X-Ray Reflectivity Studies of Membrane-Bound Configurations of PKCα-C2 and KIF16B-PX Domains

BY

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THESIS

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This thesis is dedicated to my mother Shiu-Yueh Yu, my father Chin-Yao Chen, my younger brother Chiu-Chi Chen, and my wife Tzu-Ying Hung without them this would never have been possible.
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<td>C1</td>
<td>Protein kinase C Conserved 1</td>
</tr>
<tr>
<td>C2</td>
<td>PKC Conserved region 2</td>
</tr>
<tr>
<td>DAG `</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab1/YOTP/Vac1/EEA1</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
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<td>PH</td>
<td>Pleckstrin homology</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
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<td>PtdIns(3)P</td>
<td>Phosphoinositide (3) phosphate</td>
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<tr>
<td>PtdIns(4)P</td>
<td>Phosphoinositide (4) phosphate</td>
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<tr>
<td>PtdIns(4,5)P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphoinositide (4,5) bisphosphate</td>
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<td>PtdIns(5)P</td>
<td>Phosphoinositide (5) phosphate</td>
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<td>PX</td>
<td>Phox homology</td>
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<tr>
<td>SH2</td>
<td>Src-homology 2</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<td>PCRL</td>
<td>Positively charged-rich layer</td>
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SUMMARY

Upon cellular stimulation, a large number of cellular proteins reversibly translocate to their proper membrane surfaces to form complex arrays of protein-protein and lipid-protein molecular interactions. These proteins are collectively known as peripheral proteins (or amphitrophic proteins) that interact with and penetrate only one leaflet of the membrane bilayer. Understanding the membrane binding mechanism utilized by peripheral proteins is essential to reveal how they efficiently execute their biological functions by cooperating with their biological molecular partners. In this work, synchrotron X-ray reflectivity is used to investigate the membrane-binding mechanism for protein kinase Cα (PKCα-C2) and KIF16B-PX domains.

X-ray reflectivity measurements are carried out to determine the configuration of the C2 domain of PKCα-C2 bound to a lipid monolayer of a 7:3 mixture of SOPC and SOPS supported on a buffered aqueous solution. The reflectivity was analyzed in terms of the known crystallographic structure of PKCα-C2 and a slab model representation of the lipid layer. The configuration of lipid-bound PKCα-C2 is described by two angles that define its orientation, $\theta = 35^\circ \pm 10^\circ$ and $\phi = 210^\circ \pm 30^\circ$, and a penetration depth ($PEN = 7.5 \pm 2$ Å) into the lipid layer. In this structure the longest $\beta$-sheet of PKCα-C2 are nearly perpendicular to the lipid layer and the domain penetrates into the headgroup region of the lipid layer, but not into the tailgroup region. This configuration of PKCα-C2 determined by our x-ray reflectivity is consistent with many previous findings, particularly mutational studies, and also provides new molecular insight into the mechanism of PKCα enzyme activation. Our analysis method, which allows us to test all possible protein orientations, shows that our data cannot be explained by a protein that is orientated parallel to the membrane, as suggested by earlier work.
KIF16B is a Kinesin-3 family motor protein that transports early endosomes (PtdIns(3)P enriched compartment) along microtubules in the cell biosynthetic and signaling pathway. The binding of C-terminal PX domain of KIF16B to a mixed lipid monolayer that contains PtdIns(3)P is investigated. The membrane-bound configuration of KIF16B-PX domain to this monolayer is quantitatively characterized by $\theta = 30^\circ (\pm 10^\circ)$ and $\phi = 20^\circ (+15^\circ/-35^\circ)$, with $\sim 17 \pm 2\AA$ penetration depth into SOPC/SOPS/PtdIns(3)P mixed monolayer by X-ray reflectivity measurements. The configuration suggests that the binding mechanism was initiated by electrostatic interactions between anionic PtdIns(3)P headgroup and two conserved basic motifs of the PX domain, and then proceeded by hydrophobic insertion to further enhance the binding affinity. The binding configuration of KIF16B-PX also suggests a spatiotemporal model for the mechanism of transport of cargo by KIF16B. In addition, the different membrane-bound configurations of two PX domains, KIF16B-PX and p40$\text{phox}$-PX, to membranes containing PtdIns(3)P lipids are compared and analyzed. The analysis of these two PX domains indicates that the binding configurations of these domains to the PtdIns(3)P molecule are correlated to their biological functions.
CHAPTER 1: Introduction to the Binding Mechanism of Peripheral Proteins
Introduction

In living organisms, the cell is the basic unit in terms of functionality and structure. Cell membranes are composed of a phospholipid bilayer and an array of proteins. Membranes are flexible, selectively permeable to ions, and self-sealing. Membranes form a barrier to physically separate the interior of cells from the rest of environment. Hence, membranes define the boundary of the internal and external space for a cell. The lipid bilayer is made of two layers of lipid molecules and sterols. In the lipid bilayer, most of the lipid molecules are phospholipids, which have a hydrophilic headgroup and two hydrophobic tails. The nonpolar tailgroups of lipid molecules in each layer face the core of the bilayer while their headgroups are exposed to the aqueous environment on either side. To allow cell to cell communication and cell response to environmental stimulus, the cell requires a mechanism to enable cell signaling across the cell membrane. Proteins embedded in and attached to the membrane bilayer play an essential role in cell signaling and membrane trafficking; such proteins are called membrane proteins and divided operationally into two groups. One group consists of integral membrane proteins that are firmly embedded in and span the lipid bilayer (transmembrane proteins). Integral membrane proteins comprise numerous receptors, ion channels and respiration complexes (see Figure 1). The other group consists of peripheral membrane proteins (see Figure 1) that associate with the membrane reversibly. Their binding can be driven by a variety of interactions, such as electrostatic, hydrophobic, and even entropic. Peripheral proteins carry out many cellular processes including cell signaling and membrane trafficking. They include signaling lipid-binding domains, membrane-associated enzymes, and antimicrobial peptides. In this work, the binding configuration of lipid-binding domains of peripheral proteins to the lipid membrane is investigated.
Figure 1: Illustrations of transmembrane protein and peripheral protein. (A) A structure of transmembrane protein of the ligand-free G-protein coupled receptor opsin (PDB: ID 3ACP). The protein penetrates from one leaflet of a membrane bilayer through to the other side of the bilayer. (B) The C2 domain of PKCa (PDB: ID 1DSY) binds to one leaflet of the membrane bilayer that is initiated by binding of two Ca\textsuperscript{2+} ions (green spheres) to its binding pocket.
Cells have developed many different strategies to regulate cell signaling and many important cellular events, including recruiting peripheral proteins to specific binding sites, thereby increasing the probability for interaction with appropriate partners. For instance, lipid-binding domains of peripheral proteins utilize distinct membrane binding mechanisms to strictly locate the proteins and execute their biological functions. The discovery of lipid-binding domain (membrane-targeting domain) can be traced back to the identification of conserved domains of C1 and C2 domains among protein kinase C (PKC) in the late 1970s[1]. Since then, a growing number of modular membrane-targeting domains have been identified including C1, C2, PH, PX BAR, PDZ, FYVE, ENTH, ANTH, FERM and tubby domains[2].

It is essential for protein membrane-targeting domains to properly interact and strictly localize to the membrane surface in order to ensure the execution of specific biological functions. For example, mutations of phosphoinositide 3-kinase 3-kinase (PI3K) that alter its signaling pathways can produce tumorigenesis in cancers [3]. Knockout of the kinesin 13 family motor protein (KIF2A) in mice produces abnormal axon branching in their brains and the mice die within one day of birth [4]. These cases indicate that alterations of cell signaling and transduction of membrane-targeting proteins are unable to maintain cellular growth and metabolism. Thus, it is of great importance to understand how membrane-targeting domains reversibly interact with specific lipid molecules in biological processes. Investigations of interactions of membrane-targeting proteins with their targeting lipids provide invaluable information for drug developments in order to cure diseases associated with them. We briefly highlight and discuss membrane-binding mechanisms of some common membrane-targeting domains (except C2 and PX domain) in this chapter. The membrane-binding mechanism of C2 and PX domain are discussed in more details in chapter 3 and 4, respectively.
The membrane-targeting proteins must translocate to the appropriate cell location such as the plasma membrane, endosomes, and trans-Golgi network, as well as bind to lipid molecules to regulate their biological activities. To elucidate their activation mechanism, the spatial positions of peripheral proteins in the lipid bilayer have been studied by many experimental techniques, including site-directed spin labeling, X-ray reflectivity, X-ray diffraction, chemical labeling, mutagenesis, fluorescence spectroscopy, electron paramagnetic resonance (EPR), surface plasmon resonance (SPR), and solution or solid state NMR spectroscopy. Moreover, the positions of membrane-targeting proteins have also been studied by computational approaches, including molecular dynamic simulations (MD), optimization of the electrostatic interaction energy for a protein near a charged planar membrane surface, and minimization of the energy of transfer of proteins from water to lipid bilayer. In this work, the lipid-protein interaction is studied by X-ray reflectivity measurements of proteins bound to a Langmuir lipid monolayer.

Since the 1960s, the characterization of lipid-protein interactions for proteins that bind to only one leaflet of the membrane bilayer have been extensively studied by utilizing lipid monolayers at the air-water interface [5-8]. There are two major steps in the preparations of the monomolecular lipid film at the air-water interface for investigating lipid-protein interactions. Initially, the lipid solution is spread on the aqueous subphase to a desired initial surface pressure $\pi_i = \gamma_w - \gamma_m$ (where $\gamma_w$ and $\gamma_m$ are the interfacial tensions of the clean aqueous subphase and a lipid monolayer supported on the aqueous subphase, respectively). This step is followed by the injection of proteins into the aqueous subphase. After the injection of proteins, in general, the subphase is slowly stirred (~60 rpm) for ~50 minutes in order to equilibrate the protein partitioning between the lipid monolayer and the bulk. The equilibrated (final) surface pressure of the monolayer with bound proteins is denoted $\pi_f$. The study of lipid-protein interactions by the
lipid monolayer model can be carried out either at constant surface area or at constant surface pressure. An observed increase of surface pressure difference \( \Delta \pi = \pi_f - \pi_i > 0 \) is due to binding and penetration of proteins into the monolayer. The value of \( \Delta \pi \) is dependent upon the initial surface pressure of the lipid monolayer, the initial bulk protein concentration, and the binding affinity of proteins to the monolayer. In addition, the adsorbed amount of specially prepared proteins that contain radioactive isotopes (such as \(^{13}I, ^{14}C, ^{35}S, ^{32}P, ^{3}H\)) can be determined by measuring the surface radioactivity[9].

The binding of peripheral proteins to the monolayer can be associated with a three or four-step process. The first step involves 3-dimensional diffusion of proteins to the surface of the monolayer at the air-water interface. The adsorption of proteins to monolayer can be driven by nonspecific electrostatic interactions in the second step. Specific interactions (such as hydrogen bonding and ionic pairing) between proteins and their targeting lipid molecules are achieved in the third step. The insertion of hydrophobic and/or aromatic residues into the hydrocarbon region of the monolayer can occur in the last step.

Membrane-targeting proteins can be classified into three types as H, I, and S (see Figure 2), depending upon their location in the membrane bilayer[2]. H-type proteins penetrate deeply into hydrocarbon regions, whereas I-type proteins locate between the lipid headgroup/tailgroup interface and the level of the phosphate group of the lipid headgroup. S-type proteins interact superficially with the lipid headgroup between the lipid headgroup/buffer interface and the level of phosphate group of lipid headgroup. It has been proposed that peripheral membrane proteins are driven to the membrane by stimulus-induced nonspecific (long-range) electrostatic interactions, which strongly depend on ionic strength. This electrostatics interaction was reported for the S and I type of C2A domain for synaptotagmin I[10]. The binding of this C2 domain to
Ca\(^{2+}\) ions increases the electrostatic potential surrounding the lipid binding pocket of the domain and thereby promotes association of the protein to anionic membranes. According to results of a computational study[11], lysine and arginine residues at the lipid binding regions of I-type and H-type proteins form ionic pairs and hydrogen bonds with the phosphate of the lipid headgroup. The membrane penetration of lipid-binding loops that contain hydrophobic and aromatic residues then follows for H-type proteins as a result of the reduction of desolvation penalty from neutralization of the electrostatic potential in the binding pocket. For example, the membrane-binding mechanism of the PX domain of p40\(^{phox}\) was investigated by a mutational and computational study[12]. The study reported that membrane adsorption was initiated by nonspecific electrostatic interactions between the cationic surface of the lipid-binding pocket of the PX domain and the anionic membrane. This is then followed by specific interactions of Arg58, Arg60, and Arg105 residues of the PX domain and the PtdIns(3)P headgroup. The interaction of the lipid binding pocket with PtdIns(3)P greatly reduced the strong positive electrostatic potential of the pocket and the desolvation penalty associated with membrane penetration of hydrophobic residues. The binding affinity of this PX domain was further enhanced by the penetration of hydrophobic residues (Phe35, Tyr94 and Val95) located in the binding pocket. Furthermore, the membrane binding mechanism for some common membrane-targeting domains, including PH, FYVE, C1 and FERM, is briefly introduced and categorized by H, I and S types below.
Figure 2: The penetration depth of peripheral proteins into the membrane is related to their binding mechanism. Peripheral proteins are classified into three categories (H, I and S type) based on their penetration depth in the membrane [2]. H-type proteins penetrate deepest into the hydrocarbon region. The red line indicates the level of the lipid phosphate group. H-type and I-type proteins penetrate beyond this level, whereas S-type proteins show little penetration.
Pleckstrin homology (PH) domains are composed of ~120 amino acids and one of the most common membrane-targeting domains. PH domains are found to strongly or weakly interact with various phosphoinositides. Among PH domains, ~15% of them bind phosphoinositides with relative high affinity (the dissociation constant for the head group from μM to nM range). PH domains exhibit similar core structure containing a β-sandwich and a C-terminal α-helix. The PH domains of Bruton’s tyrosine kinase (Btk) (PDB ID: 1B55) and general receptor for 3-phosphoinositides (Grp1) (PDB ID: 1FGY) were found to specifically bind PtdIns(3,4,5)P₃, whereas PLCδ₁-PH domain selectively interacts with PtdIns(4,5)P₂. The loop located between β₁ and β₂ forms a platform for interactions with phosphoinositides and binds to PtdIns(3,4)P₂ and/or PtdIns(3,4,5)P₃ with high affinity[13]. Highly conserved basic residues [KXₙ(K/R)XR] at β₁-β₂ loop were identified in alignments of PH domains and shown that those residues are critical to specifically interact with phosphate-group of phosphoinositides[13]. In general, the membrane binding mechanism of PH domains does not penetrate deeply into hydrocarbon tail regions.

FYVE domain, a small cysteine-rich Zn²⁺ binding domain, is named after the four cysteine-rich proteins Fab 1, YOTB, Vac 1, and EEA1. Majority of FYVE domains are characterized strictly binding to PtdIns(3)P and many FYVE domain-containing proteins are localized to PtdIns(3)P-enriched membrane compartments such as endosomes. FYVE domains contain 70-80 amino acids and consist of two β-hairpins and a C-terminal α-helix. A conserved basic motif R(R/K)HHCR in the first β-strand contributes to a positively-charged lipid binding pocket which specifically recognizes the headgroup of PtdIns(3)P. NMR and crystallographic studies of FYVE domain of EEA1 indicated that 1 and 3 phosphates of inositol ring of PtdIns(3)P specifically interact with residues from three conserved motifs: WXXD,
R(K)HHCR, and RXC[14, 15]. The binding to PtdIns(3)P greatly reduces the positive electrostatic potential surrounding the lipid binding pocket of the FYVE domain and thereby promotes the hydrophobic insertion of aromatic residues in the lipid binding loop. This binding mechanism is further supported by using the finite difference Poisson-Boltzman (FDPB) method to calculate the interaction energy of FYVE domains with phospholipid membranes[16].

C1 domains are named after conserved region 1 from protein kinase C (PKC) and found to interact with DAG and phorbol ester which are embedded in membranes. C1 domains of conventional and novel PKCs are further classified into C1A and C1B domains, respectively. The C1 domain (~ 50 amino acids) has a cystein-rich compact structure that contains five short $\beta$ strands, one short $\alpha$-helix, and two zinc ions. A polar binding pocket for DAG/phorbol ester of the C1 domain is located at the tip of the molecule with hydrophobic and aromatic residues surrounding at the binding pocket. The penetration depth and orientation of PKC$\delta$-C1B domain binding to phorbol ester in the lipid bilayer were investigated by a computational study by minimizing energy of protein transfer from water to the lipid bilayer[11]. The computational study showed that the hydrophobic side chains of residues M239, P241, L250, W252, G253, L254 and V255 penetrated into hydrocarbon membrane core, while W252, L254, V255 were identified to specifically interact with phorbol ester. In the same study, the hydrophobic interactions of PKC$\delta$-C1B domain with the membrane bilyare were found to increase the binding energy from -5 to -6 kcal/mol.

The FERM domain is found in the N-terminal region of the ezrin/radixin/moesin (ERM) family which regulates cross-linkers connecting actin filaments to membranes. The FERM domain comprise 300 amino acids and binds to PtdIns(4,5)P$_2$. The crystallographic study of the radixin FERM domain shows that this domain is of three subdomains, labeled A, B, and C and
the phosphoinositide-binding site is located in a basic cleft between subdomains A and C[17, 18]. The FERM domain is not expected to penetrate into the membrane hydrocarbon core due since the membrane binding region is not surrounded by hydrophobic residues. This notion is consistent with the findings by a computational study which showed that the membrane binding region of radixin-FERM penetrated into the lipid headgroup and Lysine and Arginine residues of the lipid binding loops formed ionic pairs with lipid phosphate group[11].
CHAPTER 2: Data Analysis of X-Ray Reflectivity
2.1 The Measurement of Reflectivity

X-ray reflectivity has been demonstrated to be a good technique to characterize the surface structure on a microscopic or atomic length scale. The specular reflection of X-ray is measured as a function of the wave vector transfer \( Q_z = k_{\text{scat}} - k_{\text{in}} = (4\pi/\lambda)\sin\alpha \) for wavelength \( \lambda \) and reflection angle \( \alpha \) (see Figure 3). The wave vector transfer of the reflected X-rays is solely in the \( z \)-direction, which is normal to the buffer surface. Therefore, X-ray reflectivity probes the structure as a function of depth along the interface.

Figure 3: Illustration of the geometry of X-ray reflectivity measurements in the plane of incidence. The incident beam strikes the interface of a sample at an angle \( \alpha \) and the reflected X-rays are collected by a detector positioned at the same angle \( \alpha \) in the plane of incidence. The total wave vector transfer \( Q = K_{\text{scat}} - K_{\text{in}} = Q_z = (4\pi/\lambda)\sin(\alpha) \), where \( \lambda \) is the wavelength of the incident X-rays.
2.2 Two Slab Model

X-ray reflectivity measurements as a function of the reflection angle are usually analyzed by (a) assuming a model (a functional form) for the electron density as a function of depth $z$ into the surface, but averaged over the in-plane $x$-$y$ direction (the so-called electron density profile), (b) computing the reflectivity from this model, and (c) comparing the computed reflectivity to the measured reflectivity by the use of a non-linear least squares fitting procedure that adjusts parameters in the model to yield a best fit to the data. As an example, the electron density profile of the lipid monolayer supported on aqueous buffer is often modeled as consisting of two slabs of uniform electron density on top of the bulk electron density of the aqueous buffer[19]. The two slabs model the two regions of the monolayer that correspond to the lipid tailgroups and lipid headgroups. In this case, the fitting parameters would be the thickness and electron density of each slab (and possibly also the interfacial roughness) since the electron density of the bulk buffer is known. The slab for the lipid headgroup region is characterized by a constant electron density $\rho_{\text{head}}$ throughout its thickness $L_{\text{head}}$ (see Figure 4). The other slab represents the lipid tailgroup (acyl group) region with a constant electron density $\rho_{\text{tail}}$ throughout its thickness $L_{\text{tail}}$ (see Figure 4). Each slab is associated with a constant electron density $\rho$ and its thickness $L$. In a physical system, the step function electron density profile in a two slab model is smeared by the thermal excitation of capillary waves. The electron density profile smeared by an interfacial roughness $\sigma$ yields a smooth crossover between slabs. The smooth electron density profile is the profile that is measured by X-ray reflectivity. In our X-ray data analysis, the discrete electron density profile from two slab model is convoluted by a Gaussian function with an interfacial width $\sigma$ determined from capillary wave theory[20] (equation 2.1).
The smeared electron density profile along the surface normal is given by equation 2.1, where \( \text{erf}(z) \) is an error function. The smooth electron density profile \( \rho(z) \) is further sliced into \( M \) layers with 0.2 Å thickness along \( z \). The calculated reflectivity \( R_{\text{cal}}(Q_z) \) for the profile \( \rho(z) \) with \( M+1 \) layers, including subphase, is calculated by using the Parratt formalism [21]. To make the features of the reflectivity curve more evident, the measured reflectivity \( R(Q_z) \) is divided by \( R_f(Q_z) \), which is the Fresnel reflectivity predicted for a perfectly sharp and flat interface. Deviation of the measured reflectivity, \( R(Q_z) \), from the Fresnel reflectivity, \( R_f(Q_z) \), reveals the presence of interfacial structure as a function of position along the normal to the surface. Therefore, the measured reflectivity data is analyzed in the format of \( R(Q_z)/R_f(Q_z) \). The interfacial roughness due to capillary waves can be calculated using equation 2.2 [22]

\[
\sigma^2 = \frac{k_B T}{2\pi} \int_{q_{\text{min}}}^{q_{\text{max}}} \frac{q \, dq}{\gamma q^2 + \Delta \rho_m g} \approx \frac{k_B T}{2\pi \gamma} \ln \left( \frac{q_{\text{max}}}{q_{\text{min}}} \right)
\]

where \( k_B T \) is Boltzmann’s constant times the temperature, \( \gamma \) is the measured interfacial tension, \( \Delta \rho_m \) is the mass difference of the air and subphase in this work, \( g \) is the gravitational acceleration, and the term \( \Delta \rho_m g \ll \gamma (q_{\text{min}})^2 \). The variable \( q \) is the in-plane wave vector of the
capillary waves. The limit \( q_{\text{min}} = (2\pi/\lambda) \Delta \beta \sin(\alpha) \) is determined by the instrumental resolution that sets the longest in-plane capillary wavelength resolved by the X-rays. The limit \( q_{\text{max}} \) is the short-wavelength cutoff for the smallest wavelength capillary waves that the interface can support. We have chosen \( q_{\text{max}} = 2/a \), where \( a \) is the molecular size of lipid molecular ~ 8Å. Note that \( \sigma^2 \) depends on \( q_{\text{max}} \) logarithmically and is not very sensitive to its value.

Figure 4: Two-slab model is applied to the analysis of X-ray reflectivity data for Langmuir lipid monolayers. \( L_{\text{tail}} \) and \( \rho_{\text{tail}} \) represent the thickness of the hydrocarbon region and the constant electron density through this region, respectively. Similarly, the thickness of lipid headgroup region and its corresponding uniform electron density are described by \( L_{\text{head}} \) and \( \rho_{\text{head}} \), accordingly.
2.3 Two Slab Model Plus Protein Layer

In studies of a monolayer plus protein system (see Figure 5), other authors have described the protein as an additional slab of uniform electron density[23, 24]. However, proteins like the C2 domain of protein kinase Ca (PKCa-C2) and PX domain of the KIF16B (KIF16B-PX) have robust structures with a well-defined arrangement of atoms and, therefore, a specific electron density profile for a given orientation. Describing this protein electron density profile as a single slab of uniform electron density results in a loss of information when reflectivity is analyzed. Previously, we introduced the use of the protein structure, taken from crystallography or NMR studies of protein domains, into the analysis of x-ray reflectivity from cPLA2α-C2 and p40phox-PX domains adsorbed onto Langmuir monolayers of lipids[25, 26]. The use of a crystal structure has recently been applied to a neutron reflectivity study of hemolysin channels, though the orientation was fixed[27].

![Figure 5: Illustration of the model for protein-membrane binding.](image)

The intrinsic electron density profile $\rho_i(z)$ consists of $N + 2$ layers with $N + 1$ interfaces. Two of these layers are the bulk air and buffer; the remaining $N$ layers describe the lipids and proteins. The positive $z$-axis is above the lipid layer; positions within the lipid layer, protein, or buffer are indicated by negative values of $z$. The fitting parameter of $d_p$ indicates the position of the topmost of the protein box along the $z$-direction. This value is negative since proteins are located below the interface of the air/tail-region. Protein penetration into the lipid layer “PEN” can be calculated whenever $d_p$ is determined.
Sarka Malkova had previously used the commercial crystallography software *CERIUS* to compute electron density profiles of proteins for the analysis of reflectivity for the lipid monolayer-protein system[25, 26]. In the current work on PKCa2-C2 and KIF16B-PX domains, we scrapped the commercial software and wrote new analysis software that starts directly from the protein data bank crystallography coordinates. This software is described in detail in Appendix A and B. Our method of constructing the electron density profile for a protein in a particular orientation is described in detail below. Application of these methods allows us to quantitatively characterize the penetration depth and orientations of proteins bound to a lipid monolayer at air-water interface.

### 2.3.1 Overview of the Analysis Methodology

Figure 5 illustrates the model used to fit X-ray reflectivity data in order to determine the penetration depth and lipid-bound orientation of a protein domain. The purpose of our analysis is to determine the orientation of the protein with respect to the plane of the lipid layer, the penetration depth of the protein into the lipid layer, the fraction of interface covered by the protein, as well as to characterize the thickness and electron density of the lipid tailgroup and headgroup ($L_{\text{tail}}$, $\rho_{\text{tail}}$, $L_{\text{head}}$ and $\rho_{\text{head}}$, respectively). The figure shows a protein at a particular orientation that has penetrated partially into the headgroup region of the lipid monolayer, but not into the tailgroup region. It illustrates $N$ layers, each of uniform electron density that is used to describe the electron density profile of the interface. The first layer is used to model the electron density of the tailgroup with two fitting parameters – its average electron density $\rho_{\text{tail}}$ and thickness $L_{\text{tail}}$. The tailgroup is located between the interfacial positions $z = 0$ and $z = -L_{\text{tail}}$. The second layer models the top part of the headgroup that is not penetrated by the protein. Note, however, that this model allows for the possibility that the protein domain penetrates into the
tailgroup region or does not penetrate the lipid monolayer at all, though we have discussed only
the case illustrated in Figure 5. Subsequent layers that extend down to the position of the
headgroup/buffer interface (at $z = -L_{\text{tail}} - L_{\text{head}}$) model a region of the interface occupied by both
the top part of the protein and lipid headgroups. The remaining layers model a region occupied
partially by the bottom part of the protein and partially by the aqueous buffer, though we do not
exclude the possibility that the protein can fully cover the interface.

Proteins are characterized by the atomic coordinate PDB files that were obtained from the
RCSB protein data bank. Water molecules were removed from the PDB files. The software
Molprobity was used to modify the PDB files by restoring the missing hydrogen atoms[28]. We
chose three atoms in the protein to define a protein coordinate system, then rotated the protein
with respect to a coordinate system defined by the lipid layer (equivalently, the buffer/air
surface). To account for the fact that part of the protein is located in the buffer and part in the
lipid layer, a confining mathematical box is drawn around the protein. In practice, two boxes are
used. In one of the boxes the empty space is filled with aqueous buffer by assigning to that
volume the electron density of the buffer. In the other box the empty is assigned zero electron
density. Part of the box with buffer is used to describe the corresponding part of the protein that
is in the buffer below the lipid layer. The remainder of the protein that inserts into the lipid layer
is described by the corresponding part of the box whose empty space was left empty; our
analysis method fills this empty space by electron density from the lipid layer (e.g., the
headgroup in the case illustrated in Figure 5).
For a given orientation, the electron density profile of the box with protein is calculated by slicing the box into thin layers along the surface normal (z-axis). The coordinate $z_p$ is denoted for the axis parallel to the z-axis in the protein box in (B) and (C) that is used to generate electron density profile for proteins in these two boxes. (A) To construct the electron density profile in lipid monolayer-protein model, it is required to compute the electron density profile for the protein. Two electron density profiles are input to construct the electron density profile for protein binding to the lipid monolayer. (B) The electron density profile in a box with the space (not occupied by protein) left empty represents the electron density profile for the region of the protein that penetrates into lipid monolayer. (C) The electron density profile for the rest of the protein that is submerged in the bulk phase is determined by the electron density profile of the box whose empty space (that is, the volume not occupied by the protein) is filled with aqueous buffer.

For a given orientation, the electron density profile of the box with protein is calculated by slicing the box into thin layers along the surface normal (Figure 6), then counting the number of electrons in each layer and dividing by the layer volume. These profiles are produced for a complete range of protein orientations. The protein profile for a given orientation is combined with a 2-slab model of the lipid monolayer in a non-linear least squares fitting to the x-ray reflectivity data in which six parameters are fit ($L_{\text{tail}}$, $\rho_{\text{tail}}$, $L_{\text{head}}$ and $\rho_{\text{head}}$, the displacement of the protein $d_p$ from the surface (Figure 5), and the coverage $COV$, i.e., the fraction of surface covered
by the protein-filled boxes, as defined below). This procedure yields a goodness of fit parameter $\chi^2$ for each protein orientation. Comparison of these $\chi^2$ values determines the best-fit orientation and the accompanying best-fit values for the six fitting parameters.

X-ray reflectivity data were fit to the entire range of orientational angles for proteins. The angle $\theta$ measures the angle between the protein’s $z'$-axis and the surface normal, whereas the angle $\phi$ is an azimuthal rotation about the direction of the $z'$-axis (Figure 7). Initially, fitting was carried out for values of $\theta$ spaced by 5° over the range from 0 to $\pi$ and for values of $\phi$ spaced by 20° over the range from 0 to $2\pi$. This procedure determined the approximate location of the best-fit orientations. Then, a finer 1° spacing of $\theta$ and $\phi$ values was used to locate the best-fit orientations precisely. Contour plots of the goodness of fit parameter $\chi^2$ as a function of $\theta$ and $\phi$.

Figure 7: Three reference atoms A, B, and C are represented by red, blue, and green spheres, respectively. The original PDB file from Protein Data Bank is translated and rotated to place A, B, and C atoms at $A=[0, 0, 0]$, $B=[0, 0, B_z]$ and $C=[C_x, 0, C_z]$, where this orientation is defined as starting orientation ($\theta=0^\circ$, $\phi=0^\circ$). All other orientations of proteins are generated via two Euler Angles ($\theta$ and $\phi$) from the starting orientation. In the starting orientation, the molecular coordinate $(x', y', z')$ is parallel to the laboratory coordinate $(x, y, z)$. See explanation in the text for the distinction between the primed and unprimed coordinate systems.
2.3.2 Calculation of Protein Electron Density Profiles

The surface coordinate system \((x, y, z)\) is defined by a \(z\)-axis that is normal to the average plane of the surface (or, equivalently, to the average plane of the lipid layer) given by the \(x-y\) plane. The primed coordinate system \((x', y', z')\) is fixed to the protein and is used to describe rotations of the protein with respect to the surface coordinate system \((x, y, z)\). In the initial orientation of the protein the two coordinate systems \((x, y, z)\) and \((x', y', z')\) are coincident (Figure 7). The \((x', y', z')\) coordinate system is specified by locating three reference atoms \(A, B,\) and \(C\), at positions \([0,0,0], [0,0,B_z]\) and \([C_x,0,C_z]\) (Figure 7). In the case PKCa-C2 domain, the reference atoms \(A, B,\) and \(C\), are chosen to be the \(\alpha\)-carbons of residues Leu173, Ala180 and Ala271, respectively. Subsequent orientations of the protein were characterized by the Euler angles shown in Figure 7. The azimuthal angle \(\psi\) (not shown in Fig. 7) and the angle \(\phi\) vary from 0 to \(2\pi\) and the angle \(\theta\) is varied over the range 0 to \(\pi\). Rotation of the protein by the angle \(\psi\), which corresponds to an azimuthal rotation within the plane of the surface, does not change the electron density profile of the protein along the surface normal (the \(z\) axis) and, therefore, does not change the x-ray reflectivity. Hence, only \(\theta\) and \(\phi\) angles are used to specify the protein orientation. The polar angle \(\theta\) measures the angle between the protein’s \(z'\)-axis and the surface normal \(z\), whereas the angle \(\phi\) is an azimuthal rotation about the direction of the \(z'\)-axis (Figure 7). For example, the initial orientation, described above, is given by \(\theta = 0^\circ\) and \(\phi = 0^\circ\). Subsequent orientations are generated by applying \(A = ([R(\theta)]^t[R(\phi)]^t)A'\), where \(A\) and \(A'\) are vectors in \((x, y, z)\) and \((x', y', z')\), respectively. The superscript \(t\) refers to the transpose of the rotation matrices given by
\[
R(\theta) = \begin{bmatrix}
1 & 0 & 0 \\
0 & \cos \theta & \sin \theta \\
0 & -\sin \theta & \cos \theta
\end{bmatrix}
\text{ and } R(\phi) = \begin{bmatrix}
\cos \phi & \sin \phi & 0 \\
-\sin \phi & \cos \phi & 0 \\
0 & 0 & 1
\end{bmatrix}
\]

(2.3)

Once the protein has been rotated and the coordinates of all its atoms determined in the surface coordinate system \((x, y, z)\), the primed coordinate system is no longer used in the calculation. For each orientation, the smallest possible rectangular box with sides parallel to the \(x\)-, \(y\)-, and \(z\)-axes is drawn about the protein (Figure 6).

In proteins, the electron density for an atom is assumed to be uniformly distributed throughout the volume of a sphere whose radius is equal to the van der Waals radius of the atom. The box dimensions are determined by the van der Waals radii of the atoms on the protein’s periphery[29]. As previously described, two identically sized boxes are considered for each orientation. These boxes are referred to as the “empty” (Figure 6 B) and “buffer” boxes (Figure 6 C). The box dimensions vary with protein orientation. Note that the PDB files include water molecules that form a hydration layer on the protein surface. We did not include these in the X-ray reflectivity data analysis.

It is convenient to define a \(z_p\)-axis that is parallel to the \(z\)-axis, but displaced from it by \(d_p\) such that \(z = z_p + d_p\), for the purpose of calculating the electron density profile of the protein in either the empty or the buffer box (see Figure 5, which illustrates \(d_p < 0\)). We define \(z = 0\) as the position at the top of the tailgroup (the tailgroup/air interface) and \(z_p = 0\) at the top of the protein box. In the fitting program, \(d_p\) is one of six fitting parameters. It is directly related to the protein penetration “\(PEN\)” that is of physical relevance. Since \(d_p\) and \(PEN\) are related via \(PEN = L_{\text{tail}} + L_{\text{head}} + d_p\), the original fitting parameter \(d_p\) was replaced by \(PEN\).
The electron density profile along the $z_p$-axis of a box was computed by partitioning the box into a cubic grid of cell dimension ~0.5 Å (Figure 6). Electrons from an atom are assigned to a given cell if the van der Waals sphere of that atom overlaps a pre-chosen corner of the cell (where the set of pre-chosen corners form a rectangular, though nearly square, lattice). The number of assigned electrons is then determined by the ratio of the cell volume to the atomic volume. The total number of electrons in a given cell $n_{cell}$ is determined by the sum over all atoms $k$ whose van der Waals volume $V_k$ overlaps the pre-chosen corner of that cell,

$$n_{cell} = \sum_k \frac{V_{cell}}{V_k} n_k$$  

(2.4)

where $n_k$ is the number of electrons in atom $k$ and $V_{cell}$ is a cell volume. The total number of protein electrons in a single layer of cells at height $z_p$ is computed by summing the electrons in all cells at a given height $z_p$. The electron density $\rho_{ep}(z_p)$ (at height $z_p$ in the empty box with protein) is then computed by dividing this total by the volume of a grid layer. In a buffer box, otherwise empty cells are assigned the value of the buffer’s electron density. When the number of electrons from the buffer at height $z_p$ are added to the number of electrons from the protein at the same height, the electron density $\rho_{bp}(z_p)$ (of the buffer box with protein) can be calculated. Although this procedure to calculate the electron density profile of the protein is not exact for an arbitrary cell size, it is computationally fast and converges to an accurate profile for a small enough cell size.
2.3.3 Calculation of the Total Electron Density Profile

The electron density profile along the $z$-axis for the lipid monolayer with bound proteins is computed by combining the electron densities $\rho_{ep}(z_p)$ and $\rho_{bp}(z_p)$ with the electron density of the lipid tailgroup and headgroup (characterized by $L_{\text{tail}}, \rho_{\text{tail}}, L_{\text{head}}$ and $\rho_{\text{head}}$). The electron density in the region of the interface occupied by the lipid monolayer is given by

\[
\rho_i(z) = COV \times \rho_{ep}(z_p) + \rho_{\text{tail}} \quad \text{for} \quad -L_{\text{tail}} \leq z < 0
\]
\[
\rho_i(z) = COV \times \rho_{ep}(z_p) + \rho_{\text{head}} \quad \text{for} \quad -(L_{\text{tail}} + L_{\text{head}}) \leq z < -L_{\text{tail}} \quad (2.5)
\]

where $z = z_p + d_p$, as previously discussed. The protein coverage $COV$ is the fraction of surface area occupied by protein-filled boxes. Similarly, in the buffer region below the lipid monolayer:

\[
\rho_i(z) = COV \times \rho_{bp}(z_p) + (1 - COV) \times \rho_{\text{buffer}} \quad \text{for} \quad -L_{\text{protein}} + d_p \leq z < -(L_{\text{tail}} + L_{\text{head}}) \quad (2.6)
\]

where $L_{\text{protein}}$ is the height of the protein box for a given orientation. In the region of bulk buffer for $z < -L_{\text{protein}} + d_p$ the electron density is given by $\rho_{\text{buffer}}$.

The penetration depth $PEN$ of the protein into the lipid layer is calculated as Figure 5,

\[
PEN = L_{\text{tail}} + L_{\text{head}} + d_p \quad (2.7)
\]

The interfacial area per protein $A_p$ bound to the lipid layer can be calculated as

\[
A_p = A_{\text{box}} / COV \quad (2.8)
\]

where $A_{\text{box}}$ is the cross-sectional area of the box in the $x$-$y$ plane. As mentioned previously, the parameters $d_p$ and $COV$ are fitting parameters.
The intrinsic electron density profile $\rho_i(z)$ describes the arrangement of lipid, protein, and buffer molecules, but does not fully describe the electron density profile probed by x-ray reflectivity because we have left out the description of the thermally induced roughness $\sigma_0$ of the interface due to capillary waves. This roughness is calculated from the measured interfacial tension as previously described [30]. In the hybrid theory of capillary waves the intrinsic profile $\rho_i(z)$ oscillates up and down with the capillary waves [31, 32]. X-ray reflectivity probes the profile averaged over the $x$-$y$ plane, which is smeared in the $z$-direction by capillary waves. This profile $\rho(z)$ is determined by convoluting the intrinsic profile $\rho_i(z)$ with a Gaussian function of width $\sigma$.

\[
\rho(z) = \int_{-\infty}^{\infty} G(z - \zeta) \rho_i(\zeta) d\zeta = I_0 + \sum_{j=1}^{N} I_j + I_{N+1}
\]  

(2.9)

\[
G(z) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{z^2}{2\sigma^2}\right)
\]

\[
I_0 = \frac{\rho_{\text{topphase}}}{2} [1 + \text{erf}\left(\frac{z}{\sqrt{2\sigma^2}}\right)]
\]

\[
I_j = \frac{\rho_j}{2} \left[ \text{erf}\left(\frac{z + \sum_{t=1}^{j} L_t}{\sqrt{2\sigma^2}}\right) - \text{erf}\left(\frac{z + \sum_{t=1}^{j-1} L_t}{\sqrt{2\sigma^2}}\right) \right]
\]

\[
I_{N+1} = \frac{\rho_{\text{buffer}}}{2} \left[ 1 - \text{erf}\left(\frac{z + \sum_{t=1}^{N} L_t}{\sqrt{2\sigma^2}}\right) \right]
\]

where $\rho_j$ is the electron density of the $j$-th layer of the intrinsic profile $\rho_i(z)$. These equations are valid for the general case in which the lipid monolayer is situated at an interface between two
phases. In the present case, the lower phase is the aqueous buffer and the upper phase is air, so 
\[ \rho_{\text{top phase}} = 0 \] and \[ I_0 = 0. \] The continuous electron density profile \( \rho(z) \) of the interface is then sliced into \( M + 1 \) layers, including subphase, of thickness 0.2 Å for the purpose of calculating the reflectivity using the Parratt formalism\[21\]. The zero-th layer is the upper phase (air), whereas the \((M+1)\)-th layer represents the aqueous buffer. In our geometry, the \( z = \infty \) is terminated in the bulk (air) and \( z = -\infty \) is truncated in the lower phase (aqueous buffer).

\[
R_{n-1,n} = \frac{F_{n-1,n} + R_{n,n+1} \exp(2ik_{n,z}d_n)}{1 + F_{n-1,n}R_{n,n+1} \exp(2ik_{n,z}d_n)}
\]

\[
F_{n-1,n} = \frac{k_{n-1,z} - k_{n,z}}{k_{n-1,z} + k_{n,z}}
\]

To calculate the specular reflectivity for the \( M \) layer sitting on top of an infinitely thick substrate (buffer), one start with \( R_{m+1,m+2} = 0 \) as the aqueous buffer is infinitely thick. Thus, the amplitude reflectivity from the interface between the bottom of the \( M \) –th layer and the aqueous buffer

\[ R_{m,m+1} = F_{m,m+1} \]  Here, \( F_{m,m+1} \) is the Fresnel reflection coefficient at the interface between the bottom of the \( M \) –th layer and the aqueous buffer. The thickness of \( n \)-th layer is denoted as \( d_n \) and \( k_{n,z} \) is the \( z \)-component of the wave vector in the \( n \)-th layer. The amplitude reflectivity \( R_{0,1} \) for the interface between 1–th layer and the zero-th layer (air) can be obtained by recursively applying Equation (2.10). The corresponding intensity reflectivity \( R = (R_{0,1})(R_{0,1})^* \).
In the analysis of the X-ray reflectivity data for the monolayer-protein system, the interfacial roughness is fixed to the value calculated from the capillary wave theory using measured surface pressure and temperature. The capillary wave theory shown in Equation 2.2 assumes that interfacial fluctuations are described by distortions that increase the interfacial area and therefore raise the interfacial free energy in proportion to the interfacial tension[33].

Alternatively, interfacial distortion can occur without increasing the interfacial area in a manner similar to bending a piece of paper. The energy required for this distortion is proportional to the bending rigidity of the lipid monolayer in this case [33]. Equation 2.11 indicates that the bending rigidity $\kappa$ is associated with $q^4$, whereas the interfacial tension $\gamma$ is proportional to $q^2$. Hence, the effect of a bending rigidity is to reduce the smaller wavelength interfacial fluctuations and make the interface smoother. To test the influence of including the bending rigidity, we also analyze our data of monolayer-protein by using a typical value of $\kappa = 10 k_BT$ as measured for bilayers in Equation 2.11[34]. The orientation and penetration of protein are not changed by including the bending rigidity in the calculation of interfacial roughness.

$$
\sigma_{gen\_cap}^2 = \frac{k_BT}{4\pi^2\gamma} \int \frac{d^2q}{(\kappa q^4/\gamma + q^2 + \xi^2)} \approx \frac{k_BT}{2\pi\gamma} \frac{\ln(\gamma/\kappa)^{1/2}}{q_{min}}
$$

(2.11)
CHAPTER 3: Membrane-Bound Configuration of PKCalpha-C2[35]
3.1 Introduction

Peripheral membrane proteins that are important for cell signaling and vesicle trafficking are specifically targeted to different cell membranes in response to various stimuli, including calcium and lipid mediators [36]. Since the function and regulation of these proteins depends upon their interaction with the membrane [11, 37], recent work has focused on determining their membrane-bound orientation and depth of membrane penetration [38-41]. These structural parameters have been determined by mutational studies, fluorescence measurements, EPR measurements, and x-ray reflectivity measurements of peripheral membrane proteins bound to model membranes, such as lipid bilayer vesicles or lipid monolayers [24, 25, 38, 42]. In particular, we have shown recently that X-ray reflectivity can provide a direct, detailed and quantitative determination of the membrane bound configuration of lipid binding domains, including C2 and PX domains [25, 26]. Here, we demonstrate that an improved method of analysis of the X-ray reflectivity allows us to efficiently analyze the entire space of all protein orientations. This yields a more complete and accurate determination of the bound configuration. Application of this technique to the C2 domain of protein kinase Cα (PKCα-C2) bound to mixed lipid monolayers resolves a controversy about the bound configuration of this domain.

PKCα is a member of the classical PKC family that is important in cell signaling [43-45]. The C2 domain of PKCα is an independent membrane-targeting module that is composed of an eight stranded β sandwich with flexible loops on either end (Figure 8).
Three Ca\(^{2+}\) binding loops (CBL1, CBL2, CBL3), located at one end of the domain structure, bind two or three calcium ions in a highly cooperative manner due to the presence of five highly conserved Aspartate residues [39, 40, 46]. The coordination of calcium ions alters the electrostatic potential of the C2 domain [47], which accelerates its association to the plasma membrane where it recognizes phosphatidylserine (PS) [40, 48, 49] and phosphatidylinositol-4,5-bisphosphate (PIP2) [50-52]. Recently, a mutational study reported that the interaction of the Ca\(^{2+}\) binding loops of PKC\(\alpha\)-C2 with Ca\(^{2+}\) and PS drives its membrane binding while the interaction of its cationic \(\beta\)-groove residues with PIP2 augments the membrane binding [53]. These findings indicate that the direct interaction of PKC\(\alpha\)-C2 with PS is a critical step in the mechanism of cellular plasma membrane translocation of PKC\(\alpha\).

The CBLs of PKC\(\alpha\)-C2 domain have been recognized as a critical docking region that interacts with anionic PS molecules in the membrane [47, 54, 55]. It has been suggested that PKC\(\alpha\)-C2 domain can bind to PS-containing lipid bilayers by two distinct orientations, i.e.,
perpendicular and parallel to the membrane surface [56]. Both are consistent with the critical role of the PKCα-C2 CBLs as a PS docking region. In the perpendicular model (Figure 9A), the docking surface is localized to the CBLs and the β-strands lie nearly perpendicular to the membrane surface. In contrast, in the parallel model (Figure 9B), the β-strands are oriented approximately parallel to the membrane surface, which allows Lys205, as well as Ca\(^{2+}\) ions, to interact with PS headgroups [40].

![Figure 9: Perpendicular and parallel PKCα-C2 domain with Ca\(^{2+}\) (magenta spheres) bound to a lipid layer. (A) In a perpendicular model, the β strands orient essentially perpendicular to the membrane surface and the Ca\(^{2+}\) binding pocket provides the primary interaction between the domain and the lipids. (B) In a parallel model, the β strands are essentially parallel to the membrane surface. This allows interactions between lipids and other residues, such as those in β3-β4 sheets, in addition to interactions with the Ca\(^{2+}\) binding pocket.](image)

A crystallographic study of PKCα-C2 complexed with a short-chain PS, 1,2-dicaproyl-sn-phosphatidylserine (DCPS) and Ca\(^{2+}\) ions led to the suggestion that PKCα-C2 would orient in the parallel model when bound to a membrane [40]. Two recent EPR measurements of site-specific
spin-labeled PKCα-C2 have also suggested that PKCα-C2 is oriented parallel to the membrane [56, 57]. However, the parallel model is not consistent with many mutational studies [46, 48, 58], as described in further detail in the Discussion section. Collectively, the existing studies do not determine unambiguously the membrane bound orientation of the PKCα-C2 domain. Since the reported discrepancy between EPR and mutational studies may derive from the introduction of bulky unnatural spin labels that may disturb the system, we performed a detailed structural analysis of membrane bound orientation of unlabeled PKCα-C2 by our x-ray reflectivity analysis. To determine the membrane-bound configuration of PKCα-C2, we carried out X-ray reflectivity measurements [25, 59, 60] from a mixed lipid monolayer (7:3 SOPC:SOPS) supported on the surface of a buffered aqueous solution that contains PKCα-C2 domain. X-ray reflectivity determines the variation of electron density with depth through the surface that, in this case, consists of a layer of lipids along with bound PKCα-C2 domains. This electron density is then interpreted in terms of the arrangement of PS lipids and PKCα-C2 domains. Significant conformational rearrangement of the internal structure of the C2 domain upon binding to phospholipids is not expected [40-42, 61]. Therefore, we incorporated the known structure of PKCα-C2 from the Protein Data Bank (ID 1DSY) [40] into our analysis of the x-ray reflectivity. Our results demonstrate that the reflectivity data are consistent with two slightly different bound configurations, both falling within the confines of the perpendicular model. Parallel orientations of PKCα-C2 [40, 56, 57] are not consistent with our x-ray reflectivity measurements. The preferred configuration has an orientation given by $\theta = 35^\circ \pm 10^\circ$ and $\phi = 210^\circ \pm 30^\circ$ and penetrates a distance of $7.5 \pm 2$ Å into the lipid headgroup. The PKCα-C2 domain does not insert into the hydrophobic region of the lipid layer. The calcium binding loops CBL1 and CBL2 penetrate into the lipid headgroup while CBL3 is located adjacent to the headgroup.
Furthermore, this configuration allows us to postulate a mechanism for the activation of protein kinase Ca.

3.2 Materials and Methods

3.2.1 Materials

KCl, CaCl₂ and HEPES (N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid) from Fisher Scientific (Hampton, NH) and EGTA (ethyleneglycol-O, O’-bis(2-aminoethyl)-N, N, N’, N’-tetraacetic acid) from Sigma (St. Louis, MO) were used as obtained. Stock solutions of 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) and 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoserine (SOPS) (7:3 molar ratio) in chloroform and 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzo[dia]zol-4-yl)aminop]-hexanoyl}-sn-glycero-3-phosphocholine in chloroform were purchased from Avanti Polar Lipids and used without further purification. Spreading solutions were prepared by diluting the stock solution with freshly opened bottles of chloroform (Sigma). Expression and purification of the C2 domain of PKCa were carried out as described previously [46]. The domain sequence of the purified protein is composed of M¹⁵²DHH¹⁵⁵ (additional residues from purification protocol), T¹⁵⁶ to N²⁸⁷ (from 1DSY PDB file [40]), and L²⁸⁸EHHHHHH²⁹⁵ (additional residues from purification protocol). The method for modeling the additional residues was described in the previous work [25].

3.2.2 Sample Preparation and Surface Pressure Measurements

To prepare a sample for study by X-ray reflectivity approximately 10 μl of 1 mM SOPC and SOPS (7:3) in chloroform was added drop wise onto the surface of a pH 7.0 aqueous solution containing aqueous 20 mM HEPES buffer, 0.1 M KCl and 0.1mM CaCl₂ in a circular Teflon trough of 72 mm diameter and ~40 ml total volume. The ratio of 7:3 SOPC:SOPS was
chosen to be consistent with earlier biochemical measurements on similar systems [62]. The resulting lipid monolayer was equilibrated for 2 h and the reflectivity was measured. An amount of PKCα-C2 slightly greater than the amount required to saturate the lipid layer (i.e., >240 μg) was then injected into the subphase, the system equilibrated for 1 hr with continuous slow stirring, the stir bar stopped, the system allowed to briefly relax, and the reflectivity was measured. The surface pressure was monitored throughout the experiment, including equilibration and x-ray measurement, with a filter paper Wilhelmy plate and a Nima surface pressure sensor PS-4. Typical variations in surface pressure were ±0.5mN/m and appeared as random fluctuations during the period (i.e. 6-8 h) of the measurement of the lipid layer with and without bound PKCα-C2. Here, we report on data from three separate experiments on monolayers with similar initial surface pressures (24.6, 26.7, and 24.9 mN/m) and similar changes in pressure upon adding PKCα-C2 (Δπ= 2.0 ± 0.5, 2.6 ± 0.5, and 3.1 ± 0.5 mN/m, respectively). Note that fluorescence microscopy was used to verify the absence of micrometer-scale or larger domains.

3.2.3  **X-ray reflectivity measurements**

X-ray reflectivity experiments were carried out at beamline X19C at the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY) with a liquid surface reflectometer described in detail elsewhere [63]. Reflectivity is measured as a function of the wave vector transfer \( Q_z \) by varying the incident angle \( \alpha \) and measuring the intensity of x-rays reflected at the angle \( \alpha \). The wave vector transfer of the reflected x-rays, \( Q \), is solely in the \( z \)-direction normal to the buffer surface with \( Q_z = (4\pi/\lambda) \sin(\alpha) \), where \( \lambda = 1.54 \pm 0.003 \) Å is the x-
ray wavelength. Reflectivity probes variations in electron density as a function of depth into the surface.

The reflectivity $R(Q_z)$ represents the reflected x-ray intensity divided by the x-ray intensity measured before the sample. In addition, background scattering is measured and subtracted as described elsewhere[63]. To make the features of the reflectivity curve more evident, $R(Q_z)$ is divided by $R_f(Q_z)$, the Fresnel reflectivity calculated for an ideal, smooth and flat interface [64]. Deviations of the measured reflectivity, $R(Q_z)$, from the Fresnel reflectivity, $R_f(Q_z)$, reveal the presence of interfacial structure as a function of surface depth. In this case, the structure is due to the lipid monolayer supported on the buffer surface and the PKCα-C2 domains bound to the lipid monolayer. No radiation damage was detected during the measurements, as indicated by the surface pressure stability and the reproducibility of the x-ray reflectivity data after repeated measurements on the same sample (data not shown) [25].

3.3 Data Analysis

X-ray reflectivity measurements as a function of the reflection angle are usually analyzed by (a) assuming a model (a functional form) for the electron density as a function of depth $z$ into the surface, but averaged over the in-plane $x$-$y$ direction (the so-called electron density profile), (b) computing the reflectivity from this model, and (c) comparing the computed reflectivity to the measured reflectivity by the use of a non-linear least squares fitting procedure that adjusts parameters in the model to yield a best fit to the data [19, 64, 65].

We model the electron density profile of the lipid monolayer as consisting of two slabs of uniform electron density that correspond to the lipid tailgroups and headgroups [64]. In studies of a monolayer plus protein system, other authors have described the protein as an additional slab
of uniform electron density [23, 24, 60, 66-69]. However, a protein like PKCα-C2 has a robust structure with a well-defined arrangement of atoms and, therefore, a specific electron density profile for a given orientation. Describing this protein electron density profile as a single slab of uniform electron density results in a loss of information when reflectivity is analyzed.

Previously, we introduced the use of the protein structure, taken from crystallography or NMR studies of protein domains, into the analysis of x-ray reflectivity from cPLA₂α-C2 and p40phox-PX domains adsorbed onto Langmuir monolayers of lipids (see Figure 5 and Figure 6)[25, 26].

The purpose of our analysis is to determine the orientation of the protein with respect to the plane of the lipid layer, the penetration depth of the protein into the lipid layer, the fraction of interface covered by the protein, as well as to characterize the thickness and electron density of the lipid tailgroup and headgroup (L_{tail}, ρ_{tail}, L_{head} and ρ_{head}, respectively) in Figure 4. A PKCα-C2 domain at a particular orientation has penetrated partially into the headgroup region of the lipid monolayer, but not into the tailgroup region (see in Figure 5). It also illustrates N layers, each of uniform electron density in the x-y plane that is used to describe the electron density profile of the interface. The first layer is used to model the electron density of the tailgroup with two fitting parameters – its average electron density ρ_{tail} and thickness L_{tail}. The tailgroup is located between the interfacial positions z = 0 and z = -L_{tail}. The second layer models the top part of the headgroup that is not penetrated by the protein. Subsequent layers that extend down to the position of the headgroup/buffer interface (at z = -L_{tail}-L_{head}) model a region of the interface occupied by both the top part of the protein and lipid headgroups. The remaining layers model a region occupied partially by the bottom part of the protein and partially by the aqueous buffer, though we do not exclude the possibility that the protein can fully cover the interface. Our model
also allows for the possibility that the protein domain penetrates into the tailgroup region or does not penetrate the lipid monolayer at all.

The protein is characterized by using coordinates from the PDB file [40] of the PKCα-C2 domain (1DSY) plus additional residues from the purification protocol. The software Molprobity was used to restore the hydrogen atoms onto the protein [28]. We chose three atoms in the protein to define a protein coordinate system, then rotated the protein with respect to a coordinate system defined by the lipid layer (equivalently, the buffer/air surface). The surface coordinate system \((x, y, z)\), is defined by a \(z\)-axis that is normal to the average plane of the surface (or, equivalently, to the average plane of the lipid layer) given by the \(x-y\) plane. The primed coordinate system \((x', y', z')\) is fixed to the protein and is used to describe rotations of the protein with respect to the surface coordinate system \((x, y, z)\). In the initial orientation of the protein the two coordinate systems \((x, y, z)\) and \((x', y', z')\) are coincident (Figure 10). The \((x', y', z')\) coordinate system is specified by locating three reference atoms \(A\), \(B\), and \(C\), at positions \([0,0,0]\), \([0,0,B_z]\) and \([C_x,0,C_z]\) (see Fig. 10), which correspond to physical locations \([0,0,0]\), \([0,0,20.992 \text{ Å}]\), and \([8.292 \text{ Å}, 0, 7.816 \text{ Å}]\). The reference atoms \(A\), \(B\), and \(C\), are chosen to be the \(\alpha\)-carbons of residues Leu173, Ala180 and Ala271, respectively.
To account for the fact that part of the protein is located in the buffer and part in the lipid layer, a confining mathematical box is drawn around the protein. In practice, two boxes are used. In one the empty space in the box is filled with aqueous buffer by assigning to that volume the electron density of the buffer, in the other it is left empty. Part of the box with buffer is used to describe the corresponding part of the protein that is in the buffer below the lipid layer. The remainder of the protein that inserts into the lipid layer is described by the corresponding part of the box whose empty space was left empty; our analysis method fills this empty space by electron density from the lipid layer illustrated in Figure 6.
For a given orientation, the electron density profile of the box with protein is calculated by slicing the box into thin layers along the surface normal (in Figure 6), then counting the number of electrons in each layer and dividing by the layer volume. These profiles are produced for a complete range of protein orientations. The protein profile for a given orientation is combined with a 2-slab model of the lipid monolayer in a non-linear least squares fitting to the x-ray reflectivity data in which six parameters are fit ($L_{\text{tail}}$, $\rho_{\text{tail}}$, $L_{\text{head}}$, and $\rho_{\text{head}}$, the distance $PEN$ that the protein penetrates into the lipid layer (in Figure 5), and the coverage $COV$, i.e., the fraction of surface covered by the protein-filled boxes). This procedure yields a goodness of fit parameter $\chi^2$ for each protein orientation. Comparison of these $\chi^2$ values determines the best-fit orientation and the accompanying best-fit values for the six fitting parameters.

X-ray reflectivity data were fit to the entire range of orientational angles of the PKCa-C2 domain. The angle $\theta$ measures the angle between the protein’s $z'$-axis and the surface normal $z$-axis, whereas the angle $\phi$ is an azimuthal rotation about the direction of the $z'$-axis (in Figure 7). Initially, fitting was carried out for values of $\theta$ spaced by 10° over the range from 0 to $\pi$ and for values of $\phi$ spaced by 30° over the range from 0 to 2$\pi$. This procedure determined the approximate location of the best-fit orientations. Then, a finer 1° spacing of $\theta$ and $\phi$ values was used to locate the best-fit orientations precisely. Contour plots of the goodness of fit parameter $\chi^2$ as a function of $\theta$ and $\phi$, similar to that shown in Figure 11 (discussed below), were produced for the three data sets.
Figure 11: Contour plot of the goodness of fit parameter $\chi^2$ (by color) of fits to the x-ray reflectivity data for the PKCa-C2 domain oriented at angles $\theta$ and $\phi$. This plot was produced by averaging the $\chi^2$ contour plots from measurements of three samples. The best fits had $\chi^2 = 5.9$ as determined by our use of counting statistics to calculate error bars on individual data points, which must have underestimated the error bars since the best fits are excellent even though $\chi^2 > 1$. The two best-fit orientations are indicated by the position of the numbers “1” ($\theta = 35^\circ$ and $\phi = 210^\circ$) and “2” ($\theta = 35^\circ$ and $\phi = 0^\circ$). The position of the numbers “3” ($\theta = 68^\circ$ and $\phi = 300^\circ$) and “4” ($\theta = 90^\circ$ and $\phi = 300^\circ$) indicate models proposed in the literature Malmberg et al. (2005) [57] and Verdaguer et al. (1999)[40], respectively. The four lowest bands of $\chi^2$ (see legend) correspond to deviations of one to four standard deviations (SD) from the best fits. For example, the literature conformation whose angles are indicated by position 3($\theta = 68^\circ$ and $\phi = 300^\circ$) is more than four SDs away from the best fit.
3.4 Results

Analysis of the x-ray reflectivity data was carried out for measurements from three separate samples, which differed slightly in the initial value of the surface pressure of the monolayer and in the change in surface pressure upon adsorption of the protein domain (see Materials and Methods). Figure 10 shows the $\chi^2$ contour plot that results from averaging the $\chi^2$ contour plots from analyses of the three separate measurements. Two best-fit orientations were obtained whose $\chi^2$ values were within one statistical standard deviation (SD) of each other. These orientations are given by $\theta=35^\circ \pm 10^\circ, \phi = 210^\circ \pm 30^\circ$ and $\theta=35^\circ +3^\circ/-8^\circ, \phi = 0^\circ +10^\circ/-5^\circ$. The fitting parameters for each of these two orientations are listed in Table 1, where the uncertainties were computed by the appropriate mapping of $\chi^2$-space [70]. Fits to the reflectivity data for the two best-fit orientations for one of the samples are shown in Figure 13, which demonstrates that they are nearly identical. This is a consequence of the fact that the electron density profiles for the two best-fit orientations, one of which is shown in the inset to Figure 12, are essentially identical.
Representations of the two best-fit monolayer-bound structures of the PKC-α-C2 domain are shown in Figure 13. In the $\theta = 35^\circ$, $\phi = 0^\circ$ orientation (Figure 13A), the residues Asp187, Pro188, Asn189 and Gly190 in CBL1 penetrate into the lipid headgroup. The residue Pro188 penetrates most deeply into the lipid monolayer. The location of polar and charged residues of the PKC-α-C2 domain with respect to polar or charged regions of the lipids can be judged approximately by assuming an average orientation of the lipids in the monolayer. This orientation is given by $x$-

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
 & $\theta = 35^\circ \pm 10^\circ$ & $\theta = 35^\circ (+3^\circ / -8^\circ)$ \\
 & $\phi = 210^\circ \pm 30^\circ$ & $\phi = 0^\circ (+10^\circ / -5^\circ)$ \\
\hline
Protein displacement & $-13.6 \pm 1.7$ & $-15.9 \pm 1.0$ \\
\hline
Coverage (COV) & 0.44 $\pm 0.04$ & 0.46 $\pm 0.04$ \\
\hline
$L_{\text{tail}}$ (Å) & 10.7 $\pm 0.1$ & 10.8 $\pm 0.1$ \\
\hline
$L_{\text{head}}$ (Å) & 10.4 $\pm 0.4$ & 10.9 $\pm 0.4$ \\
\hline
$\rho_{\text{tail}}$ (electrons/Å$^3$) & 0.21 $\pm 0.01$ & 0.23 $\pm 0.01$ \\
\hline
$\rho_{\text{head}}$ (electrons/Å$^3$) & 0.44 $\pm 0.01$ & 0.45 $\pm 0.01$ \\
\hline
Penetration (PEN) (Å) & 7.5 $\pm 2$ & 5.8 $\pm 1.5$ \\
\hline
Roughness σ (Å) & 3.38 & 3.38 \\
\hline
$A_{\text{box}}$ (Å$^2$) & 1663 & 1815 \\
\hline
$A_p$ (Å$^2$) & 3800 $\pm 350$ & 4000 $\pm 350$ \\
\hline
\end{tabular}
\caption{Parameters that describe the adsorption of PKC-α-C2 domain to a mixed lipid layer of SOPC and SOPS for the two best-fit orientations determined from x-ray reflectivity measurements. The parameters PEN, COV, $L_{\text{tail}}$, $L_{\text{head}}$, $\rho_{\text{tail}}$, and $\rho_{\text{head}}$, were fit.}
\end{table}
ray and neutron diffraction studies of multi-lamellar DOPC bilayers [71, 72] and is assumed to be similar to the average orientation of the SOPC and SOPS lipids that are used in this study. This orientation places the lipid phosphate groups approximately 5 Å [71-73] above the lipid/buffer interface, as indicated by the red dashed line Figure 13. Under the assumption that the region of the protein that penetrates the lipid layer does not change its conformation from that given by crystallography, Figure 13A shows for example that the polar residue Asn189 is close to the phosphate plane. Similarly the residues Thr250 and Thr251 are close to the lipid/buffer interface. Also, the Ca$^{2+}$ ion Ca2 is very close to the lipid-buffer interface, placing it near the negatively charged COO$^-$ of the lipid. The Ca$^{2+}$ ion Ca1 is further away by several angstroms. The approximate relative locations that we have identified allow for hydrogen bonding and favorable electrostatic interactions between the C2 domain and the SOPS headgroups.
Figure 12: Fitting plot of C2 X-ray reflectivity normalized to the Fresnel reflectivity from one sample. Lines represent fits to the two best-fit orientations, \((\theta = 35^\circ, \phi = 210^\circ)\) and \((\theta = 35^\circ, \phi = 0^\circ)\), where the latter was displaced by +0.5 for clarity. Inset shows the electron density profile as a function of interfacial depth for the \(\theta = 35^\circ, \phi = 210^\circ\) orientation. The dashed line represents the intrinsic electron density profile (with zero interfacial roughness) for this orientation in order to illustrate the underlying features of the model.
Figure 13: Two best fit orientations. Backbone representation of the two best-fit configurations. The dashed line indicates the average level of the lipid phosphates that lies close to the mid-plane of the lipid headgroup. (A) $\theta = 35^\circ$, $\phi = 210^\circ$ orientation with a protein penetration of $7.5 \pm 2 \, \text{Å}$, and (B) $\theta = 35^\circ$, $\phi = 0^\circ$ orientation with a protein penetration of $5.8 \pm 1.5 \, \text{Å}$. Calcium binding loops CBL1 consists of residues 187 – 193, CBL2 consists of 216 – 219, and CBL3 consists of 245 – 254. In configuration (A) CBL1 and CBL2 penetrate the lipid layer, but in configuration (B) only CBL1 penetrates the layer. Residues R252, F255, and E282 are involved in inter-domain interactions with C1A domain and labeled by blue color. The residues K197, K199, K209, and K211, colored in red, are a lysine-rich cluster. Residue K205 colored in purple. The green spheres indicate the $\text{Ca}^{2+}$ ions.
In the $\theta=35^\circ$, $\phi=210^\circ$ orientation (Figure 13A), the residues that penetrate into the lipid headgroup region are Asp187, Pro188, Asn189, Gly190 and Leu191 in CBL1 and Arg216, Ser217, Thr218 and Leu219 in CBL2. The deepest penetrating residue is Asn189 from CBL1. Again, we identify approximate locations of atoms that may lead to favorable interactions such as H-bonding or electrostatic interaction. Residues Asn189 and Ser217 lie close to the phosphate plane. Even the $\eta$-nitrogen atoms in Arg216 and Arg249 might reach to the lipid phosphate although the alpha-carbon of these residues appears to be at the lipid-buffer interface. The polar Thr218 is located between the lipid phosphate and the lipid/buffer plane. The calcium ions Ca1 and Ca2 are located slightly closer to the negatively charged respectively COO$^-$ of the seryl group near the lipid-buffer interface than in the conformation shown in Figure 13A.

The structures in Figure 13 and the description of the relative positions of the lipids and residues in the PKC$\alpha$-C2 domain indicate that these two monolayer-bound structures interact differently with the lipid layer. These differences provide a qualitative basis to suggest that the PKC$\alpha$-C2 domain in the $\theta=35^\circ$, $\phi=210^\circ$ orientation is likely to have a more favorable interaction with the lipids because of the greater number of possibilities for hydrogen bonding and attractive electrostatic interactions. In support of this, the area of the docking surface for the $\theta = 35^\circ$, $\phi = 210^\circ$ orientation (600 Å$^2$) is almost twice as large as that for the $\theta = 35^\circ$, $\phi = 0^\circ$ orientation (340 Å$^2$), which also indicates a greater potential to interact with the lipids. On the basis of these considerations, and biochemical mutational studies described below in the Discussion section, we have chosen the $\theta = 35^\circ$, $\phi = 210^\circ$ orientation to describe the lipid-bound structure of the PKC$\alpha$-C2 domain. In this orientation, the three CBLs dominate the membrane docking surface and among them CBL1 penetrates most deeply into the lipid monolayer.
3.5 Discussion

3.5.1 Comparison to Other Results

In the proposed orientation ($\theta=35^\circ$, $\phi=210^\circ$) the angle $\theta$ between the $\beta_2$-strand vector and the membrane normal is $35^\circ$, which indicates that the monolayer-bound PKC$\alpha$-C2 domain is oriented essentially perpendicular to the lipid layer as illustrated in Figure 13. This orientation is inconsistent with the parallel orientation model proposed by crystallography and EPR measurements of PKC$\alpha$-C2 domain binding to PC/PS membranes (Figure 11) [40, 56, 57]. X-ray crystallography studies of the PKC$\alpha$-C2 domain bound to a short-chain PS, DCPS suggested that only the central part of CBL3, i.e., the side chains from residues Trp247 and Arg249, is inserted into the lipid bilayer [40]. This study also suggested that the $\beta_3$-$\beta_4$ connection of the PKC$\alpha$-C2 domain, especially the residue Lys205, approaches the membrane surface, but does not penetrate. Based on these crystallography results, Verdaguer and coauthors proposed the parallel model in the docking of PKC$\alpha$-C2/Ca$^{2+}$/DCPS complex [40]. EPR studies [56, 57] determined that the polar and charged Asn189, Arg249 and Arg252 side chains interact with polar and anionic groups inside the headgroup layer, but CBL2 does not penetrate the membrane. Results of the EPR study were used to suggest that the PKC$\alpha$-C2 domain lies nearly parallel to the lipid layer with the longest $\beta_2$-strand tilted at $68^\circ\pm7^\circ$ from the membrane normal [57].

As discussed, our analysis of the x-ray reflectivity allows us to consider all orientations of the PKC$\alpha$-C2 domain and, in particular, allows us to test if the orientations of the PKC$\alpha$-C2 domain proposed by other authors would be compatible with our x-ray reflectivity data. In our notation, the orientation of the model the orientation of the model proposed from x-ray crystallography is $\theta=90^\circ$, $\phi=300^\circ$ and the orientation proposed from the EPR measurement [57] is $\theta=68^\circ$, $\phi=300^\circ$. As shown in Figure 11, these two orientations fit our X-ray reflectivity
data unacceptably poorly, with their values greater than four standard deviations away from our best fits. In general, any approximately parallel orientation of the PKCα-C2 domain cannot provide an adequate fit to our data.

Although our proposed orientation for PKCα-C2 docking disagrees with that proposed from the x-ray crystallography and EPR studies, a number of important observations from the crystallography and EPR measurements agree with our proposed orientation. For example, the x-ray crystallography study observed that Asn189 and Arg216 residues interact with the lipid headgroup [40], which is consistent with our measurements. EPR measurements indicated that the polar and charged side chains of Asn189 and Arg249, respectively, interact with polar and anionic groups inside the headgroup [56], also consistent with our measurements. Furthermore, our proposed docking model ($\theta=35^\circ$, $\phi=210^\circ$, PEN = 7.5 Å) is supported by many mutational studies. In our model CBL1 penetrates deeply into the lipid headgroup, whereas CBL3 is positioned immediately adjacent to the lipid headgroups, but on the buffer side, as opposed to the parallel models for which the inverse is true. Our result is in agreement with a previous report showing that mutations of CBL1 ligands that coordinate to Ca1 had a more significant effect on vesicle binding than did mutations of CBL3 ligands that coordinate to Ca2 [46]. Our result that CBL2 also penetrates the lipid headgroup, though to a lesser extent than CBL1, is consistent with the mutational study by Conesa-Zamora and co-workers that observed that R216A of CBL2 affected both membrane binding and enzyme activation [58]. CBL2 is not expected to interact with the membrane in the parallel models. Conesa-Zamora et al. ’s results that R249A and T251A of CBL3 affected both membrane binding and enzyme activation [58] are also consistent with our results because these residues are located immediately adjacent to the lipid layer in our model. An important distinction between our model and the parallel models is the role of
Lys205. In our model, the Lys205 located in β3-β4 is far from the lipid monolayer and, therefore, is not important for the Ca\(^{2+}\)/PS-dependent binding. This arrangement is consistent with recent EPR [57] and mutagenesis [58] studies. For example, Conesa-Zamora et al. concluded that Lys205 is essential neither for \textit{in vitro} cellular membrane interaction of PKC\(\alpha\) nor for PS-dependent enzyme activation [58].

It is sensible to expect that polar and charged residues that penetrate into the region of the lipid headgroup, and are therefore in direct contact with the headgroup, will have a stronger interaction with the membrane than those in the aqueous phase even if they are close to the lipid-water interface. In the \(\theta=35^\circ, \phi=210^\circ\) orientation, Asn189 in CBL1 penetrates into the lipid layer, indicating that it is likely to play some role in either the binding or the activation of the protein. In fact, Conesa-Zamora et al. found that N189A mutant inhibited enzyme activity of PKC\(\alpha\)[58]. Bolsover and co-authors showed that mutations of the side chains of Asn189 and Arg216 to Ala led to a weaker membrane association of the protein than did the same substitutions in Arg249 and Thr251[48]. An independent mutational study also showed that Asn189 plays a critical role in PS selectivity of PKC\(\alpha\)[49]. In addition, Arg216, Arg249 and Thr251 are also involved in PS binding [49, 58]. Later studies indicated that the mutation of Arg249 to Ala had no significant effect on the monolayer penetration [37]. These results are all consistent with our bound structure shown in figure 13A, which places Asn189 and Arg216 within the headgroup region and Arg249 and Thr251 just outside. The importance of Arg216 provides further support for our choice of the bound structure shown in figure 13A over that in figure 13B because Arg216, along with the rest of CBL2, does not penetrate the lipid layer in configuration B.
3.5.2 **Enzyme Activation**

PKCα contains three lipid binding domains, C1A and C1B in addition to C2. A recent computational docking study of C1A and C2 interdomain interactions revealed a highly complementary interface that consists of Asp55-Arg252 and Arg42-Glu282 ion pairs and a Phe72-Phe255 aromatic pair [37] (where the first residue of each pair is in C1A and the second is in C2). Mutation of Arg252, Phe255, and Glu282 to Ala suggested that these residues do not bind directly to the membrane, although they are involved in the interdomain interaction [37]. As seen in figure 13, the backbone positions of Arg252, Phe255, and Glu282 (labeled in blue) do not penetrate into the lipid layer and are in a favorable position to interact with the C1A domain. As suggested by Stahelin et al. this juxtaposing of the C1A domain with the membrane by the C2 domain should allow for further interaction of the C1A domain with the membrane and subsequent enzyme activation [37]. Such arrangement is not easily achieved with parallel models.

A lysine-rich cluster, consisting of Lys197, Lys199, Lys209 and Lys211 located in the area formed by the β3-β4 strands, has been recognized as a non-specific binding site for phosphoinositides including PIP2 [53, 74-76]. Figure 6 shows that this lysine-rich cluster is located on the periphery of the PKCα-C2 domain slightly more than halfway down its side. If the C2 domain would bind to PIP2 in the membrane, then it is expected that the angle $\theta$ would change from 35° (without PIP2) to approximately 50° [51, 77] to yield a tilted orientation. The importance of this rotation is the possibility that it will push the C1A domain further into the lipid layer, allow C1A domain binding to DAG in the membrane, and lead to enzyme activation. In the $\theta=35^\circ$, $\phi=210^\circ$ orientation, the C1A-C2 complementary pair that will interact first with the membrane as a result of the C2 rotation is likely to be Asp55-Arg252 because it is located closest
to the lipids. Interaction of the Asp55 and/or Arg252 with the membrane may lead to the untethering of the C1A domain and consequent enzyme activation. This is consistent with an earlier computational and mutational study [37, 53]. Direct evidence for such rotation could be provided by future x-ray reflectivity studies in which both PIP2 and PS are incorporated into the lipid layer.

3.6 Summary

We have used x-ray reflectivity to determine the configuration of PKCα-C2 domains bound to a mixed monolayer of SOPC and SOPS lipids. A modification of our recently introduced methodology for analysis of x-ray reflectivity, that incorporates information from crystallographic studies, allowed us to consider efficiently all orientations of the protein domain in the analysis of the reflectivity data. This analysis led to two different bound structures illustrated in Figure 13. Both are oriented nearly perpendicular to the lipid layer and penetrate partially into the lipid headgroup. Although these two configurations cannot be distinguished by X-ray reflectivity, qualitative consideration of the number and type of likely favorable interactions between the protein and the lipid headgroup led us to propose that the configuration in figure 13A is the better representation of the two. Configuration A is consistent with many details of earlier EPR, crystallographic, and mutational studies, albeit in disagreement with the parallel bound orientation proposed from earlier studies. This configuration also exposes a lysine-rich cluster and other residues in a favorable location for further interactions with membrane PIP2 and the C1A domain, which would lead to tighter membrane binding and activation of PKCα.
CHAPTER 4: Membrane-Bound Configuration of KIF16B-PX domain
4.1 Introduction

Three large superfamilies, kinesins, dyneins and myosins, have been identified to be involved in intracellular transport[78, 79]. Among these, the kinesin superfamily proteins (KIF) use the chemical energy of ATP to transport organelles and vesicles along microtubule rail networks within cells [80]. KIF16B is a motor protein in the kinesin-3 family with an amino-terminal motor domain that transports a cargo toward the plus end of the microtubule. The C-terminal PX binding domain of KIF16B was shown to bind to phosphatidylinositol-3-phosphate (PtdIns(3)P)-enriched early endosomes (see figure 14)[81]. Spatial localization of KIF16B to the appropriate intracellular membrane compartment is essential for its biological functionality. We assume that it is biologically important for the KIF16B PX binding domain to bind tightly to early endosomes so they are not lost during transport along microtubules. Questions arise as to how KIF16B recognizes, binds, and unloads its cargo throughout the transport. Characterization of the membrane-bound configuration is required in order to answer those questions and fully understand the transport mechanism of KIF16B, but this structural information is still not available.

Figure 14: Illustration of full length of KIF16B. The motor domain is at the N-terminal of the protein. Thus this protein walks along from minus end to the plus end along the microtubule. Binding to PtdIns(3)P-enriched membrane organelles is through its C-terminal PX domain.
The Phox homology (PX) domain is a membrane-targeting domain that recruits peripheral proteins to membranes in different intracellular compartments. The PX domain is composed of ~130 amino acids and was first identified in two cytosolic components, p47\textsuperscript{phox} and p40\textsuperscript{phox}, of NADPH oxidase\[82\]. Since then, the PX domain has been found in 15 yeast protein and 47 mammalian proteins, which have diverse functions in different cell types\[83\]. In addition, most PX domains are found in sorting nexin (SNX) proteins that participate in membrane trafficking. Many PX domains, including those from Vamp7\[84\], p40\textsuperscript{phox}\[85\], sorting nexin 3 (SNX3)\[86\] and KIF16B\[81\], have been determined to specifically bind PtdIns(3)P. On the contrary, the PX domain of cytokine-independent survival kinase (CISK) preferentially binds to PtdIns(3,5)P\textsubscript{2} and PtdIns(3,4,5)P\textsubscript{3}\[87\], whereas that of p47\textsuperscript{phox} binds to PtdIns(3,4)P\textsubscript{2}\[88\]. PX domains have been shown to exhibit similar structural folds consisting of the N-terminal three-stranded \(\beta\)-sheet on one side of the module and three to four \(\alpha\)-helices on the other side. Furthermore, the binding mechanism of PX domains for KIF16B and p40\textsuperscript{phox} has been revealed to use two highly conserved basic motifs of PX domain, RR(F/Y)S (basic motif I) and (R/M)R(S/I) (basic motif II), to specifically target the inositol headgroup of PtdIns(3)P via electrostatic interactions. The initial targeting is followed by insertion of hydrophobic residues at the membrane binding loops \[12, 26, 89\]. The similarity of the secondary structures and the membrane binding mechanism of KIF16B-PX and p40\textsuperscript{phox}-PX, as well as the availability of the membrane-bound configuration of p40\textsuperscript{phox}-PX \[26\], has led to the suggestion that the membrane-bound configuration of KIF16B-PX is similar to that of p40\textsuperscript{phox}-PX\[26, 89\]. In this chapter we show that the membrane-bound configuration of these two binding domains is very different and provide evidence that subtle structural variations lead to this difference.
Since functions and regulations of membrane-bound proteins is highly correlated to their interactions with membranes, techniques such as EPR, NMR, X-ray crystallography, X-ray reflectivity measurements and mutagenesis have been implemented to determine and characterize the geometry of docking configurations, especially in their orientation and penetration. EPR measurements on a spin-labeled C2 domain of Protein Kinase Cα (PKCα) determined quantitatively the orientation and penetration, though extensive labeling of the protein was required [56, 57]. Brunecky et al. utilized NMR spectroscopy to investigate the binding geometry of PtdIns(3)P-bound FYVE domain of early endosome antigen 1 (EEA1) [90]. A membrane-bound model was proposed by crystallographic study of PKCα-C2 complexed with a short-chain PS, 1,2-dicaproyl-sn-phosphatidylserine (DCPS) and Ca^{2+}[40]. A mutational study of the PX domains of NADPH Oxidase p40^{phox} and p47^{phox} studied the binding mechanism and further proposed their membrane binding modes. The membrane-bound configurations of CPLA2-C2 domain and PKCα-C2 were quantitatively determined by X-ray reflectivity measurements [25, 91]. Some limitations about these techniques to characterize membrane-bound configurations need to be noticed. NMR and X-ray crystallography studies of these proteins do not have a lipid monolayer or a bilayer in the system. It is required intensively labeling in the lipid binding pocket to precisely interpret the membrane binding configuration in EPR measurements. It is very difficult for mutational studies to identify and decide the membrane bound configurations since many possible configurations satisfy and explain mutational results. Among these, X-ray reflectivity is an excellent tool to study the membrane-bound configuration of proteins with a lipid monolayer since no labeling is needed for the proteins. X-ray reflectivity measurements quantitatively determined membrane-bound protein
configuration and provide the information at the residue scale on lipid-protein and protein-protein interactions.

For peripheral membrane proteins that penetrate only partially into the biomembrane, a lipid monolayer at the water/air interface provides an excellent model for studying lipid-protein and protein-protein interactions [92]. X-ray reflectivity measurements on proteins bound to a lipid monolayer at the aqueous/vapor interface determine electron density profiles along the surface normal and do not require proteins or lipids to be labeled or crystallized. This technique had been used to study peripheral proteins interacting with a lipid monolayer at the water/air interface[25, 26, 91]. Here, we report the quantitative determination of the membrane-bound configuration for KIF16B-PX, including its penetration and orientation, at the liquid/vapor interface by X-ray reflectivity measurements. Methods for analyzing this type of data have been described in detail elsewhere[35, 91]. In this study, we demonstrate that the KIF16B-PX domain penetrates deeply into the lipid layer. Our best fit orientation reveals a positive-charge rich layer (PCRL) that plays a critical role for PtdIns(3)P stereospecific recognition and binding. We discuss the consequences of the membrane-bound orientation and penetration of KIF16B-PX for its targeting to PtdIns(3)P and its interaction with other proteins. Our results, when combined with those from a previous cell study [93], suggest a model of a spatiotemporal transport mechanism of intracellular organelles by KIF16B. Finally, comparison of the bound configuration of KIF16B-PX to that of p40phox-PX[26] suggests a correlation between their bound configuration and their biological functions.
4.2 Material and Methods

Materials—KCl and HEPES were purchased from Fisher Scientific and KCl were roasted before use. Stock solutions of SOPC and SOPS in chloroform and PtdIns(3)P in powder form were ordered from Avanti Polar Lipids and Cayman Chemical, respectively.

cDNA cloning and vector construction—The full length gene was prepared as described previously. The PX domain (from residues 1179 to 1312) was subcloned as a BamHI and EcoRI fragment into pGEX4T-1 vector (Novagen). The plasmid was sequenced afterwards for verification.

Protein expression and purification—The PX domains containing an N-terminal GST tag were overexpressed in BL21 (DE3) pLysS cell (Novagen). Cells were grown in Luria broth at 37 °C until an absorbance of 0.6 at 600nm was reached. The expression was then induced by 0.2 mM isopropyl β-D-galactopyranoside (IPTG) and underwent 6 hours shaking at 25 °C. The cells were pelleted at 2500rpm for 10 min. To purify the GST-tagged KIF16B-PX domain, the cell pellet was re-suspended in 20 mL of 10 mM HEPES buffer, pH 7.4, containing 0.16 M KCl, 50 µM phenylmethylsulfonylfluoride, and 1 mM dithiothreitol. After sonication, the supernatant was collected by a 30 min centrifugation at 39,000 rpm at 4 °C and was incubated with 500 µl of glutathione S-transferase tagTM resin (Novagen, Madison, WI) for 1 hour. The resin was washed with 10 mM HEPES, pH 7.4, containing 0.16 M KCl and re-suspended in 1 ml of 10 mM HEPES buffer, pH 8.4, containing 160 mM KCl, 25 mM CaCl₂, and 1 µl thrombin. After 12 hours incubation, the protein was eluted with 10 mM HEPES buffer, pH 7.4 and was then purified to homogeneity by a S-Sepharose column. The purified protein was concentrated in 10 mM HEPES, pH 7.4, containing 0.16 M KCl. Protein concentration was determined by the bicinchoninic acid method (Pierce).
Brewster angle microscope—A lipid monolayer containing SOPC/SOPS/PtdIns(3)P (63:27:10) was spread onto ~40 ml subphase (0.16M KCl and 10mM HEPES buffer, pH 7.3) until a surface pressure of ~25 mN/m was reached. Once the pressure stabilized, 300µg of KIF16B-PX domain was injected into the subphase. After injection of the protein, 45 minutes were allowed for the surface pressure to stabilize while the subphase was stirred at 60rpm with a Teflon stir bar. The change of surface pressure upon the binding of the protein to the monolayer was 5 ± 0.5 mN/m. An in-house Brewster angle microscope (BAM) was used to verify the absence of domains greater than 5 µm in size for the SOPC/SOPS/PtdIns(3)P monolayer and the SOPC/SOPS/PtdIns(3)P/KIF16B-PX domain system.

Sample preparation and surface pressure measurements—To prepare a mixed lipid solution, 100 µg powder of PtdIns(3)P was dissolved in 200 µl of a mixture of chloroform : methanol : water (20:20:3) and the SOPC and SOPS from stock solutions were further diluted and dissolved in a mixture of chloroform : methanol (1:1) with 7:3 molar ratio. The 200 µl of PtdIns(3)P solution was injected into the 7:3 mixture of SOPC and SOPS in chloroform and methanol (1:1) to make a mixed lipid solution of SOPC:SOPS:PtdIns(3)P (63:27:10).

A sample for X-ray reflectivity measurements is prepared by adding dropwise ~20 µl of 560 µM SOPC:SOPS:PtdIns(3)P (63:27:10) onto the surface of a 10 mM HEPES buffer (pH 7.3) containing 0.16 M KCl in a 72 mm diameter circular Teflon trough and ~40 mL total volume. The resulting lipid monolayer was equilibrated for 20 minutes while stirring at 60 rpm. After X-ray reflectivity of just the lipid monolayer is measured, 300 µg of KIF16B-PX domain is injected into the subphase, and the system was equilibrated for 1 h with continuous stirring at 60 rpm. The X-ray reflectivity was then measured again. We report on data from two separate
experiments on monolayers with similar initial surface pressure (25.2 mN/m and 24.4 mN/m) and similar changes in pressure upon adding KIF16B-PX ($\Delta \pi = 5.0 \pm 0.5$ mN/m and $5.6 \pm 0.5$ mN/m, respectively).

4.3 Results

Measurements of X-ray reflectivity, $R(Q_z)$, determines the average electron density profile $\langle \rho(z) \rangle$ along the surface normal, defined as the $z$-axis. X-ray reflectivity data from Langmuir monolayers of lipids are typically analyzed by two slabs of uniform electron density that correspond to the lipid tailgroups and headgroups and characterize by four parameters the thickness of the tailgroup region ($L_{\text{tail}}$), the electron density of tailgroup region ($\rho_{\text{tail}}$), the thickness of the headgroup region ($L_{\text{head}}$), and the electron density of the headgroup region ($\rho_{\text{head}}$) [25, 26, 91]. In a real physical system, the step-function electron density profile generated by the previous procedure is smeared at the border of each slab by the surface roughness, $\sigma$, that is due to thermal fluctuations. This produces a smooth electron density profile $\langle \rho(z) \rangle$ that is the electron density profile averaged over the $x$-$y$ plane of the interface, which is measured by X-ray reflectivity.

X-ray reflectivity data for a mixed lipid monolayer of SOPC/SOPS/PtdIns(3) at a surface pressure of 25.2 mN/m are shown in Figure 15. The data are presented as reflectivity, $R(Q_z)$, divided by the Fresnel reflectivity, $R_F(Q_z)$. The latter is calculated for a sharp and flat interface between the bulk aqueous buffer and vapor [25]. The reflectivity is fitted by the Parratt recursive relation with four fitting parameters ($\rho_{\text{head}}, \rho_{\text{tail}}, L_{\text{head}}, L_{\text{tail}}$) using a nonlinear chi-square minimization algorithm[21, 94]. The best-fit values of the four fitting parameters are listed in
Table 2. In the analysis, the interfacial width, $\sigma$, due to thermally excited capillary waves was calculated using capillary wave theory[31, 95].

The methodology for including protein crystallographic information in to the analysis of X-ray reflectivity measurements from proteins adsorbed onto a Langmuir lipid monolayer was previously described[91]. Hydrogen atoms were added by Molprobity software and water molecules were removed from the original PDB file (PDB 2V14). In this study, three reference atoms were chosen as $A = C_\alpha$ of T1209, $B = C_\alpha$ of H1201, and $C = C_\alpha$ of I1188 to construct

![Figure 15: PX data. Normalized X-ray reflectivity and interfacial electron density profile of the pure SOPC/SOPS/PtdIns(3) monolayer colored in red and monolayer-bound KIF16B-PX colored in black. The black line is the best fit for the $\theta = 30^\circ$, $\phi = 20^\circ$ orientation. Inset shows the electron density profiles as a function of interfacial depth for best fits of monolayer (red) and monolayer-protein (black) systems.](image)
coordinate axes \((x',y',z')\) in the protein frame. The original PDB file was translated and rotated to place the reference atoms in the desired positions to define the orientation \(\theta = 0^\circ\) and \(\phi = 0^\circ\), so that the coordinate axes \((x,y,z)\) of the laboratory (monolayer) frame are parallel to the protein frame \((x',y',z')\). Rotation matrices are applied to rotate the protein to given values of the two Euler angles \(\theta\) and \(\phi\), as illustrated in Figure 16. The range of \(\theta\) and \(\phi\) are 0 to \(\pi\) and 0 to \(2\pi\), respectively; however, we are primarily interested in values of \(\theta\) that vary from 0 to \(\pi/2\), which allows the binding pocket of the PX domain to interact with the monolayer.

Figure 16: Starting Orientation of PX. Starting Orientation \(\theta = 0^\circ, \phi = 0^\circ\). Please note the view at different direction in this two coordinates. The selected reference \(C_\alpha\) atoms A, B and C in residues Thr1209, His1201, Ile1188 are colored by red, blue and green spheres, respectively. The atoms A, B, and C are located at \([0, 0, 0]\), \([0, 0, 25.787]\) and \([5.633, 0, 10.333]\), respectively.
X-ray reflectivity data of KIF16B-PX domain adsorbed to the mixed lipid monolayer is shown in Figure 15. For each protein orientation, these data are fit with 6 parameters: protein coverage (COV), protein penetration (PEN) and the four lipid parameters (ρ_{head}, ρ_{tail}, L_{head}, L_{tail}). A contour plot (Figure 17) of the goodness of fit for this analysis shows the global minimum, labeled “n”, for this space of protein orientations. Values of the best-fit parameters are listed in Table 2.

![Contour Plot of PX domain](image)

Figure 17: contour plot of PX domain. Contour Plot of the goodness of fit parameters $\chi^2$ of fits to the x-ray reflectivity data of the KIF16B-PX domain oriented at angles $\theta$ and $\phi$. The orientation “n” ($\theta = 30^\circ$, $\phi = 20^\circ$) fits the reflectivity data of mixed monolayer-protein best with $\chi^2 = 5.13$. The orientation “m” ($\theta = 30^\circ$, $\phi = 220^\circ$) was previously proposed to be the membrane-bound orientation for the KIF16B-PX domain based upon analogy with measurements of the p40^{phox}-PX domain. However, the orientation “m” disagrees with our reflectivity data on KIF16B-PX by more than four standard deviations ($\chi^2 = 11.44$).
The best-fit orientation of the KIF16B-PX domain is characterized by the orientation angles $\theta = 30^\circ \pm 10^\circ$, $\phi = 20^\circ (+15^\circ/ -35^\circ)$ and a penetration of $17\pm 2$ Å into the lipid monolayer with 38% surface coverage. The backbone as well as a molecular surface representation of the $\theta = 30^\circ$ $\phi = 20^\circ$ orientation is shown in Figure 18. In the best-fit configuration, the segments R1190-E1203, K1246-R1260, and R1219-Y1221, defined as lipid binding loops 1, 2, and 3 (BL1, 2, 3), respectively, are found to interact with the lipid monolayer. The loops BL1 and BL2 penetrate deeply into the hydrophobic region, as shown in Figure 18, while BL3 is superficially in contact with the headgroup/buffer interface. The loop BL1 penetrates most deeply into the lipid monolayer with its residues Q1196, G1197, and K1198 in the hydrophobic tail region. The part of the backbone containing F1249, G1250 and K1252 in BL2 are positioned at the tailgroup/headgroup interface, and R1219, R1220 and Y1221 in BL3 are situated at the headgroup/buffer interface. Interestingly, positively charged and polar residues R1190, Y1191,
R1219, R1220, Y1221, K1246, K1247, R1260, R1261 are in an ~8Å thick layer that straddles the headgroup/buffer interface. This layer is referred to as the positive-charge rich layer (PCRL).
Figure 18: Backbone representation of membrane-bound configuration of KIF16B-PX in $\theta = 30^\circ$, $\phi = 20^\circ$, with 17 Å penetration into the mixed lipid monolayer. The polar and hydrophobic residues that penetrate into the monolayer are colored in cyan and violet, respectively. Residues from 1190 to 1203, from 1246 to 1260, and from 1219 to 1221 are defined as lipid binding loops 1, 2, 3 (BL1, BL2, BL3), respectively. Residues in two conserved basic motifs are labeled in red. R1219 and R1220 are in motif I and R1260 and R1261 are in motif II. (B) Molecular surface of lipid binding pocket of KIF16B-PX in $\theta = 30^\circ$, $\phi = 20^\circ$ is illustrated to show how the binding pocket faces and interacts with the lipid monolayer.
In addition to X-ray reflectivity measurements, an accurate and efficient docking program, Autodock Vina\[96\], was used to investigate the docking geometry for the PtdIns(3)P-bound KIF16B-PX domain at the atomic scale. In the docking software, a headgroup of PtdIns(3)P (ligand) and a PX domain of KIF16B (PDB 2V14) as the rigid body (receptor) were input and the grid box for docking was set to a 24×26×28 (Å$^3$) centered at the lipid binding pocket of the PX domain to perform a binding energy calculation and to predict the binding geometry. Of the nine possible docking conformations, the docking geometry that corresponds to the tightest binding is shown in Figure 19 and the binding energy is -6 kcal/mol. Since results from Autodock Vina did not directly provide the protein binding orientation, the geometry of the predicted binding complex was further rotated to align with the binding configuration, $\theta = 30^\circ$, $\phi = 20^\circ$, determined by X-ray reflectivity measurements and is shown in Figure 19. In the predicted conformation, the 3-phosphate (P3) of PtdIns(3)P interacts with the charged pocket formed by R1190,R1220,Y1221,R1260 and R1261 and is locked into this pocket. Figure 19 also illustrates the steric arrangement of the PtdIns(3)P headgroup in the binding pocket and the optimization of electrostatic interactions with the pocket. Furthermore, the tailgroup was added and aligned with the position of the lipid headgroup in the predicted docking conformation. After the addition of the tailgroup, one can see in Figure 19 the result of the hydrophobic attraction between the tailgroup and the hydrophobic protrusion at the top of the protein (i.e., the F1249 and L1248 residues, see Figure 18).
Figure 19: Docking geometry of KIF16B- PX binding to PtdIns(3)P headgroup was predicted by docking software “AutoDock Vina”. The binding energy of this interaction is -6 kcal/mol. The stereospecific recognition of PtdIns(3)P by the lipid binding pocket of PX domain is illustrated. The hydrocarbon tail was added in order to visualize one arrangement of the tailgroup.
4.4 Discussion

Analysis of X-ray reflectivity data demonstrates that the KIF16B-PX binds to SOPC/SOPS/PtdIns(3)P in the orientation given by $\theta = 30^\circ$ and $\phi = 20^\circ$ with a penetration of $17 \pm 2$ Å. The uniqueness of this orientation is demonstrated in Figure 17 by the presence of a single well-defined minimum in the $\chi^2$ contour plot. Assuming that the tertiary structure of the residues of KIF16B-PX in the monolayer-bound configuration is essentially the same as in the crystal structure, the relative positions of residues of KIF16B-PX with respect to the lipid monolayer can be determined, as illustrated in Figure 18A. The molecular surface of the protein is shown in Figure 18B in order to visualize the orientation of the lipid-binding pocket of the PX domain in the monolayer-bound configuration. In the orientation given by $\theta = 30^\circ$ and $\phi = 20^\circ$, the extent of the lipid-binding pocket along the $z$-axis is maximized. In this orientation the lipid-binding pocket of the PX domain is open and accessible to interact with the targeting lipid molecule.

In spite of the large penetration of the KIF16B-PX into the mixed monolayer, the structure within the lipid monolayer regions of the interface is not dramatically altered. The thickness of the lipid headgroup region after binding, $12.8 \pm 0.4$ Å (see Table 2), is just slightly larger than the value before binding ($11.8 \pm 0.4$ Å). The thickness of the tailgroup region is essentially unchanged (Table 2). The tailgroup thickness is consistent with the computational prediction of the docking geometry of the PtdIns(3)P in the binding pocket of KIF16B-PX. The docking geometry shown in Figure 19 was predicted by minimizing the binding energy computed by “Autodock Vina” software [96]. It shows that the orientation of the monolayer-bound KIF16B-PX determined by X-ray reflectivity is qualitatively consistent with the expected orientation of PtdIns(3)P in the lipid monolayer. In addition, the projection of the hydrocarbon tail shown in
Figure 19 along the z-axis is 12 Å, which is in quantitative agreement with the thickness (11.75 ± 0.13 Å) of the lipid tailgroup region determined by X-ray reflectivity (Table 2).

4.4.1 The Docking Configuration Predicted by Autodock Vina and Electrostatic Interactions

The lipid-binding pocket, composed of binding loops BL1, BL2 and BL3, mimics the shape of a clamp and allows KIF16B-PX to clamp its targeting lipid molecule, i.e., PtdIns(3)P (Figure 18). Autodock Vina predicts that the inositol ring is fully extended. Consideration of the residues adjacent to the PtdIns(3)P lipid suggests the importance of favorable electrostatic interaction between the PtdIns(3)P and the lipid binding pocket of PX (Figure 18 and Figure 19). Figure 19 illustrates that Autodock Vina predicts that the headgroup of PtdIns(3)P prefers the side of the pocket formed by BL1 with its 3-phosphate (P3) positioned at the bottom of the binding pocket and 1-phosphate sitting at the top of the binding pocket.

The positively charged residue layer (PCRL) is expected to play an important role in the electrostatic attraction between the inositol ring of PtdIns(3)P and the lipid-binding pocket. Two highly conserved basic motifs, motif I [RR(Y/F)] and motif II [R(R/K)], have been identified in the family of PX domains [83, 97]. In the case of KIF16B-PX, R1219 and R1220 are positively charged residues in motif I [RR(Y/F)] and R1260 and R1261 are the positively charged residues in motif II [R(R/K)]. In the calculated docking geometry, the P3 phosphate of PtdIns(3)P is positioned adjacent to the molecular surface of these two conserved basic motifs [RR(Y/F)] and [R(R/K)]. More specifically, the P3 phosphate of PtdIns(3)P is closer to motif I [RR(Y/F)] in BL1 than to motif II [R(R/K)] in BL2. This finding supports the notion that motif I plays a more critical role than the conserved motif II in interactions with phosphoinositides (PIs) [98].
position of the inositol ring proximity to the side of BL1 could be attributed to stronger
electrostatics repulsion by D1253, E1254 and E1259 from BL2 than the repulsion generated by
D1199 and E1203 in BL1.

4.4.2 **Hydrophobic Interactions**

In the best-fit configuration illustrated in Figure 18, the hydrophobic region that includes
the F1249 and L1248 residues is located close to the tailgroup/headgroup interface. In this
position, the hydrophobic side chain of F1249 can interact directly with the lipid tailgroups. The
deep penetration of this hydrophobic region is most likely aided by the reduction of the strong
positive electrostatic potential produced by the residues in the positively charged residue layer in
the binding pocket as a result of the binding of the anionic lipid headgroup of PtdIns(3). Figure
19 also shows that the hydrophobic F1249 can interact with the tailgroup of the bound PtdIns(3)
in the best-fit configuration. This result is consistent with observations by biochemical
mutational studies[89]. For example, a SPR binding affinity measurements and monolayer
penetration analyses with the mutation of F1249A show that the membrane binding affinity is
significantly reduced and the penetration activity is weakened [89].

The deep penetration (by 17 ± 2 Å) of KIF16B-PX into the lipid monolayer that is
demonstrated by our X-ray reflectivity measurements is consistent with the high affinity binding
of KIF16B-PX that one imagines is necessary to maintain its hold on large cargos such as early
endosomes during the process of transporting them[89].

4.4.3 **Comparison of KIF16B-PX with p40\textsuperscript{phox} -PX**

The PX domains of KIF16B and p40\textsuperscript{phox} have been identified to specifically interact with
PtdIns(3)P[12, 81]. The PX domains from those proteins are believed to bind via the same
mechanism, i.e., membrane association initiated by electrostatic interactions with the two conserved basic motifs of the PX domains that is then followed by membrane insertion of the surface of hydrophobic residues at the α1-α2 loop [12, 97, 99]. Thus, it might be argued that the membrane-bound configuration of the PX domains of KIF16B and p40phox should be very similar because they share the same binding mechanism and their secondary structures are very similar, as shown later in Figure 21A. However, Figure 20 shows that the membrane-bound configuration of the PX domain of p40phox is very different from that of KIF16B. This configuration was determined previously by Malkova et al. and is re-labeled as $\theta=30^\circ$ and $\phi=220^\circ$, in order to compare it directly to our KIF16B analysis [26]. The position labeled with an “m” in Figure 17 shows that fits of the KIF16B data to this orientation deviated from the best fit by more than four standard deviations and, therefore, the $\theta=30^\circ$ and $\phi=220^\circ$ orientation cannot describe the bound configuration of KIF16B-PX.
The different membrane-bound configurations of PX domains of KIF16B and p40\textsuperscript{phox} could be the result of subtle differences in the shapes of their lipid binding pockets. Panels B and C of Figure 21 illustrate an important difference in the shape of these binding pockets that occurs at the position where the PtdIns(3)P head group is expected to enter the binding pocket in the $\theta = 30^\circ$ and $\phi = 20^\circ$ orientation. The shape illustrated in Panel C of Figure 21 indicates that the binding pocket of p40\textsuperscript{phox}-PX in this orientation will block the entrance of PtdIns(3)P by residues T36, S37, F39 and K98, assuming that the PtdIns(3)P will enter from above as dictated by the position of the lipid monolayer (see Figure 18 or Figure 19). If, however, p40\textsuperscript{phox}-PX is rotated in $\phi$ to the $\theta = 30^\circ$ and $\phi = 220^\circ$ orientation determined previously by Malkova et al. [26], then its binding pocket is sterically accessible to PtdIns(3)P.
As shown in Figure 20, the $\theta=30^\circ$ and $\phi=220^\circ$ orientation of the bound $p40^{phox}$-PX allows residue D76 in its longest $\alpha_2$ helix to interact with the surface of the lipid monolayer. As a result a large fraction of the surface of $p40^{phox}$-PX is involved in membrane binding and the accessible surface area for domain-domain or protein-protein interactions is proportionally reduced. The bound orientation of $\theta=30^\circ$ and $\phi=20^\circ$ of KIF16B-PX allows it to optimize its accessible surface area because only the region near the lipid binding loops interacts with the lipid layer. These differences between the bound configuration of KIF16B-PX and $p40^{phox}$-PX may have implications for their interactions with other proteins and, therefore, for their biological function, as suggested in the next section.
Figure 21: (A) The similarity of secondary structures of KIF16B-PX (green) and p40phox-PX (magenta) (B) The molecular surface of lipid binding pocket of KIF16B-PX in $\theta=30^\circ \phi=20^\circ$ orientation is showed and highlighted by a yellow circle. This binding pocket is sterically accessible to interact with PtdIns(3)P . (C) The molecular surface of lipid binding pocket of p40phox-PX in $\theta=30^\circ \phi=20^\circ$ orientation is showed and highlighted by a yellow circle. This binding pocket is “not” accessible to interact with PtdIns(3)P if the lipid layer is positioned above the PX domain. The p40phox-PX needs to be further rotated by $200^\circ$ in the angle $\phi$ to attain the $\theta=30^\circ \phi=220^\circ$ orientation in order for PtdIns(3)P to be able to enter the binding pocket.
4.4.4 Protein-Protein interactions

It has been discovered that KIF16B binds directly to a GTP-bound active form of Rab14 through amino acids 1068-1323, which contain the PX domain, and that this complex is critical for KIF16B to perform its function in intracellular transport[93]. This suggests that the PX domain of KIF16B acts as a molecular scaffold that interacts with RasGTPases. We suggest the following spatiotemporal model for KIF16B, though we emphasize that further experiments are required to substantiate this model. KIF16B binds PtdIns(3)P enriched cargos (organelles) through its PX domain. The binding of the PX domain of KIF16B to the membrane surface of the organelle enables the GTP-bound Rab14 to target it. The binding of KIF16B -PX with GTP-bound Rab14 initiates the transport of early endosomes along the microtubule. Upon reaching its destination, the cargo is unleashed from the PX and Rab14 complex by switching from the GTP-bound active form to the GDP-bound inactive form of Rab14. Note that a similar trafficking pathway was reported for the Rab protein Rab5 and its effector early endosome antigen 1 (EEA1)[100, 101].

The membrane-bound orientation $\theta = 30^\circ$ and $\phi = 20^\circ$ for the p40phox-PX domain[26] showed that R58 and R60 basic residues directly interact with the inositol ring of PtdIns(3)P headgroup. Therefore, these two residues are not accessible for domain-domain or protein-protein interactions. This observation is in good agreement with the results obtained by a small angle x-ray scattering (SAXS) and cell study, which showed that intramolecular PX–PB1 domain interactions via residues R58 and R60 of the PX domain and E259 and D269 of the PB1 domain prevent the p40phox-PX domain from interacting with PtdIns(3)P and maintain the protein in its inactive state. A disruption of the PX-PB1 interaction in p40phox leads to binding of the PX domain to PtdIns(3)P and activates the PtdIns(3)P-binding switch[102]. In conclusion, the
different biological functions of p40^{phox} and KIF16B are closely related to the membrane-bound configurations of their PX binding domains to the lipid layer.
CITED LITERATURE

APPENDICES
Appendix A

COMPUTATION OF THE ELECTRON DENSITY PROFILES FOR PROTEIN IN TWO BOXES

NAME: edpx.cpp (electron density profile of KIF16B-PX domain)

TYPE: C++

DESCRIPTION:
This program is used to rotate a protein to any desired orientation denoted as $\theta$ and $\phi$, and compute electron density profiles for the protein in each orientation. Before compiling this program, a few things need to be done first. Three reference atoms A, B, and C described in more detail in chapter 2 have to be selected in the input PDB file and input their coordinates into this program. The [x, y, z] coordinates of A, B, and C reference atoms are input as [ax, ay, az], [bx, by, bz] and [cx, cy, cz]. Then, one important parameter “fslice” for determining the slicing size for the electron density profiles of the protein. The parameter “fslice” can be varied to check the convergence of the computed electron density profiles to decide what should be set for the value of the parameter fslice. In general, fslice is set to be 0.2 Å. The smaller the fslice, the longer computing time takes. After inputs of coordinates of A, B and C atoms and the value of fslice, the program can be compiled via the following comments.

```
c++ edpx.cpp -o edpx
```

The executable file of edpx.cpp is named as edpx. In the program, the three reference atoms A, B and C from the input of a PDB file are translated and rotated to the starting orientation ($\theta = 0^\circ$, $\phi =$
Appendix A (Continued)

\(\phi = 0^\circ\). In the starting orientation, A, B, and C atoms are located in \([0, 0, 0]\), \([0, 0, B_z]\) and \([C_x, 0, C_z]\). From the starting orientation, the protein is rotated as a rigid body via \(\theta\) and \(\phi\) angles to any desired orientation. In the program, \(\theta\) and \(\phi\) are denoted as rot1 and rot2, respectively. In each orientation, the protein will be placed in two boxes described in Chapter 2 to generate the electron density profiles for the analysis of X-ray reflectivity data. Two output files are generated by this program. One is the coordinate file of the protein in an orientation \(\theta\) and \(\phi\). The file name associated with this file is t\(\times\times\)p\(\times\times\).pdb. The “t” is the value of theta angle and the “p” is the value of phi angle. The other output file is the electron density profiles for the protein in two boxes with the empty space filled by buffer and left as empty. The file name t\(\times\times\)p\(\times\times\).ed contains these two electron density profiles. An example of this file is shown below. It consists of three columns, \(z_p\) (height in \(z_p\) axis parallel to the normal of the interface), \(\rho_{ep}\) (electron density profile of protein in the box with empty space remaining empty), \(\rho_{bp}\) (electron density profile of protein in the box with empty space filling with buffer).

\[
\begin{array}{ccc}
38.18692944 & 0.00013395 & 0.33480915 \\
37.98112666 & 0.00070406 & 0.33458121 \\
37.77532388 & 0.00157348 & 0.33439273 \\
37.56952110 & 0.00262181 & 0.33445740 \\
37.36371833 & 0.00400295 & 0.33493841 \\
37.15791555 & 0.00564889 & 0.33554502 \\
36.95211277 & 0.00738880 & 0.33622702 \\
36.74630999 & 0.00957392 & 0.33719650 \\
36.54050721 & 0.01171331 & 0.33823159 \\
36.33470443 & 0.01352178 & 0.33918632 \\
36.12890166 & 0.01568911 & 0.34043496 \\
35.92309888 & 0.01803732 & 0.34165103 \\
35.71729610 & 0.01980488 & 0.34262053 \\
35.51149332 & 0.02174489 & 0.34330777 \\
35.30569054 & 0.02325032 & 0.34389451 \\
35.09988776 & 0.02506783 & 0.34425508 \\
\end{array}
\]
Appendix B

NAME: fitpkcm.5.c

TYPE: Cplot fitting function

DESCRIPTION:
The fitting function is utilized to fit the x-ray reflectivity data for the systems of monolayer and monolayer + protein. It requires for input a file of electron density profiles that is generated by using the program in the Appendix A. An output parameter file from this fitting function is shown below.

\[ \chi^2 = 4.491 \]  
\[ \rho(0), R/Rf(1), R(2), Rf(3) = 1 \]  
\[ pp(< 0) = -8.74215 \]  
\[ cov(\text{cov}=0 \text{ lipid only}) = 0.396585 \]  
\[ \text{tail} = 11.7239 \]  
\[ \text{head} = 13.3988 \]  
\[ \rho_{\text{tail}} = 0.19725 \]  
\[ \rho_{\text{head}} = 0.395782 \]  
\[ QC = 0.0218 \]  
\[ \lambda = 1.54 \]  
\[ Q_{\text{rough}} = 3.1 \]  
\[ topphase = 0 \]  
\[ botphase = 0.335 \]  
\[ e \text{ step size} = 0.2 \]

The description of parameters is started from parameter 0 (p0) and listed below.

p0: There are 3 different modes you can choose based on what you want to know about the systems. p0 = 0 provides the relative electron density profile, normalized to the electron density of subphase, of the system. p0 = 1 generates the calculated data of reflectivity divided by Fresnel reflectivity. p0 = 2 generates the calculated reflectivity data. p0 = 3 shows the Fresnel reflectivity.
Appendix B (Continued)

p1: This parameter is one of the fitting parameters. The value of this parameter indicates the position of the topmost of the protein projected in the z direction of the laboratory coordinate. $z = 0$ is located at the air/lipid-headgroup interface (see Figure 5 in the chapter 2). This parameter is one of the parameters associated with protein penetration.

p2: This indicates the coverage of protein at the interface. The area per protein can be calculated via equation (2.8) with the values of this parameter and the box area in the $x$-$y$ plane of protein that is provided by the program in appendix A. This is also one of the fitting parameters for the monolayer-protein system.

p3: This parameter represents the length of the hydrocarbon region of the lipid monolayer. The unit is in Å. This is a fitting parameter for the lipid monolayer.

p4: This parameter represents the length of the lipid headgroup region of the lipid monolayer. The unit is in Å. This is a fitting parameter for the lipid monolayer.

p5: This parameter represents the electron density of the lipid hydrocarbon region of the lipid monolayer. The unit is in $e^-/(Å)^3$. This is a fitting parameter for the lipid monolayer.

p6: This parameter represents the electron density of the lipid headgroup region of the lipid monolayer. The unit is in $e^-/(Å)^3$. This is a fitting parameter for the lipid monolayer.

p7: This parameter is the critical $Q_z$ which is calculated from critical angle on the knowledge of the electron densities of the bulks from the upper and lower phases. In general, this is not a fitting parameter.

p8: It is the wavelength of the x-ray in the measurements and determined by the photon energy of the x-ray. This is not a fitting parameter.
Appendix B (Continued)

p9: It is the interfacial roughness of the system. And it is calculated either from capillary theory (equations 2.2) or including the bending rigidity (equation 2.11) in the calculation of surface roughness. The temperature of the sample and the surface pressure of the interface are measured during the experiments in order to calculate the surface roughness.

p10: It is the electron density of the bulk of the upper phase. In the case of air/water interface, p10 = 0 for the air.

p11: It is the electron density of the bulk of the lower phase. In the case of air/water interface, p11 is the electron density of the buffer.

p12: It is the slicing thickness for the smeared electron density profile along the $z$-axis. This value has to be set smaller than the roughness. This is not a fitting parameter. The smaller the value is, the longer computing time takes.
Appendix C

NAME: fitpkc.5.c

TYPE: Cplot fitting function

DESCRIPTION:

This fitting function is further modified from the fitting function described in appendix B in order to fit all the orientations of the protein automatically. Two input files are required to execute this program correctly. The two input files are created by the program described in appendix D. The output from this program is associated with many parameter files t×××p×××.par which are corresponding to the input files of electron density profiles at orientations.
Appendix D

NAME: gfits.cpp

TYPE: C++ program

COMPILE COMMENT: c++ gfits.cpp -o gfits

DESCRIPTION:

This program generates two files for the fitting function described in appendix C. One is fitin.txt and the other is fitin.do. Two files are shown below.

File of fitin.txt contains names of many .ed files, which are generated by the program in appendix A. This file is one of the required input file for the fitting function in appendix C.

One example of the file of fitin.txt is shown below. Please note the first name of the .ed file has to repeat to ensure the fitting program read the 1st .ed file successfully.

t005p000.edt005p000.edt005p020.edt005p040.edt005p060.edt005p080.edt005p100.edt005p120.
Appendix D (Continued)

One example of the file of fitin.do is also shown below. In this example, the 1st fitting parameter file is saved as t005p000.par which is corresponding to the 1st .ed file in the fitin.do.

```
rp readin.par    (read values of parameters from the file named readin.par)
wt i                  (weighting data with instrument weight)
gd2 refl.dat      (read and get data from the file of refl.dat)
fi                     (fit fitting parameters)
sp t005p000.par   (save fitting results to a file with the name t005p000.par)
rp readin.par
wt i
gd2 refl.dat
fi
sp t005p020.par
rp readin.par
wt i
gd2 refl.dat
fi
sp t005p040.par
rp readin.par
wt i
gd2 refl.dat
fi
sp t005p060.par
rp readin.par
wt i
gd2 refl.dat
fi
sp t005p080.par
```
Appendix E

Preparation of SOPC/SOPS/Pl(4,5)P2 (63:27:10) lipid mixture for PKCα-C2

Concentration of the mixed lipid solution is 563 μM. It requires to spread ~ 20μl of mixed lipid solution with this concentration to reach the surface pressure of 25mN/m for the sample cell of 72 mm diameter cylindrical Teflon trough. Based on the design of the total concentration of the mixed lipid solution, the mixed lipid solution is composed of 56.3 μM PI(4,5)P2, 354.7 μM SOPC and 152 μM SOPS. The procedure of making the mixed lipid solution is described below step by step.

Step 1. Make stock solution of SOPC, SOPS and PI(4,5)P2
(A). Stock solution of SOPC: 25mg of SOPC powder dissolved in 4ml Chloroform. The concentration of SOPC stock solution is 7.93mM.
(B). Stock solution of SOPS: 10mg of SOPS powder dissolved in 3.62ml Chloroform. The concentration of SOPS stock solution is 3.40mM.
(C). Stock solution of PI(4,5)P2:
   I. Make a Chloroform: Methanol: Water (4:4:1) mixed solution. First, have 500μl chloroform in a clean vial. Then, add 125μl water into the same vial. Final step, add 500μl Methanol. The mixed solution is shaken for one minute to ensure the solution well mixed.
   II. Take 300 μl of mixed solution and inject into a vial which contains 100μg PI(4,5)P2 powder.

Step 2. Make Chloroform: Methanol (1:1) solution
Inject 1000μl of Chloroform and 1000μl of Methanol into a clean vial.

Step 3. Take 1261μl of Chloroform: Methanol (1:1) solution and inject it into a clean vial denoted as “finalvial”.

Step 4. Extract 300μl lipid solution which contains 100μg in step 1(C) and inject it into the “finalvial”.
Appendix E (Continued)

Step 5. Take 76μl of the stock solution of SOPC in step 1(A) and inject into the “finalvial”.

Step 6. Take 76μl of the stock solution of SOPS in step 1(B) and inject into the “finalvial”.

Step 7. The total volume of the mixed lipid solution is 1713μl and the concentration of the solution is 563μM.
Appendix F

One example of a macro for X-ray reflectivity measurements at beamline X22B at SNLS at Brookhaven National Laboratory is shown in this appendix. In X22B beamline, the slit in the upstream is usually set to vdiv = 0.14 mr and hdiv = 0.3 mr for the X-ray reflectivity measurements. The slit in the incident arm is set to cvslit = 0.03 mm in vertical and chslit = 1mm in horizontal directions. Two slits, one slit is manual and the other is motorized, are in the output arm. The manual slit which is closed to the sample stage is open 3 mm in the vertical and 4 mm in the horizontal directions. The motorized slit which is right in front of the detector is open 2 mm in the vertical and 2 mm in the horizontal directions.

## This is a macro of reflectivity measurements for lipid-protein system

```plaintext
def protein_1 '{
    local fr, buf, bar4_norm, sh_nom, stth_nom

    shut_off

    bar4_norm = 8.298*8.297*8.37*8.428
    vdiv 0.14
    hdiv 0.3
    cvslit 0 0.03
    chslit 0 1
    dcvslit 0 2
    dchslit 0 2
    DET=det
    MON=monc
    plotselect det
    _sleep=0
    umv trans 4

    ## direct beam
    umv bar 6
    _sleep=0
    ubr 0 0 0
    umv sh -1
    umvr phi 0.005
    umv bar 4
```
Appendix F (Continued)

c t 60

## disable "etrans"
motor_par(etrans,"disable",1)

fr=SCAN\_N\+1
buf=sprintf("%d %d %d %d %d %d %d %d %d %d %d %d %d %d %d %d %d %d \n",fr,fr\+1,fr\+2,fr\+3,fr\+4,fr\+5,fr\+6,fr\+7,fr\+8,fr\+9,fr\+10,fr\+11,fr\+12,fr\+13,fr\+14,fr\+15,fr\+16,fr\+17,fr\+18,fr\+19,fr\+20,fr\+21,fr\+22,fr\+23,fr\+24,fr\+25,fr\+26,fr\+27,bar4\_norm*S[det]/S[monc])

fprintf("./ttt","w\n\C trans = %f\n\C ",A[trans])
u date >> ./ttt
unix("cat ./ttt >> /home/schlossman/jul2011/makeref.do")
close("./ttt")
uinix("rm ./ttt")

fprintf("./ttt","do makeref.md %s %s\n",DATAFILE,buf)
unix("cat ./ttt >> /home/schlossman/jul2011/makeref.do")
close("./ttt")
uinix("rm ./ttt")

## reflectivity scans


## reflectivity scans

cvslit 0 0.02
dcvslit 0 4
shut\_on

umv bar 4
ubr 0 0 0.019 ; sleep(3)
lscan 0.019 0.021 3 20
dcvslit 0 2
umv bar 4
lscan 0.022 0.028 6 20

umv bar 3
lscan 0.028 0.048 10 30
cvslit 0 0.05
umv bar 2
qz\_to\_stthdev 0.064
umv stth stthdev;sleep(8)
lscan 0.048 0.08 8 40
Appendix F (Continued)

umv bar 1
qz_to_stthdev 0.105
umv stth stthdev;sleep(8)
lscan 0.08 0.13 10 20

umv bar 1
qz_to_stthdev 0.17
umv stth stthdev;sleep(8)
lscan .14 .20 12 40

qz_to_stthdev 0.23
umv stth stthdev;sleep(8)
refc .21 .25 2 180

qz_to_stthdev 0.26
umv stth stthdev;sleep(8)
refc .25 .27 1 180

umk 0 0 0.27
qz_to_stthdev 0.29
umv stth stthdev;sleep(8)
refc .27 .31 1 180

umv bar 1
umk 0 0 0.32
qz_to_stthdev 0.34
umv stth stthdev;sleep(8)
refc .32 .36 1 120

_sleep=3
umk 0 0 0.37
qz_to_stthdev 0.39
umv stth stthdev;sleep(8)
refc .37 .41 1 120

umk 0 0 0.42
qz_to_stthdev 0.44
umv stth stthdev;sleep(8)
refc .42 .46 1 180

umk 0 0 0.48
qz_to_stthdev 0.50
Appendix F (Continued)

```plaintext
umv stth stthdev; sleep(8)
refc .48 .52 1 360

umv bar 3

## enable "etran"
motor_par(etrans,"disable",0)

umk 0 0 0.05
_sleep=0
    shscan 0.3 20 3
    umv sh CEN; set sh -4.1586
    umv bar 6
    ubr 0 0 0; umv sh -1
    shut_off'

)}
```
Appendix F (Continued)

The image below is for the sample setup in X22B.
VITA

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- *The influence of lipid composition on PKC binding*
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- *Lipid Monolayer at an Electrified Liquid/Liquid Interface*

- *Ion Distributions at the Electrified Liquid Liquid Interface: Comparison of X-ray reflectivity and Excess Surface Charge Measurements*

- *Configuration of PKCa-C2 Domain Bound to Mixed SOPC/SOPS Lipid Monolayers*

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