Welcome to the 11th Southeast Enzyme Conference

Saturday, April 10, 2021

We are Virtual!

Enjoy the 11th SEC at your favorite spot!
Southeast Enzyme Conference (SEC)

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Eleventh Southeast Enzyme Conference

Saturday, April 10, 2021

Supported by generous contributions from:
Schedule (Time: EDT)

8:00-8:30:  Online Mixer/Interactions

8:30-8:40:  Opening Remarks - Douglas Goodwin (Auburn University)

Session 1

Discussion Leader:  David White (Emory University)

8:40-9:10:  Kylie Allen (Virginia Tech)
A moonlighting Diiron Oxygenase in Folate Biosynthesis: Enzyme, Substrate, or Both?

9:10-9:30:  Jinping Yang (Washington University in St Louis)
In Vitro Reconstitution of Fimsbactin Biosynthesis from Acinetobacter Baumannii

9:30-9:50:  Daniel Kneller (Oak Ridge National Laboratory)
Structure-guided antiviral design of SARS-CoV-2 main protease using X-rays, neutrons, and supercomputers

10:00-10:45:  Poster Session 1

10:46-11:30:  Poster Session 2

Session 2

Discussion Leader: Jessica Krewall (Auburn University)

11:40-12:10:  Breann Brown (Vanderbilt University School of Medicine)
Structure-Based Insights into Control of Heme Biosynthesis

12:10-12:30:  Marley Brimberry (University of Georgia)
HutW from Vibrio cholerae is an Anaerobic Heme Degrading Enzyme with Unique Functional Properties

12:30-12:50:  Sydney Johnson (Virginia Tech)
Kinetic Characterization of Novel N-Monooxygenases, CreE and PcxL, involved in Nitro and Oxime Functional Group Formation

12:50-1:50:  Lunch Break followed by Group Chats
Session 3

Discussion Leader: Jenny Kim (Kennesaw State University)

1:50-2:10: Chad Brambley (Middle Tennessee State University)
Stress conditions stimulate conformational regulation of mitochondrial ATPase YME1L

2:10-2:30: Ramesh Karki (The University of Alabama)
A conserved histidine residue is required for a functional fold-switch mechanism in retaining GT-B glycosyltransferases

2:40-3:25: Poster Session 3

3:26-4:10: Poster Session 4

Session 4

Discussion Leader: Douglas Goodwin (Auburn University)

4:20-5:20: Giovanni Gadda (Georgia State University)
The Importance of Being Positive: How Flavoprotein Oxidases Achieve Oxygen Activation

5:20-5:30: Concluding Remarks - Douglas Goodwin (Auburn University)

5:30-6:30: Networking Mixer
Session 1:

Discussion Leader
David White (Emory University)
A moonlighting diiron oxygenase involved in folate biosynthesis in *Chlamydia trachomatis*:
Enzyme, substrate, or both?

*Rowan S. Wooldridge*, †, *Yamilet Macias-Orihuela*, ‡, *Valérie de Crécy-Lagard*, §, *Robert H. White*, †, and *Kylie D. Allen*†

†Department of Biochemistry, Virginia Tech, Blacksburg, VA; ‡Biological Systems Engineering, Virginia Tech, Blacksburg, VA; §Department of Microbiology and Cell Science, University of Florida, Gainesville, FL

Folate (vitamin B9) is a tripartite molecule consisting of a pteridine ring, *p*-aminobenzoate (pABA), and glutamate residues. The fully reduced form, tetrahydrofolate (H₄F), is the active cofactor that facilitates C1 transfer reactions in central metabolic pathways. The biosynthetic pathway for H₄F is well-established; however, select microbes that are known to biosynthesize H₄F are missing key genes required for the canonical pathway. One such organism, *Chlamydia trachomatis*, lacks the known genes required for the biosynthesis of the pABA moiety of H₄F. Previous studies revealed a single gene from *C. trachomatis*, ct610, that rescues *E. coli* Δ*pabA*, Δ*pabB*, and Δ*pabC* mutants.

CT610 and its homologs are annotated as “PqqC”, but also share low sequence similarity to non-heme diiron oxygenases and the previously solved crystal structure of CT610 revealed a diiron active site. Interestingly, CT610 was originally named *Chlamydia* protein associating with death domains (CADD) and was shown to have a signaling role in regulating host cell apoptosis; thus, this “moonlighting” protein appears to have two distinct functional roles.

Here, we discuss our progress towards uncovering the substrate and mechanism involved in CT610-dependent pABA biosynthesis. Isotope feeding studies with *E. coli* Δ*pabA* cells expressing CT610 revealed that the aromatic portion of tyrosine was highly incorporated into pABA, indicating that tyrosine is a precursor for CT610-dependent pABA biosynthesis. Interestingly, *in vitro* enzymatic experiments revealed that purified CT610 exhibits low pABA synthesis activity under aerobic conditions in the absence of tyrosine or other potential substrates, where only the addition of a reducing agent appears to be required for pABA production. Further, site-directed mutagenesis studies revealed two conserved active site tyrosine residues are essential for the pABA synthesis reaction. Thus, the current data suggest that CT610 is a self-sacrificing enzyme that utilizes its own tyrosine residue(s) as a substrate for pABA biosynthesis in a reaction that requires O₂ and a reduced metallocofactor. More recently, we have confirmed the oxygenase activity of CT610, which is significantly enhanced after *in vitro* reconstitution with Fe(II). However, the increased oxygenase activity after Fe(II) reconstitution does not result in increased pABA production and actually dramatically decreases the pABA synthesis activity. Thus, we currently propose that CT610 may employ a different metal cofactor for pABA synthesis.

This work was supported by the Virginia Tech the Department of Biochemistry, the College of Agriculture and Life Sciences, and Fralin Life Sciences Institute.


Iron is an essential element for cell activity and its acquisition is crucial for bacteria to establish virulence. In order to scavenge scarce ferric ion from host environment, one common strategy for bacteria is to synthesize and secrete iron chelating small molecules termed siderophores. Strains of human pathogenic Acinetobacter baumannii produce up to three types of siderophores: acinetobactin (1), baumannoferrin (2), and fimsbactin (3). While fimsbactin operon (fbs) is less common in A. baumannii strains than acinetobactin and baumannoferrin, fimsbactin can outcompete acinetobactin for binding to the periplasmic acinetobactin binding protein BauB and impede cell growth (4). This property renders fimsbactin an attractive scaffold for optimization of sideromycins and/or siderophore derivatives. The fimsbactin biosynthesis pathway is thus of particular interest as a promising chemoenzymatic approach for the production of siderophore-based therapeutics. Fimsbactin is biosynthesized by a nonribosomal peptide synthetase (NRPS) assembly line consisting of a standalone aryl adenylation domain, FbsH, three multimodule enzymes FbsE, FbsF, and FbsG, and a standalone thioesterase domain FbsM (3). The biosynthetic building blocks include 2,3-dihydroxybenzoic acid (2,3-DHB), L-serine, and N-acetyl-N-hydroxyputrescine (AHP). While 2,3-DHB and L-Ser are supplied by endogenous metabolic pathways, AHP is derived from L-ornithine via the catalytic action of enzymes encoded in the fimsbactin biosynthetic operon. Here we establish the order of sequential reactions catalyzed by monooxygenase FbsI, decarboxylase FbsJ, and acetyltransferase FbsK to convert L-Orn to AHP for ultimate use as the terminating nucleophile to release the mature fimsbactin scaffold from the enzymatic assembly line. We also describe the complete in vitro reconstitution of the fimsbactin NRPS assembly line from human pathogenic A. baumannii ATCC 17978. We demonstrate a new role for the thioesterase FbsM as to release the shunt biosynthetic product 2,3-DHB-oxazolecarboxylic acid (DHB-oxa), which may serve as a relevant metabolite in iron acquisition. Exploration of substrate preference and potential of fimsbactin biosynthetic enzymes for chemoenzymatic synthesis of fimsbactin analogs supports the potential of this pathway for producing new siderophore-based antibiotics targeting A. baumannii.

This study was supported by NSF CAREER Award 1654611.  

REFERENCES:  
**Talk 3**

**Structure-guided antiviral design of SARS-CoV-2 main protease using X-rays, neutrons, and supercomputers**

Daniel Kneller1,2, Stephanie Galanie3,4, Gwyndalyn Phillips1,2, Leighton Coates1,2, Andrey Kovalevsky1,2

1Neutron Scanning and 2Biosciences Divisions, Oak Ridge National Laboratory, Oak Ridge, TN, 3783
2National Virtual Biotechnology Laboratory, US Department of Energy, Washington DC

SARS-CoV-2 infection leads to COVID-19 which has resulted in a once a century global health and economic catastrophe claiming almost 2.5 million lives worldwide in a year. The viral main protease (M$^{\text{pro}}$) is an essential enzyme for SARS-CoV-2 replication and thus makes an attractive target for small-molecule drugs. Structure-guided drug-design strategies typically base atomic scale understanding of enzymes from cryogenic synchrotron X-ray diffraction. Conventional protein X-ray crystallography studies are hindered by cryo-artifacts and the inability to measure protonation states. Neutrons provide an ideal probe to determine protonation states of ionizable residues, such as the Cys-His catalytic dyad of M$^{\text{pro}}$, at near-physiological temperatures. This series of studies follows efforts to generate the large protein crystals required for neutron diffraction.

Pre-requisite X-ray experiments rapidly produced insights contributing to drug-design and clinical inhibitor repurposing efforts in the early days of the pandemic. The catalytic cysteine can be trapped in the rare peroxydisulfenic acid oxidation state at physiological pH while surface cysteines remain reduced signifying high reactivity of the catalytic cysteine. Ligand-free M$^{\text{pro}}$ compared with low-temperature structures demonstrates an inherent structural plasticity in the active site suggesting the room-temperature model is a relevant template for in silico docking studies. Clinical HCV protease inhibitors narlaprevir, boceprevir, and telaprevir are low-micromolar inhibitors of SARS-CoV-2 M$^{\text{pro}}$ by binding to the active site cavity. Structural comparisons indicate the malleability of active site enables adaptation to the size of inhibitor moieties.

The SARS-CoV-2 M$^{\text{pro}}$ neutron crystal structure was determined, providing the direct observation of protonation states of the active site in a cysteine protease for the first time. The catalytic Cys-His dyad of M$^{\text{pro}}$ exists in the reactive zwitterionic state at rest, with charged thiolate and doubly protonated imidazole side chains. A second neutron structure reveals the overall +1 charge of the active site is maintained in a covalent inhibitor complex via rearrangements of protonation states.

Finally, experimental validation of a lead inhibitor identified out of a multimillion compound supercomputer docking screen will be described. This research is providing real-time data for atomistic design and discovery of M$^{\text{pro}}$ inhibitors to combat the COVID-19 pandemic and prepare for future coronavirus pathogens.

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Session 2:

Discussion Leader
Jessica Krewall (Auburn University)
Protein assembly drives many aspects of mitochondrial physiology. We are interested in uncovering and characterizing the protein interactions that support proper heme biosynthesis. Heme is a critical biomolecule that carries out several functions in all life forms, including humans where its most widely-known role is mediating oxygen transport in blood. It is imperative that heme production is tightly controlled as aberrations in cellular heme levels can have drastic consequences for human health. The first and rate-limiting enzyme controlling heme biosynthesis is aminolevulinic acid synthase (ALAS). ALAS is conserved in α-proteobacteria and non-plant eukaryotes; there are two isoforms in vertebrates with mitochondrial protein ALAS2 being responsible for heme synthesis during erythropoiesis. Curiously, two separate diseases result from one of over 50 mutations in the ALAS2 gene. Mutations decreasing optimal activity lead to the disease X-linked sideroblastic anemia (XLSA), whereas separate mutations causing hyperactivity underlie X-linked protoporphyria (XLPP). Importantly, several disease-causing mutations are located in a eukaryote-specific C-terminal extension, a region absent from bacterial ALAS enzymes. Certain mutations also result in an inability of ALAS2 to assemble with other proteins, including proteins involved in separate metabolic pathways. However, we lack an understanding of how these mutations lead to a change in ALAS2 structure, and therefore, function. We seek to understand how ALAS interacts with accessory proteins as well as organism-specific differences in assembly that may alter regulation of heme production. Therefore, we use X-ray crystallography combined with in vitro activity assays of various eukaryotic ALAS enzymes to parse apart the role of this key regulatory region in eukaryote ALAS function. Our work is beginning to reveal key structure-function relationships between the orientation and molecular contacts mediated by the C-terminus and overall ALAS enzyme function, thus controlling heme biosynthesis.

This study was supported in part by a grant from The Jane Coffin Childs Memorial Fund for Medical Research and a Burroughs Wellcome Postdoctoral Enrichment Program Fellowship award 1015092 (B.L.B). This work is also based upon research conducted at the Northeastern Collaborative Access Team beamlines, which are funded by the National Institute of General Medical Sciences of the NIH (P41GM103403).
Talk 5

HutW from *Vibrio cholerae* is an Anaerobic Heme Degrading Enzyme with Unique Functional Properties

*Marley Brimberry¹, Marina Ana Toma¹, Kelly M. Hines², and William N. Lanzilotta¹*¹

¹Department of Biochemistry and Molecular Biology & Center for Metalloenzyme Studies, and the ²Department of Chemistry University of Georgia, Athens GA 30602.

Increasing antibiotic resistance and a growing recognition of the importance of the human microbiome, demand that new therapeutic targets be identified. Characterization of metabolic pathways, that are unique to pathogens, represent a promising approach. Iron is often the rate-limiting factor for growth and *Vibrio cholerae*, the causative agent of cholera, has been shown to contain numerous genes that function in the acquisition of iron from the environment. Included in this arsenal of genes are operons dedicated to obtaining iron from heme and heme containing proteins. Given the persistence of Cholera, an important outstanding question is whether or not *V. cholerae* is capable of anaerobic heme degradation as was recently reported for the enterohemorrhagic *Escherichia coli* O157:H7. In this work, we demonstrate that HutW from *V. Cholerae* is a radical *S*-adenosylmethionine (SAM) methyl transferase involved in the anaerobic opening the porphyrin ring of heme. However, in contrast to the previously characterized enzyme ChuW, found in the enterohemorrhagic *E. coli* O157:H7, there are notable differences in the mechanism and products of the HutW reaction. Of particular interest are data that demonstrate HutW will catalyze ring opening as well as tetrapyrrole reduction and can utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron source. A complete understanding of the mechanism, catabolites, and the modulation of the enzyme in anaerobic heme degradation will provide useful insight for future targeted pathogen antibiotic development.
Talk 6

Kinetic Characterization of Novel N-Monoxygenases, CreE and PcxL, involved in Nitro and Oxime Functional Group Formation

*Sydney Johnson*¹ and *Pablo Sobrado*¹

¹Department of Biochemistry and Center for Drug Discovery, Virginia Tech, Blacksburg, VA, 24061

CreE and PcxL are N-monoxygenases (NMOs), which belong to the flavin-dependent monoxygenase family (E.C. 1.14.13). Within this enzyme family, there are eight subclasses (A-H), and the NMOs are grouped in Class B. NMOs are single-component enzymes, where reduction occurs within a single polypeptide chain using an external reductant such as NADPH¹. NMOs typically perform a single-hydroxylation of an amino acid and are implicated in both virulence and natural product biosynthesis. Recently, NMOs that catalyzed multiple hydroxylations, on the same N-atom have been identified. CreE is responsible for the multiple-hydroxylation of L-aspartic acid to nitrosuccinate² (Scheme 1A). PcxL also catalyzes a multiple-hydroxylation from 2-aminoethyl phosphonic acid to 2-hydroxyiminoethyl phosphonic acid³ (Scheme 1B). CreE is part of the cremeomycin biosynthetic pathway and PcxL, the phosphonocystoximate pathway²,³. These natural products have been shown to have antimicrobial, antifungal and anticancer activities⁴. The observed multiple oxidation reaction and the medical application of NMOs make these enzymes important targets for mechanistic and structural studies.

Bioinformatic analyses show that PcxL and CreE share low sequence identity between them and other class B enzymes. Potentially, these enzymes perform multiple N-hydroxylations utilizing new chemistry and kinetic mechanisms. Thus far, we have successfully expressed and purified both recombinant CreE and PcxL. Steady-state and pre-steady state kinetics have been elucidated using an oxygen consumption assay and anaerobic stopped-flow spectroscopy. Our preliminary results suggest that both enzymes have a high dissociation constant for NADPH, leading to a slow flavin reduction. Unexpectedly, this slow reduction does not match the steady-state kinetics displayed by both enzymes. However, thermal shift experiments revealed that both CreE and PcxL undergo favorable conformational changes when reduced and bound to NADP⁵ as indicated by an increase in melting temperature. Detailed characterization of the catalytic cycle will be presented including both half reactions and kinetic isotopic effects.

This study was supported with funding from the National Science Foundation grant CHE 2003658.


Session 3:

Discussion Leader
Jenny Kim (Kennesaw State University)
Stress conditions stimulate conformational regulation of mitochondrial ATPase YME1L

Chad A. Brambley, Justin D. Marsee, and Justin M. Miller

Department of Chemistry
Middle Tennessee State University
1301 E Main St, Murfreesboro, TN 37132, United States

The ATPases associated with various cellular activities (AAA+) represent a super family of ATP-powered molecular motors critical for cellular function. These enzymes couple the energy of ATP hydrolysis to conformational changes that may be used to drive enzymatic motions in a variety of roles, including DNA replication, membrane fusion, vesicle formation, and protein quality control. To accomplish the latter, multimeric ring-shaped AAA+ domains are often paired with proteolytic domains to facilitate binding, unfolding, translocation, and degradation of protein substrates. YME1L is a representative AAA+ protease charged with such proteolytic processing within the mitochondrial inter membrane space, where the prevalence of oxidative stress may lead to increased incidence of damaged proteins. Dysfunction of YME1L may impede the clearance of such oxidatively damaged proteins, and thus may contribute to diseases of protein aggregation, including age-related neurodegeneration, cardiovascular disease, and cancer.

Here, we have utilized a variety of biophysical techniques, including stopped-flow fluorescence, molecular dynamics (MD) simulation, and steady-state fluorescence methods to develop a kinetic, thermodynamic, and structural understanding of YME1L behavior in the presence of oxidative stress. Stopped-flow fluorescence methods demonstrate that exposure to hydrogen peroxide (H$_2$O$_2$) results in an increase in the ATP binding rate constant from $(8.9 \pm 0.2) \times 10^5$ M$^{-1}$ s$^{-1}$ to $(1.5 \pm 0.1) \times 10^6$ M$^{-1}$ s$^{-1}$. To better understand the structural nature of these observations, we calculated free energy surface models from MD trajectories and performed steered MD of nucleotide dissociation for conditions both with and without H$_2$O$_2$. The results suggest oxidative environments may influence the conformational sampling of YME1L, leading to increased access to the nucleotide binding site. To further refine this hypothesis, we applied similar methods toward a study of the isolated AAA+ domain of YME1L. Our data suggest comparable conclusions for YME1L-AAA+ under the same oxidative conditions. We additionally have reported that ATP and Mg$^{2+}$ exhibit a negative heterotropic linkage effect. These data show a decrease in the affinity of YME1L-AAA+ for ATP from ~30 μM to ~60 μM for conditions examining 0 mM and 10 mM MgCl$_2$ respectively. To further explore the structural basis of the observed linkage, we performed principal component analysis on conformational ensembles generated from equilibrium MD trajectories. These data show that the presence of Mg$^{2+}$ promotes unique YME1L-AAA+ conformational populations. Taken together, our data describe a model by which YME1L conformational dynamics, and thus behaviors such as nucleotide binding, are tightly regulated by the local environment, which may present a mechanism to ensure continued maintenance of protein homeostasis under intracellular stress conditions.

This work was supported in part by funding from MTSU Faculty Research and Creative Activity Grant 17-17-215, a generous startup package from Middle Tennessee State University, the MTSU Molecular Biosciences Doctor program to JMM, and the Department of Chemistry Graduate Teaching Assistantship to CAB and JDM. Additional thanks to all members of the Miller Lab Group as well as faculty collaborators to this work.

References
A conserved histidine residue is required for a functional fold-switch mechanism in retaining GT-B glycosyltransferases

Ramesh Karki and Patrick A. Frantom

Department of Chemistry & Biochemistry, The University of Alabama, Tuscaloosa, AL 35487

Glycosyltransferases (GT) synthesize oligosaccharides and glycoconjugates by linking activated sugar donors to a variety of acceptor molecules. These enzymes are critical for biological energy storage, signaling pathways, and cell-wall construction. On the basis of the anomeric configuration of the newly synthesized glycosidic bond, GTs can be described as using inverting or retaining mechanisms. Structurally, GTs are primarily divided into GT-A and GT-B folds. Surprisingly, there is no correlation between overall fold and mechanism, as both retaining and inverting mechanism are catalyzed by both folds. GT-B enzymes are especially interesting as they appear to utilize both an open/close conformational change and a fold-switch mechanism in catalysis. To explore the relationship between the GT-B structural fold and retaining catalytic mechanisms, a Hidden Markov Model logo of the N-terminal domain (NTD) of the GT4 family (Pfam ID: PF13439), the largest retaining family with a GT-B fold, was used to identify conserved residues. These residues were mapped onto MshA from Corynebacterium glutamicum (CgMshA), a model member of the GT4 family. The identified residues (D104, H107, H109, T131, and H133) were subjected to alanine – scanning mutagenesis followed by kinetic characterization of the CgMshA variants. D104A, H107A, H109A, and T131A CgMshA have activities similar to the wild type enzyme. In contrast the H133A substitution increased the Michaelis constant for both the donor and acceptor substrate by ~100 fold but did not affect the $k_{cat}$ value relative to the wild-type enzyme. Structural alignment of NTDs of other retaining GT-B families revealed a histidine analogous to H133 in all retaining GT-B folds. This residue was not found in any inverting GT-B enzyme families, suggesting it is unique to the retaining mechanism. Circular dichroism spectroscopy revealed that the H133A substitution in CgMshA results in changes to the $\alpha/\beta$ structure. Hydrogen-deuterium exchange mass spectrometry studies on H133A CgMshA revealed changes in dynamics to several regions in the NTD of the enzyme, consistent with the circular dichroism spectroscopy results. Most importantly, substitution of H133 results in increased dynamics in a region shown to be involved in fold-switching from an inactive state to active state in another retaining GT-B enzyme, PimA. Overall these results suggest that the highly conserved histidine residue in the retaining GT-B enzymes is essential for a proper fold-switching mechanism and may serve as a key determinant of retaining vs. inverting mechanisms in the GT-B fold.
Session 4:

Discussion Leader
Douglas Goodwin (Auburn University)
Keynote Presentation

Giovanni Gadda Ph.D.

Distinguished University Professor & Associate Chair of Chemistry
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Molecular oxygen (O₂) is abundant in the Earth's atmosphere and is a potent oxidizing agent. The triplet ground state of O₂, which is the predominant form in air and solution, prevents spontaneous combustion of organic molecules, making O₂ relatively inert. Redox catalysts can overcome O₂ sluggish reactivity by reducing O₂ to superoxide (O₂⁻) through an obligatory electron transfer. Among a limited number of organic (and inorganic) biocatalysts, flavin hydroquinones are very effective in their reaction with O₂ to produce O₂⁻. O₂ reduction by flavins in bulk solution occurs with a second-order rate constant of 250 M⁻¹s⁻¹ determined at pH 6.5. After the formation of O₂⁻ and flavin semiquinone, a radical pair's spin inversion permits further reduction of O₂⁻ to H₂O₂ in flavoprotein oxidases. O₂ reduction is highly enhanced in flavin-dependent oxidases with second-order rate constants as high as 10⁵–10⁶ M⁻¹s⁻¹. Moreover, O₂ reduction is tightly controlled with enzymes to avoid the escape of radical intermediates like O₂⁻ that react nonspecifically with many cellular compounds.

While O₂ accessibility to the enzyme reaction site, its desolvation and geometry, and other factors such as flavin stereochemistry must also be considered, the 10²-10³-fold rate enhancements observed in flavoprotein oxidases require stabilization of the transition state for the initial, obligatory electron transfer that generates the O₂⁻/flavin semiquinone pair. Mechanistic studies have established that a positive charge in the enzyme active site is vital for O₂ activation in flavoprotein oxidases. In some cases, the positive charge is provided by a histidine or lysine side chain. In others, it is harbored on the organic product of the enzymatic reaction, which is released from the enzyme active site after reduction of O₂.
Poster List

Poster Sessions 1 - 4
(10:00 am – 11:30 am; 2:40 pm – 4:10 pm)
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Poster Abstracts

Poster Session 1
(10:00 am – 10:45 am)
Properties of a CYP102A2 Predicted to Participate in Plantazolicin Biosynthesis

Jahangir Alam and Douglas C. Goodwin
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The cytochromes P450 from Bacillus species have been of particular interest since the first discovery of a catalytically self-sufficient P450 known as P450BM3 (CYP102) in Bacillus megaterium in 1985. In spite of extensive investigation for nearly 4 decades, the functional role of CYP102 in Bacillus is still unknown. Our comprehensive mining of 1,562 Bacillus genome sequences identified 1,487 CYP102 genes. Thirteen of these were identified to be associated with a biosynthetic gene cluster that encodes the production of plantazolicin, a narrow spectrum antibacterial produced only by Bacillus species. All 13 P450s were identified as CYP102A2. To make further inroads in determination of a functional role for CYP102, a representative CYP102A2 protein from Bacillus amyloliquefaciens FZB42 (BaCYP102A2) (NCBI accession: WP_012117030) was selected for in silico and in vitro characterization. At present, there are no experimentally determined atomic-resolution structures for a full-length P450BM3, including both its heme-bearing catalytic domain and its reductase domain. Accordingly, a high-resolution 3D model of the full length BaCYP102A2 protein was constructed using RosettaCM followed by structural refinement and validation. In parallel, the gene encoding CYP102A2 enzyme was synthesized, cloned into a pET21 vector, and used to transform E. coli BL21 (DE3) for expression. The enzyme was purified using Ni-NTA affinity, anion-exchange, and size-exclusion chromatography followed by extensive dialysis. The identity of full-length BaCYP102A2 protein was confirmed by the presence of a major band at ~120 KDa in SDS-PAGE gel, a Soret band at 418.5 nm in UV-vis spectrum, and a type-I spectral shift in the presence of sodium dodecyl sulfate (SDS), a substrate for CYP102. Steady-state kinetic analyses of BaCYP102A2 with respect to the substrate SDS produced a sigmoidal response that yielded a $K_{1/2}$ of 156 ± 6 µM and a $k_{cat}$ of 79 ± 3 min$^{-1}$. Further investigation into the interactions of BaCYP102A2 with plantazolicin, other azole drugs, and long-chain fatty acid substrates as evaluated by molecular docking analyses, steady-state kinetic, and substrate binding assays will be discussed.
Investigating Structural Features Utilized by Two-component Flavin-dependent Systems

Chioma H. Alooh and Holly R. Ellis

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Many enzymes exist in multiple oligomeric states, but the role of these oligomeric changes are often not investigated.1 Changes in oligomeric states serve a myriad of functions such as exposing new active sites at the interface of interacting subunits, regulating protein activity, integrating different pathways by serving as a crosstalk between them, and enhancing protein stability by strategic arrangement of subunits to enable noncovalent interactions. Some bacteria depend on the two-component alkanesulfonate monooxygenase system for sulfur acquisition when inorganic sulfate is limiting in the environment. In the alkanesulfonate monooxygenase system reduced flavin is transferred from the reductase (SsuE) to the monooxygenase (SsuD), which catalyzes the desulfonation of alkanesulfonates to the corresponding aldehyde and sulfite. Pseudomonas aeruginosa has a more complex mechanism for acquiring sulfur. In addition to the SsuE/SsuD system, the organism also encodes a flavin-dependent reductase (MsuE) and two monooxygenases (MsuC/MsuD) that together convert methanesulfinate to formaldehyde and sulfite. In the alkanesulfonate monooxygenase system, SsuE has been found to exist in different oligomeric states. SsuE exists as a tetramer (dimer of dimers) but can shift to a dimer in the presence of flavin and/or substrate.2 MsuE shares 30% amino acid sequence identity with SsuE, but the oligomeric state of MsuE has not been investigated.

Studies were performed to investigate the oligomeric state of SsuE and MsuE in the presence and absence of substrates. Both SsuE and MsuE had similar melting temperatures, demonstrating the comparable stability of the two proteins. Results from native gel electrophoresis revealed that MsuE and SsuE exist in different oligomeric states. In the absence of substrates or the MsuD partner, a dimer and monomer were the primary oligomeric states observed for MsuE. However, SsuE existed in a tetramer/dimer equilibrium. Hydrogen-deuterium exchange with mass spectrometry (HDX-MS) was utilized to evaluate the oligomeric state of SsuE and MsuE alone and in the presence of oxidized flavin. Flavin acted as a regulator of both SsuE and MsuE, as there were observable changes in their oligomeric states in the presence of flavin compared to the enzymes alone. The tetramer interface region of SsuE includes a p-helix, which forms hydrogen bonds across the interface.3 Conserved regions located at the tetramer interface also form protein-protein interactions with SsuD. Results from HDX-MS showed that the tetramer to dimer switch in the presence of FMN occurs across the dimer-dimer interface in the p-helix region. The result from these studies demonstrates the importance of oligomeric changes to serve as a regulatory mechanism for flavin transfer.

This study was supported in part by Grant 1808495 from NSF (HRE).

Synthesis of Photo-Affinity Probes for the Investigation of Siderophore Binding Sites in Bacterial Fe(III) Shuttle Proteins

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Iron uptake is vital to pathogenic bacteria’s virulence. Siderophores are small, biosynthetic, iron-chelating molecules that are essential for iron sequestration by pathogenic bacteria. In Gram-positive bacteria, iron import is mediated by siderophore binding proteins (SBPs) which complex with siderophores and ABC-type transporters on the cell surface. The siderophore mediated iron uptake pathway represents an attractive target for the development of antibiotics as siderophores are a critical virulence factor for bacteria.

Our lab recently identified a catalytic role for an S. aureus SBP, FhuD2, in sequestration of iron from host donor sources. FhuD2 is a surface displayed lipoprotein that uses ferrioxamine siderophores as cofactors for stripping ferric iron from human defense proteins including transferrin. We named these catalytic SBPs the siderophore-dependent ferrichelatases (SDFs) (1). Structural studies on apo- and holo-FhuD2, revealed a previously unrecognized binding cleft with unknown substrate and function (2). We believe this secondary binding site binds non-ferric siderophores and plays a crucial role in catalytic iron-sequestration by FhuD2 through dynamic conformational manipulation of the bound siderophore cofactor. We have synthesized a photo-affinity labeled ferrioxamine siderophore to examine FhuD2 ligand binding modes in the presence and absence of ferric iron to inform our understanding of FhuD2’s functional role as a SDF in siderophore-mediated iron uptake.

Work thus far has focused on the synthesis of the siderophore desferrioxamine B (DFOB) and its iron-chelated form, ferrioxamine B (FOB), linked to a photo-reactive diazirine moiety. Additional work has been done to determine whether modification of DFOB and FOB affects FhuD2’s binding affinity for the siderophores and the siderophores’ binding affinity for iron(III); data from these binding studies will be included in the poster. Current work is focused on determining the efficacy of FhuD2 labeling using these probes, as well as developing additional probes for tagging FhuD2.

Figure 1. Photo-affinity labeled DFOB. The photo-reactive dizirine moiety is shown in red.

Figure 2. The open form of FhuD2 with residues lining the secondary binding site shown in red.

This study was supported in part by the Washington University Chemistry Department and NSF Career Award 1654611.

1. Nathaniel P. Endicott, Gerry Sam M. Rivera, Jinping Yang, and Timothy A. Wencewicz, “Emergence of Ferrichelatase Activity in a Siderophore-Binding Protein Supports an Iron Shuttle in Bacteria,” ACS Central Science 2020 6 (4), 493-506 DOI: 10.1021/acssciencias.9b01257

Preliminary Studies on Newly Discovered Sugar-phosphate $F_{420}$-dependent glucose-6-phosphate dehydrogenases

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University of Texas at Arlington, Department of Chemistry and Biochemistry, Arlington, TX, 76019

The $F_{420}$-dependent glucose-6-phosphate dehydrogenases (FGD1) have been studied extensively within Mycobacteria. During catalysis, the oxidized cofactor $F_{420}$ becomes reduced when FGD1 catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone (Figure 1). Our kinetic studies on FGD have led to important mechanistic determinations to enzymes that utilize this lesser known cofactor. However, FGD from Mycobacteria utilizes only one substrate, which is G6P. Recently, Mascotti et. al. described new $F_{420}$-dependent dehydrogenases which have a broader specificity of sugar substrates, known as $F_{420}$-dependent sugar-6-phosphate dehydrogenase (FSDs) [1]. We have selected to study FGD from Nocardioidaceae bacterium (FGD-Noca) and FGD from Cryptosporangium arvum (FGD-Cryar) using steady-state and pre-steady-state methods. We aim to characterize the hydride transfer reaction mechanism after optimizing the expression and purification of these enzymes. Here, we will discuss our proposed experiments and provide preliminary data.

Effects of ACCase-targeting herbicides on the detection southern crabgrass resistance using malachite green colorimetric assay

Suma Basak1, Jahangir Alam2, J. Scott McElroy1, and Douglas Goodwin2

1Crop, Soil, and Environmental Sciences Department, Auburn University, Auburn, AL 2Chemistry and Biochemistry Department, Auburn University, Auburn, AL

Acetyl-CoA carboxylase (ACCase; EC.6.4.1.2) is an important enzyme that catalyzes the first step of de novo fatty acid biosynthesis for the formation of malonyl-CoA. The sethoxydim and clethodim, two well-known ACCase targeting herbicides belonging to cyclohexanedione chemical family that is widely used in annual and perennial cropping systems for controlling grass weeds. Southern crabgrass (Digitaria ciliaris (Retz.) Koeler) was previously identified as resistant to sethoxydim of R1 and R2 biotypes were utilized as the resistant ACCase source compared to known susceptible (S) ACCase. Studies were conducted to detect the sensitivity of ACCase activity in the resistant and susceptible biotypes. Increasing concentration of ACCase-targeting herbicides (0.63 to 40 μM) was evaluated for ACCase enzyme-herbicides interaction of R1, R2, and S using an in-vitro malachite green colorimetric assay. The results from this assay revealed that the resistant biotypes, R1 and R2 had high ACCase activity against both ACCase-targeting herbicides as compared to S. The IC50 values for each herbicide, therefore, were higher in resistant biotypes than those of the S biotype, indicating reduced herbicides binding to the resistant ACCase.
Acetyl CoA carboxylase (ACC) is an enzyme that catalyzes the first committed step in fatty acid synthesis; the formation of malonyl-CoA. ACC performs two half reactions; the ATP dependent carboxylation of biotin, and the transfer of a carboxyl group from carboxy-biotin to acetyl-CoA to form malonyl-CoA. Many have proposed ACC to be a drug target for the treatment of diabetes and obesity, but little is known about the mechanism of the carboxyl transferase reaction, as well as the conformational changes involved. Our lab has developed novel stable malonyl-CoA thioester and carboxylate analogs to probe this enzyme and gain insight into its reaction. These analogs are less susceptible to hydrolysis and decarboxylation and have been able to reveal catalytic details of various other enzymes through x-ray crystallography. My goal is to use these malonyl-CoA analogs to probe the carboxyl transferase domain of ACC to gain insight into the mechanistic details of its catalysis.
Investigating the production of coenzyme F$_{430}$ variants in methanogenic archaea

Kaleb Boswinkle and Kylie Allen
Virginia Polytechnic Institute and State University, Department of Biochemistry

Methanogenesis is the biological production of methane and is utilized by methanogenic archaea (methanogens) as a form of anaerobic respiration to generate energy. Methanogens are found in virtually any anaerobic environment and are responsible for over 70% of total atmospheric methane, a greenhouse gas 84 times more potent than carbon dioxide over a 20-year period. Due to the abundance of methane and its powerful heat-trapping capability, it is among the most significant greenhouse gases. In addition to methane’s importance as a greenhouse gas, it is a valuable and clean energy source that emits 50% less carbon dioxide than coal combustion. An attractive possibility for further exploiting the chemical energy stored in methane is to convert it to more useable liquid fuels and other value-added chemicals. This could be accomplished by producing a methanogenic strain capable of reversing methanogenesis and converting methane to methanol. Before this can be realized, however, the chemistry of methanogenesis must be more fully understood. The final, rate-determining, methane-forming step of methanogenesis is catalyzed by methyl-coenzyme M reductase (MCR) and its prosthetic group, cofactor F$_{430}$, a unique nickel-containing tetrapyrrole that is the essential catalytic component of MCR. Recently, multiple F$_{430}$ variants have been discovered in several methanogenic species, including the two model methanogens *Methanococcus maripaludis* and *Methanosarcina acetivorans*. Our research suggests that one of these variants, mercaptopropionate-F$_{430}$, (F$_{430}$-3) is produced by *M. maripaludis* almost exclusively in stationary phase of growth, suggesting that nutrient deprivation induces the production of this variant. We hypothesize that hydrogen deprivation is involved in inducing this expression, but future experiments will be conducted to confirm this. We further have implicated a likely enzyme involved in the first step of F$_{430}$-3 biosynthesis, the insertion of a sulfur atom. This research sets the stage for further investigation into how these two variants modulate MCR catalysis.
Stress conditions stimulate conformational regulation of mitochondrial ATPase YME1L

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The ATPases associated with various cellular activities (AAA+) represent a super family of ATP-powered molecular motors critical for cellular function. These enzymes couple the energy of ATP hydrolysis to conformational changes that may be used to drive enzymatic motions in a variety of roles, including DNA replication, membrane fusion, vesicle formation, and protein quality control. To accomplish the latter, multimeric ring-shaped AAA+ domains are often paired with proteolytic domains to facilitate binding, unfolding, translocation, and degradation of protein substrates. YME1L is a representative AAA+ protease charged with such proteolytic processing within the mitochondrial inter membrane space, where the prevalence of oxidative stress may lead to increased incidence of damaged proteins. Dysfunction of YME1L may impede the clearance of such oxidatively damaged proteins, and thus may contribute to diseases of protein aggregation, including age-related neurodegeneration, cardiovascular disease, and cancer.

Here, we have utilized a variety of biophysical techniques, including stopped-flow fluorescence, molecular dynamics (MD) simulation, and steady-state fluorescence methods to develop a kinetic, thermodynamic, and structural understanding of YME1L behavior in the presence of oxidative stress. Stopped-flow fluorescence methods demonstrate that exposure to hydrogen peroxide (H$_2$O$_2$) results in an increase in the ATP binding rate constant from $(8.9 \pm 0.2) \times 10^5$ M$^{-1}$ s$^{-1}$ to $(1.5 \pm 0.1) \times 10^6$ M$^{-1}$ s$^{-1}$. To better understand the structural nature of these observations, we calculated free energy surface models from MD trajectories and performed steered MD of nucleotide dissociation for conditions both with and without H$_2$O$_2$. The results suggest oxidative environments may influence the conformational sampling of YME1L, leading to increased access to the nucleotide binding site. To further refine this hypothesis, we applied similar methods toward a study of the isolated AAA+ domain of YME1L. Our data suggest comparable conclusions for YME1L-AAA+ under the same oxidative conditions. We additionally have reported that ATP and Mg$^{2+}$ exhibit a negative heterotropic linkage effect. These data show a decrease in the affinity of YME1L-AAA+ for ATP from ~30 μM to ~60 μM for conditions examining 0 mM and 10 mM MgCl$_2$ respectively. To further explore the structural basis of the observed linkage, we performed principal component analysis on conformational ensembles generated from equilibrium MD trajectories. These data show that the presence of Mg$^{2+}$ promotes unique YME1L-AAA+ conformational populations. Taken together, our data describe a model by which YME1L conformational dynamics, and thus behaviors such as nucleotide binding, are tightly regulated by the local environment, which may present a mechanism to ensure continued maintenance of protein homeostasis under intracellular stress conditions.

This work was supported in part by funding from MTSU Faculty Research and Creative Activity Grant 17-17-215, a generous startup package from Middle Tennessee State University, the MTSU Molecular Biosciences Doctor program to JMM, and the Department of Chemistry Graduate Teaching Assistance to CAB and JDM. Additional thanks to all members of the Miller Lab Group as well as faculty collaborators to this work.

References
Ribonucleotide Inclusion Error Correction is Strongly Dependent on Flanking Nucleotides.

Steven T. Brenden, Sara Nguyen, & Markus W. Germann

Department of Chemistry, Georgia State University, Atlanta, GA 30303

Ribonucleotide inclusion is by far the most prevalent DNA damage. Incorporation of a single ribonucleotide impacts the local structure and dynamics of the affected double stranded DNA. Our previous work has shown that for an rG intrusion the perturbation is strongly dependent on the flanking nucleotide flanking sequence. To investigate this ribo-adulterated DNA varying only by their neighboring nucleotides were reacted with an RNA repair enzyme, RNase HII targets and repairs single ribonucleotide inclusions by cutting the DNA backbone. The repair efficiency/recognition of the damaged DNA duplexes has been examined for RNase HII. Preliminary results reveal that RNase HII activity on these substrates strongly depends on the flanking sequence.

Synthesis and Activity of Triazole-Containing Adenosine Analogs as Protein Arginine Methyltransferase 5 Inhibitors

Tyler Brown, Mengtong Cao, Y. George Zheng

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, Georgia 30602, United States.

Protein Arginine Methyltransferase 5 (PRMT5) is an attractive molecular target in anti-cancer drug discovery due to its extensive involvement in transcriptional control, RNA processing, and other cellular pathways that are causally related to tumor initiation and progression. In recent years, various compounds have been screened or designed to target either the substrate or cofactor binding sites of PRMT5. To expand the diversity of chemotypes for inhibitory binding to PRMT5 and other AdoMet-dependent methyltransferases, in this work, we designed a series of triazole-containing adenosine analogs aimed at targeting the cofactor binding site of PRMT5. Triazole rings have commonly been utilized in drug discovery due to their ease of synthesis and functionalization as bioisosteres of amide bonds. Herein we utilized the electronic properties of the triazole rings as a novel way to specifically target the cofactor binding site of PRMT5. A total of about 30 compounds were synthesized using a modular alkyne-azide cycloaddition reaction. Biochemical tests showed that these compounds exhibited inhibitory activity of PRMT5 at varying degrees and several showed single micromolar potency. A large number of these compounds displayed selectivity for PRMT5 over PRMT1. Docking-based structural analysis showed the triazole ring plays a key role in binding to the characteristic residue Phe327 in the active pocket of PRMT5, explaining the compounds’ selectivity for this type-II enzyme. Overall, this study provides new structure-activity relationship information on designing AdoMet analogs for selective inhibition of PRMT5. Further structural optimization work will further improve the potency of the top leads.

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A Kinetic Prelude to \(F_{420}\)-dependent sugar-6-phosphate dehydrogenases

Juan Corrales, Alaa Aziz and Kayunta Johnson-Winters

University of Texas at Arlington, Department of Chemistry and Biochemistry, Arlington, Texas, 76019

\(F_{420}\)-dependent glucose-6-phosphate dehydrogenase (FGD) catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone using \(F_{420}\) as the hydride transfer acceptor (Figure 1). This enzyme has been studied extensively in \textit{Mycobacterium tuberculosis} and recently discovered in \textit{Rhodococcus jostii}, with crystal structures solved from both sources. Recently, a new class of \(F_{420}\)-dependent glucose-6-phosphate dehydrogenases have been identified by Mascotti \textit{et. al.} in \textit{Nocardia} and \textit{Cryptosporangium} that have a broader specificity of substrates, which can catalyze reactions with fructose-6-phosphate and mannose-6-phosphate in the presence of \(F_{420}\) and therefore classified as \(F_{420}\)-dependent sugar-6-phosphate dehydrogenases (FSDs). \(^1\) The initial goal of our work is to optimize the expression and purification of wild-type FGD from \textit{Nocardia} (FGD-Noca) and then kinetically characterize these enzymes using crystallography, steady-state and pre steady-state kinetic methods.

![Figure 1: FGD catalyzed reaction](image.png)

Elucidating the catalytic mechanism of $F_{420}$-Dependent Glucose-6-Phosphate Dehydrogenase: Evidence of a cofactor-based intermediate

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$F_{420}$-dependent glucose-6-phosphate dehydrogenase (FGD1) is present within *Rhodococcus jostii* RHA1 as well as *Mycobacteria tuberculosis* (*Mtb*). Because *Mtb* is the causative agent of tuberculosis disease (TB) the FGD1 catalyzed reaction has great biomedical relevance. FGD1 catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone using the $F_{420}$ cofactor, which becomes reduced during catalysis. The initial proposed mechanism, suggests that the conserved His40 serves as the active site base, abstracting a proton from G6P facilitating the transfer of a hydride to the $F_{420}$. The conserved Glu109 acts as the acid donating a proton to $F_{420}$ (Figure 1). While this mechanism was plausible, it lacked experimental evidence, until our recent work, which confirmed that Glu109 is the active site acid. However, our pH profiles suggest that His40 does not serve as the active site base, losing an acid limb, rather than a basic limb. The overarching goal was then to identify which amino acid serves as the active site base. This present work aims to address this by utilizing substrate binding studies, steady-state and pre steady-state kinetics, pH dependence profiling, kinetic isotope effects, and global analysis. The FGD variants, H40A, H40Q, H260A, H260N, E13A, and E13Q have all been fully characterized in order to understand the role of these amino acids during turnover. Using global analysis, we have observed the accumulation of an intermediate during catalysis. While this intermediate is present within wild-type FGD and some FGD variants, it is not observed in all variants, including the H40A and E13A variants. Here, we present the data obtained from global analysis, fluorescence binding experiments, steady-state and pre steady-state data that seemingly points to H40 serving as the active site base.

Figure 1: proposed mechanism.


Poster Abstracts

Poster Session 2
(10:46 am – 11:30 am)
Increased Mobility of the Gating Loop in NADH:Quinone Oxidoreductase Alters Lid Dynamics while Preserving Enzyme Function.

Benjamin D. Dratch‡, Archana Iyer‡, Yang-Fang Wang‡, Irene T. Weber‡§, Donald Hamelberg‡, and Giovanni Gadda‡§

Departments of ‡Chemistry, §Biology, and δThe Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302-3965

Enzymes are essential for all living organisms and often require a series of dynamic motions to maintain efficient catalytic rates. Dynamics ranging from large and prominent domain motions to small and subtle amino acid fluctuations play a key role in catalysis. However, the link between protein dynamics and catalysis is currently insufficiently well understood. The crystal structures of NADH:quinone oxidoreductase from Pseudomonas aeruginosa (NQO; EC 1.6.5.9), an FMN-dependent enzyme, show that loop 3 (residues 75-86) of the TIM-Barrel domain is displaced by 5.5 Å towards the active site with NAD⁺ bound. Thus, loop 3 is proposed to act as a gate that stabilizes the enzyme-substrate complex by forming hydrogen bond interactions with the substrate and the extended domain of NQO. Loop 3 contains three proline residues, of which only P78 is conserved in NQOs.

In this study, P78 was mutated to glycine to increase the protein backbone's internal flexibility in loop 3. The crystal structure of the variant enzyme NQO-P78G was solved to establish that the mutation did not alter the overall fold and positions of the active site residues in NQO. UV-visible absorption spectroscopy was consistent with the P78G mutation not affecting the active site microenvironment surrounding the flavin. Molecular dynamic simulation trajectories from NQO-P78G and NQO-WT with and without NAD⁺ showed that P78G samples wider conformations at the extended domain and loop 3 in the ligand-free form, which opens up the gating region in NQO. Steady-state kinetic parameters for NQO were determined at varying concentrations of NADH and Coenzyme-Q₀ (CoQ₀) at pH 6.0 and 25 °C. The results established a 3.5-fold increase in the $K_{\text{CoQ}_0}$ value, a 2.0-fold decrease in the $k_{\text{cat}}$ value, and a 1.8-fold increase in the $k_{\text{cat}}/K_{\text{CoQ}_0}$ value in NQO-P78G compared to NQO-WT. Overall, the results suggest an increased flexibility of loop 3 does not affect the active site environment or rate of substrate capture but instead plays an essential role in domain-domain interactions that facilitate gate dynamics in NQO.

An Arg switch and the formation of a protein-based cofactor in catalase-peroxidase (KatG)

Madeleine Forbes, Jessica R. Krewall, Laura E. Minton, and Douglas Goodwin
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Catalase-peroxidase (KatG) is a multifunctional, heme-dependent enzyme that catalyzes a unique combination of catalase and peroxidase activities. The enzyme serves as a key virulence factor for a variety of bacterial and fungal pathogens as it aids in the detoxification of threatening peroxides. The enzyme’s bifunctional nature is achieved by two novel active-site structures: 1) a covalently-linked structure formed among a Met, a Tyr, and a Trp (i.e., the MYW cofactor) and 2) an pH-dependent Arg residue near the active site (i.e., the Arg switch). While both structures are vital to KatG’s catalase mechanism, the autocatalytic formation of the MYW cofactor and the specific role of the Arg switch plays in the adduct’s formation are unresolved.

Investigation of the MYW formation entailed comparing two forms of KatG: 1) enzyme expressed in the apo form and then reconstituted enzyme with its heme cofactor (rKatG), and 2) enzyme expressed in the presence of heme and then purified in its holo (hKatG) state. Consistent with the wild-type enzyme, MS analyses of hR418N KatG showed the clear presence of the MYW cofactor, but only trace amounts of this structure in rR418N KatG. Treatment of rR418N KatG with H₂O₂ increased MYW cofactor signals to be nearly identical to the hR418N form of KatG. Reactivity of rR418N with H₂O₂ was monitored by stopped-flow spectroscopy. Reactions of ferric r and hR418N KatG with H₂O₂ both occurred with rate constants similar to the wild-type enzyme. For rWT and rR418N KatG but not hWT and hR418N, a subsequent step in the reaction sequence was observed which was characterized by an increase in absorption at ~315 nm. This is consistent with absorption characteristics of the MYW adduct. Fitting of the reaction sequence data revealed that the first-order rate constant for this step was three-fold slower with rR418N KatG than the wild-type enzyme. Taken together, these data suggest that the arginine switch (R418) is not essential for the formation of KatG’s MYW cofactor. However, diminished rates of putative MYW cofactor formation in R418N KatG would suggest that it plays an accessory role in the process as it does in the enzyme’s catalase activity.

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The Interaction of Porcine Dihydropyrimidine Dehydrogenase with the Chemotherapy Sensitizer: 5-Ethynyluracil

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Dihydropyrimidine dehydrogenase (DPD) is a dimeric flavoenzyme that catalyzes the reduction of the 5,6-vinyl bond of the pyrimidines, uracil and thymine. 5-fluorouracil (5FU) is a substrate for DPD and a chemotherapeutic used to treat a variety of cancers. Reduction by DPD of 5FU to 5,6-dihydro-5-fluorouracil nullifies its toxicity and efficacy. DPD activity is therefore a primary mitigating factor for cancer treatment by 5FU. 5-Ethynyluracil (5EU) is an inhibitor of DPD that covalently links to the pyrimidine active site general-acid cysteine (Cys671). Inactivation by 5EU occurs with the simultaneous binding of NADPH and the pyrimidine to DPD. Binding recognition of these two ligands induces DPD to undergo reductive activation by taking up two electrons from NADPH¹. We show that 5EU-based inactivation coincides with reductive activation with a rate constant of ~0.2 s⁻¹. The rate constant is associated with the reduction of a single flavin per enzyme subunit and the localization of a mobile loop that positions the active site cysteine proximal to the ethynyl group of 5EU at the pyrimidine site. We present three X-ray crystal structures that together capture this proposed sequence of events. These structures confirm 5EU crosslinks to Cys671 uncoupling proton-coupled electron transfer which transfers two electrons 60 Å from the NADPH/FAD site to the pyrimidine/FMN site. We also show that NADPH binding is required for efficient inactivation, but non-reductive NADPH analogs are observed to cause partial inhibition suggesting that there is also a conformational component to inactivation by 5EU.

Tetracyclines are an important group of antibiotics used in agricultural and clinical settings. They function by binding to the 30S subunit of the prokaryotic ribosome to effectively halt translation. The emergence of tetracycline resistant strains of pathogenic bacteria has threatened the continued use of the drug. One mechanism of tetracycline resistance is the degradation of the drug by the flavin dependent enzyme tetracycline monooxygenase (TetX). Only a limited number of studies of the enzyme have been conducted to date due to the limited amounts of pure protein that can be obtained from current protocols (~6 mg/L of cell culture). The current report describes the development of optimized expression and purification protocols to obtain larger amounts of pure, soluble enzyme suitable for detailed kinetic studies to deduce its chemical mechanism. During the initial phase of this work the pET22a+ plasmid with the gene encoding for TetX was used to transform either DH5α or Rosetta (DE3) pLysS Escherichia coli cells. Expression trials of these cell lines in which both the temperature and times of incubation with isopropyl β-D-1-thiogalactopyranoside were then carried out. Of the conditions, tested optimal expression of TetX was found with a 20 h induction period at 30 °C using E. coli DH5α cells. Ammonium sulfate precipitation trials were then conducted where it was concluded that a 20 fold purification of the enzyme is achieved through treatment with 40% saturation of salt and collection of the supernatant after centrifugation. A nickel affinity chromatographic protocol was also developed, which purified the enzyme to high levels as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the specific activity of the purified sample. The work reported here resulted in ~36 mg/L of cell culture, which is a 6-fold improvement over published protocols.

This study was supported by the University Research Award from Texas A&M University-Kingsville Texas Compressive Research Fund, a Departmental Research Grant from The Robert A. Welch Foundation (AC-0006) and the Ronald E. McNair Program.
Heterologous expression and assembly of methyl-coenzyme M reductase from anaerobic methanotrophs

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Methyl-coenzyme M reductase (MCR) is the key rate-determining enzyme of methanogenesis and anaerobic methane oxidation, the essential energy metabolisms of methanogenic archaea and anaerobic methanotrophs (ANME), respectively.\(^1\) Much interest lies in the possibility of using MCR for the bioconversion of methane into liquid fuels and other value-added chemicals. Although methanogenic MCR has been extensively studied, the enzymatic properties of ANME MCRs remain unclear since its activity has never been studied in vitro. Given the opposite in vivo functions, we hypothesize that ANME MCRs are evolutionarily optimized to perform methane oxidation compared to methanogenic MCRs. To study MCR, it is normally purified from the native organism. However, ANME are very difficult to culture in a laboratory setting due to their slow doubling times (~three months) and their characteristic dependence on bacterial partners, thus hampering any MCR related studies from these organisms. Thus, our approach is to utilize the model methanogen, Methanococcus maripaludis, for the heterologous expression of ANME MCRs. We are studying two types of ANME MCRs: the ANME-1 MCR for which the crystal structure has been solved and an ANME-2d MCR from Ca. Methanoperedens nitroreducens (MnMCR).

MCR is a dimer of heterotrimer with a 2α, 2β, 2γ configuration, that requires coenzyme F\(_{430}\) and proper assembly for enzymatic activity, rendering it a difficult enzyme to be successfully heterologously expressed. In methanogens, MCR is encoded in the highly conserved MCR gene cluster mcrBCDG. In addition to the MCR encoding genes (mcrB, mcrG, and mcrA), the cluster contains two other genes that encode accessory proteins McrD and McrC. These two proteins are hypothesized to be involved in the assembly and activation of MCR.\(^2,4\) Interestingly, most ANME lack one or more of these accessory proteins in their MCR gene clusters. The ANME-1 MCR gene cluster contains only the MCR-encoding genes (mcrBGA) and the MnMCR gene cluster lacks mcrC. We have created several MCR expression constructs containing the full MCR operons for ANME-1 and Ca. M. nitroreducens as well as synthetic hybrid MCR operons. The constructs include the constitutive promoter (phmva) and an N-terminal his-tag on McrA for subsequent purification by metal-affinity chromatography. After transforming into M. maripaludis, large batch cultures allow for the expression and purification of the respective ANME MCRs. For ANME-1 MCR, we were only able to purify the his-tagged McrA, which required solubilization with urea and unfortunately did not contain McrB and McrG. MnMCR also required solubilization with urea, however, the MnMCR was assembled with all three subunits as well as F\(_{430}\). Since instances of MCR being potentially associated with the membrane have been reported in many methanogens, we next carried out the purification of MnMCR in the presence of 0.5% Tween 20. Excitingly, this resulted in the successful purification of the assembled soluble enzyme containing F\(_{430}\). This result now sets the starting point for optimizing protein expression and eventual methane oxidation assays. Notably, the successful assembly of MnMCR in the heterologous host compared to the so far unsuccessful assembly of ANME-1 MCR points towards the potential importance of including mcrD within the expression construct. McrD has been hypothesized to serve as a chaperone that aids in the assembly of the enzyme, as well as potentially in delivery of cofactor F\(_{430}\).\(^3,4\) Taken together, this work lays the foundation for future biochemical studies of anaerobic methane oxidation catalyzed by ANME MCRs.

From negative to no cooperativity: A change in inner-subunit communication within F_{420}H_{2}:NADP^{+} Oxidoreductase

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F_{420}H_{2}:NADP^{+} Oxidoreductase Figure 1: Fno catalyzed reaction. (Fno) is a multi-subunit enzyme, found within methanogenic and sulfate-reducing archaea. Fno catalyzes the reversible reduction of NADP^{+} to NADPH, using reduced F_{420} as the hydride donor (Figure 1). NADPH production within these cells as well as the oxidation of the reduced F_{420} cofactor are linked to many metabolic processes, such as glycolysis, and methanogenesis. Our prior pre steady-state kinetic research on wild-type Fno has revealed biphasic kinetics with an initial burst phase followed by a slow phase. The burst phase corresponded to 50% of F_{420} cofactor reduction, which is indicative of half site reactivity, while the steady-state kinetic data suggests negative cooperativity. These data suggest that Fno is a regulatory enzyme. Therefore, the goal of this work is to explore which amino acids participate in communication between the two subunits at the Fno interface. We have identified five candidates that potentially serve in this role. These amino acids are, R186, T192, S190, T09 and His133. We have created a library of Fno variants to test our hypothesis. The Fno variants, R186Q, R186K, R186I, T192A, T192V, T192A, H133A, H133N and S190A were characterized using binding, steady-state and pre steady-state kinetic experiments. Unlike wild-type Fno, which displayed negative cooperativity binding and negative cooperativity kinetics, the Fno variants R186Q, R186K, R186I, T192A, and S190A displayed no cooperativity. Lineeweaver-Burke plots derived from the steady-state kinetic data showed linear Lineeweaver-Burke plots, which confirmed that no negative cooperativity was observed for these variants. Pre steady-state kinetics revealed that hydride transfer has become rate-limiting in catalysis for the R186Q, R186I, T192A and S190A Fno variants. Here, we report kinetic evidence that indicates cooperativity between the two Fno subunits has been eliminated and communication has been interrupted as a result of these Fno variants.
Kinetic characterization of a coupled-enzyme system for D-arginine conversion to L-arginine from Pseudomonas aeruginosa

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L-arginine dehydrogenase (PaLADH) and D-arginine dehydrogenase (PaDADH) from Pseudomonas aeruginosa are part of a two-enzyme system that catalyzes the irreversible conversion of D-arginine to L-arginine. PaDADH is an FAD-dependent enzyme that catalyzes the oxidation of D-arginine into 2-ketoarginine and ammonia. (1) PaLADH catalyzes the conversion of 2-ketoarginine and ammonia to L-arginine in a reversible manner using NAD(P)H as a substrate in the forward direction. The coupled-enzyme system enables P. aeruginosa to survive solely on D-arginine as a nitrogen and carbon source. (2) PaDADH can oxidize most D-amino acids except D-aspartate and D-glutamate, with the highest rate constants for substrate capture, $k_{cat}/K_m$, observed with D-arginine and D-lysine as substrates. (1)

We hypothesized that PaLADH and PaDADH might form a protein-protein complex for converting D-arginine to L-arginine. The formation of the protein-protein complex was probed using analytical ultracentrifugation and size-exclusion chromatography, suggesting that PaLADH and PaDADH form a complex in a 1:1 stoichiometry. The turnover number, $k_{cat}$, for PaLADH with L-arginine and NAD$^+$ as substrates was measured using a stopped-flow instrument and had a value of 8.0 ± 0.2 s$^{-1}$ at pH 8.0 and 25 °C. In contrast, a $k_{cat}$ value of 1.3 ± 0.1 s$^{-1}$ was determined in a coupled-enzyme assay in which both enzymes were mixed with saturating concentrations of D-arginine, benzoquinone at varying concentrations of NADH (Scheme 1). Preliminary homology modeling and CAVER analysis will be discussed to investigate the transport of ligand(s) from the active site of PaDADH to PaLADH. The utilization of the coupled enzyme system to metabolize D-arginine has given P. aeruginosa an edge over other bacteria to survive in nutrient-limiting conditions, and further studies will help us further understand the role of this novel system.

Scheme 1. The conversion of D-arginine to L-arginine by PaDADH and PaLADH in a coupled-enzyme system.

CreE and PcxL are N-monoxygenases (NMOs), which belong to the flavin-dependent monooxygenase family (E.C. 1.14.13). Within this enzyme family, there are eight subclasses (A-H), and the NMOs are grouped in Class B. NMOs are single-component enzymes, where reduction occurs within a single polypeptide chain using an external reductant such as NADPH. NMOs typically perform a single-hydroxylation of an amino acid and are implicated in both virulence and natural product biosynthesis. Recently, NMOs that catalyzed multiple hydroxylations, on the same N-atom have been identified. CreE is responsible for the multiple-hydroxylation of L-aspartic acid to nitrosuccinate (Scheme 1A). PcxL also catalyzes a multiple-hydroxylation from 2-aminoethyl phosphonic acid to 2-hydroxyiminoethyl phosphonic acid through multiple rounds of oxidation. CreE is part of the cremeomycin biosynthetic pathway and PcxL, the phosphonocystoximate pathway. These natural products have been shown to have antimicrobial, antifungal and anticancer activities. The observed multiple oxidation reaction and the medical application of NMOs make these enzymes important targets for mechanistic and structural studies.

Bioinformatic analyses show that PcxL and CreE share low sequence identity between them and other class B enzymes. Potentially, these enzymes perform multiple N-hydroxylations utilizing new chemistry and kinetic mechanisms. Thus far, we have successfully expressed and purified both recombinant CreE and PcxL. Steady-state and pre-steady state kinetics have been elucidated using an oxygen consumption assay and anaerobic stopped-flow spectroscopy. Our preliminary results suggest that both enzymes have a high dissociation constant for NADPH, leading to a slow flavin reduction. Unexpectedly, this slow reduction does not match the steady-state kinetics displayed by both enzymes. However, thermal shift experiments revealed that both CreE and PcxL undergo favorable conformational changes when reduced and bound to NADP$^+$ as indicated by an increase in melting temperature. Detailed characterization of the catalytic cycle will be presented including both half reactions and kinetic isotopic effects.

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In previous research, through measurement of torsional values in Protein Data Bank files of enzyme/protein-inhibitor complexes and averaging those values to estimate a mean conformation, subtilisin and trypsin were found to favor extensive alignments of atomic orbitals in their active-serine-bearing strand and in the bound inhibitor strand. It was also shown that long oligopeptide esters are significantly more reactive in chemical acyl-transfer than short ones.

Now, chymotrypsin has been analyzed similarly and found to have extensive orbital alignments similar to those of both systems. Seven files of chymotrypsin/inhibitor complexes were analyzed; the selected files were all of resolutions at or below 2.5 angstroms. As with the subtilisin system, the mean conformation of the bound inhibitor strand shows orbital alignments from the P$_2$’ to P$_4$ positions. At least four extensive alignments of orbitals are evident from the research. The alignments are located in the following places: upstream and downstream of the targeted linkage in the enzyme-bound loop, from serine 195 to glycine 196, and from serine 195 to proline 198. All four alignments converge at the targeted linkage of the bound strand, as they also do in the trypsin and subtilisin systems. These orbital alignments may help to induce and optimize the charge dynamics of acyl-transfer during proteolysis. In serving that role, the alignments would also be consistent with the known kinetic substrate-length dependences of all three enzymes.

This research and the previous results suggest that there is a kinetic benefit for enzymes and substrates to engage in extensive orbital alignments during proteolysis. The breadth of these findings predicts that such orbital alignments will also be present in other kinds of enzyme/substrate complexes.
Structure-guided antiviral design of SARS-CoV-2 main protease using X-rays, neutrons, and supercomputers

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SARS-CoV-2 infection leads to COVID-19 which has resulted in a once a century global health and economic catastrophe claiming almost 2.5 million lives worldwide in a year. The viral main protease (Mpro) is an essential enzyme for SARS-CoV-2 replication and thus makes an attractive target for small-molecule drugs. Structure-guided drug-design strategies typically base atomic scale understanding of enzymes from cryogenic synchrotron X-ray diffraction. Conventional protein X-ray crystallography studies are hindered by cryo-artifacts and the inability to measure protonation states. Neutrons provide an ideal probe to determine protonation states of ionizable residues, such as the Cys-His catalytic dyad of Mpro, at near-physiological temperatures. This series of studies follows efforts to generate the large protein crystals required for neutron diffraction.

Pre-requisite X-ray experiments rapidly produced insights contributing to drug-design and clinical inhibitor repurposing efforts in the early days of the pandemic. The catalytic cysteine can be trapped in the rare peroxysulfenic acid oxidation state at physiological pH while surface cysteines remain reduced signifying high reactivity of the catalytic cysteine. Ligand-free Mpro compared with low-temperature structures demonstrates an inherent structural plasticity in the active site suggesting the room-temperature model is a relevant template for in silico docking studies. Clinical HCV protease inhibitors narlaprevir, boceprevir, and telaprevir are low-micromolar inhibitors of SARS-CoV-2 Mpro by binding to the active site cavity. Structural comparisons indicate the malleability of active site enables adaptation to the size of inhibitor moieties.

The SARS-CoV-2 Mpro neutron crystal structure was determined, providing the direct observation of protonation states of the active site in a cysteine protease for the first time. The catalytic Cys-His dyad of Mpro exists in the reactive zwitterionic state at rest, with charged thiolate and doubly protonated imidazole side chains. A second neutron structure reveals the overall +1 charge of the active site is maintained in a covalent inhibitor complex via rearrangements of protonation states.

Finally, experimental validation of a lead inhibitor identified out of a multimillion compound supercomputer docking screen will be described. This research is providing real-time data for atomistic design and discovery of Mpro inhibitors to combat the COVID-19 pandemic and prepare for future coronavirus pathogens.


Kinetic Modelling of Cephalexin Synthesis by α-Amino Ester Hydrolase (AEH) from Xanthomonas campestris pv. campestris: How Substrate Inhibition Affects Reactor Design

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β-lactam antibiotics, especially those derived from penicillins and cephalosporins, are continually the most prescribed antibiotics in the world [1]. Semi-synthetic β-lactam antibiotics can be produced enzymatically as an environmentally conscious and cost-effective alternative to traditional chemical synthesis [2]. The enzymatic reaction couples an activated acyl side chain such as D-phenylglycine methyl ester (PGME) or p-hydroxyphenylglycine methyl ester (HPGME) with a β-lactam nucleus such as 6-aminopenicillanic acid (6-APA) or 7-aminodesacetoxycephalosporanic acid (7-ADCA). Different combinations of these moieties produce unique antibiotics (cephalexin from 7-ADCA and PGME, amoxicillin from HPGME and 6-APA, ampicillin from PGME and 6-APA, and cefadroxil from 4-HPGME and 7-ADCA).

α-Amino ester hydrolases (AEHs) comprise a small class of enzymes capable of enantioselective production of semi-synthetic β-lactam antibiotics and are a potential alternative to the industrially used penicillin G acylases. Despite their rapid kinetics and high selectivity, AEHs have had limited use primarily due to their low stability and rapid deactivation [3]. In addition, although AEH substrate specificity and reactivity has been thoroughly studied [4-6], the full kinetic mechanism of AEH catalyzed synthesis of β-lactam antibiotics has yet to be fully realized. While the general mechanism for β-lactam antibiotic synthesis by Youshko and Svedas [7] is applicable under low reactant concentrations, we demonstrate experimentally that AEH suffers from substrate inhibition which has not been shown previously.

Based on our experimental findings, we present a new kinetic model herein to fully describe the AEH catalyzed synthesis of cephalexin which includes the synthesis pathway as well as reactant hydrolysis (primary hydrolysis) and product hydrolysis (secondary hydrolysis) pathways also catalyzed by AEH. More specifically, we demonstrate substrate inhibition by PGME as well as byproduct inhibition by phenylglycine and the impact on cephalexin synthesis rate and selectivity. Based on this newly derived model, we predict the optimum reactor conditions for efficient synthesis of cephalexin on a pilot plant scale using AEH.

Evaluating the function of tryptophans near KatG’s heme-dependent active site

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A common defense of higher eukaryotes against invading pathogens is to generate H$_2$O$_2$ as a precursor to highly microbicidal agents like HOCl. Many bacterial and fungal pathogens use catalase-peroxidase (KatG) as a central defense against host-derived peroxide stress. KatG is a bifunctional heme-dependent enzyme known for its robust ability to decompose H$_2$O$_2$ by catalase and peroxidase mechanisms. KatG has a remarkably large number of oxidizable amino acids, and these are concentrated in the N-terminal domain which contains the enzyme’s sole active site. Indeed, 4.3% of the residues in the M. tuberculosis KatG N-terminal domain are tryptophans. This is fourfold greater than a typical protein. It has been hypothesized that these participate in off-catalase electron transfer; reactions that may serve to protect KatG from inactivation. By proximity to one another, these oxidizable amino acids divide into sectors, each of which has a representative in close proximity to the active-site heme center. These include Tryptophans 91, 321, and 412. To investigate the role that these tryptophans may play in off-catalase electron transfer and in KatG catalysis in general, we have generated KatG variants that replace each of these tryptophans with a non-oxidizable phenylalanine (i.e., W91F, W321F, and W412F KatG). Each of these proteins has been successfully expressed and purified in two forms: one in which heme has been incorporated during expression, and a second where KatG, expressed in its apo-form, is reconstituted with heme during purification. We refer to these forms as holo and reconstituted and designate them as hKatG and rKatG, respectively. Each of the variants in its r- and hKatG forms showed robust catalase activity similar to the wild-type enzyme. The rKatG forms of each variant reacted with H$_2$O$_2$ with second-order rate constants near 2 $\times$ 10$^6$ M$^{-1}$s$^{-1}$, but only rW321F KatG accumulated an intermediate resembling Fe$^{IV}$=O[porphyrin]$^+$ (i.e., compound I) prior to the appearance of a Fe$^{IV}$=O (i.e., compound II-like) state. For rW91F and rW412F only the Fe$^{IV}$=O (i.e., compound II-like) intermediate was observed. The catalase-dependent extent of O$_2$ production by all three variants showed that each one was inactivated prior to the full consumption of H$_2$O$_2$, but W321F KatG generated one-half the O$_2$ observed for W91F and W412F KatG, suggesting a greater propensity toward H$_2$O$_2$-dependent inactivation. Our results suggest that W321 may have a particular role to fill in KatG function, helping to prevent inactivation of the enzyme by directing off-catalase electron transfer events.
Poster Abstracts

Poster Session 3
(2:40 pm – 3:25 pm)
Evidence of Negative Cooperativity in Cytosolic Taurocyamine Kinase from Arenicola brasiliensis and its Implication in the Evolution of the Phosphagen Kinase Family

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Phosphagen kinases (PKs) play an important role in energy homeostasis by catalyzing the reversible phosphoryl transfer reaction between MgATP and a specific guanidinium substrate. One interesting structural feature is the occurrence of some PK dimers that exhibit negative cooperativity between the subunits in the binding of its substrates. Since negative cooperativity was observed in some creatine kinase (CK) derived PKs, it was thought that PKs proposed to have diverged from CK-ancestors would exhibit cooperativity. However, negative cooperativity was not present in an annelid glycocyamine kinase (GK)3, which has been proposed to also derive from a CK ancestor. This observation complicates the evolutionary relationship between the annelid specific PKs and other CK-derived PKs, both of which are proposed to have a common CK ancestor. An equally intriguing question concerns the origin and divergence of negative cooperativity within the PK family.

To better understand the evolutionary context for this negative cooperativity, a recombinant form of another annelid PK, cytosolic taurocyamine kinase (CytoTK) from Arenicola brasiliensis, was purified and examined for negativity cooperativity in forming a quaternary transition state analogue complex (TK – MgADP – NO₃⁻ – taurocyamine), where nitrate effectively mimics the phosphoryl-group in transition between the two substrates. Fluorescence quenching of the CytoTK saturated with taurocyamine and nitrate by added MgADP determined an overall dissociation constant ($K_d^{MgADP}$) of 70 ± 2 μM. The change in the extent of quenching by added nucleotide was observed to depend non-linearly on the taurocyamine concentration, suggesting that the two subunits were not forming the same complex with MgADP. A similar experiment, done instead with isothermal titration calorimetry to measure the binding of MgADP to TK saturated with nitrate and taurocyamine, provides direct evidence of two distinct sites with each site showing a different affinity for MgADP: $K_{d1} = 5.2 (± 1.3) μM$ and $K_{d2} = 470 (± 110) μM$. The first $K_d$ likely reflects MgADP dissociation from the more tightly bound TSAC, whereas the second $K_d$ likely reflects MgADP dissociation from a binary $E^{MgATP}$ complex. These results provide clear evidence of negative cooperativity in the binding of MgADP by the CytoTK.

Since other PKs that have shown negative cooperativity are thought to have evolved late from a CK ancestor, the observation of negative cooperativity in CytoTK suggests such character may have arisen late in CK evolution. Considering GK does not show negative cooperativity, GK and other annelid PKs might have diverged from CK rather early in comparison to CytoTK. Given that A. brasiliensis has a second, mitochondrial, TK, which has sequence similarity more closely related to the CKs, it will be interesting to determine whether the MiTK also displays cooperativity.

Mechanistic Studies using an Inactive F420-dependent Glucose-6-phosphate Dehydrogenase Variant from M. tuberculosis

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*Mycobacterium tuberculosis* (Mtb) is the causative agent of one of the deadliest diseases in the world, Tuberculosis (TB). F420-dependent Glucose-6-Phosphate Dehydrogenase (FGD) is a prime target for TB-related treatments because it is found within Mtb and utilizes the F420 cofactor, which is not found in humans. The conversion of glucose-6-phosphate (G6P) to 6-phosphogluconalactone and concurrent reduction of the F420 cofactor is carried out by FGD (Figure 1). Generally, F420-dependent enzymes have not been subjected to rigorous enzymological investigation until our work. Although several key pieces of information regarding FGD’s hydride transfer mechanism have been unraveled, the binding location of G6P within FGD’s active site has yet to be found. Past x-ray crystallographic studies of the Mtb-derived FGD were solved in the presence of the competitive inhibitor, citrate. To date, the position of the native substrate, G6P is unknown. Therefore, the objective of this project is to solve the crystal structure of the enzyme-substrate complex in the presence of F420 cofactor, or with the G6P analog, 1,5-anhydro-D-glucitol 6-phosphate. We have optimized crystallization conditions using the inactive FGD variant, H40A. Several conditions of interest, using the screens provided by Hauptman Woodward Medical Research Institute, will be discussed.

Figure 1. This figure delineates the conversion of G6P to 6 phosphogluconalactone and the concurrent reduction of the F420 cofactor using FGD.
Anaerobic Heme Degradation by E. coli O157:H7

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Often, iron acquisition drives bacterial virulence. In host environments, heme represents a viable iron source; consequently, many bacterial pathogens have adapted high-affinity heme acquisition systems. Targeting these pathways remains an active research area, yet studies focused towards heme utilization have overlooked an anaerobic pathway until the recent discovery of anaerobic heme degradation in enterohemorrhagic Escherichia coli.

Within the E. coli heme utilization (Chu) operon, ChuW, a Radical S-Adenosyl-L-Methionine (SAM) Methyltransferase, catalyzes the heme ring-opening reaction. Most radical SAM enzymes initiate catalysis through the reductive cleavage of SAM to produce L-methionine and a 5′-deoxyadenosine radical (5′-dAdo•), and all Radical SAM enzymes contain a [4Fe-4S] cluster responsible for the reductive cleavage reaction. ChuW catalyzes methyl addition to a methine bridge of heme, but further investigation is required to understand how this facilitates ring opening.

We have previously shown the heme-iron is not essential to catalysis using LC-MS and UV-Vis Spectroscopy. More recent work aims to characterize anaerobilin, the tetrapyrrole turnover product, more carefully using an engineered bacterial phytochrome. Crystallographic capture of the oxygen-sensitive product will provide further insight into the heme degradation mechanism. Towards characterizing ChuW structurally, limited proteolysis reveals a potential second domain which will be investigated further.

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Biochemical Characterization of the Radical SAM Methylase Involved in Tetrahydromethanopterin Biosynthesis

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Radical S-adenosyl methionine (SAM) enzymes utilize SAM and a [4Fe-4S] cluster to catalyze complex radical reactions throughout various biochemical processes. Methylases are one of the largest subgroups of radical SAM enzymes, which add a methyl group to otherwise unreactive substrates. Our previous work identified the radical SAM methylase, MjMptM, from Methanocaldococcus jannaschii, which likely catalyzes the addition of methyl groups to the C7 and C9 positions of tetrahydromethanopterin, a central C1-carrier cofactor in methanogenic archaea. MjMptM is a unique methylase since it contains multiple [4Fe-4S] clusters and our current experimental evidence indicates that it does not utilize SAM as the methyl group donor. Here, we heterologously expressed and purified MjMptM for in vitro enzymatic and spectroscopic studies. We demonstrated that the enzyme catalyzes two methylation reactions to produce a dimethylated folate species likely using methylenetetrahydrofolate as the C1 source for the methylation reactions. Site-directed mutagenesis and UV-Vis spectroscopic analysis indicate that MjMpt harbors three [4Fe-4S] clusters, two of which reside in the two canonical CX3CX2C radical SAM motifs in the N-terminus. Interestingly, our results indicate that both of the [4Fe-4S] clusters within radical SAM motifs catalyze the reductive cleavage of SAM to produce 5’deoxyadenosine, the key first step in radical SAM enzyme catalysis. The exact location and function of the third auxiliary cluster is yet to be determined, but we propose it is bound by conserved cysteine residues in the C-terminus. Current work is focused on understanding the role of this auxiliary cluster as well as details of the methylation reaction mechanism.

Inhibiting Heptosyltransferase I: Discovery of a first-in-class nanomolar inhibitor of a glycosyltransferase enzyme

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Gram-negative bacteria have been known to build biofilms to protect against environmental stressors and antimicrobial agents1. A major cell surface component that contributes to the network of intermolecular interactions in the biofilm is the lipopolysaccharide (LPS). The LPS is a large glycolipid moiety found on the exterior of the cell that is essential for adhesion to surfaces, which also impedes the penetration of hydrophobic antibiotics into the cell2. Biosynthesis of the LPS core region begins with a glycosylation reaction catalyzed by Heptosyltransferase I (HepI)3. Extensive research investigating the kinetics, dynamics, folding, thermal stability, intrinsic protein tryptophan fluorescence, and substrate binding of Hep I has been previously performed4, 5. This work laid the foundation for identifying, and characterizing inhibitors of Hep I. A series of compounds were identified by computational collaborators at the University of Minnesota, and have been tested as inhibitors of HepI. Through analysis of the kinetics of inhibition, all five compounds were shown to impair HepI catalysis and two of these inhibitors bind with nanomolar affinity to the enzyme. Previous studies demonstrated that conformational changes induced by the substrate can impact thermal stability and intrinsic tryptophan fluorescence of HepI6. These inhibitors were investigated for their ability to alter these substrate-induced conformational changes, in addition to thorough investigation of their inhibition kinetics to allow determination of their mechanism of inhibition. In vivo growth challenge assays are underway to validate their ability to alter the growth of *Escherichia coli*. Extracts of LPS from these cell based studies are being analyzed by gel electrophoresis to further confirm cellular activity. Preliminary studies suggest cell death and truncation of the LPS result from Hep I inhibition, which demonstrates the feasibility of these compounds for a robust and targeted treatment of Gram-negative bacterial infections.

#30
Importance of a Glutamate Residue on Amine Oxidation in D-Arginine Dehydrogenase from Pseudomonas aeruginosa

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D-Arginine dehydrogenase from Pseudomonas aeruginosa (PaDADH; EC 1.4.99.6) is an FAD-dependent enzyme that catalyzes the oxidative deamination of D-arginine to α-ketoarginine and ammonia.1 PaDADH has broad substrate specificity being able to oxidize all D-amino acids except D-aspartate and D-glutamate.1 The three-dimensional structure of PaDADH shows four loops (L1, L2, L3 and L4) that are part of the active site.1 Molecular dynamics studies of loop L1 were consistent with residue Y53 adopting open and closed conformations to allow substrate access to and product egress from the active site.2 Loop L2, comprised of residues 244-248, is positioned at the entrance of the active site pocket. In loop L2, only E246 points toward the active site and interacts with the guanidinium moieties of the iminoarginine product and R222, whose guanidium group stacks that of R305. Thus, E246 might contribute to stabilizing the overall net charge of the active site residues to optimize catalysis in PaDADH.

In this study, site-directed mutagenesis was used to generate the E246L, E246G, and E246Q variant enzymes to investigate the role of the E246’s negative charge in the enzyme. The steady-state kinetics with D-arginine as substrate for the E246G/Q/L enzymes showed that the $k_{cat}$ values increased by 2- to 4-fold with respect to the wild-type enzyme; however, the $k_{cat}$ values were 2-fold lower with D-leucine as substrate. The $k_{cat}/K_m$ values with both substrates were 2- to 4-fold lower compared to the wild-type enzyme. The results are consistent with the negative charge of E246 participating in the stabilization of the electrostatic network of the active site residues for substrate accommodation and catalysis. The kinetic implications of the variant enzymes with D-arginine and D-leucine as substrates will be discussed.

The flavoenzymes are involved in a wide range of biological processes. However, the reaction mechanism of many flavoenzymes is still poorly understood. Fourier-transform infrared difference spectroscopy (FTIR DS) is an important tool for obtaining detailed mechanistic information such as changes in the flavin’s intermolecular interactions with nearby amino acids. However, interpreting FTIR DS experiments would require understanding how hydrogen bonding, electrostatics, and other intermolecular interactions affect normal mode vibrational frequencies and intensities of molecular groups of the flavin isoalloxazine ring. To start to address this issue, we have modeled the effect of hydrogen bonding interactions on prominent vibrational modes in 1450 – 1800 cm\(^{-1}\) range, where intense \(\nu_{C=C}\), \(\nu_{C=N}\), \(\nu_{C=O}\), and \(\nu_{C_4=0}\) stretching modes of flavin’s isoalloxazine ring are found. The computations indicate that \(\nu_{C=C}\) and \(\nu_{C=N}\) mode frequencies are not sensitive to any hydrogen-bonding interactions, but their relative intensities are affected by the solvent’s dielectric environment. On the other hand, while the \(\nu_{C_2=0}\) and \(\nu_{C_4=0}\) mode frequencies are strongly downshifted by direct (both \(\nu_{C_2=0}\) and \(\nu_{C_4=0}\)) and indirect (only \(\nu_{C_2=0}\)) hydrogen-bonding interactions, their relative intensities are less sensitive to the dielectric environment. The calculated vibrational mode frequencies agree with experimentally determined frequencies in solution when cluster models with multiple water molecules are used. The peak at ca. 1624 cm\(^{-1}\) that is typically assigned to the \(\nu_{C_2=0}\) vibrational stretching mode has a complicated shape that suggests multiple underlying contributions. Indeed, our calculations show that this band has contributions from both the \(C_6-C_7\) and \(C_2=O\) stretching vibrations.
Mechanistic Study of a Radical SAM GTP 3’,8-cyclase MoaA in Molybdenum Cofactor Biosynthetic Pathway

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Molybdenum cofactor (Moco) is a ubiquitous cofactor essential for lives of most organisms. In humans, Moco is essential for healthy development of brain, and lack of ability to produce Moco causes a fatal Moco deficiency (MoCD) disease. Moco biosynthesis is initiated by a unique transformation from GTP to cyclic pyranopterin monophosphate (cPMP). In bacteria, two enzymes, MoaA and MoaC, are responsible for this conversion. MoaA is one of the founding members of the radical S-adenosyl-L-methionine (SAM) superfamily, harbouring two catalytically essential [4Fe-4S] clusters. Previously, our lab has identified that MoaA catalyzes the transformation of GTP into a unique intermediate, 3’,8-cyclo-7,8-dihydro-GTP (3’,8-cH2GTP), by an as yet poorly understood free-radical mediated C-C bond formation mechanism. Here, we report our detailed characterization of MoaA focused on the mechanism of the chemically challenging 3’,8-cyclization reaction. By using electron paramagnetic resonance (EPR) spectroscopy, we identified an organic radical species, 5′-deoxyadenos-4′-yl radical (5′-dA-C4′•), accumulated during MoaA catalysis. Characterization of this radical species allowed us to propose the kinetic model of MoaA catalysis and provide the first insights into the kinetic landscape of the radical mediated C-C bond formation in MoaA. We also evaluated the rate acceleration effects by the enzyme and proposed that R17 stabilizes the transition state by interacting with 3’-OH of GTP. These studies provided insights into the understanding of the mechanism of radical SAM enzymes and will contribute to the enzymological understanding of Moco biosynthesis and rational development of MoCD therapeutics.

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The structure and mechanism of D-glucosaminate-6-phosphate ammonia-lyase: A novel octameric assembly for a pyridoxal-5’-phosphate dependent enzyme, and unprecedented stereochemical inversion in the elimination reaction of a D-amino acid

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D-Glucosaminate-6-phosphate ammonia-lyase (DGL; EC 4.3.1.29) is a pyridoxal-5’-phosphate (PLP) dependent enzyme that produces 2-keto-3-deoxygluconate-6-phosphate (KDG-6-P) in the metabolism of D-glucosaminic acid by Salmonella enterica serovar typhimurium (1, 2). We have determined the crystal structure of DGL by SAD phasing with selenomethionine to a resolution of 2.58 Å. The sequence has very low identity with most other members of the aminotransferase (AT) superfamily. The structure forms an octameric assembly as a tetramer of dimers that has not been observed previously in the AT superfamily. PLP is covalently bound as a Schiff’s base to Lys-213 in the catalytic dimer at the interface of two monomers. The structure lacks the conserved arginine that binds the α-carboxylate of substrate in most members of the AT superfamily. However, there is a cluster of arginines in the small domain that likely serves as a binding site for the phosphate of the substrate. The deamination reaction performed in D2O gives a KDG-6-P product stereospecifically deuterated at C-3; thus, the mechanism must involve an enamine intermediate that is protonated by the enzyme before product release. NMR analysis demonstrates that the deuterium is located in the pro-R position in the product, showing that the elimination of water takes place with inversion of configuration at C-3, unprecedented for a PLP-dependent dehydratase/deaminase. Based on the crystal structure and the NMR data, a reaction mechanism for DGL is proposed.

Figure 1. Left, the reaction of DGL. Right, the structure of DGL.

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Zn$^{2+}$ Activates *Pseudomonas aeruginosa* D-2-Hydroxyglutarate Dehydrogenase and Enhances Flavin Reduction

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*Pseudomonas aeruginosa* D-2-hydroxyglutarate dehydrogenase (PaD2HGDH) oxidizes D-2-hydroxyglutarate to 2-ketoglutarate in the L-serine biosynthesis pathway that is necessary for *P. aeruginosa* survival.$^{1,2}$ PaD2HGDH is a potential therapeutic target against *P. aeruginosa*.$^3$ Besides D-2-hydroxyglutarate, the enzyme also oxidizes D-malate with a $k_{cat}$ value of 24 s$^{-1}$ at pH 7.4 and 25 °C.$^3$ Inductively coupled plasma mass spectrometry (ICP-MS) analysis of the purified enzyme after recombinant expression in Rosetta (DE3) pLysS showed that the active enzyme contained 0.2 mol/mol Fe$^{2+}$, 0.5 mol/mol Zn$^{2+}$, and 2.5 mol/mol Mg$^{2+}$. Treatment with EDTA yielded inactive enzyme with a 2.0 mol/mol ratio of Mg$^{2+}$, 0.08 mol/mol of Zn$^{2+}$, and 0.07 mol/mol of Fe$^{2+}$, consistent with either Zn$^{2+}$ or Fe$^{2+}$ being required for enzyme activity and Mg$^{2+}$ being a potential structural component of the enzyme. Enzyme activity assays with 5 mM D-malate as a substrate at pH 7.4 showed that 100 mM Zn$^{2+}$ activated the enzyme by 6-fold, 100 mM Mg$^{2+}$ had no effect, and 100 mM Fe$^{2+}$ inhibited the enzyme by 2-fold. Initial rates of reactions with D-malate and PMS at pH 7.4 were the same in the presence of Zn$^{2+}$ between 2.5 mM and 100 mM, suggesting a tight affinity of the enzyme for Zn$^{2+}$. Upon purifying and storing PaD2HGDH in the presence of 10 mM Zn$^{2+}$, the $k_{cat}$ and $K_m$ values at pH 7.4 were 39 s$^{-1}$ and 5.7 mM, respectively. The rate constant for flavin reduction, $k_{red}$, and the $K_d$ for D-malate were determined at pH 7.4 in a stopped-flow spectrophotometer, with values of 60 s$^{-1}$ and 4 mM, respectively. The data are consistent with PaD2HGDH being a zinc-dependent flavoprotein, for which the chemical step of D-malate oxidation and flavin reduction is almost fully rate-limiting for enzyme turnover at pH 7.4.


Synthesis and Validation of Substrates for PRMT1 using Plate-Based Screening Assay.

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PRMT (Protein Arginine Methyltransferase) is a mammalian enzyme that catalyzes methylation of arginine residues in a polypeptide chain. PRMT is categorized as 3 different types. The methylation can occur as asymmetric dimethylation (ADMA, PRMT 1, 2, 3, 4, 6, and 8), symmetric dimethylation (SDMA, PRMT 7, 5 and 9) or monomethylation (MMA, PRMT 7), Type I, II and III respectively. PRMT1 generates ADMA on arginine residues of the Histone H4 N-terminal tail, which can lead to transcription of cancer-related genes. Alternatively, PRMT5 can modify the same arginine residue to produce SDMA, which represses the development of those same cancer-related genes. A better understanding of the substrate specificity of these enzymes can assist in the development of novel isozyme-specific pharmaceuticals.

To identify these differences, we synthesized a 96-well plate of peptides based on the Histone H4 N-terminal tail, screened them against PRMT1 using a screening method previously developed in the Knuckley lab. This medium-throughput screen identified 7 “hit” peptide sequences and consensus sequences based on the “hit” peptides were synthesized by solid-phase peptide synthesis. Each of these consensus sequences varied at the N-terminus, while retaining the more distal positive charges of H4-16 peptide. The peptides were validated using a MTase-Glo™ Methyltransferase Assay to determine if they were indeed substrates for PRMT1. The kinetic values indicate their efficiency as PRMT1 substrates and further investigations are being conducted to identify the differences in the substrate specificity regarding PRMT4 and PRMT5. These continued efforts will help us gain a better understanding of the role PRMT isozymes play in the onset of cancer, while assisting in the design of novel pharmaceuticals to battle this disease.

This study was supported in part by the UNF Chemistry Department 2020 Summer Research Fellowship Grant & the UNF Office of Undergraduate 2021 Research Grant.

2. Nguyen, Hao C.; Wang, Min; Salsburg, Andrew; Knuckley, Bryan; Development of a Plate-Based Screening Assay to Investigate the Substrate Specificity of the PRMT Family of Enzymes; ACS COMBINATORIAL SCIENCE. 2015 17(9):500-505
In vivo and in vitro studies of ErmE suggest three regions are critical for function in the RRAD protein family

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Erythromycin resistant methyltransferases (Erm) belong to the ribosomal RNA adenine dimethylases (RRAD) protein family. Erm proteins dimethylate A2058 of 23S ribosomal RNA via a SAM dependent methylation reaction. This methylation blocks binding of several classes of antibiotics, thus conferring a multidrug resistance phenotype known as MLS\(_b\)K named for macrolide, lincoasmide, streptogramin B, and ketolide antibiotics. \(^{(1)}\) Methyltransferase genes conferring multidrug resistance have been acquired by several pathogens, which represents a serious threat to public health. \(^{(2)}\)

ErmE is a model from the macrolide antibiotic producer, *Saccharopolyspora erythraea*. Guided by previous alanine-scanning mutagenesis of ErmC \(^{(3)}\), the crystal structure of ErmE \(^{(4)}\), and sequence conservation data among the family, we hypothesize three regions are crucial for ErmE function: the adenine pocket, the \(\alpha4\) cleft, and the basic patch. From the three regions, we have generated and characterized several site-directed mutants to test their role in the molecular mechanism. The mutant enzymes were assayed to determine the minimum inhibitory concentration (MIC) of erythromycin for each variant. Several of the sensitive strains were then purified to analyze methylation kinetics and RNA binding. The three functional regions establish a model that may be broadly applied to the RRAD family and allow for investigation of inhibitors of methyltransferases in pathogenic bacteria.

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Characterizing the isomerase and lyase mechanisms of MST enzymes

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Bacteria, fungi and plants use the shikimate pathway to generate chorismate. Chorismate is the branch point for biosynthetic pathways that generates aromatic amino acids, folate, quinones, and siderophores. Humans do not have these biosynthetic pathways, and acquire these essential metabolites from the diet. Therefore, the enzymes of the shikimate pathway and those required for the production of metabolites derived from chorismate are important targets for herbicides and antimicrobials. The MST enzymes (menaquinone, siderophore, tryptophan) from Escherichia coli, Pseudomonas aeruginosa, Yersinia spp., and Mycobacterium tuberculosis are structurally homologous, magnesium-dependent, chorismate-utilizing enzymes that initiate the pathways at the chorismate branch point. Evidence suggests that the general acid and base residue, which are responsible for catalyzing chorismate isomerization to isochorismate, exist in reverse protonation states. To investigate whether the required magnesium is responsible for inducing the reversed protonation states, we introduced histidine in place of neutral amino acids in the active site. These mutations will be tested to determine whether catalysis is possible in the absence of magnesium through the introduction of positive charges. As well as isomerizing the chorismate ring, a subset of these MST enzymes from Yersinia spp., and M. tuberculosis have lyase activity to cleave pyruvate from the chorismate ring. The chemical mechanism of lyase activity is still debated. Interestingly, P. aeruginosa requires a second non-MST enzyme of the AroQ chorismate mutase structural family to perform the lyase activity. To resolve the mechanistic ambiguities for two enzyme classes of distinct structural classes that perform identical chemical reactions, we will measure kinetic isotope effects for the lyase reaction using isotopically labelled substrates. By reconstituting the shikimate pathway in vitro we will produce differentially deuterated chorismate and isochorismate for these kinetic studies.
Poster Abstracts

Poster Session 4
(3:26 pm – 4:10 pm)
Riboflavin Biosynthetic Pathway: Unexpected Catalytic Characteristics of GTP Cyclohydrolase II from Escherichia coli

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The first step in the riboflavin biosynthetic pathway is the conversion of guanosine triphosphate (GTP) to 2,5-diamino-6-ribosylpyrimidinone 5′-phosphate (DARPribose) and is catalyzed by the enzyme GTP cyclohydrolase II (RibA). RibA catalyzes the removal of a C8 methylene from the guanosine purine ring and the cleavage of the distal phospho group, resulting in the release of formate, pyrophosphate (PPi) and DARPribose (1, 2). A byproduct of the RibA reaction is guanosine monophosphate (GMP). HPLC analysis of the RibA reaction mixture was used to determine that ~10% of the total GTP concentration is converted to the GMP byproduct. As GMP is not a substrate of RibA, it is proposed that the hydrolysis of the pyrophosphate must occur prior to the deformylation and that the initial reaction step results in the formation of a covalently tethered enzyme-GMP complex (2). Kinetic experiments were performed to probe the individual steps of each catalytic activity using the WT enzyme and three RibA variants: two adjacent to the Zn ion and associated with the deformylation activity (K101M and Y105F) and one adjacent to a Mg ion and thought to be relevant to the formation of the enzyme-GMP covalent complex (R128K) (2). R128K does not consume GTP, while K101M and Y105F convert all available GTP to GMP. Using stopped-flow spectrophotometry, K101M and Y105F were analyzed under transient-state conditions. Both reveal several phases when observed at 252 nm, a wavelength which reports changes in the guanine spectrum. A titration of K101M with PPi, with and without GTP, revealed that similar spectrophotometric changes occur for both, implicating conformational changes of the enzyme as the source of the transient phases observed. We proposed that RibA undergoes conformational changes in response to binding GTP that can be induced also with PPi. Our kinetic analysis would argue that prior reports did not observe an intermediate species specifically related to the guanosine spectrum but is instead spectrophotometric changes associated with the enzyme’s absorption spectrum. When titrating PPi with a high concentration of the RibA WT enzyme, we also observed triphasic kinetics. Unexpectedly, all traces appear to return to their initial absorption rather than coming to an equilibrium, suggesting that along with binding the PPi, RibA is also exhibiting pyrophosphatase activity. We have utilized the enzyme Purine Nucleoside Phosphorylase (PNP) and the guanosine analogue 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to verify the release of phosphate (Pi) from the RibA reaction (3).

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Characterization of a nitro-forming enzyme involved in fosfazinomycin biosynthesis

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N-hydroxylating monooxygenases (NMOs) are a well-studied subclass of flavin-dependent monooxygenases that hydroxylate alkyl amines. Recently, NMOs that are involved in the formation of nitro-functional groups have been identified (1). One such NMO is FzmM, which is involved in fosfazinomycin biosynthesis in some Streptomyces sp (Scheme 1)(2,3). Reported here is the first kinetic analysis of a nitro-forming NMO, focusing on the first catalytic turnover performed by FzmM. Steady-state kinetic analyses show that FzmM performs a highly coupled reaction with Aspartate (Asp) ($k_{cat}$, $3.0 \pm 0.01 \text{s}^{-1}$) forming nitrosuccinate that can be converted to fumarate and nitrite by FzmL, the next enzyme in the pathway. FzmM displays a 70-fold higher $k_{cat}/K_M$ value for NADPH compared to NADH. It has a narrow range of optimal pH (7.5-8.0) and is not affected by solvent viscosity showing that molecule diffusion is not rate-limiting. The kinetic mechanism of FzmM was studied using rapid-rate analysis. Contrary to other NMOs where the $k_{red}$ is rate-limiting, the $k_{red}$ for FzmM is $>100 \text{s}^{-1}$ with NADPH at room temperature. NADPH binds at a $K_D$ value of $\sim400 \mu\text{M}$ and hydride transfer occurs with pro-R stereochemistry. Oxidation in the absence of Asp displays a spectrum with a shoulder at $\sim370 \text{nm}$ consistent with the formation of C(4a)-hydroperoxyflavin intermediate which decays into oxidized flavin and water much slower that the $k_{cat}$. In the presence of Asp, the oxidation is enhanced with a $k_{ox}$ similar to the $k_{cat}$. Flavin dehydration is the rate limiting step in the catalytic cycle.

This study was supported by in part by Grant NSF-CLP 2003658 from the NSF (P.S)

Scheme 1: (A) Structures of fosfazinomycin A/B (1). (B) FzmM performs a six electron oxidation on L-aspartate (Asp) with NADPH and oxygen as substrates to produce nitrosuccinate (2). Nitrosuccinate is then converted to nitrite and fumarate by the fumarase-like enzyme FzmL (2,3). The nitrogen labeled red represents the nitrogen incorporated into fosfazinomycin A/B.

References
Mechanistic Strategies and Conformational Changes in the Mobile Loop of the two-component FMN-dependent alkanesulfonate monooxygenase system

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The two-component alkanesulfonate monooxygenase system, consisting of a flavin reductase (SsuE) and an alkanesulfonate monooxygenase (SsuD), enables bacterial organisms to utilize a broad range of alkanesulfonates when sulfur is limiting. SsuE supplies reduced flavin to SsuD for the desulfonation of sulfonated compounds through the formation of a flavin oxygenating intermediate. Commonly, flavin monooxygenases utilize a C4a-(hydro)peroxyflavin as the oxygenating intermediate. However, unlike other flavin monooxygenase enzymes, this intermediate has not been spectrally observed in SsuD. Recent studies reported the formation of a flavin-N5-oxide which forms as an intermediate during turnover or as the final product in some flavin monooxygenases. All SsuD homologs have a TIM-barrel structure as well as an insertion region located over the active site. This dynamic loop region is proposed to stabilize reduced flavin as well as flavin oxygenating intermediates by excluding bulk solvent from the active site.

Molecular dynamics simulations and site-directed mutagenesis were performed to examine different conformations of the mobile loop in the presence of reduced flavin, the C4a-peroxyflavin intermediate, and octanesulfonate. The findings from these studies revealed that substrate-free SsuD possessed an open conformation, whereas binding of reduced flavin resulted in a closed conformation thereby preventing unproductive oxidation reactions. Two salt bridges, Asp111-Arg263 and Glu205-Arg271 were formed in the SsuD closed conformation. SsuD variants generated to disrupt the observed salt bridges (R263A, R271A and R263A/R271A SsuD) had similar activity as wild-type; however, R271A SsuD was found to be more susceptible to proteolytic digestion in the presence of reduced flavin. Substitution of Arg271 with alanine resulted in more open conformation leading to increased proteolysis. A semi-closed conformation was observed when both reduced flavin and octanesulfonate were bound to SsuD. This conformation would be suitable for molecular oxygen to enter the active site to promote oxygenolytic cleavage through the formation of a putative flavin-N5-oxide.

Previous studies identified amino acid residues that were found to be critical for the formation and stabilization of the flavin-N5-oxide. Some of these residues are conserved in other bacterial flavin monooxygenases including SsuD, and therefore we hypothesize that SsuD employs a flavin-N5-oxide during catalytic turnover. The conserved residues Leu48, Ala77, Val108, Gly117, and Leu195 found in SsuD have been proposed to control the interaction of flavin-N5 with molecular oxygen, whereas the conserved Asn106 residue stabilizes the formation of the N5-peroxyflavin through hydrogen bonding. The role of these residues was further examined through site-directed mutagenesis. The V108T SsuD variant had similar activity to wild-type, but there was no measurable activity observed with the N108T SsuD variant. These results support the role of Asn in stabilizing the proposed N5-peroxyflavin intermediate. Mass spectrometry and stopped-flow kinetic analyses were also performed in order to further examine the formation of flavin intermediates during catalytic turnover. The results presented in this study highlight the importance of the dynamic loop in protecting the unstable intermediates formed by SsuD during catalytic turnover.

This study was supported in part by Grant 1808495 from NSF (HRE).

Quantitative proteomic studies have identified thousands of acetylation substrates and acetylation sites in both eukaryotic and prokaryotic cells, but our knowledge of the acetylation marks contributed by individual lysine acetyltransferases (KATs) is still far from being complete. A distinct example is that functional studies on the histone acetyltransferase 1 (HAT1), one of the first identified KAT members, have been largely focused on the nuclear histones. Limited information on the activities of HAT1 on non-histone substrates exists, thus greatly hinders our understanding of the functions of this important enzyme in regulating cellular physiology and pathology. Recently, acyl-CoA reporters have emerged as effective tools to label KAT substrates in complex proteomic settings, especially in extracted cell lysates. However, it was commonly believed that non-cell permeability of acyl-CoA molecules prevents their application for performing protein labeling in living cells. Here, we report that the clickable acyl-CoA analog, 3-azidopropanoyl CoA (3AZ-CoA), showed remarkable cell permeability and efficiency for in-cellulo protein labeling. We rationally engineered the active site of HAT1 to obtain a HAT1 mutant that showed excellent activity with 3AZ-CoA as a cofactor to effectively label HAT1 substrates. Importantly, we were able to conduct the HAT1 substrate labeling in the living cells. The reactive azide head group further allowed for the correlated protein substrates to be selectively pulled down following conjugation with alkyne-biotin through copper-mediated bioconjugation chemistry. Proteomic analysis revealed hundreds of novel HAT1 substrate candidates, which offers a new and comprehensive view of HAT1 functionality in mammalian cells. In light of this novel cell-permeable reporter, orthogonally matched with engineered enzyme forms, we envision that the bioorthogonal approach provides a powerful chemical biology tool for further identification of other KATs substrates in live cells.

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UV/vis Absorption Intensities: A Comparison of Theory and Experiment

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UV-vis spectroscopy is widely employed in enzymology to characterize biomolecules, particularly when they contain a conjugated co-factor (e.g., flavoproteins). The UV-vis absorption bands are typically characterized by their absorption wavelengths and their intensities. Absorption intensities in experiments are typically reported as extinction coefficients, while in computational chemistry they are more often reported as oscillator strengths. The two quantities are related. In this study, oscillator strengths are computed using approximate quantum mechanical calculations and compared with oscillator strengths derived from experimental UV-Vis spectra for 100 organic compounds. The widely used quantum chemical method, B3LYP, a density function theory (DFT) method with Hartree-Fock exchange, and the 6-31+G* basis set are employed to calculate oscillator strengths. Integration of systematically fitted Gaussian curves is used to derive the experimental oscillator strengths. By expressing experimental and computed intensities using a common unit, we are able to directly quantify the accuracy of quantum chemically computed oscillator strengths. Our goal is to determine if computed oscillator strengths provide a reliable predictions of oscillator strengths, and to estimate the typical error associated with these predictions.

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Heme is a critical biomolecule involved in gas transport, chemical catalysis, and electron transfer in the cell. Aminolevulinic acid synthase (ALAS) is a mitochondrial enzyme that catalyzes the first and rate-limiting step of heme biosynthesis. ALAS exists as two isoforms. These are a ubiquitously expressed isoform, ALAS1, and an erythroid-specific isoform, ALAS2. ALAS2 has different protein binding partners and different regulatory mechanisms than ALAS1. There are specific mutations in ALAS2 that result in the blood disease X-linked sideroblastic anemia, which is characterized by decreased ALAS2 activity resulting in decreased heme production. The disease-causing ALAS2 mutations in this study also disrupt the assembly of ALAS2 with the known binding partner succinyl-CoA synthetase (SCS), a TCA cycle enzyme. I define these mutations as disassembly mutants. It is unclear why these two proteins bind in the cell and why disassembly of the ALAS2-SCS protein complex leads to decreased ALAS2 activity and decreased heme production resulting in X-linked sideroblastic anemia. I hypothesize that SCS promotes ALAS2 activity through conformational changes, through altering enzyme kinetics or both. My recent data indicates that WT ALAS2 in vitro activity increases in the presence of SCS. Using biochemical assays and site-directed mutagenesis, I have characterized the activity and binding of WT and ALAS2 disassembly disease mutants with SCS. My results indicate that the S568G ALAS2 mutant exhibits decreased enzymatic activity compared to WT ALAS2, and SCS is unable to rescue this phenotype. I will unveil how SCS affects the individual steps of the ALAS2 catalytic cycle to explain how ALAS2-SCS disassembly results in X-linked sideroblastic anemia. The results will aid the development of more effective treatment options for patients harboring the ALAS2 mutations that disrupt assembly with SCS, which currently do not respond to standard X-linked sideroblastic anemia therapeutics.

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Biochemical Characterization of Aminomutases Involved in Salt Tolerance in Methanogenic Archaea

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Lysine-2,3-aminomutase (LAM) catalyzes the conversion of L-α-lysine to L-β-lysine, which entails the transfer of an α-amino group to the β-carbon alongside the migration of a β-hydrogen to the α-carbon. This enzyme plays a role in combating salt stress in methanogenic archaea by carrying out the first step in the biosynthesis of $N^\alpha$-acetyl-β-lysine, a well-studied osmolyte synthesized and accumulated in methanogens. LAM is of special interest as it belongs to the radical S-adenosyl-L-methionine (SAM) superfamily of enzymes which perform diverse and complex chemistry that often produces valuable compounds. Radical SAM enzymes utilize a reduced $[4Fe-4S]$ cluster to initiate the homolytic cleavage of SAM, yielding a highly reactive 5'-deoxyadenosyl radical that then abstracts a hydrogen atom from an otherwise unreactive substrate. Although the bacterial LAM involved in lysine degradation in Clostridium subterminale has been well-characterized, the archaean LAM involved in osmolyte biosynthesis has never been studied in vitro.

Here, we report on the recombinant expression, purification, and enzymatic properties of LAM from Methanococcus maripaludis. The gene encoding LAM from M. maripaludis C7 (MmarC7_0106) was cloned and the his-tagged protein was overexpressed in Escherichia coli. After purification by metal-affinity chromatography under strictly anaerobic conditions, the protein was brown in color, had UV-Vis spectrum characteristics of a $[4Fe-4S]$ cluster that was capable of being reduced to an active state, and had 3.9 mol of iron per monomer. A wide ranged temperature profile study revealed that enzymatic activity was highest at 37°C. The enzyme reaction in the presence of L-lysine, SAM, and dithionite at 37°C had a $K_{cat} = 0.42 \text{ s}^{-1}$ and a $K_m = 17.8 \text{ mM}$. Finally, alternative substrates for the aminomutation reaction were investigated.

There exists another gene annotated as LAM in M. maripaludis C7 due to its close sequence similarity with the bacterial LAM; however, this gene (MmarC7_1783) lacks lysine binding residues and has a 164 amino acid N-terminal extension and as such is referred to as a “long-LAM”. The presence of a “long-LAM” such as MmarC7_1783 in a methanogen is not uncommon as there are several annotated in thermophilic methanogenic species that are known to solely use β-glutamate, another beta-amino acid, as their osmolyte. Given that the only known pathway for the biosynthesis of β-glutamate is through the use of a glutamate-2,3-aminomutase (GAM) and that these thermophilic methanogens do not have an annotated GAM, we proposed that these long-LAMs were in fact GAM which we have now confirmed through heterologous expression studies. Thus, in addition to the biochemical characterization of an archaean LAM (MmarC7_0106) described above, we have determined that long-LAMs are in fact GAMs required in the biosynthesis of an alternative osmolyte used for survival in high salt concentrations by halotolerant methanogenic archaea.

Structural and Biochemical Characterization of the Flavin-Dependent Siderophore-Interacting Protein from *Acinetobacter Baumannii*

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*Acinetobacter baumannii* is an opportunistic pathogen with a high mortality rate due to multidrug-resistant strains. The synthesis and uptake of the iron chelating siderophores acinetobactin (Acb) and pre-acinetobactin (pre-Acb) have been shown to be essential for virulence. Here, we report the kinetic and structural characterization of BauF, a flavin-dependent siderophore-interacting protein (SIP) required for the reduction of Fe(III) bound to Acb/pre-Acb and release of Fe(II). Stopped-flow spectrophotometric studies of the reductive half-reaction show that BauF forms a stable flavin neutral semiquinone intermediate. Reduction with NAD(P)H is very slow ($k_{\text{obs}}$, 0.001 s$^{-1}$) and commensurate with the rate of reduction by photobleaching, suggesting that NAD(P)H are not the physiological partners of BauF. Reduced BauF was oxidized by Acb-Fe ($k_{\text{obs}}$, 0.02 s$^{-1}$) and oxazole pre-Acb-Fe (ox-pre-Acb-Fe) ($k_{\text{obs}}$, 0.08 s$^{-1}$), a rigid analogue of pre-Acb, at a rate 3-11 times faster than with molecular oxygen alone. The structure of FAD bound BauF was solved at 2.85 Å, and was found to share similarity to *Shewanella* SIPs. The biochemical and structural data presented here validate the role of BauF in *A. baumannii* iron assimilation and provide information important for drug design.

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Non-Canonical Use of Monocyclic β-Lactam Scaffolds as Folate Inhibitors Against Dihydrofolate Synthase

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In canonical β-Lactam antibiotics, such as penicillin, the strained nature of the β-Lactam ring leads to acylation of the catalytic serine in the active site of transpeptidases. In 2018, our lab reported a novel mechanism for monocyclic β-Lactam antibiotics in the inhibition of glutamine synthetase (GS) by a natural product, tabtoxinine-β-Lactam (TβL). In TβL, the 3-hydroxy moiety of the β-lactam ring is phosphorylated by GS, mimicking the tetrahedral transition state of GS with a stable tetrahedral species. TβL is a non-covalent inhibitor; whereas, β-lactam antibiotics are canonically covalent inhibitors. A question arises: can the 3-hydroxy-β-lactam warhead of TβL be generalized to act as a tetrahedral transition state mimic of ATP dependent carboxy-amine ligase enzymes.

The folate biosynthetic pathway is a clinically significant target for antibiotic activity, with dihydropteroate synthase (DHPS) / dihydrofolate deductase (DHFR) inhibitor combinations providing synergistic effects used to treat bacterial infections as a frontline antibiotic. In the folate biosynthetic pathway, DHPS and DHFR are the enzymes which act directly before and directly after dihydrofolate synthase (DHFS) respectively. DHFS is an ATP dependent carboxy-amine ligase enzyme which catalyzes the condensation of 7,8-dihydropteroate and L-Glu. We hypothesize that the 3-hydroxy-β-lactam warhead from TβL can be repurposed to form a non-covalent inhibitor of DHFS.

In this study we conceptualized retrosynthesis of a 7,8-dihydropteroate-like molecule with the 3-hydroxy-β-lactam in place of the terminal carboxylic acid. We preformed forward synthesis and achieved a pure 3-hydroxy-β-lactam warhead, which was both chemically bound and chemoenzymatically bound to different pteridine mimics. We tested these molecules against DHFS activity and discovered novel 3-hydroxy-β-lactam inhibitory activity. These findings represent a significant expansion of the role that β-lactam antibiotics play against the bacterial proteome.

Figure 1: Comparison of TβL activity and novel folate inhibitory activity

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The recent advancements of bioinformatic techniques have revealed a wealth of protein sequence-function information that has yet to be fully utilized for fundamental or biocatalytic applications. For example, ene reductases from the ‘old yellow enzyme’ (OYE) family catalyze the asymmetric trans-hydrogenation of activated C=C bonds with enhanced stereoselectivity. These are key transformations for the sustainable production of important pharmaceutical and industrial intermediates. Despite almost 90 years of study, the OYE family remains relatively underexplored with the majority of characterized OYEs displaying similar substrate profiles. Significant efforts have been directed to expand OYE activity. Here, we employ a platform of integrated bioinformatics and synthetic biology to systematically organize and sample the natural diversity of the OYE family. Using protein similarity networks, we broadly explored the known and unknown regions of the >70,000 members of the OYE family while also identifying phylogenetic and sequence-based trends. From this analysis, we characterized 118 novel enzymes across the family to greatly expand the biocatalytic diversity of OYEs: 11 enzymes exhibited enhanced catalytic activity or altered stereospecificity when compared to previously characterized OYEs. In addition, we discovered 4 OYEs capable of reverse, oxidative chemistry at ambient conditions. Our study expands the known functional and chemical diversity of OYEs while identifying superior biocatalysts for sustainable chemical processes.

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Structure-Based Design of Tetracycline Destructase Inhibitors

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Tetracyclines are an important class of antibiotics that are threatened by an emerging new resistance mechanism, enzymatic antibiotic inactivation. Tetracycline-inactivating enzymes, also known as tetracycline destructases (TDases), are class A flavin monooxygenases that catalyze hydroxyl group transfer and oxygen insertion reactions on tetracycline substrates. Semi-synthetic modification to the tetracyclic scaffold has proven effective in evading resistance mechanisms, such as ribosomal protection and efflux (1). The D-ring is of particular interest because of ease of semi-synthetic modification and increase in potency, as shown with tigecycline. One approach to overcoming enzymatic inactivation is co-administration of an adjuvant that inhibits the activity of TDases. Anhydrotetracycline (aTC) is an intermediate in tetracycline biosynthesis and was found to rescue the activity of tetracyclines in antibacterial assays against E. coli expressing TDases (2–4). The crystal structure of aTC bound to a TDase showed that the inhibitor forces the FAD cofactor out of the active site, stabilizing the inactive conformation (2). We hypothesized that modification of aTC aromatic substituents would provide π-π stacking with the FAD cofactor and stabilize the inactive out conformation. Here we report that aTC analogues modified with amides and amines at C9 of the D-ring serve as mechanism-based inhibitors of TDases. Systematic variation of D-ring aromatic substituents and use of a hydrogen peroxide detection assay provided insight into inhibitor binding mode, potency, and mechanism. Efficacy in whole cell rescue assays utilizing TDase-expressing E. coli was found to vary based on linker flexibility and distance of the substituted aromatic ring from the aTC D-ring. These findings allow for the rational structure-based design of new TDase inhibitors for use as adjuvants to prevent the enzymatic degradation of life-saving tetracycline antibiotics.

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A suicide diiron oxygenase in p-aminobenzoate biosynthesis in Chlamydia trachomatis

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Folate is an essential cofactor required for several processes including DNA and amino acid biosynthesis. Folate molecules are made up of three components: a pteridine ring, p-aminobenzoate (pABA), and a variable number of glutamate residues. Chlamydia trachomatis synthesizes folate de novo; however, several genes encoding enzymes required for the canonical folate biosynthesis pathway are missing, including PabA/B and PabC, which are normally required for pABA biosynthesis from chorismate. (1) Previous studies have found that a single gene in C. trachomatis, CT610, functionally replaces the canonical pABA biosynthesis genes. Interestingly, CT610 does not use chorismate as a substrate. (1,2) Instead, the CT610-route for pABA biosynthesis incorporates isotopically-labeled tyrosine into the synthesized pABA molecule. However, in vitro experiments revealed that CT610 produces pABA without any added substrates (including tyrosine) in the presence of a reducing agent and molecular oxygen. CT610 shares low sequence similarity to non-heme diiron oxygenases and the previously solved crystal structure revealed a diiron active site. (3) Taken together, CT610 is proposed to be a self-sacrificing “suicide” enzyme that uses one of its active site tyrosine residues as a precursor to pABA in a reaction that requires O2 and a reduced diiron cofactor. Here, we discuss our recent progress towards understanding CT610-catalyzed pABA synthesis. We have confirmed the oxygenase activity of CT610, which requires only a reducing agent and is not stimulated by the presence of tyrosine or other possible substrates. Further, we developed an in vitro Fe(II) reconstitution procedure, where the iron content of CT610 is increased from about 0.05 mol of iron per mol of protein in as-purified CT610 to around 1.5 mol of iron per mol of protein in the reconstituted protein, consistent with the expected diiron cofactor. The reconstituted enzyme exhibits a drastic increase in oxygenase activity. The rate of oxygen consumption of the reconstituted enzyme has proven to be up to 30 times greater than that of the freshly purified non-reconstituted enzyme.

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Pharmacophore-based screening of diamidine small molecule inhibitors for protein arginine methyltransferases

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Protein arginine methyltransferases (PRMTs) are essential epigenetic and post-translational regulators in eukaryotic organisms. Dysregulation of PRMTs is intimately related to multiple types of human diseases, particularly cancer. Based on the previously reported PRMT1 inhibitors bearing the diamidine pharmacophore, we performed virtual screening to identify additional amidine-associated structural analogs. Subsequent enzymatic tests and characterization led to the discovery of a top lead K313 (2-(4-((4-carbamimidoylphenyl)amino)phenyl)-1H-indole-6-carboximidamide), which possessed low-micromolar potency with biochemical IC50 of 2.6 μM for human PRMT1. Limited selectivity was observed over some other PRMT isoforms such as CARM1 and PRMT7. Molecular modeling and inhibition pattern studies suggest that K313 is a nonclassic noncompetitive inhibitor to PRMT1. K313 significantly inhibited cell proliferation and reduced the arginine asymmetric dimethylation level in the leukaemia cancer cells.

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