Welcome to the 13th Southeast Enzyme Conference

Saturday, April 22, 2023

Georgia State University

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Southeast Enzyme Conference (SEC)

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I	2010	Giovanni Gadda	Will Lovett	GSU
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X1	2021	Douglas Goodwin	Giovanni Gadda / Joanna A. Quaye / Daniel Ouedraogo / Kedayne King / Malin Frazel / Benjamin Yun-Pin Hsieh	Online
XII	2022	William Lanzilotta	Giovanni Gadda / Joanna A. Quaye / Bilkis Mehrin Moni/Bobby Sikri	GSU
XIII	2023	Kylie Allen	Giovanni Gadda / Joanna A. Quaye / Bilkis Mehrin Moni/Jessica Kugblenu	GSU

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Saturday, April 22, 2023

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Session 1:

Discussion Leader

Xu Liu *(Emory University)*

Talk 1

N-glycan degradation by Actinobacteria

Melanie Higgins[‡]

[‡]Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487

Glycans are the most abundant and diverse natural biopolymer and are involved in virtually all physiological processes, from structural roles to intrinsic and extrinsic recognition. They decorate a variety of proteins and lipids that are collectively referred to as glycoconjugates. N-Glycosylation is the most common form of protein glycosylation and occurs when an oligosaccharide is attached to the amide nitrogen of an aspargine residue and can play pivotal roles in many different functions. Microbes have evolved numerous mechanisms to depolymerize N-glycans for nutrients, colonization, and pathogenesis, with focus primarily on the mechanisms of human associated pathogenic and commensal bacteria. Interestingly, each bacterial species has a unique pathway to remove N-glycans from the glycoconjugate, import the oligosaccharides into the cell, and depolymerize the oligosaccharide into monosaccharides for energy generation. We recently discovered a family 5 subfamily 18 glycoside hydrolase (GH5_18) found in both gut- and soil-associated Actinobacteria that is involved in deglycosylating the N-glycan core.[(1)] Structure-function analysis of GH5_18 provided details on its specificity and active site architecture. Further bioinformatics analysis led to the discovery of distinct polysaccharide utilization loci (PULs) likely dedicated to importing and metabolizing N-glycans, suggesting diverse N-glycan degradation pathways between these Actinobacteria species.

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Talk 2

Development of Improved Glycosynthase Enzymes

***Tala Azzam*[1]**, *Jonathan J. Du*[1], *Eric J. Sundberg*[1]
[1]Department of Biochemistry, Emory University School of Medicine, Atlanta, GA

Understanding IgG-specific endo-β-N-acetylglucosaminidases (ENGases) enables chemoenzymatic remodeling of the N-linked glycans on the Fc region. These glycans are important for antibody-mediated effector functions such as antibody-dependent cell mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC).[(1)] Accordingly, they provide an important point of regulation that can be used to improve monoclonal antibody therapeutics. One such IgG-specific ENGase, EndoS2, acts on complex, high-mannose, and hybrid type glycans by cleaving between the 1st and 2nd core N-acetylglucosamine (GlcNAc) saccharides[(2)]. EndoS2 can be made into a glycosynthase by mutating one of the residues in the catalytic triad, D184, to a methionine. EndoS2$_{D184M}$ can transfer an oxazoline-linked glycan onto IgGs. However, this glycosynthase still retains some hydrolytic activity[(3)]. Thus, we sought to improve the glycosynthase function of EndoS2$_{D184M}$.

We applied our Specificity of Enzymatic Activity and Kinetics (SEAK) method to study the transglycosylation reactions of recombinantly expressed EndoS2$_{D184M}$, using deglycosylated Rituximab and oxazoline linked sialylated bi-antennary complex type (SCT) N-glycan (ox-S2G2) as substrates. We established that introducing seleno-methionine in the active site to produce EndoS2$_{D184SeMet}$ increases the rate of transglycosylation, while decreasing the residual hydrolysis rate. In addition, we found that mutations in a non-catalytic residue in the active site, I185, also affect glycosynthase activity. These effects are likely due to subtle conformational remodeling of the enzyme's active site that results in improved transglycosylation and reduced residual hydrolysis.

This study was supported by the NIH grant Rationalizing glycoengineering strategies for immunotherapeutic antibodies (R01AI149297).

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Talk 3

Total Turnover Number Prediction of an Aggregating Biocatalyst: amino ester hydrolase (AEH)

Colton E. Lagerman, *Janna K. Blum, Thomas A. Rogers, Martha A. Grover, Ronald W. Rousseau, and Andreas S. Bommarius*

School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, 30332

Amino ester hydrolase (AEH; E.C. 3.1.1.42) from *Xanthomonas campestris* is a promising candidate for β-lactam synthesis but suffers from low thermostability, rapid deactivation with a half-life of 30 minutes at 25 °C, and aggregation (i.e. higher order deactivation). Stabilization via protein engineering raised the temperatures of optimum activity T_{opt} and melting T_m but did not alleviate the issue of order of the deactivation kinetics [(1)]. AEH features two transitional temperatures: (1) from native 'N' to partially unfolded, inactive intermediate 'I' around the temperature of optimum activity T_{opt}, and (2) from partially unfolded to fully unfolded entity 'U' at the melting temperature T_m. CD and light scattering data suggest aggregation near the temperature of optimum activity.

To model these complex deactivation kinetics and determine the total turnover number *TTN*, the key parameter of merit for stabilization, AEH was deactivated by imposing a temperature gradient and recording instantaneous rates of cephalexin hydrolysis. The *TTN* increased at a maximum of 5-fold from the wild-type (WT) for the quadruple variant N186D/A275P/E143H/V622I (QVH) at 25 °C and 10 nM AEH but varied significantly with AEH concentration and temperature [(2)]. This work provides insight into complex deactivation kinetics of enzymes, particularly those with multiple transition states and/or aggregation.

The methods for determining the deactivation kinetics and *TTN* for a given enzyme give additional tools for comparing variants in process design or protein engineering campaigns. Several thermostabilized AEH variants are currently being developed and have apparent changes in their unfolding thermodynamics. The work outlined here coupled with ongoing work on thermostable variants could be used to demonstrate not only improvements in thermostability, but also show improvements in their deactivation kinetics and operational stability.

This work was supported by the U.S. Food and Drug Administration, Center for Drug Evaluation and Research through Grant U01FD006484 and the National Institute of Health (Grant # 5R01AI064817-05), which is gratefully acknowledged. C.E.L. and J.K.B gratefully acknowledge funding by the U.S National Science Foundation through the Graduate Research Fellowship Program (GRFP) under Grant No. DGE-1650044. T.A.R. gratefully acknowledges funding from the GAANN (Graduate Assistance in Areas of National Need) Fellowship.

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2. Lagerman, C. E., Blum, J. K., Rogers, T. A., Grover, M. A., Rousseau, R. W., and Bommarius, A. S. Submitted.

Talk 4

Thermodynamic Dependencies of Reaction Intermediate Formation of M379A Mutant Tyrosine Phenol-lyase

Olivia Bauer[‡] *and Robert S. Phillips*[§,‡]

Departments of Biochemistry and Molecular Biology[‡], and Chemistry[§], University of Georgia, Athens, GA 30602

Tyrosine phenol-lyase (TPL) is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the reversible β-elimination of L-tyrosine into phenol and ammonium pyruvate as well as the irreversible elimination other amino acids with good leaving groups on the β-carbon. Due to the versatility of the PLP cofactor in stabilizing carbanionic reaction intermediates and the intrinsic ability of TPL to catalyze an array of substrates with good leaving groups, TPL has been used to synthesize numerous L-tyrosine analogs that are biochemically and pharmaceutically relevant, like L-DOPA.[1] Thus, engineered TPL variants with wider substrate specificity and improved catalytic efficiency are of great interest. Our lab has recently shown the M379A TPL variant to be a robust catalyst with an increased range of substrates, specifically for 3-substituted tyrosine analogs which cannot be accommodated by native TPL.[2] However, the M379A mutation also alters the conformational dynamics that are coupled with TPL catalysis.[2,3] Here, we further characterize how the M379A mutation affects the protein dynamics that play a key role in TPL reaction mechanism. The rate of formation of the M379A aminoacrylate intermediate shows an obvious nonlinear temperature dependence which is also seen with native TPL indicating that aminoacrylate formation is dependent on active site conformational dynamics. Additionally, investigation into the effect of hydrostatic pressure on the formation of the M379A TPL quinonoid intermediate shows the presence of multiple distinct processes, a unique behavior that is not seen in native TPL. Current work is focused on further elucidating M379A TPL thermodynamic activation parameters and how M379A mutation has affected vibrational coupling in the transition states.

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2. Phillips, R. S., Jones, B., & Nash, S. (2022). M379A Mutant Tyrosine Phenol-lyase from *Citrobacter freundii* Has Altered Conformational Dynamics. *Chembiochem*, *23*(13), e202200028.
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Session 2:

Discussion Leader

Endang Purwantini *(Virginia Tech)*

Talk 5

Temporal regulation of electron transfer via dynamic protein-protein interactions

Rahul Banerjee[‡], *Jason C. Jones*[*], *Ke Shi*[*], *Manny M. Semonis*[&], *Hideki Aihara*[*], *William C. K. Pomerantz*[#], *and John D. Lipscomb*[*]

[‡]Department of Chemistry and Biochemistry, Auburn University, Auburn AL-36849; Departments of [#]Chemistry, [*]Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Twin-Cities, Minneapolis, MN-55455; Department of Molecular Biology and Biochemistry, University of California, Irvine, CA-92697

Soluble methane monooxygenase (sMMO; E.C 1.14.13.25) catalyzes the oxygen dependent oxidation of methane to methanol in the C1 metabolism of methanotrophs. In order to catalyze the cleavage of the strong C-H bond of methane, sMMO furnishes a highly oxidizing oxygen-bridged dinuclear iron(IV) intermediate within the active site contained in the hydroxylase protein (sMMOH).[1] This dinuclear iron cluster is converted to the resting dinuclear iron(III) state after the hydroxylation reaction. In order to start another round of catalysis, the iron cluster is reduced to the dinuclear iron(II) state by a NADH-dependent reductase protein (MMOR). It stands to reason that this electron delivery from MMOR to sMMOH must be carefully timed for otherwise a mid-catalytic cycle transfer would reductively quench the high-valent iron intermediate and result in a futile cycle. This intricate coupling of electron delivery, O_2 activation, and methane oxidation is reliant on another sMMO protein partner termed the regulatory protein (MMOB). MMOB has many effector roles, chief among which is the organization of a small-molecule tunnel for O_2 and CH_4 delivery into the sMMOH active site.[2] In order to study the protein-protein interactions responsible for this regulation, a Protein-Observed ^{19}F-NMR (PrOF) study was pursued by selectively replacing the native tryptophan residues of MMOB and MMOR with fluoro-tryptophan analogues.[3] This methodology sidesteps the artifacts arising from large fluorophore labels perturbing native binding interactions at protein interfaces. This PrOF study indicates that MMOB and MMOR bind to sMMOH in a competitive fashion with similar affinities that do not change with the oxidation state of the iron cluster. A regulatory model has been proposed that describes how this equilibrium of protein complexes is driven forward through the catalytic cycle by the irreversible steps of electron transfer and O_2 activation. The observation that the catalytic cycle is complete during the lifetime of the sMMOH:MMOB protein complex helps explain how MMOR is prevented from reductively quenching the reactive intermediates during catalysis.

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Talk 6

The iron-dependent oxygenase FlcD in fluopsin C biosynthesis.

William C. Simke[‡], *Morgan E. Walker*[‡], *Logan Calderone*[#], *Andrew T. Putz*[‡], *Caitlin N. Vitro*[‡], *Jon B. Patteson*[‡], *Matthew R. Redinbo*[‡§⊥], *Maria-Eirini Pandelia*[#], *Tyler L. Grove*[ζ], *and Bo Li*[‡§]

Department of [‡]Chemistry, [§]Microbiology, [⊥]Biochemsitry and Biophysics, University of North Carolina, Chapel Hill, NC 27599; [#]Department of Biochemistry, Brandeis University, Waltham, MA 02453; [ζ]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461

A unique family of iron-dependent enzymes have been discovered that contain a heme oxygenase-like domain (heme oxygenase domain-containing oxygenases, HDOs). These enzymes catalyze diverse reactions, including *N*-hydroxylation, carbon scission, and nitrile formation, many of which are challenging to perform synthetically. We discovered a novel family member, FlcD, in the biosynthesis of the copper-containing antibiotic fluopsin C, which is produced by the opportunistic pathogen *Pseudomonas aeruginosa*.[1] FlcD catalyzes excision of a methine carbon from an oxime and releases formic acid as a byproduct. FlcD lacks two of six conserved iron binding residues in the HDO family. We identified residues essential for the catalysis of FlcD using site-directed mutagenesis. Isotopic labelling of the FlcD reaction shed light on the methine excision mechanism. Bioinformatic analysis shows that the iron binding sequence of FlcD is uncommon in the HDO family and reveals other possible iron binding motifs. Our findings provide biochemical and mechanistic insights into an unusual HDO enzyme and advances the understanding of the HDO enzyme family.

This study was supported by grants 1R01GM148685-01, DP2HD094657, and the UNC Program in Molecular and Cellular Biophysics training grant T32GM008570 from the NIH.

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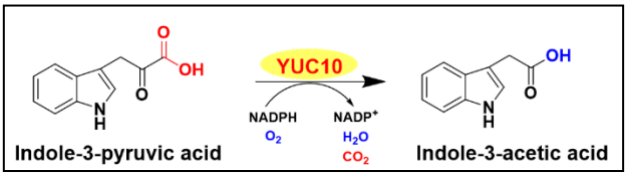
Talk 7

Kinetic Characterization of the Auxin-Producing Flavin-Dependent Monooxygenase YUC10

Noah Lyons[1] *and Pablo Sobrado*[1,2]
[1]Department of Biochemistry, Virginia Tech, Blacksburg, Virginia
[2]Virginia Tech Center for Drug Discovery, Virginia Tech, Blacksburg, Virginia

Auxins are major plant hormones involved in seed embryogenesis, growth, and homeostatic activities. The main auxin found in plants is indole-3-acetic acid (IAA).[1] IAA is synthesized via the oxidative decarboxylation of indole-3-pyruvic acid (IPA) by a YUCCA enzyme (Scheme 1). The YUCCAs, first identified in *Arabidopsis thaliana*, are members of the flavin-dependent monooxygenase (FMO) family. In particular, the YUCCAs are a distinct clade within Class B FMOs, which feature two Rossman-fold domains to bind FAD and NAD(P)H.[2] Studies *in planta* have shown that upregulating expression of *YUCCA* genes in *A. thaliana* produces phenotypes consistent with increased auxin levels, such as stem elongation, increased biomass, and drought tolerance.[3,4] However, research into the kinetic mechanism of the YUCCAs have been limited, largely due to issues obtaining recombinant, soluble protein. With homologues in nearly every food crop, understanding the biochemical mechanism of the YUCCAs will allow new insights into crop engineering and green chemistry, a necessity for the agricultural industry.



Scheme 1. Reaction catalyzed by the enzyme YUC10.

In this work, we present the kinetic characterization of YUC10 from *A. thaliana*. Following purification of recombinant, flavin-bound YUC10, we used steady-state and rapid-reaction kinetic techniques to characterize this enzyme. Oxygen consumption assays show that YUC10 catalyzes the rapid oxidative decarboxylation of IPA into IAA, with a k_{cat} of 2.9 s^{-1}. Additionally, we report that YUC10 has a narrow substrate specificity, as the α-keto acid moiety is necessary for catalysis while the aromatic group makes the reaction more efficient. Stopped-flow spectroscopy experiments reveal that YUC10 can be reduced with NADPH at a k_{red} of 5.4 s^{-1} and a K_D <10 μM, which does not significantly change in the presence of IPA. Furthermore, we detected the formation of a reactive C4a-(hydro)peroxyflavin intermediate when reduced enzyme is titrated with oxygen. In summary, we show that YUC10 is able to catalyze the oxidative decarboxylation of IAA derivatives, is reduced by NADPH via a "bold" mechanism of reduction, and forms a reactive oxygenating intermediate that is characteristic of Class B FMOs. Future studies probing the active site of YUC10 and the protonation state of the flavin during catalysis will help us further develop the kinetic and chemical mechanism of this enzyme.

This work is supported by the Fralin Life Science Institute. N.S.L. was supported, in part, by the Cunningham Doctoral Fellowship from the Graduate School at Virginia Tech.

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Session 3:

Discussion Leader

Wen Zhu *(Florida State University)*

Talk 8

Initial Characterization of *Bacillus subtilis* Fe-S cluster assembly protein SufB

Juliano Ribeiro[‡], *Ashley Edwards*[§], *Patricia dos Santos*[‡],
Department of Chemistry[‡], Wake Forest University, Winston-Salem-NC, 27108.

Iron-sulfur (Fe-S) clusters are essential protein cofactors performing a variety of catalytic reactions ranging from the synthesis of ammonia from gaseous nitrogen in nitrogen-fixing bacteria to the conversion of isocitrate to citrate in the Krebs cycle. The synthesis of these protein cofactors requires specialized pathways that involves the activation of sulfur from cysteine, the assembly of the clusters onto scaffold entities, and the transfer of nascent cluster into apo-proteins. *Bacillus subtilis* and other Gram-positive bacteria utilize the SUF pathways composed of SufCDSUB components. This pathway has not been fully characterized but is similar to the *Escherichia coli* SufABCDSE pathway involved in the biogenesis of nascent clusters during oxidative stress. In previous studies, we have characterized the function of *B. subtiltis* SufS and SufU as a cysteine desulfurase and a zinc-dependent sulfurtransferase, respectively. These functions are proposed to be analogous to those of *E. coli* SufSE in sulfur mobilization. Given sequence similarity to the *E. coli* orthologous components, the *B. subtilis* SufBCD has been proposed to serve as the scaffold complex of this system. However, experimental validation of this proposal has been hindered by challenges in obtaining soluble forms of SufB suitable for biochemical experimentation.

In this study, we have overcome this challenge by improving the protocol for expression and purification of this protein. Using an optimized method to extract SufB from inclusion bodies under urea-denaturing conditions and a refolding process, we successfully obtained soluble and active forms of this protein. SufB purified from this approach is able to enhance the ATPase activity of SufC with a tight binding activation profile. Furthermore, initial in vitro reconstitution experiments showed that SufB is able to accumulate Fe-S cluster species supporting its proposed function as a scaffold. Future work will probe the involvement of SufB in receiving sulfur from SufSU as persulfide or sulfide as well as in donating nascent clusters to apo-forms of Fe-S target enzymes.

This study was supported by the National Science Foundation (MCB- ***1716535)*** and the Wake Forest Center for Molecular Signaling.

Talk 9

Perturbation of Fe-Homeostasis During Sulfur Starvation Leads to Marked Suppression of The Virulence Pathways of *Pseudomonas aeruginosa* PAO1

Chidozie G. Ugochukwu[1], *Tonia S. Schwartz*[2], *Tonya N. Zeczycki*[3], *Douglas C. Goodwin*[1], *And Holly R. Ellis*[3]

1. Department of Chemistry and Biochemistry, Auburn University, AL
2. Department of Biological Sciences, Auburn University, AL
3. Department of Biochemistry and Molecular Biology, Brody School of Medicine, East Carolina State University, NC

Sulfate is the preferred sulfur compound for growth and metabolism in bacteria.[1] However, when sulfate is scarce, bacteria can switch to utilizing non-preferred organosulfur compounds through elaborate metabolic reprogramming that includes the expression of sulfur-scavenging and antioxidant genes.[2] The mechanistic detail behind the expression of antioxidant genes during sulfur starvation has remained to be determined. In this study, we investigate the effects of sulfur starvation on *Pseudomonas aeruginosa* (PA) an opportunistic human pathogen causing life-threatening infections.

Six samples of PA, three replicates grown in a sulfur-free media (treatment), and three replicates supplemented with 500 µM sulfate (control) were subjected to RNA sequencing (RNAseq) and label-free proteomics for transcriptome and proteome quantification, respectively. This was followed by enrichment and network analyses of the detected differentially expressed features to gain insight into overrepresented biological pathways.

RNAseq analysis identified 5658 genes, with 1053 differentially expressed (DE) in the treatment versus control. Similarly, our proteomics result showed 659 DE proteins from 2321 reliable quantified proteins. Notably, sulfur starvation resulted in a significant depression of Fe-uptake genes and pathways, including the pyoverdine (*pvd*), pyochelin (*pch*), and heme-uptake (*phu* and *has*) operons in the treatment group. We also observed significant depression of genes involved in virulence pathways, including quorum sensing, multidrug-resistant (MDR) efflux pumps, phenazine biosynthetic genes, and hydrolytic enzymes regulated by the ferric uptake regulator (Fur). Conversely, there was a significant upregulation of genes involved in Fe-storage (*bfrB*), reactive oxygen species (ROS) metabolism (*sodB, lsfA, ohR, and ahpC*), and sulfur acquisition and assimilation (*msu*, *ssu*, and *cys* operons).

Our study highlights how sulfur metabolism is intricately linked to Fe homeostasis in PA. The results suggest that sulfur starvation triggers an Fe-replete response in PA, inducing antioxidant genes to combat the oxidative effects of free intracellular Fe. More so, the study uncovers how sulfur starvation suppresses virulence pathways in PA, opening exciting possibilities for clinical investigations.

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Talk 10

Two dynamic, N-terminal regions are required for function in Ribosomal RNA Adenine Dimethylase family members.

Danielle A. McGaha[1], *Alexandrea S. Collins*[1], *Jack A. Dunkle*[1]
[1]Department of Chemistry and Biochemistry, University of Alabama, Tuscaloosa, Alabama 35487, USA

Erythromycin resistance methyltransferase (Erm) dimethylates A2058 of 23S rRNA to $m^6{}_2$A2058 using methyl donor S-adenosylmethionine (SAM), resulting in a multidrug-resistant phenotype in bacteria posing a significant threat to public health. Through our utilization of random mutagenesis and phenotypic assays, two lesser-known areas abundant with altered phenotypes were identified in the N-Terminal region: a basic patch, and a region referred to as "Motif X", both of which are highly conserved across the ribosomal rRNA adenine dimethylase (RRAD) family. To further investigate this new discovery, we designed site-directed mutants of both ErmE and KsgA to assess the effects of the two regions on the activity across the RRAD family. The resulting mutants were characterized using *in-vivo* phenotypic assays in conjunction with *in-vitro* assays, which include single turnover kinetics and RNA affinity binding assays. Our results suggest that the basic patch and Motif X of the N-terminal contribute to rRNA methylation and are required for function in the RRAD family.

This work was supported by NIAID, National Institutes of Health grant R15AI131159.

Session 4:

Discussion Leader

Kylie Allen *(Virginia Tech)*

Keynote Presentation

Amy C. Rosenzweig received a BA in chemistry from Amherst College and a PhD in inorganic chemistry from Massachusetts Institute of Technology. After postdoctoral research at Harvard Medical School, she joined the faculty of Northwestern University where she is the Weinberg Family Distinguished Professor of Life Sciences. She is a fellow of the American Academy of Arts and Sciences and a member of the National Academy of Sciences. The Rosenzweig laboratory uses structural, biochemical, and biophysical approaches to attack problems at the forefront of bioinorganic chemistry. Their work has been honored recently by the American Chemical Society Alfred Bader Award in Bioinorganic or Bioorganic Chemistry and The Protein Society Hans Neurath Award.

Amy C. Rosenzweig

Weinberg Family Distinguished Professor of Life Sciences
Depts. of Molecular Biosciences and of Chemistry
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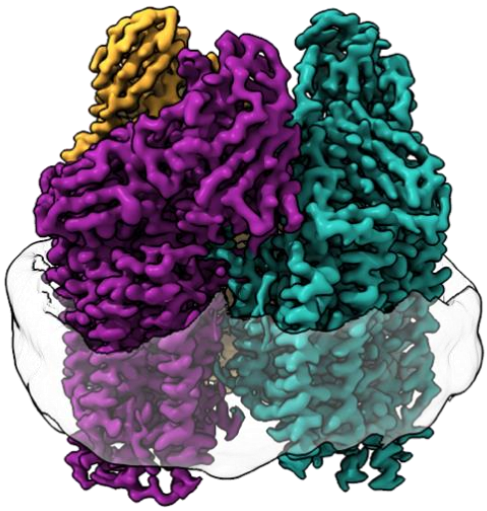
Talk 11

Copper-dependent biological methane oxidation

Amy C. Rosenzweig
Departments of Molecular Biosciences and of Chemistry, Northwestern University, Evanston, IL 60208, USA

Under the mounting threat of climate change, increasing atmospheric methane concentrations are a constant source of concern and debate. As the second most abundant greenhouse gas after carbon dioxide, methane accounts for at least 25% of current global warming. Conversion of methane to desirable fuels and chemicals could simultaneously mitigate global warming and meet increasing energy demands. However, current catalysts that can selectively activate the 105 kcal mol^{-1} C-H bond in methane require high temperatures and pressures, along with significant capital expenses. In nature, aerobic methane oxidation is accomplished under ambient conditions by bacteria called methanotrophs. In most methanotrophs, this chemically-challenging reaction is catalyzed by particulate methane monooxygenase (pMMO), a copper-dependent, integral membrane enzyme. pMMO is composed of three protein subunits, PmoA, PmoB, and PmoC, arranged in a trimeric complex. Despite extensive research and the availability of multiple crystal structures, the location and nature of the pMMO copper active site remain controversial. Studies are further complicated by issues with retaining enzymatic activity and uncertainties regarding the possible involvement of additional protein components. Our quest to address these questions will be discussed.



Cryoelectron microscopy map of pMMO in a lipid nanodisc. The three protomers in the trimer are shown in magenta, teal, and gold.

Work in the Rosenzweig lab on pMMO is supported by NIH grant R35GM118035.

Poster List

Poster Sessions 1 &2

(10:00 am – 11:30 am; 2:45 pm – 4:15pm)

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Poster Abstracts

Poster Session 1
(10:00 am – 11:30 am)

#1

Role of the *Pseudomonas aeruginosa mdo* Operon in Hydrogen Sulfide Scavenging

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Pseudomonas aeruginosa is an opportunistic human pathogen that is especially problematic for individuals with cystic fibrosis and immunocompromised patients. It is a leading cause of nosocomial infections and is responsible for 10% of all hospital-acquired infections. Treatment of ~~the~~ infections caused by this organism has been complicated by its ability to develop resistance to multiple classes of antibacterial agents. Several virulence factors contribute to *P. aeruginosa* pathogenicity including hydrogen sulfide. Sulfide (HS^-) at sub-micromolar concentrations protects *P aeruginosa* from antibiotic-induced oxidative damage and host-produced reactive oxygen species. However, elevated HS^- levels results in cellular toxicity. Therefore, HS^- concentrations must be tightly regulated to balance the potential toxicity with bacterial virulence.

In *P. aeruginosa*, the *mdo* operon expresses a sulfurtransferase (ST) and a thiol dioxygenase, 3-mercaptopropionate dioxygenase (MDO). Even though MDO is expressed from the same operon as an annotated ST, the functional role of the ST enzyme has not been recognized. MDO has been previously reported to oxidize 3-mercaptopropionate (3MPA) to 3-sulfinopropionate (SPA)[1], but no physiological role has been attributed to this product. Although the mechanism and final product of sulfide detoxification vary among bacteria, thiol dioxygenases and STs expressed from the same operon often oxidize sulfide to usable sulfur forms.

MDO was able to oxidize 3-mercaptopyruvate, which is a physiologically relevant substrate and can also be linked with ST activity. Expanding the substrate versatility of MDO served as the foundation to establish the mechanistic properties of the ST. In ST enzymes, a conserved cysteine residue mediates sulfur transfer from a sulfur donor to a sulfur acceptor. Often the sulfur donor and acceptor in ST reactions are low molecular weight thiols. The ST expressed from the *mdo* operon has two cysteine residues, Cys191 and Cys435, that could facilitate sulfur transfer through the formation of a persulfide intermediate. The Cys435 amino acid residue was the accessible cysteine in DTNB assays. Similarly, there was increased deuterium–hydrogen exchange (HDX) in the region surrounding Cys435 compared to Cys191 in HDX–MS investigations. These results suggest that the thiolate of Cys435 is readily accessible to react with sulfur donors. Cys435 was able to form a persulfide intermediate, but only thiosulfate was able to serve as the sulfur donor. Both 3-mercaptopriopionate and 3-mercaptopyruvate showed a similar binding affinity for ST in fluorescent titration studies, suggesting both metabolites could serve as sulfur acceptors. A putative disulfide bond was identified in ST protein preparations. The location of and flexibility around Cys435 could facilitate transient disulfide bond formation with Cys191 during catalysis. However, further studies revealed the disulfide bonds were artifacts and not relevant to overall sulfur transfer by ST. These investigations confirm the potential of the enzymes of the *mdo* operon to mobilize sulfur from sulfide in *P. aeruginosa* thereby enhancing its viability and pathogenicity.

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#2

Evolutionary insights into the unique modes of auto-regulation in the DCLK family kinases

Aarya Venkat[1], **Grace Watterson[1]**, Dominic P. Byrne[2], Brady O'Boyle[1], Safal Shrestha[3], Nathan Gravel[3], Emma E. Fairweather[2], Leonard A. Daly[2,4], Claire Bunn[1], Wayland Yeung[3], **Ishan Aggarwal[1]**, Samiksha Katiyar[1], Claire E. Eyers[2,4] Patrick A. Eyers[2]*, and Natarajan Kannan[1,3]*

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[3]Institute of Bioinformatics, University of Georgia, Athens, GA 30602, USA
[4]Centre for Proteome Research, Department of Biochemistry, Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Liverpool, L69 7ZB, UK + equal contributions *Correspondence to: Natarajan Kannan, Email: nkannan@uga.edu or Patrick Eyers, Email: patrick.eyers@liverpool.ac.uk

Doublecortin Like Kinases (DCLKs) are a conserved family of microtubule-associated proteins that regulate diverse cellular processes. The human genome encodes three DCLK Ser/Thr kinases (DCLK1-3), which are abnormally regulated in human cancers and neurodegenerative disorders. However, an incomplete knowledge of their unique sequence, structure and regulatory features presents a major bottleneck in the development of selective DCLK inhibitors. Here we define the hallmarks of DCLK family evolutionary divergence and functional specialization using a combination of statistical sequence analysis, structural comparisons and molecular dynamics simulations. We find that DCLK family members have evolutionarily diverged from other CAMKs through a unique tethering mode employed by the C-terminal tail, which prevents ATP binding in DCLK1 by docking to the ATP binding pocket. The 'acceptor' tethering sites in the kinase domain, including a divergent G-loop, are unique to the DCLK family and facilitate conformational regulation of the kinase domain by the C-terminal tail. Our studies provide new insights into DCLK autoregulatory mechanisms and open new avenues for the design of selective DCLK inhibitors.

#3

QM/MM Investigation of Light-Oxygen-Voltage (LOV) sensing domains

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The temporal cost of quantum calculations grows exponentially or, even, factorially, with the size of the system being investigated. Thus, in the consideration of bulky, dynamic systems, it is wise to select atoms germane to properties of interest and consider them quantum mechanically (QM) while viewing others without primary function through the lens of molecular mechanics (MM). Our system of interest here is the class of flavin-binding photoreceptor proteins known as Light-Oxygen-Voltage (LOV) domains responsible for blue light responses and circadian control. To observe the mechanism of activation and structural variation of this photoreceptor we apply a specialized hybrid QM and MM methodology (QM/MM) that treats the flavin quantum mechanically and the rest of the LOV protein molecular mechanically.

Our QM/MM procedure takes an Average Protein Electronic Configuration (APEC) of the protein around the flavin. In this presentation, we investigate how changes in the flavin oxidation state modifies the dynamics of the protein. These simulations can potentially give insight into how light-induced electronic changes in LOV domains ultimately lead to a cascade of changes downstream.

#4

Biochemical Investigation of a Specialized Inositol Dehydrogenase

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Antimicrobial resistance has become a global threat. This has birthed a need to uncover novel enzyme mechanisms in biosynthetic pathways, which play a vital role in the synthesis of antimicrobial agents. Hygromycin A (HygA) is a broad-spectrum antibiotic produced by *Streptomyces hygroscopicus* that inhibits ribosomal peptidyl transferase. [(1)] HygA contains three distinctive functional groups: furanose, cinnamic acid, and aminocyclitol. A structure-activity study has revealed that the cinnamic acid and aminocyclitol moieties are vital for target inhibition. [(2)] Through isotope-labeling and mutagenesis experiments, a pathway has been proposed for the biosynthesis of the aminocyclitol. [(3,4)] This pathway originates from glucose-6-phosphate, which is converted to *myo*-inositol-1-phosphate (MIP) by Hyg18. A *myo*-inositol phosphatase, Hyg25, then dephosphorylates MIP to give *myo*-inositol, which is then oxidized to *neo*-inosamine by the *myo*-inositol dehydrogenase Hyg17, followed by a series of enzymatic reactions that lead to the formation of the modified aminocyclitol. This study focuses on the *myo*-inositol dehydrogenase Hyg17, which catalyzes a distinct oxidation of *myo*-inositol on the C5 position, which is unlike known *myo*-inositol dehydrogenases which oxidize the C2 position.[(5)] We have performed bioinformatics and biochemical analysis on Hyg17 to gain a better understanding of this unique activity. Our findings will also verify one step of the proposed biosynthetic pathway for the aminocyclitol moiety of HygA, which plays a critical role in antimicrobial activity.

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#5

Conjugates of Antiviral-Cell Penetrating Peptides Emerged as Promising Therapeutics Targeting the Main Protease of SARS-CoV-2

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Peptides play an important role in the immune defenses of the host against several distinct types of infections. Hence, peptides represent a promising therapeutic approach for the treatment of SARS-CoV-2 infections.[1] The Mpro is the key enzyme involved in the viral replication of SARS-CoV-2.[2] Temporin L, known for its antimicrobial characteristics, has an alpha-helical structure that has been found to interact with the cytoplasmic membrane and induce the formation of pores.[3] *In-vitro* assessment of the Temporin L inhibitor was found to exhibit moderate activity against Mpro of SARS-CoV-2.[4] Notably, the conformational analysis suggests that the rigid structure of Temporin L (TL) facilitates favorable binding to Mpro's active site.

Herein, we introduce a new approach to the design, synthesis, and characterization of the conjugation of Temporin L1 to various cell-penetrating peptides (CPP) inhibitors targeting the main protease (Mpro) of SARS-CoV-2. This conjugation can be able to enhance 4- to 16-fold antiviral activity. Our theoretical study of the SARS-CoV-2 Mpro suggests that the TL1-CPP inhibitor can bind the active site of Mpro in the predicted manner. The structural insights observed from Molecular Dynamics (MD) simulations provide a clearer understanding of how the conjugate TL1-CPP peptide inhibitor could interact with Mpro's active site. The active site of the Mpro protease contains the catalytic dyad (His41 and Cys145) as well as additional amino acids necessary for substrate recognition and binding.[5] One of our TL1-CPP conjugate peptides showed that the Asp142 and Glu 166 are close to the active site and may participate in substrate recognition and binding along with the catalytic dyad. Liquid chromatography coupled with mass spectrometry (LC-MS) was used to characterize the peptide, demonstrating that experimental m/z values corresponded with theoretical m/z values. In addition, our *in-vitro* main protease assay indicates that one of the TL1-CPP peptide inhibitors is responding as an inhibitor against the Mpro. Subsequently, the results of our study provide crucial insight into the therapeutic approach of developing peptide inhibitors against the main protease of SARS-CoV-2. Furthermore, the findings of this study provide a groundwork understanding of the *in-vitro* main protease assay validation to identify the lead conjugate TL1-CPP peptides targeting SARS-CoV-2.

This study is supported by Funds to Sustain Research Excellence**,** College of Science and Mathematics, Kennesaw State University, for funding this research work.

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5. Jahirul Islam M, Halim MA. et al. A Review on Structural, Non-Structural, and Accessory Proteins of SARS-CoV-2: Highlighting Drug Target Sites. *Immunobiology* 2023;228(1):152302.

#6

Oligomeric Changes of FMN-reductases and play a Critical Role in Protein-protein Interactions in Two-component FMN-dependent Systems.

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Bacterial two-component FMN-dependent systems are responsible for sulfur acquisition when inorganic sulfate is limiting in the environment. Two-component FMN-dependent systems are comprised of a reductase (SsuE/MsuE) that catalyzes flavin reduction and the monooxygenase (SsuD/MsuD) that catalyzes the desulfonation of organosulfur compounds for sulfur acquisition. The unique reactions catalyzed by these systems are facilitated by several distinct structural properties: oligomeric changes, π-helix, and protein-protein interactions.[1] However, it is not clear how these features work synergistically to enhance catalysis.

In this study, we investigated the role oligomeric changes of FMN-reductases play in promoting protein-protein interactions between the FMN-reductase and monooxygenase. Results from previous studies utilizing different approaches reveal that SsuE exists in several oligomeric states which may modulate the transfer of reduced flavin to the partner monooxygenase. Studies were performed to determine if the oligomeric changes of the reductases were initiated by substrate binding. Apo SsuE was a tetramer but shifted to a dimer-tetramer equilibrium with either NADPH or FMN present in analytical ultracentrifugation studies. Conversely, apo MsuE was a dimer but shifted to a tetramer in the presence of FMN. Hydrogen-deuterium mass exchange spectrometry was utilized to investigate the accessibility of the tetramer and dimer interface regions of SsuE and MsuE. The tetramer interface of SsuE showed more deuterium accessibility in the presence of flavin which correlates to the shift from a tetramer to a tetramer-dimer equilibrium. However, MsuE with flavin present was less accessible due to the switch from a dimer to a tetramer. Results from these studies reveal that SsuE and MsuE show divergent oligomeric states in the presence of substrates. The mode of reduced flavin transfer from SsuE to its partner monooxygenase SsuD occurs through protein-protein interactions. Investigations were made to determine if the oligomeric changes of SsuE are linked to protein-protein interactions with SsuD. The complex (SsuE/SsuD) was formed in the presence of flavin and the dimeric form of SsuE was the predominant form. The SsuE dimer would expose the protein-protein interaction sites for the safe transfer of reduced flavin to SsuD. Conversely, MsuE has to provide reduced flavin to both MsuC and MsuD, and formation of a tetramer could regulate this dual transfer. The dissimilar oligomeric states observed in these systems in response to substrate binding is directly related to their different functional roles in flavin transfer.

This study was supported by Grant 2105998 from NSF (HRE)

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#7

Deciphering the Mechanism of TlyA-Mediated Ribosomal RNA Methylation: Insights into Ribosome Subunit Recognition and Interdomain Signaling

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The *Mycobacterium tuberculosis* ribosomal RNA (rRNA) 2'-O-methyltransferase TlyA (EC:2.1.1.227) is a dual specificity enzyme responsible for methyl group transfer from cosubstrate *S*-adenosyl-L- methionine (SAM) to nucleotides C2158 and C1402 of the 50S (23S rRNA) and 30S (16S rRNA) ribosomal subunits, respectively.[1, 2] Contrary to the more common rRNA methylation-dependent antibiotic resistance exploited by drug-producing bacteria and acquired by some drug-resistant pathogens, rRNA methylation by TlyA is ***required*** for optimal binding and thus action of capreomycin, a potent second-line anti-tuberculosis drug.[1] Previous studies from our lab have shown that the TlyA structure comprises an N-terminal domain (NTD) responsible for rRNA recognition and a C-terminal Class I methyltransferase domain (CTD), connected by an interdomain linker (tetrapeptide with sequence RAWV).[3] However, the mechanism by which TlyA recognizes its two structurally distinct substrates is not well understood. Based on the unexpected dependence of SAM binding on the RAWV motif,[3] we have speculated that the interdomain linker may play a critical role in domain coordination, with the NTD recognizing and binding the ribosome subunit followed, by transmission of a signal via the linker to the CTD for methylation once correctly positioned on the substrate.

To define the mode of TlyA binding to the Mycobacterial ribosome (using *M. smegmatis* as a model system), we used cryogenic electron microscopy combined with complementary mutagenesis and methyltransferase assays to identify TlyA residues responsible for the 50S/30S binding and methylation.[4] Additionally, to decipher the role of the linker, we proteolytically cleaved TlyA into His-NTD and CTD^{RAWV} components, that could be separated by Ni^{2+}-affinity chromatography. TlyA activity assays with 50S subunit as the substrate showed that the mixture of His-NTD and CTD^{RAWV} domains (pre-separation) retains ~20% activity compared to intact full- length TlyA, supporting a role for the interdomain linker in signal transfer. Interestingly, however, a mixture of the separated His-NTD and CTD^{RAWV} failed to methylate the 50S subunit, suggesting that optimal His-NTD and CTD^{RAWV} association is also critical in methylation. In conclusion, our findings highlight the TlyA residues necessary for ribosome binding and modification and reveal a potential role for the interdomain linker in signal transduction from the NTD to CTD.

This study was supported by NIH award R01-AI088025 (G.L.C) and in part by NIH award T32-AI106699 (Z.T.L.)

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#8

Biochemical characterization of MtaB from two Archaeal species

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Methylthiotransferases (MTTases) are a subclass of radical *S*-adenosylmethionine (RSAM) enzymes, a large and diverse group of enzymes that typically activate unreactive carbon centers via radical initialization. Three MTTases are known; RimO acts upon aspartate 88 of bacterial ribosomal S12 protein, whereas MiaB and MtaB act upon tRNA substrates. MtaB methylthiolates the universally conserved N^6-threonylcarbamoyladenosine (t^6A) to 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A). Both RimO and MiaB have been studied extensively, and both have reported structures and in vitro activities reported. In contrast, in vitro activity of MtaB has not been reported. Further, a related hypermodified tRNA modification, N^6-hydroxynorvalylcarbamoyladenosine (hn^6A) and its methylthiolated derivative (ms^2hn^6A) have been reported in various bacteria and archaea, and it is unclear whether MtaB is also responsible for this methylthiolation reaction. Here, we report in vitro activity of an MtaB ortholog from an anaerobic methanotroph (ANME) purified from *E. coli*. ANME are widely distributed archaea that oxidize methane to carbon dioxide in anoxic environments. Further, we expressed and purified a second MtaB ortholog encoded by *Methanocaldococcus jannaschii* from a closely related host, *Methanococcus maripaludis*. Lastly, we show evidence that *Methanosarcina acetivorans* produces only ms^2hn^6A, in contrast to ms^2t^6A. Interestingly, *Methanococcoides burtonii* total tRNA was reported to also contain ms^2hn^6A but not ms^2t^6A, while other tRNA pools that have been reported from various methanogens within the *Methanococcales* contain either only ms^2t^6A or both ms^2t^6A and ms^2hn^6A, suggesting a phylogenetic relationship in the distribution of methylthiolated tRNA species. An effort to produce an *mtaB*$^-$ *M. acetivorans* strain is currently underway, which will provide tRNA for in vitro assays to test activity of MtaB with hn^6A and thus clarify the substrate specificity of archaeal MtaB enzymes.

#9

Evaluation of Peptide Inhibitors Targeting the Main Protease of SARS-CoV-2 Using Liquid Chromatography and Mass Spectrometry Based Method

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The SARS-CoV-2 is a positive-sense single-stranded RNA (ssRNA) virus and a member of the SARS-CoV and MERS-CoV (Middle East respiratory syndrome) family. This novel RNA virus translates 29 proteins including 16 non-structural, 4 structural and 9 accessory factor proteins.[1] Among all SARS-CoV-2 viral proteins, the main protease (3CLpro) has an important role for viral polyproteins maturation and replication. Several small molecules drugs have showed promising results for covid-19 treatment and a Pfizer (PF-07321332) drug is approved for emergency use. However, small molecule drugs often induce harmful off-target effects and lead to therapy resistance on prolonged use. Peptide therapeutics, in contrast, are highly target specific and thus induce less toxic effect compared to small molecule drugs.[2] Peptides have several advantages including stimulation, low resistance, low toxicity, high specificity, effectiveness, easy to synthesize, high potency, fewer side effects, and low accumulation in tissue. Using solid phase synthesis one can synthesize peptide inhibitors, substrate, and even modified substrate for the main protease. Most methods used to quantify inhibitor affinity for the protease employ FRET based assay due to their availability and low cost. However, background fluorescence of fluorogenic substrate from compound interference with the optical signal can lead to false positive readings of inhibition. Liquid chromatography coupled with mass spectroscopy (LC-MS) offers greater sensitivity and robustness when compared to its fluorogenic counterparts. In this study, we developed a simple LC-MS method to detect the substrate degradation and product formation rates to obtain accurate IC50 values for the inhibitors of main protease of SARS-CoV-2. The assay developed in this study was validated with known inhibitors of 3CLpro, like the peptidomimetic agent GC-367. In addition, the inhibition efficiency of the various peptide inhibitors was evaluated with the LC-MS methods.

This study is supported by Funds to Sustain Research Excellence**,** College of Science and Mathematics, Kennesaw State University, for funding this research work.

1. Jahirul MI, Halim MA. et al. A Review on Structural, Non-Structural, and Accessory Proteins of SARS-CoV-2: Highlighting Drug Target Sites. *Immunobiology* 2023;228(1):152302.

2. Uddin MJ, Halim MA. et al Large-Scale Peptide Screening against Main Protease of SARS CoV-2. *J Comput Chem* 2023;44(8):887–901,

#10

Flanking Nucleotide Sequences Modulate RNase HII Activity

Steven T. Brenden, *and Markus Germann*
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Ribonucleotide inclusion (RNP) is the most prevalent form of DNA damage. RNases H are responsible for identifying and excising the ribonucleotide at the phosphate group 5′ of the lesion. Incorporation of a single RNP greatly impacts the local structure and dynamics of the double stranded DNA. Our previous work has shown that for an rG intrusion there is a strong perturbation on the flanking nucleotide sequences.[1]

We have prepared a series of duplexed DNA oligonucleotides all containing a single rG or rC inclusion in different flanking sequence environments to test the degree to which RNase HII's reaction efficiency is impacted. For the rG DNA substrates we have examined all possible nearest neighbor flanking sequence combinations. We found that the reactivity strongly depends on the flanking sequence by an up to 20-fold difference.

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#11

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

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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 site 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 ring as a novel way to specifically target the cofactor binding site of PRMT5. A total of about 30 compounds were synthesized using the 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 with clear 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 work 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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#12

Expression and Purification of Dimethyl Sulfide Monooxygenase from its Native Source, *Arthrobacter globiformis*

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Dimethyl sulfide (DMS) is a compound that is extremely important to the continuance of the sulfur cycle. The products from the oxidation and breakdown of DMS are the initial sites of cloud formation and atmospheric concentrations of DMS have been directly linked to environmental cooling. Breakdown of DMS occurs by the 2-subunit enzyme DMS monooxygenase. This enzyme utilizes a flavin-reducing DmoB subunit coupled to a DMS DmoA monooxygenase subunit. Much is still unknown about how the two subunits interact, native expression, and native cofactors, specifically divalent metals required for activity. DMS monooxygenase is found in a soil bacteria called *Arthrobacter globiformis*.[1, 2]

In this project, *A. globiformis* is grown, starved in carbon-depleted media, and supplemented with DMS to upregulate its production of DMS monooxygenase. Growth is monitored by cell microscopy. Growth media formulations have been screened, cells harvested, and tested for DMS monooxygenase activity. The cultures have been upscaled, a protein purification strategy is underway, as well as metal analysis and enzyme kinetics to determine the natively expressed DMS monooxygenase from *A. globiformis* optimal cofactor and substrate identity. Recent ICP-OES data has shown a surplus of iron in the lysate but has yet to be linked to enzyme activity. Results from this work will build upon the research in the field understanding this complex enzyme system and how divergent the enzyme is from different organisms.

This project is supported in part by the Office of Student Research and the Department of Chemistry and Fermentation Sciences at Appalachian State University.

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#13

Protein-Protein and Protein-Peptide Interactions in RiPP Biosynthesis.

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As more ribosomally synthesized and post-translationally modified peptides (RiPPs) have been discovered through advancements in bioinformatics and genome sequencing, attraction to the field grows due to the application of antimicrobial drug discovery by bioengineering these modified peptides from the simple biosynthesic pathways.(1) RiPP-tailoring enzymes, such as SPASM-domain containing radical SAM enzymes, modify the core sequence of the RiPP precursor peptides, resulting in a wide variety of structural features and distinct biological functions.(3) Majority of current research focuses on the chemical mechanism of these tailoring enzymes, but interest also lies in protein-peptide interactions (PPIs) among the tailoring enzyme, the RiPP recognition element (RRE), and the precursor peptides. Here, I will present a bioinformatics study on the potential PPIs between RRE domains and precursor peptides that facilitate the formation of different RiPPs. Experimental evidence also supports the hypothesis that RiPP tailoring enzymes utilize the dynamic nature of the PPIs between RRE and precursor peptides to achieve challenging chemical conversions.(2) This study shed light on further engineering the PPIs to modulate the production of the new-to-nature RiPPs.

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#14

Spin Trapping Radicals in Biological Samples for EPR Analysis

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Reactive oxygen species (ROS) are short-lived chemical species that, because of their reactivity, can have dramatic deleterious consequences for biological systems. ROS can be produced in cells as by-products of normal metabolic processes or as a result of exposure to environmental stressors such as radiation, pollution, or toxins. ROS can cause damage to cellular components, including lipids, proteins, and DNA. This can lead to cell death, tissue damage, and contribute to the development of many diseases *(1)*. Because of these reasons, detection of ROS in biological samples taken in the medical field are of great interest.

Samples with excess ROS could be at risk of irreversible degradation over time. Electron paramagnetic resonance (EPR) spectroscopy is a spectroscopy technique which allows for the study of many paramagnetic species, such as transition metal complexes and radical molecules. By reacting ROS with so-called spin trap molecules, we can monitor the amount of ROS formed. Two commonly used spin traps in EPR studies are DMPO (5,5-dimethyl-1-pyrroline N-oxide) and DEPMPO (5-(diethoxyphosphoryl)-5- methyl-1-pyrroline N-oxide). We employed DMPO as a spin trap in our proof-of-concept experiments using potassium superoxide, however as DEPMPO exhibits a longer lifetime, it was chosen as the ideal spin trap for our tissue-based studies. *(2)*

We report here a robust and reliable spin trapping protocol and EPR spectrum acquisition methodology that allows us to assess ROS formation and speciation in model systems for tissue degradation.

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#15

Two dynamic, N-terminal regions are required for function in Ribosomal RNA Adenine Dimethylase family members.

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Erythromycin resistance methyltransferase (Erm) dimethylates A2058 of 23S rRNA to $m^6{}_2$A2058 using methyl donor S-adenosylmethionine (SAM), resulting in a multidrug-resistant phenotype in bacteria posing a significant threat to public health. Through our utilization of random mutagenesis and phenotypic assays, two lesser-known areas abundant with altered phenotypes were identified in the N-Terminal region: a basic patch, and a region referred to as "Motif X", both of which are highly conserved across the ribosomal rRNA adenine dimethylase (RRAD) family. To further investigate this new discovery, we designed site-directed mutants of both ErmE and KsgA to assess the effects of the two regions on the activity across the RRAD family. The resulting mutants were characterized using *in-vivo* phenotypic assays in conjunction with *in-vitro* assays, which include single turnover kinetics and RNA affinity binding assays. Our results suggest that the basic patch and Motif X of the N-terminal contribute to rRNA methylation and are required for function in the RRAD family.

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#16

Tuning the redox activity of flavins in the Electron Transfer Flavoprotein of *Acidaminococcus fermentans*

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Flavin based electron bifurcation produces low-potential energy-rich reducing agents able to drive highly demanding reactions in anaerobes.[1] Electron bifurcation couples an endergonic electron transfer to a more exergonic one, making the overall reaction exergonic. The bifurcating electron transfer flavoprotein (ETF) from *Acidaminococcus fermentans (Afe)*has two FADs: the electron transfer FAD (ET-FAD) and the bifurcating FAD (Bf-FAD).[2, 3] In the elucidated mechanism of bifurcation, NADH donates a pair of electrons to the Bf-FAD which bifurcates one electron to an exergonic pathway via the ET-FAD and the other electron endergonically to a low potential acceptor such as ferredoxin. A domain-scale conformational change and $1e^-$ reactivity of ET-FAD have been proposed to gate electron transfer and ensure that only one electron per pair accesses the exergonic path. The ET-FAD's resting state as a semiquinone, allows it to accept one more electron, so the second electron of each pair must use the endergonic pathway. Thus, the stability of ET-flavin's semiquinone is critical to the efficiency of bifurcation. The ET site environment tunes the ET-flavin to adopt the anionic semiquinone (ASQ) state, although ASQ only accumulates to 1% of the population in free FAD.[2, 3]

The unusually high $E^o_{(OX/ASQ)}$ of ET-FAD has been attributed in part to a 99% conserved Arg and a 100 % conserved Ser or Thr. However, replacement of these does not suffice to suppress the ASQ of the ET-FAD, indicating that the site employs additional interaction(s) as well.[4] To obtain a comprehensive description of tuning applied by the ET site, we have combined the use of QM/MM computation with substitution of residues that are highly conserved among Bf-ETFs. We have demonstrated a conserved His (H290) to be critical, for the stability of ET-FAD_{ASQ}. Moreover, we can explain *why* this residue so important, based on our computations. Natural Bond Order (NBO) partial charge distributions within the two FADs in each observed oxidation state demonstrate that a H-bond from the His side chain to the ET-flavin O2 stabilizes excess negative charge, especially in the ASQ state and exclusively in the ET site.

H290 variant demonstrated lower accumulation of ET-FAD_{ASQ} and perturbation of ET-flavin's $E^{o'}_{Ox/SQ}$ by 150 mV and $E^{o'}_{SQ/HQ}$ by 100 mV. Thus, our complementary experiments and computation can explain the unusual and critical redox tuning of the ET-flavin substantially in terms of an individual interaction.

This study is supported by **National Science Foundation CHE 210814**

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#17

Construction of Protein Nanodiscs to study Cytochrome P450 27A1

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Cytochrome P450 27A1 is a mitochondrial peripheral membrane protein. It is able to catalyze the hydroxylation of cholesterol and vitamin D3. One method of studying membrane proteins in solution is to produce phospholipid bilayer nanodiscs. This allows researchers to insert a target protein into a kind of lipid membrane raft and mimic the native environment of membrane proteins. When exposed to water the hydrophobic regions on a protein membrane can misfold causing the enzyme to not function in vitro.

Our approach to synthesizing membrane scaffolding protein (MSP) in the lab was to utilize recombinant expression in *Escherichia coli* for protein expression. After the presence of MSP is determined in solution through gel staining, the His-tag is cleaved and the nanodiscs are constructed through self-assembly. The formation of the nanodiscs provides a membrane-like environment for the protein of interest which allows proteins to be studied in solution in a more native-like state. Nanodiscs are composed of two copies of a membrane scaffolding protein (MSP), phospholipids, and the target protein. The use of Nanodiscs technology is a method to study proteins in their native environment. We have successfully produced membrane scaffolding protein in the lab however, the steps associated with the extraction and purification of MSP needs to be optimized.

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#18

Tagging Human adrenodoxin with a fluorophore to investigate the dissociation constant with cytochrome P450 27A1

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Cytochrome P450 27A1 (P450 27A1) is an enzyme found on the mitochondrial membrane and is responsible for hydroxylating cholesterol and Vitamin D3, enabling them to be solubilized, excreted, or act as signaling molecules. Little is known about how it interacts with its redox partner Adrenodoxin (Adx) and its dissociation constant (K_d) with Adx. Fluorescent titration spectroscopy is a tool that can be used to determine K_d if an environmentally sensitive probe is used. Adx was fluorescently labeled with Alexa-Fluor 488 maleimide C_5. P450 27A1 was then titrated into a solution containing this tagged Adx. It was expected that an increase in fluorescent emission signal would provide the basis for a binding isotherm, similar to an experiment done with cytochrome P450 27C1, a member of the same family.[1] However, no signal increase was observed. Due to P450 27A1 and P450 27C1 being in the same protein family, it was hypothesized that the proteins would act similarly when binding to Adx. The lack of signal increase suggests that Adx and P450 27A1 interact differently than what has been observed with Adx and P450 27C1.

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#19

Elucidating the functions of putative nickel metallochaperones in *Methanococcus maripaludis*

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Methanogens are a group of archaea that utilize methanogenesis to convert simple carbon compounds to energy and a methane byproduct. This process is responsible for the production of over 1 billion tons of methane each year,[1] making it the most prominent source of methane on earth. This microbially-produced methane has great potential to be used as a source for renewable, sustainable energy. To be able to effectively utilize methanogens for efficient methane production, their essential metalloenzymes need to be produced in high yields, requiring a mechanistic understanding of metallocenter biosynthesis. This study focuses on the maturation process of [NiFe] hydrogenases in methanogens, a group of enzymes that are essential for hydrogenotrophic methanogenesis.[2] Previous work in bacterial systems has demonstrated the involvement of at least two metallochaperones – HypA and HypB – which are important for the delivery of nickel to the active site of [NiFe] hydrogenase.[3,4] Interestingly, most methanogen genomes, including the model hydrogenotrophic methanogen *M. maripaludis*, contain at least two genes that are annotated as HypB (referred to here as HypB1 and HypB2). Only one is likely to be a true HypB ortholog, while the other may be involved in the maturation of a different nickel-dependent enzyme. Alternatively, each annotated HypB may have distinct [NiFe] hydrogenase targets as *M. maripaludis* has multiple different [NiFe] hydrogenases.

We have recombinantly expressed and purified strep-tagged versions of the two putative *M. maripaludis* HypBs in *M. maripaludis*. Upon concentrating the purified protein, HypB1 appeared to have a grey-ish metal color. This suggests that HypB1 was purified with bound metal, although the identity and the quantity of the metal bound need to be further determined. Cells expressing recombinant HypBs were further subjected to *in vivo* cross-linking with either formaldehyde or dithiobis(succinimidyl propionate) to detect proteins that interact with each putative metallochaperone. Both methods of *in vivo* cross-linking performed were able to capture proteins that associated with the methanogenic HypBs. Future work will include identifying the metal bound to the purified HypBs and identifying the proteins captured in the *in vivo* cross-linking experiments, followed by further detailed investigation of the function and importance of HypBs metallochaperones in *M. maripaludis*.

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#20

Stereochemistry and Regiochemistry in Dinoflagellate Bioluminescence

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Dinoflagellate luciferase catalyzes the bioluminescent oxidation of an open-chain tetrapyrrole substrate using molecular oxygen and produces light, an oxidized product, and water. Dinoflagellate bioluminescence is 'simple' bioluminescence, requiring no other cofactors or cosubstrates. The tetrapyrrole substrate, called dinoflagellate luciferin, has two unresolved stereocenters, one at the C4 chiral carbon and one at the C15=C16 double bond. Different bioluminescent reaction mechanisms have been proposed for each substrate stereochemistry. Importantly, luciferase is pH-dependent, optimally active at pH ~6 and inactive above pH 8. Mechanistic studies of the bioluminescent reaction have been hindered by the lack of a crystal structure of the active form of the enzyme; however, recent computational studies have produced a proposed active conformer. Using the calculated open conformer of the enzyme, molecular dynamics simulations were conducted in order to understand the binding of each potential stereoisomer of the luciferin substrate and oxyluciferin product, as well as potential stereoisomers of proposed reaction intermediates implicated in the catalytic mechanism. The enzymatic interactions of each were investigated and implications for the stereochemical course of the bioluminescent mechanism are presented. Our results predict that the bioluminescent reaction utilizes the (*E,S*) stereoisomer of luciferin, and molecular oxygen can bind before or after deprotonation. The putative catalytic base, Glu1105, then abstracts the pro-*S* hydrogen from $C13^2$ of the substrate to create an enolate intermediate, which radically couples to oxygen resulting in superoxide addition to the *re* face of the enolate intermediate. Proton abstraction by the substrate then produces a hydroperoxy intermediate facilitated by the deprotonation of Glu1105. Each intermediate is stabilized by hydrogen bonding to a key residue, Arg1142. The hydroperoxy species undergoes radical rearrangement to an open-shell singlet *gem*-diolate state which emits a photon. Finally, we predict that the post-emission *gem*-diolate species is stereospecifically protonated by Tyr1168 resulting in the elimination of water, which predicts that dinoflagellate luciferase is a monooxygenase. We identify further residues facilitating each step in the proposed mechanism as candidates for mutagenesis experiments.

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#21

Constant pH Accelerated Molecular Dynamics Investigation of the pH Regulation Mechanism of Dinoflagellate Luciferin Binding Protein

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Dinoflagellates utilize a unique bioluminescence system both from a cellular and molecular perspective. The production of light occurs in sub-cellular particles termed scintillons, which contain the luciferin substrate (LH_2), the luciferase enzyme (LCF), and a luciferin binding protein (LBP). The activity of LBP is dependent on the pH of the system, where it binds to and protects LH_2 from autoxidation at alkaline pH ~8 and releases it at acidic pH ~6 when the activity of LCF is maximal.(1) LBP has been proposed to exist as a dimer that binds one equivalent of LH_2 and releases it through a conformational change below pH 7.(2) However, the structure and mechanism by which acidification induces this conformational change are unknown. Here, constant pH accelerated molecular dynamics (CpHaMD) coupled with molecular docking studies are applied to gain insight into the conformational dynamics of a structural model of LBP as a function of pH and the mode of binding/release of LH_2 to/from LBP.

This study is being funded by the NSF and calculations were performed using Alabama Supercomputer.

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#22

Spin State Characterization of Metalloenzymes via X-ray Emission Spectroscopy

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Enzyme reactivity is often enhanced by changes in oxidation state, spin state, and metal-ligand covalency of associated metallocofactors. The development of spectroscopic methods for studying these processes coincidentally with structural rearrangements is essential for elucidating metalloenzyme mechanisms. Herein, we demonstrate the feasibility of collecting X-ray emission spectra of metalloenzyme crystals at a third-generation synchrotron source.

In particular, we report the development of a von Hamos spectrometer for the collection of Fe Kβ emission optimized for analysis of dilute biological samples. We further showcase the application of this high-resolution, "DIY" mini-spectrometer in crystals of the immunosuppressive heme-dependent enzyme indoleamine 2,3-dioxygenase (IDO).[1]

Spectra obtained from reference compounds were compared to catalytically relevant states of IDO via integrated absolute difference analysis to determine their spin numbers, a method traditionally used in characterizing inorganic compounds.[2,3] Complementary density functional calculations assessing covalency support our spectroscopic analysis and identify active site conformations that correlate to high- and low-spin systems. These experiments validate the suitability of an X-ray emission approach for determining spin states of previously uncharacterized metalloenzyme reaction intermediates.

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#23

The β-latch structural element of the SufS cysteine desulfurase mediates active site accessibility and SufE transpersulfurase positioning.

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Under oxidative stress and iron starvation conditions, *Escherichia coli* uses the Suf pathway to assemble iron-sulfur clusters.[(1)] The Suf pathway mobilizes sulfur via SufS, a type II cysteine desulfurase. SufS is a pyridoxal-5′-phosphate–dependent enzyme that uses cysteine to generate alanine and an active-site persulfide (C_{364}-S-S^-). The SufS persulfide is protected from external oxidants/reductants and requires the transpersulfurase, SufE, to accept the persulfide to complete the SufS catalytic cycle. Recent reports on SufS identified a conserved "β-latch" structural element that includes the α_6 helix, a glycine-rich loop, a β-hairpin, and a *cis*-proline residue.[(2)] To identify a functional role for the β-latch, we used site-directed mutagenesis to obtain the N99D and N99A SufS variants. N99 is a conserved residue that connects the α_6 helix to the backbone of the glycine-rich loop via hydrogen bonds. Our x-ray crystal structures for N99A and N99D SufS show a distorted beta-hairpin and glycine-rich loop, respectively, along with changes in the dimer geometry. The structural disruption of the N99 variants allowed the external reductant TCEP to react with the active-site C364-persulfide intermediate to complete the SufS catalytic cycle in the absence of SufE. The substitutions also appear to disrupt formation of a high-affinity, close approach SufS–SufE complex as measured with fluorescence polarization. Collectively, these findings demonstrate that the β-latch does not affect the chemistry of persulfide formation but does protect it from undesired reductants. The data also indicate the β-latch plays an unexpected role in forming a close approach SufS–SufE complex to promote persulfide transfer.

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#24

Functional implications of partner proteins in the thioredoxin system of hyperthermophilic methanogens

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Methanocaldococcus jannaschii (*Mj*) is a phylogenetically deeply rooted hyperthermophilic methanogen that lives in deep-sea hydrothermal vents where the environmental conditions mimic those of early Earth. In this habitat, *Mj* experiences about 60,000-fold changes in the availability of hydrogen (H_2), the only energy source it can utilize, and this translates to a major redox shock. Accordingly, it is highly likely that *Mj* possesses fast-acting and efficient response systems to manage these redox stresses and thrive in such an extreme environment. We hypothesize that *Mj* utilizes a highly specialized thioredoxin (Trx) system as part of its stress response infrastructure. It is composed of two thioredoxins, *Mj*Trx1 and *Mj*Trx2, and an unusual Trx reductase that utilizes coenzyme F_{420} as an electron carrier and called deazaflavin-dependent flavin-containing thioredoxin reductase (*Mj*DFTR) (1, 2). *Mj*DFTR carries a flavin unit with a low mid-point redox potential value (E'_0, –389 mV) and this feature would allow the activation system to operate only when the H_2 level is suitable for energy production and growth. Recently, we have discovered that this system has another layer of complexity. The genes of *Mj*Trx1 and *Mj*Trx2 are co-expressed with that of a set of partnering proteins, *Mj*Trx1-p and *Mj*Trx2-p, that likely control the activities of the former. Our results suggest that *Mj*Trx1-p would enhance the activity of *Mj*Trx1 and *Mj*Trx2-*Mj*Trx2-p complex would serve in an iron-sulfur cluster biogenesis role under low redox conditions that are conducible to growth, and these activities will stop if the organism faces a high redox situation.

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#25

Structural and functional studies of epoxyqueuosine reductase QueH in the biosynthesis of queuosine modified tRNA

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A large number of post-translational modifications are found in the anticodon stem-loop (ASL) of transfer RNAs (tRNAs), prominently located at the key wobble position. Queuosine (Q) is a representative hypermodified nucleoside found in the wobble position of tRNAs encoding for Asn, Asp, His or Tyr.[1] The base has been shown to be broadly distributed in bacteria and eukaryotes. Epoxyqueuosine (oQ) reductase QueG is responsible for the final step of the biosynthetic pathway to Q, catalyzing a reduction of oQ to Q.[2] However, some eubacteria that retain Q biosynthesis lack QueG orthologs. Using comparative genomics approaches and analysis of opposite distribution patterns, QueH was identified as a nonorthologous replacement for QueG.[3]

In this study, we determined a series of high-resolution structures of QueH from *Thermotoga maritima* by X-ray crystallography to define how QueH is able to perform epoxide reduction. The structure of QueH showed that the enzyme contains two metal binding sites: an iron-sulfur cluster coordinated with a four-cysteines, and an unpredicted single metal binding site formed two cysteines and an aspartate ligand.[4] To understand what the single metal is and how QueH catalyze the deoxygenation of epoxyqueuosine, a mutant was prepared and the structure shows that the two cysteines are indispensable for the single metal binding site. Chemical composition analysis and inductively coupled plasma mass spectrometry (ICP-MS) analysis were employed confirming the [4Fe-4S] cluster along with a single metal zinc. Electron paramagnetic resonance (EPR) was also used to characterize the [4Fe-4S] cluster and demonstrate the redox properties. The information in these studies provides insight into the mechanism of the final step of the Q biosynthetic pathway catalyzed unique enzymology.

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#26

Robust Chemoenzymatic Synthesis of the Keratinimicin Aglycone Facilitated by the Specificity and Structure of OxyB

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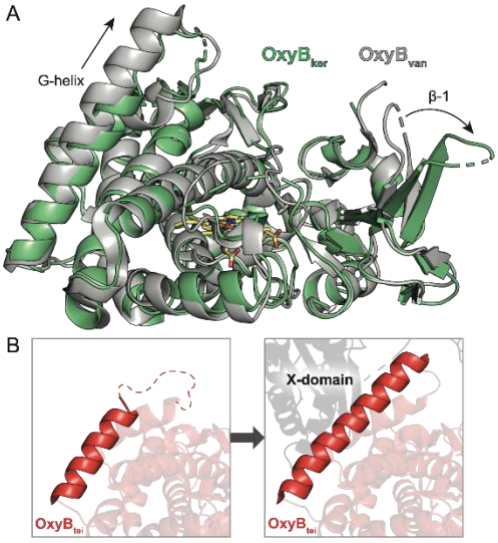
The emergence of multidrug-resistant pathogens poses a threat to public health and requires new antimicrobial agents. As the archetypal glycopeptide antibiotic (GPA) used against drug-resistant Gram-positive pathogens, vancomycin[1] provides a promising starting point. Peripheral alterations to the vancomycin scaffold have enabled the development of new GPAs. However, modifying the core remains challenging due to the size and complexity of this compound family. The recent successful chemoenzymatic synthesis of vancomycin[2] suggests that such an approach can be broadly applied. Herein, we describe the expansion of chemoenzymatic strategies to encompass type II GPAs bearing all aromatic amino acids through the production of the aglycone of keratinimicin A, a GPA that is five-fold more potent than vancomycin against *Clostridioides difficile*.[3] In the course of these studies, we found that the cytochrome P450 enzyme $OxyB_{ker}$ boasts both broad substrate tolerance and remarkable selectivity in the formation of the first aromatic crosslink on the linear peptide precursors. The X-ray crystal structure of $OxyB_{ker}$, determined to 2.8 Å, points to structural features that may contribute to these properties. Our results set the stage for using $OxyB_{ker}$ broadly as a biocatalyst toward the chemoenzymatic synthesis of diverse GPA analogues.



Crystal structure of $OxyB_{ker}$ and the importance of the G-helix. (A) X-ray crystal structure of $OxyB_{ker}$ (PDB accession code 8F91, green) overlaid with $OxyB_{van}$ (PDB accession code 1LFK, gray), with the heme cofactor shown in yellow; main structural differences are found in peripheral loops and the G-helix, which interacts with the X-domain in the teicoplanin system. B) Elongation of the G-helix upon X-domain binding to $OxyB_{tei}$ (PDB accession code 4TVF [left] and 4TX3 [right]).

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#27

Shikimate Pathway Enzymes as candidates for Multitarget Inhibitor Development

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Tuberculosis (TB) is one of the preeminent causes of death among infectious agents. In 2021, an estimated 1.6 million deaths were attributed to TB, with around 10.6 million new cases emerging in the same year. The most used drugs for TB treatment are rifampin, isoniazid, pyrazinamide, and ethambutol. Due to the substantial increase in strains with multi and extensive drug resistance, it is crucial to identify novel anti-tubercular agents.[1] For the last three decades "one disease, one target, one drug" paradigm has been the primary approach to new drug discovery. Because drugs are often developed targeting a particular bacterial molecule, especially proteins, the emergence of resistance only requires modification of a single target. Accordingly, this approach is partly liable for the surge in antibiotic resistance observed across multiple infectious agetents. So, the search for multitarget inhibitors has accelerated greatly in recent years.

The shikimate pathway is essential for the survival of *Mycobacterium tuberculosis (Mt),* the causative agent of TB. This pathway produces chorismate, a precursor for aromatic amino acids and other aromatic cellular metabolites. Fortuitously, the shikimate pathway is not present in mammals. Because several downstream substrates and products of this pathway share similar scaffolds, enzymes involved in this pathway are promising for novel anti-tubercular drug development.[2] We are evaluating shikimate dehydrogenase (SDH; EC 1.1.1.25) and shikimate kinase (SK; EC 2.7.1.71), the fourth and fifth enzymes of the pathway, respectively, as consecutive targets for a multitarget inhibitor. Both enzymes were expressed following a similar approach.[3] *M. tuberculosis* H37Rv *aroK* and *aroE* genes were optimized for expression in *E. coli* and synthesized for the separate expression of each enzyme, *M. tuberculosis* shikimate kinase (*Mt*SK) and *M. tuberculosis* shikimate dehydrogenase (*Mt*SDH). The genes were incorporated into the pET vectors to encode a 6X-His tag on the terminal end of the protein products. *E. coli* (BL21-Gold[DE3]pLysS) transformants were selected based on ampicillin and chloramphenicol resistance. Enzymes were purified using nickel-nitrilotriacetic acid (Ni-NTA) chromatography (subsequently, size exclusion chromatography for *Mt*SDH). Steady-state kinetic analyses were carried out for each enzyme to enable estimation of kinetic parameters with respect to the substrates utilized by each enzyme.

To identify and validate potential candidates for multitarget inhibition of *Mt*SK and *Mt*SDH, multiple *in silico* approaches (molecular docking and dynamics) were utilized. A small-molecule database of 9523 compounds was constructed using ZINC15 and PubChem. PDB codes 4P4G and 2IYQ were used as the crystal structures of *Mt*SK and *Mt*SDH, respectively. Molecular docking and virtual screening were carried out using the UCSF DOCK 6.9 available on the HPC systems of the Alabama Supercomputer Authority (ASA). Based on the descriptor score and footprint similarity score, the top 50 screened compounds for both enzyme systems were selected. Crosschecking the top 50 compounds provided 19 overlaps which were then subjected to fragment similarity score (FragFp) analysis using DataWarrior. FragFp analysis eliminated three compounds, providing the top 16 potential candidates which were further subjected to ADMET analysis using SwissADME and pkCSM. Thirty-two sets of the protein-ligand complex (generated during the docking) were analyzed using LigPlot^{+} and the Ligand Contact tool to determine the interacting residues and corresponding bond types. Molecular dynamics (MD) analysis was carried out using AMBER to calculate the binding free energies. The ff14SB force field with the TIP3P water model was utilized for these analyses. Additional *in vitro* analyses will be carried out to elucidate the kinetic mechanisms and mode of action of the multitarget inhibitor candidates.

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#28

Rational Design of Amino Ester Hydrolase (AEH) for Improved Thermostability

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α-Amino ester hydrolases (AEHs) are enzymes of interest for their ability to rapidly synthesize several β-lactam antibiotics with particular interest in cephalexin synthesis. AEHs have stronger substrate specificity and higher activity than that of penicillin G acylase (PGA), the enzyme currently used in industrial production of several β-lactam antibiotics; however, one of the major shortcomings of AEHs is their low thermostability. AEH rapidly deactivates near its temperature optimum of activity at 25 ℃ and has a half-life of 30 minutes at 27 ℃. Previous attempts to stabilize AEH using B-FIT analysis and structure-guided consensus methods resulted in a quadruple variant (QVH, E143H/A275P/N186D/V622I) which has a 30 minute half-life at 34 ℃ [1]. While this variant is an improvement over wild-type AEH, this work aims to improve the thermostability of AEHs further with the aid of rational design techniques.

For β-lactam synthesizing enzymes such as AEH, directed evolution campaigns are not possible as a high-throughput assay for the desired synthesis reaction does not currently exist. We focused instead on employing two computational techniques for improvement of AEH thermostability: FireProt [2] and PROSS [3]. Single mutations predicted from both PROSS and FireProt were first tested for thermostability improvements relative to QVH. While marginal improvements were made, we found that whole variant design through PROSS (up to 15% of residues mutated) rapidly enhanced AEH thermostability; however, most variants were found to be inactive. A mutated calcium binding site was found to cause inactivation and changes in AEH oligomeric state. Several rounds of PROSS variants were designed with increasingly stringent restrictions.

The most active variant designed, coined QVH.P.3.2, has 4% of residues mutated and greatly improved AEH thermostability as measured by nanoscale differential scanning fluorimetry (nanoDSF). For all active variants designed by PROSS, the optimal temperature of activity does not appear to be greatly improved, implying that improved thermostability measured by unfolding thermodynamics does not necessarily translate to improved operational stability.

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#29

On the kinetic mechanism of dimethylarginine dimethylaminohydrolase (DDAH)†

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Dimethylarginine dimethylaminohydrolase (DDAH, EC 3.5.3.18) catalyzes the hydrolysis of asymmetric Nω,Nω-dimethyl-L-arginine (ADMA), an endogenous inhibitor of human nitric oxide synthases. The active-site Cys residue has been proposed to serve as the catalytic nucleophile, forming an S-alkyl thiourea reaction intermediate, and serving as a target for covalent inhibitors. Inhibition can lead to ADMA accumulation and downstream inhibition of nitric oxide production. Prior studies have provided experimental evidence for the formation of this covalent adduct but have not characterized it kinetically. Here, rapid quench-flow is used with ADMA and the DDAH from Pseudomonas aeruginosa to determine the rate constants for formation (k_2 = 17 ± 2 s^{-1}) and decay (k_3 = 1.5 ± 0.1 s^{-1}) of the covalent S-alkyl thiourea adduct. A minimal kinetic mechanism for DDAH is proposed that supports the kinetic competence of this species as a covalent reaction intermediate and assigns the rate-limiting step in substrate turnover as hydrolysis of this intermediate. This work helps elucidate the different reactivities of S-alkyl thiourea intermediates found among the mechanistically diverse protein superfamily of guanidine-modifying enzymes and provides information useful for inhibitor development.

#30

Investigating the Structure-Function Correlation of Carotenoid Cleavage Dioxygenase BCO1

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Carotenoid Cleavage Dioxygenases (CCDs) constitute a diverse family of non-heme iron-dependent enzymes. These evolutionarily conserved enzymes regulate carotenoid metabolism in various organisms and play vital roles in pigmentation, photoprotection, photosynthesis, visual health, cell signaling, etc. However, the catalytic mechanism of CCDs is poorly understood. Fundamental biochemical questions such as how CCDs activate oxygen and target specific C=C double bonds in carotenoids are obscure. CCDs are structurally distinct from most non-heme iron-dependent oxygenases by having a 7-bladed β-propeller fold, 4-His metal coordination, and three Glu residues as the second-sphere coordination, although it is unclear how the unique structural features endow the enzymatic functions of CCDs. To better understand these physiologically essential enzymes, a representative CCD, BCO1, was chosen for structure-function investigations. BCO1 symmetrically cleaves β-carotene in the presence of O_2, serving as the initial step in the biosynthesis of Vitamin A. Dysfunctions of BCO1 lead to hypercarotenemia and Vitamin A deficiency, which can affect vital processes such as vision, embryonic development, cell differentiation, and membrane and skin protection. BCO1 from *Caenorhabditis elegans* (CeBCO1) was tested for activity assays through UV-vis absorption spectroscopy and HPLC analysis. CeBCO1 showed activity in the degradation of β-carotene, which was not previously reported. Furthermore, EPR was employed for analysis of perturbations of the iron center upon substrate binding. Nitrosyl complexes were observed in both high- and low-spin regions. Through this research, we aim to characterize the catalytic mechanism of BCO1, along with expanding the knowledge of the CCD superfamily and providing a pathway for treatment of Vitamin A deficiency-related disorders.

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#31

Study of protein dynamics of retaining GT-B glycosyltransferases using hydrogen-deuterium exchange mass spectrometry

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Glycosyltransferases (GTs) catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, resulting in the formation of new glycosidic bonds, and are involved in a wide range of biological processes, including cell-cell recognition, immune response, and protein folding and stability. Structurally GTs are classified into two major folds: GT-A and GT-B. Both folds can catalyze a reaction with the formation of new glycosidic bonds with either retention or inversion of configuration as compared to the configuration of sugar donors. In GT-B enzymes, substrates bind in the cleft resulting from two Rossmann-like domains connected by a linker region. The formation of the binding cleft involves a conformational change where the *C*-terminal domain (CTD) undergoes rigid body rotation relative to the *N*-terminal domain (NTD) to bring the donor and acceptor substrates together. In inverting GT-Bs, catalytic residue helps to generate a nucleophile from the acceptor substrate. In contrast, the retaining GT-Bs utilize a substrate-assisted mechanism where the donor substrate itself acts as a catalytic base to generate a nucleophile from the acceptor. Thus, correct positioning of the acceptor and donor substrate is critical in retaining GT-B enzymes. For proper orientation of substrates, the local and global dynamics of retaining GT-Bs may be important. In this research, the change in dynamics of the retaining GT-B enzyme CgMshA (MshA from *Corynebacterium glutamicum*) in the presence of substrates (uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc)) and inositol-1-phoshphate (I1P) and product (uridine diphosphate (UDP)) was investigated using hydrogen deuterium-exchange mass spectrometry. The acceptor substrate (I1P) binding decreased the deuterium uptake in residues 26-34, 77-85, 159-198, and 392-398, which are mostly from the second half of the NTD and the linker region. The donor substrate (UDP-GlcNAc) binding decreased deuterium uptake in the second half of both the NTD and the CTD. Identical deuterium uptake at the NTD in the presence of either I1P or UDP-GlcNAc suggests that the binding of UDP-GlcNAc creates the binding site for I1P. UDP binding resulted in a loss of deuterium uptake in the majority of residues from both the NTD and the CTD as compared to the presence of I1P or UDP-GlcNAc suggesting a less dynamic conformation when UDP was bound. The binding of UDP-GlcNAc resulted in the EX1 exchange kinetics of residues 323-334, 335-351, and 369-381. Within this set of peptides, E324 contributes to binding the ribose of UDP-GlcNAc and may play important role in conformational change. While UDP-GlcNAc showed protection in regions of the CTD, the NTD experienced identical protection in the presence of UDP-GlcNAc at early exchange time points (15 s) to that of the apo state. To identify if UDP-GlcNAc caused any changes in dynamics on a faster timescale, rapid-quench HDX-MS was used. However, from the 50-2000 ms exchange reaction time, the decrease in deuterium uptake was observed only at the residues from the second half of CTD.

#32

The role of the m1G37 RNA modification in tRNAProL in regulating mRNA reading frame maintenance and EF-P recruitment

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Maintenance of the mRNA reading frame ensures accurate protein synthesis and prevents the increased fitness costs associated with nonfunctional, mistranslated proteins[1]. When the mRNA reading frame is not maintained, this event is called frameshifting where the three nucleotide codon shifts towards either the 5'(-) or 3'(+) end of the mRNA. This frameshift event alters what is translated from the remainder of the mRNA. To prevent unwanted frameshift events from occurring, the cellular translation machinery contains several modified nucleic acids and different translation factors to help preserve the correct mRNA reading frame. One highly modified subset of molecules used in translation are tRNAs, which decode codons to determine which amino acid will be incorporated into the growing protein chain[2]. tRNA molecules contain over 100 of the known 150 nucleic acid modifications, with many of the modifications located in or near the anticodon, the region of the tRNA that interacts with the mRNA codon.

The RNA modification m^1G37 is located adjacent to the 3' end of the anticodon and is known to help prevent +1 frameshifts in the $tRNA^{Pro}$ family of isoacceptors. These tRNAs are prone to frameshifts due to the slow rate of peptide synthesis involved with proline, especially on slippery, polynucleotide codons[3, 4]. Here, we determined structures of the 70S ribosome in different elongation states to determine how the absence of modification m^1G37 in $tRNA^{ProL}$ impacts reading frame maintenance. Additionally, elongation factor P (EF-P) is recruited to ribosomes stalled on polyproline codons and we solved a structure of EF-P bound to the 70S, preventing $tRNA^{Pro}$-mediated frameshift errors[5].

This study was supported by the NIH under award number R01GM093278. Cryo-EM datasets were collected at the National Center for Cryo-EM Access and Training (NCCAT) and the Simons Electron Microscopy Center located at the New York Structural Biology Center. These centers are supported by the NIH Common Fund Transformative High Resolution Cryo-Electron Microscopy program (U24 GM129539) and by grants from the Simons Foundation (SF349247) and NY State Assembly.

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#33

H^{421} modulates substrate capture and catalysis in *Pseudomonas aeruginosa* D-2-Hydroxyglutarate Dehydrogenase

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Pseudomonas aeruginosa is an opportunistic gram-negative bacterium responsible for 10% of all hospital-acquired infections in the US, including ventilator-associated pneumonia, incision-related and nosocomial infections[1]. The bacterium requires the flavoenzyme D-2-hydroxyglutarate dehydrogenase (*Pa*D2HGDH), which converts D-2-hydroxyglutarate to 2-ketoglutarate during L-serine biosynthesis, for survival[2]. Knockout of the gene that codes for *Pa*D2HGDH inhibits *P. aeruginosa* growth, making *Pa*D2HGDH a potential therapeutic target against *P. aeruginosa*[3]. Recent studies have identified Zn^{2+} as a required metal cofactor that orients and activates the *Pa*D2HGDH substrate for catalysis[4,5]. Amino acid sequence comparison of *Pa*D2HGDH to other closely related enzymes reveals a fully conserved H^{421} residue[3]. In the *Pa*D2HGDH homology model, H^{421} aligns with a topologically conserved histidine, which functions as a catalytic base to initiate the hydride transfer from the substrate to the flavin N^5 atom in most glucose-methanol-choline (GMC) enzymes[6]. However, the role of H^{421} in *Pa*D2HGDH has not been established[3].

In this study, H^{421} was replaced with glutamine ($H^{421}Q$), cysteine ($H^{421}C$), or phenylalanine ($H^{421}F$) by site-directed mutagenesis. The resulting variant enzymes were purified and characterized using biochemical and biophysical approaches. The flavin extinction coefficient (ε_{450}) value for the $H^{421}Q$ mutant was identical to that of the wild-type, with a value of 12.5 ± 0.4 $mM^{-1}cm^{-1}$. In contrast, the $H^{421}C$ and $H^{421}F$ variants had ε_{450} values of 11.7 ± 0.2 $mM^{-1}cm^{-1}$ and 11.2 ± 0.2 $mM^{-1}cm^{-1,}$ respectively. The flavin N^3 atom's pK_a value decreased from 11.9 in the wild-type to 10.7 in the $H^{421}Q$ mutant. Of the three mutant enzymes, only $H^{421}Q$ was active with D-malate as substrate, allowing for the determination of the apparent k_{cat} value of 0.81 ± 0.03 s^{-1}, which was 15x slower than the turnover rate of the wild-type with a k_{cat} of 12.3 ± 1.2 s^{-1}. Additionally, there was an observed 35-fold decrease in the k_{cat}/K_m parameter from 5,000 ± 1,000 to 140 ± 10 $M^{-1}s^{-1}$ upon H^{421} mutation to Q^{421}. The data suggest that the fully conserved H^{421} residue is important for substrate capture and catalysis in *Pa*D2HGDH.

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#34

Divalent Metal Ion

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2-methyl-4-amino-5-hydroxymethylpyrimidine phosphate (HMP-P) synthase is a radical S-adenosyl-methionine (SAM) enzyme that uses a reduced tris-cysteine-coordinated [4Fe-4S] cluster to reductively cleave the 5'C─S bond to produce a highly reactive 5'-deoxyadenosyl (5'-dAdo●) radical. 5'-dAdo● then abstracts a hydrogen atom from aminoimidazole ribonucleotide (AIR) as the first step toward its conversion into HMP-P, a crucial intermediate for the production of thiamin (vitamin B1). HMP-P synthase (ThiC) is distinct from conventional radical SAM enzymes as the carboxylato and amino termini of the methionine fragment are tethered to a divalent metal ion site as opposed to the unique iron of the [4Fe-4S] cluster. This divalent metal ion is bound to the enzyme by two histidine sidechains at positions 426 and 490. In this study we expressed and purified single and double histidine ThiC mutants to better understand the effect of the divalent metal ion site on enzyme functionality. When these histidines were replaced with alanines, we observe changes in the ability of ThiC to produce deoxyadenosine, a product of SAM cleavage, and HMP-P, the product of the native reaction. We discuss the relevance of these findings on the importance of the divalent metal ion to the mechanism of ThiC.

#35

Insights into the Interconversion of Activities in Radical SAM Enzymes ThiC and BzaF

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Radical SAM enzymes (RSEs) are ubiquitous in nature, occurring in all domains of life. They perform a wide variety of reactions all initiated by radical cleavage of S-adenosyl-L-methionine (SAM) to yield a 5'-deoxyadenosyl (5'-dAdo) radical and L-methionine, with the former typically abstracting a hydrogen atom from the substrate to begin a cascade of reactions toward forming product.[1] ThiC is an RSE which catalyzes the radical rearrangement of 5-aminoimidazole ribonucleotide (AIR) to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P), an important biosynthetic precursor to thiamin.[1] BzaF is another RSE which also catalyzes a radical rearrangement of AIR, but instead converts it into 5-hydroxybenzimidazole (HBI), a precursor to dimethylbenzimidazole (DMB), the lower axial ligand of vitamin B12/cobalamin.[2] ThiC and BzaF both catalyze radical rearrangement of AIR, yet each creates a very different product. Interestingly, the active sites of Arabidopsis thaliana ThiC (*At*ThiC) and Desulfuromonas acetoxidans BzaF (*Da*BzaF) differ by only three amino acids in the first shell.[2]

We sought to determine whether conversion of these AAs in *At*ThiC to their *Da*BzaF counterparts would also convert the activity of *At*ThiC to that of *Da*BzaF. To that end, variants of *At*Thic were produced with every combination of one, two, or all three of the mutations D383N, N228S, and T320A, and each variant was assessed for relative 5'-dAdo production, AIR consumption, HMP-P production, and HBI production using HPLC-UV-Vis. All variants exhibited 5'-dAdo production, although only the T320A single mutant appeared to retain any level of HMP-P production. Some variants seemed to consume AIR without producing HMP-P, implying that they may be forming a novel product.

#36

Investigating the Substrate Binding and Enzymatic Activity of a Heme Enzyme in Pyrrolnitrin Biosynthesis

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Pyrrolnitrin is an antifungal metabolite whose biosynthetic pathway consists of a conversion of tryptophan in four steps. The second step is a ring rearrangement reaction of 7-Cl-tryptophan catalyzed by a histidine-ligated heme-dependent enzyme PrnB. This reaction includes indole cleavage, pyrrole formation, and decarboxylation. PrnB belongs to the enzyme family of indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), and it is recently expanded to histidine-ligated heme-dependent aromatic oxygenases (HDAO). The activity of PrnB has only been observed in *vivo*. Previous studies using O_2 as a co-substrate of PrnB were unable to establish product formation. In this work, we probe the binding of Trp and its analogs by using UV-vis and EPR spectroscopies. Trp and Tryptamine covalently bound to the oxidized heme cofactor of PrnB through the amine groups, forming six-coordinate low-spin heme centers, but Tryptamine has a decreased binding affinity. However, 3-indolepropionic acid (3-IDPA) only bound to the distal pocket with a K_D comparable to that of Trp. The results indicate that the $-COO^-$ group is more critical for binding, but the $-NH_3^+$ group has a more profound impact on the heme cofactor. As shown by HPLC and stopped-flow experiments, the direct coordination blocked the heme center and prevented the activation of small molecules, leading to the absence of the activities. The change of pH and the extra space gained from the removal of COO^- could allow NH_3^+ to be displaced by the oxidant to some extent. The substrate binding and enzymatic activity of PrnB will also be explored in the reduced state. Solving the mystery of the PrnB activity is expected to expand the heme catalysis with a new chemistry and provide a template for enzyme engineering to facilitate the production of antifungal biomolecules.

This work is supported by NIGMS R35GM147510 and the start-up funds provided by UGA.

#37

Active Site Residues Involved in the Interaction of 2,4'-Dihydroxyacetophenone Dioxygenase with its Substrate.

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2,4'-Dihydroxyacetophenone dioxygenase (DAD) catalyzes the oxidative cleavage of 2,4'-dihydroxyacetophenone (DHA) to form 4-hydroxybenzoic acid (4HB) and formic acid. Computational docking simulations suggest an important hydrogen bonding interaction between the 4'-OH group of DHA and residues W62 and D64 in DAD. The importance of these residues in the formation of the DAD-DHA complex was tested by expressing, transforming, and purifying DAD mutants D64N, W62F, and W62F/D64N. We have successfully expressed and purified these three mutants. DHA consumption assays were performed to determine the kinetic parameters of the mutants compared to wild-type. Kinetic analyses of D64N and W62F/D64N show an identical but crippling effect on the kinetics (on both k_{cat} and k_{cat}/K_M), while W62F shows nearly identical kinetics to wild-type. These results strongly suggest that D64 is important for interaction with DHA, but W62 appears to have no effect on the kinetics.

#38

Identification and characterization of the FMN-dependent DMS monooxygenase subunit from *Oceanobacillus piezotolerans*.

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The goal of this project is to identify and characterize novel forms of dimethyl sulfide (DMS) monooxygenase enzyme. DMS monooxygenase is essential to the degradation of DMS volatile sulfur compounds, which play a role in generating clouds and decreasing solar radiation.[1] *Hyphomicrobium sulfonivorans* is a known DMS monooxygenase that has already been isolated and purified. There are two subunits that are functionally required in DMS monooxygenase: a monooxygenase DmoA subunit in charge of DMS breakdown and a flavin reductase DmoB subunit. Bioinformatics using the National Center for Biotechnology Information and the Joint Genome Institute/Integrated Microbial Genomes were used to identify alternative species containing this enzyme in order to study divergence in the phylogenetic tree.[2,3] Candidates that exhibited highly conserved amino acid sequences, a good query coverage, and had both subunits on the same operon, were selected for further characterization. *Oceanobacillus piezotolerans*, a deep soli bacterium found in the Pacific Ocean, was identified as a potential candidate. Characterization of the DmoB subunit from this organism synthesized and transformed into *E. coli* is the focus of this work. The subunit has been successfully expressed in the soluble lysate and purified. Current characterization on the DmoB subunit includes protein crystallization, cofactor and substrate specificity, and subunit interactions. Data collected from successfully synthesizing the enzyme will contribute towards our understanding of how structure dictates chemical mechanism as it relates to climate regulation.

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Poster Abstracts

Poster Session 2
(2:45 pm – 4:15 pm)

#39

Computational Investigations of F430 Biosynthesis Suggest a Cooperative Mechanism of Catalysis by the Nitrogenase Homolog CfbCD

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Functional expression of methyl-coenzyme M reductase (MCR), the enzyme responsible for the terminal step of archaeal methanogenesis, requires a host of accessory proteins for the successful biosynthesis and insertion of its Ni-containing tetrapyrrole cofactor F430.[1] Previous work in the Mansoorabadi laboratory revealed the gene cluster responsible for F430 biosynthesis, denoted *cfb*.[2] F430 stands out as the most highly reduced natural tetrapyrrole, a characteristic achieved by the protein complex CfbCD via an unprecedented ATP-dependent reaction involving the addition of 6 electrons and 7 protons to Ni-sirohydrochlorin *a*,*c*-diamide (Ni-s-acd) and the cyclization of its *c*-acetamide side chain to a γ-lactam ring to form 15,17^3-seco-F430-17^3-acid.[2-3]

CfbCD itself is a member of the nitrogenase superfamily and consists of a CfbC dimer (homologous to the nitrogenase Fe protein) and a CfbD dimer (homologous to the nitrogenase MoFe protein), with both components possessing intersubunit [4Fe-4S] clusters.[3-4] CfbD diverges significantly from the heteromeric MoFe protein, which possesses an intersubunit [8Fe-7S] P cluster in place of the CfbD [4Fe-4S] cluster, in addition to the [7Fe-9S-C-Mo-homocitrate] cofactor at its singular active site. It is unclear if the CfbCD "dimer of dimers" itself dimerizes to form a heterooctamer analogous to fully assembled nitrogenase.[4]

In this study, we have performed constant pH accelerated molecular dynamics (CpHaMD) on an AlphaFold-predicted heterotetrameric model of CfbCD from *Methanosarcina thermophila* docked with Ni-s-acd and proposed reaction intermediates to make predictions of catalytic residues and substrate docking poses.[5-7] Preliminary data demonstrates conformational asymmetry in the CfbD subunits in the absence of substrate. Simulations in the presence of substrate suggest the presence of one active site per CfbD monomer that binds substrate in an asymmetric manner suggestive of catalytic cooperativity between active sites.

This work was supported in part by grants from the U.S. Department of Energy (DE-SC0023451 and Early Career Award DE-SC0018043) and by a grant of high-performance computing resources and technical support from the Alabama Supercomputer Authority.

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#40

Mechanism of Macrolide-Induced Ribosomal Frameshifting

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Antibiotic resistance is a significant and growing threat to public health, with deaths from drug-resistant infections projected to increase from 700,000 per year currently to over 10 million annually by 2050 without preventive action[1]. Therefore, understanding how bacteria gain resistance to antibiotic treatment is critical for ensuring the continued usefulness of these drugs. Macrolide antibiotics bind the bacterial large ribosomal subunit and disrupt peptide bond formation, and their modulation of ribosome function can be exploited by bacteria to control resistance gene expression. Previous studies have demonstrated that translation of the bacterial macrolide resistance gene *ermC* can be induced through a mechanism involving a macrolide-dependent shift in the messenger RNA (mRNA) reading frame at a crucial frameshift-prone mRNA sequence[2]. However, the underlying mechanism of this frameshifting phenomenon is unknown.

The loosening of the ribosome's control over the mRNA reading frame in response to macrolides implies the presence of allosteric communication between the large ribosomal subunit, which houses the macrolide binding site, and the small ribosomal subunit, which handles the mRNA during translation. However, the path of frameshift signal transmission and the nature of this communication are unknown. Structural and biochemical studies to uncover the mechanism of macrolide-induced frameshifting are therefore important for understanding how bacteria regulate resistance to macrolides. Primer extension inhibition ("toeprint") assays to determine the position of the mRNA demonstrate the ability of macrolide-bound ribosomes to frameshift *in vitro*. High-resolution single-particle cryogenic electron microscopy (cryo-EM) studies of actively translating ribosome complexes containing frameshift-prone mRNA and a macrolide antibiotic will demonstrate the mechanism by which macrolide-induced frameshifting proceeds during the elongation cycle of translation. The new understandings generated through this study will reveal how macrolide antibiotics modulate ribosomal activity to perturb mRNA reading frame maintenance and, more generally, how the bacterial ribosome can sense and respond to changes in cellular chemical conditions to regulate translation.

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#41

Role of redox partner concentration on cytochrome P450 27A1 kinetics

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Human mitochondrial enzyme cytochrome P450 (P450) 27A1 oxidizes vitamin D3 into calcifediol using adrenodoxin as a redox partner. Adrenodoxin acts as an electron donor although it may also affect the substrate binding to the enzyme. If electron donation was the rate-limiting step of the reaction, we would expect the measured k_{cat} value of the reaction to change with changing adrenodoxin concentration. If adrenodoxin significantly modifies the ability of the enzyme to bind substrate, we would expect to see an effect on the K_M of the reaction. Michaelis-Menten steady-state kinetics have been performed to measure the effect of adrenodoxin on reaction kinetics. Product formation was quantified using High Performance Liquid Chromatography. Our results show that increasing adrenodoxin concentrations caused a minor increase in P450 27A1 and vitamin D3 k_{cat} and increase the measured K_M. This result is different than what has been reported previously when changing adrenodoxin concentrations in reactions involving human P450s 11B1[1] and 11B2[2]. In those cases, increasing adrenodoxin concentration significantly increased measured k_{cat} values and significantly decreased K_M values. Therefore, despite a conserved role of adrenodoxin to provide reducing equivalents, our data indicates that adrenodoxin may have additional effects on enzyme kinetics that vary from enzyme to enzyme.

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#42

The three-step lanosterol 14α-demethylation reaction catalyzed by human cytochrome P450 51A1 is highly processive

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Cytochrome P450 family 51 enzymes catalyze the 14α-demethylation of sterols, a function that is conserved among all kingdoms of life. To achieve a net demethylation, these enzymes catalyze a sequential, three-step oxidation of sterols (proceeding via 14α-hydroxymethyl and 14α-aldehyde intermediates) that culminates in the removal of C32 (as formic acid) and desaturation of the C14-C15 bond. In this study, the concerted nature of the multi-step sterol oxidation (e.g. the kinetic processivity) was addressed with the human enzyme (P450 51A1), whose substrate is lanosterol and whose product is follicular fluid meiosis-activating sterol (FF-MAS), a key intermediate in cholesterol biosynthesis.

24,25-Dihydrolanosterol (and its 14α-hydroxymethyl and 14α-aldehyde intermediates, natural substrates in the Kandutsch-Russell cholesterol pathway) were synthesized, and an HPLC-UV assay was developed to monitor the formation of demethylated product (FF-MAS). Evidence for reaction processivity was found in steady-state kinetic parameters and binding constants, dissociation rates of P450-sterol complexes, and a rapid time course of lanosterol oxidation. The 14α-hydroxymethyl and 14α-aldehyde intermediates were oxidized at rates 1-2 orders of magnitude greater than the competing rate of dissociation (k_{off}) of the respective P450-intermediate complex, strong evidence for a concerted (processive) mechanism.

With each successive step of (dihydro)lanosterol oxidation, one C-H bond is broken. The synthesis of 32-trideuterated dihydrolanosterol afforded the opportunity to further study the kinetics of the oxidative mechanism, although the absence of a kinetic isotope effect when used in steady-state experiments indicated that C32 C-H bond breaking is not rate-limiting. Then, as processive mechanism requires the sterol to be tightly bound in the active site for the duration of the reaction, the active site tolerance of P450 51A1 was probed with epi-dihydrolanosterol (the 3α-hydroxy analog) and the common lanosterol contaminant, dihydroagnosterol. Both sterols were found to be substrates of the enzyme, with >100% and ~50% the activity of dihydrolanosterol, respectively, suggesting the P450 51A1 active site is relatively promiscuous.

Overall, these results indicate that the reaction of P450 51A1 with dihydrolanosterol is rapid and highly processive. Such a mechanism increases the efficiency of the reaction, and also renders it less sensitive to inhibition. As some fungal and protozoan P450 51 enzymes are drug targets in treatment of deadly systemic infections, the concerted nature of sterol demethylation is also of general interest in the fields of drug metabolism and public health.

This work was supported by grants from the National Institute of General Medical Sciences of the National Institutes of Health (NIH grant numbers R01 GM118122 [to F.P.G.] and R01 GM067871 [to G.I.L.]). This material is also based upon work supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. 1937963 (K.D.M.).

#43

Directing metalloenzyme movies with light: Developing new methods in kinetic crystallography

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Structural enzymology is largely limited to static structures of substrate and product-bound complexes that provide little information about intermediate states along an enzyme's mechanistic path. Additionally, trapping intermediates can prove technically difficult. It is therefore desirable to develop time-resolved approaches capable of tracking dynamics that facilitate reactivity. We are particularly interested in metalloenzymes, as the interplay between metal ions/cofactors can enable otherwise inaccessible chemistries. Our work focuses on developing tools to photo-trigger metalloenzyme catalysis via metal reduction or substrate uncaging, thereby facilitating downstream crystallographic characterization of reaction intermediates. In this study, we showcase our use of the photoreductant acriflavine to bring metalloenzymes to their reactant state and kick-start reactions. Proof of concept studies targeting heme-dependent protein systems, such as myoglobin, were first conducted in solution. Using a home-built UV-vis microspectrophotometer, we were also able to observe the accumulation of reaction intermediates *in crystallo*. Caged substrates (including caged NO[1] and caged O_2[2]) that can initiate catalysis via substrate binding were also investigated. Our aim with this study is to further develop these tools and utilize them in designing time-resolved crystallographic experiments that set the foundation for "metalloenzyme movies."

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#44

Interactions Shaping the Contrasting Reactivities of the Flavins of Bifurcating ETFs

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Flavin-based bifurcation accomplishes the feat of producing highly reducing electrons at the expense of a less potent source. In bifurcating electron transferring flavoproteins (BfETFs) a *pair* of modestly reducing electrons is acquired from NADH, by a flavin (the bifurcating flavin, Bf-flavin). Favorable transfer of *one* electron to a lower-energy acceptor (higher midpoint potential, E°) is mediated by a second flavin, called the electron transfer (ET) flavin. Consistent with this role, the ET-flavin has a stable anionic semiquinone state (ASQ), which is not the norm for free flavin.[1] However for the Bf-flavin, loss of this first electron must produce a semiquinone that is sufficiently unstable to reduce the high-energy electron acceptor. Thus, interactions with the protein must tune the two flavins to very different reactivities.

To identify residues responsible for the flavins' contrasting reactivities, we are replacing residues that interact with each of the flavins, characterizing their effects on flavin redox energetics via measurement of E°s,[2] and effects on electronic structure via the flavin optical signatures.[3, 4] We complement these measurements with quantum chemical calculations to examine the roles of electrostatics, geometry and individual hydrogen bonds.[3, 5] Our computations replicate observed behavior confirming their validity, and allow us to probe states that cannot be observed experimentally. Computed charge distributions suggest how the ET site stabilizes ASQ. Our work paves the way for modifying flavin sites to achieve desired reactivity.

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#45

Biochemical analysis of a *β*-N-acetylgalactosaminidase involved in mucin degradation from a gut commensal bacteria

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An emerging probiotic comes in the form of the gut commensal bacteria *Akkermansia muciniphila*. Previous studies have shown it's potential to lower high blood pressure, improve insulin resistance and protect against food additive-induced irritable bowel syndrome. (1) One-way *A. muciphila* is hypothesized to improve overall gut-barrier function is through its influence on goblet cell proliferation and subsequent mucus production induced by mucin degradation. (2) *A. muciniphila* resides in the loose outer mucus layer of the host and can break down complex O-glycan mucin structures, using a variety of enzymes including glycoside hydrolases. Transcriptomics data comparing gene expression during growth in mucin versus glucose suggests that specific enzymes are involved in mucin degradation (3), however, many of these enzymes have yet to be biochemically characterized. One such enzyme is a member of glycoside hydrolase family 123 (GH123). There are three characterized GH123 enzymes that function as β-N-acetylgalactosaminidases and act on glycosphingolipids. (4) However, their ability to degrade mucin structures have not been shown. Here, we present biochemical analysis of the recombinantly expressed GH123 from *A. muciniphila* ATCC BAA-835 (AmGH123) to assess its possible role in mucin or glycosphingolipid degradation.

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#46

The Impact of Alternative Metals on the Biophysical and Kinetic Properties of Metalloflavoprotein D2-Hydroxyglutarate Dehydrogenase from *Pseudomonas aeruginosa* PA01

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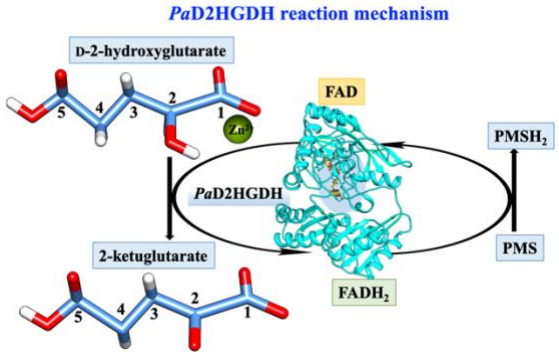

D-2-hydroxyglutarate dehydrogenase (*Pa*D2HGDH) is an enzyme from *Pseudomonas aeruginosa*, a bacterium that is commonly associated with opportunistic infections. (1,2) The enzyme catalyzes the conversion of D-2-hydroxyglutarate (D2HG) to alpha-ketoglutarate (α-KG), which is a key step in the tricarboxylic acid cycle in *Pseudomonas aeruginosa*. (1,2) *Pa*D2HGDH is a metalloflavoprotein that contains a metal ion, typically Zn^{2+}, essential for its function. (3,4) Understanding the effects of alternative metal ions such as Ni^{2+}, Co^{2+}, Mn^{2+}, or Cd^{2+} on the activity and stability of *Pa*D2HGDH can provide insight into the role of the metal ion in the function of the enzyme. To investigate the impact of alternative metal ions on the biophysical and kinetic properties of *Pa*D2HGDH, recombinant His-tagged *Pa*D2HGDH was purified to high levels in the presence of 1 mM chloride salts of Ni^{2+}, Co^{2+}, Mn^{2+}, or Cd^{2+}. The UV-Visible absorption spectrum of the enzyme-treated with Ni^{2+}, Co^{2+}, Mn^{2+}, or Cd^{2+} demonstrated a 1-6 nm red shift of the maximal wavelengths, and the flavin extinction coefficient at 450 nm (ε_{450}) was comparable to that of the enzyme with no metals. The presence of Co^{2+}, Mn^{2+}, or Cd^{2+} increased the flavin N_3 atom pK_a value by approximately 1 unit, while Ni^{2+} decreased the pKa value by about 1 unit compared to the enzyme with no metal. The spectroscopic data indicate that the alternative metals influence the biophysical properties of *Pa*D2HGDH. The steady-state kinetics of E-Zn^{2+} demonstrate a k_{cat} value of 38 s^{-1} and a k_{cat}/K_m value of 7,000 $M^{-1}s^{-1}$. The data indicates that the substrate binding and catalysis are affected by the properties of the metal, as evidenced by the 1-8-fold difference in k_{cat} values and 2-20-fold difference in k_{cat}/K_m values among various metal (Ni^{2+}, Co^{2+}, Mn^{2+}, or Cd^{2+}) bound enzymes compared to E-Zn^{2+}.

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#47

A Comparison of Focused Orbital Alignments in Aspartic vs. Serine Protease Complexes

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A geometric analysis of known PDB structures of aspartic proteases was performed. HIV-protease was chosen for analysis due to its biomedical relevance and the high number of available PDB files. It was previously determined that the active aspartic segments of HIV-1PR and its bound strand contain extensive orbital alignments that converge at the targeted linkage. Glycine is conserved in HIV-1PR and other proteases in the AA clan at the Asp+2 position. In HIV-1PR, the glycine is likely conserved due to potential steric interference with the active aspartic residues. Analysis of the AD and AF clans also indicates focused orbital alignments, but the limited number of available files makes this conclusion more tentative. In subtilisin, a serine protease, the orbital alignments of the catalytic triad and the bound ligand seem to be preorganized for splicing during proteolysis. When comparing subtilisin with HIV-1PR, the evidence of orbital alignments involving the active Asp segments, a bound water molecule, and the enzyme-bound strand suggests a similar splicing process. We think this transitional splicing helps to distribute the electron densities of the reaction center more favorably.

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#48

Sequence and Structure Analysis of Asparagine Synthetase and its Related Enzymes.

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Asparagine synthetase (AsnB) is one of the enzymes in the biosynthetic pathway of the primary metabolite, asparagine, utilized by organisms for protein production from all kingdoms of life. It contains two domains connected by a hydrophobic tunnel. The ammonia produced at the N-terminal is transferred to the C-terminal active site for amidating aspartic acid in the presence of ATP. [(1)] Unlike many other bi-domain enzymes in its family, such as glutamine-dependent NAD+ synthetase, *E. coli* AsnB does not exhibit tight activity coordination between two active sites to produce asparagine. However, a recent study suggested that ASNS, the human ortholog of AsnB, could have a structurally 'built-in' reactivity control embedded in its protein structure. [(2)] In this poster, I will present the sequence and structural analysis of enzymes that are functionally or structurally related to AsnB.[(3)] My analysis suggests that the selective utilization of these structural features enabled AsnB to become a versatile protein scaffold that has been adopted by a wide range of enzymes involved in secondary metabolisms. My work demonstrates a tight relationship between primary and secondary metabolisms in microorganisms.

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#49

Expression and Characterization of the Luciferase of *Noctiluca scintillans*

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Noctiluca scintillans is a species of bioluminescent dinoflagellates that is widely distributed across the world's oceans.(1) The unique light emission of this species is the result of the oxidation of dinoflagellate luciferin by the dinoflagellate luciferase (LCF).(1) The LCF of *N. scintillans* (NS-LCF) is distinct compared to the LCF of other dinoflagellate species, sharing characteristics of a canonical LCF and a luciferin binding protein (LBP).(1) Using molecular cloning techniques via polymerase chain reaction (PCR) the NS-LCF gene was inserted into the Champion™ pET-SUMO vector. The constructed plasmids were expressed in BL21 *E. coli* cells and purified by immobilized metal affinity chromatography, followed by characterization utilizing SDS-PAGE. SDS-PAGE analysis shows possible dimerization or even higher oligomerization occurring. Current results match our AlphaFold calculations, which show higher order oligomer complexes, such as dimers, trimers, and dodecamers are possible.

This work was supported in part by National Science Foundation CAREER Award CHE-1555138 (S.O.M.) and an Auburn University Undergraduate Research Fellowship (S.P.O.)

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#50

Negative cooperativity and the atypical kinetics of hUGDH.

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Human UDP-Glucose Dehydrogenase (hUGDH) is the only enzyme that catalyzes the formation of UDP-glucuronic acid (UDP-GlcA), the essential substrate in Phase II metabolism of drugs. Attaching GlcA to drugs increases solubility, which promotes the excretion of the resulting glucuronides. Glucuronidation is also a common drug resistance mechanism found in some cancers. Thus, regulating the availability of UDP-GlcA is a promising strategy in cancer treatment. Key to this goal is a detailed understanding of the reaction mechanism of hUGDH. The hUGDH mechanism uses two molecules of NAD^+ to catalyze the stepwise oxidation of UDP-Glc to UDP-GlcA. Kinetic analysis reveals several atypical features, the most striking being negative cooperativity with respect to NAD^+. Negative cooperativity typically arises from molecular asymmetry or a mixture of folded and misfolded proteins, both of which can result in two or more active sites with different affinities for NAD^+. However, we have found no evidence supporting either mechanism despite numerous crystal structures and biophysical studies. We hypothesize that negative cooperativity in hUGDH originates from the unique kinetic mechanism of the enzyme. Specifically, if the second NAD^+ binding step is rate limiting at low [NAD^+], then it is possible for a subsequent kinetic step to become rate limiting at high [NAD^+]. This NAD^+-dependent shifting of rate limiting steps could result in negative cooperativity in a substrate saturation curve. To test this, we have simulated a kinetic model which reproduces the negative cooperativity seen in hUGDH. This model also predicts several other unusual features of hUGDH, including: (i) substrate inhibition by UDP-Glc at sub-saturating [NAD^+]; (ii) hyperbolic binding of UDG at saturating [NAD^+]; and (iii) biphasic progress curves. Current work is focused on collecting experimental data of each step in the mechanism to refine the accuracy of the model.

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#51

Insights into the Mechanism of DNA Translocation by Xeroderma Pigmentosum Group D Protein: A Theoretical Study

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The Xeroderma Pigmentosum Group D (XPD) protein, an intrinsic part of the transcription factor IIH, has a helicase activity that is highly crucial to the Nucleotide excision repair (NER) process [1]. XPD consists of four distinct domains namely, two RecA-like helicase domains (HD1 and HD2), the 4Fe-S and Arch domains. XPD translocases along the 5'-3' direction in ssDNA, acting as a lesion-scanning protein, where the 4Fe-S domain plays an important role in its helicase activity [2]. Despite extensive efforts, a detailed molecular-level understanding of the translocation mechanism of XPD remains at large. An oversimplified two-step mechanistic model was previously proposed [3]. But it lacks a detailed mapping of the translocation process onto the XPD structure. Here we set ourselves the objective of using molecular dynamics simulation to gain detailed insights into the mechanism.

The translocation of XPD along ssDNA was computationally reproduced in three consecutive cycles with one base per ATP hydrolyzed, using the partial nudged elastic band (PNEB) method. The underlying free energy surface was sampled by launching unbiased trajectories from the PNEB path. The ensemble hence obtained was projected onto the surface spanned by independent components 1 and 2 (IC1 and IC2), obtained as linear combinations of distances between CA-atoms of protein and P-atoms of the ssDNA. The mechanism was realized in terms of transitions between ten distinct metastable states, identified following the Markov states assumption with suitable lag time.

A careful study of the mechanism reveals the following details: 1) Sliding of the HD2 domain along ssDNA is facilitated by ATP binding and is the rate-determining step (RDS). 2) The 3' end of ssDNA was held tightly to the Arch and 4Fe-S domain during the RDS. 3) Upon ATP hydrolysis, the HD2 grips ssDNA strongly at the 5'-end and initiates the opening of the cleft between the Arch and 4Fe-S domain. 4) On its return to the initial conformation, the Arch domain pushes the DNA forward along the 3'-5' direction facilitating translocation of the ssDNA and consequently restoring the HD2 domain to its initial conformation.

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#52

Structure and Dynamics of *Proteus vulgaris* Tryptophan Indole-lyase Complex with L-Ethionine

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Tryptophan indole-lyase (TIL; E.C. 4.1.99.1) is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the reversible β-elimination of indole from L-tryptophan. The mechanism of elimination of indole from L-tryptophan starts with formation of an external aldimine of the substrate and PLP, followed by deprotonation of the α-CH of the substrate forming a resonance stabilized quinonoid intermediate. Proton transfer and carbon-carbon bond cleavage of the quinonoid intermediate gives indole. L-Ethionine is a TIL competitive inhibitor that forms a stable quinonoid intermediate with λ_{max}=508 nm. We have now determined the X-ray crystal structure of the TIL complex with ethionine to 1.88 Å. In 3 of 4 chains, the active site is in a closed conformation, and the ethionine is in a quinonoid complex. In the other chain, the active site is open, and the ethionine is in an external aldimine complex.

The TIL-ethionine complex is affected by hydrostatic pressure (Figure 1). As pressure increases, the peak at 508 nm decreases, and a new peak at 335 nm appears. These changes are reversible when pressure is released. The 335 nm species could be either a *gem*-diamine or an enolimine tautomer of the external aldimine. We measured the fluorescence spectrum of the complex under pressure to differentiate these structures. When excited at either 290 or 335 nm, the 335 nm species emits at 400 nm, establishing that it is a *gem*-diamine complex. Pressure jumps result in relaxations that can be fit as a single exponential. The relaxation rate constant is pressure dependent, showing an activation volume, $\Delta V^{\ddagger}$, of about +50 mL/mol, and and transition state compressibility, $\Delta\beta^{\ddagger}$, of -19 mL/mol-kbar.

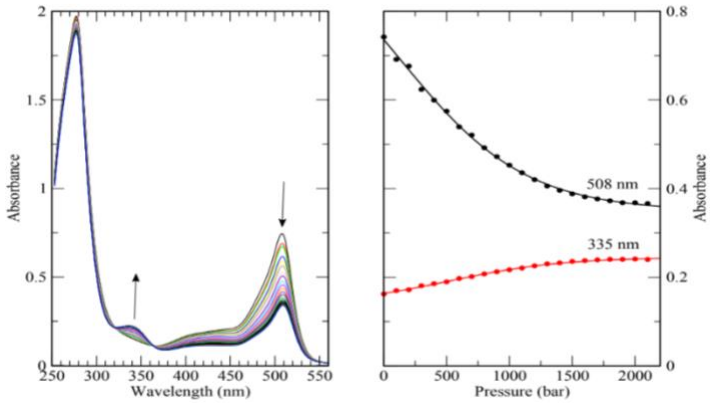

Figure 1. Effect of hydrostatic pressure on the TIL-ethionine complex. A. Spectra of the TIL-ethionine complex at pressure from 1 – 2100 bar. B. Plot of absorbance at 508 and 335 nm as a function of pressure. The lines are the fits to a Boltzmann function.

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#53

Calorimetric and Stability Study of Tryptophan and PrnA from *Burkholderia ambifaria*

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Burkholderia ambiferia is a member of the *Burkholderia cepacian* complex, a combination of over twenty different *Burkholderia* species, which poses a serious risk to individuals with cystic fibrosis and related diseases. This member of the *Pseudomonadota* phylum carries a four-gene pathway that is homologous with known pyrrolnitrin biosynthetic pathways (prnABCD). The first step of this pathway utilizes a flavin dependent oxygenase enzyme termed PrnA, which catalyzes the chlorination of the seventh position of tryptophan (Trp) utilizing a FAD-dependent active site. This activates O_2 and a chloride ion, where the regioselectivity of this system is controlled by carefully orienting the substrate for a specific C-H activation. The intermolecular forces between protein and substrate have been identified by X-ray crystallography, and these interactions are thought to govern the selectivity of this process. Here, we report our efforts to characterize the thermodynamics associated with a Trp substrate and its substrate analogues (e.g. alternative amino-acids like Ala and indole) in relation to their binding to PrnA using both spectroscopic and calorimetric methods. Correlating association constants (K_a) and related thermodynamic terms from these studies with steady-state kinetic affinity measurements (K_M) will allow a higher understanding of the mechanism of this system. Improving upon our understanding of PrnA opens the door for further experimentation toward developing biochemical systems for targeted new halogenation pathways.

#54

Optimization of the Production and Expression of Recombinant Human Adrenodoxin in *Escherichia Coli*

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The enzyme cytochrome (CYP) P450 27A1 catalyzes the metabolism of vitamin D and cholesterol derivatives, which contributes to sterol homeostasis and cancer signaling. To better understand the functions of this enzyme, it is pertinent to understand how it interacts with its redox partner, adrenodoxin (Adx). Adx is an iron-sulfate ferredoxin protein that delivers electrons to cytochrome P450 enzymes.[(1)] Adx may support CYP P450 reactions beyond the delivery of electrons. Understanding Adx and optimizing its production is essential to the complementary characterization of P450 27A1. The purpose of this study is to determine the most effective methods of expression and purification of adrenodoxin.

Adrenodoxin purity levels were analyzed to measure the efficiency of various purification methods. Adx expressed in *E.coli* BL21 was purified using size exclusion chromatography and ammonium sulfate precipitation. Measurements of Adx purity and yield were obtained using Bradford assays, ultraviolet-visible (UV-Vis) spectroscopy at a wavelength of 414 nm, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Optimization of the purification protocol began with running a test to compare the purification efficiency of ammonium sulfate precipitation and size exclusion chromatography. The results suggested that neither method resulted in a greater yield and higher purity of Adx. Simultaneously, western blotting is utilized to measure Adx quantity . This will allow for a better understanding of the most productive cell line (*E.coli* BL21, C41, C43, or Tuner (DE3)) used to optimize the production of adrenodoxin.

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#55

The *Pseudomonas aeruginosa* D-arginine dehydrogenase's gating residue E246 controls the rate of enzyme turnover.

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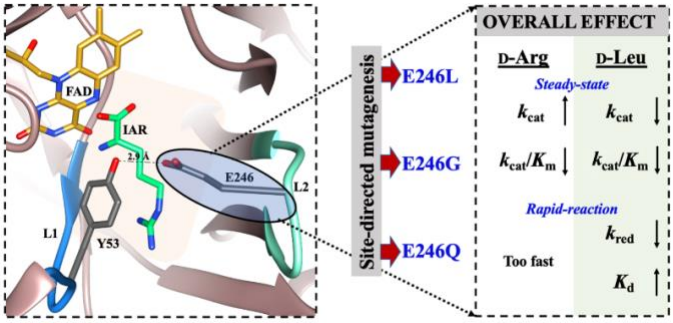

Enzymes are proteins that assume multiple conformations during their catalysis of biochemical reactions.[1] In recent years, the bioengineering of enzymes to generate faster biological catalysts has proved difficult due to the limited understanding of enzyme motions and loop dynamics that are critical for catalysis.[2,3] Commercial food and L-amino acid industries depend on bioengineered D-amino acid oxidizing enzymes to detect and remove D-amino acid contaminants.[4,5] The *Pseudomonas aeruginosa* D-arginine dehydrogenase (*Pa*DADH) oxidizes most D-amino acids[6] and is a good candidate for application in the L-amino acid and food industries. The *Pa*DADH crystal structure reveals four active site loops (L1-L4), with loop L2 located at the entrance of the active site pocket.[6] The side chain of the loop L2 E^{246} residue points toward the active site and potentially secures the substrate upon binding. Additionally, E^{246} likely secures the closed conformation of the active site gating loop to facilitate catalysis after substrate binding. This study used site-directed mutagenesis, steady-state, and rapid reaction kinetics to generate the glutamine, glycine, and leucine variants and investigate the role of the E^{246} residue in *Pa*DADH. With the physiological substrate D-arginine, the mutation increased the enzyme turnover k_{cat} from 122 s^{-1} to 500 s^{-1} when the E^{246} residue was mutated to glycine. Likewise, there was a ~2-fold increase in the k_{cat} values when E^{246} was replaced with glutamine or leucine. Thus, we have engineered a faster biocatalyst that can be applied in the food and L-amino acid industries. However, the engineered variant forms of *Pa*DADH's did not have faster turnover rates with D-leucine as a substrate. The study shows that residue E^{246} controls the rate of the iminoarginine product release in *Pa*DADH and is essential for maintaining the closed conformation of the active site gating loop during catalysis.

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#56

The Key to Allosteric Inhibition in Human UDP-glucose 6-dehydrogenase is a Buried and Unsatisfied Polar Group

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Human UDP-glucose 6-dehydrogenase, hUGDH, is an essential enzyme in the synthesis of extracellular matrix components like hyaluronic acid. The overproduction of hyaluronic acid has been implicated in the development of aggressive prostate cancers and their metastasis. Understanding how hUGDH is regulated may lead to new strategies in cancer treatment. The activity of hUGDH is regulated through allosteric feedback inhibition by UDP-Xylose. The binding of UDP-Xylose alters the conformation of hUGDH from an active hexamer (E-state) to a horseshoe-shaped, inactive complex called the EΩ-state. The conformational change between these states is slow and can be observed as a lag in progress curves (hysteresis). Our structural studies on hUGDH allostery and hysteresis have identified a buried, unsatisfied polar group (Thr127) within the hydrophobic core of the E-state. In the EΩ-state, the hydroxyl group of Thr127 resides within a small cavity just large enough to accommodate a water molecule. We hypothesize that Thr127 will stabilize the EΩ-state and favor the allosteric transition. Consistent with our hypothesis, the residue corresponding to Thr127 in *C .elegans* UGDH is an isosteric valine, and the enzyme has a 16-fold lower affinity for UDP-Xylose. We confirmed this observation with a T127V substitution in hUGDH ($hUGDH_{T127V}$). The resulting construct had similar K_M and k_{cat} values but a 10-fold lower affinity for UDP-Xylose. Additional support for the role of Thr127 can be seen in a heavily diminished hysteresis, suggesting that the equilibrium has shifted away from the EΩ-state. Our results show how a buried residue plays a key role in the allosteric mechanism of this essential enzyme.

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#57

Characterization of Putative Dms Monooxygenase from *Oceanobacillus piezotolerans*

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The goal of this project is to characterize a new form of the enzyme dimethyl sulfide (DMS) monooxygenase. This enzyme plays an essential role in degrading the volatile sulfur compound DMS, which acts as a foraging cue for top predators, coral reef health and most notably, a nucleation site for the formation of clouds. There is a single report on the isolation and purification of DMS monooxygenase from *Hyphomicrobium sulfonivorans*. This project aims to characterize additional DMS monooxygenases from alternative species in order to study the divergence among this family of enzymes. DMS monooxygenase requires two subunits for its function; a monooxygenase DmoA subunit where DMS breakdown occurs and a flavin reductase DmoB subunit. A candidate was identified from the organism *Oceanobacillus piezotolerans*, a deep soil bacterium found in the Pacific Ocean. Both the *dmo*A and *dmo*B genes from this organism were synthesized and transformed into an *E. coli* host for recombinant protein expression. After screening expression conditions, both subunits were successfully expressed in the soluble lysate. Protein purification and characterization is underway. Characterization includes protein structure determination, cofactor and substrate specificity, and subunit interactions. This work will contribute towards our understanding of how structure dictates chemical mechanism as it relates to climate regulation.

#58

Recombinant expression of methyl-coenzyme M reductase to understand the importance of accessory proteins for assembly.

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Methanogenesis and the anaerobic oxidation of methane (AOM) are the essential energy metabolisms of methanogens and anaerobic methanotrophs (ANME), respectively. Methyl-coenzyme M reductase (MCR) catalyzes the final methane-forming step in methanogenesis and the first methane activation step in AOM. MCR is a dimer of heterotrimers with a 2α, 2β, 2γ configuration, and requires the nickel tetrapyrrole prosthetic group, coenzyme F_{430}. The requirement of a unique cofactor, various unusual post translational modifications, and many remaining questions surrounding assembly and activation of MCR has so far largely limited *in vitro* experiments to native enzymes. The development of heterologous expression systems that result in assembled and active MCR would be desirable to investigate the enzymatic properties of diverse MCRs, especially those from ANME.

The α, β, and γ subunits of MCR are encoded by the genes *mcrA*, *mcrB*, and *mcrG* respectively. In methanogens, the three genes are part of a gene cluster, *mcrBDCGA*, which encodes two additional accessory proteins, McrC and McrD. In ANME, however, one or more of these accessory proteins are often absent from the MCR gene cluster. Additionally, in methanogens with a MCR isozyme II, one or more of the accessory proteins are also missing. McrC and McrD are thought to be involved in the activation and assembly of MCR, where McrD is specifically implicated in the delivery of F_{430} to the apo-enzyme. We created recombinant MCR expression constructs containing the MCR operons from *Methanosarcina acetivorans*, *Methanocaldococcus jannaschii*, and *M. maripaludis* with or without accessory proteins. After expressing these constructs in *M. maripaludis*, we then assessed the importance of having the two accessory proteins in the recombinant gene cluster by purifying the resulting MCR and analyzing F_{430} content. We have successfully purified recombinant *M. jannaschii* and *M. maripaludis* MCR without the accessory proteins, showing that the accessory proteins do not need to be present within the gene cluster for assembly. However, the recombinant *M. acetivorans* MCR expressed in *M. maripaludis* shows low expression and incomplete assembly, suggesting that the native *M. maripaludis* accessory proteins are not compatible with the more distantly related *M. acetivorans* MCR.

#59

TEV Protease Cleavage of His-Tagged Cytosolic Abundant Heat Soluble Protein D

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Cytosolic abundant heat soluble (CAHS) proteins originate from tardigrades, microorganisms known for their ability to withstand a variety of stressors, including desiccation. CAHS D has been shown to stabilize desiccated enzymes such as lactate dehydrogenase and lipoprotein lipase, and could potentially be used to stabilize protein based pharmaceuticals.[(2)] CAHS D has traditionally been purified using cation exchange chromatography but co-purifies with a contaminating protein and requires several rounds of purification. CAHS D is thought to aggregate or oligomerize into a hydrogel during the desiccation process[(4)], so adding a his-tag to aid in purification may interfere with the ability of CAHS D to stabilize other proteins. We are investigating the Tobacco Etch Virus protease (TEV protease) as a way to remove the his-tag[(3)] to compare the effectiveness of his-tagged CAHS D vs. cleaved CAHS D with no his-tag. The TEV protease was prepared for this experiment by transforming *E. coli* and purifying the protein using immobilized metal affinity chromatography. CAHS D was also purified using affinity chromatography. The cleavage reaction of CAHS D by TEV protease was optimized, with small scale reactions at 4 °C overnight determined to be the most effective. His-tagged and cleaved CAHS D with no his-tag will be used to stabilize alcohol dehydrogenase at elevated temperatures.

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#60

Exploring the Catalytic Mechanism of Two-component $FMNH_2$-dependent Monooxygenases Involved in Sulfur Acquisition

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All organisms require sulfur for diverse metabolic and physiological processes. Bacteria primarily use inorganic sulfate as their sulfur source; however, during sulfur starvation bacteria can also utilize a variety of organosulfur compounds to obtain sulfur.(1) Preliminary proteomic studies revealed that the expression of different genes involved in sulfur acquisition, sulfur assimilation, and oxidative stress were upregulated during sulfur starvation. These genes also include the flavin reductases (SsuE and MsuE) and $FMNH_2$-dependent monooxygenases (SsuD, MsuC, and MsuD) found in the opportunistic human pathogen *Pseudomonas aeruginosa*. Conversely, several virulence factors of *P. aeruginosa* were found to be downregulated during sulfur starvation. These findings highlight the importance of sulfur for the survival and the pathogenicity of *P. aeruginosa*.

The flavin reductases (SsuE and MsuE) and the monooxygenases (SsuD, MsuC, and MsuD) are part of two-component flavin-dependent alkanesulfonate systems which enable bacteria to utilize a broad range of organosulfur compounds during sulfur limitation. The flavin reductases (SsuE and MsuE) and the monooxygenases (SsuD and MsuD) catalyze the coordinated desulfonation of alkanesulfonates. SsuD and MsuD share ~65% amino acid sequence identity and have been proposed to use a flavin-N5-adduct during catalysis. In previous studies, the flavin-N5-oxide was formed as an oxygenating flavin adduct or as the final product during catalysis.(2) We were unable to identify the flavin-N5-oxide as the final product for SsuD, and currently propose that the enzyme uses a flavin-N5-peroxyflavin as the oxygenating intermediate.

Unlike SsuD and MsuD, MsuC is structurally distinct and adopts the acyl CoA dehydrogenase fold. MsuC, with reduced flavin provided by MsuE, catalyzes the oxidation of methanesulfinate to yield methanesulfonate. The MsuC enzyme was shown to have a higher affinity for the reduced flavin form and prefers $FMNH_2$. Interestingly, rapid reaction kinetic analyses revealed that reduced flavin was protected by MsuC in the absence of methanesulfinate when evaluating the reduced flavin transfer from MsuE. Changes in the oligomeric states of different flavoenzymes in the presence of substrates has been shown to regulate activity as well as efficient flavin transfer from the reductase partner. The presence or absence of substrates and products was not seen to affect the quaternary structure of MsuC in native-PAGE and analytical ultracentrifugation analyses. The thermal stability of MsuC was also similar in the presence and absence of substrates. Altogether, these findings suggest that unlike other flavoenzymes, the presence of substrates and products do not alter the oligomeric state and the structural stability of MsuC. Since bacterial antibiotic resistance continues to be a global threat, the mechanistic information obtained from these studies could represent a notable target for drug development.

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#61

30S subunit recognition and G1405 modification by the aminoglycoside-resistance 16S ribosomal RNA methyltransferase RmtC

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Acquired ribosomal RNA (rRNA) methylation has emerged as a significant mechanism of aminoglycoside resistance in pathogenic bacterial infections. Modification of a single nucleotide in the ribosome decoding center by the aminoglycoside-resistance 16S rRNA (m^7G1405) methyltransferases effectively blocks the action of all 4,6-deoxystreptamine ring-containing aminoglycosides, including the latest generation of drugs. To define the molecular basis of 30S subunit recognition and G1405 modification by these enzymes, we used a S-adenosyl-L-methionine (SAM) analog to trap the complex in a post-catalytic state to enable determination of a 3.0 Å cryo-electron microscopy structure of the m^7G1405 methyltransferase RmtC bound to the mature *E. coli* 30S ribosomal subunit. This structure, together with functional analyses of RmtC variants, identifies the RmtC N-terminal domain as critical for recognition and docking of the enzyme on a conserved 16S rRNA tertiary surface adjacent to G1405 in 16S rRNA helix 44 (h44). To access the G1405 N7 position for modification, a collection of residues across one surface of RmtC, including a loop that undergoes a disorder to order transition upon 30S subunit binding, induces significant distortion of h44. This distortion induces flipping of G1405 into the enzyme active where it is positioned for modification by two almost universally conserved RmtC residues. These studies expand our understanding of ribosome recognition by rRNA modification enzymes and present a more complete structural basis for future development of strategies to inhibit m^7G1405 modification to re-sensitize bacterial pathogens to aminoglycosides.

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#62

The Southeastern Center for Microscopy of MacroMolecular Machines (SECM^4), and the Future of Structural Biology

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Cryogenic electron microscopy (cryo-EM) has grown by leaps and bounds in recent years. Despite this growth, challenges remain for many researchers including lack of access to instrumentation and intractable problems with preparing samples. The Southeastern Center for Microscopy of MacroMolecular Machines (SECM^4) at Florida State University is a newly established NIH supported service center that seeks to overcome these problems by offering free access to cryo-EM sample preparation, imaging, and training for users in the greater Southeast. The center will: 1) enable users at universities across the Southeast to gain entry the cryo-EM field without making multi-million dollar investments in instrumentation, 2) offer screening and specimen optimization services that will address bottlenecks in cryo-EM such as preparation of cryo-EM samples that will reconstruct to high-resolution, and 3) offer in person training that will enable interested users to gain entry into the field of cryo-EM without having to seek out a collaboration with an already saturated cryo-EM expert. I will describe the available services at the SECM^4 and how interested users can apply for access to the center.

#63

Orbital alignments and glycine conservation in cysteine proteases.

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Cysteine proteases utilize thiol functional groups as their active nucleophile, allowing for more regulable chemistry compared to serine and aspartyl proteases. Cysteine proteases are important in viral systems as they assist in viral replication. In mammals, cysteine proteases in the lysosome contribute to protein degradation. Cysteine proteases usually maintain a catalytic dyad of cysteine and histidine and an oxyanion hole formed by one or more hydrogen-bonding groups. Other residues also help to accommodate optimal functional character in the binding site. Glycine allows for closer packing between strands and for the optimization of orbital alignments along the strand of the active cysteine. The focus of this study is on clans CE and PA, which tend to conserve a glycine at the Cys + 1 position. Our goals were to identify orbital alignments in their active sites and understand the patterns of glycine conservation for any stereo-electronic effects. We argue that extensive orbital alignments develop to facilitate proteolysis and that some of the conserved glycines amongst cysteine proteases of clans CE and PA favor orbital alignments that would not be favored by a larger side-chain.

#64

The Sacrificial Lamb: CADD from Chlamydia trachomatis is a Mn/Fe-dependent oxygenase that synthesizes p-aminobenzoate using its own amino acid residues as substrates.

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Folate is an essential cofactor for one-carbon transfer reactions in various biochemical pathways including DNA and amino acid biosynthesis. Most bacteria and plants can synthesize folate *de novo*, unlike animals that obtain folate from their diet. An established pathway for folate biosynthesis exists in most bacteria; the pterin portion is produced from FolEQBK enzymes, *p*-aminobenzoate (pABA) is typically produced by PabA/B and PabC enzymes, and FolPCA joins these two portions and performs the final glutamylation steps.[1] However, some bacteria such as *Chlamydia trachomatis* do not contain the canonical folate biosynthesis genes, despite still being able to produce folate *de novo*.[1]

Genomic investigation of C. trachomatis revealed *ct610* to be clustered with other folate biosynthesis enzymes.[1] The gene product of *ct610* is "CADD" and previous characterization of this enzyme showed that it associated with receptors that induced apoptosis in mammalian cells; thus, it was named Chlamydia protein Associating with Death Domains (CADD).[2] Interestingly, genetic studies showed that CADD can replace the function of PabABC for pABA synthesis.[3] Subsequent isotopic labelling experiments indicated pABA was derived from tyrosine and *in vitro* studies demonstrated the ability of this enzyme to produce pABA when incubated with molecular oxygen and a reducing agent alone.[4] These results are consistent with CADD acting as a self-sacrificing pABA synthase.

Here, we further investigated the details of CADD-dependent pABA synthesis. Although the protein was implicated to contain a diiron cofactor, CADD expressed from *E. coli* consistently contained sub-stoichiometric Fe content and addition of Fe to enzyme assays resulted in decreased pABA synthesis.[4,5] Enzymatic assays in the presence of various metals revealed that CADD retains the highest pABA synthase activity with Mn, and a hetero-dinuclear Mn/Fe cofactor is likely the physiological cofactor. Further, mass spectrometry-based proteomics analysis revealed modified peptides with a glycine substitution at Tyr27, which provides strong evidence that Y27 is the sacrificial tyrosine for CADD-derived pABA. Additionally, aminoadipic acid was found in place of K152, suggesting this reside is the required amino group donor. Finally, isotopic labelling has demonstrated that the oxygens in the carboxylic acid portion of pABA are from molecular oxygen.[6]

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#65

Examining a natural catalytic residue variant amongst endo-β-N-acetylglucosaminidases

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Asparagine-linked glycosylation (N-glycan) is an important post-translational modification in eukaryotic proteins. N-glycans play a pivotal role in protein folding, stability, and scaffolding, which can have numerous biological effects such as with the immune response, cell signaling, and blood coagulation. N-glycans share a common core α-D-Man-(1→6)-[α-D-Man-(1→3)]-β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc structure (Man3GlcNAc2) and can be divided into three types depending on the carbohydrate composition of the of extensions: high mannose, complex, and hybrid.[(1)] Bacterial endo-β-N-acetylglucosaminidases from glycoside hydrolase family 85 (GH85) are widespread enzymes that are responsible for the hydrolysis of the β(1,4) linkage between the two N-acetylglucosamine (GlcNAc) residues in the core N-glycan, releasing the N-glycan from its glycoconjugate. Currently, there are 12 characterized GH85 enzymes that are active on various N-glycan types using a retaining mechanism through a catalytic glutamate residue.[(2,3)] We have identified a putative GH85 from *Streptomyces* sp. NRRL F-5122 (SspGH85) whose catalytic glutamate has been replaced by an aspartate. Using sequence similarity networks, we have observed that the majority of GH85 sequences have a catalytic aspartate instead of glutamate. However, functional data for GH85s with a catalytic aspartate is lacking. Here, we aim to perform biochemical and structural analysis of SspGH85 to investigate the activity of GH85s that contain of the catalytic aspartate, which is more ubiquitous among GH85 enzymes.

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#66

The C-terminus of the Antitoxin RelB is Essential for Inhibiting the Toxic Activity of the RelE Toxin *Escherichia coli* Enzyme.

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Toxin-antitoxin systems are linked gene pairs found in most bacteria, with many bacteria containing dozens of family members. These macromolecular complexes have bifunctional roles and act as molecular switches: they are part of a negative feedback loop that regulate their own expression during exponential, non-stress growth and supress growth during stress to protect bacteria. In type II toxin-antitoxin systems, both the toxin and antitoxin are proteins, and the antitoxin physically binds to and inhibits the toxin under normal growth conditions. However, during stress, the antitoxin is selectively degraded, and the toxin is freed. Toxins are thought to confer protection against stress by halting cell growth by targeting either cell membrane stability or gene expression. Many type II toxins are endoribonucleases that target important cellular RNAs that are undergoing translation.

One such type II toxin is RelE. The RelE endoribonuclease toxin is ribosome dependent and binds the *E. coli* ribosomal aminoacyl (A) site to cleave mRNAs that are actively undergoing translation. In conjunction with the ribosome, RelE catalyses mRNA cleavage via transition-state charge stabilization and leaving-group protonation through lysine 52 and arginine 81.(1) RelE exhibits very little codon specificity cleaving mostly between two purine codons.(2) RelE has been shown to be activated by the bacterial stringent response.

While much is understood about RelE's mechanism of action, not much is understood about RelE's activation, specifically how RelE is supressed by RelB and released during conditions of stress (e.g., the stringent response). The structure of the RelB-RelE heterotetramer (3) indicates that the RelB C-terminus is responsible for the RelB-RelE interaction. A series of C-terminal truncations in RelB show that RelE is active indicating that the RelB C-terminus is necessary for suppression of RelE. Additionally, deletion of as few as four C-terminal RelB amino acids reduces RelB-RelE binding affinity 10-fold. Thus, the RelB C-terminus is necessary for RelB-RelE macromolecular formation. This study provides further insight into RelE activation and allows for further studies into the interactions of other related toxin-antitoxin systems.

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#67

Binding of fluorophore-labeled cytochrome b_5 to cytochrome P450 17A1 and stimulation of pregnenolone 17,20-lyase activity

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Cytochrome b_5 (b_5) is a small hemeprotein that involves a variety of biochemical reactions, including some oxidations catalyzed by cytochrome P450 (P450) enzymes. Although the mechanism of b_5 functions is still unclear, it is well established that b_5 stimulates the catalytic activity of P450, especially for the 17,20 C-C bond cleavage (17,20-lyase) reactions catalyzed by P450 17A1, which is one of the steroid biosynthetic P450s. P450 17A1 catalyzes 17α-hydroxylation of progesterone and pregnenolone as well as the 17,20-lyase reactions with 17α-hydroxyl progesterone and pregnenolone to produce androstenedione and dehydroepiandrostenedione (DHEA), respectively. The 17α-hydroxylation activities can be observed without b_5 and are not enhanced much by its addition, but P450 17A1 requires b_5 for the 17,20-lyase activities and stimulates them in a concentration-dependent manner. [(1)] These phenomena provide a strong interest in understanding the protein-protein interaction between b_5 and P450 17A1, which can also be a potential drug target for the treatment of prostate cancer.

In our previous work, we reported the development of a green fluorophore (Alexa 488)-conjugated mutant b_5 to visualize the b_5-P450 17A1 interaction.[(1)] In the course of further work, we found that that multiple lysines in b_5 were modified with the fluorophore. Hence, site-directed mutagenesis was conducted to construct a series of Lys-to-Cys b_5 variants for selective labeling. The fluorescence quenching of Alexa 488-conjugated b_5 mutants, when titrated with P450 17A1, varied depending on the labeled position. Labeling with the fluorophore did not generally impair the function of b_5 in the pregnenolone lyase reaction. Fluorescence titration data using apo-b_5 (without the heme prosthetic group) suggested the relevance of the heme for the fluorescence attenuation, indicating it uses a fluorescence resonance energy transfer (FRET)-like mechanism. A fluorescence polarization assay was also developed to study the binding, and the K_d value of the binding was estimated to be ~ 100 nM. Although 1000 times weaker than the binding to P450 17A1 protein, modified b_5 variants bound to a peptide mimicking the putative b_5 binding region of P450 17A1, which can be dissociated by non-labeled b_5. These findings provide fluorescence-based methodologies to study the b_5–P450 17A1 interaction.

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#68

Stabilization of Monoclonal Antibodies using Cytosolic Abundant Heat Soluble Protein D

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Tardigrades are microscopic eukaryotes that have survived outer space, extreme temperatures, and many other high stress environments. Tardigrades retract into a tun state upon dehydration and emerge again when exposed again to water. Cytosolic Abundant Heat Soluble Protein D (CAHS D) is one of the intrinsically disordered proteins present within tardigrades thought to be partially responsible for the ability of tardigrades to survive desiccation. This protein has already shown promise in the stabilization of biologics in literature. Monoclonal antibodies are of particular interest for stabilization due their therapeutic and diagnostic applications, and we aim to use CAHS D for this purpose.

CAHS D was purified recombinantly from bacteria using metal-chelate affinity chromatography on an FPLC system. CAHS D was used to stabilize desiccated monoclonal antibodies under heat stress. Stabilization was verified via Western blotting using chemiluminescence to measure antibody functionality. Current work is focused on the protection of monoclonal antibodies in solution without the need for desiccation. The stabilization of monoclonal antibodies using CAHSD will allow for better stress tolerance, resulting in increased shelf stability. This would potentially lower transportation cost and allow for the use of antibody-based technologies in areas where refrigeration is not available.

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#69

Investigating Subunit Interactions Between an Enzyme Involved in Sulfur Cycling

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The volatile sulfur compound dimethyl sulfide (DMS), CH_3-S-CH_3, plays an essential role in connecting aquatic and terrestrial forms sulfur to the atmosphere. DMS is formed predominately from the breakdown of a compound generated by phytoplankton; DMSP. Once formed, DMS serves as a signaling cue for feeding and level effect coral reef health. At warm temperatures DMS readily evaporates to the atmosphere where its degradation products lead to increased cloud nucleation sites. Increased levels of DMS result in more cloud formation, which result in solar radiation backscattering and observed decreasing temperatures, especially at the local scale. The biochemistry regarding the enzymatic metabolism of DMS is not well known. This project focuses on the enzymatic degradation of DMS to methanethiol (MeSH) catalyzed by dimethyl sulfide monooxygenase, a two-subunit enzyme. This enzyme is part of the $FMNH_2$-dependent two-component monooxygenase family of enzymes. In this family, a flavin reductase B subunit provides electrons to a monooxygenase A subunit necessary for substrate conversion to occur. The transfer of $FMNH_2$ reduced cofactor is different for different enzymes in this family. We aim to investigate how $FMNH_2$ is transferred in the DMS monooxygenase; DmoB and DmoA subunits; specifically, whether $FMNH_2$ transfer occurs by a diffusion or a channeling mechanism between each subunit. In addition, we aim to determine the subunit interaction specificity with regards to primary sequence, species divergence, and protein structure.

#70

Structure of Yeast ALA Synthase Reveals Divergent Mechanisms of Enzyme Autoregulation Governing Heme Biosynthesis

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Heme is an essential cofactor involved in numerous biological processes, ranging from oxygen transport to cellular differentiation.[1] Biosynthesis universally begins with the rate-limiting production of aminolevulinic acid (ALA), which is catalyzed by the pyridoxal phosphate (PLP)-dependent enzyme ALA synthase (ALAS) in α-proteobacteria and non-plant eukaryotes.[2] ALAS homologs feature a highly conserved homodimeric core, but across eukaryotes, a divergent C-terminal extension (Ct-ext) has emerged with differential regulatory functions.[3] Previous work has shown that in *Saccharomyces cerevisiae* ALAS (Hem1), the Ct-ext uniquely interacts with conserved active-site elements, and Ct-ext truncation leads to a 25% loss of function. However, the mechanism by which the Hem1 C-terminus controls enzyme activity was unknown.[4]

We developed a mutant enzyme lacking a portion of the C-terminus (Hem1 ΔCT) to elucidate how this divergent extension modulates enzyme function.[5] We determined the Hem1 ΔCT crystal structure to 2.1 Å resolution (PDB ID 8EIM), which revealed increased disorder and altered PLP binding compared to wildtype. Using hydrogen-deuterium exchange mass spectrometry, we confirmed that these disordered regions exhibit increased solvent accessibility and correspond to conserved motifs critical for activity of all ALAS enzymes. This greater flexibility leads to a decrease in substrate binding and cooperativity that significantly lowers catalytic efficiency. Further, our data point toward a novel role of the Hem1 Ct-ext in mediating functional asymmetry within the homodimer. Collectively, these findings demonstrate that the Hem1 Ct-ext directly stabilizes protein structure to maintain functionality, representing a new means of eukaryotic ALAS autoregulation.[5]

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#71

Investigating the Sequence Specificity of Type II Ribosome-dependent Toxins RelE and YoeB from *Escherichia coli*

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Toxin-antitoxin modules are a pair of genes ubiquitous in bacteria, where one gene encodes a toxin protein that inhibits cellular growth, and the other gene encodes the corresponding antitoxin that inhibits the toxin. The types of toxin-antitoxin modules are classified according to the antitoxin and its mode of inhibition. Type II toxin-antitoxin modules are characterized by the toxin and the antitoxin being both proteins and the antitoxin directly binding the toxin to inhibit it. Ribosome-dependent type II toxins (~10 kDa) can cleave mRNA while it is being translated. These endoribonucleases have low sequence homology (~15%) yet share a RNase-like fold where they contain an antiparallel β-sheet flanked by 2-4 α-helices.

One of these endoribonucleases, HigB, prefers adenosine-rich sequences and recognizes its target sequence based on the third nucleotide of the A-site codon. [(1)] Additionally, a pre-cleavage structure of HigB bound to the ribosome illustrated that an asparagine residue (N71) of HigB stabilizes the interaction between C1054 residue of the 16S rRNA and the third position in the A-site codon. Mutation of the N71 residue to an alanine reveals that the toxin retains its activity yet loses its codon specificity, suggesting that this specificity can be modulated by a single amino acid. RelE and YoeB are also ribosome-dependent endoribonucleases, but in contrast to HigB, cleave a broad spectrum of codons: RelE cleaves UAG, UAA, and CAG codons while YoeB cleaves UAA, AAA, AAU, CUG codons. [(2)]

We propose that RelE and YoeB also recognizes their target sequences based on the third nucleotide of the A-site codon, similar to HigB. To investigate RelE and YoeB specificity, we mutated residues that interact with the 16S rRNA C1054 residue and the third position of the A-site codon. The growth of these variants was monitored to look at *in vivo* toxin activity, and the results show that the variants cause a growth defect similar to wildtype. A single-turnover kinetic assay is being developed to test these variants against a panel of A-site codon substrates.

Understanding how these enzymes interact with the ribosome for selection of its target codons may provide new avenues for antimicrobial development, since the ribosome is a major target for antibiotics. [(3)]

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#72

The One-Pot Reaction of Pyruvate Carboxylase and Phosphoenolpyruvate Carboxykinase for the Production of Isotopically Labled Phosphoenolpyruvate

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The MST enzymes are a family of bacterial enzymes involved in producing menaquinone, siderophore, and tryptophan. Due to their presence in several pathogenic bacteria, including *Pseudomonas aeruginosa*, *Yersinia pestis,* and *Mycobacterium tuberculosis,* the MST enzymes are attractive antibiotic targets. However, the mechanism of the lyase reaction for the MST enzymes is not fully understood, making drug design difficult. Kinetic isotope effect experiments would help elucidate the mechanism but producing deuterated chorismate at scale is a limiting factor. Enzymatic synthesis of deuterium-labeled chorismate through a series of three, modular one-pot reactions is being undertaken as an alternative to standard synthesis. This approach involves the *in vitro* reconstitution of the shikimate pathway which converts phosphoenolpyruvate (PEP) and erythrose-4-phosphate to chorismate through a series of seven enzymes.

This work describes the first of three modules which will be used to produce labeled chorismate, namely the conversion of deuterated pyuvate to phosphosphoenolpyruvate. To accomplish this, a one-pot reaction of pyruvate carboxylase (PycA; E.C. 6.4.1.1) from *Staphylococcus aureus* and PEP carboxykinase (PckA; E.C. 4.1.1.49) from *Escherichia coli* is being developed. In this study, PycA and PckA were expressed and purified, and their enzymatic activity was verified. Additionally, several trials of the one-pot reaction have been performed. The products from these reactions have been separated using hydrophobic interaction liquid chromatography (HILIC). Preliminary HPLC chromatograms and mass spectroscopy data suggests that PEP is successfully being produced. Furthermore, when linked to the first enzyme of the shikimate pathway, DAHP synthase (AroG; E.C. 2.5.1.54), data indicates successful production of DAHP.

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#73

Uncovering the Biological Function of a Nitrating Heme Enzyme in Rufomycin Biosynthesis

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Rufomycins constitute a class of cyclic heptapeptides that are isolated from *Streptomyces atratus*. Several rufomycins are found to inhibit the proliferation of *mycobacterium tuberculosis* through a novel target which regulates protein homeostasis. Hence, rufomycins offer promising anti-TB treatment, especially potent regarding drug-resistant TB strains. It was proposed that the unnatural amino acids in rufomycins, including a 3-nitro-tyrosine, are first enzymatically modified, and then assembled by a heptamodular non-ribosomal peptide synthetase (NRPS). RufO, a cytochrome P450-type enzyme, was proposed to catalyze the formation of the 3-nitro-tyrosine in the presence of O_2 and $^{\bullet}NO$. CYP-dependent nitration is rarely seen, and its catalytic mechanism is obscure. The only other reported example is TxtE which nitrates Trp in thaxtomin biosynthesis. To investigate the biological function of RufO, interactions of RufO with potential ligands, including the proposed substrate Tyr and peptides mimicking intermediate products along the biosynthetic pathway, have been investigated using spectroscopies and activity assays. However, there is no evidence of binding or reactivity among the tested ligands. A low- to high-spin transition and a dramatic increase in redox potential that are commonly found in CYP enzymes were not shown. X-ray crystal structures of RufO reveals a hydrophobic distal pocket and large substrate access tunnel that are inappropriate for tyrosine binding. Our recent progress suggests that nitration of tyrosine likely occurs during the peptidyl assembly, which shifts our attention to the third module of the NRPS that incorporates the 3-nitro-tyrosine. Our study will define the biological function of RufO and add new knowledge to non-ribosomal peptide biosynthesis.

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#74

Stabilization of Insulin with a Tardigrade Specific Cytosolic Abundant Heat Soluble Protein

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Protein-based therapeutics are susceptible to temperature-induced inactivation. To make proteins more heat tolerant, we are using a tardigrade-specific intrinsically disordered protein called CAHS D. Cytosolic Abundant Heat Soluble (CAHS) proteins are required for tardigrades to survive desiccation, and previous work has shown that CAHS D can also protect enzymes from desiccation and lyophilization-induced inactivation in vitro.[(1), (2)] In addition to preserving the activity of enzymes, this project aims to use CAHS D to stabilize ligands without interfering with the ligand-receptor interaction. Specifically, we show that CAHS D can protect insulin from inactivation due to heat stress. Future work will test insulin stabilization with CAHS D at room temperature and will verify functionality of stabilized insulin in a mouse model. We hope that, using this novel stabilization strategy, insulin can be stored and transported without the need for refrigeration.

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#75

A Non-bactericidal approach to inhibit biofilm formation in *Streptococcus mutans* using glucosyl transferase inhibitors.

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Dental plaque consists of more than 700 different bacterial species living in complex bacterial communities called biofilms. *Streptococcus mutans* is implicated as the major etiological agent in the initiation and development of dental caries (tooth decay). The formation of tenacious biofilms is the hallmark of *S. mutans* induced cariogenesis. *S. mutans* form biofilms readily on the tooth surface and rapidly produce lactic acid from the dietary sugars. To develop non-bactericidal biofilm inhibitors, we have targeted the *S. mutans* cell surface secreted enzyme called glucosyltransferases (Gtfs). These enzymes metabolize sucrose into water insoluble glucans, which play a critical role in mediating irreversible attachment of bacterium to the tooth and providing an extracellular matrix, shielding the bacteria from the host immune responses, and antimicrobial agents. A group of small molecule biofilm inhibitors are synthesized based on an identified lead compound and SAR studies. The emerged lead compound from this study inhibited Gtfs, reduced the formation of *S. mutans* biofilms *in vitro* and reduced the incidence of smooth-surface caries *in vivo* in rats at low micromolar non-bactericidal concentrations.

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#76

Structural Basis of Docking Interactions among the Kinase Domains within ASK1-MKK-JNK3 Cascades

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Mitogen-activated protein kinase (MAPK) cascades are highly conserved, three-tiered kinase pathways that orchestrate a wide array of vital cellular responses including differentiation, growth, survival, and apoptosis. The structural basis of these essential sign signalosomes remains unclear. The primary aim of this research is the exploration of the assembly of a three-component kinase complex through a combination of computational analyses by focusing on the complexes consisting of the kinase domains of Apoptosis-Activated Kinase I (ASK1), its downstream MAP Kinase (MKK4/7) and c-Jun N-Terminal Kinase 3 (JNK3). To achieve the putative structures of ASK1-MKK4/7-JNK3, molecular docking studies using ClusPro (Boston University, Boston, MA USA) were guided by the identification of potential docking sites based on the highly conserved, evolutionarily important and energetically "frustrated" nature of amino acids surface residues. These residues were identified using the Evolutionary Trace method (Lichtarge Lab, Baylor University, Houston, TX USA) and the Protein Frustratometer (EMBNET, University of Buenos Aires, Buenos Aires, Argentina). The docking interfaces were experimentally verified using a direct pull-down assay. The constructed ASK1-MKK4/7-JNK3 complex structure was studied using molecular dynamics (NAMD v. 2.6., University of Illinois at Urbana-Champaign, Urbana-Champaign, Illinois, USA). These all-atom simulations suggest that the complex remained stable with the interface residue contacts remaining intact throughout the simulation. The simulations and experimental verification suggest a mechanism of the sequential activation of MAPK by diverse activator biomolecules and demonstrates the structural insights into MAPK cascades.

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