹ Many of these functions involve the association of A2t with membrane surfaces. This capability of A2t has been investigated thoroughly *in vitro*. It has previously been shown that A2t can bind to and aggregate membranous bodies such as chromaffin granules in addition to analogues such as giant unilamellar vesicles (GUVs).^{16,19} The ability of A2t to associate with negatively charged phospholipid membranes can be largely attributed to a small number of basic residues at its convex surface.¹⁴⁶ A2t has also been demonstrated to bridge membranes.¹¹² Residues K279 and K281 of Annexin A2 (A2) form a phosphoinositide-specific binding site (PIP site) that contributes significantly to the specificity of A2t binding of phosphoinositide-containing membranes.¹⁴⁶ A2t is known to induce F-actin bundling and as well as recruit it to the cell surface.⁶ Exocytotic behavior has been attributed to A2t and is driven by A2t's ability to induce lipid microdomains and to bundle F-actin.¹⁴⁷ The final nine residues of A2, LLYLCGGDD, compose the F-actin binding-site.¹⁴⁸ A2t serves as a cell-surface receptor for tissue-plasminogen activator (tPa) and the zymogen of plasmin, plasminogen (Pmo). The colocalization of tPa and Pmo is an important step during the activation of plasminogen.¹¹

The upregulation of A2 in many aggressive metastatic cancers has been reported in the literature. It has been postulated that within the context of metastatic cancers the increased levels of A2 and subsequently A2t result in increased levels of cancer proliferation.² Plasmin is known to be important for the endothelial to mesenchymal transition (EMT).¹⁴⁹ EMT has implications for the activity of many metastatic cancers as it enhances the mobility and subsequently the

invasiveness of cancer cells.¹⁴⁹ Thus, increased levels of A2t lead to increased levels of activated Pmo which may promote the progression of metastatic cancers.¹¹

A2t is composed of four subunits, two A2 monomers and an S100A10 (p11) dimer.¹⁵⁰ Each p11 monomer is composed of two EF-hand motifs (EF1 and EF2) connected by a “hinge”. The EF1 motif contains a long helix (HI), a loop (L), and a short helix (HII). The EF2 motif contains a short helix (HIII), a loop (L), and a long helix (HIV). Unlike many other S100 proteins, p11 does not require Ca^{2+} -binding to adopt its “open” conformation, which can be a prerequisite for binding events.¹⁵¹ The A2 subunits consist of two domains, the N-terminal domain (A2-N) and the C-terminal domain (A2-C). A2-N contains 32 residues. A2-C has two faces (convex and concave) and is organized into four homologous repeats (I, II, III, and IV) which are each ~70 residues long. The repeats contain five α -helices (A, B, C, D, and E). Helices A, B, D, and E of each repeat are wound into a right-handed superhelix. Helix C is perpendicular to the other four helices and located toward the concave face. All Ca^{2+} -binding sites are located on the convex face of A2.¹⁵²

A2t is a highly symmetric complex. Its center is composed of a p11 dimer, with an A2 on either side. The N-terminus of each A2 associates with a cleft formed by EF1-HI from one p11 monomer, EF2-HIV from the other p11 monomer, and the hinge from the monomer that contributes EF2-HIV. A graphical representation of A2t with the discussed regions labeled is presented (see **Figure 4.1**).¹⁵³

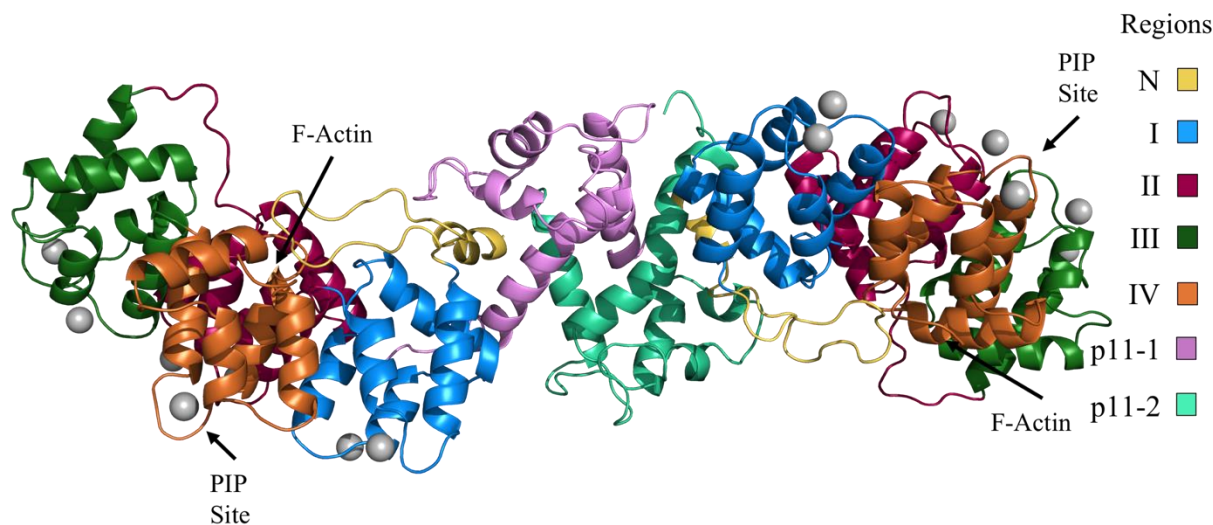


Figure 4.1. Representation of A2t with regions and important binding-sites labeled. Each monomer of A2 has the N-terminal domain (N) and the core domain composed of repeats I, II, III and IV.

There are three types of Ca^{2+} -binding sites located in A2: Type II, Type III, and type AB'. Type II sites have the canonical sequence of the endonexin fold, KGXGT-(38X)-D/E, a Ca^{2+} -binding site that is characteristic of the annexin protein family.¹⁵⁴ Coordination at Type III sites involves two peptidyl oxygens and a bidentate glutamic acid. Two peptidyl carbonyl oxygens contribute to coordination at AB' sites.⁶⁷ Type II sites appear to be the most important for A2-membrane binding.¹⁵⁵

Ca^{2+} in the context of A2 is largely understood to mediate the interaction of A2/A2t and cellular membranes. A2/A2t-membrane binding is a necessary step for many of A2/A2t's functions. Examples include A2/A2t-mediated activation of plasminogen to plasmin, bridging of membranes, endo/exocytotic events, and lipid microdomain formation.^{11,16,36,112,156} In addition to membrane-binding, Ca^{2+} is known to induce the bundling of F-Actin in the presence of A2t. The degree of bundling has been demonstrated to increase as Ca^{2+} concentration increases.⁶ Ca^{2+} is important for both membrane-binding and F-actin bundling. Thus, the focus of our study was to

investigate any Ca^{2+} -induced structural rearrangements which might occur to facilitate membrane-binding and F-actin bundling.

Previously, molecular dynamics (MD) simulations have been performed on the A2 monomer and on the p11 dimer individually but never on a complete A2t complex. Hakobyan et al.¹⁵⁷ investigated plausible orientations of A2 with respect to a membrane model. This study focused on interactions between A2 and the membrane as well as A2-induced changes in the membrane model. Another model investigated the interaction between the cytoplasmic domain of CEACAM1 (CEACAM1-SF) cytoplasmic domain and a pseudo-tetramer of A2t which consisted of only the p11-dimer with the N-termini of A2 associated.¹⁵⁸ In this study, for the first time, we performed an MD investigation for the full A2t structure, with a particular focus on the influences of Ca^{2+} on the system.

4.2. Computational Methods

4.2.1. Structure Generation

The initial structure of A2t was obtained from the PDB structure (PDB Code: 4HRE) of A2t in the complex with SMARCA3 peptide.¹⁰ A2 and p11 subunits were extracted from the structure. The Modeller software was used to alter the primary sequence of the A2 subunit to match the reported sequence for wild type A2 on Uniprot; mutations or missing residues were changed or added.^{159,160} The residues changed in the A2 monomer included P29A, V46I, A66V, A84P, D197E, P223C, and D230E. The final five residues of p11, Q93, K94, G95, K96, and K97, were absent. The missing residues for p11 and A2 were added using the Modeller software. Copies of the altered A2 and p11 subunits were aligned to the subunits of the original tetramer to generate a model of wild-type A2t. The original structure lacked coordinated Ca^{2+} ions. Ca^{2+} ions were added to the A2t model by alignment with an A2 crystal structure (PDB code: 1XJL) with

seven coordinated Ca^{2+} ions.⁶⁷ The final models generated for simulation were A2t complexes with 7 or 0 Ca^{2+} ions coordinated per A2, referred to as 7Ca and 0Ca, respectively.

4.2.2. Simulation System Construction

The tleap module of AmberTools was utilized to solvate the system and generate the required topology file.¹⁶¹ Explicit solvation was performed using TIP3P waters in an octahedral box, with a minimum of 16 Å from the protein to the box boundary for both 7Ca model and 0Ca models.¹⁶² Monovalent chlorine and sodium ions were added to neutralize the system and maintain the salt concentration of the system at 0.15 M. The total number of atoms in the simulations were 353,083 and 353,104 for the 7Ca and 0Ca models, respectively.

4.2.3. Simulation Details.

Simulations were run on a 4 - RTX 2080 Ti workstation housed at the Department of Chemistry of East Carolina University. The pmemd.MPI module of AMBER18 was used to run the energy minimization and heating steps. The pmemd.cuda module was used for the production run.¹⁶¹ The AMBER force field (ff14SB) was utilized for A2t.^{163,164} The parameters of Bartolotti et al. were utilized for describing the Ca^{2+} ions using a 12-6 potential.¹⁶⁵ Periodic boundary conditions were applied during the simulation, with a cutoff of 10 Å to truncate the Van der Waals forces. The particle-mesh Ewald method was used to treat the long-range electrostatic interactions. All bonds involving hydrogen atoms were constrained using the SHAKE algorithm, which allowed the usage of a longer timestep of 2 fs.¹⁶⁶ Prior to simulation the entire system was minimized in two steps, the first with A2t and Ca^{2+} ions restrained and the second unrestrained. For the restrained minimization, 500 steps were performed using the steepest descent method followed by 500 steps using the conjugate gradient method. For the unrestrained minimization, 2500 steps of the minimization were performed using the steepest descent method followed by

7500 steps using the conjugate gradient method. The minimized systems were then warmed to 310 K over 60 ps. Constant pressure dynamics were carried out on each of the 7Ca and 0Ca models for more than 600 ns. The stability of the simulations was determined by the conservation of energy of the simulation system and the maintenance of a constant average temperature.

4.2.4. Data Analysis.

The molecular dynamics simulation trajectories of more than 600 ns for each of the 7Ca and 0Ca models were analyzed using the CPPTRAJ program. Plotting and visualization were performed using a combination of R scripts, Python scripts, and the PyMol molecular visualization program.^{161,167}

Pairwise MMGBSA was performed for the entire system using the MMGBSA.py script of AMBER18. Additionally, a unique tool was developed for creating feature-space plots and reduced-dimensionality correlation plots. Only the plots from the latter are reported here. This tool allows for a protein system to be divided into subregions and for the cross correlations to be calculated using the center of mass of those subregions.

4.3. Results and Discussion

4.3.1. Conformational Analysis

4.3.1.1. The Gross Structural Change Between A2t Models Differs.

Principal component analysis (PCA) was conducted to generate simplified representations of the conformational changes that occurred during the 7Ca and 0Ca trajectories (see **Figures 4.2A** and **4.2C**). The 7Ca model experienced rapid transformation over the first 200

ns before reaching a stable conformation over the final 300 ns as indicated by the large blue, green, and yellow clusters (see **Figure 4.2A**).

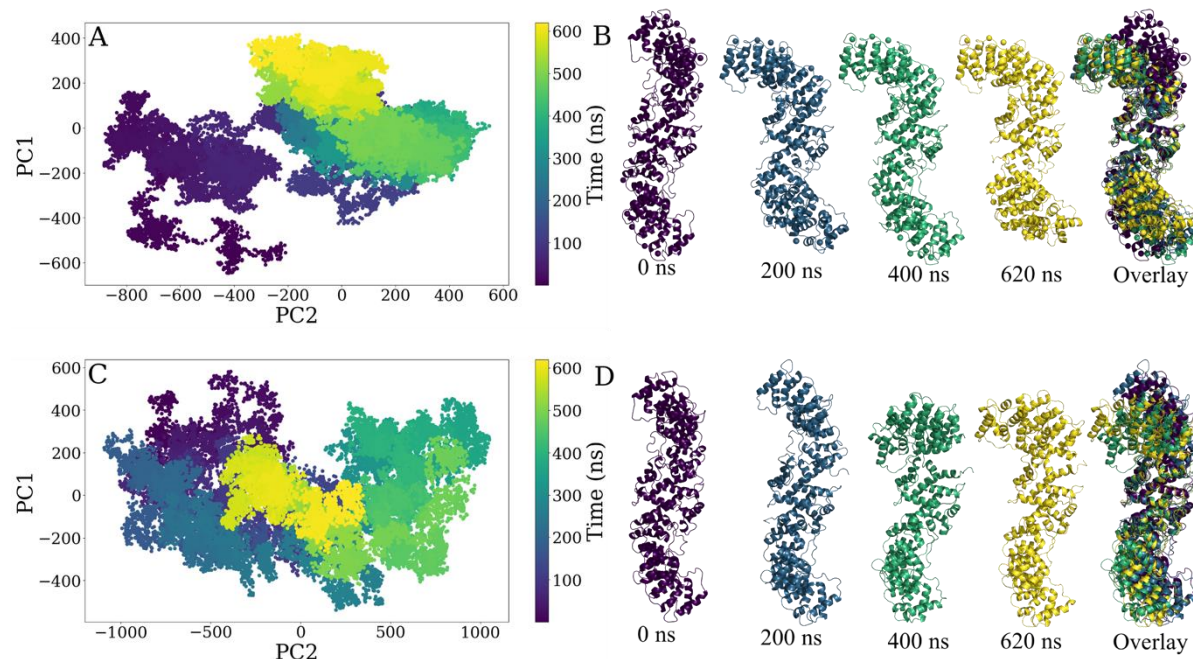


Figure 4.2. Principal component analysis (PCA) results for the (A) 7Ca and (C) 0Ca models along with representative structures from the (B) 7Ca and (D) 0Ca trajectories. The p11 of the representative structures are aligned to the first in the series of structures with the A2-2 subunit being oriented at the top of each structure.

The trajectories of the 7Ca and 0Ca models were sampled to extract representative structures for visually representing the observed conformational changes (see **Figures 4.2B** and **4.2D**). The A2-2 subunits of both models rotated $\sim 90^\circ$ from their starting conformations by the end of the simulation. This rotation occurred much more rapidly for 7Ca-A2-2, taking only 200 ns of simulation time while 0Ca-A2-2 required 400 ns to achieve a similar rotation. The behavior of the models' A2-2 subunits differed as 7Ca-A2-2 maintained a relatively stable position and 0Ca-A2-2 oscillated about the 90° position. The behavior of the A2-1 subunits exhibited much greater differences. 0Ca-A2-1 remained in a similar position relative to the initial conformation of 0Ca-A2t. Conversely, 7Ca-A2-1 underwent a more significant transformation by progressively twisting farther out with reference to the plane of the screen.

4.3.1.2. Ca^{2+} -Coordination.

Ca^{2+} coordinations in the 7Ca model were compared to those in the crystal structure model (PDB Code: 1XJL) (see **Table 4.1**). Coordinations in the crystal structure were defined as oxygen atoms less than 3.5 Å from Ca^{2+} atoms.⁶⁷ Coordinations are reported for oxygen atoms within 3.5 Å of Ca^{2+} ions for at least 1% of total simulation. Coordinations corresponding to crystal structure coordinations were reported with a percentage of coordinated frames as low as 0.1%. Few of the reported contacts are coordinated for less than 30% of the trajectory frames. The coordinations for both monomers were similar for all the Ca^{2+} atoms with some slight differences. Coordinations at sites Ca^{2+} -1,2,5,6,7 were consistent with the results reported for the crystal structure. Ca^{2+} ion parameters packaged with AMBER18, including the 12-6 IOD LJ, 12-6 HFE LJ, and 12-6-4 LJ models, were tested using A2 monomer and similar calcium coordination deviations from X-ray structure were seen for sites 3 and 4.^{168–171} This suggests that Ca^{2+} binding sites 3 and 4 might be of a lower affinity than those which reproduce coordinations more faithfully. Quantitative measures of binding affinity, such as K_d , could not be found in the literature to test this postulation. However, the observed Ca^{2+} coordination is reasonable, and most contacts present in the crystal structure were preserved for significant durations throughout the trajectory.

Table 4.1. Comparison of Ca²⁺ coordinated oxygen atoms between A2 X-ray Structure 1XJL and Simulated A2t Complex.

Ca ²⁺	Ca ²⁺ Type	A2 Repeat	1XJL	A2-1	Percentage of Coordinated Frames	A2-2	Percentage of Coordinated Frames
			Coordination	Coordination		Coordination	
1	III	I	G50@O	G50@O	77.2%	G50@O	99.6%
			V51@O	V51@O	76.8%	V51@O	99.1%
			E53@OE1	E53@OE1	97.5%	E53@OE1	99.8%
			E53@OE2	E53@OE2	98.9%	E53@OE2	95.7%
			-	D326@OD1	50.1%	-	-
			-	D326@OD2	19.4%	D326@OD2	1.7%
2	III	I	K88@O	K88@O	51.5%	K88@O	77.7%
			L91@O	L91@O	48.4%	L91@O	62.2%
			E96@OE1	E96@OE1	84.5%	E96@OE1	83.6%
			-	E96@OE2	81.1%	E96@OE2	86.9%
			-	-	-	-	-
3	II	II	-	S117@O	1.1%	-	-
			M118@O	M118@O	43.0%	M118@O	92.2%
			-	K119@O	1.4%	-	-
			G120@O	G120@O	3.6%	G120@O	8.3%
			G122@O	-	-	G122@O	3.0%
			-	T123@O	13.0%	-	-
			-	E125@OE2	35.8%	-	-
			-	D162@OD1	97.2%	D162@OD1	98.4%
			D162@OD2	D162@OD2	98.2%	D162@OD2	98.7%
			-	-	-	-	-
4	A/B	II/III	T123@O	T123@O	0.2%	-	-
			-	D124@OD1	45.1%	-	-
			-	D124@OD2	23.9%	-	-
			E125@OE1	E125@OE1	87.5%	E125@OE1	97.4%
			E125@OE2	E125@OE2	43.1%	E125@OE2	96.1%
			-	D251@OD1	95.8%	D251@OD1	99.0%
			-	D251@OD2	96.2%	D251@OD2	99.7%
			-	-	-	-	-
5	II	III	-	A201@O	77.8%	-	-
			G202@O	G202@O	95.9%	G202@O	99.1%
			R205@O	R205@O	88.4%	R205@O	98.4%
			G207@O	G207@O	95.2%	G207@O	99.2%
			-	D209@OD1	10.0%	-	-
			-	D209@OD2	99.9%	-	-
			E247@OE1	E247@OE1	99.7%	E247@OE1	99.8%
			E247@OE2	E247@OE2	88.5%	E247@OE2	99.7%
6	III	III	R245@O	R245@O	9.9%	R245@O	2.8%
			V248@O	V248@O	9.6%	V248@O	2.6%
			-	E253@OE1	91.1%	E253@OE1	98.2%
			E253@OE2	E253@OE2	97.4%	E253@OE2	94.7%
			-	N254@OD1	1.7%	N254@OD1	6.2%
7	II	IV	M278@O	M278@O	73.4%	M278@O	84.0%
			G280@O	G280@O	54.4%	G280@O	38.6%
			G282@O	G282@O	3.9%	G282@O	33.8%
			-	-	-	T283@OG1	1.0%
			-	D285@OD1	76.2%	-	-
			-	D285@OD2	79.1%	-	-
			-	-	-	Q321@OE1	4.7%
			D322@OD1	D322@OD1	99.6%	D322@OD1	99.0%
			D322@OD2	D322@OD2	99.8%	D322@OD2	97.2%

4.3.1.3. Ca^{2+} -Binding Affects the Conformation of Residues at PIP and F-Actin

Binding Sites.

The effects of Ca^{2+} on the sites of biological importance, specifically the PIP and F-Actin sites were of greatest interest during this study. The PIP site is composed of residues K279 and K281 while the F-actin site is composed of the final nine residues of A2. The solvent accessible surface area (SASA) was calculated and utilized as a metric for identifying Ca^{2+} -induced morphological changes at sites of known biological importance. The SASA values for the PIP and F-Actin sites in the 7Ca and 0Ca models were calculated over the course of the simulations, and the mean values were compared. Results for each site are reported in **Table 4.2**, and they suggest that Ca^{2+} has a discernable effect on the morphology of the F-actin and PIP sites. The cause of the morphological changes was further investigated at an atomic level.

Table 4.2. Comparison of Mean SASA (in $\text{\AA}^2$) for PIP and F-Actin Binding Sites Between 7Ca and 0Ca Models of A2t.

Site	Subunit	Model	Mean	SD
PIP	A2-1	7Ca	192	6
		0Ca	165	15
	A2-2	7Ca	180	18
		0Ca	178	11
F-Actin	A2-1	7Ca	346	8
		0Ca	402	33
	A2-2	7Ca	349	18
		0Ca	366	20

The SASA for the PIP site residues, K279 and K281, was greater in the 7Ca model when compared to the 0Ca model (see **Table 4.2**). This suggested a conformational shift occurred upon Ca^{2+} -binding that made these residues more accessible for membrane-binding. It has been previously demonstrated that A2 does not associate with membranes in the absence of Ca^{2+} .¹ However, if negatively charged Ca^{2+} -binding residues are mutated to alanine residues, A2 can

then associate with membranes. This led to the hypothesis that negatively charged residues “capture” positively charged membrane-binding residues in the absence of Ca^{2+} .¹⁷² The distances between Ca^{2+} -binding residue D322 and both K279 and K281 were calculated and reported in Table 3 for the A2 subunits. It was found that in the absence of Ca^{2+} K281 was appreciably closer to D322 (8.529 Å to 12.090 Å for A2-1 and 10.991 Å to 12.095 Å for A2-2, respectively). The K279 of A2-2 had a similar mean distance from D322 for both models (see **Table 4.3**). It should be noted that the distance distributions for K279 of the 0Ca model had long tails at smaller distances when compared to the 7Ca model (see **Figures 4.3A** and **4.3C**). This illustrated that capture of K279 by D322 did occur with some appreciable frequency in the 0Ca model. Distance vs time plots (see **Figure A.1**) corresponding to **Figure 4.3** are provided in **Appendix A**. These distance analyses support the hypothesis that membrane-binding residue capture by acidic residues occurs in the absence of Ca^{2+} , as demonstrated by the comparison of **Figures 4.3E** and **4.3F** with **Figure 4.3G**. **Figures 4.3E** and **4.3F** depict the capture of A2-K279 and A2-K281, respectively, by D322 as observed during the simulation trajectory. The absence of capture in the presence of Ca^{2+} can be seen in **Figure 4.3G**. These results agree with experimental results as the mutation of negatively-charged Ca^{2+} -binding residues has been shown to restore membrane-binding capacity in the absence of Ca^{2+} .¹⁷² The observed reduction of K279 and K281 availability in the presence of Ca^{2+} would make them less available for membrane-binding.

Table 4.3. Distances (in Å) Between the Carboxylic Oxygens of Ca²⁺-Binding Residue D322 from A2 and the Amine Nitrogen of K279 and K281.

Subunit	Group 1	Group 2	Model	Mean	SD
A2-1	D322@OD1 D322@OD2	K279@NZ	7Ca	11.19	1.36
			0Ca	10.85	2.35
	D322@OD1 D322@OD2	K281@NZ	7Ca	12.09	1.52
			0Ca	8.53	3.42
			7Ca	11.76	0.91
A2-2	D322@OD1 D322@OD2	K279@NZ	7Ca	11.76	0.91
			0Ca	11.94	1.79
	D322@OD1 D322@OD2	K281@NZ	7Ca	12.10	1.35
			0Ca	10.99	2.13
			7Ca	12.10	1.35

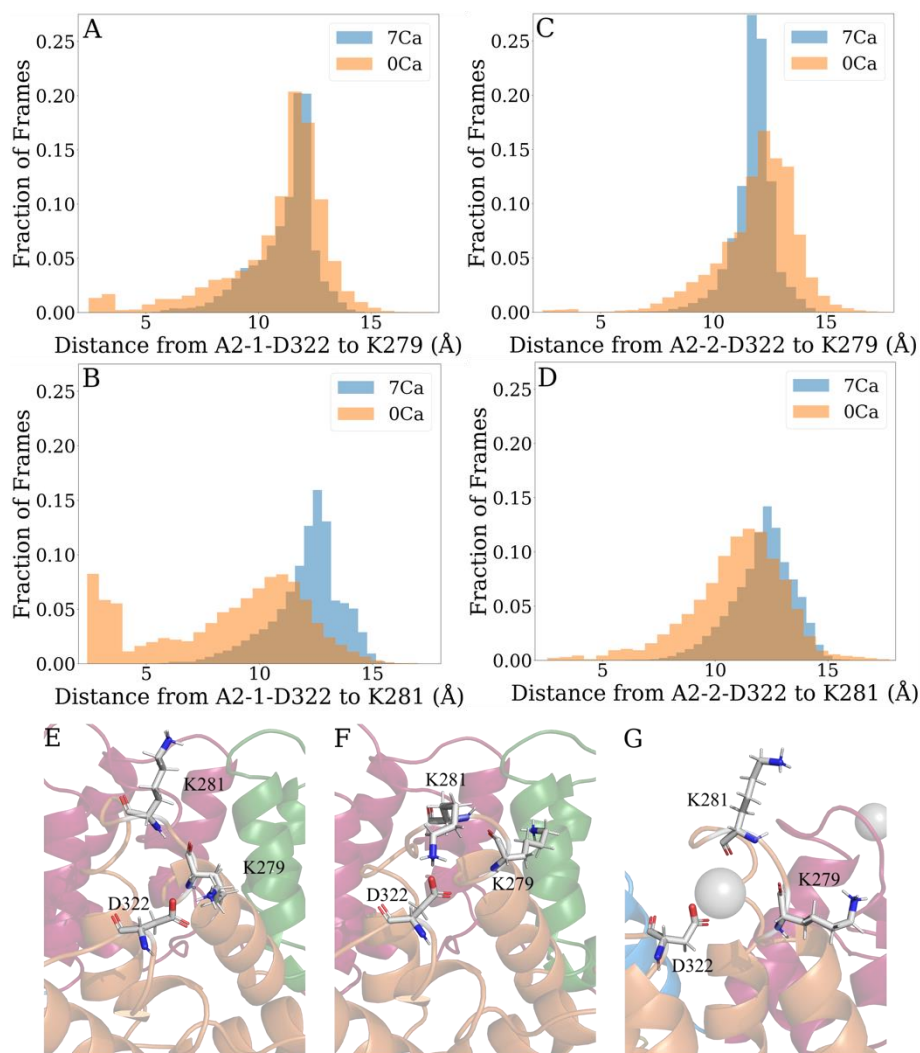


Figure 4.3. Distance histograms generated using the carboxylic oxygen of Ca²⁺-binding residue D322 and the (A) A2-1-IV-K279 (B) A2-1-IV-K281 (C) A2-2-IV-K279 (D) A2-2-IV-K281 amino nitrogen for both the 7Ca and 0Ca models. Graphical representations of the D322 R-group carboxylate associated with (E) K279, (F) K281, and (G) Ca²⁺. The (E) and (F) snapshots were taken from the 0Ca trajectory while the (G) snapshot was taken from the 7Ca trajectory.

The SASA for the final nine residues of A2, the F-Actin binding residues (L331, L332, Y333, L334, C335, G336, G337, D338, and D339), was smaller for the 7Ca model when compared to the 0Ca model (see **Table 4.2**).¹⁴⁸ Visual inspection of the trajectory revealed that this was due to the interactions of A2-1/2-D339 with A2-1/2-R304 and A2-1/2-K308 (see **Figures 4.4C** and **4.4F**). Distance calculations showed that the mean distance between A2-D339

and both A2-R304 and A2-K308 was greater in the 0Ca model than the 7Ca model (see **Table 4.4**). Two distinct distributions can be seen in the distance histograms for the 0Ca model (see **Figures 4.4A, 4.4B, 4.4D, and 4.4E**). Distance vs time plots corresponding to **Figures 4.4A, 4.4B, 4.4C, and 4.4D** are provided in **Appendix A** (see **Figure A.2**). The observed, distinct distributions correspond to exposed (see **Figure 4.4C**) and buried states (see **Figure 4.4F**). The only appreciable state in the 7Ca model was the buried state. *In vitro* studies have previously shown that A2-induced F-actin bundling increases with an increasing Ca^{2+} concentration.⁶ Combined with our reported results, this suggests that F-actin binding may prefer A2-D339 in the buried state rather than the exposed state.

Table 4.4. Distances (in Å) between Terminal Carboxylic Oxygens of D339 and Amine Nitrogen of K308 as well as the Carboxylic Oxygens of D339 and Guanidino Nitrogens of R304.

Subunit	Group 1	Group 2	Model	Mean	SD
A2-1	K308@NZ	D339@O D339@OXT	7Ca	3.23	0.75
			0Ca	5.22	1.81
		R304@NE	7Ca	3.92	0.49
	R304@NH1 R304@NH2	D339@OD1			
		D339@OD2			
			0Ca	6.16	1.71
A2-2	K308@NZ	D339@O D339@OXT	7Ca	3.25	0.69
			0Ca	3.84	1.32
		R304@NE	7Ca	3.46	0.26
	R304@NH1 R304@NH2	D339@OD1			
		D339@OD2			
			0Ca	4.23	1.25

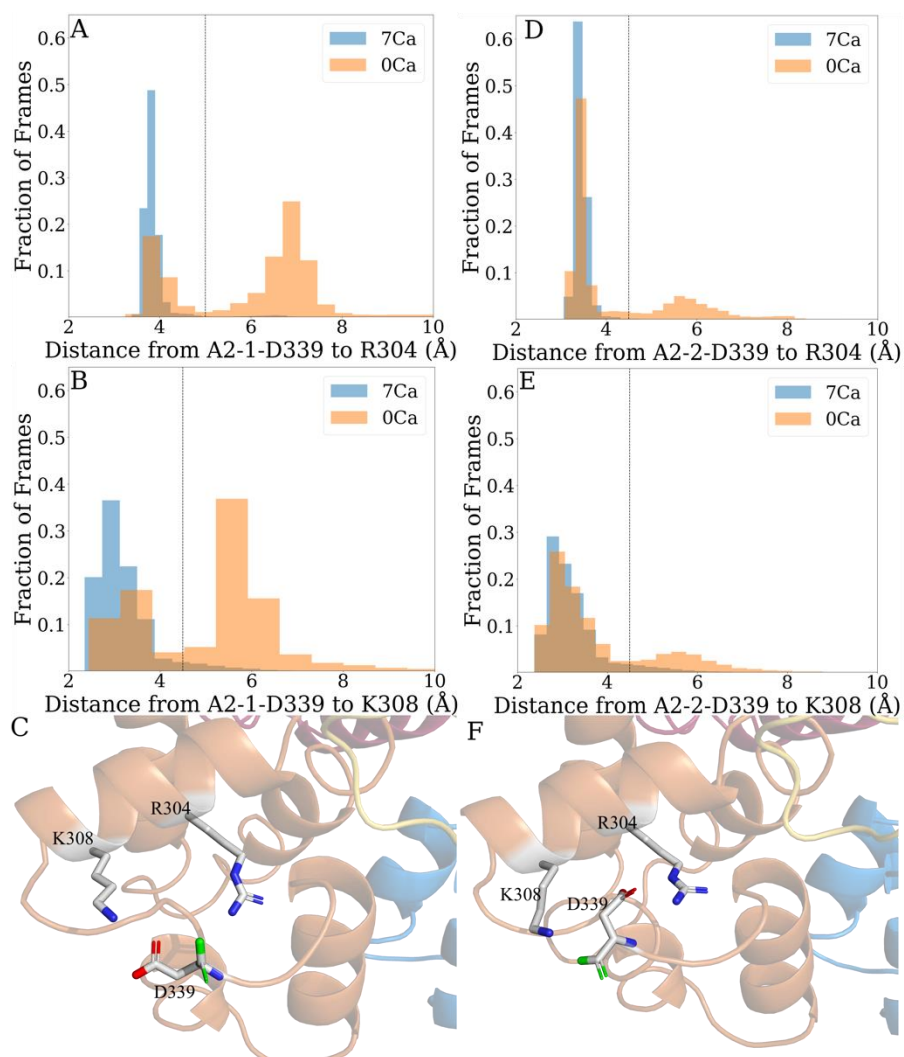


Figure 4.4. Distance histograms were generated using the distances between the (A) side-chain carboxylic acid oxygens of A2-1-IV-D339 with the guanidino nitrogens of A2-1-IV-K304, (B) terminal carboxylic acid oxygens of A2-1-IV-D339 with the amine nitrogen of A2-1-IV-K308, (D) side-chain carboxylic acid oxygens of A2-2-IV-D339 with the guanidino nitrogens of A2-2-IV-R304, and (E) terminal carboxylic acid oxygens of A2-2-IV-D339 with the amine nitrogen of A2-2-IV-K308. The lines in the center of each graph separate the buried and exposed distributions into left and right regions, respectively. Graphical representations of the (C) exposed and (F) buried states.

The presence of Ca^{2+} affected the morphology of both the F-actin and PIP binding sites. The presence of Ca^{2+} increased the accessibility of key PIP-binding residues K279 and K281, which are critical for membrane-binding. The C-terminus became less accessible in the presence

of Ca^{2+} adopting a more stable conformation largely due to interactions of D339 with K308 and R304. Previous experiments have shown that each of the functions associated with these binding sites are reduced or non-existent in the absence of Ca^{2+} .^{6,146} Our results suggest that the availability and shape of these sites are mediated by conformational changes due to Ca^{2+} binding.

4.3.2. Correlated Motion Among A2t Subunits

4.3.2.1. Correlated Motions of A2t.

Cross-correlation heatmaps were generated for the 0Ca and 7Ca models using α -carbons (see **Figure 4.5A** and **4.5D**). **Figures 4.5A** and **4.5D** were plotted using separate regions of the trajectory; the upper triangles were generated using frames corresponding to 20-320 ns while the lower-triangle was generated using frames corresponding to 320-620 ns. Most of the two regions in the separate 0Ca and 7Ca plots demonstrate excellent agreement suggesting that the simulations have converged. The most striking feature of the correlation maps was the strong positive correlation amongst the components of the central regions (p11, A2-N, and A2-1) of 0Ca and 7Ca. This central region of both complexes was negatively correlated with the components that make up the periphery (A2-II, A2-III, and A2-IV) of the complex. While generally similar, key differences can be identified across the two models indicating that the presence of Ca^{2+} has significant impacts on the organized motions of the system. Specifically, the relationships between the A2 subunits and the p11 subunits appeared more organized in the 7Ca model than in the 0Ca model with strong, clearly defined negative correlations between each A2 and the p11 dimer. To simplify the visualization of these relationships, representations of the α -carbon correlation map were generated using the centers of mass of each discrete region of the complex (see **Figures 4.5B** and **4.5E**). Here it can be seen, more easily, which components of A2 are

found in the strongly correlated central region of the complex. Further, it can be more clearly seen that the central region is not a perfect square, rather it is two overlapping squares of regions that are strongly correlated. The first is composed of A2-1-N, A2-1-I, and the p11 dimer. The second is composed of A2-2-N, A2-2-I, and the p11 dimer. The A2-1-N and A2-1-I subunits are not strongly correlated with A2-2-N and A2-2-I.

The described trends can be seen in the first normal mode of 0Ca and 7Ca. In both cases, the A2 subunits engaged in nearly mirror rotations with each repeat I of the A2 subunits rotating toward p11. The magnitudes of these motions are different; one A2 subunit was significantly more mobile than the other in both models (see **Figures 4.5C** and **4.5F**).

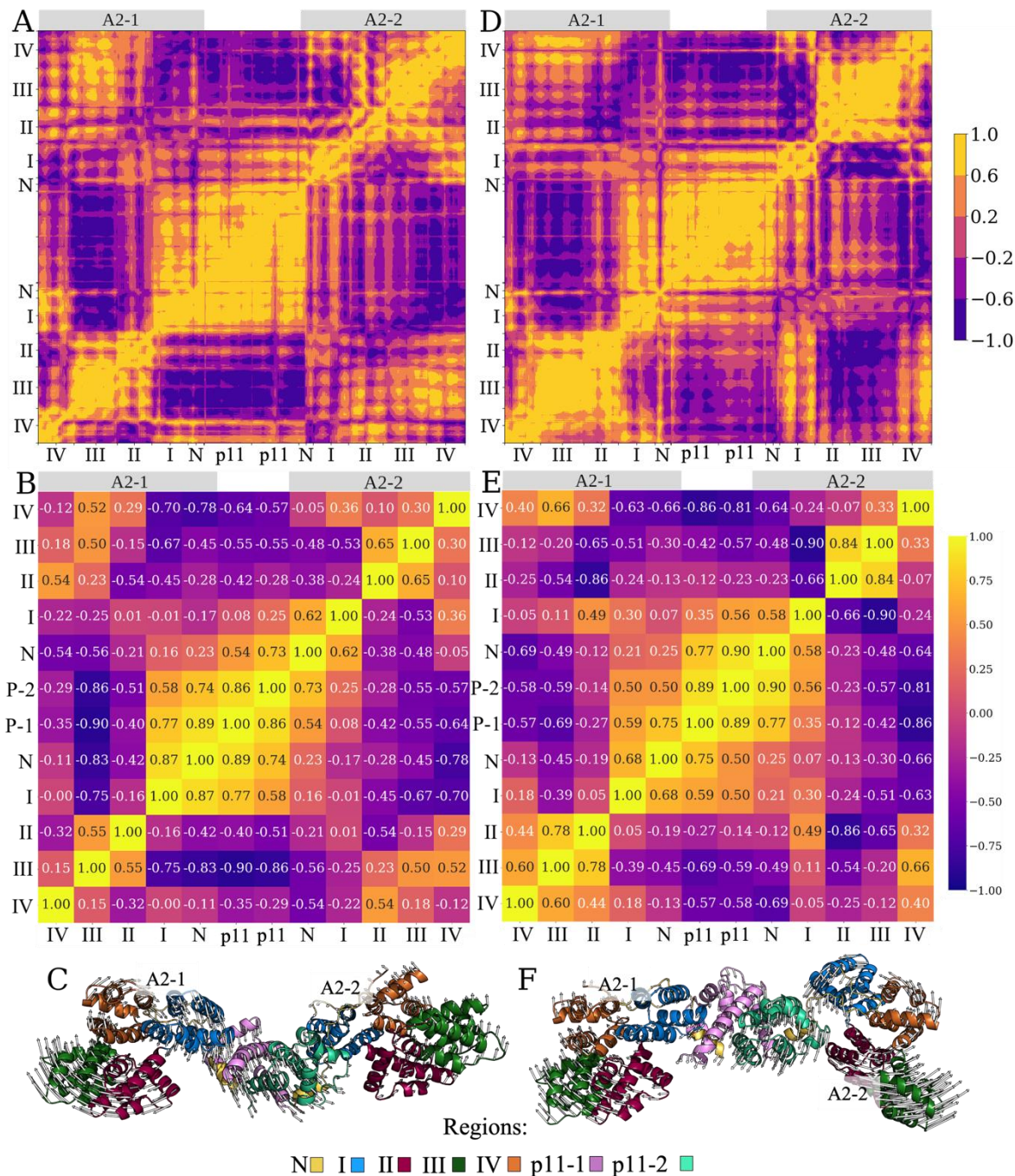


Figure 4.5. Correlation heatmap derived from α -carbons with upper triangle calculated using 20-320 ns and lower triangle calculated using 320-620 ns, (B) Correlation heatmap derived from region centers of mass, (C) and graphical depiction of normal mode 1 for the 7Ca model. (D) Correlation heatmap derived from α -carbons with upper triangle calculated using 20-320 ns and lower triangle calculated using 320-620 ns, (E) Correlation heatmap derived from region centers of mass, (F) and graphical depiction of normal mode 1 for the 0Ca model.

4.3.3. Internal Interactions

4.3.3.1. Interactions at the Interface of A2t Subunits.

Pairwise MMGBSA calculations were run to determine the strong interactions at the interface of each subunit of both the 7Ca and 0Ca models.¹⁷³ The total contributions of each residue from one subunit to the interaction with the entirety of another subunit were calculated and reported in **Table A.1** for the 7Ca model and **Table A.2** for the 0Ca model, both located in **Appendix A**. The strongest individual residue-residue interactions were also calculated and reported in **Table 4.5** for the 7Ca model and **Table A.3** for the 0Ca model. All results were ranked from strongest to weakest interactions. These results were consistent with experimental results which were found in the literature (**Table A.4**).¹⁵⁰

The top interactions of individual residues with entire subunits were compared with results from the literature detailing the important residues for A2-p11 interaction (see **Table A.4**). All A2 residues (T3, V4, I7, L8, L11) that were found to form contacts with p11 in the literature were present among the top ten strongest interactions for both A2s in the 0Ca and 7Ca models. These interactions are highlighted in bold in **Tables A.1** and **A.2**. The majority of the p11 residues (E6, M9, E10 M13, F14, F42, A82, and Y86) that formed contacts with A2 were also present among the top residues of p11 (see **Tables A.1** and **A.2**). Five residues (F39, L79, C83, F87, M91) were not present among the top interactions, suggesting they have a less significant contribution towards the total interaction between p11 and A2 subunits. Most notably, each N-terminal domain of A2 (A2-N) interacts with both p11s of the dimer; A2-N has important interactions with p11-HI of one subunit and p11-HIV of the other subunit as seen in **Table 4.5** and **S3**.

Table 4.5. Strong Interactions of Residue Pairs between Subunits of the 7Ca Model.

Sub-unit 1	Residue 1	Sub-unit 2	Residue 2	ΔG (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)	Sub-unit 1	Residue 1	Sub-unit 2	Residue 2	ΔG (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)
A2-1	K80	p11-1	D85	-9.9	3.8	p11-1	E10	p11-2	K96	-6.8	3.3
	R77		D85	-7.3	3.2		K92		H17	-5.3	2.4
	D16		K54	-4.5	2.7		E38		M5	-4.6	1.6
	K80		K94	-3.5	1.5		S3		E38	-3.4	1.2
	L13		Q46	-3.0	1.0		F69		N84	-3.2	0.6
	L8		F42	-3.0	0.4		N84		H17	-3.1	0.4
	L11		N45	-2.9	0.9		E38		Q4	-3.0	2.2
	H5		P40	-2.8	0.5		M9		T80	-2.8	0.5
	F73		Y86	-2.7	0.7		N84		F69	-2.7	0.5
	Q76		Y86	-2.5	1.4		T80		M9	-2.6	0.6
A2-1	S2	p11-2	E6	-14.7	7.8	A2-2	S2	p11-1	E6	-19.8	2.1
	S2		E10	-12.3	4.1		S2		E10	-11.5	2.2
	T3		E10	-8.6	1.8		T3		E10	-9.3	1.5
	V4		E6	-5.4	2.1		V4		E6	-6.5	1.1
	H5		E6	-3.2	1.5		H5		E6	-4.4	0.7
	V4		E10	-2.4	0.9		V4		E10	-2.1	0.6
	T3		E6	-1.4	0.9		T3		E6	-1.9	0.6
	V4		M9	-1.4	0.3		V4		M9	-1.5	0.3
	T3		F14	-0.9	0.5		T3		F14	-1.1	0.3
	V4		M13	-0.8	0.2		V4		M13	-1.1	0.2
A2-1	E6	A2-2	S2	0.0	0.0	A2-2	R77	p11-2	D85	-12.6	1.8
	K80		D16	0.0	0.0		R145		D48	-8.4	2.0
	S2		E6	0.0	0.0		D16		K57	-6.5	4.2
	R77		D16	0.0	0.0		S2		K97	-6.1	1.3
	R77		D70	0.0	0.0		E6		K97	-5.2	2.7
	E149		S2	0.0	0.0		L8		Q46	-3.5	0.6
	R77		D34	0.0	0.0		T3		K96	-3.3	0.6
	K80		D70	0.0	0.0		L11		Q46	-3.0	0.6
	R77		E36	0.0	0.0		T3		K97	-2.8	0.5
	R77		E14	0.0	0.0		L8		F42	-2.6	0.6

Not only are our results consistent with those found in the literature but our results identified some novel interactions that have not been reported in the literature. The results of pairwise residue interactions show that the repeat I of the A2 core domain (A2-I) has strong interactions with residues of p11-HIII, p11-HIV, and the flexible p11 C-terminus (see **Tables 4.5**

and **A.3**). Furthermore, the asymmetric motion of A2 observed in the first normal mode (see **Figures 4.5C** and **4.5F**) where one A2 subunit moved more than the other appears to stem from an interaction between A2-R145 and a p11 residue. In the 7Ca model, A2-2-II-R145 interacted with p11-2-hinge-D48 (see **Table 4.5**). In the 0Ca model A2-1-2-II-R145 interacted with p11-1-K97 (see **Table A.3**). In both cases, this additional interaction had a significant impact on the dynamics of the A2t complex as demonstrated by the first normal modes (see **Figures 4.5C** and **4.5F**).

4.4. Conclusions

The major objectives of this study were to computationally model the A2t tetramer, investigate the effects of Ca^{2+} -binding on sites of biological importance, qualitatively describe relative motions of the system's subunits, and describe the energetics driving the interaction amongst the A2t subunits.

It was demonstrated, using PCA, that Ca^{2+} likely has a substantial impact on the structural stability and accessible conformational space of A2t. The Ca^{2+} -binding sites and coordination were compared to those found in a previously solved crystal structure. Reasonable agreement was observed between the coordinations present in the crystal structure and those present throughout the simulation trajectory. Important sites within A2t, the F-actin and PIP binding sites, were investigated for Ca^{2+} -induced morphological differences. Differences were identified at both sites and further investigation revealed the causes of the change in their respective SASA values. The availability of membrane-binding residues A2-K279 and A2-K281 was shown to be limited by interaction with Ca^{2+} -binding residue D339 in the absence of Ca^{2+} . The presence of Ca^{2+} was also shown to impart a preference for what we refer to as the “buried”

state of a residue important for F-Actin binding, A2-D339, where it was found wedged between A2-R304 and A2-R308.

Normal mode and correlation calculations were performed to qualitatively describe the motions within A2t. The results demonstrate that the A2t complex is a highly correlated system. The A2-N, I and p11 regions of the complex form a strongly correlated central region of the system. This strongly correlated region is negatively correlated with the periphery of the complex, A2-II, III, IV. The first normal mode is presented and serves as a visual model for the relationships observed in the correlation plots. Mirror rotations of the A2 subunits toward p11 are observed in the first normal mode.

MMGBSA analysis was used to identify the strongly interacting residues among subunits of A2t complex. We compare our results to descriptions of the A2-p11 interaction found in the literature. Our results demonstrate excellent agreement regarding known interactions and provide a useful estimation of their relative strengths. They also indicate that residues from A2-I, II may interact with the p11 dimer. Residue A2-II-R145 was highlighted as having a significant effect on the dynamics of A2t via its interaction with p11.

Here, results were presented which may implicate previously unrecognized residues as important for functions of A2t. Most significantly, our results suggest that further experimental studies involving the role of A2-R145 in organizing A2t dynamics and the roles of A2-R304 and A2-K308 for facilitating the interaction between A2t and F-Actin are necessary.

CHAPTER 5: Interactions and Conformational Changes of Annexin A2/p11

Heterotetramer Models on a Membrane: A Molecular Dynamics Study

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Title: “Interactions and conformational changes of annexin A2/p11 heterotetramer models on a membrane: a molecular dynamics study”

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Abstract

Ca²⁺-dependent membrane-binding by the Annexin A2/p11 heterotetramer (A2t) plays an important role in various biological processes including fibrinogen activation and exocytosis in neuroendocrine cells. Two models where A2t associates with a single membrane surface were generated and used to perform molecular dynamics simulations. The first model mimics initial A2t-membrane binding through both Annexin A2 (A2) subunits of A2t (TS model) while the second model mimics A2t-binding through a single A2 subunit (OS model). Conformational changes were summarized using principal component analysis (PCA), simulation snapshots, and distance plots from the simulations. The full TS model, including the p11 dimer, fully associates with the membrane adopting a stable structure with little conformational variation as evidence by PCA. The unassociated subunits of the OS model moved toward the membrane. The molecular mechanics/Generalized-Born surface area (MMGBSA) method was applied to investigate the energetics of the models. The MMGBSA results demonstrated that R63 of p11 was the primary contributor to the p11-membrane interaction. The TS model results were both consistent with those found in the literature and provide novel insights about the specific residues driving the A2t-membrane interaction. Additionally, it represents the most complete model of A2t on the membrane surface available.

5.1. Introduction

Annexin A2 (A2) is a membrane-binding protein of the Annexin protein family. Annexins are characterized by their ability to bind negatively charged phospholipid bilayers in a Ca^{2+} -dependent manner.¹⁷⁴ A2 is known to form a heterotetramer (A2t) with a dimer of p11.¹⁷⁵ A2t is involved in a number of biological functions, including membrane repair, membrane bridging, and plasminogen activation.^{176–178} Additionally, A2t has been implicated in various pathologies including cancer and viral infection.^{2,3} A2 is found to be upregulated in many aggressive metastatic cancers. Downregulation of A2 or targeting A2 using A2-specific antibodies has proven effective for selectively killing or otherwise inhibiting the activity of model cancer cells *in vitro*.^{179–183} A2t appears to play an important role in the cellular infection cycle for a number of viruses.^{100,101,108,184} Targeted inhibition of A2t and A2 has potential therapeutic applications for the treatment of both cancer and viral pathologies.

A2t is found in a variety of biological contexts dependent on the function it is performing. Two such contexts are: at membrane junctions and on membrane surfaces.^{61,177} The former has been implicated in the formation of the tight junctions between epithelial cells. The latter conformation has been suggested for A2t's function as a receptor for plasminogen.¹¹ Scanning force microscopy (SFM) has been used to demonstrate that both A2 subunits of the tetramer are associated with the membrane when a single membrane surface is available for binding.⁹ A cryogenic electron microscopy (cryo-EM) study demonstrated that both A2 subunits of A2t are found on opposite sides of the p11 dimer with their convex, membrane-binding faces toward each membrane when found in-between two membranes.⁷

The A2 subunits of A2t are composed of two domains, a C-terminal domain (CD), and an N-terminal domain (ND). The ND is 32 residues long. The CD is composed of four homologous

repeats, I-IV (see **Figure 5.1A**). Each repeat contains five α -helices, A-E (see **Figure B.1A**). The p11 dimer is made of two individual p11 subunits (see **Figures 5.1A** and **B.1B**). Each p11 subunit is composed of two EF-hand repeats which each contain two α -helices (see **Figure B.1B**). The interface between A2 and the p11 dimer sees the A2-ND interact with a cleft formed by the first helix of one p11 and the final helix the other p11 (see **Figure B.1C**). See **Figure B.2B** for a simplified schematic representation of the A2t structure. Each A2 subunit contains seven calcium binding sites, three type II, three type III, and one AB type site. Type II sites are formed by four donor residues, three peptidyl carbonyl oxygens and one D/E residue. Type III sites are formed by three donor residues, two peptidyl carbonyl oxygens and one E residue. The type AB sites are formed between two donor residues, one peptidyl carbonyl oxygen and one E residue.¹

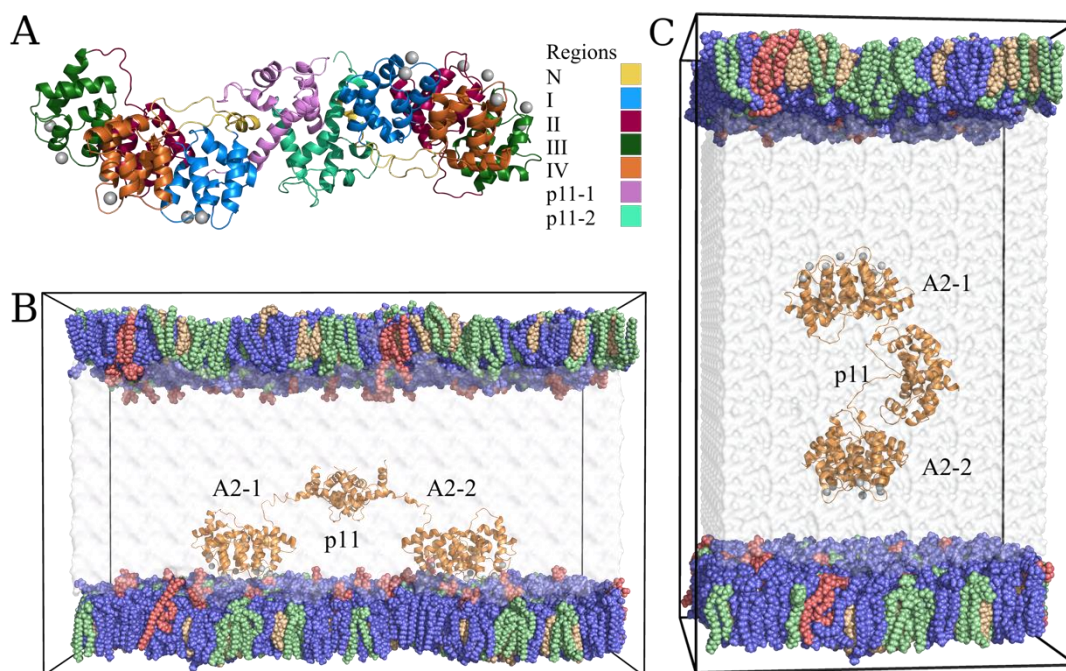


Figure 5.1. (A) Graphical representation of important regions of A2t. Graphical representations of the simulation set-ups for the (B) TS-model and (C) OS-model with the relative orientation of the subunits labeled.

Two molecular dynamics (MD) studies on the interactions of the membrane with the components of A2t were previously reported. The first utilized the CHARMM36 force-field with TIP3P water and focused on the interaction of the Annexin A2 monomer (A2) with a membrane model. It indicated that the convex face of A2 binding was the primary interface for membrane binding and demonstrated that A2 binding directly affects membrane dynamics.^{112,122} The initial orientation of A2 was varied then the binding enthalpies of each conformation were compared to identify which binding-orientation was most favorable. Additionally, lipid height differences pre and post A2-binding as well as lipid order parameters were calculated to demonstrate the direct effect of A2-binding has on the membrane. The second study applied the FF14SB force field and TIP3P waters to investigate the interaction of the CEACAM1-S cytoplasmic domain with an A2t pseudo-tetramer formed by a p11 dimer interacting with A2-ND, and the results showed that the key residue in this interaction is F454 of CEACAM1-SF.¹⁵⁸ Neither of the studies were performed using a full A2t model. The first MD study on the full A2t complex was recently performed by our group using the ff14SB force field and TIP3P waters. It focused on the effects of Ca^{2+} on membrane and F-actin binding sites. It was demonstrated that Ca^{2+} -binding makes key membrane-binding residues more accessible.¹⁸⁵ Previous MD studies involving another member of the Annexin protein family, Annexin A1 (A1), were performed by our research group. Our group investigated the interaction of both the A1 CD and A1 ND with a membrane model using the ff03 force field. MMGBSA analysis was employed to characterize the interaction between the A1 N-terminus and a membrane model.¹¹⁹ Our group also performed a nudged-elastic-band study investigating the conformational change of A1 from its ND buried state to its ND exposed state using the ff14SB force field.¹¹⁷

In this work, molecular dynamics simulations were performed to study A2t-membrane binding in the presence of a single membrane. The aims of this study were twofold. Firstly, we sought to generate full models of two A2t conformations that have been proposed in the literature¹⁶ and determine their stability and consistency with the literature within the context of a single membrane surface. The first model sees one A2 of A2t interact with the membrane (see **Figure B.2A**) while the second sees both A2 of A2t interact with the membrane (see **Figure B.2C**). Our second aim was to investigate the interaction between the membrane and A2t and determine which residues are primarily responsible for driving the interaction.

5.2. Materials and Methods

5.2.1. A2t-Membrane Model Construction.

PyMol was used to build all A2t models.¹⁶⁷ The A2t models were constructed using 1BT6, 1W7B, and 1XJL from the RCSB PDB data bank.^{151,186–188} 1BT6 was used as a scaffold as it contains the A2 ND inside the two pockets of the p11 dimer. These two ND fragments were used to align A2 monomers with p11. The coordinates for A2 were taken from 1W7B. As 1W7B contains no Ca²⁺ cations the locations of the Ca²⁺ cations from 1XJL were used by aligning the model with 1W7B then removing the non- Ca²⁺ coordinates of 1XJL. The A2 ND residues that overlapped with the residues of the A2 ND in p11 were deleted. The pmemd.MPI module of AMBER18 was used to perform a constrained minimization on the A2 ND of the resulting structure to correct any aberrations in the A2t structure.¹⁶¹ The second A2 was added to the structure by duplicating the already inserted A2 and overlaying the duplicate with the second ND fragment in the p11 structure as seen in the protein model in **Figure 5.1C**. A second A2t structure was generated by rotating the ND, without moving the portion associated with p11, such that both A2 were facing the same direction as seen in the protein model found in **Figure 5.1B**.

The membrane used for each of the models was constructed using phosphatidylinositol 4,5-bisphosphate (PIP), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POS), and cholesterol (CHL). Membrane components were built using GaussView09 and their structures were optimized using Gaussian09.¹⁸⁹ The electrostatic potential was obtained using Gaussian 09 at the HF/6-31G* level of theory. The Antechamber program of AMBER18 was then used to generate parameter libraries for each of the lipids. The lipids forcefields generated by our lab were utilized in preference of those packaged with AMBER18 to preserve consistency with the force fields utilized in previous studies conducted by our lab. The membranes used in this study were constructed using scripts written for PyMol. A 20 lipid by 20 lipid system composed of POC lipids was constructed. The lipids of each layer were placed 8 Å apart. Mixed layers were constructed by randomly replacing POC lipids by CHL, POS, or PIP to achieve a ratio of 60:20:17:3 for POC, CHL, POS and PIP, respectively, in each layer. This composition was chosen to mimic that utilized in previous *in vitro* studies.^{16,112} The second layer was generated by flipping the first layer 180 degrees and then translating it vertically such that the head-groups were 70 Å apart from each other. This set-up allowed the lipid tails to interact with each other across the periodic boundary; the membrane was set-up in the same manner as the protein-membrane models seen in **Figures 5.1B** and **5.1C**. The system was solvated using TIP3P waters with sufficient Na⁺ and Cl⁻ to bring the concentration of NaCl to 0.15 M and neutralize the system. The membrane equilibration process followed a procedure outlined in the literature.¹⁹⁰ Briefly, constant pressure dynamics were performed, after a two-step minimization and two-step warming to 310 K, until membrane surface area had been equilibrated.

The full A2t-membrane systems were assembled by combining the A2t structures as well as the equilibrated membranes. Two models were constructed to study A2t-membrane interaction using the equilibrated bilayers and the A2t models. One model is to mimic only one A2 monomer of A2t interacting with a membrane surface (designated as the OS model) seen in **Figure 5.1C**; another model is to simulate the interaction of two A2 monomers of A2t interaction with a membrane surface (designated as the TS model) seen in **Figure 5.1B**. The OS model used the originally constructed A2t model. The TS model used a modified version of the original model where the flexible A2 ND was rotated until both convex A2 CD surfaces were facing the same direction. The layers of the bilayer were separated by 160 Å in the OS model (see **Figure 5.1C**) and 120 Å in the TS model (see **Figure 5.1B**). The proteins for both models were placed 10 Å above the head groups of the bottom layer of the bilayer. The systems were then solvated using TIP3P waters with sufficient NaCl added to make the concentration 0.15 M and neutralize any extraneous charges. The OS and TS models contained 370,144 and 603,078 atoms, respectively.

5.2.2. Simulation and Analysis Details.

The simulations used the ff14SB force field for the protein including explicit TIP3P waters as a solvating medium.^{162,163} The charges and force field parameters for all the lipid molecules were generated using Gaussian 09, AMBER's Antechamber program, and the GAFF force field.^{164,191} The pmemmed.MPI module of AMBER18 was used to perform minimization and warming steps. Constant pressure dynamics were performed using the pmemd.cuda module of AMBER18. Periodic boundary conditions were applied to the system with a non-bonded cutoff of 10 Å to truncate Van der Waals interactions. The particle-mesh Ewald method was used to treat long-range electrostatic interactions, with a cubic B-spline interpolation.¹⁹² The SHAKE algorithm was used to constrain all bonds involving hydrogen atoms, which allowed the usage of

a 2-femtosecond time step. The systems were minimized in two steps, a solute-restrained step with 500 kcal mol⁻¹ force constant position restraints followed by an unrestrained step. In both steps, 500 steps of steepest descent minimization were followed by 1500 steps using the conjugate gradient method. Minimization was followed by warming to 310 K for systems containing membranes in two steps. Subsequently NPT dynamics were performed to equilibrate the density of the systems. Finally, production simulations were performed using NPT dynamics with anisotropic Berendsen regulation at 1 atm and Langevin thermostat with a collision frequency of 1 ps⁻¹.

Most analyses were performed on the simulations using the cpptraj module of AMBER18. Free energy of binding calculations were performed using the MMPBSA.py script, another component of AMBER18.¹⁶¹ MMGBSA was run using a modified generalized born model with a salt concentration of 0.15 M.¹⁹³ Decomposition was utilized to attain energies on a pairwise per-residue basis. Decomposition was not utilized for the values reported in **Figure B.4**. Data were prepared using a combination of bash shell scripts, Linux command-line utilities, and Python scripts. All figures were generated using the matplotlib Python module and the PyMol molecular visualization software.^{167,194} Movies were generated using the Molecular Nodes plugin for Blender.^{195,196} The GNU Image Manipulation Program and Microsoft PowerPoint were used to assemble image components into final visualizations.

5.3. Results and Discussion

5.3.1. Conformational Change of the TS Model

The overall conformational change of the TS model from its initial state is represented in **Figure 5.2**. The TS model undergoes a rapid conformational change (see **Figures 5.2A** and **5.2B**). Both A2 subunits of the TS model begin nearly associated with the surface of the

membrane, ~ 10 Å from the surface while the height of the p11-dimer center of mass (COM) is ~ 55 Å relative to the surface of the upper leaflet; height was determined using the difference between the COM y-coordinate of the upper-leaflet phosphate atoms and the COM of the p11-dimer. Within the first 100 ns of simulation the p11 dimer comes to associate with the surface of the membrane. The height of the p11 dimer COM relative to the membrane surface over the course of the trajectory is seen, colored blue, in **Figure 5.3**. The difference between the initial position and the equilibrium distance is ~ 35 Å. A movie of the TS simulation trajectory is provided (see **TS Movie** in **Appendix B**). The most striking feature of which is the reorganization of the p11-dimer. It can be seen that the p11-dimer reorients with respect to the A2 monomers before rapidly descending toward and associating with the membrane. Stable association of the p11-dimer with the membrane and limited internal reorganization of the A2t complex is then observed for the remainder and majority of the simulation.

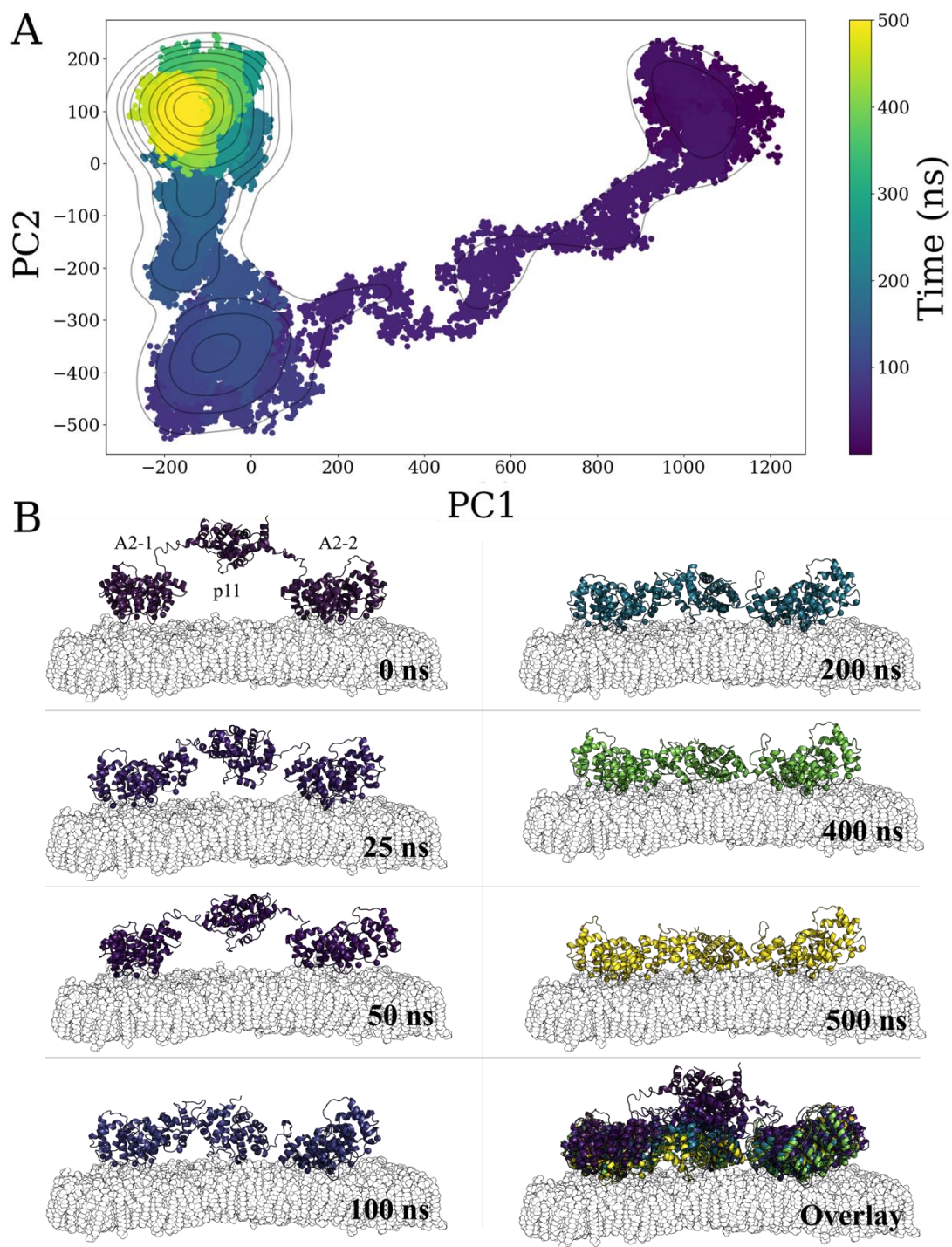


Figure 5.2. Structural summary of the TS model trajectory using (A) projections of each frame onto the first two principal components with color representing the time-scale and a kernel-density estimate plot overlain to visualize the density of points and (B) representative structures from throughout the trajectory.

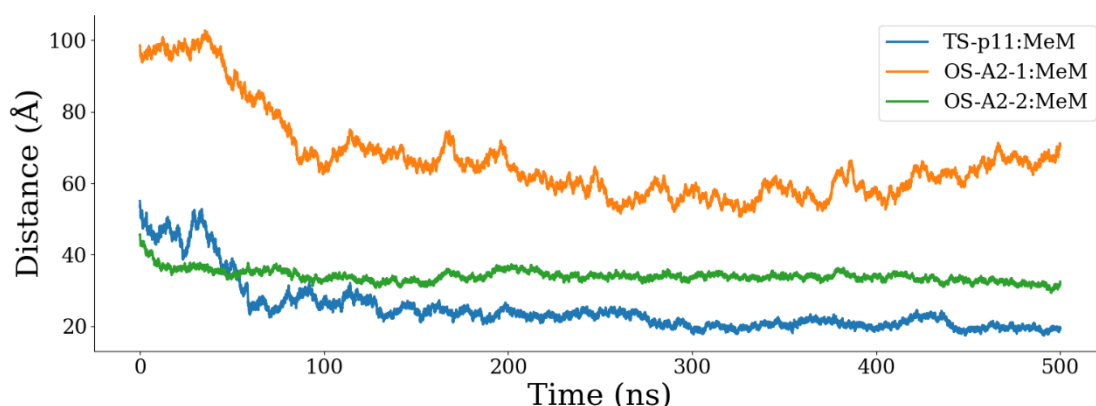


Figure 5.3. Height of A2t subunit component center of mass from the surface of the upper-leaflet of the MeM; TS-p11 (blue), OS-A2-1 (orange), and OS-A2-2 (green).

A scanning force microscopy (SFM) study investigated the height of A2t aggregated on the surface of monolayer membrane models. The determined heights ranged from ~ 18 – 30 Å with SFM mode-dependent averages of 20 and 26 Å for intermittent contact and contact modes, respectively.⁹ A reproduction of these measurements was generated using a procedure described extensively in the Supporting Information. Briefly, the solvent-accessible residues were identified using a PyMol script. The top residues were manually selected using the PyMol GUI. Then the heights of each residue over the trajectory from the upper leaflet of the membrane were calculated. The heights of each selected residue across the trajectory were averaged for each frame. The height of the p11 dimer stabilized after ~ 200 ns. The metrics for comparing to the SFM data were extracted using the average height values after 200 ns of simulation. The mean value 27.63 Å (SD = 0.83) agrees well with the contact mode measurement. This suggests that the presented TS model is a reasonable spatial representation of A2t on a monolayer membrane surface.

5.3.2. Conformational Change of the OS Model

The conformational change of the OS model during the simulation is presented in **Figures 5.4A** and **5.4B**. The OS-A2-1 subunit of the complex rapidly moves down toward the

membrane “leaning” over the OS-A2-2 subunit. It continues leaning toward the membrane until 300 ns.

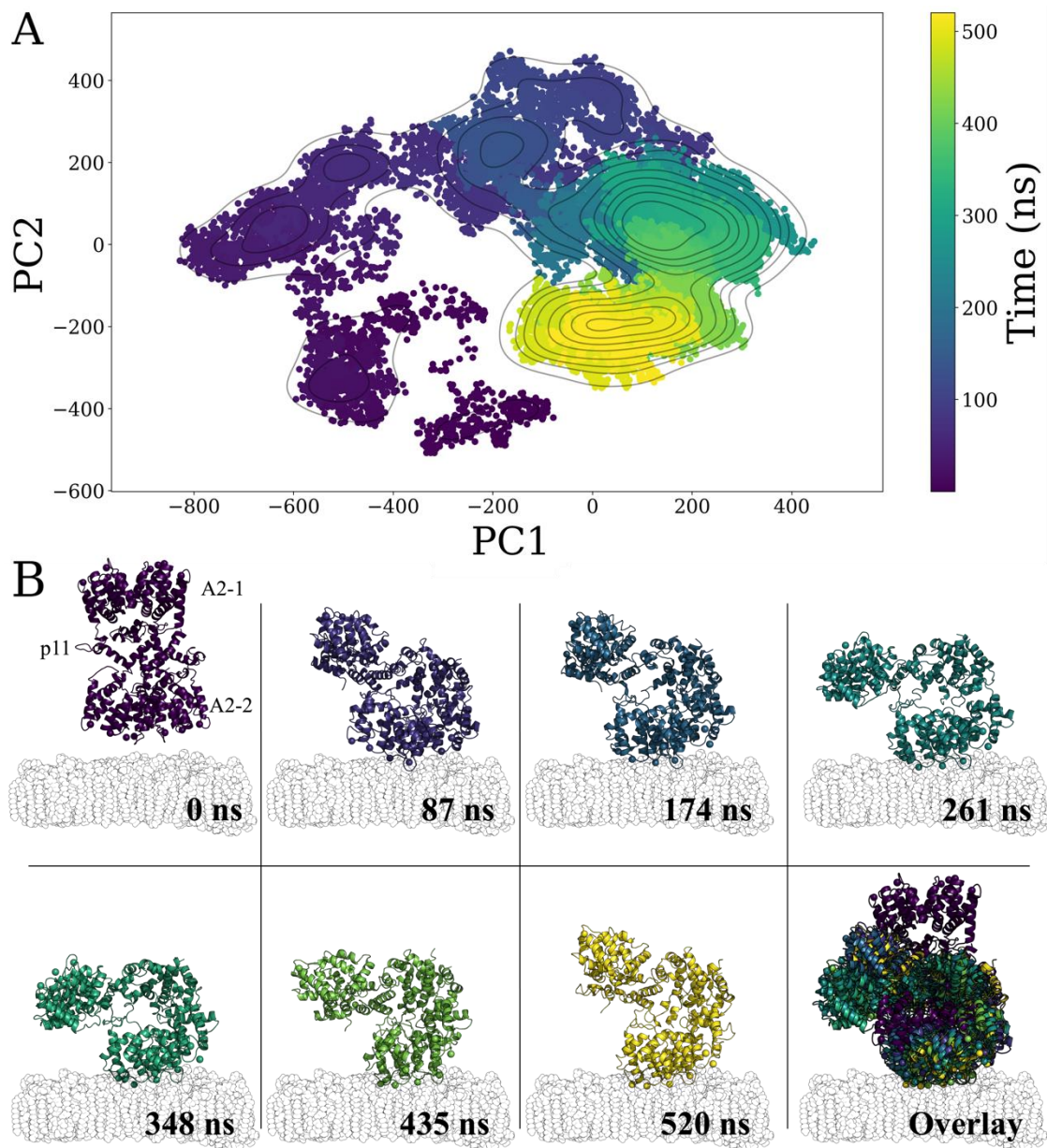


Figure 5.4. Structural summary of the OS trajectory using (A) projections of each frame onto the first two principal components with color representing the timescale and a kernel-density estimate plot overlay to visualize the density of points and (B) representative structures from throughout the trajectory.

The COM of OS-A2-2 began ~ 36 Å from the membrane surface at the start of the trajectory. It moved downward to interact with the surface of the membrane. From its initial position OS-A2-2 drops ~ 12.5 Å (see **Figure 5.3**). OS-A2-1 undergoes a more dramatic shift relative to the surface of the membrane. The COM of OS-A2-1 is initially ~ 89 Å from the upper leaflet. It drops 35 Å within the 50-100 ns time range. Subsequently, it drops another 10 Å closer to the membrane within 300 ns then drifts 10 Å away by the end of the simulation (see **Figure 5.3**). Another high-quality movie of the OS simulation trajectory is provided (see **OS Movie in Appendix B**). Movement of the A2-1 subunit towards the A2-2 subunit can clearly be observed.

The simulation of OS model illustrated that A2t tetramer gradually bends, and A2-2 moves toward the lower membrane due to the interaction with the first monomer in A2t tetramer (discussed in **Section 5.3.3**). This suggests that in the absence of a second membrane, the second A2 monomer of A2t model will move toward the membrane and may eventually adopt the TS conformation. Further, it suggests that the transition from the OS model to a TS model can be driven initially by interactions within A2t rather than interactions between A2t and the membrane.

5.3.3. Interaction of A2t Models with the Membrane Surface

All subunits of the TS model interact strongly with the membrane surface. The interaction between TS-A2-1 and the membrane is driven by lysine residues near the three types II Ca^{2+} -binding sites (TS-A2-II-K119: -18.29, TS-A2-III-K249: -35.51, TS-A2-IV-K281: -40.99; kcal mol⁻¹) (see **Figure 5.5** and **Table B.1**). This result is consistent with previous experimental findings. Residue K281 has been highlighted as important for phosphoinositide-containing membrane specificity; its absence results in an order of magnitude reduction in the affinity of A2t for membrane models.⁵

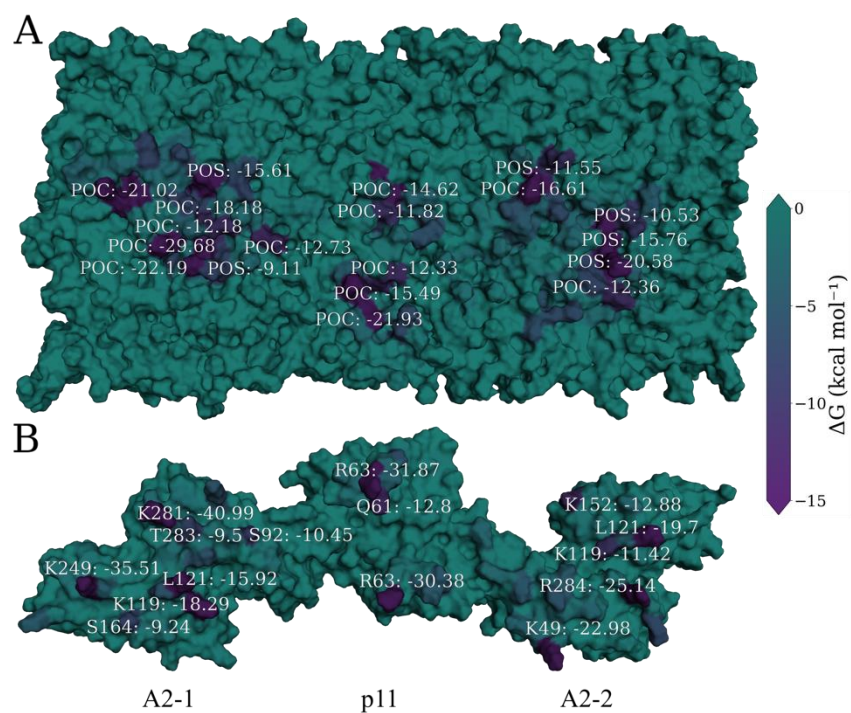


Figure 5.5. (A) Total estimated binding energy of each individual MeM lipid with the entire TS-A2t complex. (B) Total estimated binding energy of each TS-A2t residue with the entire upper-leaflet of the MeM. Residues and lipids are colored according to the binding energy. Lipid and residue names along with binding energies in kcal mol⁻¹ are provided for residues that met a cutoff threshold of $\Delta G < -9.0$.

Table 5.1. Difference Between A2t and A2t Alanine Mutant MMGBSA Binding Energy.

Subunit	Residue	$\Delta\Delta G_{\text{ala}}$ Alanine (kcal/mol)	Standard Deviation (kcal/mol)
A2-1	K279	-1.2	3.1
	K281	-16.3	7.2
A2-2	K279	0.0	0.1
	K281	-6.1	6.7
p11-1	R63	-21.9	4.5
p11-2	R63	-9.2	8.7

The interaction of the TS-p11 dimer with the membrane surface is primarily driven by the two R63 residues of the monomeric p11 subunits. These residues interact strongly with the membrane (TS-p11-1-R63: -31.87, p11-2-R63: -30.38; kcal mol⁻¹) (see **Figure 5.5** and **Table B.1**). To our knowledge, this interaction has not previously been reported in the literature.

The interaction of TS-A2-2 with the membrane is also driven primarily by lysine residues though only one is found near a type II Ca²⁺ site (TS-A2-2-II-K152: -12.88; kcal mol⁻¹) while the other two (TS-A2-2-II-K119: -11.42, TS-A2-2-I-K42: -22.98; kcal mol⁻¹) are found near type AB and type III Ca²⁺ binding sites, respectively. There are two more notable strong interactions between TS-A2-2 and the membrane (TS-A2-2-II-L121: -19.7, TS-A2-2-III-R284: -25.14; kcal mol⁻¹) (see **Figure 5.5** and **Table B.1**). To our knowledge, the contribution of non-polar residues at the surface of A2 have not been reported experimentally. Further, the R284 residue is near two other residues of known importance, K279 and K281; it may also be an important contributor to A2-membrane interactions. It should be noted that the achievable timescale of atomistic MD simulations limits the movement of lipids which can be observed; the difference between the strongest interactions for TS-A2-1 and TS-A2-2 may be explained by the difference in local compositions of lipids at sites of TS-A2-membrane interaction. Previously reported lateral diffusion coefficients for lipids in atomistic molecular dynamics simulations and experiments are on the order of 10⁻⁹ cm² s⁻¹ or more appropriately 10 Å² μs⁻¹.¹⁹⁷ This means that within the 0.5 μs of simulation the constituent lipids the simulation system would not have been able to travel significantly with respect to the A2 subunits which occupy an ~3000 Å² area.

In the OS model all residues that interact strongly with the membrane, except for one (OS-A2-II-L121: -13.25; kcal mol⁻¹), are in repeats III and IV. OS-A2-II-L121 belongs to a type II site along with three other residues found in repeats III and IV (OS-A2-III-R206: -37.04, OS-

A2-IV-279: -23.22, OS-A2-IV-281: -46.1; kcal mol⁻¹). The remaining residues that interact strongly with the membrane are found near the type III site in OS-A2-III (OS-A2-III-R245: -30.68, OS-A2-III-K246: -22.13, OS-A2-III-K249: -25.89; kcal mol⁻¹) (see **Figure 5.6** and **Table B.2**). As has been previously highlighted in this paper, residues K279 and K281 are known to be extremely important contributors to the interaction between A2t and phosphoinositide-containing membrane models used in *in vitro* experiments.

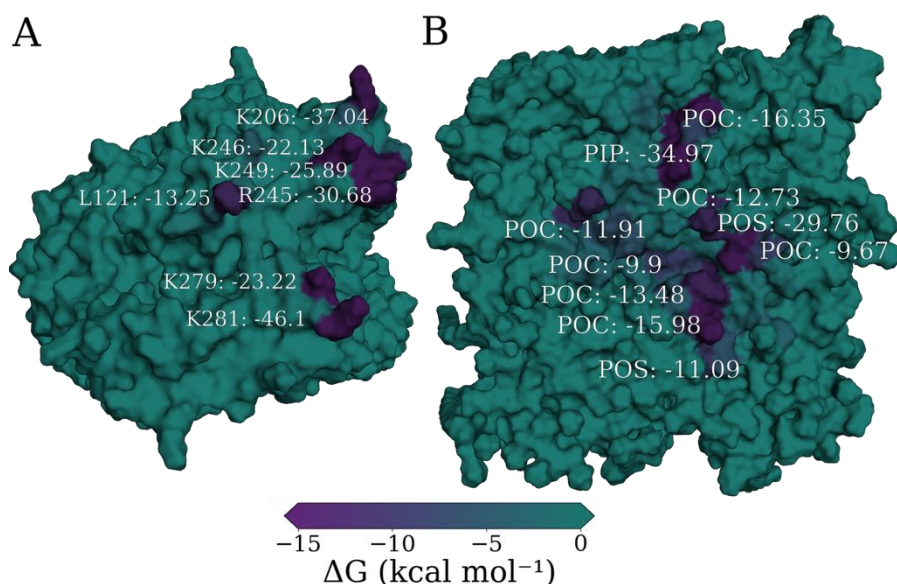


Figure 5.6. (A) Total estimated binding energy of each OS-A2t residue with the entire upper-leaflet of the MeM. (B) Total estimated binding energy of each individual MeM lipid with the entire OS-A2t complex. Residues and lipids are colored according to the size of the binding energy. Lipid and residue names along with binding energies in kcal mol⁻¹ are provided for residues that met a cutoff threshold of $\Delta G < -9.0$.

The contributions of K279, K281, and R63 to membrane binding for the TS model were further investigated via MMGBSA alanine screening. $\Delta\Delta G_{\text{ala}}$ was defined as the difference between the ΔG from an unmutated trajectory and a trajectory where the residue of interest was mutated to an alanine. It was found, as is seen in the pairwise binding affinity results, that K281 and R63 are primary contributors to A2t-membrane binding as their corresponding mutants saw significant reductions in binding energy (see **Table 5.1**). K279A mutation had little to no effect

on membrane binding. Given the known importance of K279 residue for binding it seems likely that the initial orientation of the system was such that the K279-membrane interaction was not particularly favorable. The OS model results lend further credence to this as the pairwise MMGBSA results demonstrate that A2-279 is a primary contributor to the A2t-membrane interaction.

The distinct conformational change of the TS model is primarily driven by interactions with the membrane. This is understandable within the context of the binding affinity results for the individual residues. For the OS model, no appreciable interaction was found between any residues of the OS-A2-1, the A2 which moves toward the membrane, with the membrane from the initial pairwise MMGBSA results though OS-A2-1 does move toward the membrane. Subsequently, MMGBSA was performed for each combination of individual system components, membrane, A2-1, A2-2, p11-1, and p11-2, at regular intervals throughout the simulation and revealed that the observed conformational change is driven by internal interactions within A2t. A2-1 has no interaction with the membrane throughout the simulation but does interact with A2-2 (see **Figure B.4**). Of all the OS-A2t subunits only OS-A2-2 has an appreciable interaction with the membrane. The interactions driving the downward motion of A2-2 were further probed by running pairwise MMGBSA for the residues of OS-A2t using 200 frames over the interval of 336 to 355 ns, roughly centered around image 5 in the eight-image sequence of **Figure 5.4B**. The top 10 pairwise interactions with each of the other subunits are reported in Table S3. The A2-1 interaction profile with the p11-dimer is as expected; the strongest of the pairwise interactions between residues from the p11 helices immediately adjacent to A2-1-ND. The residues that contribute to the top interactions between the A2 subunits come from A2-1-ND and the flexible loop of A2-2 which connects repeats II and III (see **Table B.3**). This is consistent with both

Figure 5.4B and the movie, **OS Movie**, found in **Appendix B**. In both visualizations these two regions can be seen reaching toward each other.

The contributions from each major amino acid with the membrane were also investigated using the results from the pairwise MMGBSA analysis. As shown in Figure S5, for both models, lysine residues contribute the most to the A2t-membrane interaction. For the OS model arginine, glycine, and leucine residues also contribute modestly to the A2t-membrane interaction. For the TS model the same residues are important contributors to the interaction, but the combined strength of their interaction is greater than that of any found in the OS model. In addition to those residues already mentioned, the TS model has major contributions from threonine and serine residues.

5.4. Conclusions

Molecular dynamics simulations for full A2t models in the presence of lipid bilayers were performed. Two models of A2t, one with a single A2 subunit associated with the membrane (OS model) and another with both A2 subunits associated with the membrane (TS model) were generated. In the OS model, the A2 farthest from the membrane surface moved ~ 35 Å closed to the membrane surface. This movement was not driven by A2t-membrane interactions but rather by intra A2t interactions between the A2-1-ND and the flexible loop connecting repeats II and III of A2-2. In the TS model, both A2 subunits began near the membrane with the p11 dimer ~ 55 Å above the membrane. The p11 dimer rapidly associated with the membrane resulting in the entire TS model being flush on the membrane. The resulting TS conformation agreed with scanning force microscopy measurements found in the literature suggesting that it is a reasonable model of A2t binding with a membrane.

Pairwise MMGBSA calculations were performed between the A2t system and the associated membrane. It was found that the A2t interaction with membrane for the OS and TS models was driven primarily by basic residues at the convex surface of A2 subunits. Agreement was seen with experimental results as well; known membrane-binding residues A2-K279 and A2-K281 demonstrated strong interaction with the membrane. The p11 dimer of the TS model also exhibited strong membrane-binding capacity. The p11-membrane interaction was driven by residue R63. Further, the movement of OS-A2-1 toward OS-A2-2 the motion was determined to be the result of ND residues interacting with the flexible loop connecting repeats II and III of A2-2.

The TS model serves as the first comprehensive model of A2t on the surface of a membrane. Both its dimensions and membrane-interactions are consistent with those that have previously been put forth in the literature. This serves to support that the TS model is a reasonable and accurate representation of A2t. The exploration of an alternative initial conformation, in the form of the OS model, serves to indicate that A2t on the surface of a monolayer will move toward a conformation where unassociated A2t subunits are closer to the membrane model. Previously unidentified interactions that appear to be critical for A2t-membrane association were also identified. All the previous serve to further the current understanding of the mechanism of A2t-membrane association and the orientation that is adopted on a membrane surface.

CHAPTER 6: Computational Investigation of Potential Annexin A2-Membrane Binding

Inhibitors

Abstract

Annexin A2 has previously been demonstrated as a viable target for the selective treatment of aggressive metastatic cancers making it a desirable target for small-molecule inhibition. Virtual screening was performed for the PI(4,5)P2-specificity site of Annexin A2 using a library of approximately 180,000 natural products and all FDA-approved drugs. Molecular dynamics simulations were performed for the top 20 ranked molecules to assess the stability of their binding. Further MD simulations were performed for five of the most promising candidates in the presence of Ca^{2+} and a lipid membrane. It was found that three of the five ligands had a reproducible impact on Ca^{2+} -binding, reliably displacing one or more Ca^{2+} -ions. Additionally, two of the ligands demonstrated some capability to interfere with the association of the PI(4,5)P2-specificity site and the membrane model. The identified ligands serve as two potential inhibitors of Annexin A2-membrane binding.

6.1. Introduction

Annexins are a superfamily of peripheral membrane-binding proteins that associate with phospholipid membranes in a Ca^{2+} -dependent manner. Annexin A2 (A2) is one of the most thoroughly studied members of the Annexin family. Human annexins all share many structural characteristics. Every annexin, except Annexin A6, is composed of four homologous repeats of approximately 70 amino acids, denoted as RI – RIV (see **Figure 6.1B**). Each repeat can be further subdivided into five alpha helices A-E (see **Figure 6.1A**). Annexins also have unique N-terminal domains (ND) which vary widely in form and function. In A2 the ND is 32 residues in length and serves as the binding interface for forming heterotetramers with S100 proteins (see **Figure 6.1B**).¹

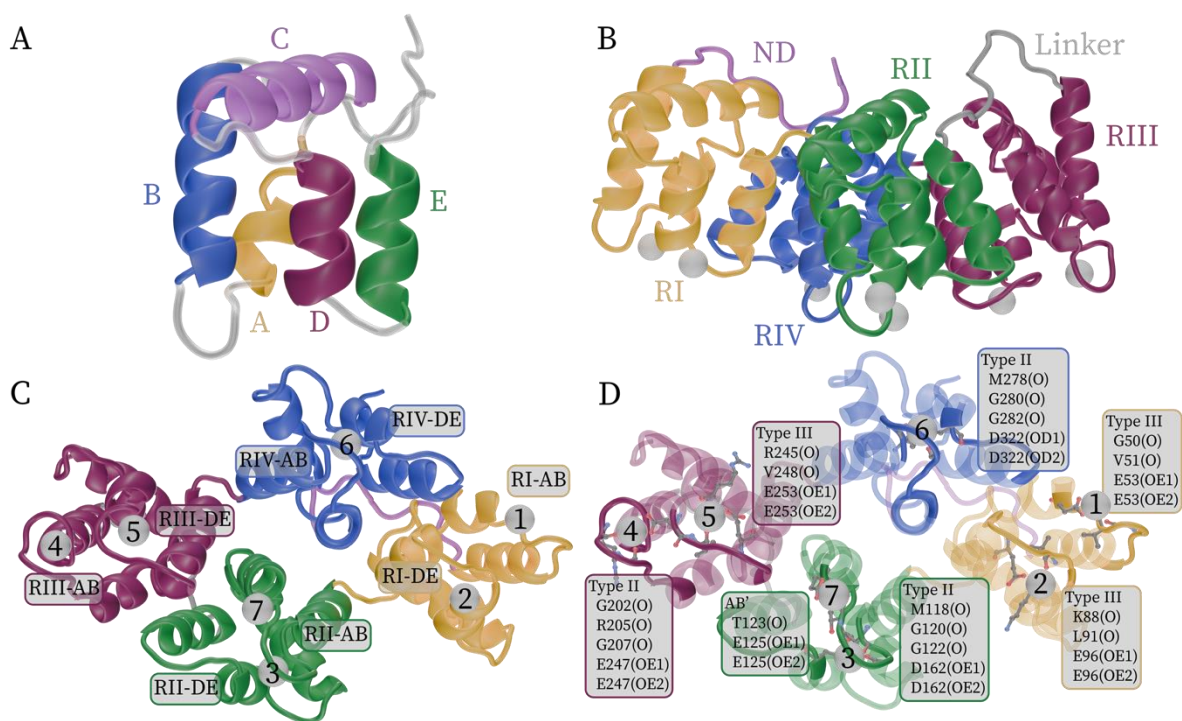


Figure 6.1. Ribbon diagrams of (A) RI of A2 with helices labeled using color coded letters. (B) Full A2 structure with the ND, RI-RIV, and Linker labeled using color-matched text. (C) Full view of the convex face of A2 with Ca^{2+} ions and loops labeled. (D) Full view of the convex face of A2 with Ca^{2+} -binding sites shown using a ball-and-stick representation. Each Ca^{2+} -binding site is labeled with its site type and a list of coordinations. Across images, Ca^{2+} ions are shown using white spheres.

Annexin A2 contains three unique types of Ca^{2+} -binding sites, types II, III, and AB'. Each site contains three, two, and one carbonyl oxygens, respectively, in addition to a single bidentate acidic residue, E/D for type II sites and E for type III and AB' sites. A2 contains three of both Type II and III sites and a single AB' site for a total of 7 Ca^{2+} -binding sites (see **Figures 6.1C** and **6.1D**).⁶⁷ It has been hypothesized that in the absence of Ca^{2+} -binding that basic membrane-binding residues are captured by acidic Ca^{2+} -binding residues.⁴ This assertion has been supported by molecular dynamics simulations conducted by our lab.¹⁸⁵

Mis-regulation of A2 has been implicated in many aggressive, metastatic cancers.² There are two primary mechanisms by which A2 has been demonstrated to aid the propagation of the cancers. The first is via usage of the redox active C8 residue to neutralize reactive oxygen species (ROS). This mechanism was observed by Madureira et al who first investigated the role of A2 in cellular redox regulation.¹⁹⁸ In subsequent studies Madureira et al demonstrated that after the introduction of ROS, A2 migrates toward the nucleus ostensibly to protect the integrity of DNA as an antioxidant.⁶⁹ Many cancer treatments such as chemotherapies rely upon the activity of ROS making upregulated A2 a barrier to effective treatment.¹⁹⁹ The second method of enhancing cancer proliferation is an A2-facilitated increase in the epithelial to mesenchymal transition (EMT).²⁰⁰ EMT is a necessary process for metastasis to occur.²⁰¹ In addition to cancers, A2 has also been implicated in the infection cycle of a number of viruses that include human papillomavirus, respiratory syncytial virus, and avian influenza A virus.^{3,100,103,107} A2 has been identified in a number of viral infection cycle steps including viral entry as well as later steps such as assembly.³ Common to the role of A2 in EMT and its role as a secondary or primary receptor in viral infection cycles is the need for A2 to associate with the cellular membrane. This makes inhibition of A2-binding an attractive method for treating multiple pathologies.

Targeted inhibition of A2 via either siRNA or A2-specific antibodies has yielded promising results. In the case of ovarian cancer, it had a significant impact on the reduction of tumor size within mouse models.²⁰² Further, A2(-/-) mouse models have been shown to have a normal developmental cycle making it an extremely promising therapeutic target; A2 inhibition has a disproportionate effect on cancer cells when compared to healthy cells.¹³ Efforts have already been made toward exploring A2-inhibitors as therapeutics by developing ligands that target the A2-S100A10 interaction. These efforts have yielded tri-substituted 1,2,4-triazoles as inhibitors of the A2-S100A10 interaction.¹⁵ They disrupt A2t formation by associating with the A2-ND binding site within the p11 dimer. Further, they have been demonstrated to prevent human papillomavirus infection in model systems.¹¹⁰

Given the importance of membrane-association for A2's functions it seems reasonable that a ligand targeting A2-membrane association might also have therapeutic applications. A site containing major contributors to A2-membrane binding, K279 and K281, has been previously identified in the literature. Additionally, this site was identified as playing a role in the specificity of A2 for phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2).⁵ This work sought to identify novel candidate ligands for disrupting the interaction between A2 and phosphoinositide-containing membranes via computational methods. We identified candidates that disrupted Ca^{2+} -binding at the PI(4,5)P2 site and the A2-membrane interaction in molecular dynamics (MD) simulations.

6.2. Methods

6.2.1. *Virtual Screening with AutoDock Vina*

Virtual screening (VS) was performed on a 160-core computer cluster composed of eight Intel Xenon processors, housed at East Carolina University. The ligand library was built by retrieving ligands in sdf format from the ZINC database.²⁰³ Openbabel was utilized to prepare the structures for docking by removing nonpolar hydrogens and converting the ligands to the pdbqt format.²⁰⁴ For those ligands which lacked a 3D representation, one was generated and optimized by Openbabel using the associated SMILES and then optimized with the gaff forcefield.¹⁶⁴ Additional information about each ligand was also collected using the curl interface for the ZINC database. Virtual screening was performed by distributing ligands in batches across processors. A structure of A2 (PDBID: 1W7B) was utilized as the receptor for all screening. 1W7B is the x-ray structure of A2 in the presence of Ca^{2+} with the best resolution, 1.52 Å, that is available on the Protein Data Bank.⁶⁷ The PyMol molecular visualization software was utilized to remove all ligands from 1W7B, which was then passed to the 'prepare_receptor' tool from the ADFR Suite to generate a pdbqt file.²⁰⁵ The Python API for Vina was utilized to perform all virtual screening.^{206,207} Exhaustiveness was set to 10 and the top ten poses for each ligand were saved. Averages and standard deviations of the binding energies for a given ligand were saved. Visual depictions of the search region are shown in **Figures 6.2A-6.2C**.

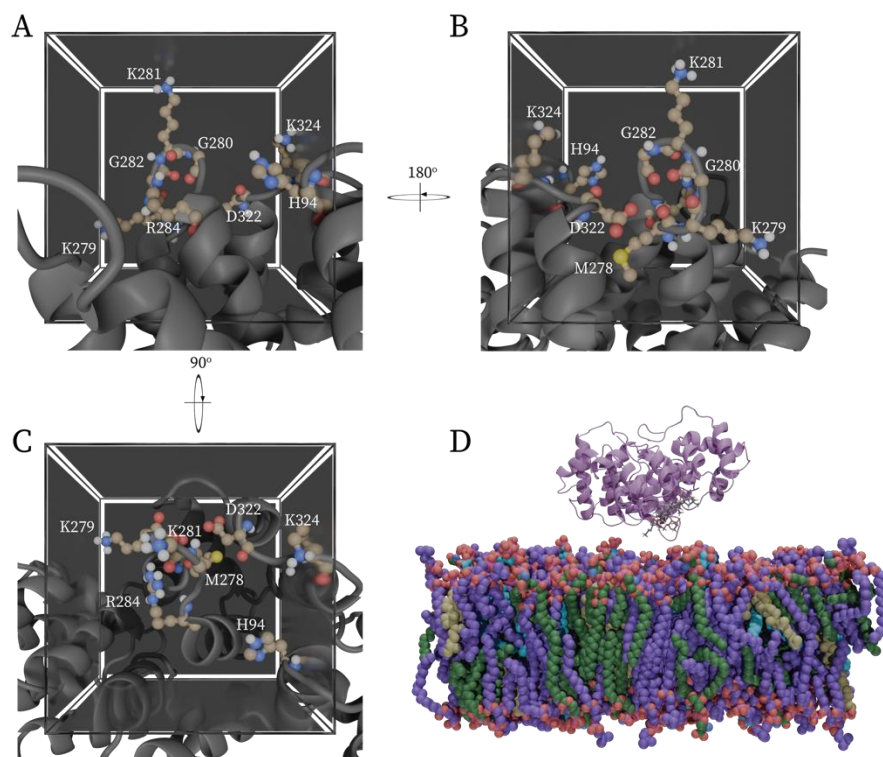


Figure 6.2. (A, B, C) Three views of a cartoon representation of A2 with a box surrounding the screening site and important PI(4,5)P₂-specificity, membrane-binding, and Ca²⁺-binding residues shown explicitly using sticks, colored according to their element identity, and labeled. (B) Structure (A) is rotated 180° about the z-axis. (C) Structure (A) is rotated 90° about the x-axis. (D) A representative example of the initial setup of the membrane system. A2 is shown as a pink cartoon with ligand L17 shown using sticks. Lipids are shown using spheres. The carbons of POPC, POPS, PI(4,5)P₂, and CHOL are colored purple, green, cyan, and yellow, respectively, and all other elements are colored according to their identity. Oxygen is colored red, nitrogen blue, and explicit hydrogens shown on the ligand are colored white.

6.2.2. Simulation Parameters

Openbabel was utilized to add hydrogens to ligand structures as well as convert from pdbqt to pdb format. Antechamber was utilized to calculate the atomic point charges according to the AM1-BCC model and assign atom types appropriate for usage with the GAFF force-field.

The parmchk utility of amber was then utilized to find suitable replacements for any missing parameters.¹⁹¹

Lipids utilized during the membrane simulation included phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and cholesterol (CHOL). Models from previous studies conducted by our lab were leveraged. Each lipid was built and optimized using GaussView09.¹⁸⁹ The electrostatic potential was obtained using Gaussian 09 at the HF/6-31G* level of theory. AMBER18's Antechamber program was then utilized to generate parameter libraries for each of the lipids. The AMBER lipid force-field was not used as it lacks phosphoinositides which are key binding-partners of A2.

6.2.3. Bilayer Generation

The membrane used in this study was constructed using a series of Python scripts for generating membrane systems that is maintained by our lab. A 110 x 110 Å lipid bilayer with 196 lipids in each leaflet was constructed. The composition of the bilayer leaflets was 60:20:17:3 for POPC, CHOL, POPS and PI(4,5)P2, respectively. This composition was chosen to mimic that utilized in previous *in vitro* studies.^{16,112}

6.2.4. A2-Ligand Simulations

. Molecular dynamics simulations were run for each of the top ligands in complex with A2 using the same procedure. The ff14SB protein and GAFF forcefields were used for protein and ligand components of the system, respectively.^{163,164} Systems were solvated with TIP3P waters in an octahedral box with a minimum of 14.0 Å from the solute to the edge of the box.¹⁶² Na⁺ and Cl⁻ ions were added to the system to neutralize it and bring the total salt concentration to 0.15 M. Minimization of the system was performed in two steps. In the first step, solute, A2 and

the associated ligand, were held fixed using 500 kcal / mol $\text{\AA}^2$ position restraints. 500 steps of steepest descent were followed by 500 steps of conjugate gradient minimization. Solute was unrestrained for the second step of minimization of which 2500 steps were performed using the steepest descent algorithm while 7500 steps were performed using the conjugate gradient method. The system was warmed over two steps using dynamics with a constant volume. In the first step the temperature was increased from 0 to 100 K over 10,000 steps with a timestep of 0.5 fs. In the second step of simulation the temperature was raised from 100 K to 310 K over 25,000 steps with a timestep of 2 fs. Heating was followed by a production run of 150 ns for each A2-ligand complex with isotropic pressure scaling. The Langevin thermostat was utilized for all simulations along with a collision frequency of 1 ps⁻¹.²⁰⁸ Hydrogen mass repartitioning was applied to allow for the usage of a 4 fs timestep.²⁰⁹

6.2.5. A2-Ligand-Bilayer Simulations

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6.2.6. Software

All virtual screening was performed using AutoDock Vina. File preparation utilized tools packaged with the ADFR Suite of programs, PyMol, and OpenBabel.^{167,204–207} All molecular dynamics simulations were performed using the AMBER Suite of programs.¹⁶¹ Simulation analysis was performed in JupyterLab using the MDAnalysis, matplotlib, and numpy modules.^{194,210–213} The Molecular Nodes plugin for blender was used to create images of the system.^{195,196}

6.3. Results and Discussion

6.3.1. Ligand Virtual Screening Against the PI(4,5)P Binding Site

Virtual screening was performed against the PI(4,5)P2 site with FDA and Natural Products ligands from AutoDock Vina. The screening site encompassed the PI(4,5)P2-specificity residues. The site contained a total of nine residues that are either directly or indirectly important for membrane binding, these include H94, M278, K279, G280, K281, G282, R284, D322, and K324 (see **Figure 6.2A-6.2C**). The importance of K279 and K281 for PI(4,5)P2 specificity has previously been studied experimentally. Residue R284 was identified as an important contributor for membrane binding in all atomic simulations conducted by our lab.²¹⁴ Being basic residues as

the convex face of A2, residues H94 and K324 could play a role in A2-membrane association.

M278, G280, G282, and D322 compose the Ca^{2+} site 7 of A2 which is important for modulating the availability of K281 and K279.^{67,185}

Table 6.1. Top 20 A2 Ligands from Screening of the FDA and Natural Products Libraries Against the PI(4,5)P2 Binding Site.

Ligand ID	ZINC ID	Average Binding Affinity (kcal/mol)	Std Dev (kcal/mol)	logP	Molecular Weight (g/mol)	H-Bond Donors	H-Bond Acceptors
L1	ZINC000004221938	-160.57	0.42	0.596	529.663	4	7
L2	ZINC000253529804	-113.34	1.10	-3.583	740.664	12	19
L3	ZINC000253389497	-112.13	1.04	0.233	957.117	11	18
L4	ZINC000195771375	-107.30	0.00	-0.002	954.664	13	26
L5	ZINC000514287741	-87.75	0.53	0.382	520.531	4	11
L6	ZINC000958322868	-80.98	0.62	2.867	589.733	6	6
L7	ZINC000253532664	-78.64	1.20	-1.139	977.034	8	15
L8	ZINC000253487389	-64.66	0.47	1.706	942.235	14	17
L9	ZINC000253500604	-62.91	0.47	1.682	941.118	10	16
L10	ZINC000255996515	-51.33	1.16	1.750	857.815	1	20
L11	ZINC000299817838	-50.99	1.02	-1.102	952.648	14	27
L12	ZINC000169356230	-42.69	0.79	2.680	862.064	4	17
L13	ZINC000028536309	-40.02	0.79	2.894	714.761	1	14
L14	ZINC001534514511	-31.98	0.00	2.405	730.937	4	14
L15	ZINC000263583469	-31.45	1.05	2.246	822.942	8	13
L16	ZINC000002157489	-29.12	0.23	2.062	379.434	3	6
L17	ZINC000253389471	-28.61	1.02	1.099	940.134	10	16
L18	ZINC000253501100	-26.19	0.95	-0.250	929.107	11	18
L19	ZINC000253532158	-26.06	0.90	-0.695	804.795	12	18
L20	ZINC000030882164	-23.80	0.56	1.865	303.424	2	3

Ligands were sorted by their average binding affinity and the top twenty ligands were selected for further study. Virtual screening results are summarized in **Table 6.1** and visual representations of all ligands can be found in **Figure 6.3**. Many of the ligands violate Lipinski's Rule of 5, though it has been demonstrated that drugs derived from natural products often violate the rules regarding molecular weight and H-Bond acceptors.^{215,216} However, the logP criterion, $\log P < 5$, and H-bond donor criterion, 5 or less donors, are still important. No ligands have a $\log P > 5$ but over half violate the criterion regarding H-bond donors.²¹⁶ With the advent of generative

deep learning models for creating molecular variants with modified properties, atomic composition, logP, H-bond donors, etc., it seems likely that lead refinement will become easier in the future making the initial lead identification the most important step during drug

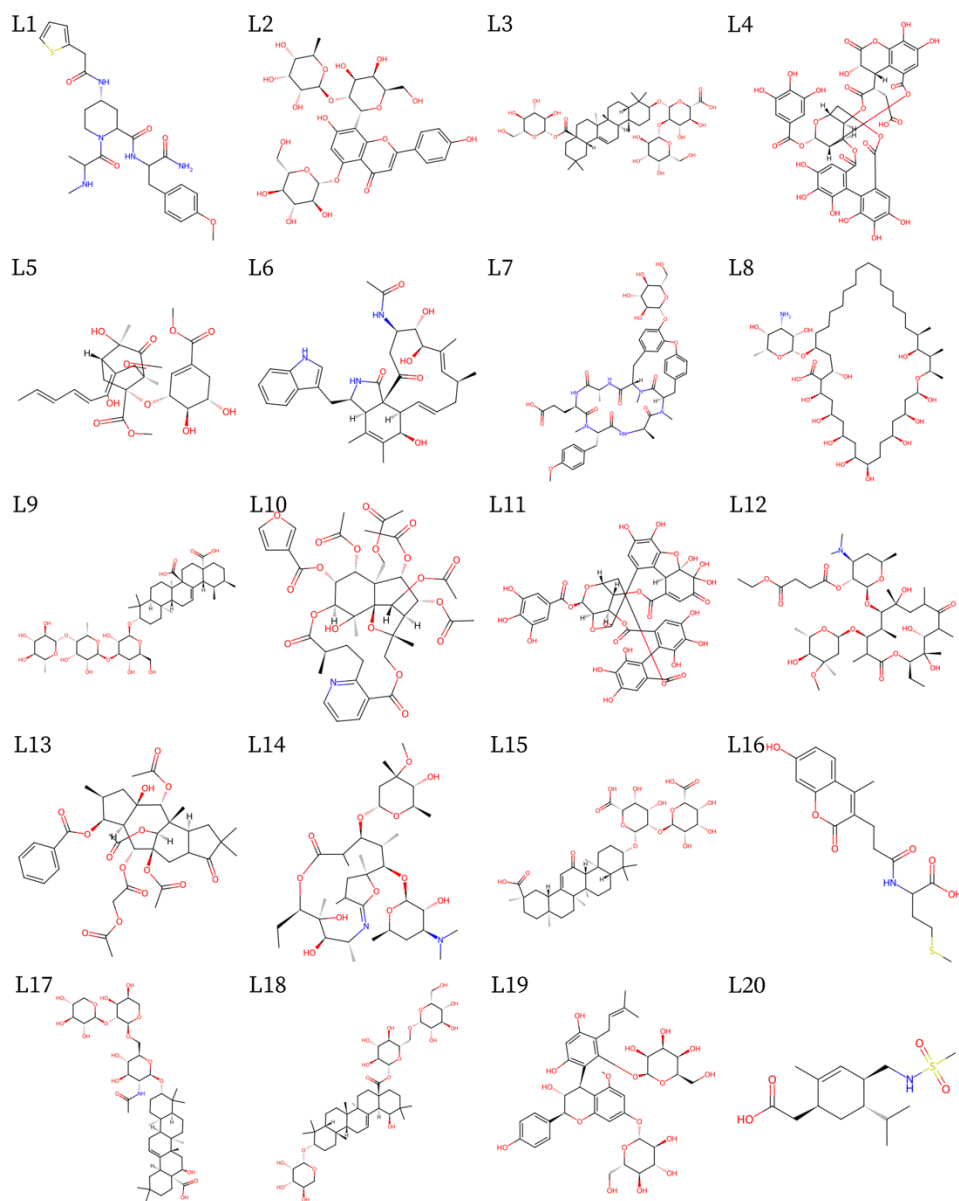


Figure 6.3. 2D Representations of the top 20 ligands identified during virtual screening.

development.²¹⁷

6.3.2. Molecular Dynamics for Assessment of A2-ligand Binding

MD simulations were performed with the top pose from the selected A2-ligand models. The stability of A2-ligand interactions was assessed via ligand RMSD, ligand distance from docking-site, visual inspection of the trajectory, A2-ligand contacts, and binding energies calculated using MMGBSA. Ligand RMSD with respect to the aligned A2 demonstrated that L2, L3, L6, L8, L9, L11, L14, L15, L16, L17, and L19 adopted relatively stable conformations for most of the simulation (see **Figure 6.4**). Ligand distance from the combined RIV-AB and RIV-DE loop center of mass was utilized to identify stably binding ligands that migrated completely away from the screening site (see **Figure C.1**). Of the identified stable binders, ligands L6, L14, and L16 were found to migrate completely away from the screening site. Due to the unstable nature of their binding, ligands L1, L4, L5, L7, L10, L12, L13, L18, and L20 were not considered further.

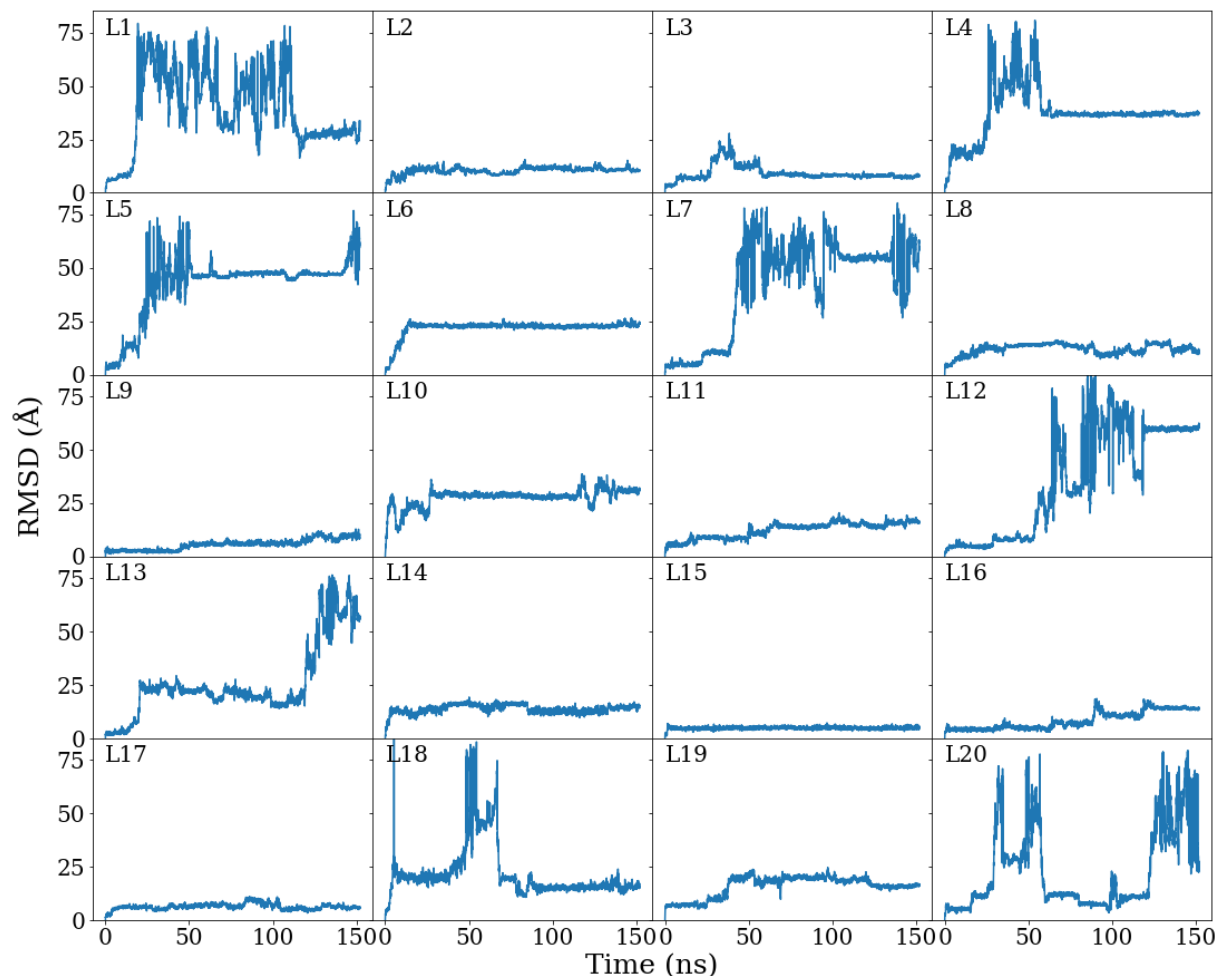


Figure 6.4. RMSD of the ligand with alignment performed using the protein backbone for ligands L1-L20.

The trajectories of the stable binders were visually inspected to verify distance results. Ligand L8 was found to adopt an unrealistic conformation with the large central ring wrapped around K281 and a portion of its associated loop (see **Figure 5**). Because of its unreasonable conformation, ligand L8 was not considered further. Ligands L6, L14, and L16 were found to migrate from their original locations (see **Figure 5**). The migration of Ligand L6 was most dramatic, leaving the convex surface entirely for the interface of helices RI-B and RIV-E where it remained in a relatively stable conformation. Ligands L14 and L16 stabilized in between helices RI-E, RII-A, and RIV-B at the interface of RI, RII, and RIV, still proximal to the

PI(4,5)P2 site. Ligands L2, L3, L9, L11, L15, L17, and L19 were found to have stayed near original docking site with ligands L15 and L17 adopting exceptionally stable conformations (see **Figure 5**).

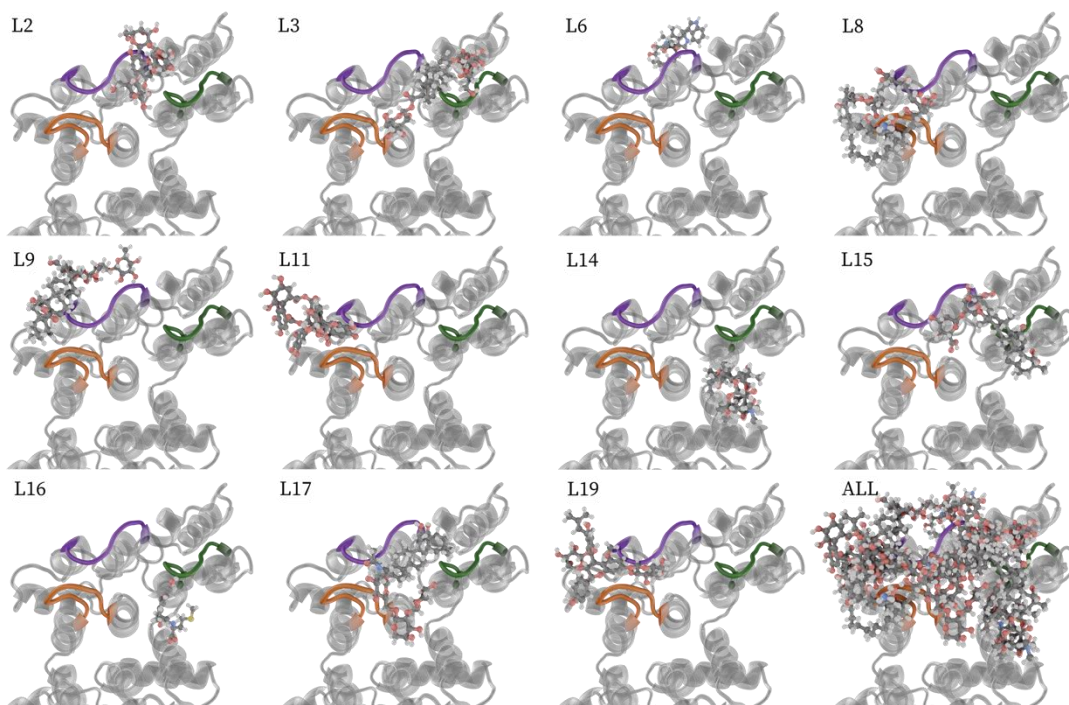


Figure 6.5. A2 in complex with ligands L2, L3, L6, L8, L9, L11, L14, L15, L16, L17, L19, and all ligands. A2 is depicted using a cartoon representation and ligands stick representations. A2 is colored grey and partially transparent except for the RI-DE, RIV-AB, and RIV-DE loops which are colored green, orange, and purple, respectively. Ligands are colored according to element with carbon, oxygen, nitrogen, and hydrogen colored grey, red, blue, and white, respectively.

Contact analysis was utilized to identify the most important residues for all the stably binding ligands (see **Figure 6.6**). Three regions of A2 account for most of the A2-ligand contacts, the RI-DE, RIV-AB, and RIV-DE loops. In addition to **Figure 6.6**, this can be seen in **Figure 6.5**, where each of the loops are highlighted individually. Each of the stably binding ligands primarily bound to the loops of interest or peripheral to the site of interest, except for ligand L6 which migrated entirely away from the convex site to adopt a stable conformation at the interface of helices RI-B and RIV-E.

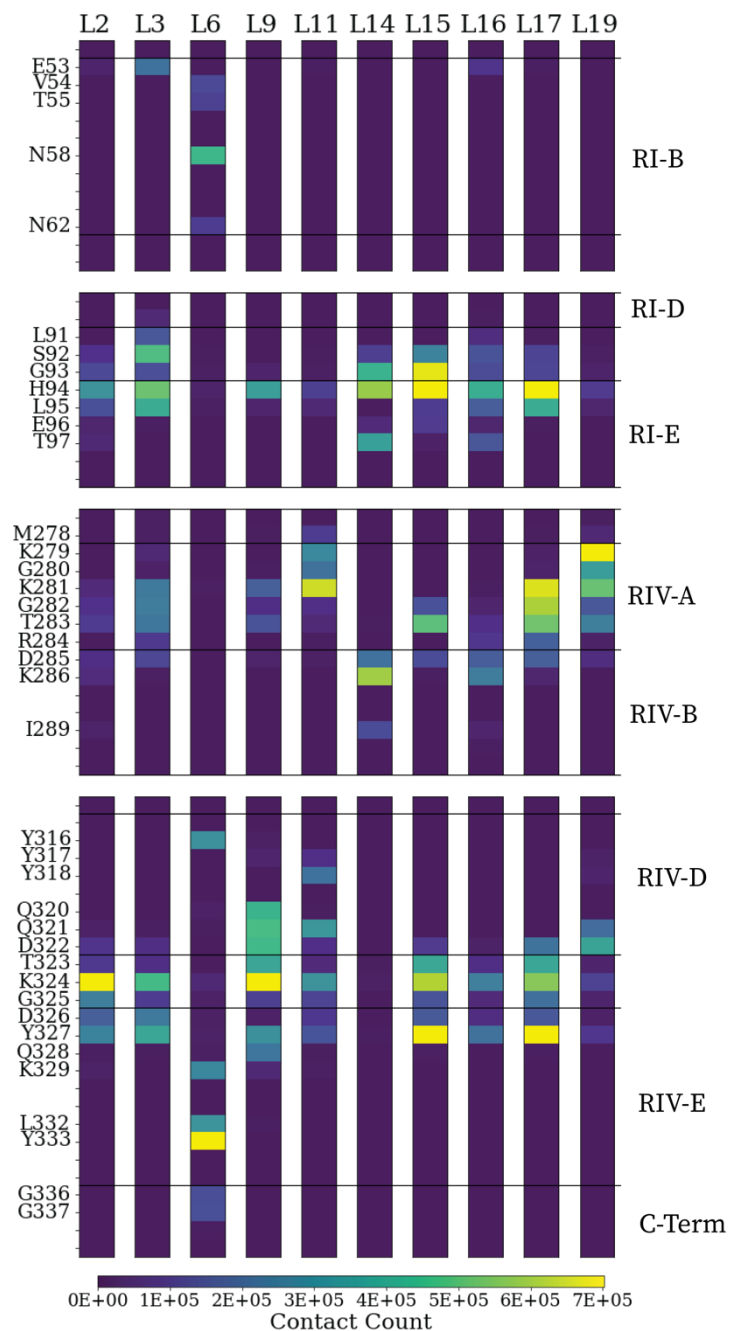


Figure 6.6. Heatmap of A2 residue contacts with ligands, for all stably binding ligands. Labels are shown only for residues that have a high number of contacts with a ligand. Ligand numbers are found at the top of each column.

Binding affinities were calculated using the MMGBSA method for 200 evenly spaced frames from the final 20 ns of simulation for each of the stable binders. The binding affinities ranged from relatively weak, -3.3 kcal/mol for L9, to very strong, -11.9 for L17 (see **Table 6.2**). Five ligands, L2, L3, L14, L15, and L17, were selected for further modeling in the presence of a membrane bilayer and Ca²⁺-ions. These ligands were selected based on the quality of the combined RMSD, distance, visual inspection, contact-analysis, and MMGBSA results.

Table 6.2. MMGSA Results for A2-Lignads Interactions.

Ligand ID	ZINC ID	MMGBSA ΔG (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)
L2	ZINC000253529804	-4.9	3.1
L3	ZINC000253389497	-6.9	3.8
L6	ZINC000958322868	-7.2	3.9
L8	ZINC000253487389	-11.7	4.6
L9	ZINC000253500604	-3.3	3.7
L11	ZINC000299817838	-4.7	4.0
L14	ZINC001534514511	-5.1	2.2
L15	ZINC000263583469	-10.7	3.5
L16	ZINC000002157489	-6.4	3.3
L17	ZINC000253389471	-11.9	3.9
L19	ZINC000253532158	-9.2	3.5

6.3.3. Molecular Dynamics Assessment of Ligand Impact on A2-Membrane Binding

Three simulations were performed for each of five ligands (L2, L3, L14, L15, and L17) in the complexes with membrane and A2. Prior to investigating the impact of the ligands on A2-membrane binding for the membrane simulations, stable A2-ligand binding was verified by calculating ligand RMSD, A2-ligand distance, and A2-ligand binding affinities. A2-ligand binding was stable for most simulation repeats, with three exceptions. Binding affinities across the three trials were calculated and averaged. The A2-ligand binding was strong for all five ligands with values ranging from -14.3 to -53.8 kcal/mol (see **Table D.1**). The stability of ligand binding was also demonstrated by tracking the ligand RMSD when the protein backbone is

aligned to the first frame (see **Figure 6.7A**). Most ligands do not migrate significantly from their starting locations. Three exceptions were observed in one repeat from each of the following: ligands L3, L14, and L17. In repeat 3 of the L3 system, the ligand dissociates briefly in favor of the membrane before partially reassociating with A2. In repeat 2 of the L14 system, the ligand migrates toward the convex face of A2 between RIII and RIV. In repeat 3 of the L17 system the ligand migrates toward the interface of RI and RII at the convex face of A2. These results are also reflected in the ligand distance from the RIV Ca^{2+} -binding loops which demonstrates that all ligands, discounting the discussed exceptions, remain near the desired binding site (see **Figure 6.7B**). It should be noted that even in the in the simulation repeats where migration was observed, all ligands remained in complex with A2.

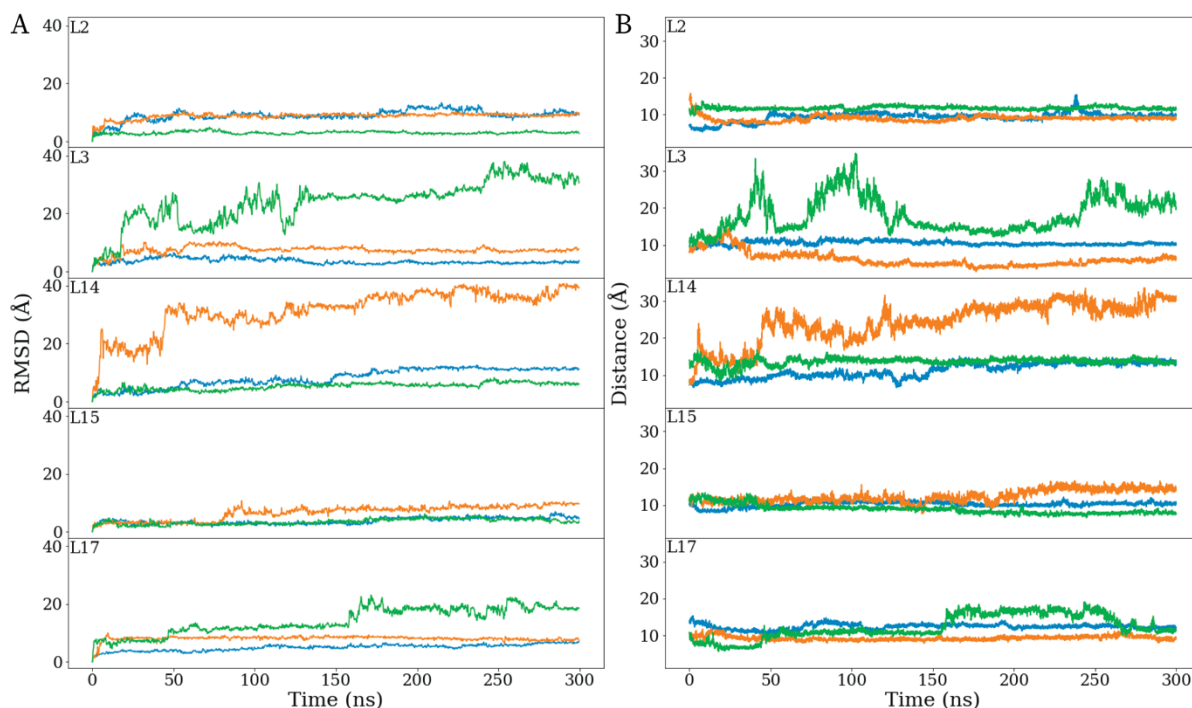


Figure 6.7. (A) RMSD of ligands from the membrane simulations with alignment performed using the protein backbone and (B) distance from the membrane simulation ligands' center of mass to the combined RIV-AB, RIV-DE center of mass. Results are reported for L2, L3, L14, L15, and L17. Simulation repeats are colored blue, orange, and green, respectively with ligands labeled in the upper left corner of each plot.

When considering the impacts of the individual ligands, two key phenomena were of interest, both relating to how the ligands affect membrane binding: the capacity for ligands to interfere with A2-Ca²⁺ binding and the direct impact of ligands on A2-membrane binding. Ca²⁺-distances from the binding sites reported by Rosengarth and Luecke were used to investigate the effects of ligands on Ca²⁺-binding. The ligand-free simulation was used as a comparison. Ca²⁺-binding was observed to be stable at all locations in the ligand-free system (see **Figure 6.8**). L2 demonstrated no capacity to significantly disrupt Ca²⁺-binding, all Ca²⁺ remained at their binding sites (see **Figure 6.8**). L3 displaced Ca²⁺-2 during one repeat but an ability to disrupt binding was not observed in the other simulation repeats (see **Figure 6.8**). L14, L15, and L17 had reliable impacts on Ca²⁺-binding at some combination of sites 1, 2, 3, and 6, making them promising candidates for the disruption of A2-membrane binding (see **Figure 6.8**). Ca²⁺ sites 4, 5, and 6, were not impacted by any ligands (see **Figure 6.8**). Ca²⁺-disruption was most consistent within RI with L14 consistently disrupting both sites and L13 and L15 consistently disrupting the Ca²⁺-2 site. This is understandable as the Type III Ca²⁺ binding sites found in RI are lower affinity than the Type II site found at RIV. L14 and L17 demonstrated an ability to disrupt Ca²⁺ site 6 found within RIV.

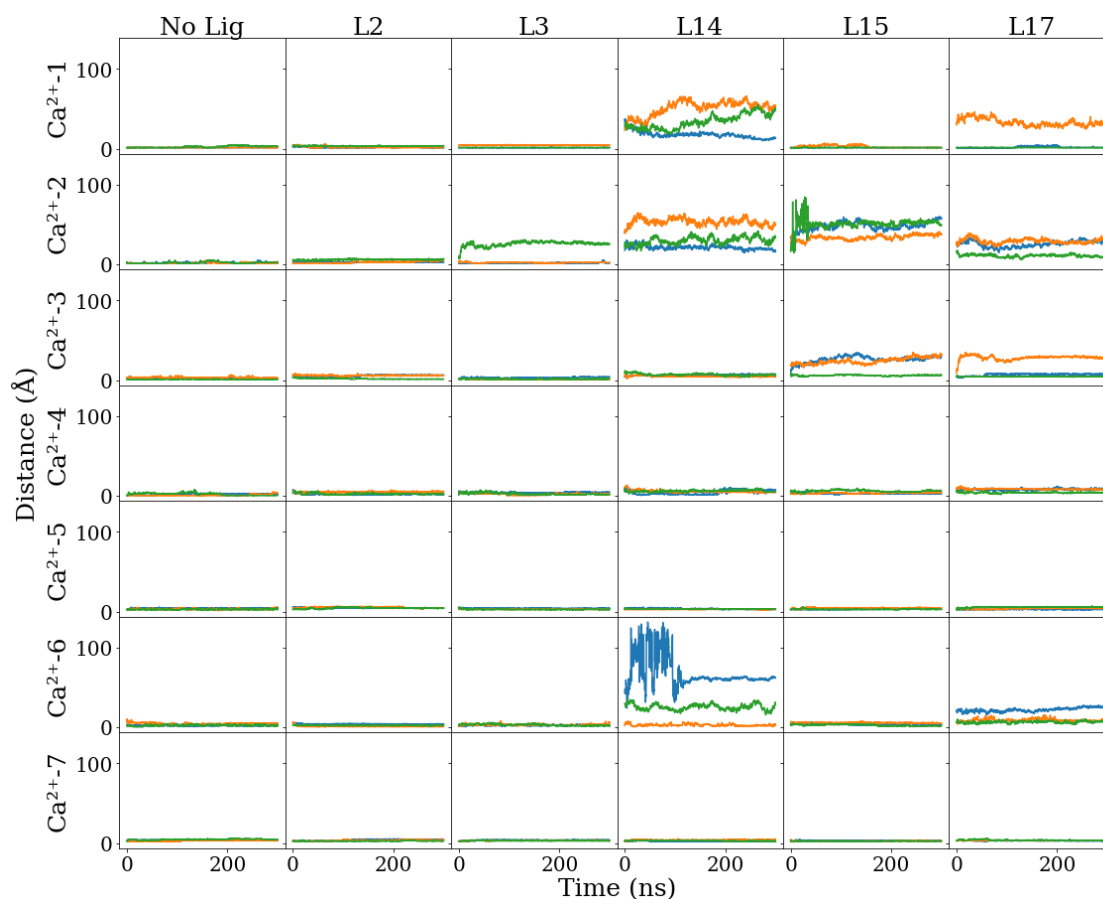


Figure 6.8. Ca^{2+} distances from their binding sites for Ca^{2+} 1-7 of the no ligand, L2, L3, L14, L15, and L17 systems. Simulation repeats 1, 2, and 3 are colored blue, orange, and green, respectively.

The effects of ligands on A2-membrane binding were investigated by examining the heights of the individual repeats above the membrane with the ligand-free A2 serving as a baseline for comparison (see **Figure 6.9**). Ligands L2 and L3 appeared to have the capability to modestly enhance A2-membrane binding (see **Figure 6.9**). Coupled with their inability to disrupt Ca^{2+} -binding, ligands L2 and L3 may be potential A2-membrane binding promoters. Ligands L14, L15, and L17 demonstrated the capacity to disrupt the RI and RIV membrane interaction (see **Figure 6.9**). This is also consistent with the analysis of Ca^{2+} -binding as all three ligands demonstrated the ability to reliably disrupt A2- Ca^{2+} binding.

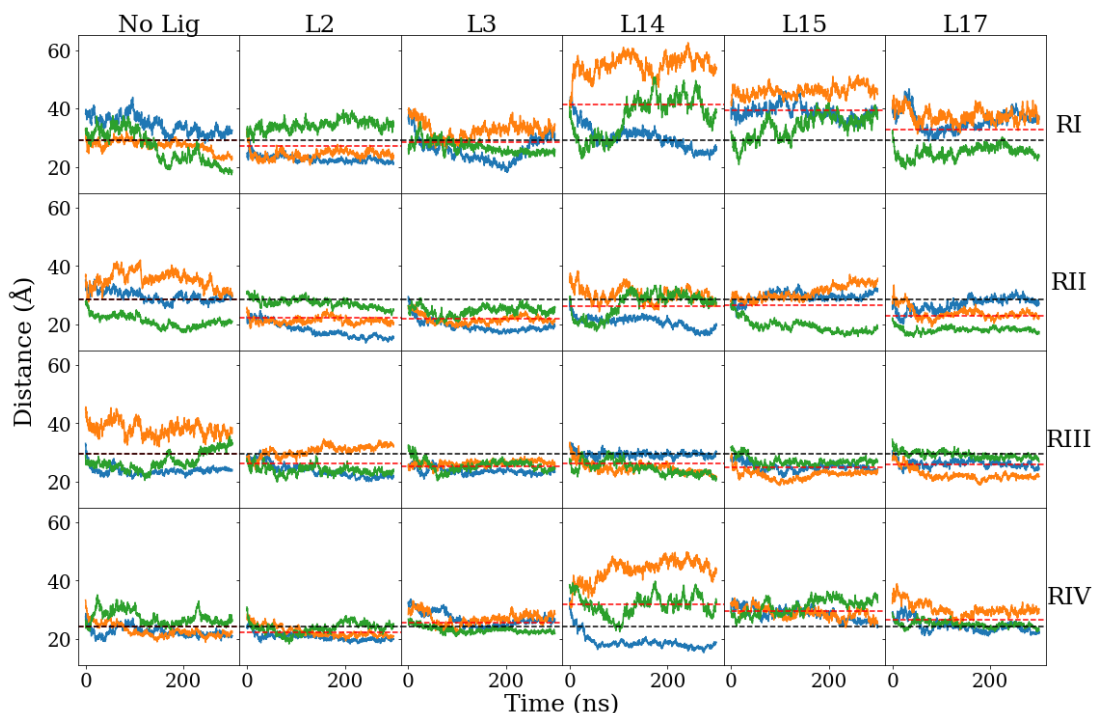


Figure 6.9. RI-RIV heights above the membrane center of mass for the membrane simulations. Simulation repeats 1, 2, and 3 are colored blue, orange, and green, respectively. The mean height across repeats for the no ligand system is shown on every plot using dashed black lines. The mean height across repeats for each ligand is shown using red dashed lines.

Height of A2 from the membrane was utilized to determine the effect of each ligand on the A2-membrane interaction. The mean height of the A2 center of mass from the membrane center of mass was compared to that of the ligand-free A2 to determine if the ligand had any effect. Ligands L14 and L15 had slightly elevated heights when compared to the ligand-free A2 while ligands L2, L3, and L17 had slightly decreased heights. This suggests that ligands L14 and L15 reduced the strength of the A2-membrane interaction while ligands L2, L3, and L17 enhanced it. MMGBSA was performed for the ligand-membrane interaction (see **Table D.2**) and the relationship to A2 height was assessed. It was found that membrane-ligand binding affinity and average A2 height have a strong correlation ($r = 0.88$, $p = 0.02$); as the membrane-ligand interaction becomes more unfavorable, the average height of A2 from the membrane increases

(see **Figure 6.10**). All ligands demonstrated strong relationships with A2 but the strength of their interactions with the membrane differed. Ligands with very strong membrane interactions, ligands L2 and L3, enhanced the ability of A2 to associate with the membrane by directly bridging the interaction between A2 and the membrane. Ligand L14 had a strong repulsive interaction which directly hindered A2's ability to associate with the membrane.

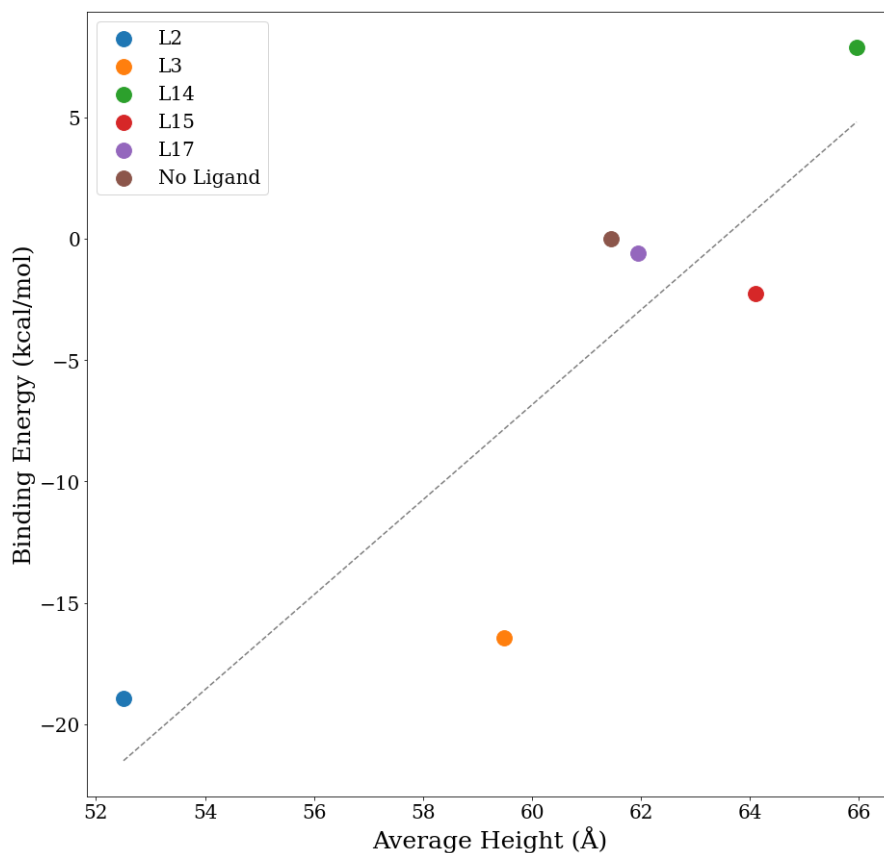


Figure 6.10. Average A2 ligand-Membrane binding energy vs Averaged Height across repeats. Ligands are colored according to the associated legend. A line of best fit is also plotted alongside the individual data points.

Contact analysis was performed on the A2-Ligand complex both to compare the A2-ligand contacts to those observed in the ligand-only simulation as well as investigate the mechanism by which Ca^{2+} -binding disruption occurs (see **Figure 6.11**). Interestingly, the mechanism of Ca^{2+} -binding disruption could not be linked directly to the capture of Ca^{2+} -binding

residues; contacts between Ca^{2+} -binding residues and ligands were not always indicative of Ca^{2+} -binding disruption. Ligands L3, L15, and L17 show some degree of capture of the important Ca^{2+} site 6 binding residue D322 but only L17 disrupts binding. Additionally, residues near Ca^{2+} -site 1 form contacts with all ligands but only ligands L14, L15, and L17 disrupt binding. Thus, it appears that the disruption of Ca^{2+} -binding is not universal but rather achieved via an overall destabilizing effect or other indirect mechanism that ligands L14, L15, and L17 have on Ca^{2+} -binding loops.

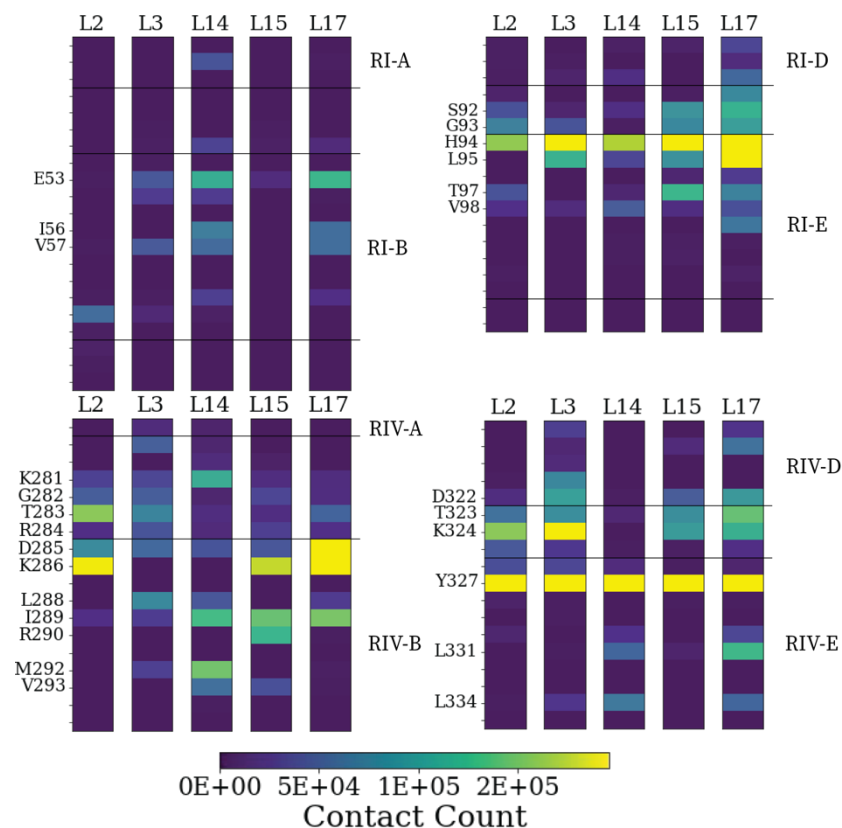


Figure 6.11. Heatmap of A2 residue contacts with ligands, for all membrane systems. Labels are shown only for residues that have a high number of contacts with a ligand. Ligand numbers are found at the top of each column.

A2-ligand contact profiles are consistent between the ligand-only and membrane simulations. Ligands L14 and L17 see new contacts around residue E53 that were previously only present in L3. Binding with the RI-DE loop remains largely consistent, with H94 being the primary point of contact for all ligands. RIV-AB loop binding experienced the most dramatic shift in coordination with the binding shifting from being centered on K281 to K286. RIV-DE binding remained largely similar, centered on Y327.

6.4. Conclusions

The PI(4,5)P2-specificity site of A2 was screened against a library of ~180,000 ligands containing both the library of FDA-approved drugs and all Natural Products libraries available via the ZINC database. Ligands were ranked based on the averaged predicted binding-affinities of the top ten poses for each ligand.

Molecular dynamics simulations were performed for the top 20 ligands. MMGBSA, distance, RMSD, and contact analysis were employed to identify ligands which stably bound to A2 at or near the PI(4,5)P2 site. By considering the discussed results, five candidates which were demonstrated to both stably associate with A2 and remain proximal to residues of the PI(4,5)P2 site, other membrane-binding residues, or Ca²⁺-binding residues.

Further MD simulations which included Ca²⁺ ions at canonical binding-sites and a PI(4,5)P2-containing membrane were run. Ligands L14, L15, and L17 were demonstrated to reproducibly impact the stability of Ca²⁺-binding. This behavior was desirable as A2-membrane binding is a Ca²⁺-dependent process. Thus, displacement of Ca²⁺ ions would likely impair the membrane-binding capacity of A2. Additionally, it was observed that ligands L14 and L15 caused the height of RIV to increase relative to the ligand-free simulation, another desirable indication that ligands were disrupting A2-membrane binding.

Ligands L14 and L15 are predicted to have a high affinity for A2 and disrupt membrane binding. Future work will investigate A2-ligand binding via direct, isothermal titration calorimetry, or indirect, membrane binding assays, means. Further computational work would be desirable as well. Libraries of similar structures will be generated using CycleGAN generative models and identified ligands. This will allow for the investigation of similar compounds that vary in properties such as logP, number of hydrogen-bond donors and acceptors, molecular weight, and elemental composition.

CHAPTER 7: MMAEVe, a Versatile and Program for Constructing Systems for Molecular Dynamics Simulations and Visualization

Abstract

Advances in Molecular Dynamics have allowed for routine access to the μ s-timescale for large, complex systems. This has made possible the simulation of protein-membrane complexes that are on the same scale as functional-biological units. Tools for constructing membrane-protein systems exist but are limited in their scope, size, and abilities. A novel Python package named MMAEVe was developed for the construction of such systems. MMAEVe can be construct bilayers, nanodiscs, nanotubes, micelles and vesicles. Protein components can be distributed around or integrated into constructed membrane models. Both lipid and protein complexes can be of arbitrary composition. This versatility allows for extremely complex systems to be constructed easily. MMAEVe is also performant, it can generate virion-sized systems of mixed membrane-protein composition in under ten seconds. Finally, MMAEVe is easily extensible with a simple organization that is meant to facilitate the extension of its capabilities.

7.1. Introduction

Advances within the past decade, at the level of hardware, software, and force field development have led to dramatic increases in achievable timescales for molecular dynamics (MD) simulations. The widespread availability of consumer-grade GPUs coupled with the porting of many popular MD codes, such as GROMACS, AMBER, and LAMMPS to GPUs has led to 10x or greater increases in throughput for all-atomic MD simulations.^{124–126} This along with methods for increasing simulation timestep such as SHAKE and hydrogen-mass-repartitioning has made simulations of μ s's in length achievable on affordable consumer-grade workstations.^{126,166,209}

At the level of force-field development, the creation of the martini coarse-grained force field and its iterations has allowed for simulation of enormous systems, approaching the scale and composition of functional biological units.^{18,218,219} This is evidenced by the work of Pezeshkian et al. and Wang et al. who each have published MD studies of a coronavirus models with experimentally accurate diameters of nearly 100 nm.^{220,221} The reasons for this speed are because of a roughly 10x reduction in the number of atoms coupled with a 10x or greater increase achievable integration timestep.¹⁸ The martini FF has recently been ported to OpenMM, and runs almost exclusively on the GPU, which may pave the way for even further increases in simulation speed.²²²

With the aforementioned advances comes the need for tools to create complex systems in a both flexible, expedient, reliable, and standardized manner. The current landscape includes many excellent membrane and mixed protein/membrane system builders however, they all have limitations which can become prohibitive when assembling large, complex systems. Some of the most popular options for building complex, biological systems containing membranes include

the CHARMM-GUI, BumPy, and Insane.^{223–225} Each of these has their merits as well as drawbacks. The CHARMM-GUI is extremely powerful in that it performs all steps of setting up a simulation, including generation of required input files for different MD codes.²²³ However, it can be difficult to programmatically generate systems of differing size/composition. This owing to its web-based interface which has prompted the development of at least one series of scripts for interacting with it without the need for the GUI. Further, it lags in speed which, given the duration of MD simulations on most workstations is a minor time investment. The time required can become prohibitive when setting up large-scale studies which investigate the programmatic variation of system composition. Additionally, the scale of systems that it can construct was found to be limited in our investigations. BumPy is another performant builder and is capable of constructing a wide variety of membrane systems including, semi-spheres, cylinder, flat bilayers, partial torus's, spheres, torus's, capped cylinders, semicylinder planes, semispherical planes, and buckled bilayers.²²⁴ Despite the wide variety of available shapes, BumPy does have some limitations. It is unable to handle the addition/construction of membrane-protein systems; it can only be used for the creation of membrane-only systems. Protein distribution would require a separate tool to be utilized. BumPy also requires a section of pre-equilibrated bilayer which adds to the required amount of investment for constructing a system. Insane is another membrane generator that is geared toward the construction of flat bilayers with a single protein inserted.²²⁵ The major advantage of Insane is that it comes with native support for and is regularly updated with additions to the body of lipids which have been parameterized for the Martini forcefield. It can also be used to solvate systems and generate topology files. Its major limitations are its restricted ability to handle proteins and restriction to the creation of flat-bilayer systems.

Given the limitations seen across all the discussed membrane/biomolecular complex builders we saw a clear need and opportunity for the creation of a tool that can quickly and easily compose massive, complex systems. We chose Python for the creation of this tool because of its widespread usage within the scientific community, the existing ecosystem of MD related packages such as OpenMM and MDAnalysis, as well as the NumPy package which can be utilized for performant array operations.^{211,213,226} The Python module has a simple syntax that can be used to create and combine complex biological systems. It is entirely agnostic to molecule type and can be used for the construction of systems containing lipids, proteins, ligands, sugars, etc. though it was created with a focus on membrane-protein systems. We call the module MMAEVe, short for “Membrane, Micelles, and Even Vesicles”. Though, as previously noted, its scope extends beyond those systems contained in the title.

7.2. Methods

7.2.1. System Description

The first step for system creation is the selection of the building-blocks, their ratios, and their polarity to generate a description of the system. Building blocks can be a pdb file of any molecule, including not just contiguous, covalently linked molecules but also multimers such as the SARS-COV2 spike protein. Ratios determine the proportion of points in each construction that will be represented. Polarity is manually specified by selecting an atom to serve as the “head” and an atom to serve as the “tail” for each molecule. An optional z-shift can also be specified. This is necessary, for example, in the construction of a system that contains integral as well as peripheral membrane components.

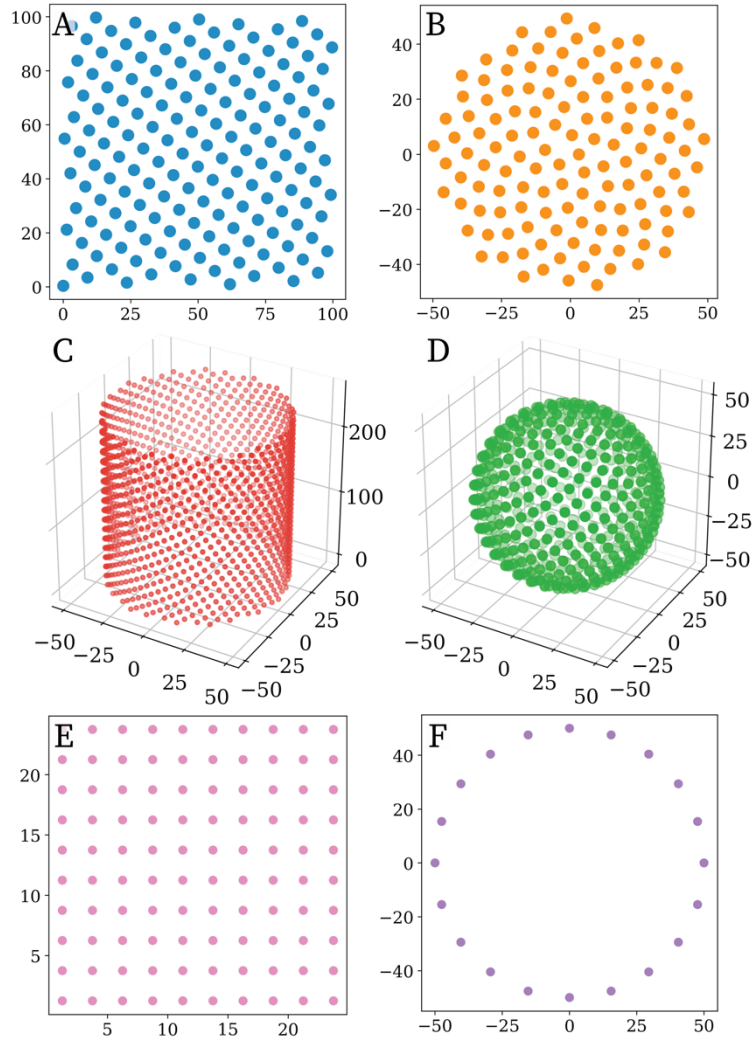
7.2.2. Scaffold Generation

After defining the system composition, the next step of the system distributes points in a specific pattern. Currently six patterns are supported: Lattices, Discs, Cylinders, Spheres, Grids, and Circles (see **Figure 7.1**). The first four shapes are derived from the Fibonacci Lattice, defined below.

$$(7.1) \quad (x_i, y_i) = \left(\frac{i}{\phi} \% 1, \frac{i}{n-1} \right) \text{ for } 0 \leq i < n$$

Where ϕ is the golden ratio and the % is the modulo operator.

$$(7.2) \quad \phi = \frac{1 + \sqrt{5}}{2}$$



For the construction of Discs, Cylinders, or Spheres, the lattice coordinates are transformed according to equations (7.3), (7.4), or (7.5), respectively.

$$(7.3) \quad (\theta, r) = (2\pi x, \sqrt{y})$$

$$(7.4) \quad (\theta, r, h) = (2\pi x, r, y)$$

Figure 7.1. Distribution of points derived from a Fibonacci Lattice to create the point distributions for the (A) Lattice, (B) Disc, (B) Cylinder, and (D) Sphere shapes. (E) Grid distribution of points. (F) Circle distribution of points.

$$(7.5) \quad (\theta, \phi, r) = (2\pi x, \arccos(1 - 2y), 1.)$$

The resulting polar, cylindrical, or spherical coordinates are then transformed into cartesian coordinates. The Grid shape consists of $n \times m$ points distributed at regular intervals over specified dimensions while the Circle consists of points placed at even increments on a circle.

7.2.3. Structure Distribution

After creation, the point collections are assigned IDs at random according to the user-specified proportions. The assigned IDs correspond to the molecule that will be placed at that point. Molecules are then oriented to their associated point in the point collection, using the specified head and tail. The method of orientation is shape dependent. Molecules in a Lattice, Nanodisc, Grid, or Circle are first aligned to the positive z-axis, then translated to their position. Molecules associated with a Cylinder shape are aligned to the projection of their associated point on the xy-plane prior to translation. Molecules associated with the Spherical shape are aligned with their associated points then translated to their final position. All rotations are completed using quaternions.

7.2.4. Combining Systems

Individual shapes can be combined with each other to form larger systems. The construction of a bilayer, for example, would involve the construction then combination of two separate Lattices. In some cases, overlap between components of two created shapes may be undesirable, as in the case of transmembrane proteins. To address this, we provide a method for removing overlap. The KDTree lookup algorithm, as implemented in the SciPy Python package

is utilized. This method is performant, $O(n \log n)$, when compared to a pairwise distance lookup, $O(n^2)$. After overlap has been removed, systems can be combined as usual.

7.2.5. Program Organization

The complete algorithm for system construction is simple yet extensible. The Python implementation relies on a parent class `BiomolComplex` which handles most of the system initialization and writing output topology, pdb, and cif files. Child classes are derived from `BiomolComplex` for each shape requiring the definition of only two supplementary class methods `generate_positions` and `distribute` which define the distribution of positions and the distribution of structures to those positions, respectively. This simple organizational paradigm makes the addition of novel shapes to MMAEVe simple.

7.2.6. System Export

Constructed systems can be exported in one of two currently supported structural formats, PDB or CIF. The former is most useful for creating systems that will be used to perform MD simulations while the second is useful for creating extremely large systems owing to its delimited nature which removes the size limits of a the fixed-width-format PDB file. Topology files can be written for gromacs which contain the counts of each molecule in the system. It does still require that the user add references to the itp files associated with each molecule.

7.3. Results and Discussion

7.3.1. MMAEVe Can Construct a Diverse Array of Systems

MMAEVe can be applied to create a variety of complex systems. Bilayers, nanodiscs, nanotubes, micelles, and vesicles are the basic membrane systems that can be constructed using MMAEVe (see **Figures 7.2C, 7.2F, and 7.2H**). Bilayers are routinely constructed or used in MD

simulations, either to approximate an appropriate environment for a protein component of a system or for investigation of lipid composition of membrane properties, dimensions, transport properties, phase separation, etc.²²⁷ Nanodiscs are an important tool leveraged by physical biochemists for the study of membrane properties. While their application in MD simulations is much more niche than that of bilayers, they have been utilized in recent years to investigate topics such as nanodisc formation, differences between nanodisc and bilayer simulations, and nanoparticle encapsulation by nanodiscs.²²⁸ Lipid nanotubes are a topic of interest in both biology and material sciences. In biological systems, lipid nanotubes have been observed linking cells together.²²⁹ In material science, lipid nanotubes can serve as a versatile method of creating scaffolding for the deposition of inorganic materials.²³⁰ A recent study by Manrho et al. leveraged coarse-grained molecular dynamics for the investigation of nanotube formation.²³¹ Micelles are observed in biological systems as a means for the absorption of fat-soluble vitamins among other nutrients.²³² Reverse micelles have found application in the study of peripheral membrane binding proteins.²³³ Both vitamin micelle interaction and reverse micelles have been the subject of molecular dynamics studies.^{234,235} Vesicles and vesicle-like systems are common throughout biology. MD studies have been applied to study both the spontaneous formation of vesicles as well as their fusion with larger membranes.^{236,237}

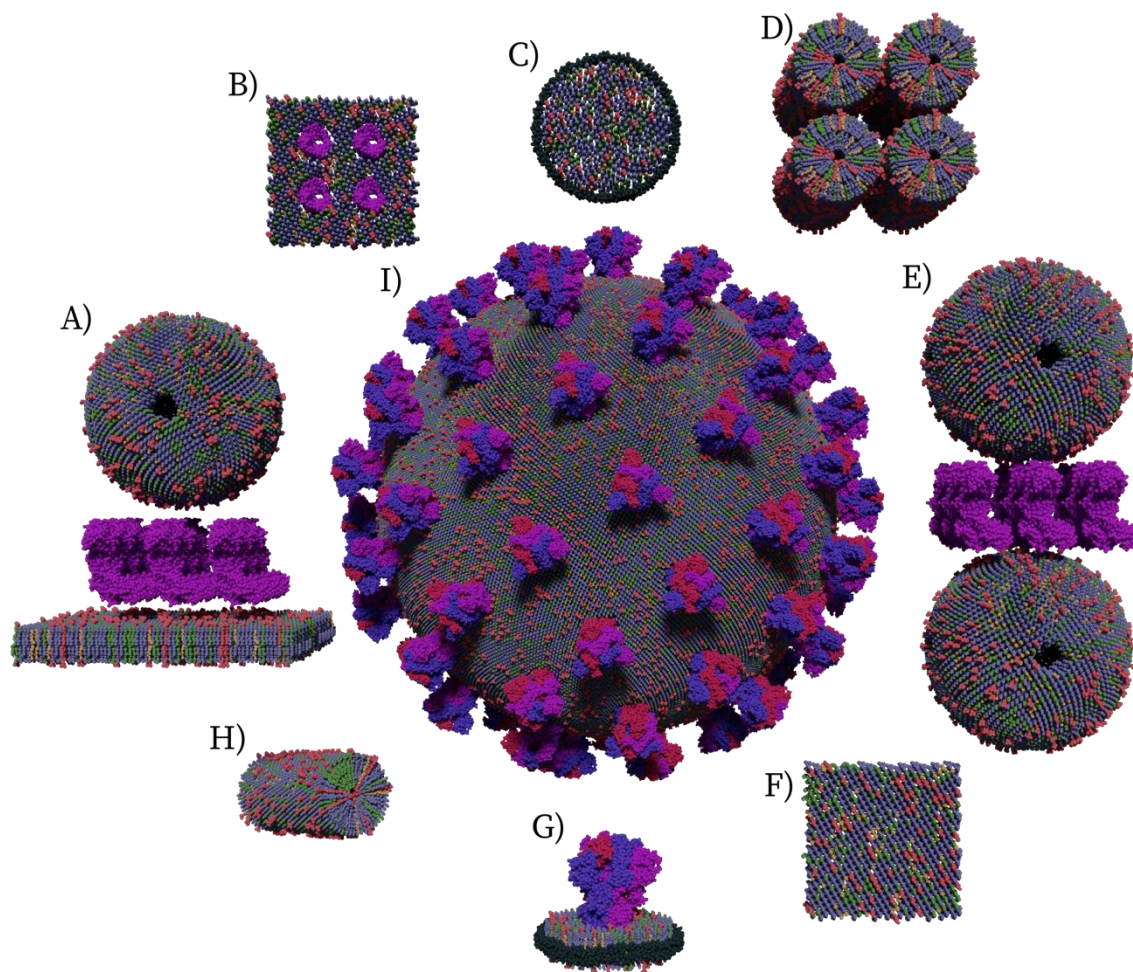


Figure 7.2. Examples of various systems constructed solely using MMAEVe. (A) 9 A2t placed in a grid formation between a membrane bilayer and vesicle. (B) Bilayer with 4 porin inserted. (C) Membrane nanodisc. (D) Array of 4 membrane nanotubes. (E) 9 A2t placed in a grid formation between two vesicles. (F) Membrane bilayer. (G) SARS-COV-2 Spike embedded in a membrane nanodisc. (H) Membrane nanotube. (I) SARS-COV-2 model containing the Spike only. All images are to-scale with reference to each other, except for (I) which had to be scaled down for its inclusion in the figure.

7.3.2. Performance Comparison

A series of benchmark systems were generated to investigate the performance of MMAEVe (see **Table 7.1**). These benchmarks were extended to include other membrane builders as well to assess its relative performance. The total required construction time of each system was reported. Not all systems could be benchmarked for each builder as BumPy does not have

the ability to handle proteins, CHARMM-GUI can only handle them in a limited fashion, and Insane was found not to handle proteins properly, completely distorting their structure, in our tests. Of the systems that were tested, BumPy and Insane demonstrated speeds that were nearly equivalent to that of MMAEVe. This was expected as all three were built using Python and make heavy usage of the NumPy module. CHARMM-GUI showed significantly worse raw performance than the other three builders when constructing systems; it was three orders of magnitude slower than the other builders. It is, however, more versatile than BumPy and Insane which could only be benchmarked for two and one of the ten systems, respectively. The simplest benchmark system was a bilayer with less than 2,000 total lipids (see **Figure 7.2F**). The most complex of the benchmark systems was a to-scale creation of a full SARS-COV2 virion model with over a million coarse-grained beads (see **Figure 7.2I**). This makes performant construction of biological-scale cellular models of small bacteria such as cocci and many envelope viruses achievable in seconds.

Table 7.1. Table of System Construction Benchmarks for MMAEVE and Other Common Membrane Builders, BumPy, CHARMM-GUI, and Insane.

System	Particle Count	Lipid Count	Protein	X Len (Å)	Y Len (Å)	Radius (Å)	Length (Å)	Builder	Avg Time (s)	Std Dev (s)
Bilayer	15744	1310	0	200	200			MMAEVe	0.110	0.006
	16708	1451	0	200	200			BumPy	0.138	0.004
	15393	1300	0	200	200			CHARMM	139.980	1.869
	13496	1140	0	200	200			Insane	0.181	0.004
Bilayer-Porin Grid	14916	1038	4	200	200			MMAEVe BumPy CHARMM Insane	0.123	0.001
Nanodisc	10388	738	2			95		MMAEVe	0.106	0.000
	11109	800	2			95		BumPy		
								CHARMM	53.566	1.632
								Insane		
Nanodisc-Spike	16229	640	3			95		MMAEVe	0.219	0.004
	17314	700	3			95		BumPy		
								CHARMM	105.496	6.038
								Insane		
Nanotube	33724	2832				50	250	MMAEVe	0.196	0.000
	32588	2832				50	250	BumPy	0.320	0.009
								CHARMM		
								Insane		
Nanotube Array	92254	11327						MMAEVe	0.631	0.004
								BumPy		
								CHARMM		
								Insane		
Vesicle	61960	5228				125		MMAEVe	0.351	0.004
	53112	4610				125		BumPy	0.546	0.006
	65796	5483				125		CHARMM	319.164	2.385
								Insane		
Vesicle-Bilayer Bridge	127166	9244	9	350	350	125		MMAEVe	0.739	0.004
								BumPy		
								CHARMM		
								Insane		
Vesicle-Vesicle Bridge	141370	10456	9			125		MMAEVe	0.726	0.013
								BumPy		
								CHARMM		
								Insane		
Covid	1578131	88723	75			500		MMAEVe	9.851	0.211
								BumPy		
								CHARMM		
								Insane		

7.3.3. Using MMAEVe for the Systematic Investigation of Cholesterol's Influence on Membrane Proteins

Here, a representative application of MMAEVe for the study of cholesterol composition on membrane dynamics and structure is presented. The effects of cholesterol composition on membrane properties, diffusion coefficients, coarse-grain lipid order parameters (LOP), and membrane thickness, were investigated. 11 CHOL/DPPC systems were generated using MMAEVe with CHOL concentrations that ranged from 0 to 30% of all lipids (see **Figure 7.3A**). 5 repeats were run for each system with a total of 1 μ s of production. Clear trends were observed between LOP, diffusion coefficients, and membrane thickness against cholesterol composition (see **Figures 7.3A-7.3D**). As CHOL composition increased the observed SCC and membrane thickness of DPPC also increased. This is consistent with a membrane in a more ordered state. The observed diffusion coefficient decreased as the cholesterol concentration decreased. The investigation of the effect of CHOL composition is not novel and its impacts on membrane properties are both well-known and have been investigated in the literature using coarse-grained MD.²³⁸ This demonstrates that in addition to application for the construction of large, complex systems, MMAEVe is also useful when constructing smaller, more routine systems.

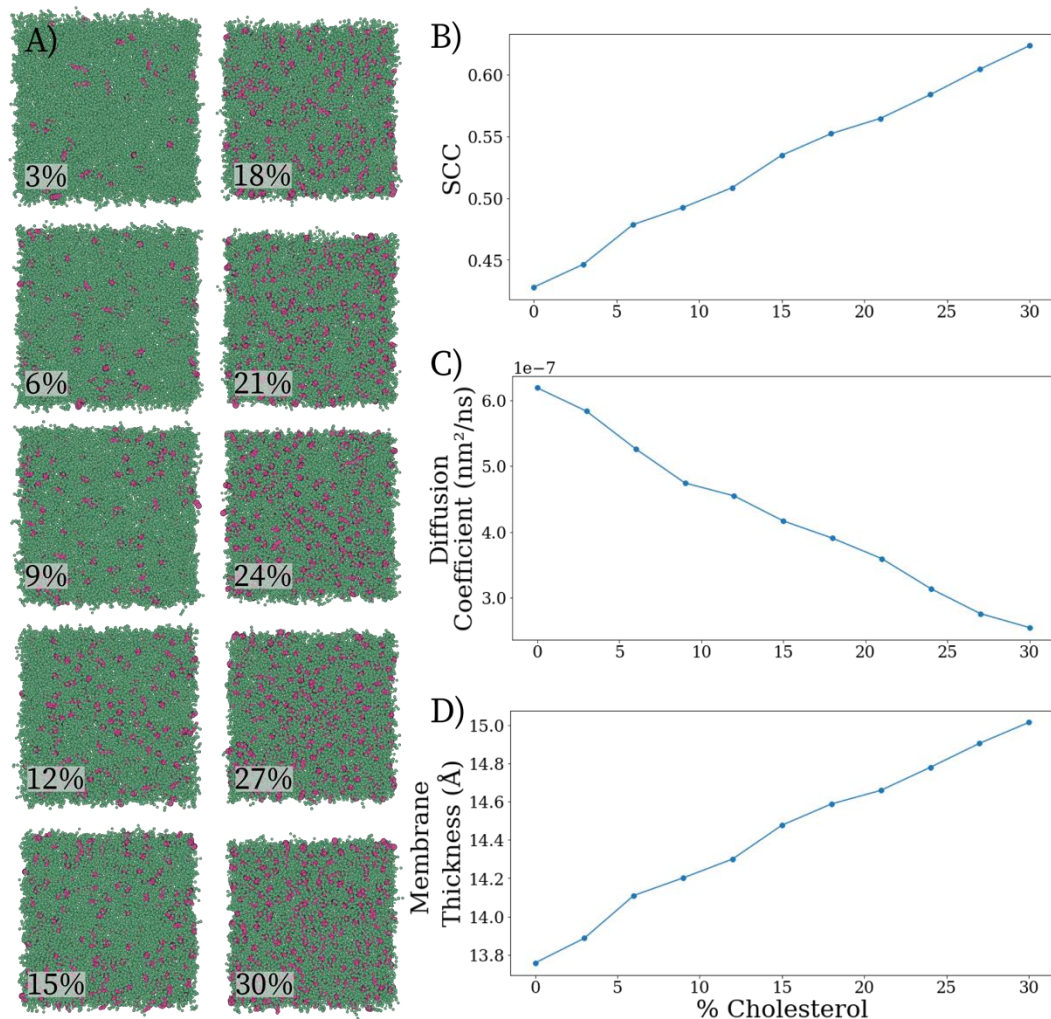


Figure 7.3. (A) Final membrane structures from the first repeat of each CHOL/DPPC system. CHOL are colored pink while DPPC are colored green. Average values of (B) coarse-grained lipid order parameters (C) diffusion coefficients and (D) membrane thickness vs CHOL composition.

7.4. Conclusions

Currently available membrane-builders such as the CHARMM-GUI, Bumpy, and Insane all have limitations when constructing complex membrane-protein system. The most prominent being their limited scope. The MMAEVe Python module was created as an alternative that addresses some of the key limitations of the other builders.

The user specifies the composition of each of the system components. These components are then distributed onto a surface. These simple pieces can be combined to create systems such as bilayers, nanodiscs, nanotubes, micelles, and vesicles with or without a complex protein component. More complex systems such as full-size virion models can also be constructed with ease.

Various systems were benchmarked and the performance compared to the CHARMM-GUI, Bumpy, and Insane, when possible. MMAEVe, Bumpy, and Insane all showed similar performance but the latter two could only be used to construct 3 and 1, respectively, of the ten example systems. The CHARMM-GUI was more versatile with the ability to construct 4 of the ten example systems but its performance lagged significantly when compared to the other builders.

MMAEVe was also applied to build systems for the study of the effect of cholesterol concentration on the dynamics of DPPC membranes. This is a well-studied phenomenon, but its investigation demonstrated that MMAEVe is suitable for more routine studies such as exploring the effects of changes in membrane composition.

While it is currently an extremely capable system, improvements to MMAEVe are planned. There are two major improvements that are planned. The first is the creation of systems where the distribution of lipids is done along an irregular mesh such as one created from a 3D-modelling software or experiments such as cryo-electron microscopy. The second is the inclusion of a polymer-packing module such as polyply.¹³⁷ This would allow for the generation and inclusion of DNA or other polymers that conform to a structure that has been generated by MMAEVe. We hope for the final form of MMAEVe to be capable of generating whole-cell models.

CHAPTER 8: Coarse-Grained Modeling of Annexin A2-Induced Microdomain Formation on a Vesicle

Abstract

Annexin A2 (A2) induced microdomain formation is a key step in biological processes such as Ca^{2+} -mediated exocytosis in neuroendocrine cells. In this work, ten 15 μs molecular dynamics simulations were performed on a vesicle model with a diameter of approximately 250 Å using the Martini2 force field. Five simulations were performed in the presence of ten A2 and five simulations in the absence of A2. Consistent results were generated among the simulations. A2-induced PIP2 microdomain formation was observed and shown to occur in three phases: A2-vesicle association, localized A2-induced PIP2 clustering, and A2 aggregation driving PIP2 microdomain formation. The relationship between A2 aggregation and PIP2 microdomain formation was quantitatively described using a novel method which calculated the variance among protein and lipid positions via the Fréchet mean. A large reduction in PIP2 variance was observed in the presence of A2 but not in its absence. This reduction in PIP2 variance was proportional to the reduction observed in A2 variance and demonstrates that the observed PIP2 microdomain formation is dependent upon A2 aggregation. The three-phase model of A2-induced microdomain formation generated in this work will serve as a valuable guide for further experimental studies and the development of novel A2-inhibitors.

8.1. Introduction

The Annexin family of Ca^{2+} -binding proteins associate with biological membranes in a Ca^{2+} -dependent manner. There are 12 human Annexins, Annexin A1-A11 and A13, with Annexin A12 currently unassigned. The human Annexins share a highly conserved core domain composed of four homologous repeats I-IV (RI-RIV) of ~ 70 amino acids each in length (**Figure 8.1B**)¹. Annexin A6 is an exception as it contains the equivalent of two core domains²³⁹. Each of the repeats, consists of five α -helices, A-E, connected by loops. Helices A, B, D, and E are parallel to each other with C being roughly perpendicular to A, B, D, and E (**Figure 8.1A**). Each annexin also has a variable N-terminal domain (ND). These ND vary significantly in both size and composition¹. The ND of Annexin A1 is held inside its core domain in a hydrophobic pocket until Ca^{2+} -binding frees it. However, The ND of Annexin A2 (A2) remains exposed independent of Ca^{2+} -binding^{67,240}. The ND of Annexin A1 and A2 are 41 and 32 residues in length, respectively while the N-terminus of Annexin A11 is 199 residues long^{67,240,241}.

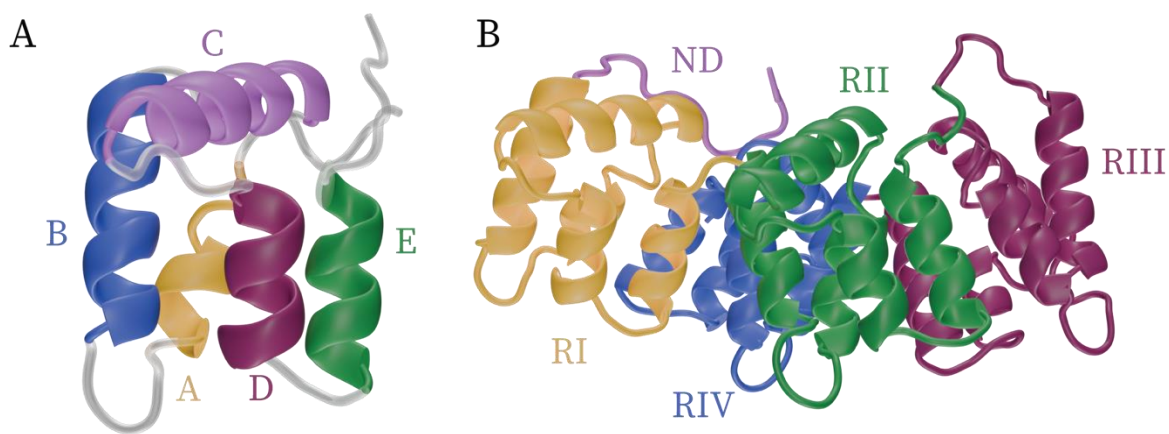


Figure 8.1. Colored and labeled cartoon representations of Annexin A2 structural features. (A) An Annexin A2 repeat and with the A, B, C, D, and E helices colored yellow, blue, light purple, maroon, and green, respectively. (B) The full Annexin A2 structure where the ND, RI, RII, RIII, and RIV, are colored dark green, light blue, gold, lavender, and red, respectively.

A2 is the most widely studied of all the Annexin proteins. It is directly involved in a number of important biological events including F-actin reorganization and plasminogen activation ^{147,150}. Further, A2 has demonstrated involvement in a range of pathologies. Multiple reviews have emphasized the role of A2 in viral infection cycles ^{3,242,243}. Aberrations in the expression patterns of A2 have been linked to the proliferation of cancers, including aggressive, metastatic cancers ². The membrane-binding capacity of A2 is critical to most of its biological functions ¹. *In vitro* studies involving A2 have investigated its ability to bridge membranes and induce microdomain formation. Both of the mentioned properties are important for the biological functions of A2 ^{7,16,112,244}. A fluorescence microscopy study conducted by Drücker et al. demonstrated that monomeric A2 both clusters on the surface of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) containing giant unilamellar vesicles (GUVs) and induces PI(4,5)P2 rich microdomains. The A2/p11 heterotetramer (A2t) was also demonstrated to exhibit the same behavior. Both A2 and A2t were shown to induce GUV aggregation and budding ¹⁶.

Membrane microdomains play a variety of roles across biology. Reviews have highlighted their importance in T-cell receptor signaling, migration of immune cells across the blood brain barrier, and exocytotic events ^{156,245,246}. Further, improper or impaired functions of microdomains have been implicated in pathologies including insulin resistance, atherosclerosis, and aging ^{247–249}. Membrane microdomains have also been shown to have significance in the assembly processes of many viruses ²⁵⁰. Given the biological relevance of microdomains and their involvement in disease, there has been much research focused on the development of drugs targeting microdomain-related activity ^{251,252}. A2 has been demonstrated to play an important role during Ca²⁺-induced exocytosis, partially owing to its ability to induce actin bundling and the formation of PI(4,5)P2-rich microdomains. Notably, the involvement of A2 in exocytotic activity

has been demonstrated as critical for the release of neurotransmitters in neuronal cells ¹⁵⁶. Given its importance, the process of A2-induced microdomain formation is an appealing target for computational modelling to further understand the process.

Lateral aggregation of A2 and A2t on membrane surfaces has been investigated via both scanning force microscopy (SFM) and mutation studies involving chemical crosslinkers ^{8,9,113}. SFM demonstrated that A2 and A2t form 2D aggregates on DPPC/DPPS membranes and that their heights are consistent with expectations; the observed heights in both cases were dimensionally consistent with the protein component of the experiment laying on top of the membrane surface in a single layer ⁹. The height of the A2t complex derived from molecular dynamics (MD) simulations reported by our lab agreed with experimentally derived values reported in the literature ²¹⁴. Crosslinking studies performed by Matos et al. have demonstrated that the charged residues on the peripheral regions of A2 are important for the aggregation observed in the aforementioned SPR studies ^{8,9}.

Our research group has previously conducted a variety of all atomic simulations studying the properties of A1 and A2t or their interaction with membrane models ^{117,119,120,185}. These studies focused on a single annexin monomer or tetramer. The available literature involving MD simulation studies of vesicles tends to focus on the process of vesicle formation or fusion ^{237,253–255}. Studies on the interaction between proteins and a vesicle model, coarse-grained or atomistic, are relatively rare; we were able to identify only a few instance in the literature most of which focused on viruses and one which investigated SNARE proteins ^{256–259}. In this work, we investigated two systems computationally: a vesicle in the presence of ten A2 (A2V system) and a vesicle in the absence of A2 (V system). Simulation runs were 15 μ s for each system and conducted in pentaplicate. The vesicle component of both systems was composed of four lipids

1-palmitoyl-2-oleoyl-glycerol-3-phosphocholine (POC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-L-serine (POS), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-(1'-myo-inositol-3',4'-bisphosphate) (PIP2), and cholesterol (CHL). During all A2V simulations, A2 aggregation and PIP2 microdomain formation were observed. The vesicle component of the systems had a diameter of 250 Å, approaching the dimensions of membrane models, specifically, small unilamellar vesicles, commonly used in experimental studies ²⁶⁰. This work is the first computational study investigating the aggregation of A2 and A2-induced membrane microdomain formation.

8.2. Computational Methods

8.2.1. Software Details

MD simulations were run using GROMACS (v 2020.6) and version 2.2 of the Martini force field ^{124,218}. Visualizations of structures were generated using either PyMol or the Molecular Nodes plug-in for Blender ^{167,195,196}. Analysis was performed using Python scripts that rely upon the Pandas, NumPy, SciPy, scikit-learn, and MDAnalysis packages ^{211–213,261–264}. All scripts were executed within Jupyter Lab ²¹⁰. Plots were generated using the Matplotlib package of Python ¹⁹⁴. Final figures were assembled using either PowerPoint or GIMP.

8.2.2. System Setup

Coarse-grained models of four lipids were utilized to generate the vesicle, 1-palmitoyl-2-oleoyl-glycerol-3-phosphocholine (POC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-L-serine (POS), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-(1'-myo-inositol-3',4'-bisphosphate) (PIP2), and cholesterol (CHL). Parameters for the lipids were readily available in the Martini Lipidome ²²⁵. The bilayer composition consisted of 60/20/8/12% POC, POS, PIP2, and CHL, respectively.

This composition was chosen to mimic experimental studies that observed A2-GUV interaction using fluorescence microscopy¹⁶. The vesicle was generated using scripts for biomolecular system generation maintained by our lab. Input files were derived from those provided by the CHARMM-GUI for coarse grained MD simulations involving vesicles^{223,265}. The radius of the generated vesicle was 125 Å. The final vesicle contained 2632, 876, 525, and 349, POC, POS, CHL, and PIP2 lipids, respectively. A crystal structure of A2 in the presence of Ca²⁺ (PDBID: 2HYW), was used to generate the A2 model structure of human A2²⁶⁶. All non-A2 molecules were removed from the crystal structure. PyMol was utilized to mutate negatively-charged Ca²⁺-binding residues, E53, E96, D162, E247, and D322, to alanine, a modification which has been demonstrated to restore A2's membrane-binding capacity in the absence of Ca²⁺⁴. The martinize2 script was utilized to convert the atomistic structure to a coarse-grained structure and the Elnedyn22 method for generating an elastic network was utilized with an upper cutoff, lower cutoff, and force constant of 0.9 nm, 0.5 nm, and 500 kJ mol⁻¹ nm⁻², respectively^{267,268}. The 2HYW model lacked the N-terminus which has been shown to be a non-essential component of A2 for its membrane-binding activity¹¹². Ten A2 were distributed across the surface of the vesicle and oriented with their convex faces toward the vesicle membrane ~5-10 Å away from the membrane surface using a suite of Python scripts for biomolecular system generation maintained in our lab. A2V systems were solvated in an octahedral box with a minimum of 15 Å of distance from the system to the box edge. Van der Waals radii were increased during solvation to prevent the insertion of water beads into the membrane. Sufficient Na⁺ and Cl⁻ ions were added to bring the concentration to 0.15 M and neutralize any residual charge in the system. See **Figure 8.2** for a visual representation of the system setup. Solvation and ion addition were performed in the same manner for the vesicle without A2 as well.

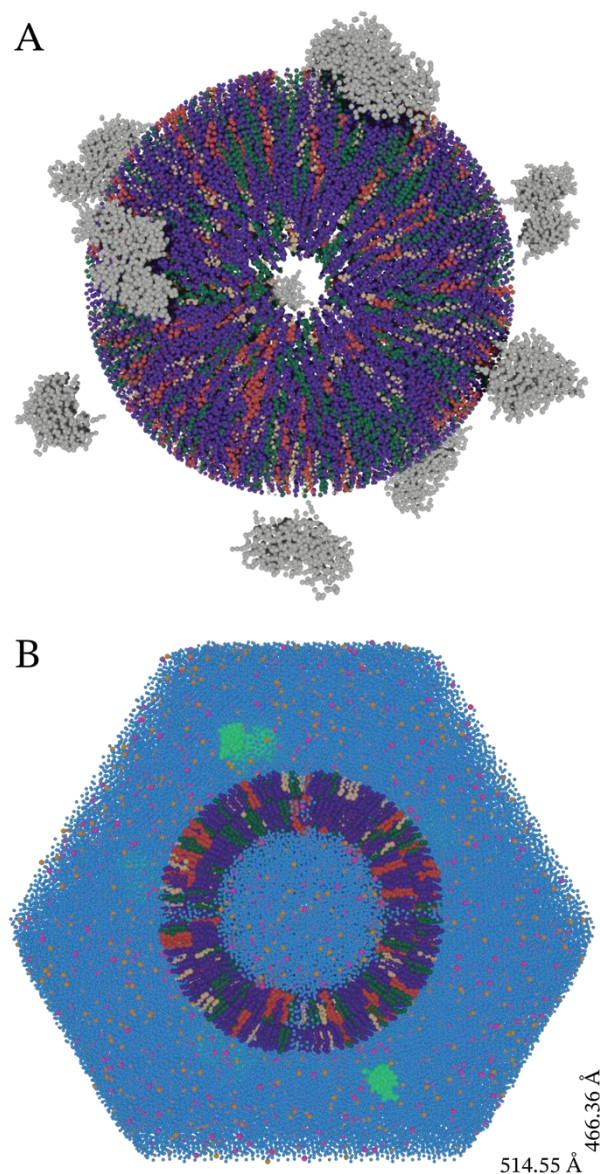


Figure 8.2. Graphical representations of the simulation setup. (A) Pre-solvation A2V after the addition of ten A2 to the vesicle. Lipids and A2 are represented as spheres. (B) Cross-section of the solvated system. All components of the system are represented using spheres. The color key for both (A) and (B) follows, POC: blue, POS: light green, PIP2: red, CHL: tan, water: teal, Na^+ : magenta, Cl^- : light orange. A2 are grey in (A) and bright green in (B).

8.2.3. Simulation Details

The procedure outlined by the CHARMM-GUI input generator was utilized with minor modifications. Minimization was performed twice. 5000 steps of soft-core minimization were performed first to remove any lipid-lipid overlap in the system. This was followed by 5000 steps of normal minimization. The steepest descent algorithm was used in both minimizations. Equilibration was performed in five steps of 10 ns each with position restraints applied to the individual A2 and cylindrical restraints applied to the lipid tails. The lipid restraints maintained 6 pores along the principal axes of the system (**Figure D.2**). The pores allowed for the water phases to equilibrate across the membrane and for lipids to move between the membrane leaflets of the vesicle. The radii of the pores were reduced incrementally with each step in the following order: 20 Å, 15 Å, 10 Å, 5 Å, 2 Å. Additionally, the timestep was increased in each equilibration step as follows: 2 fs, 5 fs, 10 fs, 15 fs, 20 fs. A2 were held restrained during equilibration for A2V. Production runs were carried out at a temperature of 310 K. A velocity-rescale thermostat was used with separate temperature coupling for the A2, lipids, and solvent. The Parrinello-Rahman barostat was used to maintain the pressure at 1 bar²⁶⁹. An isotropic pressure-coupling scheme was applied. The Verlet scheme was used to generate a pair-list with a buffer tolerance of 0.005 kJ mol⁻¹ ps⁻¹²⁷⁰. Coulombic terms were calculated using the reaction-field with a cutoff distance of 11 Å²⁷¹. A cut-off scheme with a cut-off distance of 11 Å was used for calculating van der Waals terms. For integration the md integrator with a time-step of 20 fs was utilized²⁷². The MD simulation was then run for a total of 15 μs, and a total of 5 independent MD simulation runs were performed. The same protocol was followed for V with the only difference being the absence of A2 restraints during equilibration as no A2 were present in V system.

8.3. Results and Discussion

8.3.1. A2 Association with the Vesicle

A necessary prerequisite for investigating the process of A2-vesicle association was a thorough description of the vesicle morphology provided in “Supplemental Materials” including **Figures D.1-D.5**. It was demonstrated that the vesicle component of both the A2V and V systems remains spherical, and that the vesicle’s outer radius, inner radius, and thickness were approximately 122, 81, and 42 Å across simulation runs.

The progress of A2 association with the membrane was tracked across simulation runs by plotting the average A2 height vs time (**Figure D.6**) and the height of individual A2s vs time (**Figure D.7-D.11**) for each A2V simulation run. Height was determined by subtracting the average radius of outer-leaflet lipid-heads from the magnitude of a given A2 center of mass vector with the vesicle COM representing the origin of the system. Individual A2 were assigned a letter code, A-J, such that they are distinguishable individually across all plots. All simulations saw complete association of all A2 by 4.0 μs with most A2 associating within the first μs of simulation or having associated during equilibration of the system. The height of the A2 COM from the upper leaflet has previously been reported in a cryo-electron microscopy study performed by Lambert et al. as approximately 26.0 Å. The radial distribution function was calculated for the upper leaflet and A2 after 4 μs to generate a suitable comparison. The calculated distance of A2 above the membrane surface was 26.48 ± 0.27 Å across simulation runs (**Figure D.12**). This agrees well with the value reported in the literature, ~26 Å, which demonstrates that the dimensions of A2, post-association, are in accordance with existing experimental studies.

8.3.2. PIP2 are the Primary Contributors to the A2-Vesicle Interaction

An understanding of which lipids drive A2 association was desired. The total number of A2-membrane contacts for each A2 were determined over the course of the trajectory. A contact was defined as any two CG beads that are within 6 Å of each other. These were plotted over a fraction of the total 15 μ s of simulation time, 0 – 6 μ s, to emphasize two key features of the system (**Figures D.13-D.17**). Firstly, PIP2 drives the initial association of A2. It can be seen in **Figures D.13-D.17** that PIP2 is either a significant contributor or the only significant contributor upon initial association. Secondly, the number of PIP2 contacts increases over a period of approximately 1 to 2 μ s post association. This suggests that individual A2 induce the formation of small PIP2 clusters that grow over time which is consistent with visual inspection of the trajectory (**Figure 8.3A**). It can be seen in **Figure 8.3A** that the number of PIP2 contacts between A2-I and the vesicle increases over the shown interval. This increase is consistent with the increase observed in the contact count for A2-I (**Figure D.13**). The percent contribution of each lipid to the total number of contacts per frame was also calculated for each A2 (**Figures D.18-D.22**). In **Figures D.18 – D.22**, PIP2 can clearly be seen as the primary contributor to the interaction between A2 and the membrane. The percentage of PIP2 contacts rarely drops to below 50% and tends to stay between 75% and 90% for many of the A2 across simulation runs. It should also be noted that PIP2 is the least abundant of all the constituent membrane lipids as it accounts for only 8% of all the lipids in the membrane. Thus, PIP2 had a disproportionate contribution to A2-membrane binding relative to its total number. This is reflected in experimental results which demonstrate that in the absence of a phosphoinositide membrane component the K_d for A2 for membrane-binding is increased by an order of magnitude.

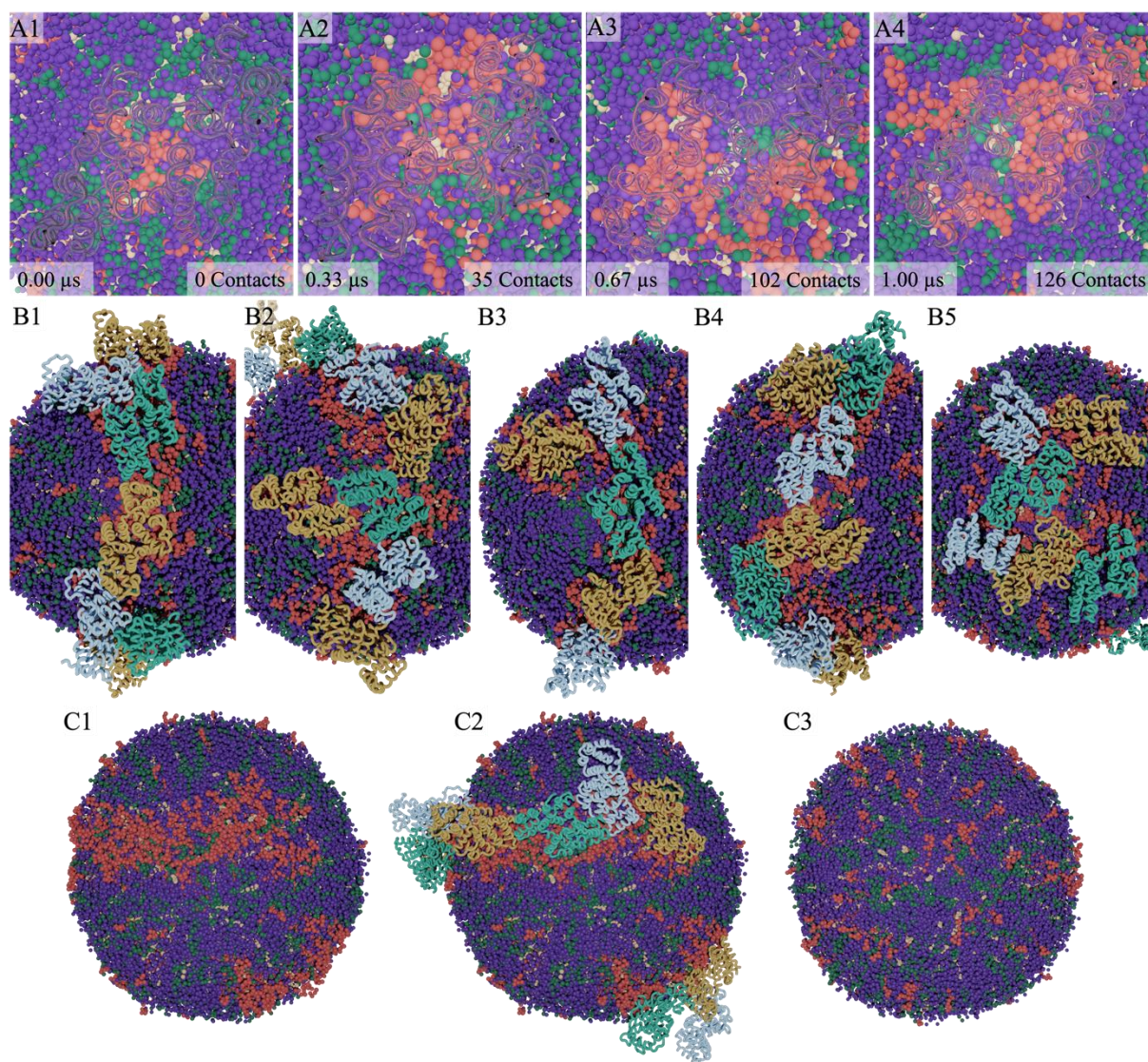


Figure 8.3. Key A2 and PIP2 clustering events. (A1-A4) Snapshots of A2-I from simulation run 1 of the A2V system highlighting the progressive formation of a PIP2 cluster underneath A2-I over the course of $\sim 1 \mu\text{s}$ of the simulation. Labels identify the time and number of PIP2 contacts with A2-I. (B1-B5) Snapshots of the largest A2 aggregate in the final frame of A2V simulation runs 1-5, respectively. (C1-C2) The final frame from simulation run 1 of the A2V simulation (C1) without A2 shown and (C2) with A2 shown. (C3) Final frame from the vesicle simulation. In all images POC, POS, PIP2, and CHL, are colored purple, green, red, and tan, respectively. In (A1-A4) A2 are rendered using a transparent material to enhance visibility of the PIP2 cluster beneath. In (B1-B4, C1-C3) A2 are colored gold, green, or light blue such that they are easily distinguishable from their neighbors.

8.3.3. Basic Residues on the Convex Face of A2 are Principal Contributors to the A2-Vesicle Interaction

It has previously been shown that basic residues on the convex face of A2 are principal contributors to its anionic membrane-binding capacity. Specifically, a study by Gokhale et al. investigating the mechanism of A2 specificity for PI(4,5)P2 demonstrated that residues K279 and K281 belong to a PI(4, 5)P2-binding motif and that they contribute significantly to the binding-capacity of A2. K279A and K281A mutations result in an order of magnitude increase of the K_d for A2 and phosphoinositide-containing membrane interactions. R284 was also identified as a potential contributor though its contribution could not be investigated owing to difficulties with R284A mutant expression ⁵. All atomic simulations of A2t-membrane systems conducted by our lab also support results reported in the aforementioned experimental studies ²¹⁴.

The most important residues for A2-membrane interaction during the simulation were identified via contact analysis. Contacts were counted after 4 μ s at which point every A2 had stably associated with the membrane or another A2; this was done to prevent bias from A2 that associated with the structure earlier than others as the contribution from an A2 that associated earlier would be over-represented. A2-membrane contacts less than 6 Å were counted and pooled. The percentage contribution of each residue was then calculated.

Results are summarized in **Figure 8.4B** and **Table D.1**. A graphical representation of the results was generated so that the distribution of top contributors across the structure could be viewed (**Figure 8.4B**). Each repeat of the core domain contained important contributors. All the top contributors are located at or immediately adjacent to Ca^{2+} -binding sites composed of loops that connect helix A to helix B and helix D to helix E. The primary contributors are largely lysine residues; 8 of the top 12 residues are lysine (**Figure 8.4B**). Important contributions are also seen

from non-lysine residues R284, H94, L121, and Y151. With R284 having been suggested as a potential contributor by Gokhale et al ⁵. To our knowledge, the contributions of H94, L121, and Y151 have not been investigated in the literature. The contact profile also agrees well with expectations; most residues that contribute to the membrane interaction are basic amino acids located on the convex surface of A2. Further, K281 and R284 were identified among the top contact contributors though residue K279 was not which suggests that K279 may play an indirect role in facilitating the A2-membrane interaction ⁵.

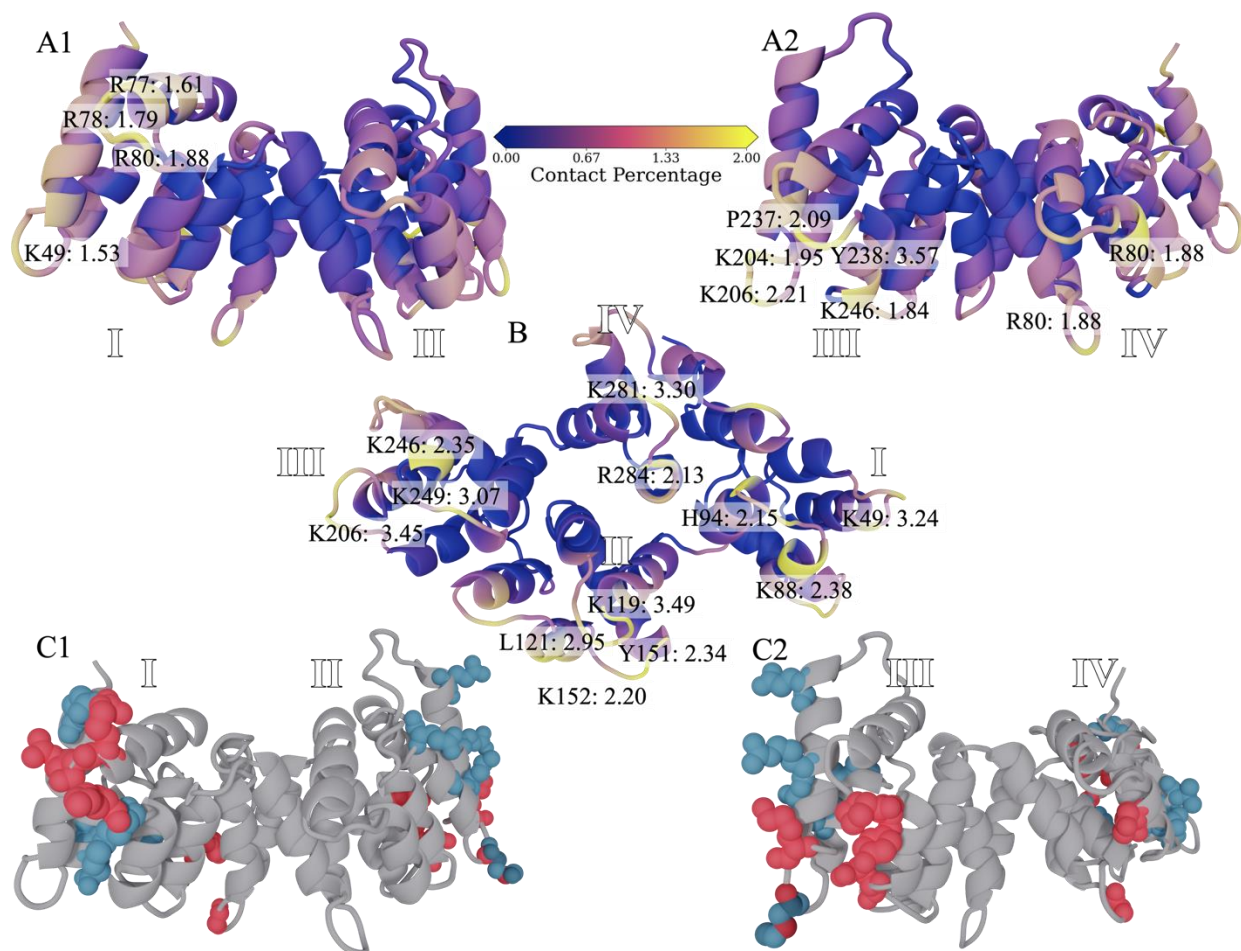


Figure 8.4. 3D representations of contact analysis. (A) Views of A2 models each residue colored according to its percent contribution to total A2-A2 contacts. Labels are shown for residues that contribute more than 1.5% of all A2-A2 contacts. (B) View of an A2 model's convex face with residues colored according to their percent contribution to total A2-membrane contacts. Labels are shown for residues that contribute more than 2.0% of all A2-membrane contacts. (C) Views of an A2 model are show with residues experimentally identified as important for A2 aggregation via cross-linking studies represented as red spheres and the top ten residues we identified as important for A2-A2 contacts colored blue. The provided color bar corresponds to both (A) and (B). Repeats of A2 are labeled in all images.

8.3.4. A2 Aggregation on the Vesicle Surface

Visual inspection of the trajectories for A2V simulation runs revealed the formation of large A2 aggregates across all simulations (**Figure 8.3B**). The largest A2 aggregates which formed in A2V simulation runs 1-5 contained 6, 7, 5, 7, and 6, A2, respectively. The other A2 not belonging to the largest aggregates were members of smaller aggregates. Only one A2 never associated with other A2 across the five 15 μ s simulation runs; only one of 50 A2 considered across all A2V simulation runs did not join an A2 aggregate within 15 μ s of simulation.

Contact analysis was performed for the 14-15 μ s times range. The method of contact analysis applied to probe the A2-membrane interaction was also applied to A2-A2 interactions throughout the simulation. Contacts were identified and pooled to be used for calculating the percentage contribution of each residue to the total number of A2-A2 contacts.

A graphical representation of the contact results was generated to visualize the distribution of contributing residues across A2 (**Figure 8.4A**). The top ten contributors are all contained within the A, C, and D helices of RI-RIV or their associated loops. In particular, the CD loops appear to be significant contributors to the A2-A2 interactions. Further inspection of the A2 structure reveals that C, D, and the CD loop form modestly outward extrusions at the “four corners” of A2. In the A2 aggregates the A2 corners appear to feature prominently at the A2-A2 interfaces (**Figure 8.3B**). These results are consistent with those reported in cross-linking studies which investigated lateral aggregation patterns among individual A2 subunits on a membrane model ¹¹³. They are not consistent in their exact identities, but rather they are similar in their proximal locations and similar characteristics i.e. charged residues located within the aforementioned locations. Most of the top residues identified as primary contributors to A2-A2 interaction are primarily basic residues (Table S2). A graphical representation showing the

locations of the residues investigated during the cross-linking study conducted by Matos et al. and the top contributors from the A2V simulation runs was generated to emphasize similarity of location (**Figure 8.4C**)¹¹³. This suggests that the process of lateral A2 aggregation may involve more residues than have been highlighted in the literature while still supporting the suggestion of Matos et al. regarding the proximal location of said residues.

8.3.5. A2 Aggregation Drives Microdomain Formation

In addition to the previously described local clustering, large and stable PIP2 microdomains were observed during the A2V simulation runs. Formation of the microdomains was identified as being driven by the aggregation of individual A2. As the A2 came together, the local PIP2 clusters underneath each A2 merged to form large microdomains. This is highlighted in **Figures 8.3C1** and **8.3C2** where the final structure from the simulation is shown with and without A2, respectively. The A2 clusters are clearly visible along with the PIP2 microdomains that formed beneath them (**Figures 8.3C1** and **8.3C2**). This stands in stark contrast to the initial setup of the A2V system (**Figure 8.2A**) as well as the final structure of the first MD simulation for the V system (**Figure 8.3C3**). In both **Figures 8.3C3** and **8.2A**, the PIP2 are much more scattered and no microdomains are observed.

A quantitative metric for describing the degree of microdomain formation was desired that could also be applied to A2 aggregation. Variance is an obvious candidate for measuring spread; however, it is difficult to calculate using standard methods because Euclidian distance calculations result in an unrealistic representation of the variance. Given the previous, a suitable Fréchet variance was defined for the system instead. The Fréchet variance and its application to the system are described in detail in the “Fréchet Mean” section of the “Supplemental Materials”. Briefly, the Fréchet variance is a general description of distance between points

which can be used to identify Karcher means; these are points that locally minimize the Fréchet variance. If a global minimum exists, then it is referred to as the Fréchet Mean. The Fréchet variance can then be calculated with respect to the Fréchet mean. For our purposes, the Fréchet variance was defined as the squared arclength between lipid centers of mass ⁵.

The Fréchet variance, henceforth variance, was calculated for the lipids in the V (**Figure 8.5C**) and A2V systems (**Figure 8.5D**). The variances from the V system are relatively stable, remaining near their initial values (**Figure 8.5C**). This differs by the number of each lipid type. POC variance is extremely stable because the POC lipids are the most populous vesicle component and have little room to come closer to each other; they are unable to aggregate any further because they already occupy so much of the available surface area. PIP2 lipid variance varies more as the PIP2 lipids, being the least frequent component, are more sensitive to shifts in relative position on the surface. As with the V system, the variances of POC, POS, and CHL are stable for the A2V system (**Figure 8.5D**). However, the PIP2 variance demonstrates a clear downward trend (**Figure 8.5D**). A clear downward trend is also observed in the A2 variance during this time. The variances of A2 and PIP2 for the A2V system were plotted together with separate scales to clearly illustrate their relationship (**Figure 8.5A**). They appeared to have a strong relationship. A plot of A2 variance vs PIP2 variance from during this time-range of the simulation was generated and demonstrates a clear relationship (**Figure 8.5B**). All variance calculations were performed for every simulation run of the A2V and V systems and the described observations hold for every simulation run (**Figures D.23 – D.26**).

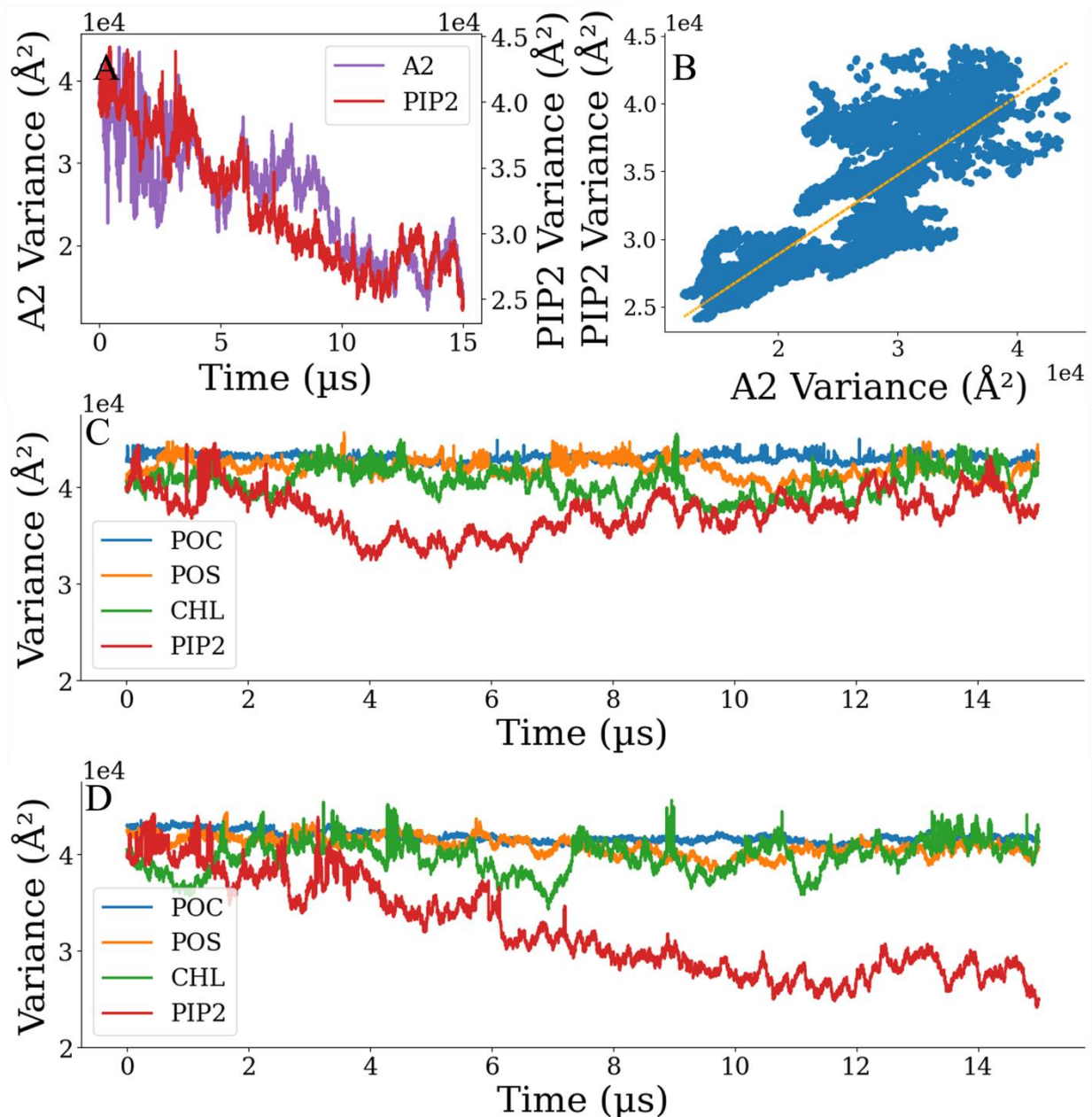


Figure 8.5. Lipid and A2 variance plots. (A) Plot of A2 and PIP2 variances vs time with separate y-axes scales specified. (B) Plot of PIP2 variance vs A2 variance with a trendline included. Variance for all lipids from (C) the V system which lacks A2 and (D) the A2V system which contains A2.

The variance clearly demonstrates the progressive formation of PIP2 microdomains in the presence of A2. Thus, it serves as a suitable quantitative metric for identifying microdomain

formation in the system. Further, it demonstrates, quantitatively, the effect that A2 aggregation has on global microdomain formation.

8.3.6. Summary of PIP2 Microdomain Formation

When combined, the presented descriptions of A2-association, A2-induced local PIP2 aggregation, and A2 aggregation, form a cohesive model of A2-induced microdomain formation. The A2 associate with the membrane via interactions with lipids, predominately PIP2. Subsequently, individual A2 recruit more PIP2 underneath themselves, forming local PIP2 clusters. The individual A2 then aggregate, causing the PIP2 beneath them to merge into larger microdomains. A graphical summary of this process is presented in **Figure 8.6**. This mechanism is largely consistent with that put forth by Gokhale et al. and expands upon it by identifying residues important for A2-membrane interaction and A2 aggregation binding as well as clearly demonstrating the order in which events occur ⁵. Further, it is consistent with other existing biophysical studies which investigated the dimensions of A2-membrane structures and the residues which associated with the membrane.

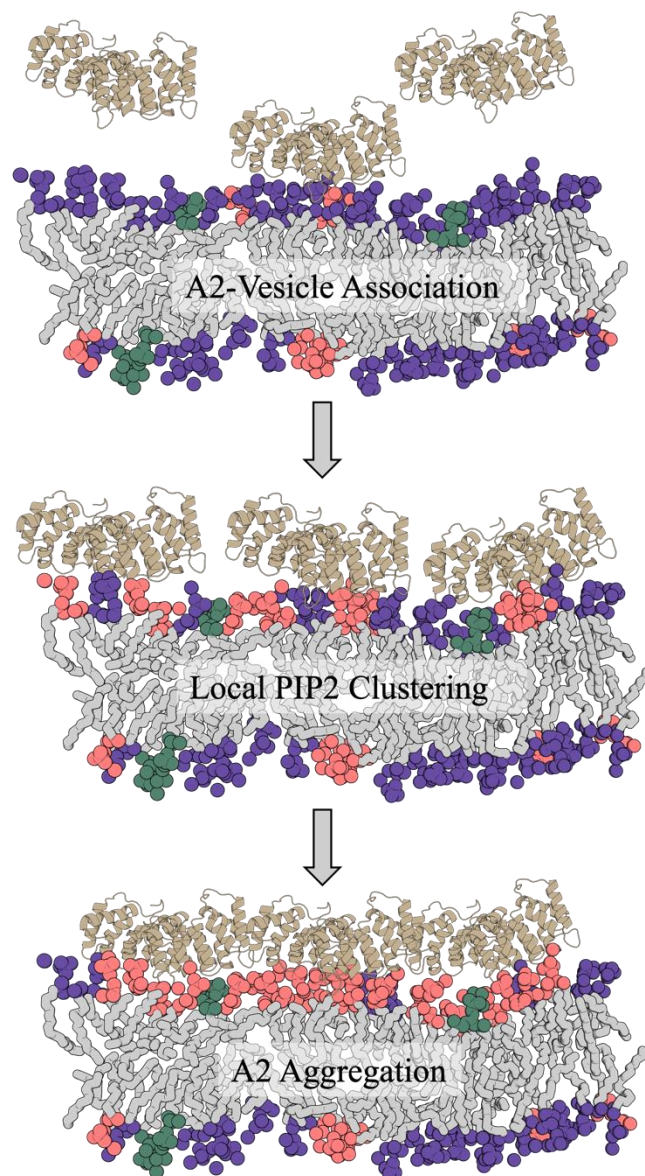


Figure 8.6. Graphical summary of A2-induced PIP2 microdomain formation. POC, POS, and PIP2 lipid heads are colored purple, green, and red, respectively. Lipid tails are colored grey. A2 is colored tan and represented as a cartoon. The first image represents the initial association of A2 with the membrane, the second image represents A2-induced local aggregation of PIP2, the final image represents A2 aggregation and the associated formation of PIP2 microdomains.

8.4. Conclusions

The processes of A2-membrane association were thoroughly described. The association of the individual A2 was demonstrated to mostly occur within the first 1 μ s of simulation with all A2 being stably associated with another A2 or the membrane surface. By 4 μ s of simulation time. The distance between the upper leaflet COM and the A2 COM of the simulation, 26.48 ± 0.27 Å, agreed well with that reported in the literature, ~ 26 Å. PIP2 lipids were found to be the most significant contributors to both the initial and sustained association of all A2 by way of both absolute and relative number of contacts. A2 contacts with the membrane were investigated and top-contributing residues were identified. All the top contributors were on the convex face of A2 and found among all repeats of the core domain. The top five contributing residues were K119, K206, K281, K49, and K249. K281 is a known contributor to the A2-membrane interaction. Further, non-lysine residues at the convex face, L121, Y151, H94, and R284, were identified as important contributors to the A2-vesicle interaction.

Aggregation of A2 was observed during the simulation. By the end of all A2V simulation runs every A2 belonged to an A2 aggregate with the exception of a single A2. Contact analysis was employed to identify the top contributors to the A2-A2 interaction. Primary contributors were located at the “four corners” of A2 within A, C, D helices or the CD loop. The top five contributors to A2 aggregation were Y238, K206, P237, Y317, and K204. Further, the location of residues driving A2-A2 interaction are largely consistent with what has been put forth in the literature.

A2 aggregation was observed to drive the merging of local PIP2 aggregates into large microdomains. The Fréchet mean was demonstrated to be an excellent quantitative metric for identifying global microdomain formation.

The presented results illustrate the process of A2-membrane association, local PIP2 aggregation, A2 aggregation, and microdomain formation. The details of the A2-A2 interaction and A2-membrane interaction were presented in detail. Further, they are consistent with but also expand upon existing experimental studies which supports their veracity. Significant insight into the process of A2-induced microdomain formation has been generated. We believe the presented model of A2-induced PIP2 microdomain formation and A2 aggregation will serve as an invaluable guide for directing further experimental studies as well as the development of novel therapeutics targeting A2.

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APPENDIX A

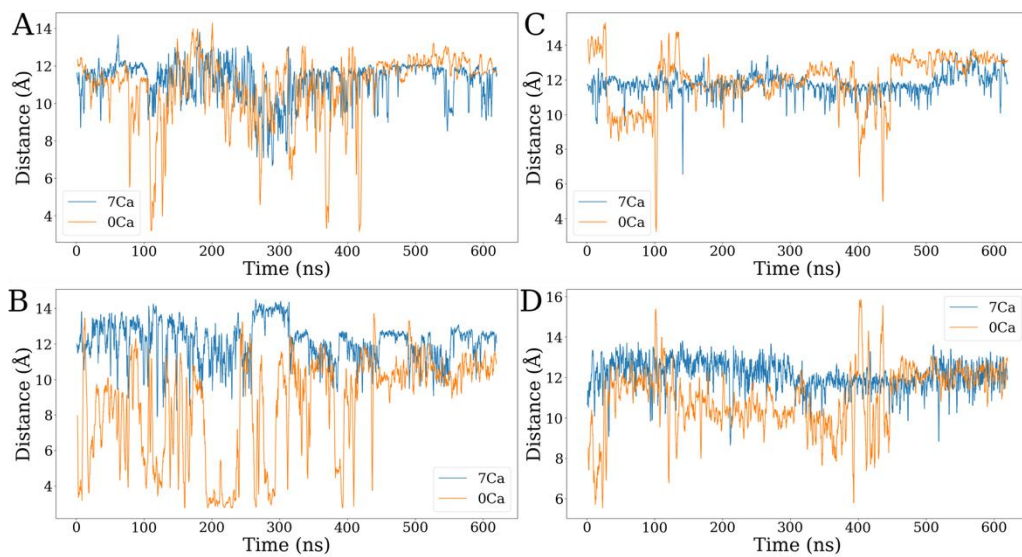


Figure A.1. Distance vs time plots generated using the carboxylic oxygen of Ca²⁺-binding residue D322 and the (A) A2-1-IV-K279 (B) A2-1-IV-K281 (C) A2-2-IV-K279 (D) A2-2-IV-K281 amino nitrogen for both the 7Ca and 0Ca models.

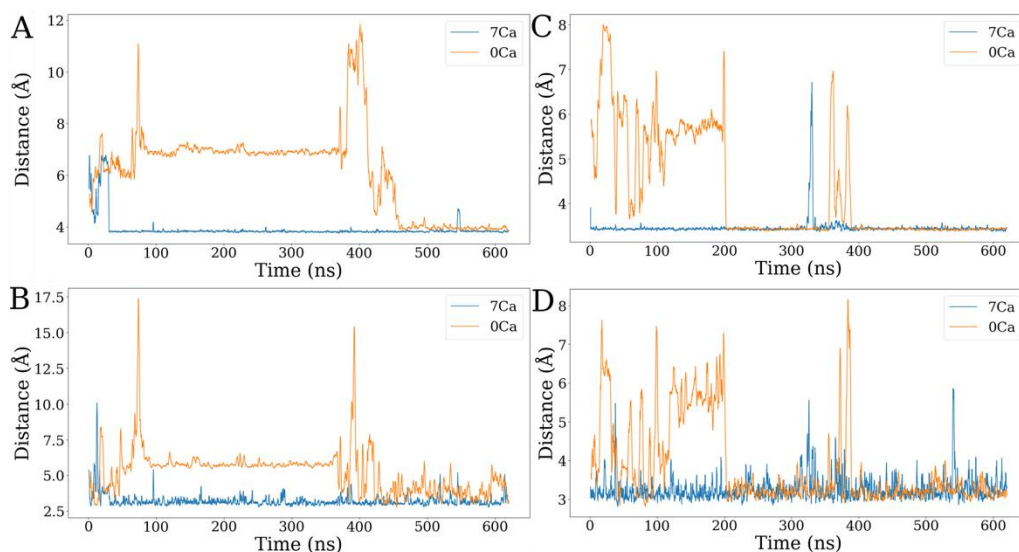


Figure A.2. Distance vs time plots were generated using the distances between the (A) side-chain carboxylic acid oxygens of A2-1-IV-D339 with the guanidino nitrogens of A2-1-IV-K304, (B) terminal carboxylic acid oxygens of A2-1-IV-D339 with the amine nitrogen of A2-1-IV-K308, (D) side-chain carboxylic acid oxygens of A2-2-IV-D339 with the guanidino nitrogens of A2-2-IV-R304, and (E) terminal carboxylic acid oxygens of A2-2-IV-D339 with the amine nitrogen of A2-2-IV-K308.

Table A.1. Top Total Binding Energy of a Single Residue with an Entire Subunit for the 7ca Model.

Residue	Residue Subunit	Interaction Subunit	ΔE (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)	Residue	Residue Subunit	Interaction Subunit	ΔE (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)
K80	A2-1	p11-1	-14.6	0.4	D85	p11-1	A2-1	-18.1	0.3
L8			-10.6	0.1	Y86			-11.5	0.1
R77			-10.4	0.3	N45			-9.0	0.1
L11			-10.0	0.1	Q46			-8.0	0.1
L13			-7.7	0.1	K47			-7.9	0.2
I7			-7.3	0.1	F42			-6.9	0.0
D16			-7.2	0.3	P40			-6.0	0.0
Q76			-6.0	0.2	V89			-5.7	0.0
E82			-4.8	0.2	C83			-5.2	0.0
F73			-4.1	0.1	K54			-5.0	0.2
S2	A2-1	p11-2	-27.0	1.0	E6	p11-2	A2-1	-25.7	0.5
T3			-11.5	0.2	E10			-23.7	0.2
V4			-10.3	0.2	M13			-1.6	0.0
HIE5			-3.7	0.2	M9			-1.4	0.0
L8			-1.3	0.0	F14			-1.3	0.0
I7			-0.6	0.0	H7			-0.9	0.0
R77			-0.2	0.0	Q70			-0.2	0.0
K80			-0.1	0.0	T11			-0.1	0.0
K10			-0.1	0.0	H17			-0.1	0.0
K152			-0.1	0.0	D22			-0.1	0.0
M5	p11-1	p11-2	-12.4	0.1	M5	p11-2	p11-1	-14.3	0.2
K92			-8.9	0.4	H17			-11.0	0.3
N84			-8.5	0.1	K96			-9.6	0.3
E38			-8.3	0.3	F73			-8.1	0.1
M9			-8.3	0.1	M9			-7.6	0.1
F73			-7.7	0.1	T80			-7.4	0.1
E10			-7.3	0.3	N84			-7.2	0.2
F69			-6.7	0.1	M13			-7.0	0.1
M12			-6.6	0.1	E38			-6.5	0.2
T80			-6.5	0.1	F69			-6.3	0.1
S2	A2-2	p11-1	-31.3	0.3	E6	p11-1	A2-2	-33.3	0.1
T3			-12.7	0.2	E10			-23.2	0.1
V4			-11.6	0.1	M13			-2.0	0.0
H5			-5.2	0.1	M9			-1.7	0.0
L8			-1.0	0.0	F14			-1.4	0.0
I7			-0.8	0.0	H7			-1.0	0.0
E6			-0.3	0.0	S3			-0.5	0.0
K10			-0.1	0.0	T11			-0.2	0.0
R77			-0.1	0.0	Q70			-0.1	0.0
C9			-0.1	0.0	F69			-0.1	0.0
R77	A2-2	p11-2	-15.1	0.2	K97	p11-2	A2-2	-14.2	0.2
R145			-10.6	0.2	D85			-14.1	0.1
D16			-10.0	0.4	Q46			-9.8	0.1
L8			-10.0	0.1	K57			-9.4	0.2
L13			-8.8	0.1	Y86			-8.5	0.1
T3			-8.5	0.1	D48			-8.1	0.1
I7			-7.8	0.1	L50			-6.9	0.1
L11			-7.7	0.1	F42			-5.9	0.0
S2			-7.0	0.1	C83			-5.8	0.0
E6			-6.6	0.3	H90			-4.9	0.1

Table A.2. Top Total Binding Energy of a Single Residue with an Entire Subunit for the Oca Model.

Residue	Residue Subunit	Interaction Subunit	ΔE (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)	Residue	Residue Subunit	Interaction Subunit	ΔE (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)
R77	A2-1	p11-1	-16.6	0.6	K97	p11-1	A2-1	-25.3	0.2
R145			-14.0	0.2	D85			-13.7	0.3
L8			-10.3	0.1	K54			-13.7	0.2
E82			-8.8	0.4	D58			-12.6	0.3
K10			-8.8	0.3	Y86			-11.2	0.1
E14			-8.6	0.3	K96			-11.0	0.3
L11			-8.4	0.1	Q46			-10.5	0.1
L13			-8.2	0.2	K94			-7.3	0.2
F73			-8.0	0.1	H90			-7.0	0.1
I7			-7.7	0.1	K57			-6.9	0.3
S2	A2-1	p11-2	-34.9	0.4	E6	p11-2	A2-1	-34.3	0.2
T3			-11.7	0.3	E10			-25.2	0.2
V4			-11.4	0.1	M13			-1.4	0.0
H5			-4.3	0.1	F14			-1.4	0.0
L8			-1.2	0.0	H7			-1.3	0.1
I7			-0.8	0.0	M9			-1.1	0.0
R77			-0.1	0.0	T11			-0.1	0.0
K80			-0.1	0.0	Q70			-0.1	0.0
E6			-0.1	0.0	H17			-0.1	0.0
K10			-0.1	0.0	Q4			-0.1	0.0
M5	p11-1	p11-2	-11.1	0.1	M5	p11-2	p11-1	-12.7	0.2
H17			-10.5	0.3	F73			-8.6	0.1
M9			-8.1	0.1	M9			-8.6	0.1
F73			-7.7	0.1	N84			-8.4	0.1
T80			-7.4	0.1	E38			-8.2	0.2
E38			-7.4	0.5	K92			-8.1	0.4
N84			-7.2	0.1	T80			-7.0	0.1
F69			-6.2	0.1	Q4			-6.7	0.4
M13			-6.2	0.1	F69			-6.1	0.1
I81			-6.0	0.1	M13			-5.7	0.1
S2	A2-2	p11-1	-20.0	0.6	E10	p11-1	A2-2	-22.6	0.3
T3			-9.9	0.2	E6			-11.8	0.2
V4			-7.2	0.2	P2			-2.1	0.1
H5			-4.4	0.2	M13			-2.1	0.0
L8			-0.8	0.0	M9			-1.8	0.0
I7			-0.5	0.0	F14			-1.5	0.0
E6			-0.2	0.0	H7			-0.8	0.0
E149			-0.1	0.0	S3			-0.3	0.0
K80			-0.1	0.0	T11			-0.2	0.0
C9			-0.1	0.0	D22			-0.1	0.0
L8	A2-2	p11-2	-10.2	0.2	K54	p11-2	A2-2	-12.4	0.1
L11			-9.7	0.1	Y86			-11.4	0.1
D16			-7.7	0.2	N45			-10.4	0.2
I7			-7.4	0.1	Q46			-8.8	0.1
R77			-7.3	0.6	V89			-6.8	0.1
F73			-6.9	0.1	F42			-6.7	0.0
L13			-6.5	0.1	D85			-6.6	0.3
H5			-6.5	0.1	H90			-5.7	0.1
Q76			-6.3	0.2	C83			-5.7	0.0
T19			-4.7	0.1	P40			-5.5	0.1

Table A.3. Top Pairwise Binding Energies for Intersubunit Interactions for the 0ca Model.

Sub-unit 1	Residue 1	Sub-unit 2	Residue 2	ΔE (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)	Sub-unit 1	Residue 1	Sub-unit 2	Residue 2	ΔE (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)
A2-1	R145	p11-1	K97	-14.5	2.1	p11-1	S3	p11-2	E38	-5.005	1.4
	R77		D58	-11.1	5.5		H17		K92	-4.927	2.6
	E82		K94	-6.8	4.1		E38		Q4	-4.710	4.2
	K80		D85	-6.8	5.3		T80		M9	-3.194	0.4
	H17		K54	-4.2	2.9		H17		N84	-3.162	0.4
	E14		K54	-4.1	1.3		N84		H17	-2.869	0.6
	D16		K57	-3.8	5.2		M9		T80	-2.828	0.5
	D70		K96	-3.8	4.4		F69		N84	-2.631	0.6
	K10		K96	-3.6	2.3		A77		F73	-2.403	0.4
	R77		D85	-3.6	3.1		I81		F69	-2.382	0.4
A2-1	S2	p11-2	G6	-20.3	2.8	A2-2	S2	p11-1	E10	-12.009	4.2
	S2		G10	-14.5	2.7		T3		E10	-7.815	1.9
	T3		G10	-8.0	2.3		S2		E6	-7.683	4.2
	V4		G6	-6.6	1.0		V4		E10	-2.491	1.5
	H5		G6	-3.9	1.3		H5		P2	-2.008	1.5
	T3		G6	-2.5	1.9		H5		E6	-1.854	0.4
	V4		G10	-2.3	0.9		V4		E6	-1.800	0.7
	V4		M9	-1.3	0.3		V4		M9	-1.502	0.4
	T3		F14	-1.0	0.5		T3		F14	-1.197	0.3
	S2		H7	-0.9	1.0		V4		M13	-1.088	0.3
A2-1	K80	A2-2	E14	0.0	0.1	A2-2	D16	p11-2	K54	-9.017	1.8
	S2		E6	0.0	0.0		R77		D85	-5.246	5.4
	E6		S2	0.0	0.0		H5		P40	-3.813	1.0
	S2		E149	0.0	0.0		Q76		V89	-3.345	1.2
	R77		E14	0.0	0.0		L8		F42	-3.342	0.5
	R78		E14	0.0	0.0		F73		Y86	-3.026	0.7
	S2		E14	0.0	0.0		L11		Q46	-3.006	0.6
	S2		E142	0.0	0.0		E82		K94	-2.833	3.9
	K80		D16	0.0	0.0		K10		Y86	-2.688	0.9
	S2		D154	0.0	0.0		Q76		H90	-2.598	1.0

Table A.4. A2-p11
Contact Pairs
Identified in the
Literature.

A2 Residue	p11 Residue
V4	E6
	M9
	E10
	M13
I7	C83
	Y86
	F87
	M91
L8	M13
	F39
	F42
	L79
L11	C83
	E6
	M9
	F42
T3	A82
	C83
	Y86
	E10
	F14

APPENDIX B

SFM Measurement Reproduction

The solvent accessible residues of A2t were selected using the FindSurfaceResidues PyMol script found on the PyMol Wiki. Non-solvent accessible residues were removed from the structure, these are represented as sticks in Figure S1A. The structure was then oriented such that the membrane-binding surface was facing downwards. Residues beneath the top of the complex were then selected and removed, these are colored red in Figure S1B. The center of mass of each residue was extracted. The center of mass of each residue was calculated over the trajectory. The center of mass of the upper membrane leaflet phosphorous atoms was also calculated. The height of each residue over the trajectory was calculated by subtracting the y-coordinate of the phosphorous center of mass from the center of mass for each residue. These calculated heights were averaged for each frame. The p11 height from the membrane had stabilized by the 200 ns point in the simulation (see Figure 4) so the heights after 200 ns were used for comparison to the SFM data. The average height was 27.63 Å (SD = 0.83). The total list of selected residues used in the height calculations follows: A2-1-M1, A2-1-S2, A2-1-T3, A2-1-H5, A2-1-E6, A2-1-C9, A2-1-K10, A2-1-P20, A2-1-P21, A2-1-S22, A2-1-A23, A2-1-Y24, A2-1-G25, A2-1-S26, A2-1-V27, A2-1-K28, A2-1-A29, A2-1-Y30, A2-1-T31, A2-1-N32, A2-1-F33, A2-1-D34, A2-1-R37, A2-1-D38, A2-1-L40, A2-1-N41, A2-1-E43, A2-1-T44, A2-1-T55, A2-1-N58, A2-1-I59, A2-1-T61, A2-1-N62, A2-1-R63, A2-1-S64, A2-1-N65, A2-1-E66, A2-1-Q67, A2-1-R68, A2-1-Q69, A2-1-D70, A2-1-A72, A2-1-F73, A2-1-R77, A2-1-R78, A2-1-L103, A2-1-T105, A2-1-P106, A2-1-A107, A2-1-Q108, A2-1-D110, A2-1-S134, A2-1-R135, A2-1-T136, A2-1-N137, A2-1-Q138, A2-1-E139, A2-1-L140, A2-1-Q141, A2-1-E142, A2-1-N144, A2-1-R145, A2-1-V146, A2-1-E149, A2-1-K176, A2-1-R178, A2-1-R179, A2-1-A180, A2-1-E181, A2-1-D182, A2-1-G183, A2-1-S184, A2-1-V185, A2-1-I186, A2-1-D187, A2-1-Y188, A2-1-E189, A2-1-L190, A2-1-D192, A2-1-Q193, A2-1-D194, A2-1-R196, A2-1-D197, A2-1-S215, A2-1-I216, A2-1-T218, A2-1-E219, A2-1-R220, A2-1-S221, A2-1-V222, A2-1-P223, A2-1-H224, A2-1-Q226, A2-1-K227, A2-1-F229, A2-1-D230, A2-1-R231, A2-1-K233, A2-1-S234, A2-1-Y235, A2-1-CYX262, A2-1-I263, A2-1-Q264, A2-1-N265, A2-1-K266, A2-1-P267, A2-1-L268, A2-1-V293, A2-1-S294, A2-1-R295, A2-1-S296, A2-1-E297, A2-1-V298, A2-1-D299, A2-1-M300, A2-1-L301, A2-1-K302, A2-1-R304, A2-1-S305, A2-1-E306, A2-1-K308, A2-1-R309, A2-1-Y333, A2-1-L334, A2-1-G336, A2-1-G337, A2-1-D338, A2-1-D339, A2-2-M1, A2-2-S2, A2-2-T3, A2-2-H5, A2-2-E6, A2-2-I7, A2-2-L8, A2-2-C9, A2-2-K10, A2-2-S12, A2-2-S18, A2-2-P20, A2-2-P21, A2-2-S22, A2-2-A23, A2-2-Y24, A2-2-G25, A2-2-S26, A2-2-V27, A2-2-K28, A2-2-A29, A2-2-Y30, A2-2-T31, A2-2-N32, A2-2-F33, A2-2-D34, A2-2-A35, A2-2-E36, A2-2-R37, A2-2-D38, A2-2-L40, A2-2-N41, A2-2-V54, A2-2-T55, A2-2-N58, A2-2-I59, A2-2-T61, A2-2-N62, A2-2-R63, A2-2-S64, A2-2-N65, A2-2-E66, A2-2-R68, A2-2-Q69, A2-2-D70, A2-2-A72, A2-2-R78, A2-2-L103, A2-2-T105, A2-2-P106, A2-2-A107, A2-2-Q108, A2-2-Y109, A2-2-CYX133, A2-2-T136, A2-2-N137, A2-2-Q138, A2-2-E139, A2-2-Q141, A2-2-E142, A2-2-N144, A2-2-R145, A2-2-V146, A2-2-K176, A2-2-G177, A2-2-R178, A2-2-R179, A2-2-A180, A2-2-E181, A2-2-D182, A2-2-G183, A2-2-S184, A2-2-V185, A2-2-I186, A2-2-D187, A2-2-Y188, A2-2-E189, A2-2-L190, A2-2-D192, A2-2-Q193, A2-2-D194, A2-2-R196, A2-2-D197, A2-2-Y199, A2-2-D200, A2-2-V203, A2-2-K204, A2-2-K212, A2-2-S215, A2-2-E219, A2-2-R220, A2-2-V222, A2-2-P223, A2-2-H224, A2-2-Q226, A2-2-K227, A2-2-F229, A2-2-D230, A2-2-R231, A2-2-K233, A2-2-S234, A2-2-Y235, A2-2-S236, A2-2-P237, A2-2-Y238, A2-2-D239, A2-2-M240, A2-2-L241, A2-2-

E242, A2-2-Q261, A2-2-I263, A2-2-Q264, A2-2-N265, A2-2-K266, A2-2-P267, A2-2-L268, A2-2-Y269, A2-2-D272, A2-2-Y275, A2-2-V293, A2-2-S294, A2-2-R295, A2-2-S296, A2-2-E297, A2-2-V298, A2-2-D299, A2-2-L301, A2-2-K302, A2-2-R304, A2-2-S305, A2-2-E306, A2-2-K308, A2-2-R309, A2-2-K310, A2-2-Y311, A2-2-G312, A2-2-K313, A2-2-S314, A2-2-Y316, A2-2-Y317, A2-2-Y318, A2-2-Q320, A2-2-K329, A2-2-L332, A2-2-Y333, A2-2-G336, A2-2-G337, A2-2-D338, A2-2-D339, p11-1-P2, p11-1-S3, p11-1-Q4, p11-1-E6, p11-1-H7, p11-1-A8, p11-1-E10, p11-1-T11, p11-1-F14, p11-1-H17, p11-1-K18, p11-1-F19, p11-1-V33, p11-1-E36, p11-1-K37, p11-1-E38, p11-1-P40, p11-1-G41, p11-1-L43, p11-1-E44, p11-1-N45, p11-1-Q46, p11-1-K47, p11-1-A82, p11-1-C83, p11-1-N84, p11-1-D85, p11-1-Y86, p11-1-F87, p11-1-V88, p11-1-V89, p11-1-H90, p11-1-M91, p11-1-K92, p11-2-P2, p11-2-S3, p11-2-Q4, p11-2-E6, p11-2-H7, p11-2-A8, p11-2-E10, p11-2-T11, p11-2-F14, p11-2-H17, p11-2-K18, p11-2-F19, p11-2-G21, p11-2-D22, p11-2-K23, p11-2-G24, p11-2-Y25, p11-2-E29, p11-2-D30, p11-2-R32, p11-2-V33, p11-2-E36, p11-2-K37, p11-2-E38, p11-2-P40, p11-2-G41, p11-2-L43, p11-2-E44, p11-2-K47, p11-2-G68, p11-2-H90

Figures and Tables

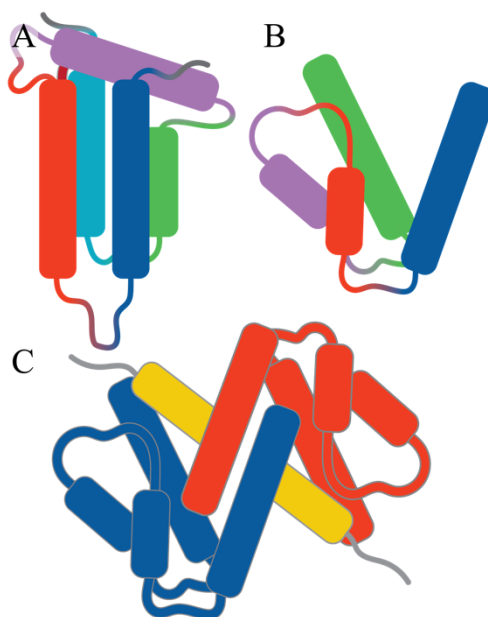


Figure B.1. Schematic representations of various A2t subunit structural components. (A) A single A2 core domain repeat with helices A-E colored dark blue, red, purple, green, and light blue, respectively. (B) A single p11 with HI-HIV colored blue, red, purple, and green, respectively. (C) A p11 dimer with the individual p11 subunits in complex with the A2 ND where the p11 are colored blue and red and the A2-ND is colored gold.

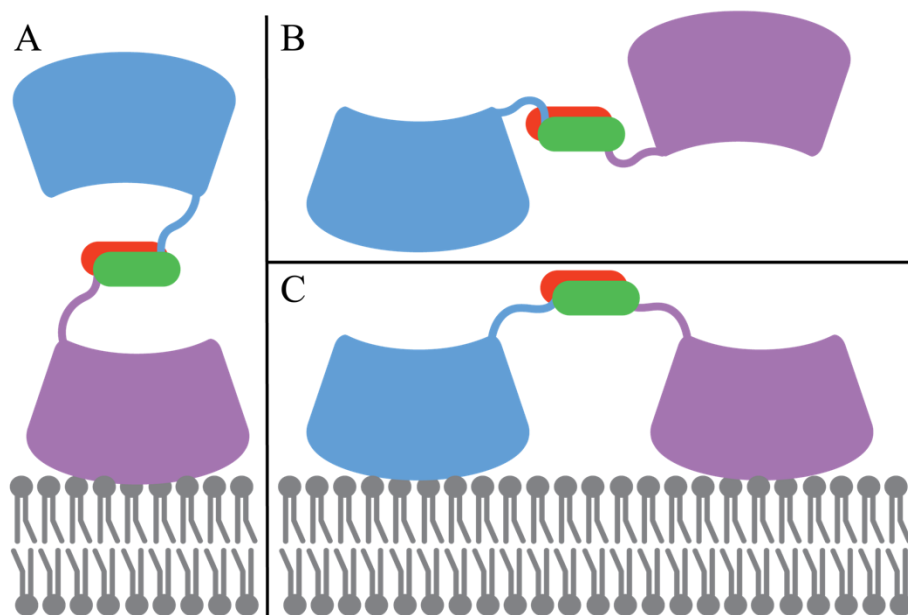


Figure B.2. Schematic representations of the (A) initial OS model, (B) A2t, and (C) the initial TS model. A2-1 is colored blue, A2-2 is colored purple, the two subunits of the p11 dimer are colored green and red, and lipids are colored grey. Two important structural features are made naturally apparent, the first being the orientation the A2 convex surface toward the membrane and the concave surface away. The second being the A2-ND interaction with the p11 dimer at the interface of both subunits.

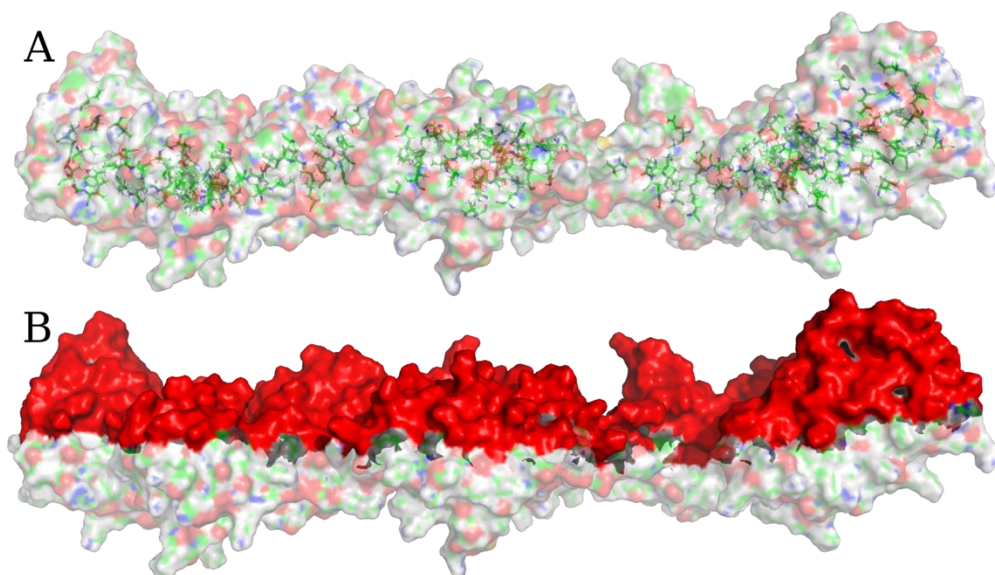


Figure B.3. Representations to demonstrate selected residues used in height calculations. (A) The solvent accessible residues are represented as a transparent surface while the solvent inaccessible residues are represented as sticks. (B) The residues used for height calculations are colored red and are not transparent while the residues that were not selected are semi-transparent.

Table B.1. Top Twenty Strongest Membrane-Interacting Residues from the TS model.

Residue	ΔG (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)	Subunit
K281	-40.99	5.62	A2-1
K249	-35.51	6.50	A2-1
R63	-31.87	4.72	p11-1
R123	-30.38	6.37	p11-2
R284	-25.14	8.45	A2-2
K49	-22.98	10.74	A2-2
L121	-19.70	2.38	A2-2
K119	-18.29	6.93	A2-1
L121	-15.92	1.89	A2-1
K152	-12.88	7.20	A2-2
Q61	-12.80	2.89	p11-1
K119	-11.42	3.00	A2-2
S92	-10.45	2.15	A2-1
T283	-9.50	2.40	A2-1
S164	-9.24	2.29	A2-1
K281	-8.28	5.33	A2-2
K66	-7.68	4.29	p11-1
G280	-7.53	1.38	A2-1
G122	-6.77	0.97	A2-1
S89	-6.75	1.20	A2-2

Table B.2. Top Ten Strongest Membrane-Interacting Residues from the OS model.

Residue	ΔG (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)	Subunit
K281	-46.10	6.30	A2-2
K206	-37.04	6.38	A2-2
R245	-30.68	3.31	A2-2
K249	-25.89	5.90	A2-2
K279	-23.22	7.88	A2-2
K246	-22.13	5.51	A2-2
L121	-13.25	1.88	A2-2
G207	-6.12	1.29	A2-2
S164	-4.94	2.65	A2-2
G120	-4.19	0.89	A2-2

Table B.3. Top Ten Pairwise-Residue Interactions of A2-2 with the other Subunits of OS-A2t.

Subunit 1	Residue 1	Subunit 2	Residue 2	ΔE (kcal mol ⁻¹)	Standard Deviation (kcal mol ⁻¹)
A2-1	R145	p11-1	D53	-14.54	2.54
	R77		E44	-14.10	2.12
	E149		R32	-13.88	1.78
	D70		K47	-9.50	2.55
	E149		K28	-7.77	1.51
	E14		Y86	-4.64	2.20
	K10		Y86	-3.73	0.58
	F73		K47	-3.11	0.88
	Q69		D48	-2.87	1.23
	E66		K54	-2.77	4.02
A2-1	T3	p11-2	E10	-10.78	2.49
	S2		E10	-8.48	2.67
	M1		E10	-8.47	1.83
	M1		H7	-1.84	0.58
	T3		M9	-1.43	0.41
	M1		E6	-1.36	0.45
	T3		M13	-1.08	0.36
	V4		E6	-0.58	0.42
	M1		P2	-0.55	0.64
	V4		E10	-0.50	0.29
A2-1	K28	A2-2	D187	-9.31	4.09
	V27		D187	-3.26	1.50
	K28		V185	-2.66	1.21
	V27		L190	-1.21	0.36
	V27		E189	-1.08	0.34
	A29		V185	-0.92	0.44
	Y30		V185	-0.89	0.83
	N32		L190	-0.74	0.44
	S26		E189	-0.64	0.81
	A29		L190	-0.59	0.24

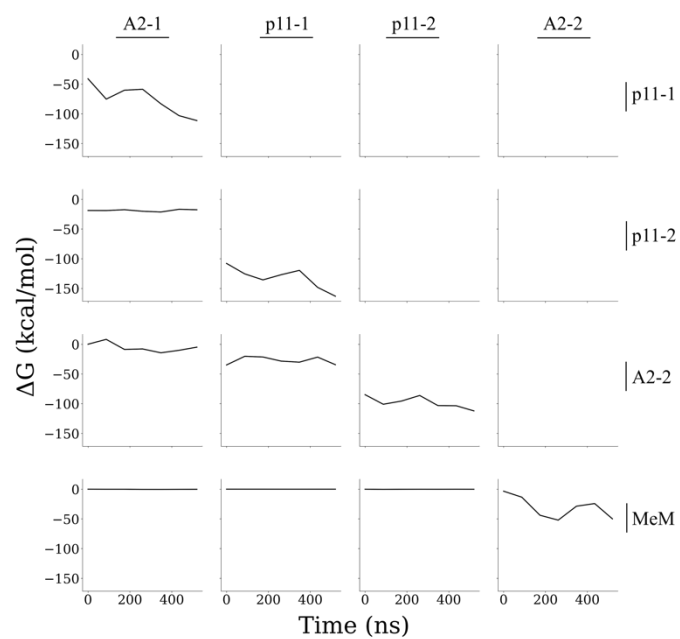


Figure B.4. Estimated binding energy of each OS subunit with each other and the MeM at regular intervals throughout the trajectory.

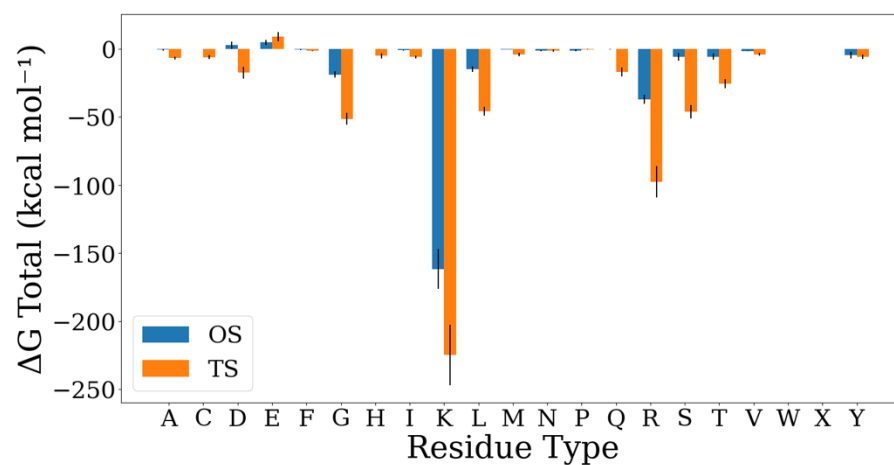


Figure B.5. Total estimated binding energy contribution from each type of residue found in the A2t models. Error bars represent the standard deviation.

TS Movie

<https://youtu.be/8OZfijPSR8Q>

OS Movie

<https://youtu.be/tWfxJJNKQSY>

APPENDIX C

Figures and Tables

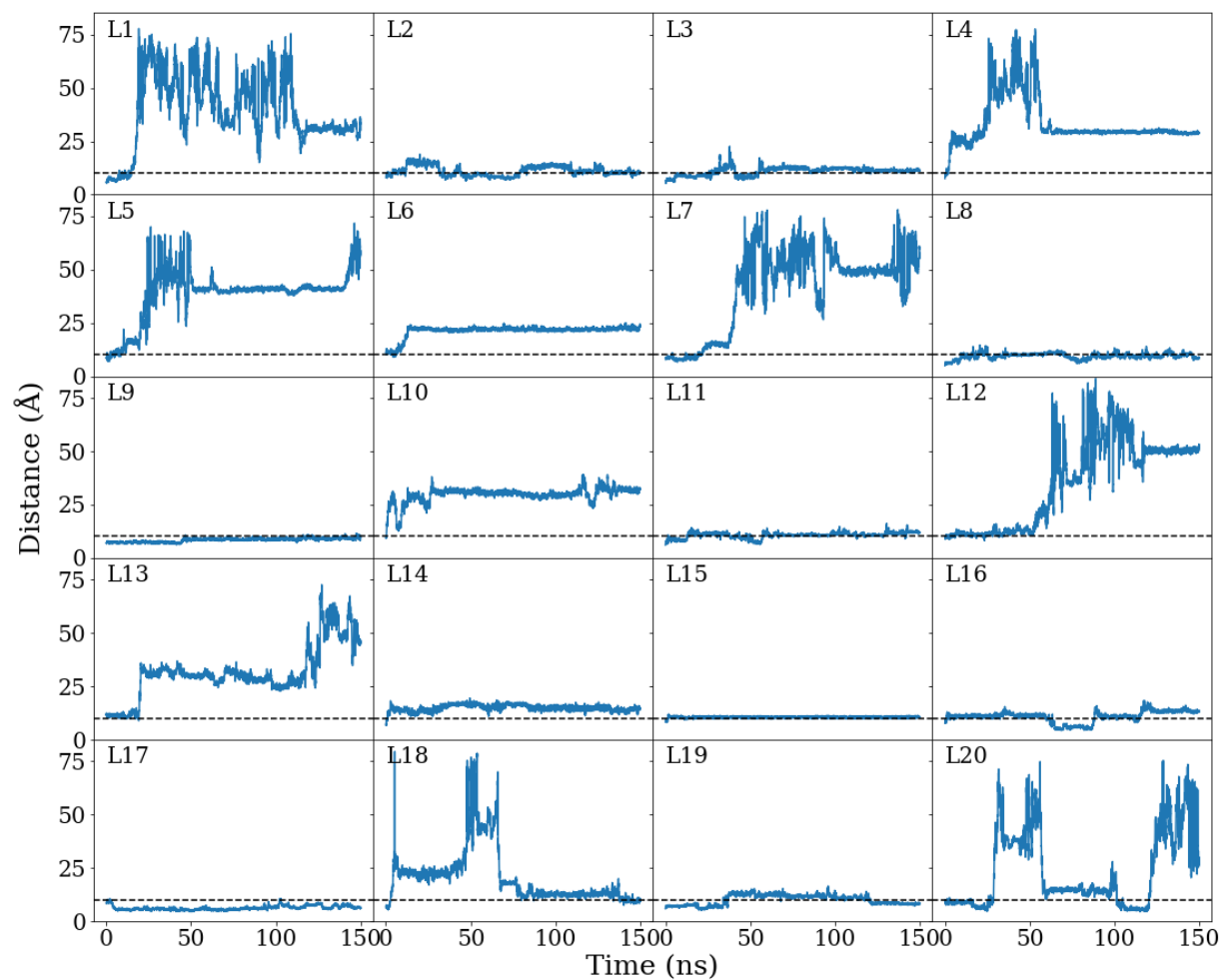


Figure C.1. Distance from the ligand center of mass to the combined RIV-AB, RIV-DE center of mass for ligands L1-L20.

Table C.1. MMGBSA Results for the A2-Ligand Interactions During Membrane Simulations.

Ligand	MMGBSA ΔG (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)
L2	-29.0	3.7
L3	-26.7	21.2
L14	-14.3	14.0
L15	-34.0	11.0
L17	-53.8	17.1

Table C.2. MMGBSA Results for the Ligand-Membrane Interactions During Membrane Simulations.

Ligand	MMGBSA ΔG (kcal mol ⁻¹)	Std Dev (kcal mol ⁻¹)
L2	-19.0	12.9
L3	-16.4	14.2
L14	7.9	10.1
L15	-2.2	4.6
L17	-0.6	5.8

APPENDIX D

Vesicle Morphology

Eccentricity of the Vesicle

Eccentricity has previously been used as a metric for describing how spherical a micellular model is over the course of an MD simulation. This method was applied to describe how spherical the vesicle component of the system remains across MD simulation runs. Calculated eccentricity values are reported as means. The eccentricities were 0.073 ± 0.003 and 0.072 ± 0.006 for the A2V and V systems, respectively. The reported eccentricity indicates that the vesicles remain extremely spherical across simulation runs for both systems. Thus, the surface of the vesicle can be reasonably approximated by a sphere of equivalent radius, an important fact for later variance calculations. The moments of inertia (MOIs) of the A2V vesicle about the principal axes vs time for the first trajectory, are shown in **Figure D.1A**. Analogous plots for the other A2V simulation runs and all V system runs were generated (see **Figures D.2** and **D.3**).

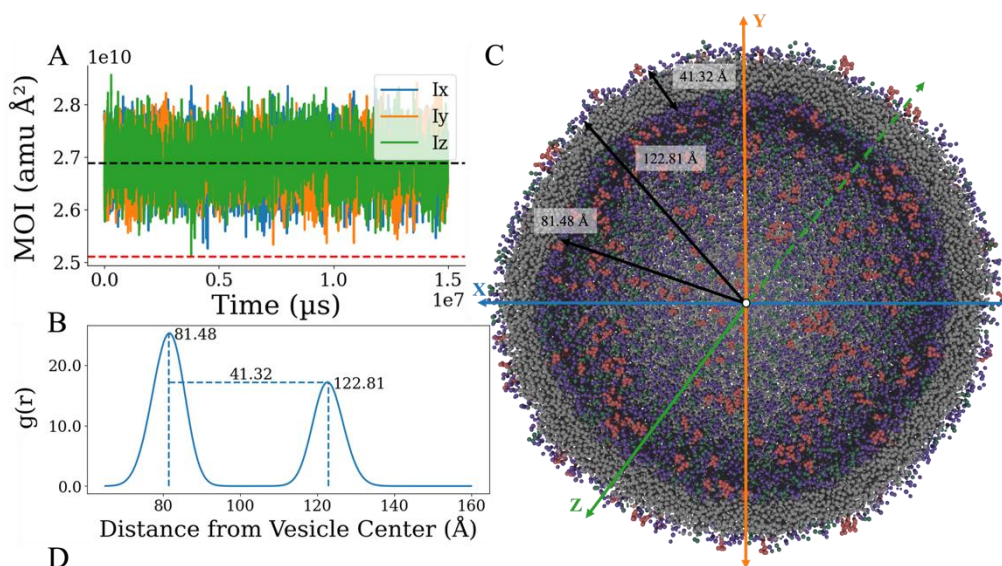


Figure D.1. All plots correspond to repeat 1 of the A2V system. (A) Plot of moment of inertia (MOI) about the three principal axes with the average and minimum MOI marked by black and red lines, respectively. The minimum and average MOIs are both used to calculate the eccentricity of the vesicle. (B) Plot of radial distribution function of lipid heads with respect to the vesicle center of mass. Inner radius, bilayer thickness, and outer radius are shown from left to right. (C) Cross-section of the A2V vesicle with principal axes labeled and colored corresponding to the legend in (A). Dimensions are also labeled. Lipids are shown as spheres with POC, POS, and PIP2 head-group CG beads colored purple, green, and red, respectively. Lipid tails and CHL are colored grey. Only the non-grey head-groups were used for calculating the radial distribution function. (D) Radial distribution functions of lipids and A2 calculated separately and plotted on top of each other to estimate A2 height above the membrane.

Dimensions of the Vesicle

The dimensions of the vesicle component of the system were determined using the radial distribution function of lipid head groups over the trajectories. The radial distribution was calculated from the bilayer center of mass (COM) using the lipid head groups of POC, POS, and PIP2 lipids, with reference to the origin, using the MDAnalysis python package. CHL was excluded from the calculation as it sat lower than the heads of the phospholipids, closer to the tails of other lipids. The peaks of the resulting plot were then used to generate estimates of the inner radius, outer radius, and bilayer thickness, which were averaged across the five repeats of

each system. These values were determined to be $81.03 \pm 0.31 \text{ \AA}$, $122.43 \pm 0.21 \text{ \AA}$, and $41.40 \pm 0.29 \text{ \AA}$, respectively for the A2V vesicle (**Figures D.1B and D.4**) and $81.16 \pm 0.48 \text{ \AA}$, $122.47 \pm 0.22 \text{ \AA}$, $41.31 \pm 0.28 \text{ \AA}$, respectively for the V system (**Figure D.5**). The high bilateral symmetry of each peak is consistent with what one would expect if the lipid heads were distributed homogenously across a spherical shell, serving as a further evidence of the vesicle's extremely spherical shape.

Fréchet Variance Calculation

The Fréchet variance, $\psi(p)$, is the sum of squared distances, d , between point, p , and N other points, x_i .

$$(D.1) \quad \psi(p) = \sum_{i=1}^N d^2(p, x_i)$$

d is defined in a manner appropriate to the system. For the vesicle system, Euclidian distance is not appropriate; taking an average using the lipid center of mass coordinates results in a mean position that is not on the surface of the vesicle but rather on the interior of the vesicle. The field of directional statistics servers to address these concerns. For our system d was defined using the arclength, σ , calculated using the radius of the outer layer of the vesicle, R , as discussed in the **Vesicle Morphology** section of **Appendix E**, and the angle between points p and x .

$$(D.2) \quad S = R\theta_{p,x}$$

Thus, the final form of $\psi(p)$, as applied to the system was:

$$(D.3) \quad \psi(p) = \frac{\sum_{i=1}^N S(p, x_i)^2}{N}$$

A scaling factor of $1/N$ was applied as well. The Fréchet mean is any point which globally minimizes $\psi(p)$.

$$(D.4) \quad m = \underset{i}{\operatorname{argmin}} \sum_{i=1}^N d^2(p, x_i)$$

For our construction:

$$(D.5) \quad m = \underset{i}{\operatorname{argmin}} \frac{\sum_{i=1}^N S(p, x_i)^2}{N}$$

The Fréchet mean was determined by minimizing (D.3). (D.3) with constraints was minimized using the Sequential Least Squares Programming optimizer (SLSQP) as implemented by the SciPy Python package. A single constraint was applied; the magnitude of the average position vector was restrained to the radius of the vesicle upper leaflet's radius. Two methods were tested for providing initial guesses to the optimizer. The first was using all lipid COMs as initial guesses and the second was using six unit vectors aligned with the principal axes scaled by the vesicle's outer radius. The second method required fewer iterations and returned the same result as using the more computationally taxing first method. The identified m was then substituted into Equation (D.3) for p . This was used for calculating the reported Fréchet variances.

Figures

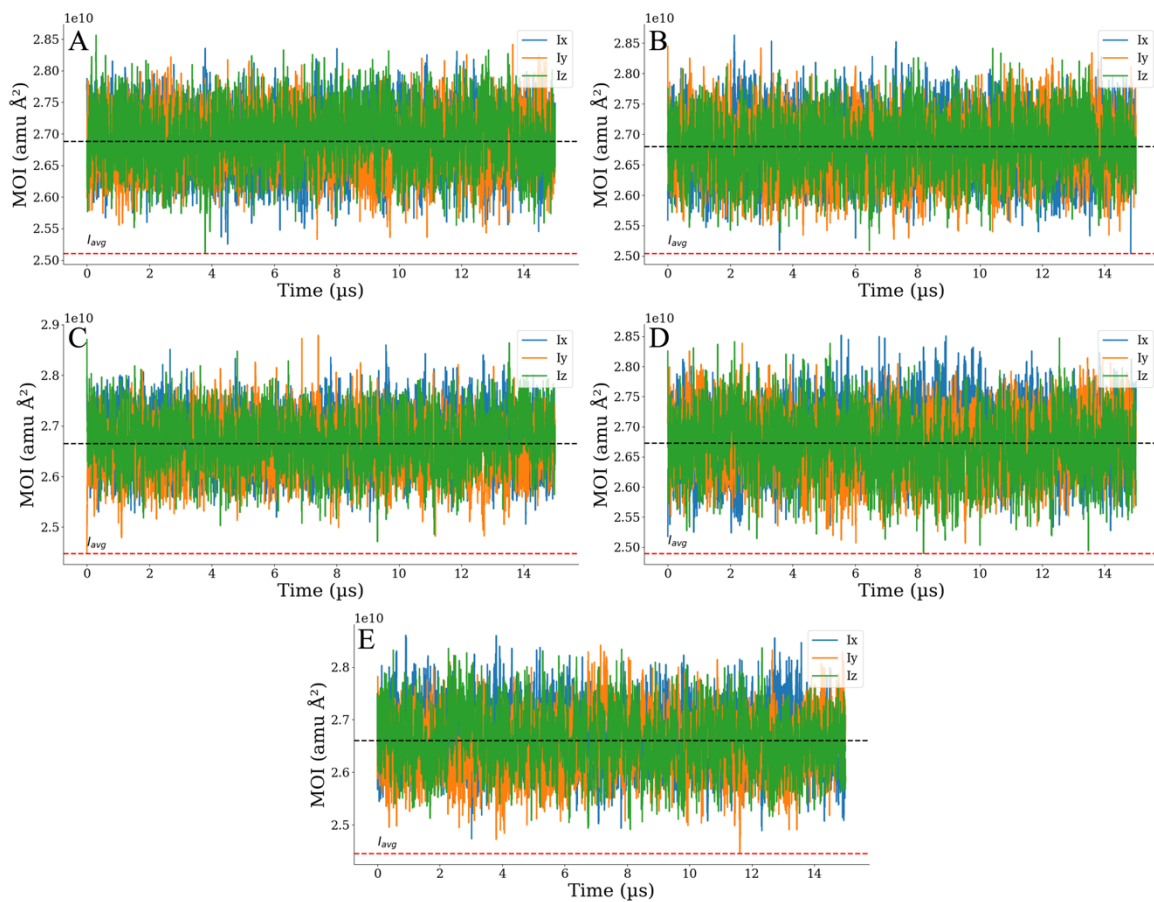


Figure D.2. Plots of moment of inertia (MOI) about the three principal axes with the average and minimum MOI marked for the vesicle component of the A2V system in black and red, respectively. Plots A-E correspond to A2V simulation runs 1-5.

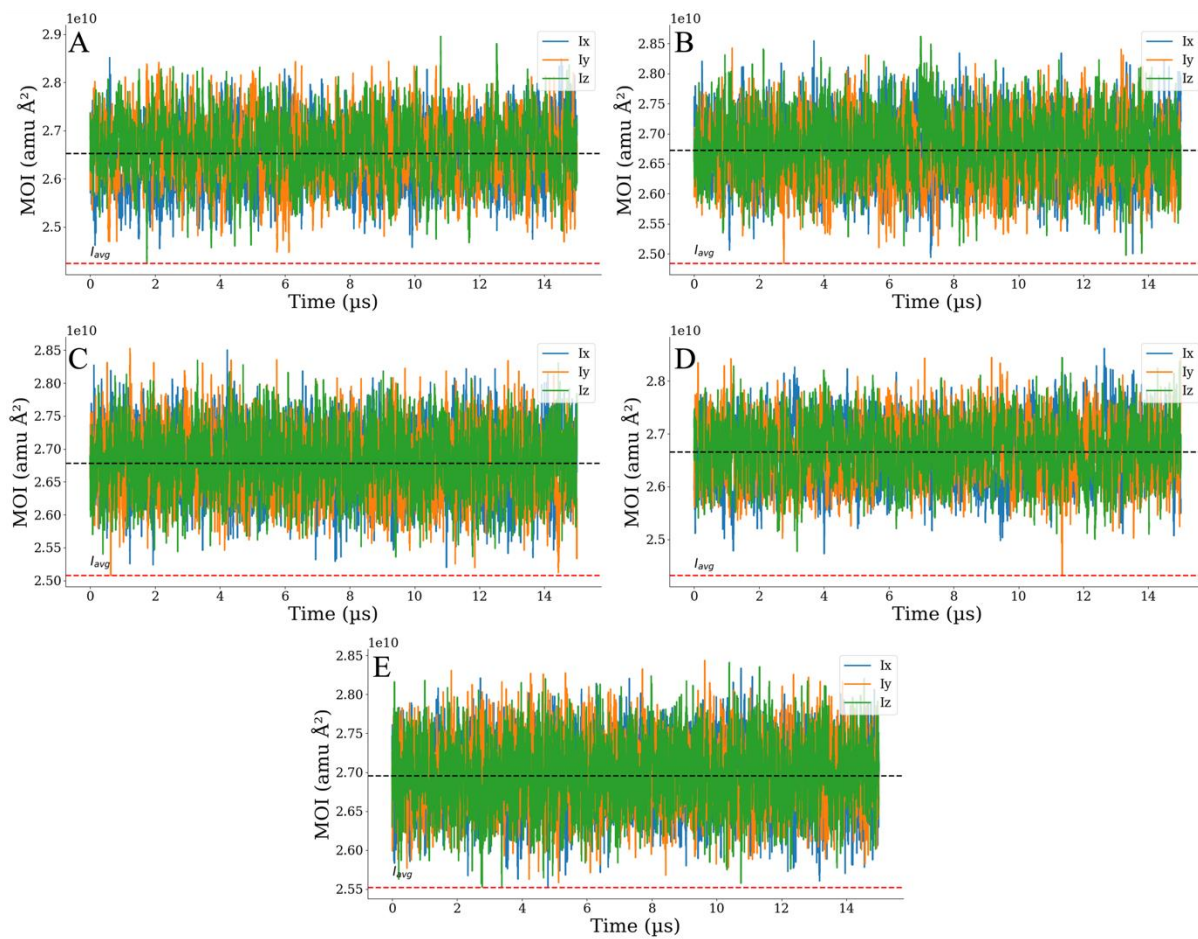


Figure D.3. Plots of moment of inertia (MOI) about the three principal axes with the average and minimum MOI marked for the vesicle component of the V system in black and red, respectively. Plots A-E correspond to V system simulation runs 1-5.

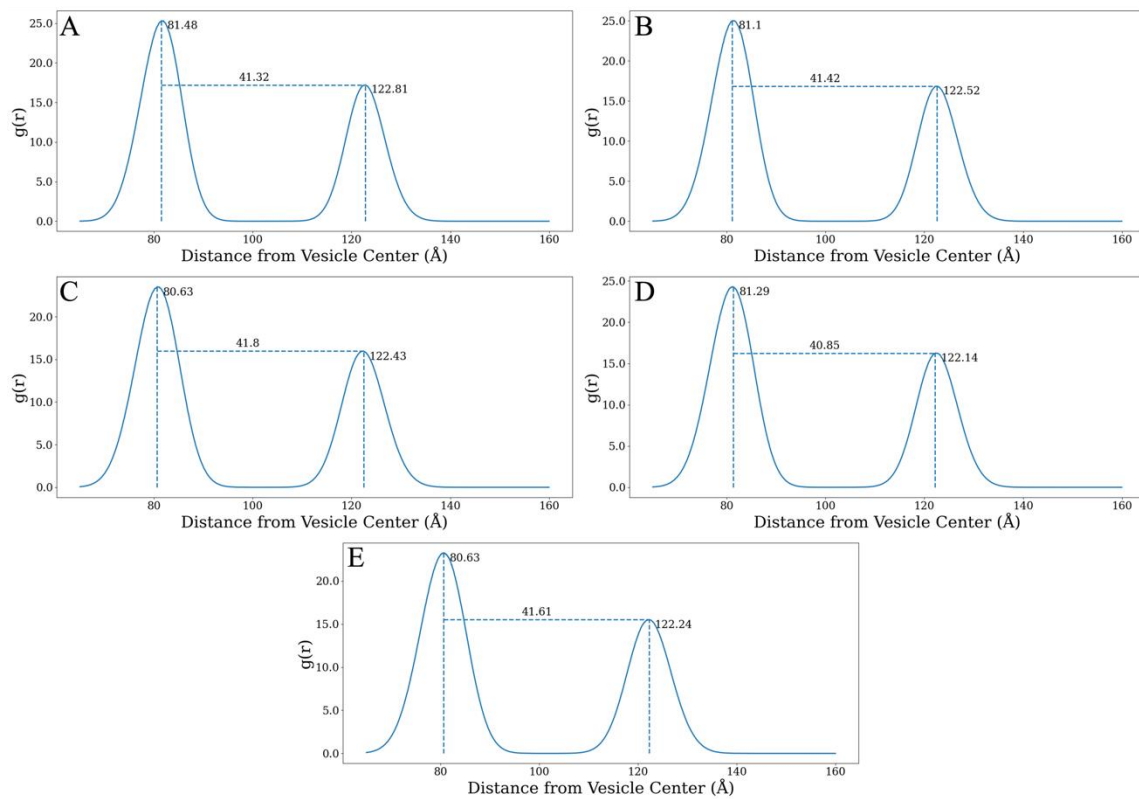


Figure D.4. Plots of radial distribution function ($g(r)$) vs distance from vesicle center of mass for the POC, POS, and PIP2 headgroups for the A2V system. A-E correspond to simulation runs 1-5.

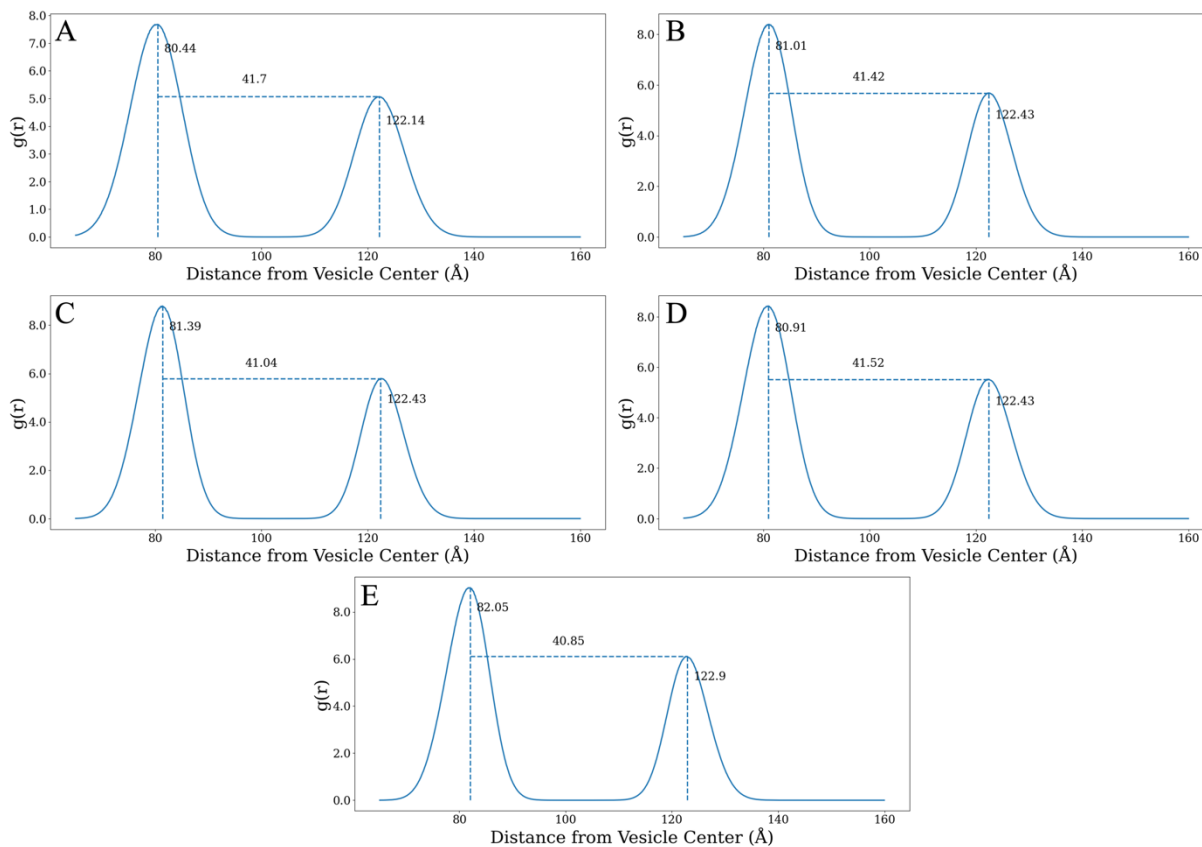


Figure D.5. Plots of radial distribution function ($g(r)$) vs distance from vesicle center of mass for the POC, POS, and PIP2 headgroups for the V system. A-E correspond to simulation runs 1-5.

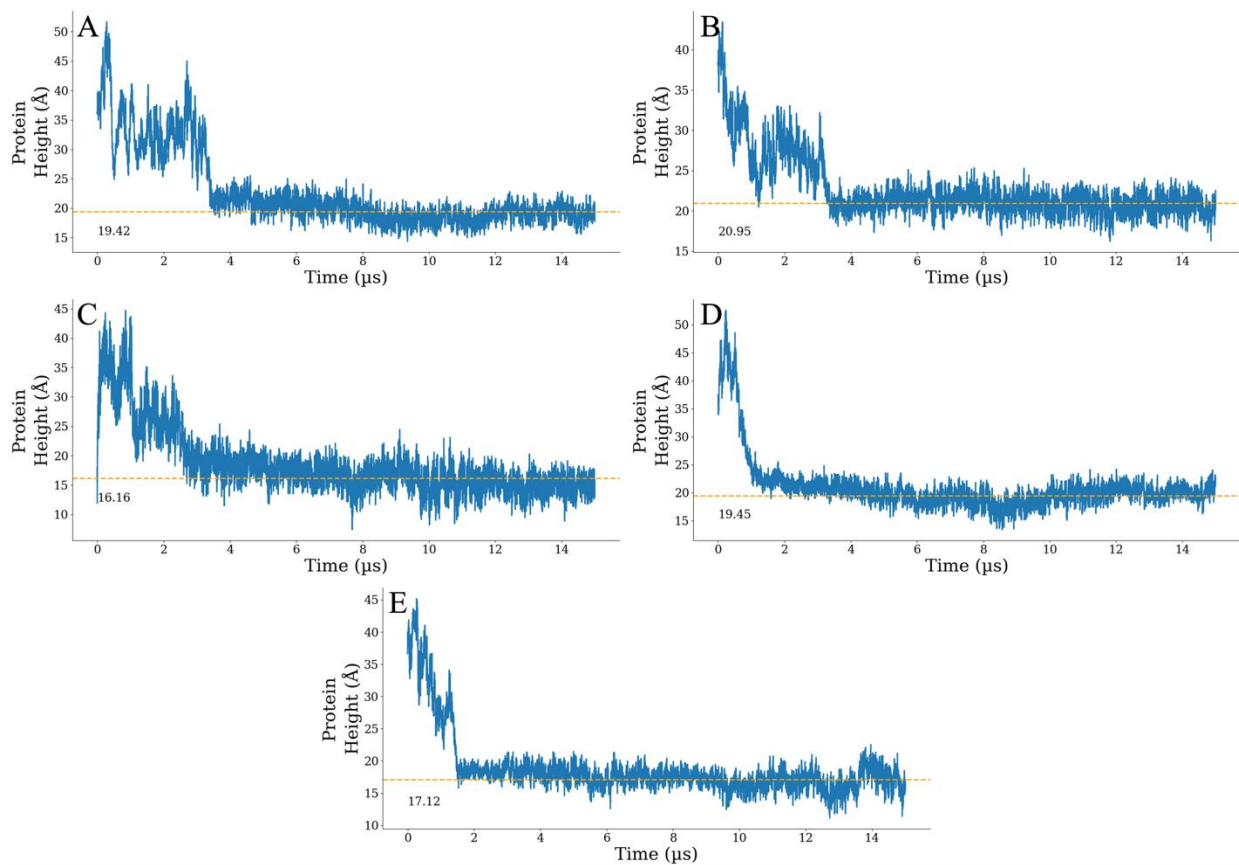


Figure D.6. Average height of the A2 COM from the vesicle surface vs time. A-E correspond to A2V simulation runs 1-5.

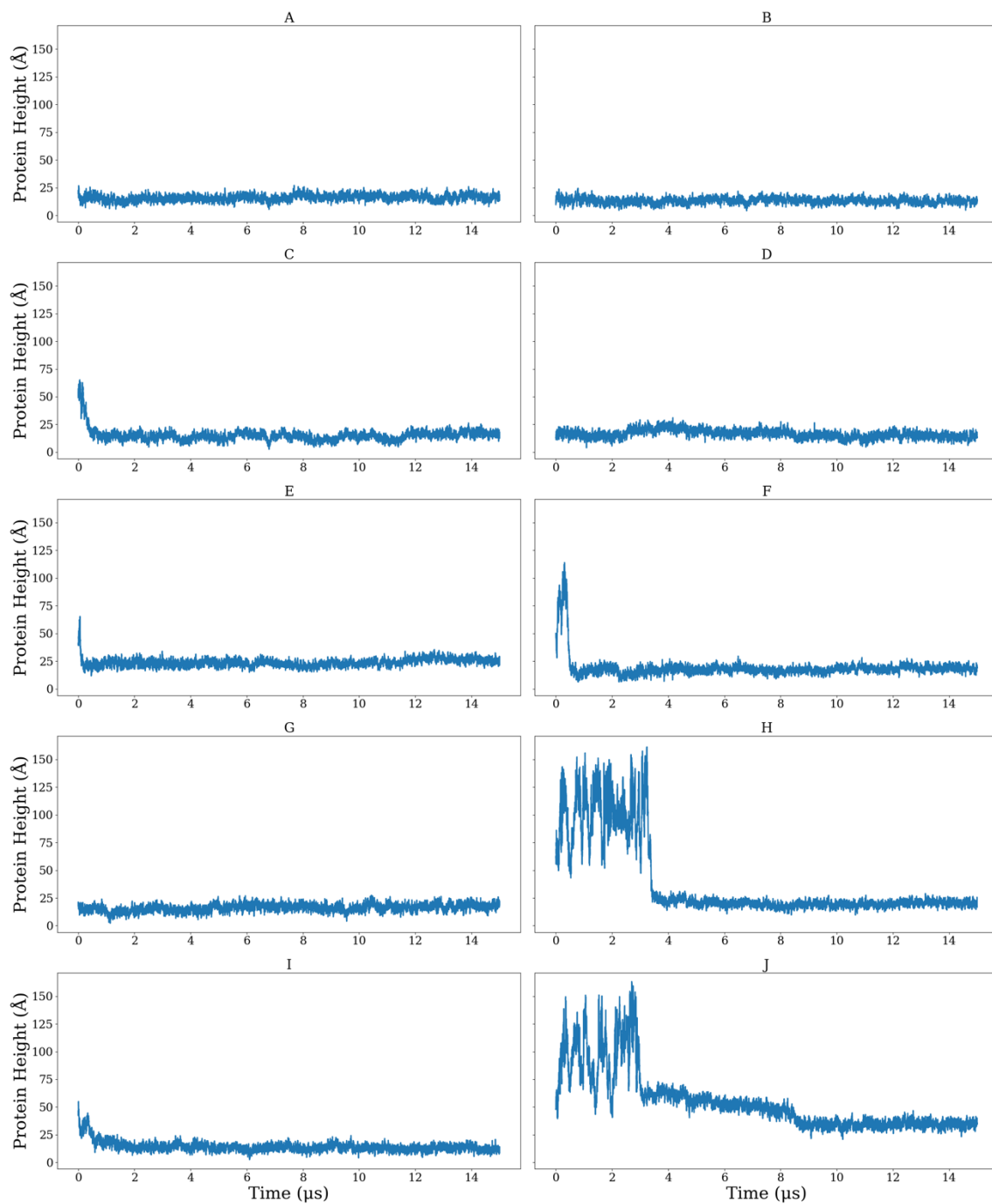


Figure D.7. Heights of individual A2 vs time for simulation run 1 of the A2V system.

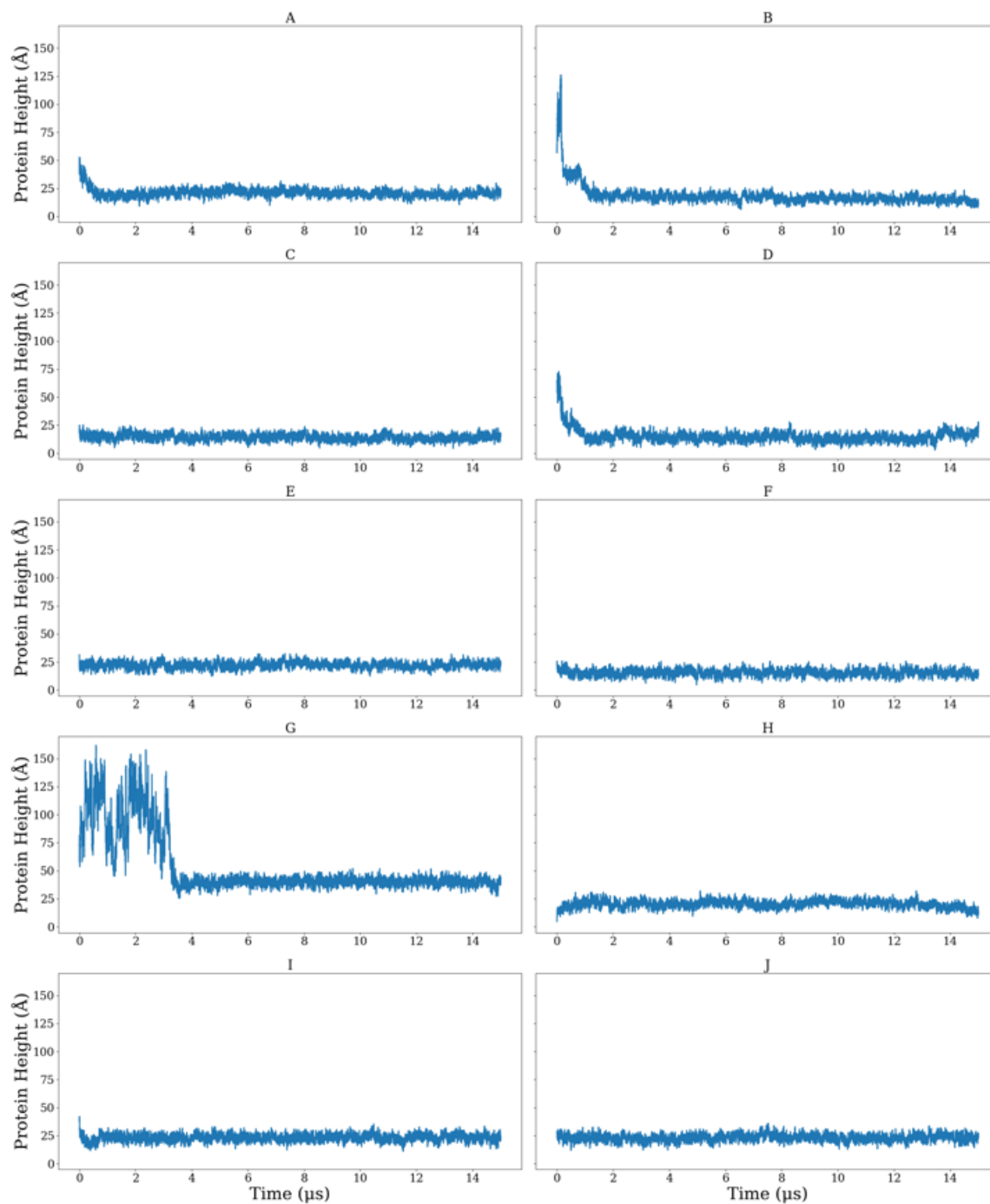


Figure D.8. Heights of individual A2 vs time for simulation run 2 of the A2V system.

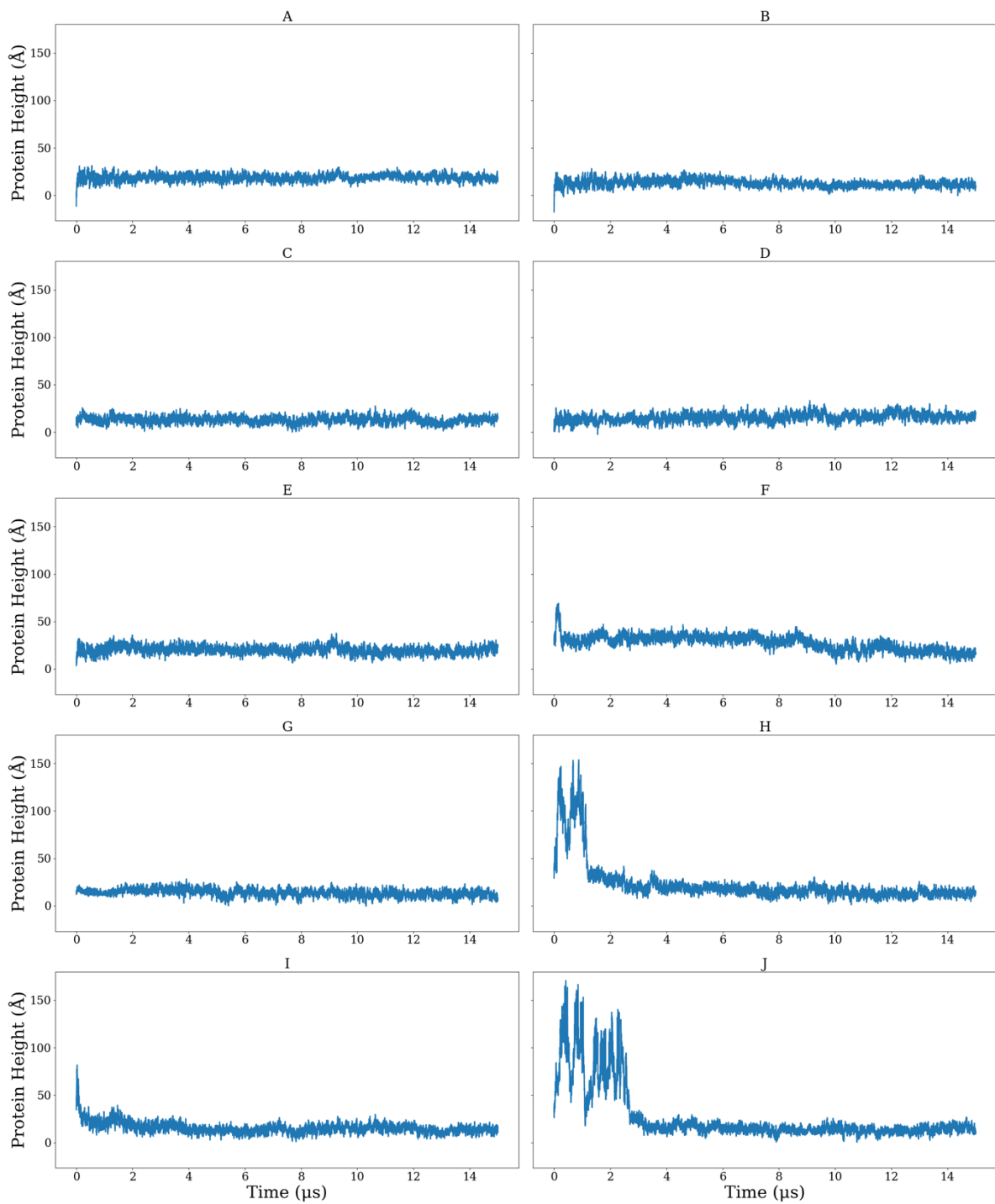


Figure D.9. Heights of individual A2 vs time for simulation run 3 of the A2V system.

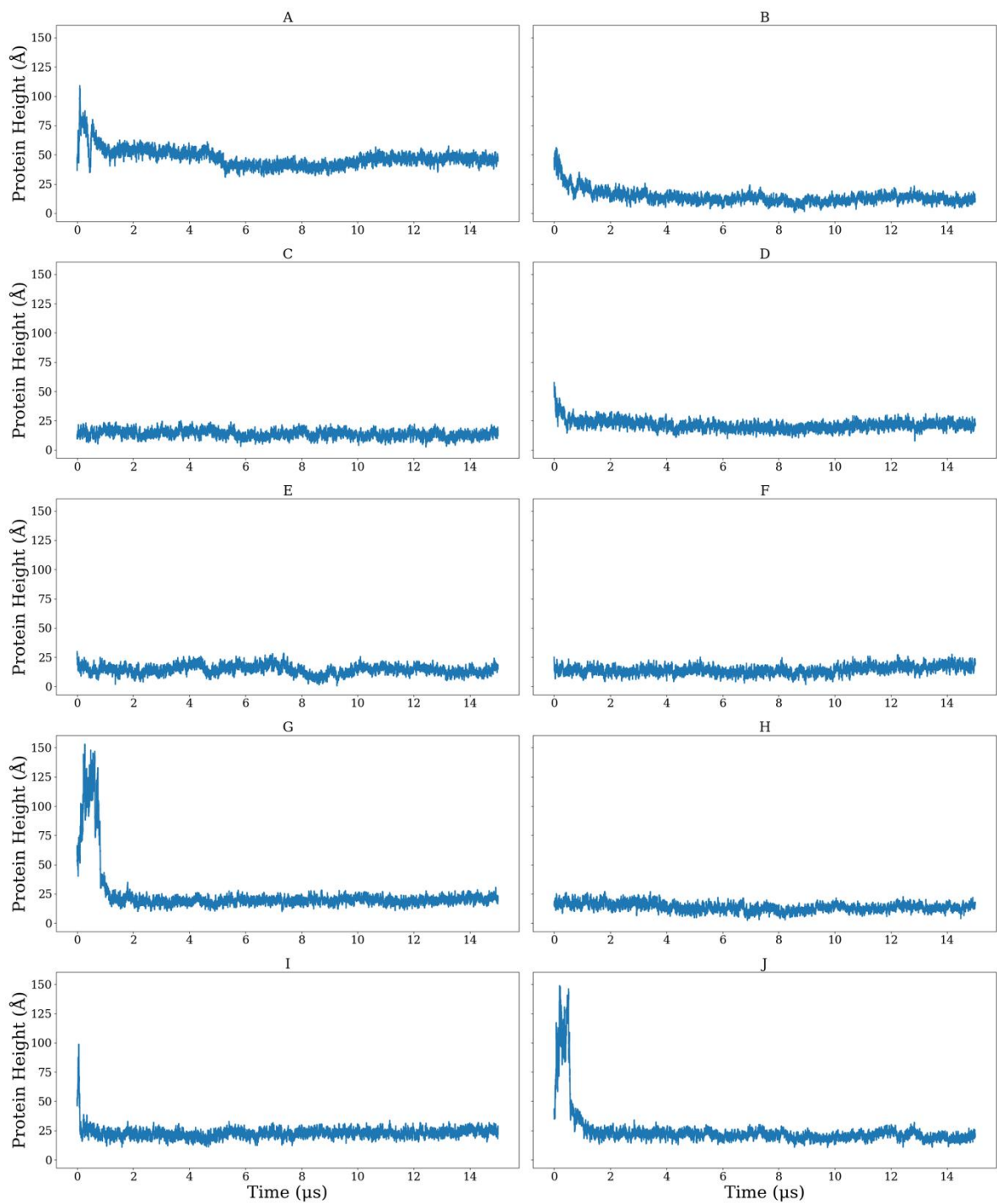


Figure D.10. Heights of individual A2 vs time for simulation run 4 of the A2V system.

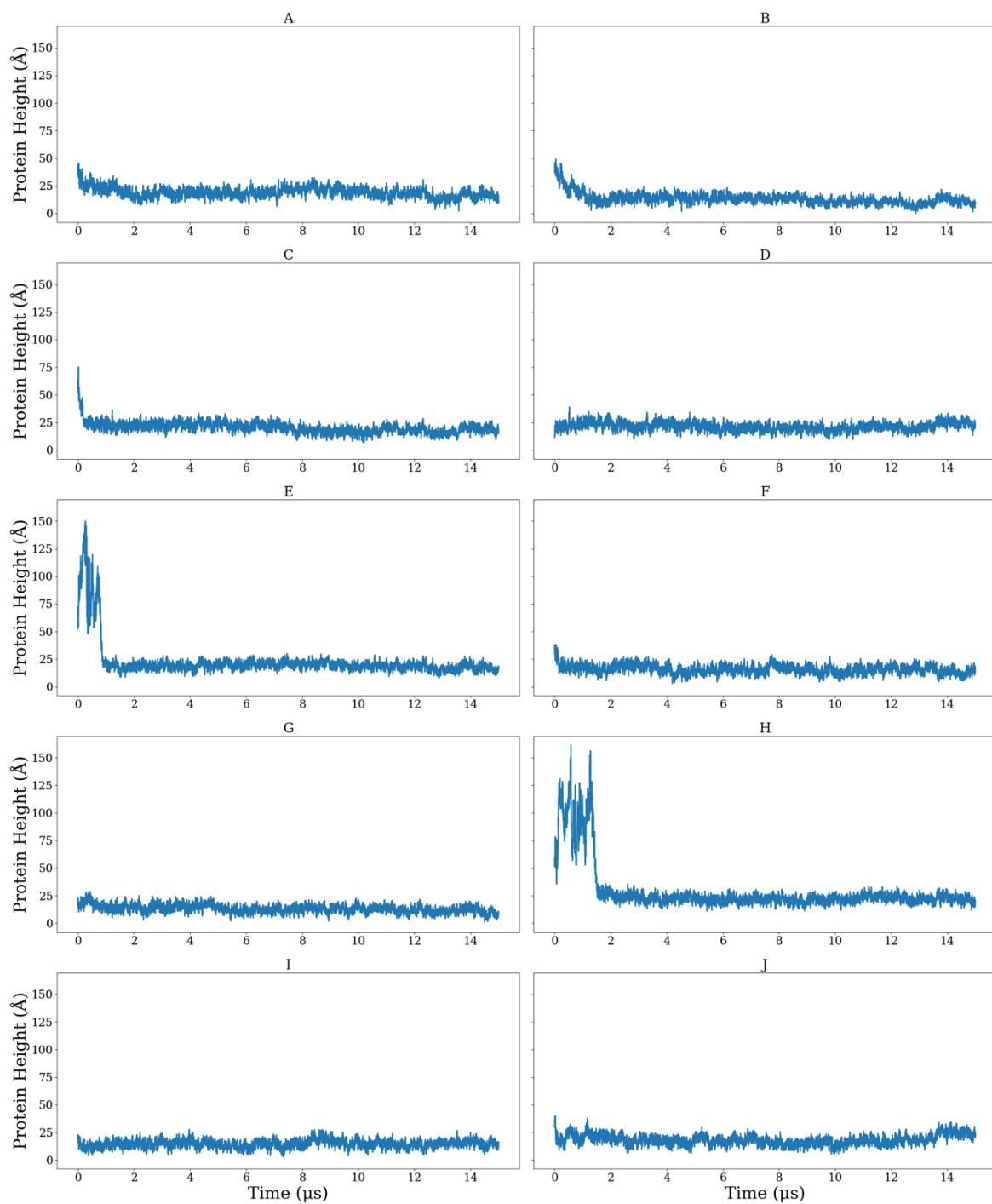


Figure D.11. Heights of individual A2 vs time for simulation run 5 of the A2V system.

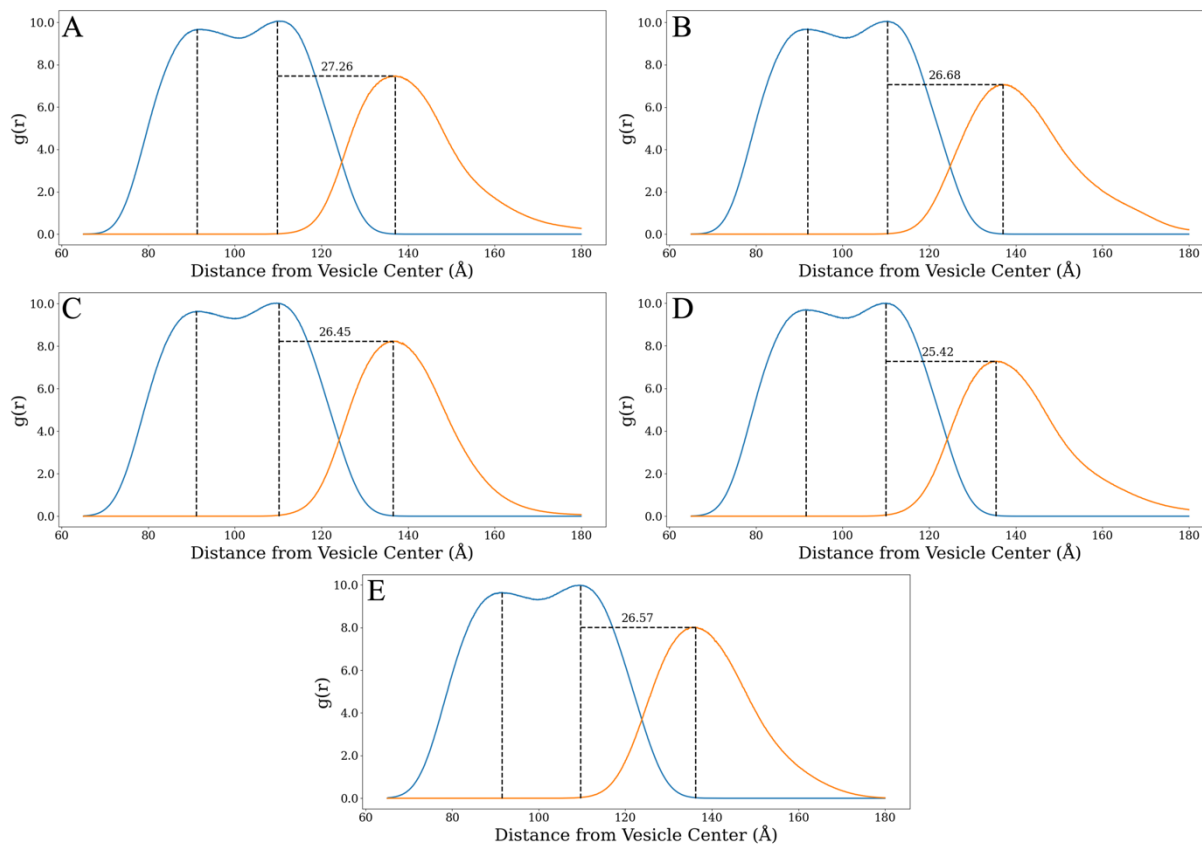


Figure D.12. Radial distribution functions of lipids and A2 calculated separately and plotted on top of each other to estimate A2 height above the membrane. A-E correspond to A2V simulation runs 1-5.

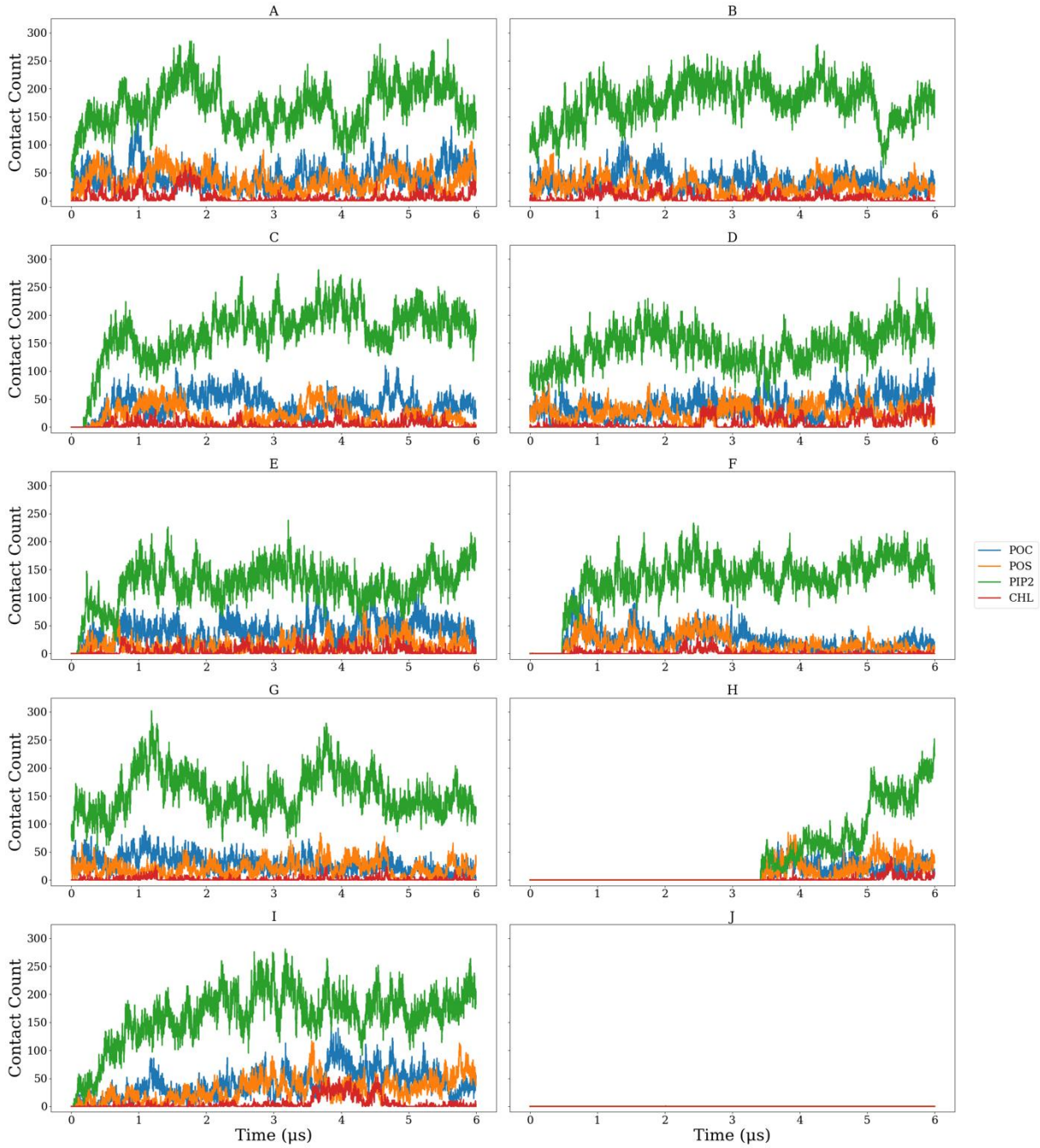


Figure D.13. Total count of A2 contacts with each component of the membrane vs times for each individual A2 over the first 6 μs of simulation time. The results correspond to simulation run 1 of the A2V system.

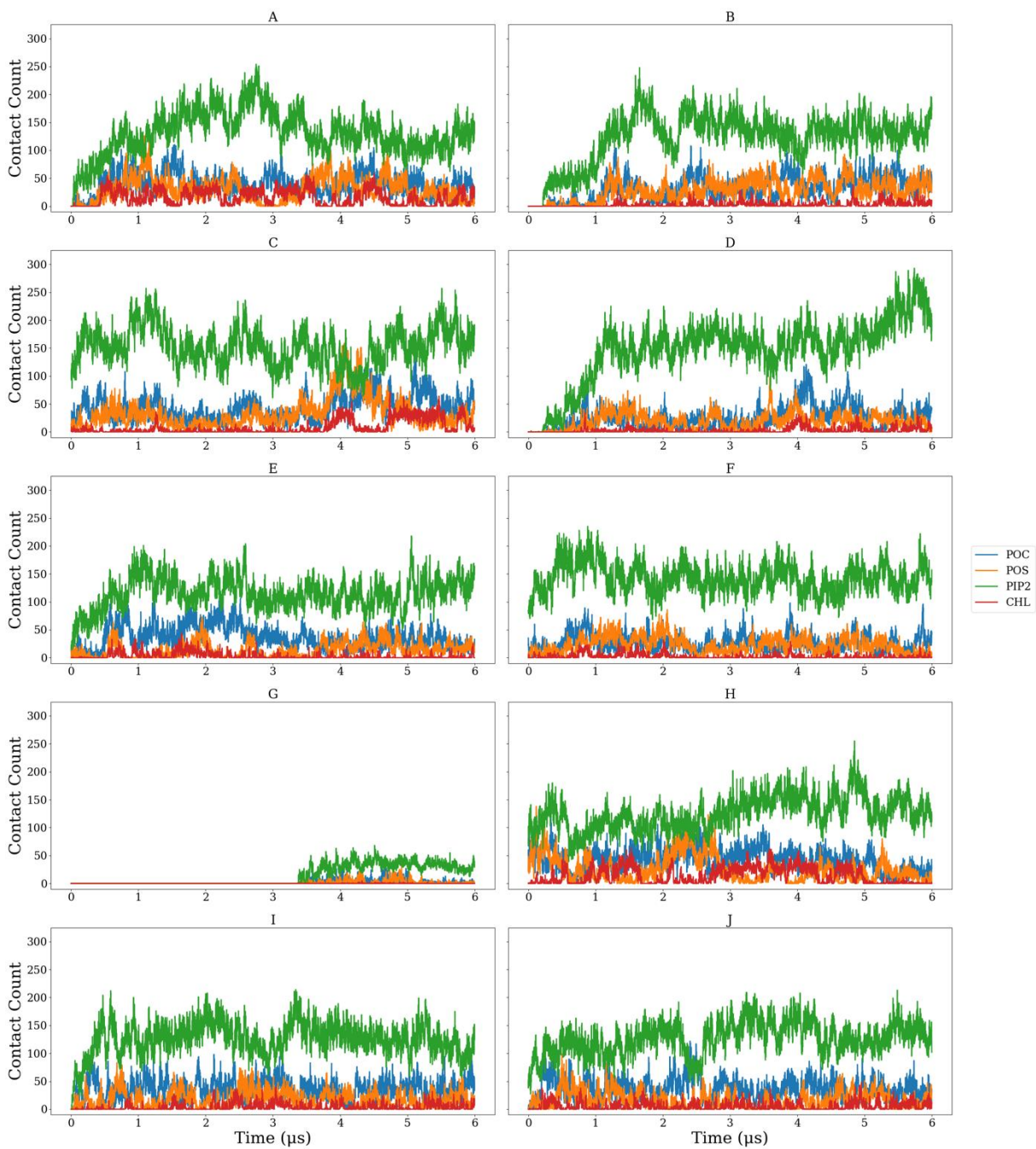


Figure D.14. Total count of A2 contacts with each component of the membrane vs times for each individual A2 over the first 6 μs of simulation time. The results correspond to simulation run 2 of the A2V system.

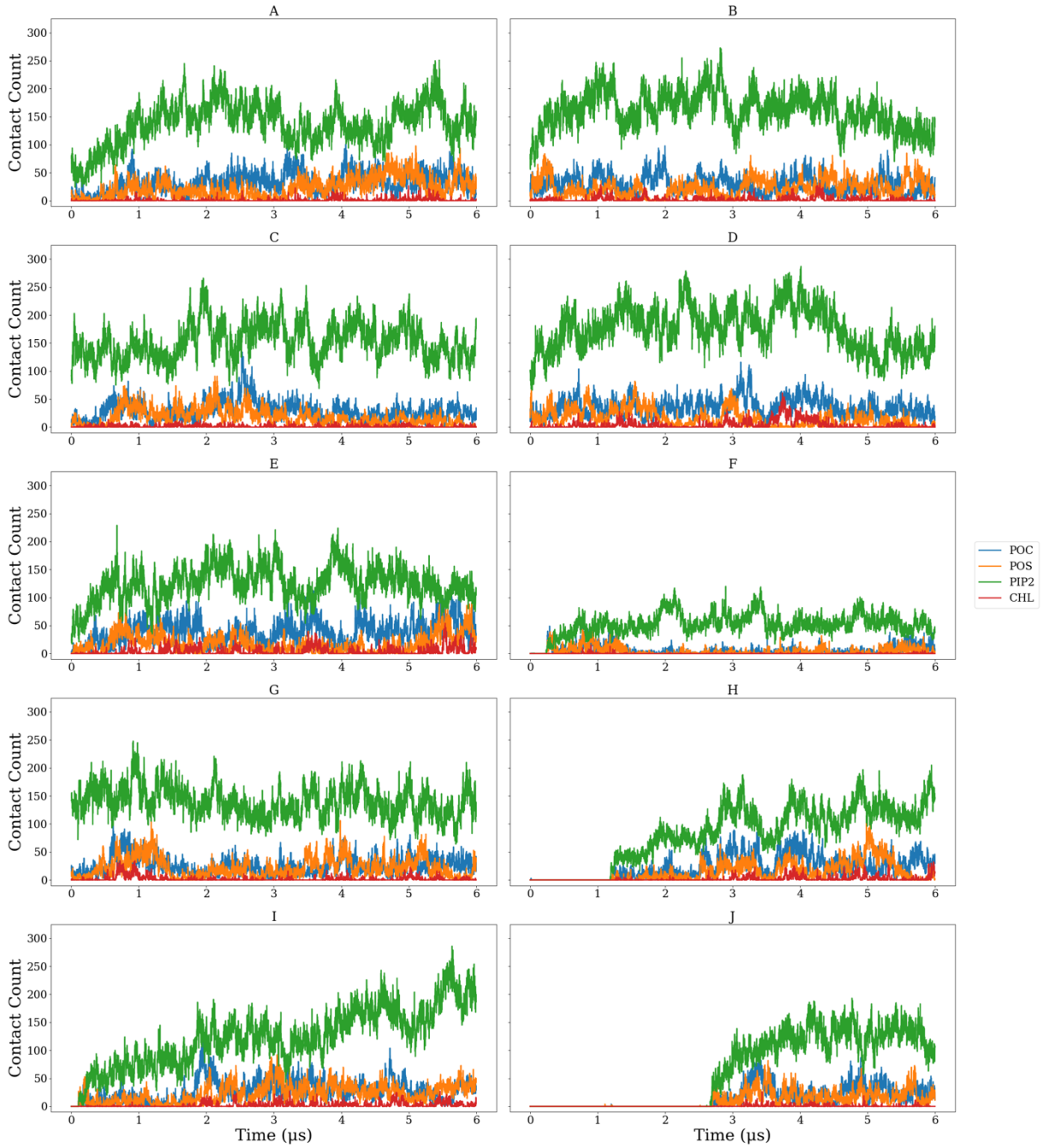


Figure D.15. Total count of A2 contacts with each component of the membrane vs times for each individual A2 over the first 6 μs of simulation time. The results correspond to simulation run 3 of the A2V system.

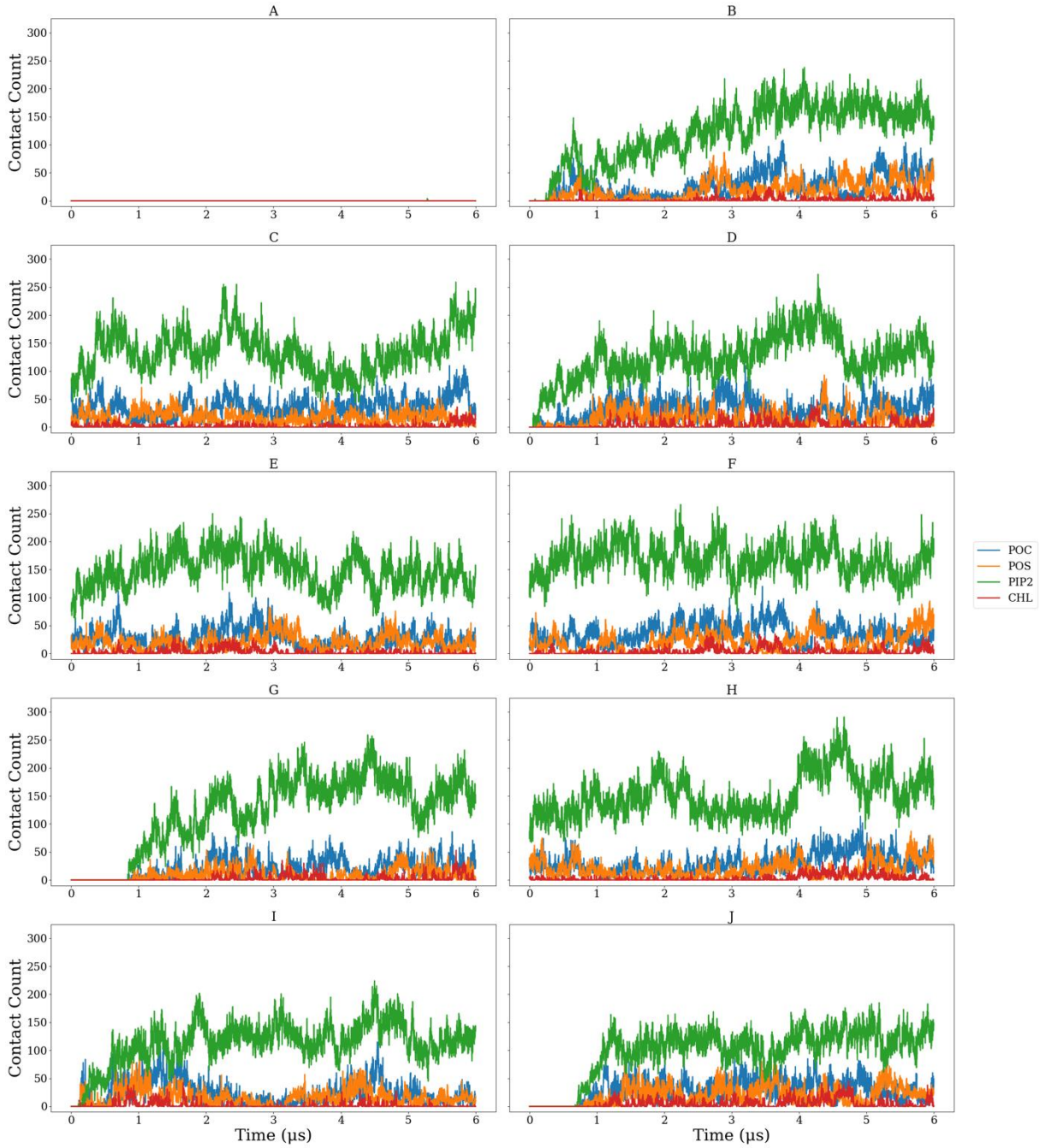


Figure D.16. Total count of A2 contacts with each component of the membrane vs times for each individual A2 over the first 6 μs of simulation time. The results correspond to simulation run 4 of the A2V system.

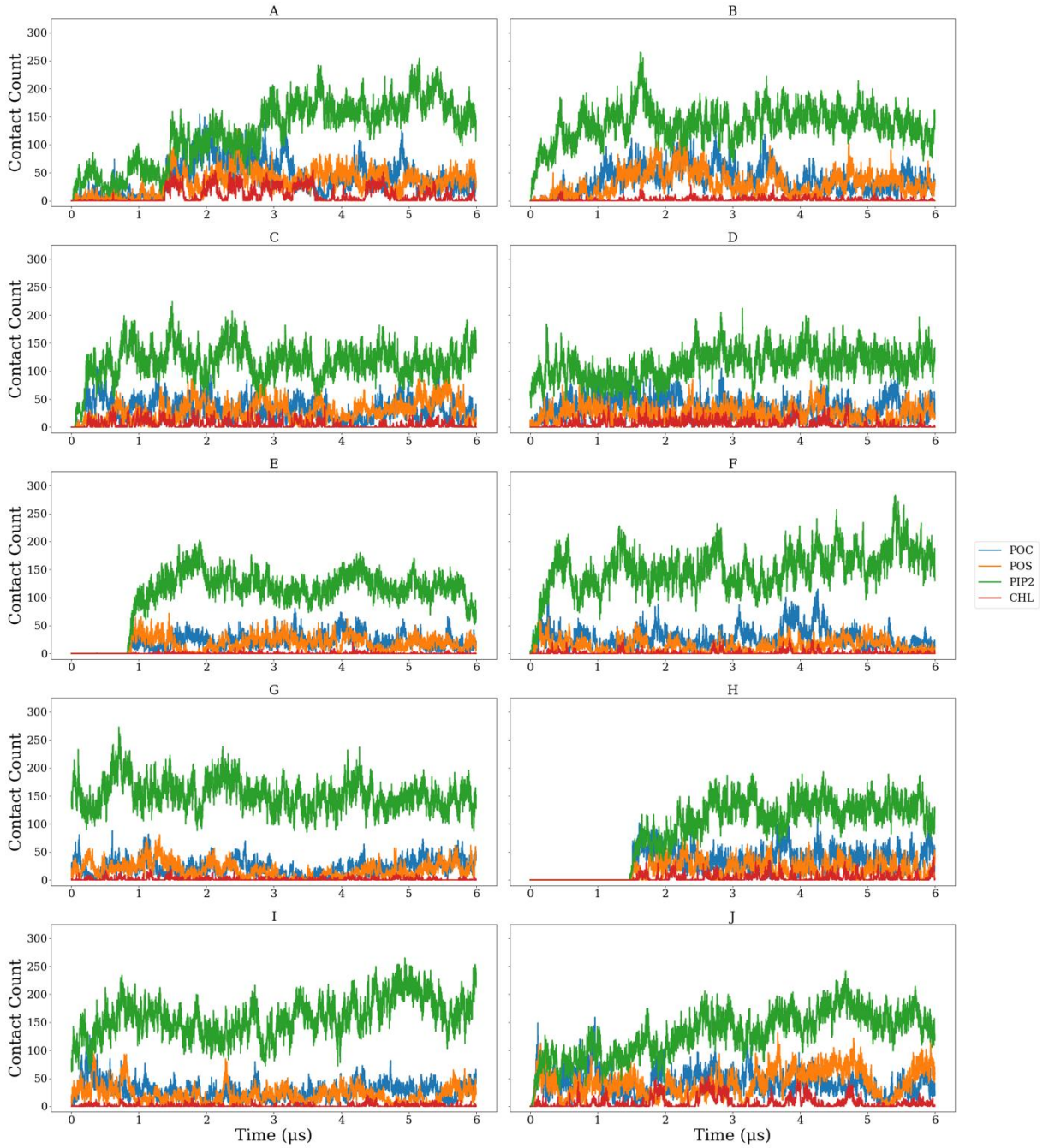


Figure D.17. Total count of A2 contacts with each component of the membrane vs times for each individual A2 over the first 6 μs of simulation time. The results correspond to simulation run 5 of the A2V system.

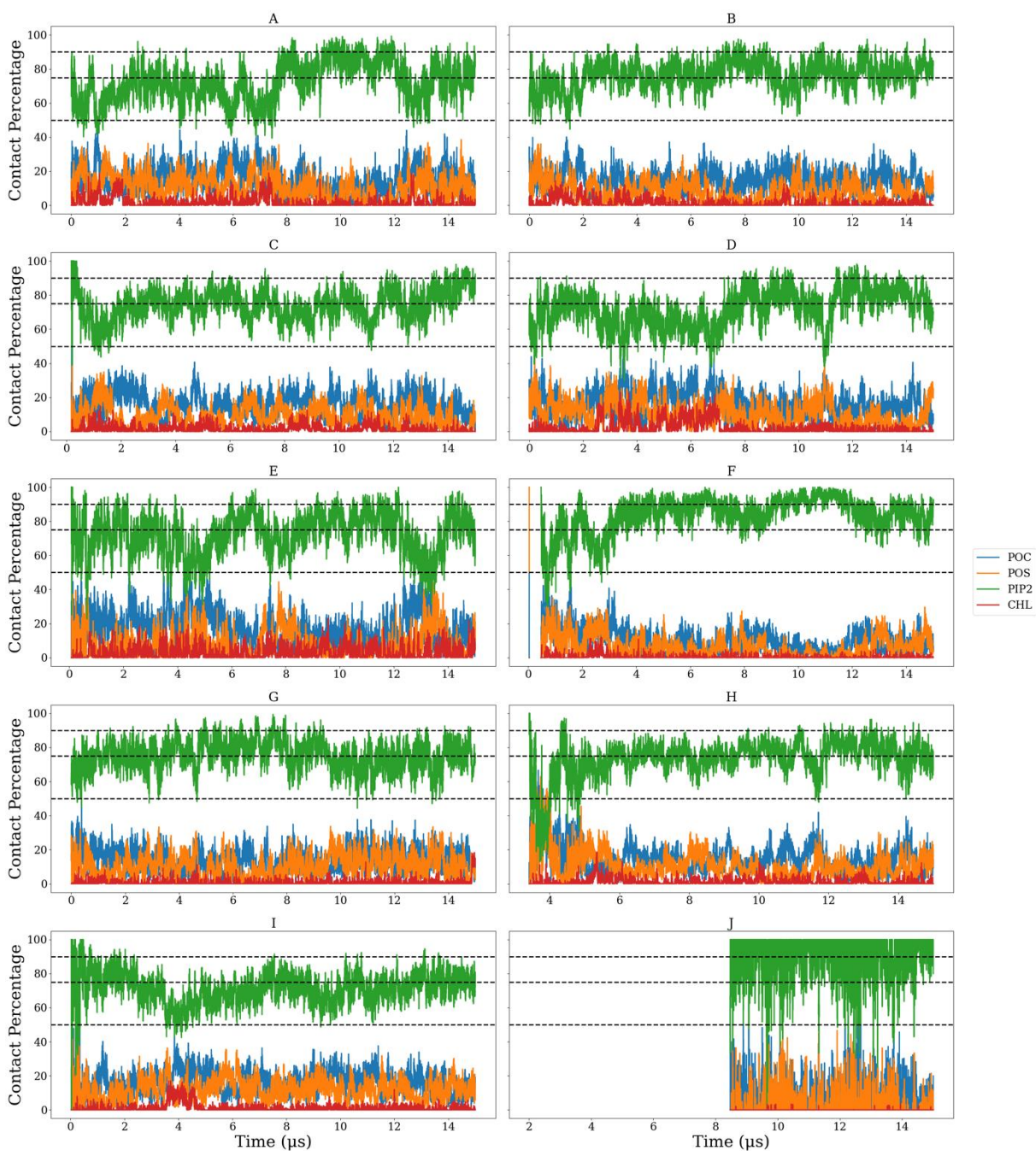


Figure D.18. Percentage of total contacts each lipid is responsible for vs time. Dashed lines are shown at 50, 75, and 90%. The results correspond to simulation run 1 of the A2V system.

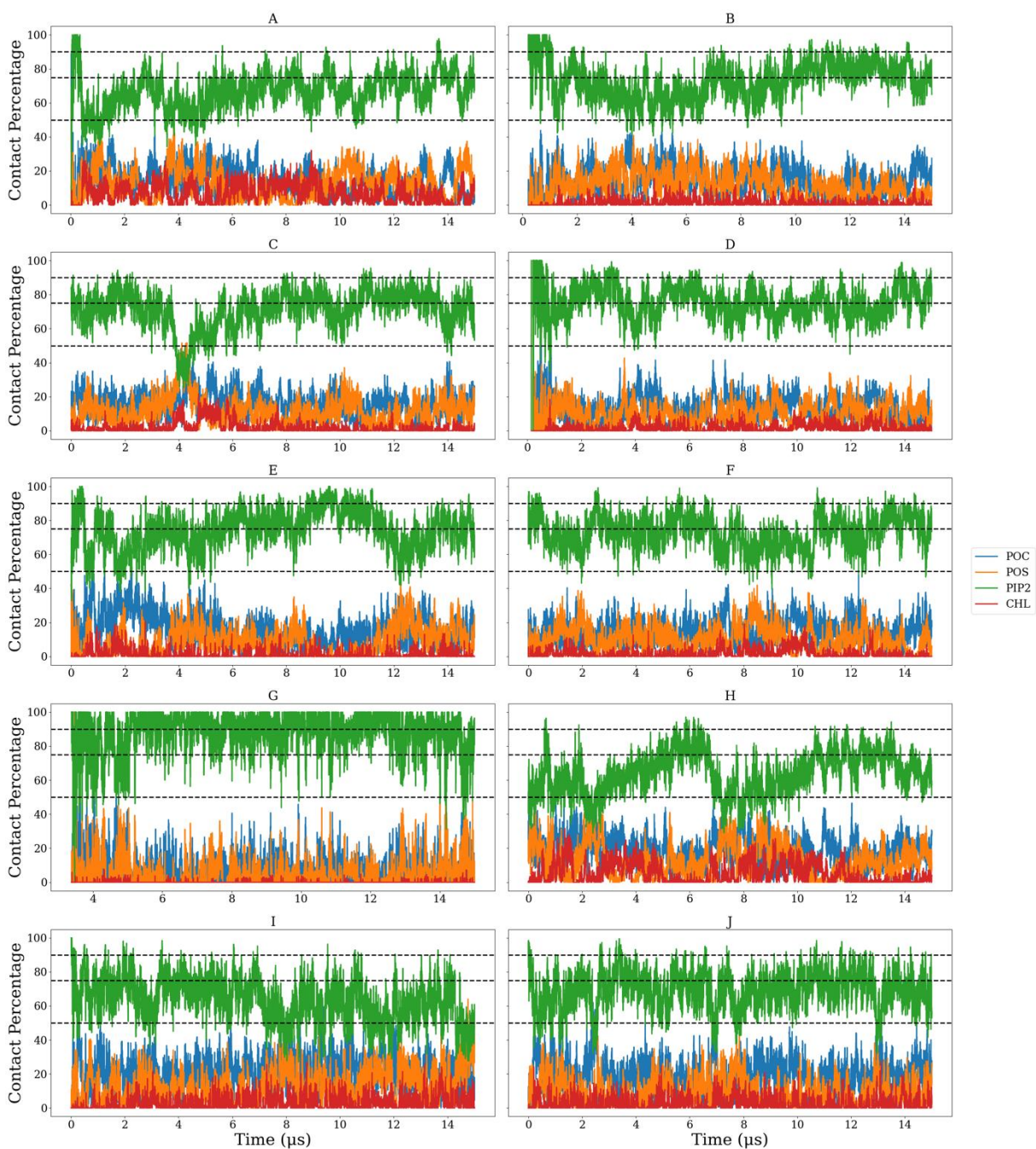


Figure D.19. Percentage of total contacts each lipid is responsible for vs time. Dashed lines are shown at 50, 75, and 90%. The results correspond to simulation run 2 of the A2V system.

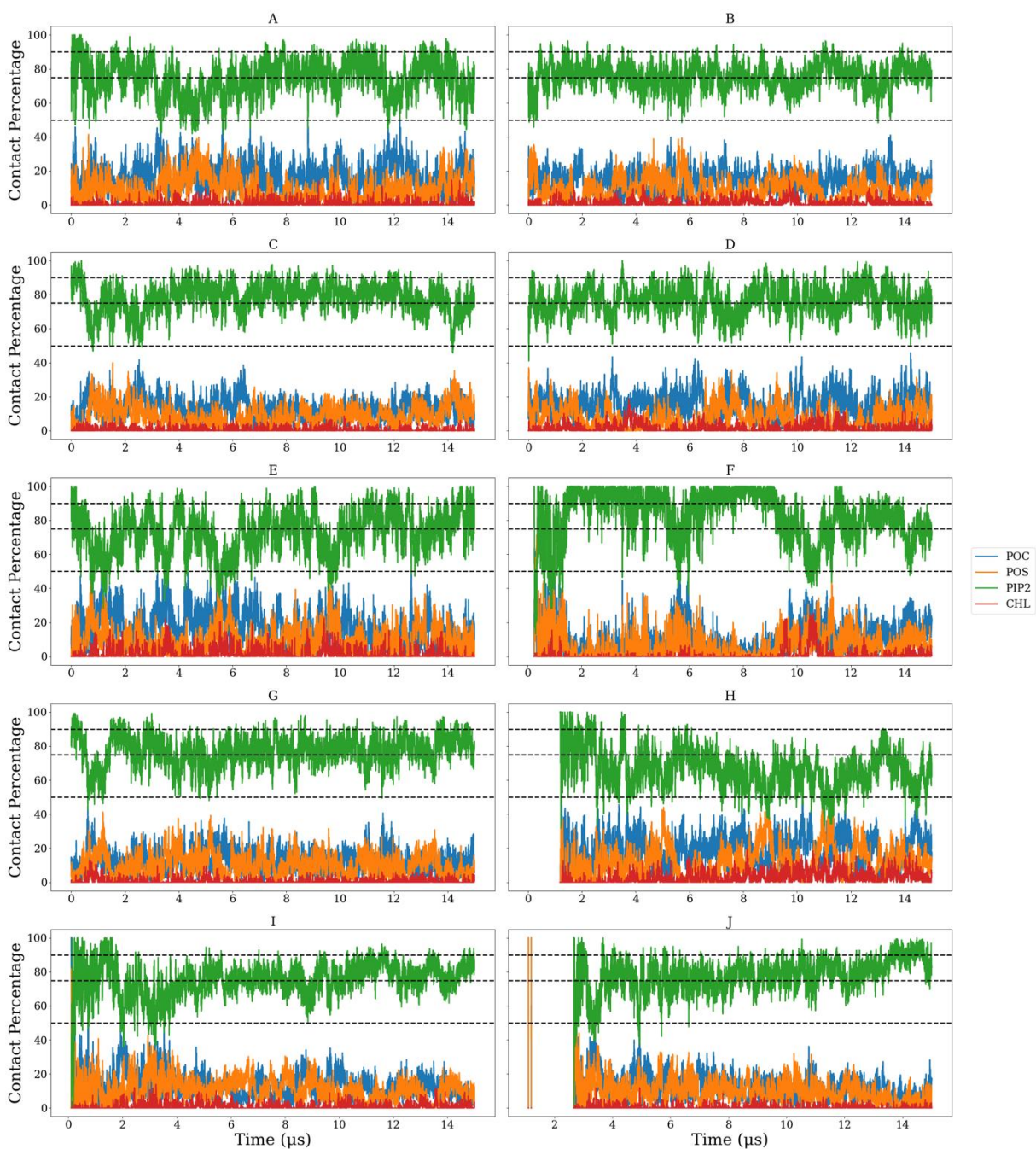


Figure D.20. Percentage of total contacts each lipid is responsible for vs time. Dashed lines are shown at 50, 75, and 90%. The results correspond to simulation run 3 of the A2V system.

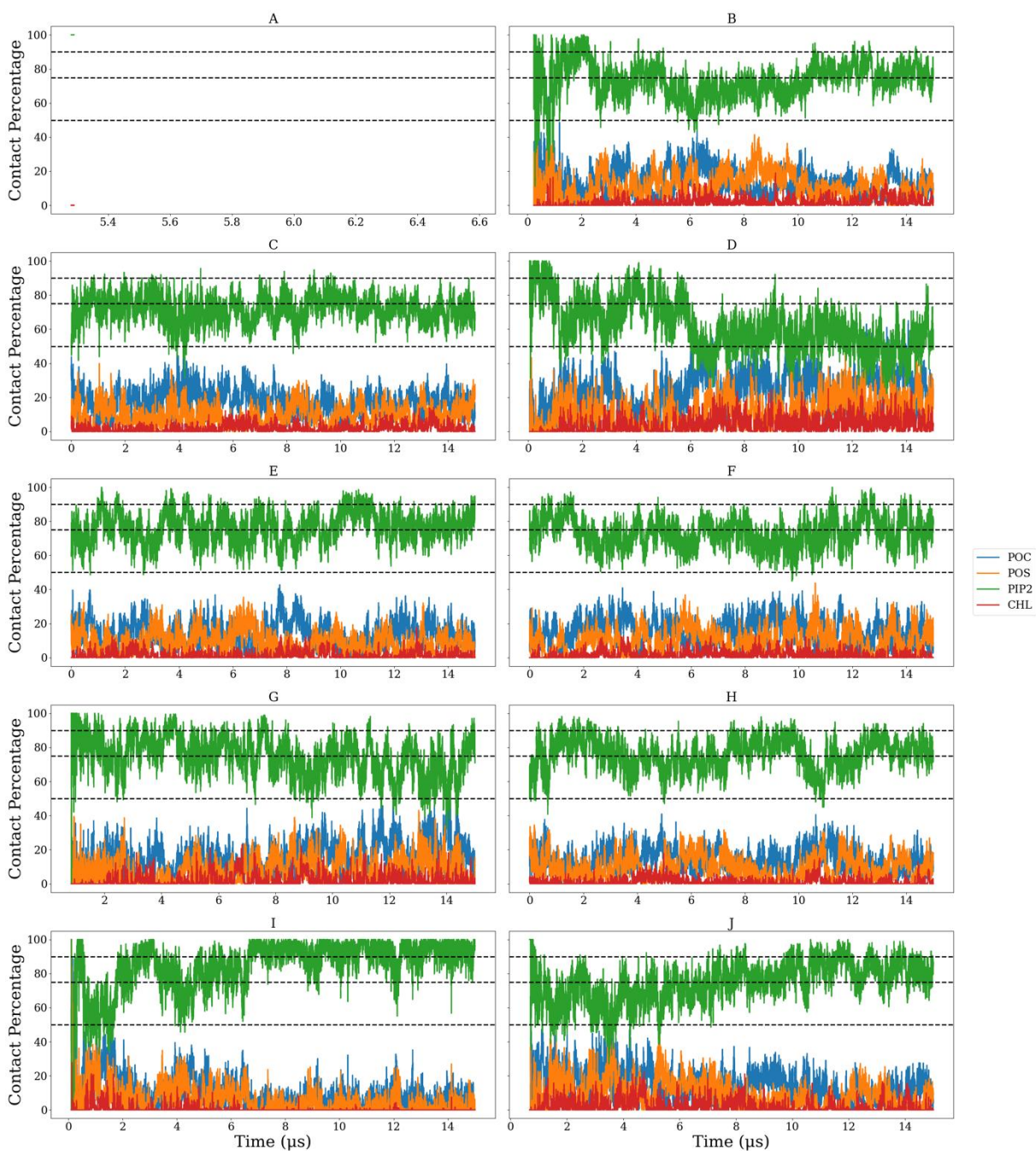


Figure D.21. Percentage of total contacts each lipid is responsible for vs time. Dashed lines are shown at 50, 75, and 90%. The results correspond to simulation run 4 of the A2V system.

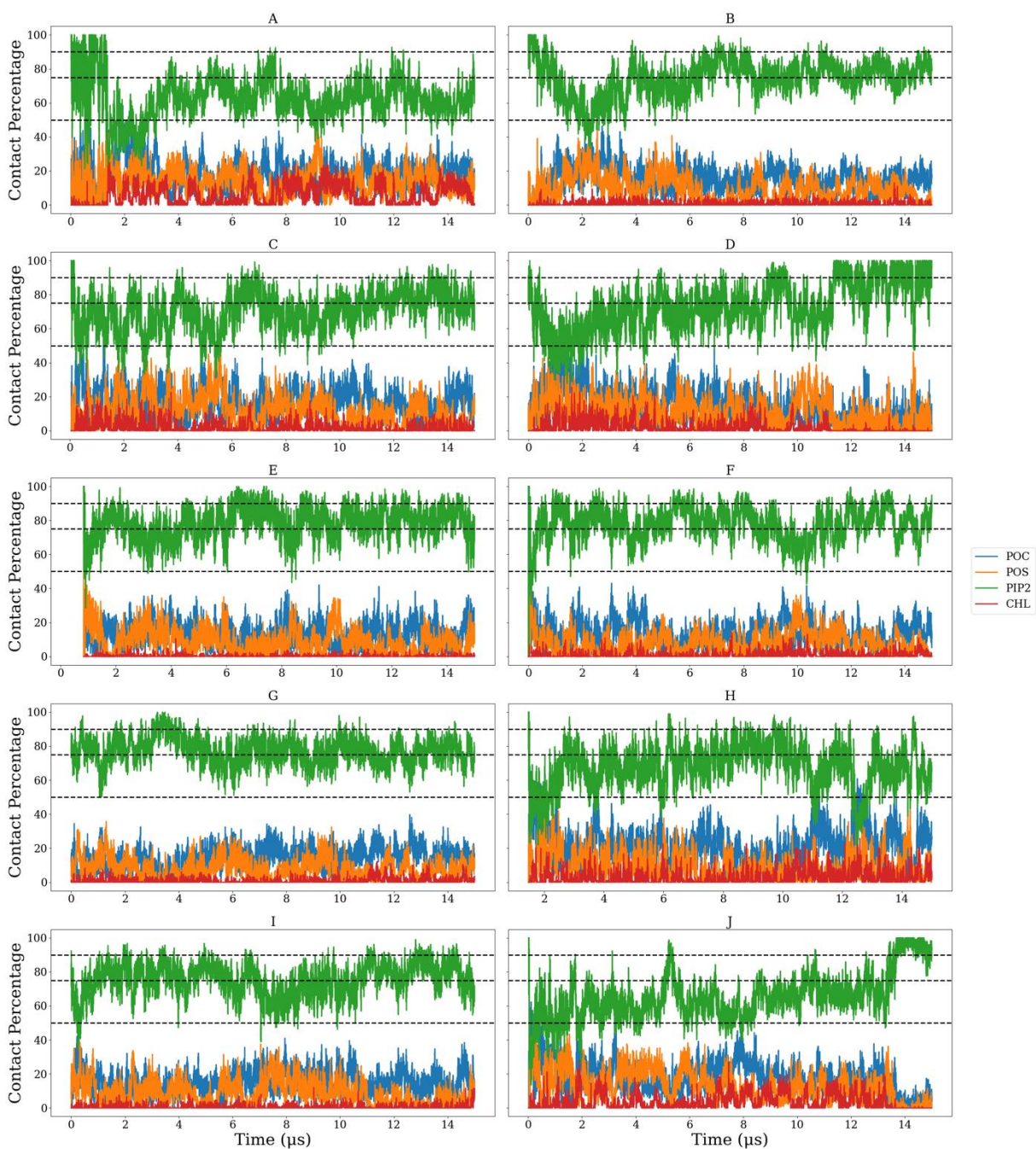


Figure D.22. Percentage of total contacts each lipid is responsible for vs time. Dashed lines are shown at 50, 75, and 90%. The results correspond to simulation run 5 of the A2V system.

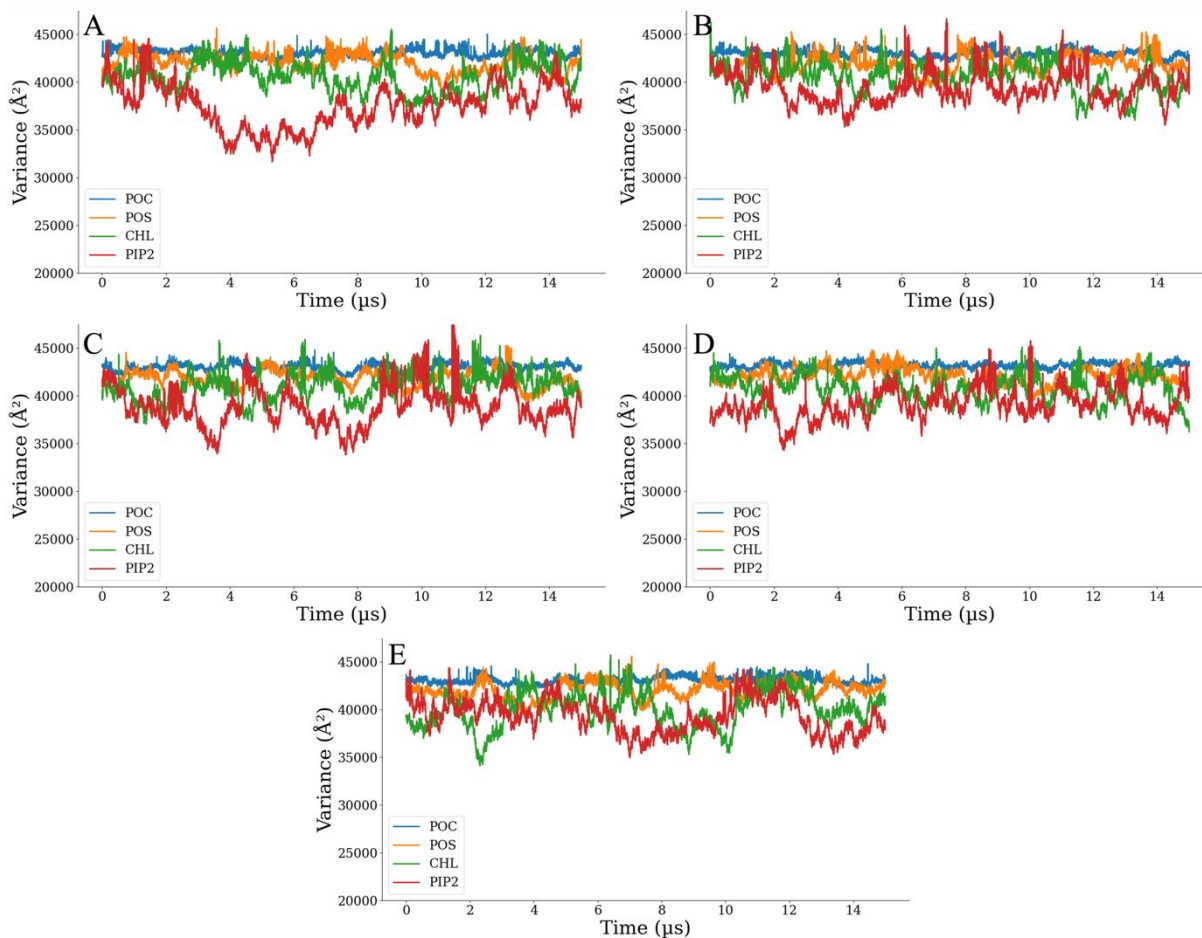


Figure D.23. Variance vs time for all lipids from the V system. A-E correspond to simulation runs 1-5.

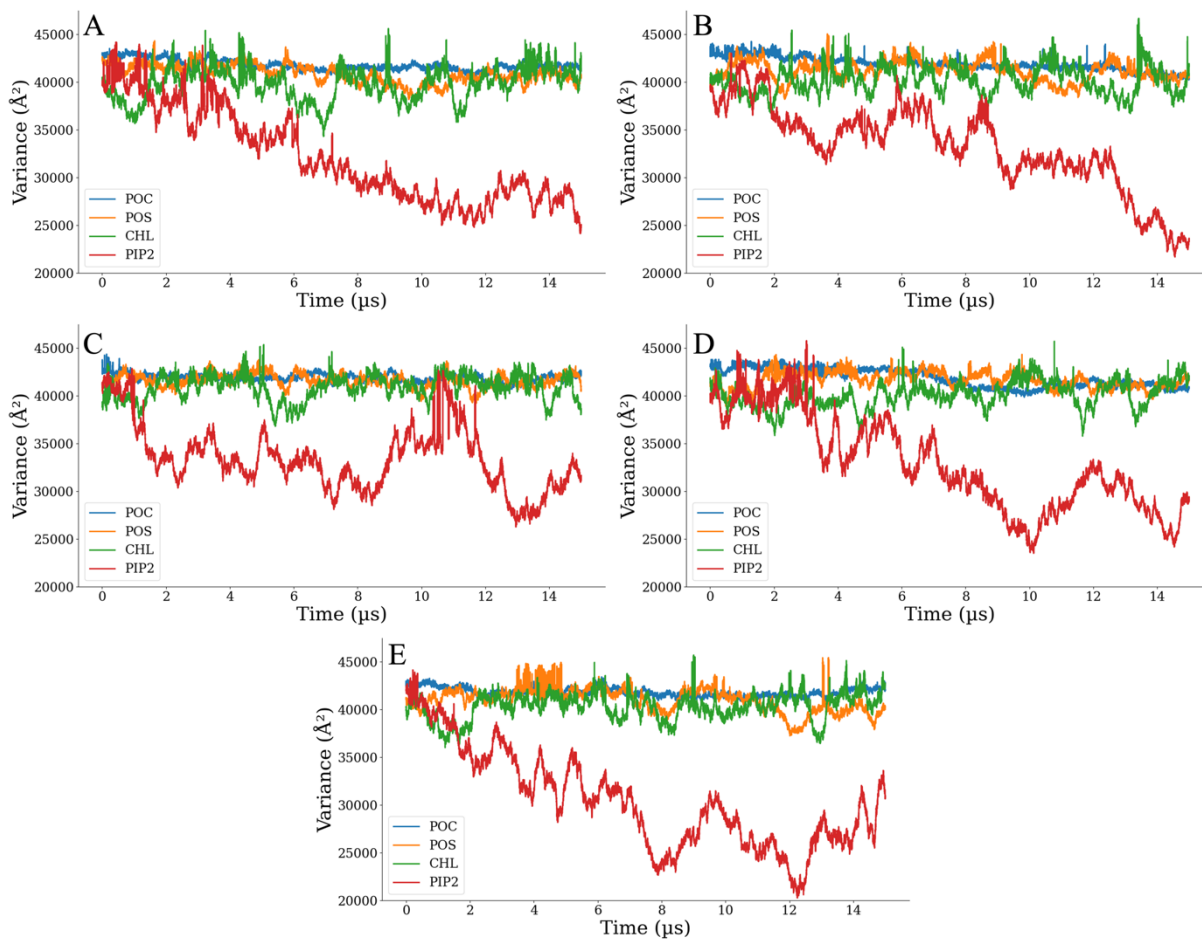


Figure D.24. Variance vs time for all lipids from the A2V system. A-E correspond to simulation runs 1-5.

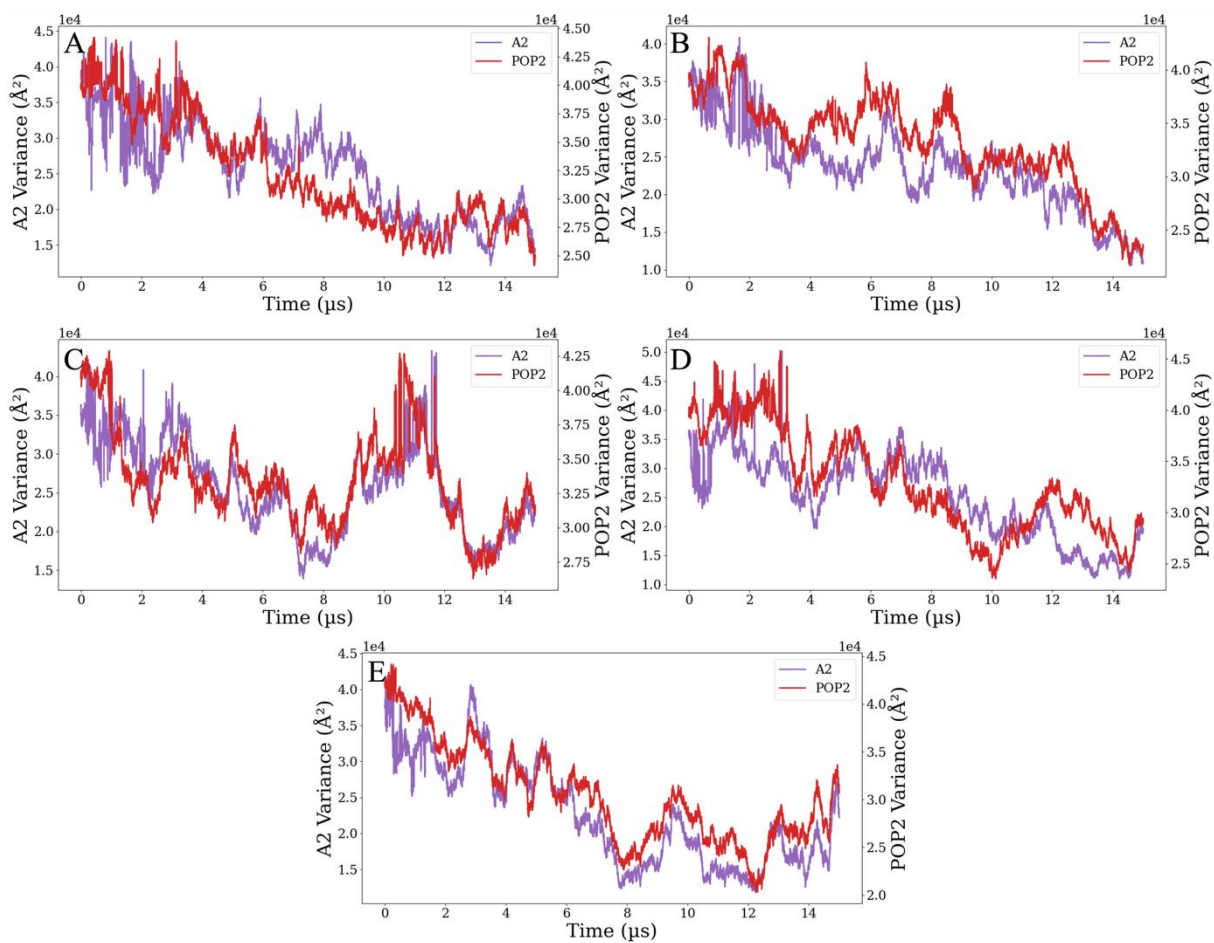


Figure D.25. A2 and PIP2 variances vs time with separate y-axes scales specified. A-E correspond to simulation runs 1-5.

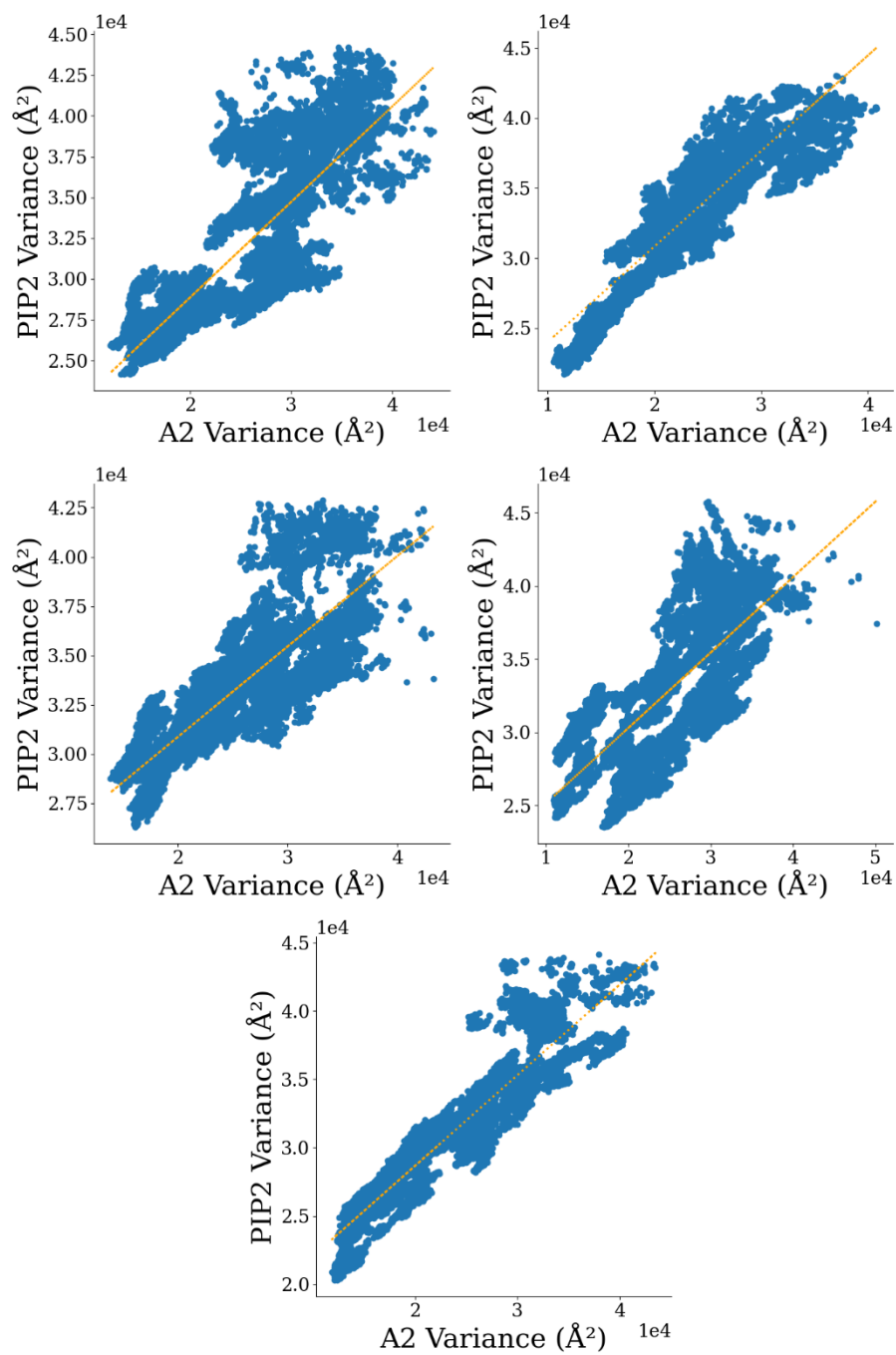


Figure D.26. Plots of PIP2 variance vs A2 variance with a trendline included. A-E correspond to simulation runs 1-5.

Tables

Table D.1. Top Residues Contributing to the Total Number of A2-Vesicle Contacts.

Residue	Repeat	Percent Contact Contribution
K119	II	3.49
K206	III	3.45
K281	IV	3.30
K49	I	3.24
K249	III	3.07
L121	II	2.95
K88	I	2.38
K246	III	2.35
Y151	II	2.34
K152	II	2.20
H94	I	2.15
R284	IV	2.13
K324	IV	1.98
S89	I	1.93
S92	I	1.88
K47	I	1.87
S161	II	1.79
K81	I	1.78
R245	III	1.78
K157	II	1.72

Table D.2. Top Residues
Contributing to the Total Number of
A2-A2 Contacts.

Residue	Repeat	Percent Contact Contribution
Y238	III	3.57
K206	III	2.21
P237	III	2.09
Y317	IV	2.01
K204	III	1.95
K80	I	1.88
K246	III	1.84
K281	IV	1.83
R78	I	1.79
R77	I	1.61
K49	I	1.53
F33	I	1.45
K324	IV	1.38
K313	IV	1.37
Y311	IV	1.36
K47	I	1.30
T79	I	1.28
F73	I	1.23
K249	III	1.21
Q321	IV	1.18