UC San Diego

MBTG 2018 Annual Retreat

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Program Book

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MBTG 2018 Annual Retreat

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The Molecular Biophysics Training Grant is supported by NIH Grant T32 GM008326.

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Engineering the Entropy-Drive Free-Energy Landscape of a Dynamic, Nanoporous Protein Assembly

Robert Alberstein
Akif Tezcan Lab

The sophistication of all living systems hinges at the molecular level on the ability of proteins and protein assemblies to alter their structures in response to physical and chemical stimuli. Although there have been notable advances in the design of protein structures, the de novo design of stimuli-responsive dynamic protein assemblies that predictably switch between discrete conformational states remains an essential but difficult goal. Here, we describe the first example of a synthetic protein assembly whose free energy landscape associated with the structural dynamics is fully determined by computer simulations, which has allowed us to predictably engineer its conformational switching behavior.

We have previously reported the design and construction of synthetic 2D lattices self-assembled from a C4 symmetric protein, C98RhuA, via disulfide bonding interactions. These crystalline assemblies displayed a unique capacity to undergo coherent conformational changes without losing crystalline order, leading to a Poisson's ratio of –1 and the modulation of porosity over a remarkably broad range (1 to 4 nm). In this study, we have carried out all-atom molecular dynamics simulations to map the free-energy landscape of these lattices, validated this landscape through extensive structural characterization by electron microscopy and established that it is predominantly governed by solvent reorganization entropy. Subsequent redesign of the protein surface with conditionally repulsive electrostatic interactions enabled us to predictably perturb the free-energy landscape, and obtain a new protein lattice whose conformational dynamics can be chemically and mechanically toggled between three different states with varying porosities and molecular densities.

Alberstein, R., Suzuki, Y., Paesani, F., Tezcan, F.A. Nature Chemistry (Accepted)

Identification of Short Catalytic Motifs from Hundreds of Ribozymes

Joshua Arriola
Ulrich Muller Lab

The RNA World hypothesis states that RNA played an important role in the emergence of life on Earth; RNA acted both as genetic storage and as the only genome-encoded catalyst. To identify catalytic RNA capable of prebiotically relevant functions, our lab has established an in vitro selection system that identified RNA capable of self-triphosphorylation from a pool consisting of 10^{14} random sequences. This in vitro selection resulted in ribozymes about 200 nucleotides long, a length that is prebiotically implausible since the prebiotic polymerization of long catalytic RNA (ribozymes) has not yet been shown. For instance, oligomers of only about 40 nucleotides long have been synthesized by polymerization on mineral surfaces under ideal conditions. To explore how the RNA world started we need to focus on the smallest possible ribozymes. We have developed a method that can identify small catalytic motifs from a pool containing hundreds of active ribozymes. Using a randomized primer, we generated truncations of the pool. One round of selection was performed in order to identify the smallest possible sub-pool that still contained active ribozymes. We identified active ribozymes 50 nt in length and were able to identify motifs that may be important for catalysis. Our method can be used to identify small catalytic motifs and short ribozymes from a pool containing active ribozymes.

Investigation of the folding of OmpA into nanodiscs by bimolecular fluorescence quenching

DeeAnn Asamoto
Judy Kim Lab

The folding and insertion of Outer membrane protein A (OmpA) into nanodiscs has been confirmed through a combination of SDS-PAGE differential mobility and digestion studies with steady-state fluorescence and UV resonance Raman spectroscopy. Similar refolding yields and kinetics were observed for the folding of OmpA into nanodiscs and small unilamellar vesicles (SUVs). UV resonance Raman spectroscopy revealed an enhanced- out- of- plane mode of OmpA folded in nanodiscs and this spectroscopic signature is only observed under refolding conditions in SUVs and detergent micelles. These data indicate that nanodiscs can be used as an alternative native-like membrane mimic that offers experimental benefits of optical clarity, greater stability, and better control of protein- to- nanodisc ratios.

Changes in protein solvation during the folding reaction of OmpA into nanodiscs was probed by Stern-Volmer fluorescence quenching experiments. Four single tryptophan mutants of OmpA (W7, W15, W57, and W143) provided site-specific information of the changes in solvent accessibility of tryptophan at the lipid-water interface (N-terminus side of β-barrel: W15, W57, and W143, periplasmic side of β-barrel: W7) during the folding reaction into nanodiscs. Stern-Volmer quenching constants, KSV, fit to double exponentials revealed a major slow component of 33-258 minutes. Shifts in the fluorescence λmax revealed a major fast component of 4-6 minutes. The fast λmax shift to lower wavelengths can be attributed to the adsorption and partial insertion of OmpA to the nanodisc bilayer surface which supports the idea that the OmpA adsorption process is fast and occurs prior to the insertion and folding processes [1,2]. The data indicate that the dehydration of tryptophan takes place after insertion and formation of native structure within the bilayer. These data provide insight into the changes of protein solvation during the folding reaction of OmpA into nanodiscs.

1. Kleinschmidt JH, Tamm LK (1996) Folding intermediates of a beta-barrel membrane protein. Kinetic evidence for a multi-step membrane insertion mechanism. Biochemistry-Us 35 (40):12993-13000
2. Kleinschmidt JH (2006) Folding kinetics of the outer membrane proteins OmpA and FomA into phospholipid bilayers. Chem Phys Lipids 141 (1-2):30-47

Cryo-EM studies of the Parkinson's disease associated protein LRRK2 and its interaction with microtubules

Colin Deniston
Andres Leschziner Lab

Currently 1% of people over 60 years of age in the US develop Parkinson's Disease (PD). Despite the abundance of cases, PD has proven difficult to study, as many cases are sporadic and not genetically inherited. However, the discovery of PD associated mutations in the protein Leucine Rich Repeat Kinase 2 (LRRK2) in 2004 marked the first known case of a protein with a role in both familial and sporadic forms of the disease. Despite a decade and a half of research into LRRK2, many questions remain unanswered surrounding the protein's molecular interactions and their relation to disease progression. To begin answering some of these remaining questions I am utilizing cryo-EM and biochemical methods to structurally and pathologically investigate both a known LRRK2 intermolecular interface as well as a novel interaction LRRK2 has with microtubules (MTs) [1].

One of the largest impediments to better characterizing interactions has been the lack of significant structural information on the LRRK2 protein. I aim to address this by first focusing on structurally characterizing LRRK2's known intermolecular oligomerization contacts, which I have begun to visualize in my 2D cryo class averages (Fig.1). Upon resolving the residues involved through a 3D structure I aim to investigate what role oligomerization may have in disease progression by interrupting the interface in various PD mutant LRRK2s *in vivo*. In parallel, I am also focusing on structurally resolving the binding interface between LRRK2 and MTs, a recently discovered novel interaction. Utilizing the same mentality as with the oligomerization site I will disrupt the interface between PD mutated LRRK2s and the MT *in vivo* to investigate any potential relationship to disease development. Ultimately these studies will start to answer if certain LRRK2 interactions are important for disease formation, potentially even going so far as to indicate specific sites for future drug targeting.

1. Kett, L.R., et al., *LRRK2 Parkinson disease mutations enhance its microtubule association.* Hum Mol Genet, 2012. **21**(4): p. 890-9.

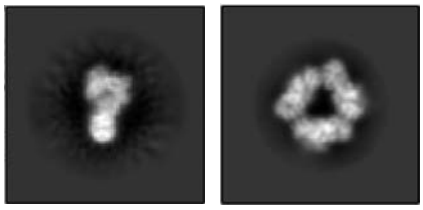


Fig.1 (Left) Monomeric LRRK2 viewed from the side. (Right) Trimeric LRRK2 viewed from above. Each side of the triangle represents one monomer rotated 90^{o} horizontally in relation to the left image. Oligomer contacts are made at each corner of the triangle where monomeric units touch.

Metal-Binding Pharmacophore Utilizing Bioisosteres as New Scaffolds for Metalloenzyme Inhibitors"

Benjamin Dick
Seth Cohen Lab

The principle of isosteres or bioisosteres in medicinal chemistry is a central and critical concept in modern drug discovery. For example, carboxylic acids are often replaced by bioisosteres to mitigate issues including limited membrane permeability, metabolic instability, and potential toxicity, while retaining acidic characteristics and hydrogen bond donor/acceptor abilities. Separately, the development of metal-binding pharmacophores (MBPs) for binding to active site metal ions in metalloenzymes of therapeutic interest is an emerging area in the realm of fragment-based drug discover (FBDD). Despite numerous investigations into bioisosteres, the direct application of the bioisostere concept to MBPs has not been well described or systematically investigated. Herein, the a MBP fragment is used as a case study for the development of MBP isosteres, or metal-binding isosteres (so-called MBIs). A library of approximately ~25 MBIs was assembled, many of which are novel ligands for metal coordination and drug development. Information on the acidity (pK_a), metal ligating properties, and metalloenzyme inhibition properties will be presented. Based on these findings, MBIs are a largely untapped source of metal binding functional groups, that allow for a new tool to modulate metalloenzyme inhibitors activity and physicochemical properties.

Remodeling the glycocalyx with sialic acid bearing glycopolymers to examine the impact of glycan presentation on Influenza A adhesion

Christopher J. Fisher
Kamil Godula Lab

Influenza A Virus (IAV) infection begins with the recognition of host cell surfaces by way of sialic acid (Neu5Ac) containing glycans presented in the cell's glycocalyx. As a result, assessing an IAV strain for its potential risk to human health commonly begins by identifying the virus's receptor specificity towards sialic acid bearing glycans. However, the multitude of sialylated glycoconjugates found both as secreted decoys and as adherent structures in this glycan-rich area of the cell makes host-recognition a complex landing event. Recent developments in the study of IAV has led researchers to begin recognizing other complicating factors that result from this host-glycan "forest" that impact IAV adhesion. The inherent heterogeneity and structural diversity of native glycoconjugates, coupled with the difficultly of genetically controlling glycan assembly, makes studying this multivalent interaction challenging. To bypass these difficulties, we utilize synthetic glycopolymers that mimic native macromolecules to remodel cell and viral surfaces to better investigate the role the glycan scaffold plays on IAV recognition, while simultaneously including the glycocalyx in its full complexity. These lipidadated and sialylated glycomaterials allow for effective and controlled remodeling of the glycocalyx, facilitating either the inhibition or restoration of IAV adhesion depending on their application. Utilizing these glycomaterials, we aim to better understand structural features of the glycocalyx that enhance IAV's primary binding event to target cells.

Regulation of Human Dynein by Lis1

John Gillies
Samara Reck-Peterson Lab

Cytoplasmic dynein-1 (dynein) is a microtubule-based motor protein. In human cells it is responsible for transporting hundreds of cargoes to the microtubule minus end, while in yeast it play a role in positioning the mitotic spindle. Lis1 is a conserved dynein regulator that is required for most dynein functions. In vitro, using purified yeast proteins, Lis1 causes dynein to bind tightly to microtubules. This is achieved by binding to a site on dynein's AAA+ motor domain. Recently, the Reck-Peterson lab identified an additional opposing mode of Lis1 regulation on dynein in yeast. Lis1 induces weak microtubule binding by dynein when it binds to both the AAA+ ring site and a novel binding site on dynein's "stalk", which leads to its microtubule binding domain.

These earlier experiments were done with yeast proteins. However, unlike yeast, mammalian dynein requires additional factors to move processively in vitro. Our lab and others have shown that Lis1 increases the velocity of mammalian dynein complexes as well as their affinity for microtubules. Using a combination of protein biochemistry and single-molecule microscopy experiments we found that the original Lis1 binding site identified in yeast is conserved and required for Lis1's effect on microtubule affinity using purified human proteins. Current experiments are aimed at understanding how Lis1 increases the velocity of human dynein complexes.

Zinc sulfinate reagent probing of RNA structure in solution

Anastassia Gomez
Navtej Toor Lab

There are currently no solution probing techniques that modify all four nucleobases in RNA. We have developed a methodology utilizing zinc bis[(phenylsulfonyl)methanesulfinate] to modify C-H groups on the Hoogsteen edge of RNA nucleobases. We can detect the sites of modification using a reverse transcription primer extension assay. Additionally, our protocol used natively folded RNA, and was conducted in open air, without organic solvents. This is the first method to probe the Hoogsteen edge of ribonucleobases. Chemicals that modify RNA are useful for a broad set of research applications. These applications include (but are not limited to) modifying RNA for the purposes of generating RNAs with longer *in vivo* lifetimes, labeling for visualization within cells, and probing 3D RNA structure.

Simulation Enabled Estimation of Kinetic Rates (SEEKR): A Hybrid Molecular Dynamics, Brownian Dynamics, and Milestoning Approach for Calculating Protein-Ligand Binding Kinetics

Benjamin Jagger
Rommie Amaro and J. Andrew McCammon Labs

We present recent developments to our SEEKR1 software, a hybrid molecular dynamics, Brownian dynamics, and milestoning approach for the calculation of association and dissociation rates as well as binding free energies for protein-ligand systems. The advantage of this approach, particularly the use of milestoning theory, is that it is highly parallel; resulting in a significant speedup and increased sampling as compared to conventional long-timescale molecular dynamics simulations. This extensive sampling, in conjunction with milestoning theory provides a statistically robust framework for the calculation of kinetic parameters. We also present recent successes including calculation of association and dissociation rates for the well-studied trypsin-benzamidine system that are in good agreement with experimental rates. Furthermore, we discuss the potential of this approach for the quantitative ranking of ligands by their kinetic parameters using the model system, ß- cyclodextrin. SEEKR reproduces the experimental ranking of a series of seven ligands by both off rates and binding free energies as effectively as conventional long-timescale molecular dynamics, with a significant reduction in computational cost.

(1) Votapka*, L. W.; Jagger*, B. R.; Heyneman, A. L.; Amaro, R. E. J. Phys. Chem. B.

Conformations of RSK activation

Evan Kobori
Susan Taylor Lab

Protein kinase have evolved to be dynamic macromolecular switches that alternate between inactive and active conformations. Although most kinase are typically phosphorylated and activated by other kinases, relatively little is known about the detailed mechanistic steps required for one kinase to activate another. The p90 ribosomal S6 kinases (RSKs) are a family of serine/threonine kinase that lie downstream of the Ras-MAPK pathway and regulates cell proliferation, cell survival, cell growth, and cell motility. RSK is an interesting model system to study kinase activation because it contains two distinct kinase domains, an N-terminal kinase (NtK), and a C-terminal kinase (CtK) in the same polypeptide chain. RSK has a unique activation mechanism that includes sequential phosphorylation events and requires two additional kinases: extracellular signal-regulated kinase (ERK) and 3-phosphoinositide-dependent protein kinase 1 (PDK1). The precise structural details of RSK kinase activation are not fully understood and a more rigorous structural characterization could provide valuable insight into how kinases activate other kinases. Two major goals of this work are as follows: to determine the structure of full length RSK bound to its activating kinase ERK using cryoEM, and to determine the structure of an active RSK NtK using x-ray crystallography.

RSK activation begins with ERK binding to and activating the CtK. A major goal of this project is to determine the structure of the full length RSK:ERK complex primarily utilizing cryoEM to gain insight into the specific interaction networks that exist within three separate kinase domains. ORF45 is a viral peptide that has been utilized to facilitate this structural study due to its unique ability to interact with the NtK and ERK simultaneously. The determination of the ORF45 binding sites on the NtK and ERK by hydrogen/deuterium exchange mass spectrometry and initial 2D class averages has provided an initial model of the RSK:ERK:ORF45 complex. A second major goal of this project is to elucidate a high resolution structure of the active conformation of RSK NtK as the currently available NtK structures are of the inactive conformation.

Markov Models of Influenza Hemagglutinin Reveal Druggable Sites

Sarah E Kochanek
Rommie Amaro Lab

Influenza virus infection continues to be a major healthcare issue, with 3-5 million cases of severe disease reported and 300,000-500,000 deaths worldwide each year. Variation in the sequences and structures of the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), from strain to strain complicate prophylactic and therapeutic approaches. Integrative modeling techniques combining cryo-electron tomography with x-ray crystallography, homology modeling, and protein-protein docking have led to the construction of a whole-virion model of influenza. Using the petascale computing machine Blue Waters, we have performed molecular dynamics (MD) simulations of this virion model. Principal component analysis reveals that simulation of the complete virion allows exploration of greater glycoprotein conformational space as compared to single glycoprotein simulations. Further, Markov state models constructed from the trajectories of the glycoproteins reveal two novel druggable pockets in HA that can be targeted for the development of novel treatments for influenza virus infection. The identification of druggable pockets previously unseen in simulations of individual glycoproteins demonstrates the utility of modeling in the sub-cellular scale and beyond to inform drug discovery.

ABC Transporter Dynamics: A New Perspective

Noah Kopcho
Geoffrey Chang Lab

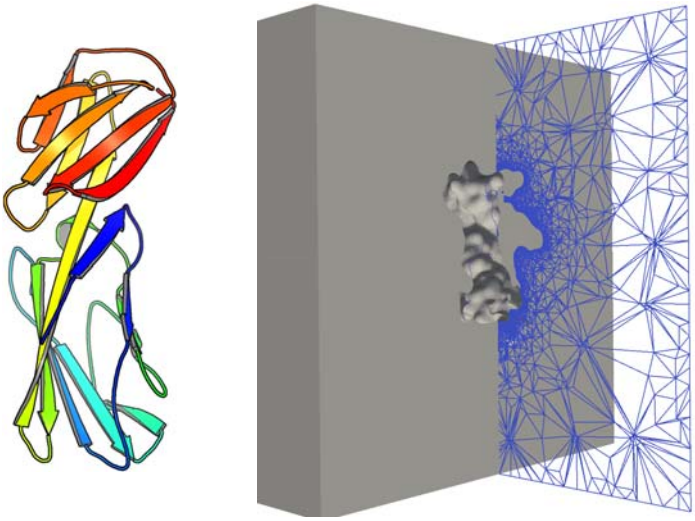
ATP Binding Cassette (ABC) transporters comprise one of the largest families of membrane proteins. These proteins couple the exergonic hydrolysis of ATP to substrate transport across a cell membrane. The first identified mammalian ABC transporter, P-glycoprotein (P-gp), transports a diverse pool of substrate molecules unidirectionally out of cells. This activity extrudes metabolites and prevents the entry of toxic molecules. P-gp has also been directly linked to numerous disease pathologies, such as tumor multidrug resistance and the progression of cerebral amyloidosis.

To date, a number of x-ray crystal structures have revealed intermediate conformations during the P-gp transport pathway. However, these images are static snapshots of an intricate mechanism. Few details are known regarding the precise molecular motions which couple ATP hydrolysis to the massive conformational changes required for transport. In order to characterize these structural changes, we have applied hydrogen-deuterium exchange mass spectrometry (HDX-MS). This technique allows the visualization of local conformational dynamics, and may reveal transient changes during the transport cycle which have remained unobserved in crystal structures.

Gamer 2.0: Software Toolkit for Adaptive Mesh Generation from Structural Biological Datasets

Christopher T. Lee
Rommie Amaro and J. Andrew McCammon Labs

Computer-aided mathematical simulation of signaling cascades and other biological phenomena have become increasingly popular. While some initial models have been built using simplified geometries, the use of high-fidelity geometric models from imaging data has the promise to improve simulation accuracy. GAMer (Geometry-Preserving Adaptive MeshER), an open-source software, was developed to generate high-fidelity mesh models from segmented image data. The original implementation suffered from lack of cross-platform support and instability. We introduce a substantial update to GAMer featuring a complete redesign to support cross-platform use across major operating systems. This version also features the automated resolution of some common mesh defects. We hope that GAMer 2.0 will enable faster and easier development of new models from imaging data.



Dynamics and Assembly of ASB-containing E3 Ubiquitin Ligases

Ryan Lumpkin
Elizabeth Komives Lab

E3 Ubiquitin Ligases (UBLs) facilitate the highly-specific covalent attachment of activated Ubiquitin (Ub) to bound substrate proteins through an isopeptide bond on an exposed lysine residue. Ub signaling regulates cellular protein degradation which is essential for proper cell functioning. The multi-subunit Cullin-RING ligase (CRL) is the largest family of UBLs, and it is responsible for up to 20% of degradation through the proteasome. CRLs share a common structure, composed (in order of arrangement) of a substrate receptor with a suppressor of cytokine signaling(SOCS) domain, one or two adapter proteins, a Cullin (CUL) protein, and a RING-box (RBX) protein that recruits E2 enzymes charged with activated Ub. Among the substrate receptors belonging to this class of UBLs, all 18 proteins in the Ankyrin Repeat and SOCS-box (ASB) family of proteins associate with CUL5 and RBX2 through the Elongin B and C (EB/C) adapter proteins.

ASB9 has been experimentally shown to tightly and specifically bind to Creatine Kinase (CK), but pulldown experiments recently suggested ASB9 may bind other substrates. (Balasubramaniam et al. 2015) Ankyrin repeat proteins have extended helix-turn-helix domains, yet previously they have only been shown to bind single substrates in vitro. By studying the ASB9-containing CRL (ASB9-CRL), I aim to further characterize the functional role of Ankyrin repeat proteins and the dynamics of the CRLs that enable ubiquitin transfer across 70 Å distances (Schiffer et al. 2016). I have assembled the full ASB9-CRL at concentrations suitable for biophysical experiments, and I demonstrated the ubiquitinylation activity of this ligase toward CK through band shift assays, anti-Ub western blots, and mass spectrometry. In collaboration with the Leschziner lab at UCSD, I have obtained a 4.5A cryo-EM structure of ASB9 in complex with CK and EB/C (Figure 1). We are currently working to obtain the structure of the full ligase. I identified Histones H2,H3 and H4 as novel substrates of this ASB9-containing CRL. I am using hydrogen deuterium exchange mass spectrometry to study the internal dynamics of the ligase and the influence of Neddylation, a post-translational modification on CUL5, on the ubiquitinylation activity of the ligase.

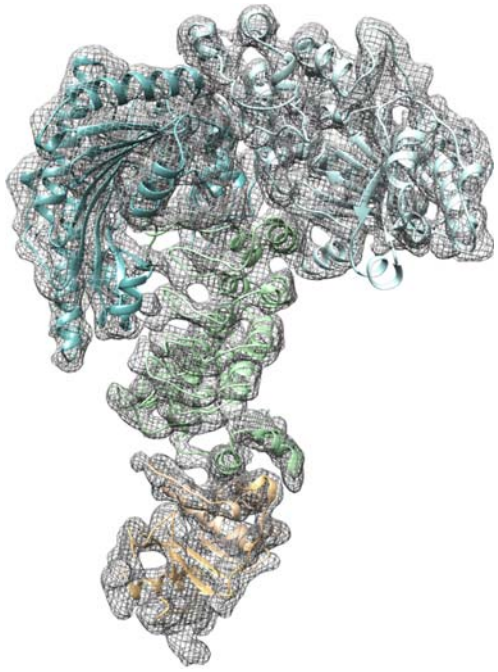


Figure 1: Model of CK (Blue), ASB9 (Green), and EB/C(Orange), overlaid with 4.5A Cryo-EM density map

A Minimal Eukaryotic Translation System for Studies of FMRP

Adam Maloney
Simpson Joseph Lab

Fragile X syndrome is a common neurological disease that stems from a gene defect in the FMR1 gene. Expanded CGG repeats in FMR1 can cause silencing of the protein FMRP, which is involved in regulation of protein translation in the brain. Previous research from our lab has suggested that FMRP may inhibit translation in neurons by interacting directly with the ribosome or mRNA during translation. A current focus of our research is to better understand this interaction through structural studies of FMRP and the translating ribosome. By investigating the mechanism through which FMRP inhibits translation we hope to gain an understanding of how translation is fundamentally regulated.

Structural studies of translation are challenging using traditional in vitro methods because of the many different protein factors required for to initiate translation. My research is focused on developing a minimal model of translation that can be used in biophysical studies of FMRP interacting with the ribosome. I am working on utilizing the CrPV IRES, a specialized RNA sequence that facilitates translation initiation, and purified protein, ribosome, and tRNA components to construct a translation system simple enough to be studied using cryo EM. At this point I have managed to purify these components and am working on producing and validating minimal translation complexes. Development of a minimal translation system will eventually be utilized to analyze the mechanism of translation regulation by FMRP using structural and biophysical methods.

Understanding Acyl Carrier Protein Interactions In Type II Systems

Jeff Mindrebo

Joseph P. Noel and Michael D. Burkart Labs

Fatty acid biosynthesis (FAS) is an elegant enzymatic assembly line that condenses and reduces two carbon keto units to produce saturated and unsaturated fatty acids for the cell. FAS is a conserved metabolic pathway in all domains of life and is essential to almost every living organism.[1] Central to FAS is the small acyl carrier protein (ACP) that contains a prosthetic phosphopantethiene (PPant) arm installed onto a conserved serine residue by a phosphopantetheinyl transferase (PPTase).[1] The ACP carries thioester tethered FAS intermediates to at least seven different partner enzymes to elongate and modify the growing fatty acid. It is still not understood how the ACP can effectively interact with multiple enzymes in a controlled and organized manner. Understanding how FAS systems function in greater detail requires the careful analysis of the ACP interaction with each partner protein as well as how the identity of the ACP tethered substrate directs traffic to different partner proteins. In the Burkart and Noel labs, we are using our ACP modification toolkit and pantetheine analog probes to develop a greater understanding of ACP dynamics and interactions with the *E.Coli* FAS ketosynthases FabF and FabB.[2–4] We have recently solved crystal structures of the ACP=FabF and ACP=FabB crosslinked complexes, providing an atomic resolution view of the ACP-ketosynthase interaction interface. Additionally, we have been able to crystallize these complexes with different acyl substrates in order to better understand the mechanism and substrate selectivity of ketosynthases. Elucidating the fundamentals of FAS can provide new approaches for antibiotic development and potentially clinical therapeutics in the future. Additionally, engineering future biosynthetic pathways will require a detailed understanding of ACP interactions with partner enzymes as well as their substrate specificity.

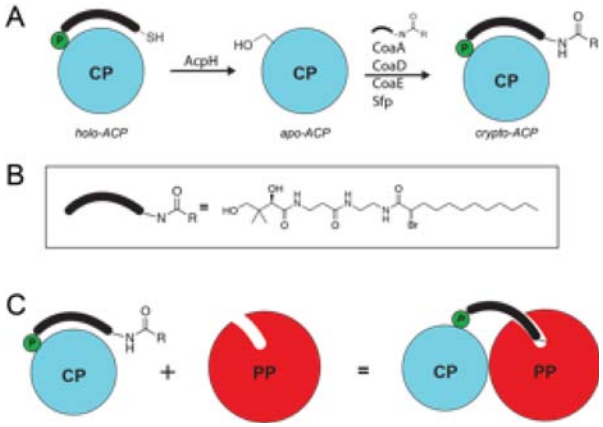


Figure 1: Workflow to study interactions between ACP and partner proteins. **A**) ACP is first converted from its *holo* form to *apo* form by removing its prosthetic pantetheine arm using the enzyme acyl carrier protein hydrolase (AcpH). ACP is then loaded with a PPant analog crosslinking probe (**B)** using a one-pot chemoenzymatic method to produce crypto-ACP. **C**) Crypto ACP is then incubated with a respective partner protein (PP) to create a crosslinked complex that can be detected by SDS-PAGE gel.

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The Dynamics Behind Thrombin Mutants with Anticoagulative Substrate Activity

Riley B. Peacock, Jessie Davis, Phineus Markwick, and Elizabeth A. Komives
Betsy Komives Lab

The serine protease, thrombin, regulates the balance between the anticoagulation and coagulation in the blood clotting pathway. Binding of thrombomodulin to thrombin switches its catalytic activity from procoagulative substrates (i.e. fibrinogen) to protein C, triggering the switch from the coagulation to the anticoagulation pathway. Recently, Ala mutants replacing Trp215, Glu217, and Phe227 were reported to lose activity towards fibrinogen without significant loss of activity towards protein C, and the double mutant W215A/E217A has been included in clinical studies, because it has an even greater preference for anticoagulative over procoagulative substrates. Our lab has obtained experimental and computational results suggesting that enzyme dynamics are important for connecting the main allosteric site on thrombin, where TM binds, to the active site. To understand how mutation of Trp215, Glu217, and Phe227 may alter thrombin specificity, hydrogen-deuterium exchange experiments (HDXMS) were carried out to compare the dynamics of W215A, W215A/E217A, W215I, F227A, and F227V with those of WT, activity assays were utilized to map out the catalytic effect of each mutant, and Accelerated Molecular Dynamics simulations of W215A were used to shed light on the phenomena behind the HDXMS results. Results indicate that mutations that affect the Trp215-Phe227 pi interaction directly destabilized the 170's, and 220's loops, and indirectly destabilized the N-terminus of the heavy chain of thrombin, resulting in misalignment of the catalytic triad. The W215A/E217A double mutant caused an even greater degree of destabilization within these regions, which extended into additional regions theorized to be important in TM-dependent allostery. Using HDXMS experiments, we show that TM-binding partially compensates for the destabilizing effects of the W215A, E217A, and F227A mutants. Our results provide a mechanism by which TM allows these mutant thrombins to cleave protein C -in the presence of TM- more effectively than procoagulative substrates.

Functional characterization of GPCRs in synthetic membranes using in situ lipid synthesis for protein reconstitution technology

Kira Podolsky
Neal Devaraj Lab

G-protein coupled receptors (GPCRs) are the largest family of transmembrane proteins involved in cell signaling. By using reductionist strategies, ligand induced signaling responses of GPCRs in vitro provide further insight into fundamental knowledge of membrane protein structure and function. Common approaches to reconstitute membrane proteins including GPCRs into membranes pose many problems such as altered protein activity and inferior biomimetic environments. Recently, our lab has developed in situ lipid synthesis for protein reconstitution technology (INSYRT), a non-enzymatic and chemoselective method to efficiently reconstitute GPCRs in phospholipid vesicles without the drawbacks of common detergent based methods. We are continuing to develop this technology for the reconstitution of additional components of the adenosine A2A receptor signaling pathway. This will allow us to characterize precise molecular events such as signaling bias and allosteric effects of ligand binding within a controlled biomimetic model setting.

Prediction of the Presence of a Seventh Ankyrin Repeat in IκBε from Homology Modeling Combined with Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS)

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The IκB protein family comprises a set of four members which function as inhibitors of NFκB transcription factors with distinct functions for each member specific to cell and tissue type. IκBα, IκBβ and Bcl-3 are the most well characterized proteins in this family and contain a shared structural architecture of a central well-folded domain of six ankyrin repeats (ARs) for IκBα/IκBβ and seven ARs for Bcl-3 flanked by disordered N- and C-termini. In 1997, a fourth IκB protein, IκBε, was discovered but the intervening two decades have yielded virtually no biochemical or biophysical characterization of the IκBε structure and function. Sequence analysis of IκBε suggests that it contains not six, but seven, ankyrin repeats similar to the non-canonical IκB family member Bcl-3. Using homology modeling, we generated structures of IκBε with each IκB protein family member as a template which all showed the conserved structure of a central AR domain and unstructured N- and C-termini. However, while the IκBα and IκBβ templates yielded models with six ankyrin repeats, the Bcl-3 template modeled a seventh ankyrin repeat as is suggested from sequence analysis of the IκBε C-terminus. In order to ascertain which homology model actually represents the solution structure of IκBε, we used hydrogen-deuterium exchange mass spectrometry (HDXMS) to probe the dynamics and level of foldedness in the C-terminus of IκBε and determined that the HDXMS data was inconsistent with a complete lack of structure in this region where only the Bcl-3 template modeled a seventh ankyrin repeat.

Investigating the Role of an Oxygen Ligand to the P-Cluster in Nitrogenase

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Nitrogenase is the only known enzyme capable of reducing dinitrogen to ammonia. In this intricate bioinorganic system, electron transfer is mediated by a complex iron-sulfur cluster (the P-cluster) which is unique to nitrogenase. It is known that an oxygen from a serine residue ligates the oxidized P-cluster in *Azotobacter vinelandii*, but this serine residue is only semi-conserved in nitrogenases. When the serine residue is not present, the oxygen from a tyrosine residue in the organism *Gluconacetobacter diazotrophicus* ligates the P-cluster, and most nitrogenases lacking the serine contain the tyrosine. I am investigating the role of the oxygen ligand in dinitrogen reduction by characterizing three *Azotobacter vinelandii* nitrogenase mutants which differ in their oxygen containing P-cluster ligands. Based on preliminary results, I hypothesize that the oxygen ligand serves to stabilize the structure of the oxidized P-cluster. These P-cluster mutants are being characterized with crystallography, electron paramagnetic resonance spectroscopy, and activity assays.

Peptide-Using Ribozymes and the RNA World

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It is estimated that life on Earth began approximately 4 billion years ago, and there is strong evidence for cellular life from at least 3.5 billion years ago. Based on molecular fossils, among other evidence, it is hypothesized that before the advent of the DNA-RNA-protein world, RNA served both as the genome and the only genome-encoded catalyst – this is the RNA world hypothesis. However, experiments by Stanley Miller and others since have shown that amino acids readily form under conditions estimated to be like those of the early Earth. Additionally, short (>10 amino acids) peptides have been shown to form under prebiotically plausible conditions. Thus, amino acids and short peptides were likely to have been present during the RNA world. We are performing an in vitro selection for ribozymes which triphosphorylate their own 5' end in the presence of peptides. The goal is to find ribozymes which use peptides as cofactors. Starting with a library of over 2.4*1014 sequences, active sequences were obtained from an intermediate round. A preliminary screen of 32 ribozymes has identified at least 5 ribozymes for which activity is improved by the presence of peptides. Each round of the ongoing selection is more stringent than the last. After it is completed, the fastest peptide-recruiting ribozymes will be identified by high-throughput sequencing analysis. Individual ribozymes will be analyzed will be analyzed biochemically and biophysically for their interactions with the peptides.

Revealing the molecular mechanisms of human 6-O-Endosulfatase specificity

Bryce Timm
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The polysaccharides that decorate proteoglycans (glycosaminoglycans – GAGs) display sulfate groups installed by sulfotransferases during GAG biosynthesis. The organization of specific sulfated (and non-sulfated) glycans within GAG chains results in unique anionic presentations that are used as recognition motifs for cytokines and growth factors. The sulfation code can be further modified by two human extracellular matrix (ECM) 6-O-endosulfatases (hSulf1 and hSulf2), which catalyze the removal of sulfate groups from the 6-O-position of heparan sulfate (HS) GAGs, mobilizing growth factors and indirectly activating signaling pathways. While the recognition mechanisms of the hSulfs remain poorly characterized, a highly charged region, termed the hydrophilic domain (HD), is thought to be the main actor in GAG recognition. Interestingly, deletion of the unique HD impairs endosulfatase catalytic activity, but does not affect the enzyme's ability to desulfate arylsulfates. This suggests the presence of necessary regulatory elements within the HD which influence the activity of the catalytic domain either directly or indirectly.

Utilizing both chemical and biophysical methodologies, I seek to develop biochemical probes that will aid in the imaging and characterization of the hSulfs, enlightening recognition mechanisms and providing necessary interrogative tools for the field. Our first step in elucidating these questions is the design of a synthetic chemical probe that enables visualization of enzyme activity through enzyme-mediated fluorescence turn-on. Additional structural features of our probe will interrogate the affinity of the enzymes toward individual glycans. Future work will use the information gathered to guide structural studies of the enzyme's recognition domain and ultimately the design of potential inhibitors.

The Msh4-Msh5 complex's role in meiotic crossover formation

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The MutS Homologs (MSH) have been identified in all organisms from E. coli to humans, and function in the initial recognition of mismatched base pairs in the conserved mismatch repair pathway. The MSH2-MSH6 and MSH2-MSH3 complexes participate in the repair of mismatches after mitotic DNA replication. In contrast to MSH2-MSH6 and MSH2-MSH3, the MSH4-MSH5 complex does not participate in mitotic mismatch repair, but plays a critical role in meiotic recombination and the segregation of homologous chromosomes during gamete/spore formation. The current model for the MSH4-MSH5 complex's function is that it binds and stabilizes an early recombination intermediate, perhaps the D-loop product of initial strand invasion or alternatively a Holliday Junction, and promotes the assembly of crossover-specific machinery. In order to further our understanding for the MSH4-MSH5 complex's binding mechanism to recombination intermediates and its roles in meiotic crossover formation, we have reconstituted the S. Cerevisiae MSH4-MSH5 complex, identified the Holliday Junction as it's strongest meiotic recombination binding partner, and through progressive truncations have identified necessary regions for DNA binding in vitro. Studies are underway to determine the structure of the protein complex bound to a Holliday Junction via Cryo-EM. Additional studies will aim to determine the complex's interaction with other proteins such as RecA/Rad51 homologs and downstream repair factors. This work will reveal how MSH4 and MSH5 interact with chromosomes and components of recombination machinery. By researching the MSH4-MSH5 complex and its downstream partners, we will be able to make a leap in understanding the physical basis of crossover formation.

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Targeted Depalmitoylation of Proteins in Living Cells by Native Chemical Ligation

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Protein S-palmitoylation is a reversible post-translational modification that is present on proteins involved in numerous biophysical processes, such as cellular trafficking, lipid raft association, and protein complex formation. Specifically, S-palmitoylation plays a significant role in the signal transduction pathway of proteins such as the oncogenic Ras proteins, which are mutated in many cancers. This post-translational modification allows Ras to traffic from internal membranes, such as the Golgi, to the plasma membrane, thus enabling its downstream signaling cascade. Mutations in Ras lead to an overactive protein that turns on downstream proteins involved in the cell growth and survival pathways, which contribute to cancer. Directly targeting the palmitoylation modification of Ras proteins can be an approach used to inhibit their oncogenic activity by preventing association with the plasma membrane using compounds capable of cleaving endogenous S-palmitoyl modifications. Our group has synthesized a class of molecules capable of chemoselective reactions with long chain thioesters, which result in the efficient release of a free thiol. Selective and reversible chemical agents for depalmitoylation would enable temporal control over protein lipidation and allow for the study of palmitoylation and trafficking kinetics in vivo. Our group is using live-cell imaging to study the changes in N-Ras protein localization and interaction with the plasma membrane as well as western blotting to study the changes in downstream oncogenic signaling pathways affected by the cleavage of the S-palmitoyl protein modification. These depalmitoylating agents could also serve as new drug candidates able to intercept and shut down cancer signaling through a novel mechanism.

Using cell permeable, cysteine-functionalized amphiphiles, we have also demonstrated the direct depalmitoylation of other cellular proteins via native chemical ligation (NCL). NCL successfully allows for depalmitoylation of mislocalized proteins in an Infantile Neuronal Ceroid Lipofuscinosis (INCL) disease model. INCL is a degenerative and fatal disease caused by mutations in the palmitoyl-protein thioesterase-1 (PPT1) gene, which encodes for a thioesterase responsible for the depalmitoylation of many S-palmitoylated proteins. Treatment of patient-derived INCL fibroblasts using depalmitoylation by NCL resulted in the release of GAP43 from ER-localized aggregates and restored a normal cellular distribution of GAP43. Using these novel depalmitoylating agents, which interact with membrane-localized proteins through the mechanism of NCL, enable direct and facile depalmitoylation of proteins in live cells and has potential therapeutic applications for diseases such as INCL, where native palmitoyl-protein thioesterase activity is deficient.