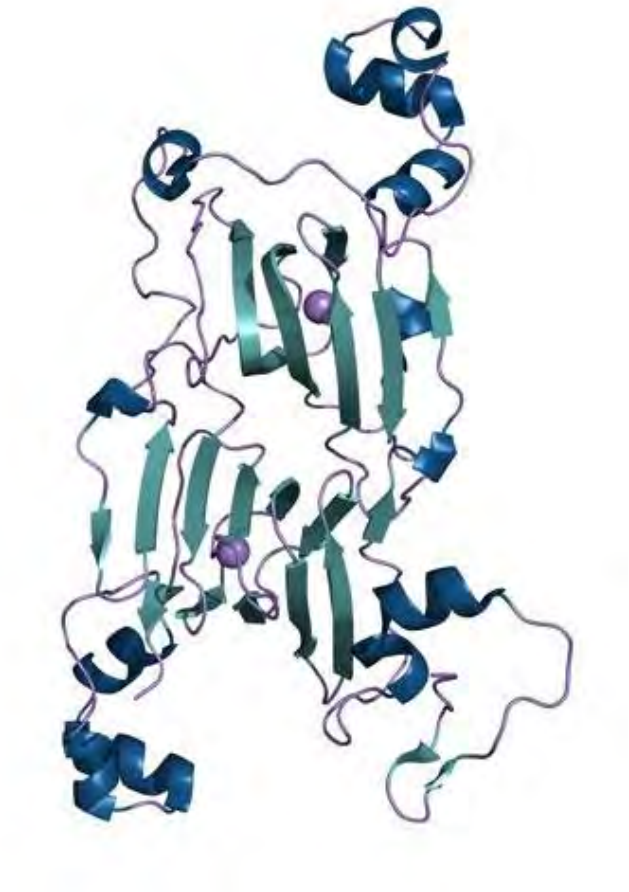
Eighth Southeast Enzyme Conference



Saturday, April 8, 2017

Georgia State University
Atlanta, GA

Urban Life Building
140 Decatur Street
Room 220

Eighth Southeast Enzyme Conference

Saturday, April 8, 2017

Supported by generous contributions from:

Kennesaw State University
College of Science and Mathematics

Kennesaw State University
College of Science and Mathematics
Department of Chemistry and Biochemistry

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Southeast Enzyme Conference (SEC)

Meeting	Year	Program Chair	Site Chair	Site
I	2010	Giovanni Gadda	Will Lovett	GSU
II	2011	Nigel Richards	Giovanni Gadda / Will Lovett	GSU
III	2012	Robert Phillips	Giovanni Gadda / Will Lovett	GSU
IV	2013	Holly Ellis	Giovanni Gadda / Neil Renfroe / Will Lovett	GSU
V	2014	Liz Howell	Giovanni Gadda / Will Lovett / Neil Renfroe / Robert Daniel	GSU
VI	2015	Anne-Frances Miller	Giovanni Gadda / Will Lovett / Gwen Kenny / Robert Daniel	GSU
VII	2016	Pablo Sobrado	Giovanni Gadda / Will Lovett / Crystal Smitherman Rosenberg / Robert Daniel / Will Thacker	GSU
VIII	2017	Ellen Moomaw	Giovanni Gadda / Will Lovett / Crystal Smitherman Rosenberg / Will Thacker / Anthony Banks	GSU
IX	2018			

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Crystal structure and functional analysis of ClbQ, an unusual intermediate-releasing thioesterase from the colibactin biosynthetic pathway
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9:20-9:40 **Shanzhi Wang**, University of Arkansas, Little Rock
Dissecting the roles of the enzymatic and scaffolding functions of EXO1 in vivo

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The thermodynamics of metal and substrate binding to taurine/α-ketoglutaratedependent oxygenase (TauD) from E. coli
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Do transitions between conformational substates help stabilize chemical transition states? Combinatorial thermodynamic cycle analysis
12:00-1:00 **Group Photo followed by Lunch Break**

Session 3 - *Discussion Leader*: **Kevin Francis**, Texas A&M University, Kingsville
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1:20-1:40 **Prashasti Kumar**, University of Tennessee, Knoxville
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3:00-4:00 **Pablo Sobrado**, Virginia Polytechnic Institute and State University, Blacksburg
Targeting siderophore biosynthesis: structure, mechanism, and inhibition of flavindependent N-monoooxygenases

4:00 **Concluding Remarks**: **Ellen Moomaw**, Kennesaw State University, Kennesaw
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Session 1:

Zac Wood

Discussion Leader

Crystal Structure and Functional Analysis of ClbQ, an Unusual Intermediate-Releasing Thioesterase from the Colibactin Biosynthetic Pathway

Naga Sandhya Guntaka[1], *Steven D. Bruner*[1]

[1]Departments of Chemistry, University of Florida, Gainesville, Florida 32611

Small molecule microbial secondary metabolites by regulating host-microbe interactions play an important role in all aspects of disease etiology and treatment. Colibactin is a secondary metabolite linked to the progression and pathogenesis of colorectal cancer (CRC) and inflammatory bowel disease (IBD) by inducing DNA damage in host cells. The chemical details of the colibactin and the biosynthetic pathway are emerging but clearly are unusual and noncanonical. Our research addresses a key aspect of colibactin biosynthesis, the occurrence of multiple metabolites and the biosynthetic rationale for this. Recent studies suggest an atypical role of ClbQ, a type II editing thioesterase in releasing pathway intermediates from the assembly line (1) and genetic deletion of ClbQ has been shown to abolish colibactin cytotoxic activity (2). Presented is an interdisciplinary approach to address the role of ClbQ, using enzyme structure, organic synthesis of substrates/intermediates and mechanistic analysis. The 2.0 Å crystal structure and biochemical characterization of ClbQ reveal that ClbQ exhibits greater catalytic efficiency toward acyl-thioester substrates as compared to precolibactin intermediates and does not discriminate between carrier proteins in the pathway. As reported in earlier studies (1), late-stage cyclized intermediates are not the preferred substrates for ClbQ. However, late-stage linear precolibactin intermediates are hydrolyzed. Our data, combined with previous reports, support a novel role of ClbQ in facilitating the promiscuous offloading of premature precolibactin metabolites and suggest novel insights into colibactin biosynthesis.

1. Li, Z.-R., Li, J., Gu, J.-P., Lai, J. Y. H., Duggan, B. M., Zhang, W.-P., Li, Z.-L., Li, Y.-X., Tong, R.-B., Xu, Y., Lin, D.-H., Moore, B. S., and Qian, P.-Y. (2016) Divergent biosynthesis yields a cytotoxic aminomalonate-containing precolibactin. *Nat. Chem. Biol*. **12**, 773–775.
2. Cougnoux, A., Dalmasso, G., Martinez, R., Buc, E., Delmas, J., Gibold, L., Sauvanet, P., Darcha, C., Déchelotte, P., Bonnet, M., Pezet, D., Wodrich, H., Darfeuille-Michaud, A., and Bonnet, R. (2014) Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype. *Gut*. **63**(12)**,** 1932-42.

Role of Loop L1 Dynamics in *Pseudomonas aeruginosa* D-Arginine Dehydrogenase

Daniel Ouedraogo[1], *Michael Souffrant*[1], *Sheena Vasquez*[1,5], *Donald Hamelberg*[1, 3, 4], *and Giovanni Gadda*[1, 2, 3, 4, *]

[1]Department of Chemistry, [2]Department of Biology, [3]Center for Diagnostics and Therapeutics, [4]Center for Biotechnology and Drug Design, Georgia State University, Atlanta, Georgia 30302, United States, [5]University of Georgia, Athens, Georgia 30602

The conformational changes of mobile loops positioned at the entrance of active sites of enzymes often participate in catalysis to control the access to the active site, to orient catalytic residues and to shield the active site from the bulk solvent.[1, 2] Previous crystallographic data on D-arginine dehydrogenase from *Pseudomonas aeruginosa* (*Pa*DADH) showed the presence of an active site loop L1 with two different peptidyl regions including residues 45-47 located at the FAD-binding site and residues 50-56 positioned at the entrance of the active site.[3] In the two peptidyl regions of loop L1, only the S45, A46 and Y53 residues adopt major conformational changes corresponding to the open (ligand-free) and closed (product-bound) conformations.[3] In the closed conformation the side chain of Y53 prevent the exit of the product from the active site whereas the side chain of A46 swings closer to the FAD and the side chain of S45 points away from the FAD.[3] In the open conformation Y53 points away from the active, while the side chain of A46 swings away from the FAD and the side chain of S45 points closer to the FAD. The alternate conformations of the S46 and A46 residues, which are not in direct contact with the substrate, have been dubbed the Ser/Ala switch.[3]

In this study, S45 was mutated to alanine and A46 to glycine. The role of the conformational change of the Ser/Ala switch in *Pa*DADH was studied through molecular dynamics, steady-state and rapid reaction kinetics techniques. Molecular dynamics of loop L1 showed higher probabilities in the S45A and A46G variant enzymes to be in the open conformation compared to the wild-type *Pa*DADH, consistent with an exposed active site to the solvent. The flavin fluorescence intensity was ~2-fold higher in the S45A and A46G variant enzymes with respect to the wild-type *Pa*DADH, with a 9 nm bathochromic shift of the emission band. The k_{cat}/K_m values with D-arginine in both variants, were ~13-fold lower in comparison with the wild-type *Pa*DADH. Moreover, a hollowed pH-profile was observed on the k_{cat} value with D-arginine consistent with restricted proton movements in catalysis. Rapid reaction kinetic data showed no saturation with the slow substrate D-leucine in the reductive half-reaction for the variant enzymes. All taken together the data indicate that the dynamics of loop L1 is important for substrate binding and catalysis in *Pa*DADH.

Support: NSF CHE-1506518 (G.G.)

(1) Hanoian, P., Liu, C. T., Hammes-Schiffer, S., and Benkovic, S. (2015) Perspectives on electrostatics and conformational motions in enzyme catalysis. *Acc. Chem. Res. 48*, 482-489.

(2) Gora, A., Brezovsky, J., and Damborsky, J. (2013) Gates of enzymes. *Chem. Rev. 113*, 5871-5923.

(3) Fu, G., Yuan, H., Li, C., Lu, C. D., Gadda, G., and Weber, I. T. (2010) Conformational changes and substrate recognition in *Pseudomonas aeruginosa* D-arginine dehydrogenase. *Biochemistry 49*, 8535-8545.

Dissecting the roles of the enzymatic and scaffolding functions of EXO1 *in vivo*

Shanzhi Wang[1], Kyeryoung Lee[2], Richard Chahwan[3], Yongwei Zhang[2], Catherine Tang[2], Sergio Roa[2], Rikke B. Morrish[3], Elisa Pesenti[2], Isabelle Lykens[1], Paula E. Cohen[4], Thomas MacCarthy[5], Winfried Edelmann[2], Matthew D. Scharff[2]

[1]Chemistry Department, University of Arkansas at Little Rock, AR 72204; [2]Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461; [3]Department of Biosciences, University of Exeter, Exeter EX2 4QD, United Kingdom; [4]Department of Biomedical Sciences, Cornell University, Ithaca, NY, 14853; [5]Department of Applied Mathematics and Statistics, State University of New York, Stony Brook, NY 11794

Activation induced cytidine deaminase (AID) generates U:G mismatches in immunoglobulin (Ig) variable-regions (V) genes during somatic hypermutation (SHM) and switch-regions (SR) during class switch recombination (CSR). Exonuclease 1 (EXO1) is essential for both processes. It has been reported that mice expressing EXO1 with the cancer associated E109K mutation (*Exo1*^{EK}) did not have the defects in SHM and CSR seen in *Exo1*^{null} mice, suggesting that the enzymatic activity of EXO1 was not required for antibody diversification. However, subsequent biochemical work revealed that the untagged full length EXO1-E109K protein retained WT exonuclease activity while and EXO1-D173A (*Exo1*^{DA}) active site mutation had negligible enzymatic activity (1, 2). We therefore examined this question by generating new mice expressing the enzymatically nonfunctional EXO1-D173A mutation (Exo1^{DA}) and comparing them to EXO1-knockout (Exo1^{null}) and WT mice. Meiosis in Exo1^{DA} and WT mice was identical while Exo1^{null} mice were sterile, suggesting that only the scaffolding role of EXO1 is essential for meiosis. Mutation accumulation in a reporter gene elsewhere in the genome was comparable between Exo1^{DA} and Exo1^{null} mice, and twice as high as the WT mice, suggesting that the enzymatic role of EXO1 is important for non-Ig canonical error free mismatch repair. Deep-sequencing of 186.2 heavy chain variable region in B cells from (4-hydroxy-3-nitrophenyl)acetyl (NP) immunized mice showed the mutation spectra of Exo1^{DA} mice was different from the Exo1^{null} mice but was not significantly different from the WT mice. This supports our previous observation in Exo1^{EK} mice that scaffolding functions of EXO1 are critical for ncMMR at the Ig variable regions of B cells, while its exonuclease activity is not required or somehow compensated for by other unknown mechanisms. In contrast, CSR efficiencies of Exo1^{DA} and Exo1^{null} mice were defective compared to WT mice, and residual switching events were slightly enriched in blunt and short SR microhomologies. This could be due to a requirement for EXO1 exonuclease activity in the resections required to create the double stranded DNA breaks. Taken together, the data suggest that EXO1 recruits other factor(s) to promote ncMMR repair in the Ig V regions during SHM, while its enzymatic role is clearly required during CSR. It will be important to identify the factor(s) that are required for the excision of AID-generated G:U mismatches in the V regions in order to elucidate how these regions are exposed to error prone repair and the introduction of A:T mutations during antibody affinity maturation.

1. Shao H *et al. Nucl. Acids Res.* 2014, 42:7104-7112.
2. Bregenhorn SJ *et al*. *Nucl. Acids Res.* 2014, 42: 7096-7103.

Session 2:

Anne-Frances Miller

Discussion Leader

FixAB at the Heart of Flavin-based Electron Bifurcation in Diazotrophic Bacteria

H. Diessel Duan[1], Carolyn E. Lubner[2], Paul W. King[2] and Anne-Frances Miller[1]

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Energy conservation is essential for all life on Earth. As the recently-discovered third fundamental mechanism of energy conservation in biology, flavin-based electron bifurcation not only conserves excess free energy of exergonic reactions, enabling organisms to survive in harsh habitats, but also provides low potential electrons to drive extremely challenging reactions such as reduction of atmospheric dinitrogen. This presentation will use the flavoenzyme component FixAB from a metabolically versatile bacterium *Rhodopseudomonas palustris* to explore the unique properties that underpin flavin-based electron bifurcation. The crucial difference between FixAB and its homolog, the canonical electron-transferring flavoprotein (ETF) resides in a second FAD in the bifurcating enzyme that replaces the AMP of its non-bifurcating counterpart. Circular dichroism (CD) is employed to distinguish the different flavin sites in FixAB allowing interrogation of the putative bifurcating FAD. Our CD data permit assignment of the three midpoint potentials of FixAB to specific flavins, elucidation of the mechanism of bifurcation in FixAB and investigation of the flavin properties upon which the mechanism rests. Transient absorption spectroscopy (TAS) further supports FixAB's potential to mediate bifucation by revealing the presence of a short-lived anionic semiquinone (ASQ), lending credence to the mechanism whereby a thermodynamically unstable ASQ intermediate may participate in electron bifurcation to the low potential acceptor [1]. FixAB is the central component of the flavin-rich Fix system that provides potent reducing equivalents to nitrogenase, making it important for global food security. However understanding of FixAB will also shed light on electron bifurcation in general, and how electron flow can be directed in a controlled manner in a protein setting.

[1] Lubner, C. E., Jennings, D. P., Mulder, D. W., Schut, G. J., Zadvornyy, O., Hoben, J., Tokmina-Lukaszewska, M., Berry, L., Nguyen, D., Lipscomb, G. L., Bothner, B., Jones, A. K., **Miller, A.-F.**, King, P. W., Adams, M. W. W. and Peters, J. W. (2017) "Mechanism of Energy Conservation by Electron Bifurcation" *In Press, Nature, Chemical Biology*

The thermodynamics of metal and substrate binding to taurine/α-ketoglutarate-dependent oxygenase (TauD) from *E. coli*

Mingjie Li[1], Kate L Henderson[1,2] Salette Martinez[3] Robert P Hausinger,[3] and Joseph P Emerson[1]*

[1]Department of Chemistry, Mississippi State University
[2]Department of Biochemistry, University of Wisconsin
[3]Department of Biochemistry and Molecular Biology, Michigan State University

Taurine/α-ketoglutarate (αKG) dioxygenase (TauD) is a nonheme iron(II) and αKG dependent metalloenzyme, which catalyzes the hydroxylation of taurine leading to its decomposition into aminoacetaldehyde and sulfite, where sulfite is a key sulfur containing metabolite in *E. coli*. The nonheme iron(II) center in TauD is formed from two histidine side chain residues and a glutamic acid coordinating to one face of the octahedral coordination geometry. This common metal binding motif has been termed the 2-His-1-carboxylate facial triad and is found in a number of nonheme manganese, iron, and cobalt containing proteins. Here we have focused our efforts to measure the thermodynamic driving forces that lead to formation of these bioinorganic centers in biology, by studying divalent metal ion coordination to TauD using isothermal titration calorimetry. Titrations of metal complexes into the metal-free (apo) TauD and the corresponding chelation experiments were performed under anaerobic environment.

The thermodynamic terms associated with cobalt(II), iron(II), and manganese(II) binding to apoTauD were deconvoluted from complex experiments, where the pH and buffer independent binding constant (K) were measured to be 2.9×10^9, 2.4×10^7, and 9.8×10^5, respectively. (The corresponding ΔG values were calculated to be -11.2 kcal/mol, -10.1 kcal/mol, and -7.1 kcal/mol, respectively.) Interestingly the measured enthalpy changes for these binding events (ΔH) are -12.7 kcal/mol, -12.8 kcal/mol, and -13.9 kcal/mol, respectively. These data are fully consistent with the Irving-Williams series, which suggest there is increasing affinity for transition metal ions from left to right across the periodic table. However, it seems this the increasing affinity is derived from increasing favorability of both the related ΔH and ΔS terms.

Do transitions between conformational substates help stabilize chemical transition states? Combinatorial thermodynamic cycle Analysis

Charlie Carter
Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7260

Enzyme mechanisms, especially those that couple NTP hydrolysis to mechanical work and information, use sophisticated dynamic networks to transduce active-site chemistry into domain motions that change binding affinities. We measured and cross-validated the energetics of such networks in *B. stearothermophilus* Tryptophanyl-tRNA synthetase (TrpRS) using both *multi-mutant* and *modular* thermodynamic cycles. Coordinated domain motions develop shear in a core packing motif conserved in >125 different protein superfamilies (2). Multi-dimensional combinatorial mutagenesis showed that four side chains from this "molecular switch" move coordinately with the active-site Mg^{2+} ion in the transition state for amino acid activation (3). A *modular* thermodynamic cycle consisting of full-length TrpRS, its Urzyme, and the Urzyme plus each of the two domains deleted in the Urzyme (4) gives similar energetics. These complementary experiments establish that catalysis and specificity in full-length TrpRS are both coupled by ~ –5 kcal/mole to: (i) the core packing region where domain movement generates shear (5), and (ii) the simultaneous motion of the two domains relative to the Urzyme (4). An Onsager-Machlup action-minimizing algorithm for the most probable trajectories through the conformational transition state ensembles estimates four computational parameters—time to the transition state, conformational free energy differences, and transition-state activation energies—for comparison to experimental kinetic rates. These computational simulations can be carried out sufficiently rapidly to use in "high throughput" mode. Correlations between those parameters, the experimental rates, and structural variations induced in the combinatorial mutants confirm that these trajectories are realistic. These results validate our previous conclusion that catalysis by Mg^{2+} ion is coupled to the overall domain motion (3). Computational free energy surfaces demonstrate that TrpRS catalytic domain motion itself is endergonic but is driven thermodynamically by PPi release (6). Comparison of the impact of combinatorial mutagenesis on both pre-steady state and steady-state rates confirm that dynamic active-site pre-organization endows TrpRS with the elusive mechanism coupling NTP utilization to domain motion, clarifying a previously puzzling aspect of free energy transduction.

1. *J. Biol. Chem.* **289**:30213 (2014)
2. *Bioinformatics* **26**:709 (2010)
3. *Structure* **20**:128 (2012)
4. *J. Biol. Chem.* **288**:34736 (2013)
5. *J Biol Chem* **289**:4367 (2014)
6. *Structural Dynamics* **3**,:012101 (2016)

Session 3:

Kevin Francis

Discussion Leader

Elucidation of the Biosynthesis Pathway for the Key Coenzyme of Methanogenesis and Anaerobic Methane Oxidation

Kaiyuan Zheng, Phong D. Ngo, Victoria L. Owens, Xue-peng Yang, Steven O. Mansoorabadi
Departments of Chemistry and Biochemistry, Auburn University, Auburn, AL 36849

Methyl-coenzyme M reductase (MCR) is the key enzyme of methanogenesis and anaerobic methane oxidation (AOM). The activity of MCR is dependent on the unique nickel-containing tetrapyrrole, coenzyme F430. We used comparative genomics to identify the coenzyme F430 biosynthesis (cfb) genes and characterized the encoded enzymes. The pathway was found to involve nickelochelation of sirohydrochlorin by the first nickel-specific chelatase, amidation to form a novel tetrapyrrole, Ni-sirohydrochlorin a, c-diamide, an un-presented a 6-electron reduction/ γ -lactamization reaction by a primitive homolog of nitrogenase, and intramolecular carboxylic ring formation by a Mur ligase homolog. This study significantly advances our understanding of coenzyme F430 biosynthesis and MCR maturation, identifies new targets for inhibitors of natural greenhouse gas emissions, and sets the stage for metabolic engineering efforts utilizing MCR (*1*).

1. K. Zheng, P. D. Ngo, V. L. Owens, X. Yang, S. O. Mansoorabadi (2016) The biosynthetic pathway of coenzyme F430 in methanogenic and methanotrophic archaea, *Science*. 354, 339–342.

INSIGHTS INTO A LOW PROMISCUOUS AMINOGLYCOSIDE MODIFYING ENZYME, AMINOGLYCOSIDE *N3* ACETYLTRANSFERASE-VIA

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1) Graduate School of Genome Science and Technology, The University of Tennessee and Oak Ridge National Laboratory, Knoxville, Tennessee 37996

2) Spallation Neutron Source, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

3) Department of Biochemistry, Cellular and Molecular Biology, The University of Tennessee, Knoxville, Tennessee 37996

Aminoglycoside modifying enzymes (AGMEs) are plasmid-encoded enzymes found in resistant bacteria. AGMEs covalently modify their substrates and thus render the drug ineffective for the bacteria. Aminoglycoside (AG) antibiotics are bactericidal agents used to treat various bacterial diseases like tuberculosis and meningitis. However, the clinical effectiveness of these drugs has been drastically affected by the emergence of AGMEs. More than 50 different AGMEs are known, having variable levels of substrate promiscuity. However, no correlation has been confirmatively observed between the sequence or structure of an AGME and its substrate profile. We aim to understand the molecular principles underlying this ligand selectivity by deciphering the thermodynamic, structural and dynamic properties of enzyme-ligand complexes.

Kinetic, thermodynamic and structural properties of the aminoglycoside *N3* acetyltransferase VIa (AAC-VIa) are described. Despite having significant sequence similarity to highly promiscuous acetyltransferase, AAC-VIa can modify only 5 aminoglycosides, with a ~4-fold difference in the k_{cat} values. Thermodynamic studies determined the binding of ligands to be enthalpically driven and entropically unfavorable. Unlike other AGMEs, the formation of binary and ternary complexes was accompanied by a net deprotonation of the enzyme, ligand or both. Another significant difference was observed in the structure of AAC-VIa and other AGMEs in solution. Analytical ultracentrifugation (AUC) studies showed that AAC-VIa exists in a monomer-dimer equilibrium, with more dimeric form appearing with increasing concentrations of the enzyme. Binding of ligands drive the enzyme to a more monomeric form. Also, dimer formation is achieved mainly through polar interactions. Crystal structures of different complexes of the enzyme showed that structures of apo-and ligand-bound forms were identical which suggests that, unlike other AGMEs, more rigid structure of AAC-VIa may limit the active site to accommodate only few selected aminoglycosides, hence low substrate promiscuity.

Keynote Presentation:

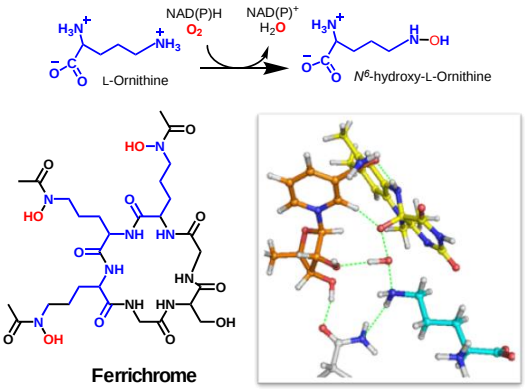
Ellen Moomaw

Discussion Leader

Targeting siderophore biosynthesis: structure, mechanism, and inhibition of flavin-dependent *N*-monoooxygenases

Pablo Sobrado
Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061

Flavin-dependent monooxygenases catalyze the NADPH-and O_2-dependent hydroxylation of a variety of substrates. At the center of the chemical mechanism of these enzymes is the formation and stabilization of the C4a-hydroperoxyflavin intermediate, which is the hydroxylating species. Siderophore A (SidA) is a flavin-dependent monooxygenase that catalyzes the hydroxylation of ornithine at the N5-position in the biosynthesis of siderophores in *Aspergillus fumigatus*. Because the function of SidA is essential for virulence, this enzyme has been identified as a target for drug development. I will present the results from biochemical[1], DFT[2, 3], molecular dynamics simulations[4], and structural approaches[5, 6] that have allowed the detailed characterization of the mechanism of hydroxylation and the role of key residues in catalysis. The results support a mechanism of coenzyme-assisted catalysis, where NADP(H) plays a dual role, flavin reduction and stabilization of oxygenated flavin intermediate. The results are relevant to other flavin-dependent enzymes and have implication for drug design[7].



Supported by National Science Foundation grant MCB 1021384.

[1] Romero, E., Fedkenheuer, M., Chocklett, S. W., Qi, J., Oppenheimer, M., and Sobrado, P. (2012) Dual role of NADP(H) in the reaction of a flavin dependent N-hydroxylating monooxygenase, *Biochim Biophys Acta 1824*, 850-857.
[2] Robinson, R., Badieyan, S., and Sobrado, P. (2013) C4a-hydroperoxyflavin formation in N-hydroxylating flavin monooxygenases is mediated by the 2'-OH of the nicotinamide ribose of $NADP^+$, *Biochemistry 52*, 9089-9091.
[3] Badieyan, S., Bach, R. D., and Sobrado, P. (2015) Mechanism of N-hydroxylation catalyzed by flavin-dependent monooxygenases, *J Org Chem 80*, 2139-2147.
[4] Shirey, C., Badieyan, S., and Sobrado, P. (2013) Role of Ser-257 in the sliding mechanism of NADP(H) in the reaction catalyzed by the Aspergillus fumigatus flavin-dependent ornithine N5-monooxygenase SidA, *J Biol Chem 288*, 32440-32448.
[5] Franceschini, S., Fedkenheuer, M., Vogelaar, N. J., Robinson, H. H., Sobrado, P., and Mattevi, A. (2012) Structural insight into the mechanism of oxygen activation and substrate selectivity of flavin-dependent N-hydroxylating monooxygenases, *Biochemistry 51*, 7043-7045.
[6] Binda, C., Robinson, R. M., Martin Del Campo, J. S., Keul, N. D., Rodriguez, P. J., Robinson, H. H., Mattevi, A., and Sobrado, P. (2015) An Unprecedented NADPH Domain Conformation in Lysine Monooxygenase NbtG Provides Insights into Uncoupling of Oxygen Consumption from Substrate Hydroxylation, *J Biol Chem 290*, 12676-12688.
[7] Martin Del Campo, J. S., Vogelaar, N., Tolani, K., Kizjakina, K., Harich, K., and Sobrado, P. (2016) Inhibition of the Flavin-Dependent Monooxygenase Siderophore A (SidA) Blocks Siderophore Biosynthesis and Aspergillus fumigatus Growth, *ACS Chem Biol 11*, 3035-3042.

Poster Session 1 – 9:40-10:50:

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6. Ball, Jacob
7. Baumert, Andrew
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9. Bester, Stephanie M.
10. Bhojane, Purva
11. Brown, Caroline
12. Bufkin, Kendra
13. Caparco, Adam
14. Carter, Charlie
15. Carter, E. Kathleen
16. Chenge, Jude
17. Chepaitis, Patrick
18. Davis, Cameron
19. Dodd, Thomas
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21. Duff, Michael
22. Dzimianski, John
23. Eckshtain-Levi, Meital
24. Endicott, Nathaniel
25. Engel, Carly
26. Erlitzki, Noa
27. Fang, Luting
28. Ferrell, Brandon L.
29. Flores, Elias
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31. Francis, Kevin
32. Franklin, Robert
33. Fuente, Gabriel
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35. Goodwin, John
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38. Gross, Phillip G.
39. Gumpper, Ryan
40. Guntaka, Naga Sandhya
41. Hagen Richard
42. Haseltine, John
43. Ho, Jessica
44. Hua, Eric
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46. Iyer, Archana
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66. Nambiar, Deepika
67. Ndungu, Joan
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Characterization of the H183F Variant of Class I Nitronate Monooxygenase from *Pseudomonas aeruginosa* PAO1

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Nitronates, such as propionate 3-nitronate (P3N), are toxic compounds that are found in fungi and plants as glycosides. Nitronate monooxygenases (NMOs; E.C. 1.13.12.16) are flavin-dependent enzymes that utilize oxygen to detoxify these toxins. P3N was discovered as the physiological substrate for *Pseudomonas aeruginosa* NMO (*Pa*NMO). P3N irreversibly inhibits succinate dehydrogenase – an important enzyme involved in energy production. Previous kinetic and spectroscopic studies have been conducted on both fungal and bacterial NMO demonstrating that P3N is oxidized with k_{cat} and k_{cat}/K_m values of 1000 s^{-1} and $\geq 10^7$ M^{-1} s^{-1}, respectively. Over 4,000 genes in a multitude of organisms are annotated as NMOs.[1] The crystal structures of both *Cyberlindnera saturnus* NMO and *Pa*NMO have been resolved and demonstrate that both enzymes have conserved active sites. Multiple sequence alignments demonstrated that Y109, H133, H183, Y254, Y299, and Y303 are highly conserved residues in various prokaryotic and eukaryotic NMO's.[1]

Utilizing polymerase chain reaction, H183 was replaced with phenylalanine. *Pa*NMO H183F was expressed and purified to high levels. The enzyme exhibits at least a 1000-fold reduction in the $^{app}k_{cat}$ value and a 25,000-fold reduction in the $^{app}(k_{cat}/K_m)$ value with respect to the wild-type enzyme. Such a large impact on these kinetic parameters for P3N suggests that His183 plays a role in substrate binding or catalysis. Further kinetic and structural characterization is undergoing.

This study was supported in part by grant CHE1506518 from the NSF (G.G.), a GSU Molecular Basis of Disease Fellowship (D.S., C.A.), an IMSD Fellowship (C.A.), and an LSAMP Fellowship (C.A.).

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Characterization of new Streptococci genes implicated in heme utilization

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Streptococcal pathogens are responsible for a variety of infections in humans. Metal iron is required for growth and virulence in pathogenic bacteria. During infection, free iron is not available, and thus, invading microbes, such as Streptococci, must develop a mechanism to compete for the metal. Heme bound to hemoglobin is the most abundant source of iron inside the human body. Here we show that addition of hemoglobin restored growth of Streptococci in iron-depleted medium, demonstrating that this pathogen can use heme to satisfy its need for iron. Transcriptome analyses of Streptococci grown with hemoglobin revealed a major shift in metabolic genes. We also identified a two-gene cluster that was upregulated 10-fold. This cluster encodes two cytoplasmic proteins; one contains an FMN binding motif. We cloned and expressed both proteins in *Escherichia coli*. Preliminary experiments indicate that both proteins bind and degrade heme *in vitro*. We hypothesize that these genes play an important role in Streptococcal heme utilization.

D-Glucosaminate-6-phosphate ammonia lyase: An aminotransferase family member with unusual D-amino acid specificity

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Salmonella enterica serovar Typhimurium is a common food-borne pathogen. *S. enterica* is theorized to survive competition for colonization of the gut through the utilization of unusual substrates such as D-glucosaminate. A catabolic pathway for D-glucosaminate has been proposed, and the genes encoding the proteins necessary for the pathway have been identified and named *dga*ABCDEF, with the genes *dga*ABCD corresponding to four permease components and the genes *dga*E and *dga*F corresponding to a dehydratase and an aldolase, respectively (1). The *dga*ABCD permease is a PTS transport system which forms D-glucosaminate-6-phosphate. The action of *dga*E and *dga*F converts D-glucosaminate-6-phosphate to the final products, D-glyceraldehyde-3-phosphate, pyruvate and ammonium. The *dga*E protein, D-glucosaminate-6-phosphate ammonia lyase (DGL), is of mechanistic interest because it is the sole enzyme in the pyridoxal-5'-phosphate (PLP) dependent aminotransferase superfamily known to catalyze reaction on a D-substrate. Furthermore, it also reacts rapidly with substrate to form a quinonoid intermediate within the dead time (ca. 2 msec) in stopped-flow kinetics experiments. Although crystals have been obtained, the structure of DGL has yet to be determined. We propose that the unusual stereochemistry of the DGL reaction requires a catalytic base poised on the opposite face of the PLP-substrate complex from the other members of the aminotransferase superfamily. Site-directed mutagenesis is being used to identify the base responsible for carrying out the reaction. Good candidates for site-directed mutagenesis were identified by comparing the primary sequences and structures of homologous proteins to that of DGL. Ascertaining the structure of DGL and the base involved in the reaction may open up new possibilities for the medical treatment of *Salmonella*.

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Fast Protein Dynamics: Allosteric Inhibition of Lactate Dehydrogenase

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Enzymes provide powerful, often synthetically inaccessible chemical transformations under mild conditions; however, the fundamental physical processes of biological catalysis are still poorly understood. While high-resolution structural details are widely available, such information only provides a static framework for enzyme-substrate interactions. To fully understand catalytic activity, dynamics must be considered. Processes on the appropriate timescale to contribute to active catalysis include rapid, femto- to picosecond vibrations. A sub-class of these motions, termed "rate promoting vibrations" (RPVs) are presumed to be low frequency, internal, compressive motions. While such dynamics have largely been explored computationally, few direct experimental handles exist to confirm their existence or contribution to catalysis. A novel approach is to position an allosteric effector near the residues implicated in the RPV and monitor the impact on the reaction being catalyzed. An allosteric binding site has been posited for human heart lactate dehydrogenase, an enzyme which contains a heavily studied RPV, and candidates for inhibition have been proposed (1). The interaction of these inhibitors with the protein have been characterized at equilibrium and via kinetic studies to further interrogate the role of protein dynamics in catalysis.

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RELATING DYNAMICS AND FUNCTION IN AN ENZYME FAMILY

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The enzymes of the ribonuclease (RNase) family share the common chemical function of catalyzing the hydrolysis of single stranded ribonucleic acid (ssRNA). These enzymes possess identical or similar active site residues and conserved fold architecture. However, their catalytic efficiency differs by 10^{-5}–10^{-6} fold. Further, preliminary evidence from nuclear magnetic resonance (NMR) indicates that the rate of dynamics (internal protein motions) amongst these enzymes range from microsecond to second time-scale, a factor 10^{6} difference. Recent investigations provide insights into the role of internal protein motions in enzyme catalysis. It is now believed that dynamics and structure together play critical role in the function of biomolecules including enzymes. Internal motions in the enzyme drive the sampling of short-lived minor population of conformations (called as *sub-states*). These conformational *sub-states* might contain features that can promote the function of an enzyme. Therefore correlating the role of time dependent dynamics in sampling conformational sub-states would enable a better understanding of the catalytic mechanism of the RNases family. However, this is challenging and beyond the reach of any single technique. Therefore we are using a combination of theoretical modeling, atomistic molecular simulations, kinetics and CPMG NMR relaxation dispersion experiments to quantitatively characterize dynamical changes that regulate the mechanism of enzyme catalysis across the members of the human RNase family. Preliminary results indicate, that the dynamical and phylogenetic classification of the members of the RNase A superfamily are alike, suggesting that dynamical motions are coupled to the designated function of the enzyme. As RNases differ in their substrate binding properties we modeled the members of the human RNase family with two tetra nucleotide substrates and observed diverse binding preferences.

Mechanistic Studies of a Flavin-dependent NADH:quinone Reductase from *Pseudomonas aeruginosa PAO1*

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PA1024 is an FMN-dependent protein that catalyzes the two-electron reduction of quinones utilizing NADH as electron donor. PA1024 was previously presumed to be a nitronate monoxygenase (NMO) based on the sequence analysis and the presence of 2-nitropropane in the crystal structure, but eventually the enzyme was proven to not be an NMO and instead possess an entirely different function. PA1024 is a novel NADH:quinone reductase with little amino acid sequence identity (< 20%) or active-site structural similarity to the well-known and studied human NADH:quinone oxidoreductase 1 (NQO1), NQO2, or lot6p from yeast.

The enzyme is active with a broad range of quinone substrates including coenzyme Q_0, a structural analogue to ubiquinone. PA1024 strictly prefers NADH over NADPH as the reducing substrate, demonstrating almost no activity with NADPH. Two-electron reduction of quinones leads to relatively stable hydroquinone compounds, whereas the one-electron reduction produces semiquinone radicals and ultimately superoxide that are deleterious to the cell. The genomic and biochemical knowledge of PA1024 thus far suggests the enzyme is implicated in cellular NAD^+ regeneration or quinone detoxification. The present study utilizes pH effects on the steady-state kinetics of PA1024 with 1,4-benzoquinone and 5-hydroxy-1,4-naphthoquinone (Juglone) as substrates. Preliminary data indicate there are two active states of the enzyme and one inactive state that all involve ionization of enzymatic residues. Two potential mechanisms are proposed accounting for the two ionizations relevant to catalysis.

This study was supported in part by grant CHE-1506518 (G.G.) from the NSF and a Molecular Basis of Disease Fellowship (J.B.) from GSU

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Subcloning Nitronate Monooxygenase from *Neurospora crassa* for Structural and Mechanistic Studies

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Nitronate monooxygenase (NMO) catalyzes the oxidation of alkyl nitronates into aldehydes and nitrite (1). A substrate of NMO, propionate-3-nitronate (P3N), is a known metabolic inhibitor that is produced by various leguminous plants and microbial species in its conjugate acid form, 3-nitropropane (3-NPA) (2). The kinetic mechanism of *Pseudomonas aeruginosa* PAO1, *Cyberlindnera saturnus* and *Neurospora crassa* NMOs have been studied extensively, revealing that the enzymes have striking differences in their substrate specificity, for example, *P. aeruginosa* NMO can only oxidize P3N and other alkyl nitronates, but not 3-NPA and other nitroalkanes, whereas *N. crassa* NMO can oxidize nitroalkanes and alkyl nitronates (1,3). Four motifs have been identified in bacterial, fungal and two animal NMOs, defining Class I NMO; *N. crassa* NMO lacks these four motifs, defining Class II NMO (3). *P. aeruginosa* and *N. crassa* NMOs share 25.6% amino acid sequence identity and 36.6% amino acid sequence similarity. Although the crystal structure of *P. aeruginosa* NMO is known, the structure of *N. crassa* NMO has not yet been resolved. *N. crassa* NMO is 40 kDa, and it exists as a homodimer with each monomer containing one mole of noncovalently bound FMN and is devoid of metal cofactors (1). To make structural and mechanistic comparisons between *P. aeruginosa* and *N. crassa* NMOs, the gene for *N. crassa* NMO, ncd-2, has been subcloned into the pET-20b(+) vector and a C-terminal His tag was added for ease of purification.

In the pET20 vector system, a XhoI site is directly upstream of the His tag sequence, and this restriction enzyme is used for the addition of a C-terminal histidine tag. Unfortunately, the 2-ncd gene contains one internal XhoI in the gene open reading frame. Thus, the internal XhoI site of 2-ncd was silently mutated. Then, the mutated 2-ncd gene was amplified using polymerase chain reaction, double digested with NdeI and XhoI and ligated into the pET-20b(+) vector and transformed into *Escherichia coli*. The protein product of the 2-ncd gene, ncNMO will be purified to high levels for use in structural and mechanistic studies.

This study was supported in part by grant CHE-1506518 from the NSF.

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Allostery and Hysteresis are coupled in human UDP-glucose dehydrogenase

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Human UDP-glucose dehydrogenase (hUGDH) is regulated by an atypical allosteric mechanism in which the feedback inhibitor UDP-xylose (UDP-Xyl) competes with substrate for the active site. Binding of UDP-Xyl triggers the T131-Loop/α6 allosteric switch, which converts the hexameric structure of hUGDH into an inactive, horseshoe-shaped complex (E^{Ω}). This allosteric transition buries residue A136 in the protein core to produce a subunit interface that favors the E^{Ω} structure. Here we use a methionine substitution to prevent the burial of A136 and trap the T131-Loop/α6 in the active conformation. We show that $hUGDH_{A136M}$ does not exhibit substrate cooperativity, which is strong evidence that the methionine substitution prevents the formation of the low UDP-Glc affinity E^{Ω} state. In addition, the inhibitor affinity of $hUGDH_{A136M}$ is reduced 14 fold, which most likely represents the K_i for competitive inhibition in the absence of the allosteric transition to the higher affinity E^{Ω} state. hUGDH also displays a lag in progress curves, which is caused by a slow, substrate-induced isomerization that activates the enzyme. Stopped flow analysis shows that $hUGDH_{A136M}$ does not exhibit hysteresis, which suggests that the T131-Loop/α6 switch is the source of the slow isomerization. This interpretation is supported by the 2.05 Å resolution crystal structure of $hUGDH_{A136M}$, which shows that the A136M substitution has stabilized the active conformation of the T131-loop/α6 allosteric switch. This work shows that the T131-Loop/α6 allosteric switch couples allostery and hysteresis in hUGDH.

Structural Insights of Stereospecific Inhibition of Human Acetylcholinesterase by VX and Subsequent Reactivation by HI-6

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Organophosphonate nerve agents, such as VX (O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate), phosphonylate the catalytic serine residue of acetylcholinesterase (AChE, E.C. 3.1.1.7), an enzyme vital for all organisms with a nervous system. AChE reactivators, which remove organophosphonates from AChE, vary in efficacy depending on the nerve agent and which stereoisomer is present. In order to understand the efficacy of HI-6 better, AChE was crystalized and soaked with VX (either racemic, -, or +), HI-6, or both HI-6 and VX (racemic, -, or +). Enzymatic assays were also performed to obtain reactivation data for HI-6, which supported the differences observed in the crystallographic structures. HI-6 appeared in three different conformations within the structures, which potentially contributes to the stereoselective efficiency of HI-6 with VX. The enzymatic data and structures also offer great insight into redesigning HI-6 to have greater efficacy with all nerve agents and their stereoisomers.

Small Angle Neutron Scattering (SANS) Studies on R67 DHFR, a Tetrameric Protein with Intrinsically Disordered N-termini

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Dihydrofolate reductase (DHFR) catalyzes the NADPH dependent reduction of dihydrofolate to tetrahydrofolate, which serves as a source for one-carbon donation reactions in cellular metabolism. R67 DHFR is a plasmid-encoded DHFR that confers resistance against trimethoprim, which is a potent inhibitor of *E.coli* chromosomal DHFR. R67 DHFR is a homo-tetramer with a single active site pore. The dimer crystal structure indicates 16-18 amino acids at the N-terminus of each monomer are intrinsically disordered. Truncation of 16 N-terminal amino acids results in almost full activity but a lowered stability.

We investigated the effect of ligand binding on the disordered N-termini that might induce a coupled binding and folding of the unstructured tails using small angle neutron scattering (SANS). The binary complex with the oxidized cofactor ($NADP^+$) and the ternary complex with the substrate (dihydrofolate) resulted in radii of gyration comparable to that of the apo protein, suggesting minimal, if any changes in the overall shape of the protein.

We did not observe compaction of the overall structure in the presence of betaine as the radius of gyration (R_g) of the protein indicated slightly higher values. A combined analysis using molecular dynamics and a program called SASSIE gives better insight into the ensemble of states sampled by the disordered tails of the apo R67 DHFR in the presence and absence of betaine. A similar analysis was done for the binary and ternary protein complexes. The disordered N-termini seem to sample collapsed as well as partially extended conformations and remain mostly disordered in all the conditions tested.

We also studied the hydration of the full length R67 DHFR in presence of osmolytes (glycine betaine and DMSO) and our results indicated around 1200 water molecules hydrating the protein in the presence of betaine as well as DMSO. Similar studies with the truncated R67 DHFR yielded around 400 water molecules hydrating the protein in the presence of betaine.

Mechanism of the enzymatic synthesis of furan-containing compound

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This research project stems from the desire to explore and eventually harness the enzymatic mechanism of 4-(hydroxymethyl)-2-furan- carboxaldehyde-phosphate synthase (MfnB). The enzyme of interest, MfnB, is found most prominently in methanogens and has the ability to catalyze five or more separate chemical transformations in a single active site. This singular enzyme takes two molecules of glyceraldehyde-3-phosphate to create a furan-containing compound 4-(hydroxymethyl)-2-furan-carboxaldehyde-phosphate. The US. Department of Energy (DOE) has published a list of the "Top 10 + 4" bio-based chemicals, with furan-containing compounds highlighted for their high potential in the production of biofuels and biomaterial compounds(1). As one can imagine, industrial applications of MfnB might usher in a new era for the synthesis of furan compounds to be used in the generation of liquid fuel or other biomaterial via enzyme-catalyzed reactions. Despite the initial characterization of MfnB and the identification of Schiff base-forming lysine, the detail mechanism of MfnB remains speculative(2). Our team seeks to understand the details of catalytic mechanism through site-directed mutagenesis, comprehensive kinetic evaluation and structural study. Uncovering the molecular basis of this catalytic mystery will help us in applying the knowledge to enzyme engineering. The engineered catalytic machinery can then be used for generating building block compounds to meet the needs of industrial biofuel and other biomaterial products.

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Hydroxamate formation in the siderophore Albachelin

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N-Hydroxylating monooxygenases (NMOs) are part of Group B flavoenzymes, which are involved in siderophore biosynthesis. Siderophores are high affinity iron- chelators composed of catechol and hydroxamate functional groups that are synthesized and secreted by microorganisms and plants [1]. It has been showed that many NMOs are essential for the virulence of many opportunistic pathogens such as *Aspergillus fumigatus [2]*. NMO hydroxylate L-Ornithine and produce N^5–hydroxyl-L-ornithine, which is ultimately incorporated into siderophores. Recently, a new siderophore named albachelin was isolated from a culture of *Amycolatopsis alba* lacking iron (Figure 1) [3]. Structural studies have shown that the NMO from *A. alba* (AMO) shares a 31% amino acid sequence identity to SidA from *A. fumigatus*, a well-characterized NMO. This work focuses on the expression, purification, and pre-steady/steady-state characterization of the unique single-component enzyme *AMO*. The enzyme was purified and characterized in its holo (FAD-bound) and apo (unbound) forms. Steady state characterization of AMO was performed by measuringN^5–hydroxyl-L-ornithine production, oxygen consumption and hydrogen peroxide formation. The two forms of AMO are highly specific to ornithine and do not catalyze its hydroxylation of lysine. Steady state kinetics show that the apo-AMO prefers NADH while holo-AMO prefers NADPH. Results from this work will enable us to better understand the NMO class of flavoenzymes and their mechanism of action.

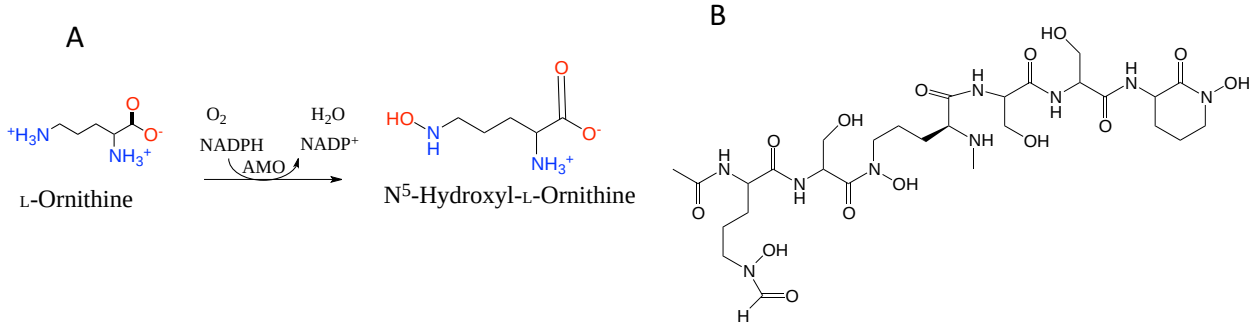

Figure 1: (A) Reaction catalyzed by AMO and (B) desferri-albachelin chemical structure.

This work was supported by grants from the National Science Foundation (NSF) CHE-1506206 and MCB-1021384.

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Design of a Heterogeneous Biocatalyst for Cofactor Regeneration and Improved Catalytic Characteristics

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Enzyme immobilization generally allows for improved lifetime and stability of catalytically active protein. Typically, immobilization systems involve adsorption to surfaces, cross-linked enzyme aggregates or entrapment of enzyme in a polymer network.[1] However, these systems often can introduce unwanted limiting effects, such as high resistance to mass transfer or distortion in the enzyme structure due to adsorption or crosslinking. To overcome these shortcomings, it is possible to use more porous materials which are imbued with immobilization domains. Supraparticles formed by a hierarchically structured self-assembly of protein-inorganic nanoflowers provide a porous and stable support for enzyme capture with a high surface area and low mass transfer resistance. In this work, we used hybrid protein-inorganic supraparticles made of calcium phosphate and protein with leucine zipper binding domains for high-affinity immobilization of fusion proteins containing the complementary binding domain and enzymes.[2] Particularly, we showed the modularity of the supraparticle system by immobilizing two distinct fluorescent proteins on the particles in tandem. The development of active "zippered" enzyme constructs, as well as their immobilized and soluble activities, is shown. This work demonstrates our ability to create an industrially relevant heterogeneous biocatalyst which can incorporate cofactor regeneration and is stable in enzymatically relevant conditions.

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Do transitions between conformational substates help stabilize chemical transition states? Combinatorial thermodynamic cycle Analysis

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Enzyme mechanisms, especially those that couple NTP hydrolysis to mechanical work and information, use sophisticated dynamic networks to transduce active-site chemistry into domain motions that change binding affinities. We measured and cross-validated the energetics of such networks in *B. stearothermophilus* Tryptophanyl-tRNA synthetase (TrpRS) using both *multi-mutant* and *modular* thermodynamic cycles. Coordinated domain motions develop shear in a core packing motif conserved in >125 different protein superfamilies (2). Multi-dimensional combinatorial mutagenesis showed that four side chains from this "molecular switch" move coordinately with the active-site Mg^{2+} ion in the transition state for amino acid activation (3). A *modular* thermodynamic cycle consisting of full-length TrpRS, its Urzyme, and the Urzyme plus each of the two domains deleted in the Urzyme (4) gives similar energetics. These complementary experiments establish that catalysis and specificity in full-length TrpRS are both coupled by ~ –5 kcal/mole to: (i) the core packing region where domain movement generates shear (5), and (ii) the simultaneous motion of the two domains relative to the Urzyme (4). An Onsager-Machlup action-minimizing algorithm for the most probable trajectories through the conformational transition state ensembles estimates four computational parameters—time to the transition state, conformational free energy differences, and transition-state activation energies—for comparison to experimental kinetic rates. These computational simulations can be carried out sufficiently rapidly to use in "high throughput" mode. Correlations between those parameters, the experimental rates, and structural variations induced in the combinatorial mutants confirm that these trajectories are realistic. These results validate our previous conclusion that catalysis by Mg^{2+} ion is coupled to the overall domain motion (3). Computational free energy surfaces demonstrate that TrpRS catalytic domain motion itself is endergonic but is driven thermodynamically by PPi release (6). Comparison of the impact of combinatorial mutagenesis on both pre-steady state and steady-state rates confirm that dynamic active-site pre-organization endows TrpRS with the elusive mechanism coupling NTP utilization to domain motion, clarifying a previously puzzling aspect of free energy transduction.

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DNA microstructure influences selective binding of small molecules designed to target mixed-site DNA sequences

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Specific targeting of protein–nucleic acid interactions is an area of current interest, for example, in the regulation of gene-expression. Most transcription factor proteins bind in the DNA major groove; however, we are interested in an approach using small molecules to target the minor groove to control expression by an allosteric mechanism. In an effort to increase understanding of sequence recognition by DNA-targeted-small-molecules a recent extensive investigation was preformed using competition mass spectrometry and surface plasmon resonance (1). We discovered that the heterocyclic diamidine, DB2277, not only forms strong monomer complexes with sequences that include both A·T and G·C base pairs, but also shows unexpected binding of two DB2277 with certain sequences. The inherent microstructural differences within the experimental DNAs were identified through computational analyses to ascertain the molecular basis for recognition. These findings emphasize the critical nature of the DNA minor groove microstructure for sequence-specific recognition and offer new avenues to design synthetic small molecules for effective regulation of gene-expression. Sequences that the start (free) and end (bound) most similarly have the most favorable binding as a result of lower deformation energy of the DNA. This implies that the sequence with the highest binding affinity already has a shape complementary to the small molecule and further suggests that inherent microstructure of the DNA strongly influences binding affinity.

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Divergence In Substrate Specificity By The vOTU Domain of Various Strains of Highly-pathogenic PRRSV And The implications To Pathogenicity

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Porcine reproductive and respiratory syndrome virus (PRRSV) is widespread with a high variation in sequence and virulence among the divergent strains and causes an economically destructive disease. A viral ovarian domain protease (vOTU) has been previously identified within the nonstructural protein nsp2 of PRRSVs. As with many vOTU family proteases, these proteases have been suggested to be a virulence factor for PRRSV through their ability to potentially cleave ubiquitin and ISG15 conjugated substrates. Recently, significantly divergence in substrate specificity between two vOTUs from PRRSV strains varying in virulence had been revealed. Here we examine the substrate differences of four PRSSV strains, the previously characterized JXwn06 and MLV strains along with strains SDSU73 and NADC31. This additional information provides a more complete view of the range PRRSV vOTU possess in substrate specificity and suggests potential correlation between substrate specificity of strains and differences in virulence.

Investigation of the free energy profile and structural mechanism for the base-flipping pathway of a 5-carboxylcytosine lesion in DNA by thymine DNA glycosylase

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The base excision repair (BER) process in DNA begins with enzymatic recognition of a lesion base or a base pair differing from the normal Watson-Crick construct. Thymine DNA glycosylase (TDG) is one such enzyme that exhibits broad interrogation of lesion bases mispaired with thymine. The mechanisms of recognition searching, base extrusion or base flipping, and glycosidic bond cleavage have begun to be investigated. However, TDG's base flipping process as an entire pathway has not been thoroughly studied to our knowledge, and experimental models have yet to provide distinct pathway energetics and mechanism. Instead, only kinetic snapshots of base flipping and simulations of base excision have been reported (1,2). In order to address the issue, we employed the partial nudged elastic band method (PNEB) to determine the minimum free energy path for flipping of the lesion base 5-carboxylcytosine by TDG. The preliminary dynamics simulations have provided a trajectory suitable for inquiry into the pathway energetics using umbrella sampling. We hope that further investigation and examination of the results will provide a mechanistic basis for and structural insight into how TDG affects the energy barrier for base flipping.

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Molecular Mechanism of JNK Attenuation Regulated by Parkin

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Parkinson's disease (PD) is characterized by the waxing pigmentation, accumulation of lewy bodies, and the ultimate degeneration of neurons in the substantia nigra pars compacta region of the brain. The most prevalent indication of hereditary PD is a mutation in the PARKIN gene. Parkin facilitates the proteasomal degradation of target proteins through its ubiquitination-catalyzing activity. The E3 ubiquitin ligase has been further implicated in attenuating the activation of the c-jun N-terminal kinase pathway, a pro-apoptotic mitogenactivated protein kinase (MAPK) signaling cascade, implying neuroprotective characteristics. However, the mechanism whereby parkin regulates the JNK pathway remains obscure. Utilizing in vitro pull-down assays in tandem with co-immunoprecipitation methods, we have identified a novel interaction between parkin and apoptosis signal-regulating kinase (ASK-1), an upstream kinase in the JNK pathway. This interaction was characterized through co-overexpression in HEK293T cells, which indicated the parkin-induced degradation of ASK1. In vitro ubiquitination assays showed an enhanced activation of parkin in the presence of ASK1. Our results suggest the interaction with ASK1 is able to disrupt the intrinsic auto-inhibition of parkin, enhancing its activation to carry out the ubiquitin-signaled degradation of ASK1.

Understanding the Allosteric Mechanism Behind the E3 Ligase Parkin

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The ubiquitin-proteasome pathway, conserved from yeast to mammals, is an important process required for the targeted degradation of most shortlived proteins in the eukaryotic cell. The main targets include regulatory proteins, as well as proteins unable to fold properly within the endoplasmic reticulum. E3 ligases facilitate the transfer of ubiquitin from an ubiquitin conjugating enzyme (E2) to a target protein, initiating the degradation process. Parkin is a RING-between-RING (RBR) E3 ligase that functions in the covalent attachment of ubiquitin to specific substrates. Mutations in Parkin are linked to Parkinson's disease, cancer and mycobacterial infection. We have modeled Parkin in complex with an E2-ubiquitin conjugate using existing crystallographic data of HOIP, an E3 ligase within the same RBR subfamily and structurally similar to that of Parkin. Initial molecular dynamics simulations of Parkin indicate that the activated ubiquitin binds to the RING2 domain of Parkin via a hydrogen-bonding network similar to that of HOIP. These data suggest that all RBRs have a catalytic domain capable of accommodating the di-Arginine motif found on ubiquitin. Through dynamic network analysis, we hope to elucidate key details behind the allosteric mechanism that allows Parkin to bind E2-ubiquitin conjugates.

Crosslinking of Dicyclotyrosine by a P450 Enzyme

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Abstract

A recently identified P450 enzyme in *Mycobacterium tuberculosis* (Mtb), CYP121, was found to be required to maintain cell viability. Additionally, the ability for CYP121 to bind tightly to known azole compounds have marked it as a potentially new target to combat Mtb infections. Characterization of CYP121 demonstrated that it catalyzes a unique C-C coupling reaction on its substrate, dicyclotyrosine (cYY). This reaction is distinct from the traditional oxygen insertion activities observed in many other P450 systems. Here, we tested the P450 catalytic shunt pathway and investigated the chemistry of crosslink formation of cYY with CYP121 using peracetic acid as the oxidant. We employ rapid kinetic methods, stopped-flow UV-Vis and rapid-freeze quench EPR (RFQ-EPR) spectroscopies to monitor the transient kinetics of the reaction. By detecting the product mycocyclosin using LC-MS, we validate the use of peracetic acid as an oxidant. We observed a new intermediate species formed upon mixing the ES-complex with peracetic acid. Our results allow us to propose a consistent mechanism for the C-C crosslinking reaction of cYY.

Folate Forms Weak Off-Target Interactions with Other Proteins

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The B9 vitamin, folate, and its derivatives are used by the cell as a one-carbon donors for the synthesis of metabolites, including thymidine and methionine. Dihydrofolate reductase (DHFR) is the initial enzyme of the cell's folate cycle, which reduces folate and dihydrofolate to tetrahydrofolate using NADPH as a cofactor. Our earlier research indicated that the catalytic efficiency, as well as ligand affinity, for two DHFRs that do not share sequence or structural homology are decreased in the presence of macromolecular crowders. As two structurally unrelated DHFRs show similar effects with crowder proteins, this leads to the hypothesis that the decrease in enzyme efficiency is due to interaction of the ligands with the crowder proteins. This is similar to the interactions between folate and osmolytes.

The potential interactions between folate and the DHFR cofactor, $NADP^+$, were explored using docking and biophysical techniques. Docking studies predict that folate and $NADP^+$ tend to interact with large clefts, such as active sites, on the crowder proteins. When the ligands were forced to dock at sites other than the large clefts, weaker interactions were predicted by Autodock Vina. This suggests that the ligands will bind to larger clefts, or pockets, on proteins. However, experimental evidence for potential interactions is still lacking. Binding studies by isothermal titration calorimetry yielded no enthalpy of interaction. Likewise, there were no shifts in the apparent T_ms for the crowder proteins in the presence of folate compared to without folate. Therefore, potential interactions between folate and crowder proteins may be weak and transient, and overestimated by docking studies.

Biochemical and structural insights into nairoviral deISGylase preferences for interferon stimulated gene product 15 originating from particular species

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Crimean-Congo Hemorraghic Fever Virus (CCHFV) is part of the *Nairovirus* genus in the family *Bunyaviridae*. Endemic to parts of Eastern Europe, the Middle East, Asia, and Africa, CCHFV causes debilitating disease with a fatality rate as high as 30%. Encoded in the L segment of the negative sense RNA tripartite genome is a homologue of the Ovarian Tumor (OTU) proteases. This viral OTU (vOTU) possesses potent activity for reversing the post-translational modification of proteins by ubiquitin (Ub) and the Ub-like molecule interferon stimulated gene 15 (ISG15), suggesting a potential role in mediating viral evasion of the innate immune response. Interestingly, while Ub is almost perfectly conserved across Eukaryotes, ISG15 shows a great degree of diversity, with sequence identities that can be less than 60% between mammals and 35% when extended to fish. This presents potential challenges for zoonotic viruses such as CCHFV and other nairoviruses that circulate within a range of vertebrate hosts. To gain a better understanding of the impact ISG15 diversity could have on nairoviruses, the vOTUs from CCHFV along with other human disease-causing nairoviruses, Erve virus (ERVV) and Nairobi Sheep Disease Virus (NSDV), were assessed for the ability to process ISG15 derivative substrates from a range of species, including human, mouse, shrew, sheep, bat, camel, and fish. These assays revealed that different ISG15s can show substantial variation in the efficiency of processing by these proteases. Additional characterization by isothermal titration calorimetry (ITC) on a subset of these ISG15s revealed not only overall differences in binding affinity, but also differences in the thermodynamic factors driving binding with some proteases. The molecular underpinnings of these differences were more thoroughly elucidated by the determination of a 2.47 Å X-ray crystal structure of the ERVV vOTU bound to the C-terminal Ub-like domain of mouse ISG15. Comparison of this structure with previously solved structures of CCHFV vOTU bound to human ISG15 or Ub not only explained sequence driven differences in intrinsic protease activity, but also unveiled specific residue positions in ISG15 that may drive protease preference for the ISG15 of one species versus another. These results provide key insights into the effect of biological diversity on virus-host interactions, with the potential to isolate important factors that may impact the host range and preference of various nairoviruses.

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Kinetic Mechanisms of the Two-component Flavin-dependent Monooxygenase Involved in Valanimycin Biosynthesis

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Two-component flavin-dependent monooxygenase enzymes are involved in oxidation reactions in numerous metabolic and biosynthetic pathways in microorganisms. These enzymes consist of a reductase, which generates a reduced flavin and a monooxygenase that utilizes the reduced flavin as a substrate for monooxygenation reactions. The reductase component is known to be unspecific for NADPH or NADH as well as for FAD or FMN (1). The free transfer of reduced FAD normally yields a highly uncouple reaction with low hydroxylation yields (1).

Here, we study the Isobutylamine N-hydroxylase (IBAH) – Flavin reductase (FRED) system from *Streptomyces viridifaciens*, both reported to be involved in the antibiotic valanimycin biosynthesis. IBAH is a flavin-dependent monooxygenase that with the help of the FRED catalyses the hydroxylation of isobutylamine (IBA), the precursor of valanimycin. We investigated the reaction mechanisms and kinetics of the reductases and the monooxygenase, as well as the mechanism of reduced flavin transfer. Furthermore, we explored the protein–protein interactions while assessing the necessity of protein complex formation for higher efficiency. This knowledge is necessary to further understand catalysis by enzymes using two-component systems.

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Structural Requirements for Receptor-Mediated Xenosiderophore Utilization by Pathogenic *Staphylococcus aureus*

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Staphylococcus aureus is a Gram-positive bacterium that has become an increasingly prominent source of infection in clinical settings in its methicillin-resistant form (MRSA), proving especially threatening to patients requiring prolonged hospital stays (1). Virulence factors associated with nutrient acquisition are critical for the proliferation of MRSA in a human host. Siderophores associated with iron acquisition are particularly important. To expand capacity for iron acquisition beyond the use of endogenously produced staphyloferrin A, staphyloferrin B, and staphylopine, MRSA also scavenges xenosiderophores produced by competing bacteria using the FhuBCDG proteins (2,3). FhuD2 is a surface-exposed lipoprotein with broad xenosiderophore binding capabilities. FhuD2 preferentially binds trihydroxamate siderophores, including desferrioxamine B (DFOB), which is commonly used as a treatment for human iron overload diseases. DFOB use is associated with an increased risk of infection by pathogens, such as MRSA, capable of utilizing DFOB as a xenosiderophore (4). Here we use a panel of synthetic DFOB analogs to establish critical structure-function relationships for trihydroxamate xenosiderophore utilization by pathogenic *S. aureus*. We explore iron-binding properties, FhuD2 receptor binding, and *S. aureus* growth promotion. Our results suggest that the DFOB scaffold can be tuned based on net charge for determining utilization by *S. aureus*.

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Analyzing Flavin-Based Electron Bifurcation in Heterodisulfide Reductase

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Daily human activities (i.e. rice production, husbandry of ruminants) has significantly increased the amount of methane, into the atmosphere (1, 2). Key enzymes in the methane synthesis pathway are studied as possible targets for novel inhibitors, including the enzyme heterodisulfide reductase (HDR; E.C. 1.8.98.1) (1). This enzyme reduces mixed disulfide of coenzyme M and coenzyme B (CoM-S-S-CoB) through flavin-based electron bifurcation, a process that creates electrons with low redox potentials without coupling the electron transfer step to ATP hydrolysis (2). Electrons are transported through numerous iron-sulfur clusters while flavin adenine dinucleotide (FAD) bifurcates these electrons into two different directions: 1) to the heterodisulfide reduction site and 2) to ferredoxin. Due to the unusually high number of iron-sulfur clusters, electron paramagnetic resonance (EPR) studies are necessary to understand how FAD performs this bifurcation process. Redox titration and rapid freeze quench experiments were performed on HDR in *Methanothermobacter marburgensis* to determine the potentials of the iron-sulfur clusters and the time at which each cluster receives the electron from FAD. Midpoint potentials were determined for several of the paramagnetic species present in HDR and their position in the electron paths will be discussed.

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Hydration properties of the DNA minor groove binder DB1976

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The disposition of hydration waters in DNA binding by minor-groove ligands is presently controversial. On the one hand, the impossibility of two atoms occupying the same space leads to the expectation that spine-of-hydration waters buried deep within the minor groove should be displaced on binding of a minor groove ligand. On the other hand, previous studies on the effect of osmotic stress on the binding of model minor groove ligands to DNA revealed that the ligand/DNA complex was destabilized compatible cosolutes, which was interpreted as an uptake of hydration waters on complex formation (1). In addition, crystallographic studies showed the presence of persistent interfacial waters that mediate ligand/DNA binding (2). Here, we investigated the role of hydration in DNA binding by the AT-specific minor groove ligand DB1976, a symmetric heterocyclic diamidine, using both ionic (NaCl) and non-ionic cosolutes (ethylene glycol, glycine betaine, maltose, nicotinamide, urea) to apply osmotic pressure. While the DB1976/DNA complex was indeed destabilized by osmotic pressure from non-ionic cosolutes, the degree to which binding was perturbed depended on the identity of the cosolute, an indication of preferential interactions between DB1976 and the cosolutes. This strong non-osmotic component limits the interpretation of osmotic sensitivity as simply increased hydration of the complex relative to the unbound constituents. To eliminate the possibility of confounding preferential interactions, we used only NaCl to exert osmotic pressure, and found that hydration waters are in fact net released on binding of DB1976 to DNA. We propose that destabilization of the DB1976/DNA complex in response to osmotic stress by non-ionic cosolutes reflects a preferential exchange of cosolute surrounding the unbound compound for water (and the coupled release of cosolute) on binding to DNA, rather than being an indication of net water uptake on complex formation.

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Tabtoxin: a Promising Antibiotic against Tuberculosis with High Reactivity and Selectivity

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There are 2 million people that die each year of tuberculosis (TB) and one-third of the world's population is currently infected by *Mycobacterium tuberculosis (Mtb)*.[1] Treatment of TB with antibiotics is very difficult because the waxy cell envelope excludes many small molecules. *Mtb* is an intracellular pathogen while it was reported that glutamine synthetase (GS) can be excreted into the cytoplasm of the host cell during infection.[2] Inhibition of glutamine synthetase can stop the growth of *Mycobacterium tuberculosis* in a guinea pig infection model, suggesting that GS is a good target for TB treatment.[3] Tabtoxin is a dipeptide prodrug composed of L-Thr and tabtoxinine-beta-lactam (tbl), a naturally occurring potent inhibitor of glutamine synthetase.[4] However, the mechanism of GS inhibition by tabtoxinine-beta-lactam is still unknown. In this project, we used mass spectrometry and solid-state NMR to analyze the enzyme-inhibitor complex. We also compared the inhibition potency of tbl against human GS and bacterial GS based on kinetic data, which revealed good selectivity of tbl for inhibiting bacterial GS. Bacterial growth curves confirmed tabtoxin to be a potent bacteriostatic antibiotic against certain bacterial strains. The result shows that tabtoxin is a potent inhibitor against glutamine synthetase, so hopefully, it explored as a potential treatment for TB.

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Heme Uptake via the ChtB Surface-anchored Protein in *Corynebacterium diphtheriae*

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Corynebacterium diphtheriae is a gram-positive, pathogenic bacterium. Pathogenic bacteria require iron as a key nutrient for survival. These bacteria use a heme biosynthesis pathway, a heme uptake pathway, or both, to obtain the necessary iron. *C. diphtheriae* utilizes a direct heme uptake system in which the heme binds to a receptor protein that transfers the heme along the pathway to an ABC transporter, which facilitates the transfer of heme into the bacterial cell. In the human body, hemoglobin serves as the source of iron for the direct heme uptake pathway of *C. diphtheriae*. This pathway is encoded partly by the cht gene locus, which includes a two-gene operon coding for ChtB (1). ChtB is proposed to have a function in transporting the heme obtained by HtaA or ChtA/C to HmuT. A sequence alignment of ChtB with other HtaA-like proteins in *C. diphtheriae* shows two conserved tyrosines (Y30 and Y202) and one histidine (H95). The heme binding domain of wild type ChtB (ChtB-CR) with a Strep tag was expressed and purified on a Strep-Tactin column. SDS PAGE of as-isolated WT ChtB-CR (≈ 60% heme loaded) showed a single band with a molecular weight of approximately 28 kD. The pyridine hemochrome technique (2) was used to calculate an extinction coefficient of 11300 M^{-1} cm^{-1} at the Soret. Thermal denaturation studies were performed to determine the stability of wild type ChtB-CR. In the presence of 1 M GdnHCl, a T_m of 62 °C was measured. Time scale of unfolding studies in the presence of 4 M GdnHCl determined a half-life of 39 min. Raman spectroscopy reveals that reducing ChtB and trapping it with CO yields two species: one with tyrosine *trans* to the CO and one with histidine *trans* to the CO, which indicates the use of tyrosine and histidine in the heme pocket. Understanding the role of ChtB in the process of heme uptake in *C. diphtheriae* may lead to developments in drug technology to combat this bacteria.

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We thank the Research Corporation for support. Rizvan Uluisik thanks the Molecular Basis of Disease Program at Georgia State University for a fellowship.

Characterization of PA1225 in *Pseudomonas aeruginosa* PAO1 as an NADPH-specific FAD:Quinone Oxidoreductase

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The product of gene *pa1225* in *Pseudomonas aerguniosa* PAO1 is currently annotated as a putative NAD(P)H-quinone oxidoreductase, based on a 29% amino acid sequence identity to human NAD(P)H dehydrogenase, quinone 2. PA1225 contains flavin adenine dinucleotide (FAD) as a cofactor, as suggested by fluorescence spectroscopy and MALDI-TOF spectrometry. The consensus sequence (GXGXXG/A) for the Rossmann fold found in FAD-dependent enzymes is absent in the PA1225 sequence. The enzyme has been hypothesized by others to participate in *P. aeruginosa* PAO1 Vitamin K cycle for 2-e^- reductions of menaquinone and phylloquinone. Also, PA1225 is repressed 89-fold in the presence of the LysR regulator PA4203, which also represses 10-fold the expression of nitronate monooxygenase, an enzyme that oxidized the mitochondrial toxin propionate 3-nitronate (1). Thus, PA1225 may be linked to oxidative stress experienced by *P. aeruginosa* in the presence of propionate 3-nitronate.

To elucidate whether PA1225 is indeed an NAD(P)H-quinone oxidoreductase, the *pa1225* gene was amplified by PCR from the genomic DNA of *P. aeruginosa* PAO1 and ligated into vector pET20(b)+. The resulting recombinant plasmid was used to transform *Escherichia coli* strain Rosetta(DE3)pLysS to express PA1225. Initial purification of the recombinant enzyme using anion exchange chromatography revealed that the FAD cofactor readily dissociates from the holoprotein. To expedite protein purification and preserve the integrity of the holoprotein, PA1225 was reengineered to incorporate an N-terminal His-tag. The stoichiometry of FAD:protein in the His-tagged enzyme increased from 0.15 to 0.35. The enzyme turned over with *p*-1,4 benzoquinone and NADPH, but not with NADH. Interestingly, NADH did not act as an inhibitor when NADPH was used as a substrate. At pH 8.0 and a fixed concentration of 0.2 mM *p*-1,4 benzoquinone, the apparent kinetic parameters were: $^{app}k_{cat}$ = 2.6 ± 0.4 s^{-1}, $^{app}K_m$ =130 ± 25 μM, and $^{app}(k_{cat}/K_m)$ = 20,000 ± 3,000 M^{-1} s^{-1}. Current results will be presented and discussed in the poster.

This study was supported in part by grant CHE1506518 from the NSF (G.G.) and an LSAMP Fellowship (E.F.).

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Identifying unique structural properties which promote flavin transfer in two-component FMN-dependent enzymes.

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Sulfur is essential to maintain normal function in bacteria; however, sulfur is often limiting in the environment. When environmental sulfur is scarce, a set of proteins are expressed allowing bacteria to acquire inorganic sulfate from various organosulfate molecules. Two of the expressed proteins (SsuD and SsuE) comprise the alkane sulfonate monooxygenase system. The alkanesulfonate system is a FMN-dependent two-component enzyme system comprised of a flavin reductase (SsuE) and a monooxygenase (SsuD) capable of desulfonating C_2-C_{10} alkanesulfonates in an effort to produce sulfite for incorporation into biomolecules [1]. Utilizing reduced flavin supplied by SsuE, SsuD activates dioxygen forming a C4a-(hydro)peoxyflavin intermediate which catalyzes the desulfonation of a wide range of organosulfonates ultimately producing inorganic sulfite and a subsequent aldehyde. Monooxygenases can acquire flavin through diffusion or through a channeling mechanism which promotes protein-protein interactions. The SsuE enzyme transfers reduce flavin to SsuD through protein-protein interactions; although diffusion can occur under less optimal conditions [2]. One argument that supports flavin transfer by a diffusion mechanism is monooxygenase enzymes can acquire reduced flavin from FMN-reductases that are not dedicated to the system. However, structural similarities exist between SsuE and other sulfur induced two-component flavin reductases that may explain this observation.

The alkanesulfonate monooxygenase system is one of several identified two-component systems activated during sulfur starvation in bacteria [3]. *Pseudomonas sp.* contain two FMN-reducatases (SfnE and MsuE) which supply reduced flavin to their partner enzymes: dimethylsulfone and methanesulfinate/methanesulfonate monooxygenases. These enzymes encompass a series of enzymatic reactions that converts dimethyl sulfide (DMS) to inorganic sulfite [4, 5]. Sequence comparisons between the two flavin reductases (SfnE and MsuE) yield ~ 30% amino acid identity with SsuE, suggesting structural features could play a role in flavin transfer. Identifying possible protein-protein interactions within these two-component systems may provide insight into common structural features needed for the nonspecific transfer of reduced flavin.

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Mechanistic Studies of Tetracycline Monooxygenase

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Tetracycline is an antibiotic that is widely used to combat a variety of bacterial infections. The recent emergence of tetracycline resistant pathogens, threatens its continued use in the clinic. Tetracycline monooxygenase (TetX; E.C. 1.14.13.231) is an enzyme that has been identified as a major source of antibiotic resistance. The long term goal of the proposed research is to deduce the chemical mechanism of TetX in order to rationally design inhibitors of the enzyme to be used in conjugation with tetracycline to treat bacterial infections. The plasmid pET28 harboring the *tetX* gene encoding for TetX has been used to transform *E. coli* BL21(DE3) and trials to optimize the expression of *N*-terminal His_6-tagged enzyme are currently underway. Once these trials are complete the enzyme will be studied using a variety of techniques. Steady state kinetic analysis of the enzyme will be conducted using a Clark-type oxygen electrode. This will allow for the determination of the steady-state kinetic mechanism of the enzyme, which will be studied by varying the concentrations of both tetracycline and oxygen. The kinetic parameters will be measured as a function of pH to determine possible roles of ionizable groups in the catalytic mechanism of the enzyme. Finally, site directed mutagenesis will be carried out based on the published structure of the enzyme in order to elucidate the roles of specific amino acid residues in binding and catalysis by the enzyme. While the studies are at the preliminary stages, this presentation is aimed at demonstrating the training that primarily undergraduate students will receive in the fields of biochemistry, microbiology, molecular biology and biophysics.

Expanding the utility of Amine Dehydrogenases by modulating substrate affinity and specificity

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The novel amine dehydrogenase (AmDH) has been recently developed and characterized[1]. Through protein engineering of the amino acid dehydrogenase scaffold, the amine dehydrogenase now catalyzes the asymmetric reductive amination of prochiral ketones to chiral amines. Further protein engineering to the phenylalanine amine dehydrogenase (F-AmDH) to expand the binding pocket has led to an increased specific activity toward its model substrate, para fluoro phenyl acetone (pFPA). F-AmDH also operates in the opposite direction to convert (R)-amines to ketones through oxidative deamination. In recently submitted work, this functionality was successfully applied to racemic mixtures of amines to isolate the (S)-amine. We report progress on two fronts, substrate affinity and breadth of specificity.

F-AmDH exhibits a very low affinity for ammonia (K_M: 650 mM), often requiring the enzyme to perform with molar quantities of ammonia for reductive amination. Such ammonia concentrations can lead to undesirable side reactions. The primary goal of the present work is to produce an AmDH with milder operating conditions by decreasing the K_M of ammonia. Detailed initial velocity studies are conducted with F-AmDH and its parent phenylalanine dehydrogenase (PheDH) from B. badius to gain mechanistic insight for these enzymes. Comparison of the kinetic mechanisms of AmDH and its parent enzyme will help to determine the best path for protein engineering. Also investigated is the role, if any, of non-enzymatic formation of an imine intermediate before access to the active site of PheDH and AmDH.

Regarding substrate specificity, we expanded the substrate specificity of the AmDHs towards relevant intermediates to pharmaceuticals. These include methyl ketones, diketones, and beta-keto nitriles. Previously published AmDHs along with a library of promising variants were tested against a range of potential substrates to identify activity using a spectrophotometric assay.

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Ligand binding studies of a plasmid encoded dihydrofolate reductase by ^{19}F NMR

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Plasmid encoded-R67 dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate. R67 DHFR is a homotetramer with a single active site pore and two tryptophans per monomer (W38 and W45). W38 and its symmetry-related partners occur at the dimer-dimer interfaces while the W45 residues occur at the monomer-monomer interfaces. We have labeled these tryptophans using ^{19}F-labeled indole with the ^{19}F atoms at different positions (4-, 5-, 6- or 7-) of the indole ring. *In vitro* ligand binding studies of $NADP^+$ to the apoprotein or a ternary complex with $NADP^+$ and DHF showed characteristic spectra for each complex. The apoprotein gave rise to a sharp and a broad peak. Upon addition of $NADP^+$, the sharp peak for W38 shows line broadening while the broad peak for W45 remains unchanged. In the ternary complex, the sharp peak in the apo and binary complex splits into three peaks while the broad peak remains. The appearance of three new resonances can be explained by how NADPH and DHF bind in the active site pore. Two symmetry related Lysine 32s at the edge of one side of the pore constrain the position of NADPH by forming ionic interactions with the phosphate group. However, on the other side of the pore, the glutamate tail of DHF is disordered and, switches between ion pairs with the K32s on that side of the pore. This results in two different environments for the nearby W38residue. Our results are consistent with NMR and computational simulations of the glutamate-tail interacting with symmetry related lysine 32 residues at the edge of the pore.

Overexpressing Methyl-Coenzyme M reductase (Mcr) on another archaea: A way to study the catalytic mechanism of Mcr

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Methane is an important compound. It is the main component of natural. It is also a potent and the second most abundant greenhouse gas after CO_2. Methane is attractive in biofuel industries, either being an end product of microbial fermentation or the starting point in engineering methane to biofuel pathways. Methanogenic archaea are the only known microorganisms that are capable of producing methane during their energy metabolism. Methanotrophic archaea anaerobically oxidases methane. Methyl-coenzyme M reductase (Mcr) is the key enzyme in both the anaerobic production and oxidation of methane. Mcr catalyzes the reversible reaction of methyl coenzyme M and coenzyme B to a mixed disulfide of coenzyme M and coenzyme B and methane. In the active site of Mcr, a unique nickel-containing tetrapyrrole, Factor 430 (F_{430}) is present. The nickel center could be found in three different oxidations states; 1^+, 2^+ and 3^+. Only Ni(I) form, named Mcr_{red1} state is catalytically active and is extremely unstable. The catalytic mechanism of the enzyme is not well understood. Site directed mutation could decipher some of the mystery in the mechanism. Recombinant Mcr from *Methanococcus okinawensis* (Mcr_{ok}) was successfully expressed in the host *Methanococcus maripaludis*. The recombinant enzyme was purified and characterized. Interestingly two distinct fractions of Mcr_{ok} were obtained. One fraction contains an additional subunit McrD, but not cofactor $F_{430,}$ while the other fraction has cofactor F_{430} and only the expected subunits McrABG. McrD could play a role in folding and F_{430} insertion to the apo-Mcr.

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Reversible Inhibition and Irreversible Inactivation of Bicupin Oxalate Oxidase in the Presence of Hydrogen Peroxide

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Kinetic investigations employing membrane inlet mass spectrometry (MIMS) and HPLC demonstrate that hydrogen peroxide is both a reversible noncompetitive inhibitor and an irreversible inactivator of the CsOxOx catalyzed oxidation of oxalate. The build-up of the turnover-generated hydrogen peroxide product leads to the inactivation of the enzyme. The introduction of catalase to reaction mixtures protects the enzyme from inactivation allowing reactions to proceed to completion. Circular dichroism spectra indicate that no changes in global protein structure take place in the presence of hydrogen peroxide. Oxalate oxidase is a manganese containing enzyme that catalyzes the oxidation of oxalate to carbon dioxide in a reaction that is coupled with the reduction of oxygen to hydrogen peroxide. Oxalate oxidase has potential applications in pancreatic cancer treatment [1], to prevent scaling in paper pulping [2], and in biofuel cells [3]. Oxalate oxidase from *Ceriporiopsis subvermispora* (CsOxOx) is the first fungal and bicupin enzyme identified that catalyzes this reaction [4]. We apply a MIMS assay to directly measure initial rates of carbon dioxide formation and oxygen consumption in the presence and absence of hydrogen peroxide. The MIMS method of measuring oxalate oxidase activity involves continuous, real-time direct detection of oxygen consumption and carbon dioxide production from the ion currents of their respective mass peaks. $^{13}C_2$-oxalate was used to allow for accurate detection of $^{13}CO_2$ (m/z 45) despite the presence of adventitious $^{12}CO_2$ [5].

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Analysis of binding interfaces between CCHF and polymeric ubiquitin substrates

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Crimean-Congo Hemorrhagic fever (CCHF) virus is a negative-sense, single-stranded RNA nairovirus that affects humans causing fever, prostration, and severe hemorrhages. CCHF has a fatality rate from 5-70% depending on viral strain, transmission-route, and available treatment. CCHF contains a viral ovarian tumor domain (vOTU) protease that plays a major role in viral proliferation by facilitation of innate immune response evasion. vOTUs are able to down regulate innate immune response by removing the post-translational modifications, ubiquitin (Ub) and Ub-like interferon stimulated gene product 15 (ISG15) from host proteins. Ubiquitin has been shown to form polymeric ubiquitin (poly-Ub) chains through an enzymatic process that joins the C-terminus at the lysine of another ubiquitin molecule. Some of these polymeric ubiquitin chains have been studied extensively and have well defined functions. The conjugation of a protein to K63-linked poly-Ub has an activation effect on immune response. In order to gain insight into which CCHF residues are involved in poly-Ub chain binding for deubiquitination, an enzymatically inactive form of CCHF was expressed with isotopic labeling for NMR analysis. K63 di-ubiquitin was titrated into ^{15}N-labeled CCHF samples at various concentration for ^{15}N-HSQC peaks shift analysis, amino acids corresponding to these peaks were identified revealing the binding interface between CCHF and K63 di-ubiquitin.

Exploring the Role of a Cysteine Gatekeeper in Cysteine Dioxygenase

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Cysteine dioxygenase (CDO) is an iron-dependent enzyme that catalyzes the oxidation of L-cysteine to L-cysteine sulfinic acid. Adjacent to the iron center is a thioether crosslink between a Cys (Cys93) residue and a Tyr (Tyr157) residue. Since the crosslink has been shown to increase catalytic activity by 5-fold compared to non-crosslinked CDO, the crosslink is proposed to assist in positioning the Cys substrate to coordinate the iron center. Located ~8 Å away from the iron center is a conserved Cys (Cys164) residue in the opening to the active site. Cys164 does not participate in any intramolecular disulfide bonds and exists as a free thiol. A set of proteins in bacteria have been identified as CDO homologs and contain either an Arg or a Met residue at a comparable position as Cys164 in mammals. Substitution of Cys164 resulted in decreased catalytic activity compared to wild-type CDO.[1] Therefore, it has been speculated that Cys164 could be contributing to the substrate specificity of CDO. Other studies have shown that Cys164 is involved in a disulfide bond with a free Cys in three-dimensional structures. Since cysteine has the ability to undergo oxidative modifications and serve as a redox switch, Cys164 may become oxidized as a regulatory response.[(2,3)] However, these hypotheses have not been adequately evaluated.

Three variants of Cys164 (C164A, C164S, and C164R CDO) were constructed and purified. Metal analysis showed an iron content of 100% for each variant compared to 39% iron for wild-type CDO. Despite the increased iron content evaluated in each of the variants, the catalytic activity of C164A, C164S, and C164R CDO displayed a decrease in activity compared to wild-type CDO. Following purification, C164A and C164S CDO existed as heterogeneous mixtures of non-crosslinked and crosslinked isoforms; however, C164R CDO was predominantly in the non-crosslinked isoform. Crosslink formation studies showed that C164A CDO could generate the fully crosslinked species at increased Cys substrate concentrations similar to wild-type CDO. Conversely, C164S and C164R CDO could not form the homogenous crosslinked species. Although Cys164 is not in the active site, these studies suggest that Cys164 likely plays a key role in both crosslink formation and Cys substrate oxidation. Since Cys164 is located at the opening of the active site, it may regulate accessibility of the Cys substrate to the active site under specified conditions.

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A Cavity-Filling Substitution Increases Flexibility of the NAD^+-Binding Domain in hUGDH

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UDP-glucuronic acid is the substrate for glucuronidation during drug metabolism, a process that often reduces drug efficacy and can produce toxic glucuronide derivatives. Thus, controlling glucuronidation is an important clinical goal. The NAD^+ dependent enzyme UDP-glucose 6-dehydrogenase (E.C. 1.1.1.22) oxidizes UDP-glucose to UDP-glucuronic acid. The human enzyme (hUGDH) is allosterically inhibited by the downstream metabolite UDP-xylose, which induces an isomerization from a 32 symmetry hexamer (E) to a lower symmetry hexamer (E^{Ω}). This isomerization requires tightly packed core residues to move, which is facilitated by cavities throughout the protein. The A225L substitution was designed to fill a cavity in the E state that is occupied by D280 in the E^{Ω} state; this would destabilize the E^{Ω} state and prevent isomerization when UDP-xylose binds. However, the crystal structure of the A225L E^{Ω} state is essentially identical to wild type. In contrast, the crystal structure of the A225L E state reveals a disordered NAD^+-binding domain, suggesting the A225L substitution increases flexibility. Consistent with this hypothesis, $hUGDH_{A225L}$ is more sensitive to trypsin digestion than wild type hUGDH. The NAD^+-binding domain is also part of the hexamer building interface, thus increased flexibility could explain the complete absence of hexamer in sedimentation velocity studies of the substituted enzyme. Kinetic analysis revealed a significant increase in NAD^+ K_M with little change in the UDP-glucose K_M or UDP-xylose K_I. While the original design of the substitution was to prevent UDP-xylose binding induced isomerization, this study has shown that cavity filling substitutions can destabilize hUGDH.

Structure and Function Studies of the Polymerase Complex in Vesicular Stomatitis Virus

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Negative sense single strand RNA viruses (NSRVs) contain some of the most deadly pathogens known to mankind—e.g. Ebola and Influenza viruses. Interestingly, for NSRVs to carry out transcription and replication, the viral polymerase must recognize the genomic RNA sequestered within the nucleocapsid. In order to study this fascinating mode of transcription and replication, Vesicular Stomatitis Virus (VSV) was chosen as the prototype. While various structures of the nucleocapsid (N-RNA) complex have been solved, the interaction between the native genomic RNA and the N protein is poorly understood. Incorporation of a native RNA sequence in the nucleocapsid protein allows us to crystallographically pinpoint relevant binding patterns and structural interactions in the protein-RNA complex. Furthermore, the structure of VSVs polymerase (L-protein) was recently solved by Cryo-EM to 3.8 Å. The structure of the L protein provides the basis for dissecting interactions between the viral polymerase and the nucleocapsid template to construct the active transcription/replication complex. Our goal is to uncover how the viral polymerase can recognize the sequestered RNA sequence to initiate transcription/replication

Crystal Structure and Functional Analysis of ClbQ, an Unusual Intermediate-Releasing Thioesterase from the Colibactin Biosynthetic Pathway

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Small molecule microbial secondary metabolites by regulating host-microbe interactions play an important role in all aspects of disease etiology and treatment. Colibactin is a secondary metabolite linked to the progression and pathogenesis of colorectal cancer (CRC) and inflammatory bowel disease (IBD) by inducing DNA damage in host cells. The chemical details of the colibactin and the biosynthetic pathway are emerging but clearly are unusual and noncanonical. Our research addresses a key aspect of colibactin biosynthesis, the occurrence of multiple metabolites and the biosynthetic rationale for this. Recent studies suggest an atypical role of ClbQ, a type II editing thioesterase in releasing pathway intermediates from the assembly line (1) and genetic deletion of ClbQ has been shown to abolish colibactin cytotoxic activity (2). Presented is an interdisciplinary approach to address the role of ClbQ, using enzyme structure, organic synthesis of substrates/intermediates and mechanistic analysis. The 2.0 Å crystal structure and biochemical characterization of ClbQ reveal that ClbQ exhibits greater catalytic efficiency toward acyl-thioester substrates as compared to precolibactin intermediates and does not discriminate between carrier proteins in the pathway. As reported in earlier studies (1), late-stage cyclized intermediates are not the preferred substrates for ClbQ. However, late-stage linear precolibactin intermediates are hydrolyzed. Our data, combined with previous reports, support a novel role of ClbQ in facilitating the promiscuous offloading of premature precolibactin metabolites and suggest novel insights into colibactin biosynthesis.

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Evolutionary Role(s) of the π-helix in FMN Reductase of the Methanesulfonate Monooxygenase System

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Two-component flavin-dependent monooxygenase systems are comprised of a flavin reductase enzyme which supplies the associated monooxygenase enzyme with reduced flavin. The monooxygenase enzyme then utilize the reduced flavin to activate dioxygen, and insert an oxygen atom into the corresponding substrate(s). A key catalytic event in two-component flavin-dependent systems is the transfer of reduced flavin from the flavin reductase to the associated monooxygenase enzymes. Certain structural properties have been identified in flavin reductases which facilitate flavin reduction and transfer to the monooxygenase enzymes. These structural features include π-helices and oligomeric state changes which have been shown to play a functional role in flavin reductases of two-component flavin-dependent monooxygenase systems.(1, 2) The π-helix initially identified in SsuE (the flavin reductases associated with the alkanesulfonate monooxygenase system) is highly conserved in other NAD(P)H: flavin reductases of two-component systems. The π-helices are characterized by a single amino acid insertion in an already established α-helix, and are attributed to enhancement of enzyme function.(3)

The π-helices in the flavin reductases of two-component systems were shown to have evolved through insertion of a Tyr residue in a α-helix associated with canonical flavoproteins. (1, 2) A conserved His residue was recently identified in a similar position as Tyr in the two-component flavin reductases MsuE and SnfE. The SnfE enzyme supplies SnfG with reduced flavin in the conversion of $DMSO_2$ to methanesulfinate, and MsuE supplies reduced flavin to both MsuC and MsuD resulting in the desulfonation of methanesulfinate. The sulfite is then incorporated in sulfur containing biomolecules. Although π-helices confer evolutionary advantages in proteins, they remain underexplored in most proteins including the flavin reductase two-component systems. Both sfnE and msuE enzymes have been expressed and purified to evaluate the role of π-helices in these flavin reductases of two-component monooxygenase systems involved in sulfur acquisition. Results from kinetic assays indicate that the wild-type MsuE (flavin free as purified) utilizes FMN but shows a substrate preference for NADH over NADPH. We have generated His variants to investigate the role of the π-helix in flavin reduction and transfer by MsuE.

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On the Generality of Extensive Orbital Alignments in Enzyme/Ligand Complexes

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Some of the most basic mysteries of enzyme structure and reactivity remain unsolved. As scientists, of course, we treat mysteries by their very nature as holding clues to a solution. We have been studying the shared detailed geometry of trypsin and subtilisin active sites for some time. Previously, we pointed out extensive orbital alignments within and between key strands of serine protease/protein inhibitor complexes (1). We discussed their possible roles in reactivity as well as kinetic evidence of through-strand electronics in a non-enzymatic reaction.

If such orbital alignments facilitate the electronics of rate-determining steps, and if they emerge by mutation and natural selection, then we might expect them to evolve in many systems. This presentation reviews key aspects of our work on trypsin and subtilisin, it will outline our ongoing analyses of HIV-1 protease and HEW lysozyme, and it will argue that extensive orbital alignments make a connection between an enzyme's active site and its global dynamics and energy. Figure 1 shows some orbital alignments in a glycosylated HEW lysozyme mutant.

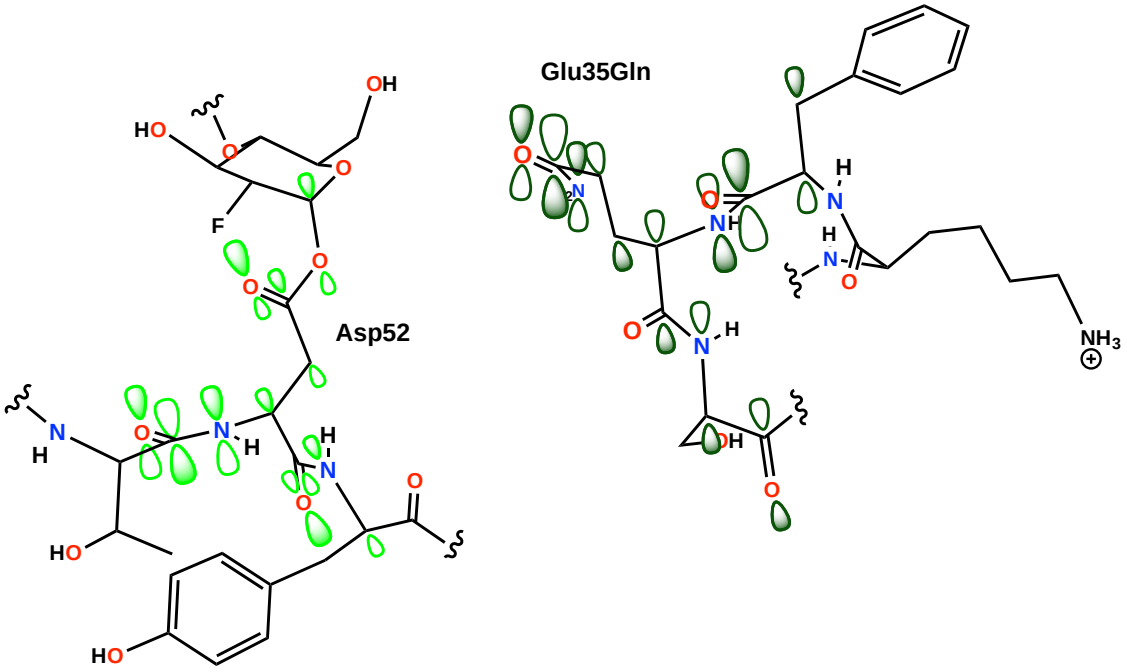

Figure 1. Extensive orbital alignments in the active site of a glycosylated HEW lysozyme mutant, from a crystal structure (PDB 1H6M) by Withers et al. (2).

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Genotype-Phenotype Correlations for POMGNTs in Congenital Muscular Dystrophy

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Congenital muscular dystrophy (CMD) is a heterogeneous family of inherited muscle disorders. A subtype of CMD known as dystroglycanopathy is classified by hypoglycosylation of alpha-dystroglycan (α-DG), which arises from defects in the protein *O*-mannosylation pathway. Two enzymes involved in that pathway are POMGNT1 and POMGNT2. Mutations in the genes encoding either enzyme have been observed in patients with various dystroglycanopathies. Our work examines the role of the R311G, R605H, P303L, and D556N mutations in POMGNT1 and the R157H, G412V, R445*, and P253L mutations in POMGNT2 to define a genotype-phenotype correlation. All but one of the POMGNT2 mutations translate to Walker Warburg Syndrome (WWS), the severest dystroglycanopathy. However, the P253L mutation manifests as high CKemia, a much milder phenotype. The POMGNT1 mutations R311G, R605H, and P303L cause muscle-eye-brain disease (MEB), which is similar to WWS but usually has a better prognosis. The D556N mutation causes limb-girdle muscular dystrophy (LGMD), which results in muscle wasting but lacks neurological comorbidities. The effects of those mutations on enzyme characteristics are not well established. Therefore, we sought to identify mutation-derived changes in enzyme kinetics and stability. To do that, HEK293F cells were transfected with mutant plasmids generated by QuikChange II Site-Directed mutagenesis. Preliminary data indicated that the R311G, R605H, and D556N POMGNT1 mutants maintained enzyme expression, but the R157H, G412V, and R445* POMGNT2 mutants did not. Radiolabel transfer assays established that the two MEB mutants were kinetically dead while the LGMD mutant still exhibited transfer. Promega's UDP-Glo^{TM} assay was performed on the LGMD mutant, which demonstrated reduced kinetic activity. A SYPRO Orange thermal shift assay revealed the aforementioned POMGNT1 mutants to be thermodynamically stable. We are currently experimenting with the P303L POMGNT1 and P253L POMGNT2 mutants and are also investigating the ability of all mutants to rescue POMGNT1 and POMGNT2 knockout cell lines. Understanding genotype-phenotype correlations in those glycosyltransferases will facilitate the design of more targeted treatments for individuals based on the mutation(s) they carry.

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A Comparison of the Catalytic Activity of Tryptophan Synthase from Salmonella and Photobacterium at Various Temperatures and Pressures

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The purpose of this experiment is to determine the effect of various temperatures and pressures on activity of samples of the enzyme tryptophan synthase isolated from two different organisms, Salmonella typhimurium (PEBA) and photobacterium profundum (Ppro). This significance of this research study lies in its capacity to enlighten our understanding of the structure and function of tryptophan synthase, which synthesizes an amino acid invaluable to the proteins and biochemical pathways essential for the sustenance of all living organisms.

With increasing temperature, the catalytic properties of enzymes typically increase. Thus, it is hypothesized that Salmonella tryp. syn. activity will be higher at increased temperatures. However, since photobacterium profundum optimally grows in the cold depths of the sea (5000m), it is hypothesized that the optimal temperature range for the Ppro tryptophan synthase enzyme will be lower than expected.

Regarding the correlation between catalytic activity and pressure, it is hypothesized that the optimal pressure for maximum enzyme activity should approximate the homeostatic pressure within the organism. Salmonella tryp. syn. should perform well at standard atmospheric pressures. Because photobacterium is acclimated to extreme depths, the ppro enzyme has been hypothesized to maintain functionality at higher pressures, while maintaining sound function at normal atmospheric pressure.

To conduct the experiment, first a sufficient amount of cells were cultivated from the cell culture. After cultivation, autoclaving, and sonication, the proteins were then purified through a phenyl sepharose column. A solution containing PLP, indole, serine, water, and a buffer was inserted in a spectrophotometer with the added enzyme, and the absorbance was measured over a ten minute period to ascertain the enzyme's activity.

Results at this time for the enzymes from both organisms are congruent with those hypothesized. Tryp. Syn. activity peaks at the climate conditions that its organism is acclimated to.

Development of an Expression System for the Investigation of Cobalamin-dependent Radical *S*-Adenosyl-L-methionine Enzymes Involved in Bacteriochlorophyll *c* Biosynthesis

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Green sulfur bacteria (GSB) produce a specialized photopigment, bacteriochlorophyll (Bchl) *c*, which enables them to grow phototrophically under extreme low-light intensity conditions. Bchl *c* has several unique structural features that allow it to self-assemble into the large nanostructures that comprise the highly efficient light-harvesting complex of GSB, the chlorosome. The biosynthetic pathway of Bchl *c* has yet to be fully characterized. Based on genetic information, three key reactions in Bchl *c* biosynthesis are thought to be catalyzed by homologous enzymes (BchE, BchQ, and BchR), which are annotated as cobalamin (B_{12})-dependent members of the radical *S*-adenosyl-L-methionine (SAM) superfamily. BchE catalyzes anaerobic O atom insertion and formation of the isocyclic ring of Bchl *c*. In contrast, BchQ and BchR are unusual methyltransferases capable of methylating unactivated C-H bonds. Specifically, BchQ transfers up to 3 methyl groups to the C8 ethyl group of Bchl *c*, while BchR catalyzes monomethylation of the C-12 methyl group. BchQ and BchR are expressed in a soluble form in the heterologous host *Escherichia coli* BL21(DE3), but the proteins are not purified in the *holo* form, even when grown in the presence of exogenous B_{12} and additional iron/sulfide. An expression system was therefore developed that simultaneously enhances the intracellular concentration of B_{12} and aids in the reconstitution of iron-sulfur clusters that can be used for the production of any cobalamin- and/or iron-sulfur cluster-dependent enzyme.

Structural Analysis of 6-OH-FAD in the Y249F Variant of D-Arginine Dehydrogenase from *Pseudomonas aeruginosa*

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Flavin-dependent enzymes are involved in a wide array of reactions usually found to contain FMN or FAD as a yellow chromophore. D-arginine dehydrogenase from *Pseudomonas aeruginosa* (PaDADH; E.C. 1.4.99.6) is an FAD-dependent enzyme that catalyzes the conversion of D-arginine to iminoarginine. Purification of a mutant variant of PaDADH in which tyrosine at 249 position is replaced with phenylalanine yielded two distinct protein fractions: one with FAD which was enzymatically active, and an unusual green fraction with a modified FAD, which was unreactive[1]. The modified green cofactor extracted from the Y249F enzyme variant was established as a FAD hydroxylated at the 6 position by various techniques i.e. NMR, mass spectrometry, high-performance liquid chromatography, and UV−visible absorption spectroscopy.

Here we report the X-ray crystal structure(s) of the Y249F variant of PaDADH to a resolution of 1.29 Å. The PaDADH Y249F variant was crystallized with 6-OH-FAD and an unexpected covalent N(5) flavin adduct with the product keto-arginine in the active site. Also obtained were crystal structures of the Y249F variants of PaDADH with 6-OH-FAD or FAD in the absence of substrate at 1.55 Å and 1.32 Å respectively. The PaDADH Y249F structure(s) shares several features with the structure of the wild-type enzyme[2] along with some key differences. As a first step towards elucidating the mechanism of the 6-hydroxylation, the Y249F PaDADH with FAD was incubated with D-alanine and the formation of 6-OH-FAD was then confirmed spectroscopically. The presence of 6-OH-FAD in the Y249F variant speaks to the significance of tyrosine 249 and the role it plays in maintaining the integrity of the flavin in the active site of PaDADH.

This work was supported in part by grant CHE1506518 from the NSF

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HmuT in the Heme Uptake Pathway of *Corynebacterium diphtheriae:* Stability and Function

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Abstract

Strategies to use heme as a source of iron are key to the survival and virulence of many bacteria; inhibition of iron uptake pathways may be a new strategy to prevent bacterial infection. *Corynebacterium diphtheriae* is a Gram-positive, pathogenic bacterium that is the causative agent of diphtheria It utilizes proteins in heme uptake pathways to obtain required iron for survival and virulence. One uptake pathway involves an ABC-type transporter encoded by the hmuTUV genes. We analyze the role of HmuT, the protein that donates heme to the ABC transporter. We hypothesize that certain residues in the heme pocket, in addition to the direct heme binding ligands, control heme binding and release in HmuT. Sequence alignment with other heme-binding proteins and I-TASSER homology modeling revealed the following possible essential residues: H136, Y235, Y272, Y349, R237 and M292. Site-directed mutagenesis was used to create alanine mutants for these residues. Mutants studied include H136A, Y235A, Y272A, and M292A. UV-visible spectroscopy was used to compare spectral signatures of the WT to the mutants. In addition, chemical and thermal unfolding experiments were performed to assess the contribution of each residue to heme binding. We have shown that H136 and Y235 are axial ligands to the heme while M292 appears to buttress the axial tyrosine. R237 is a H-bonding partner to Y235. Y272, and Y349 in the heme pocket as well, also affects heme binding. Understanding heme proteins create a possible new strategy to prevent bacterial infection by inhibiting iron uptake pathways.

Expression of Protein from Histidine Decarboxylase in Enterobacter Aerogenes

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Keywords: Histidine Decarboxylase, *E. aerogenes*, pLATE11, PCR, gel electrophoresis, transformation, sequencing,

ABSTRACT

The *HDC* gene was used to express protein. The expression processed through multiple steps of experimental procedures due to the time consumed through incubation and growth of the cells. The experimental materials were temperature and time sensitive. The prepared mixtures, such as primers, buffers, and template DNA, had to be kept on ice to transfer into sterile tubes. The pipette tips and PCR tubes were sterile when they were used for growth of cells. If not, there is a possibility of contamination or mutation. The first step is to design primers according to the sequences shown from *HDC* (1). The PCR reaction amplified the DNA with the primer then used for gel electrophoresis in order to collect the DNA from the band shown under UV light. After plasmid preparation, the mixture was processed to transformation (2). The mixture was confirmed by sequencing, which led to further procedure. If not, the experiment had to be repeated to observe expected sequence. The cells were transformed again after sequencing. Through sonication, the protein was extracted after elongated centrifugation. However, after positive results through qualitative observations, the protein did not show absorbance above 1.5. The absorbance was recorded approximately from 0.2-0.8. The obtained protein with buffer was about 5 mL. Therefore, the expected absorbance should be above 3.0. The growth of the cells in the beginning could have been altered from 37°C to 30°C because the cells were grown more efficiently at a lower temperature. The protein in the experiment was very minimal, which is the reason of a low absorbance.

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Detecting Hydrogens and Deuteriums in high resolution X-ray crystal structures of HIV-1 Protease

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Neutron crystallography of HIV protease in complex with the clinical inhibitor amprenavir has provided detailed insights into the hydrogen bonding interactions and water structure (1). We have solved, refined and analyzed the X-ray crystal structures of the same complex with perdeuterated and hydrogenated HIV protease and investigated the visibility of H/D atoms in X-ray diffraction data.

A perdeuterated HIV-1 protease complexed with amprenavir was refined to 1.08 Å resolution and compared with a 1.03 Å hydrogenated counterpart. No significant overall structural changes can be seen in the perdeuterated structure helping to confirm the validity of joint X-ray/Neutron refinement. Peaks in the difference density were observed at positions corresponding to H or D atoms. These peaks were enriched on main chain heavy atoms as well as in well-ordered motifs and hydrogen bond interactions. More hydrogen peaks were cataloged in the protonated structure than in the deuterated model and they are also associated with heavy atoms of relatively lower B-values.

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The role of solvent effect on the ligand-binding properties of the thermostable variants of Aminoglycoside Nucleotidyltransferase 4' (ANT4)

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The aminoglycoside nucleotidyltransferase 4' (ANT) is a homodimeric enzyme that detoxifies a large number of aminoglycoside antibiotics by nucleotidylating at the C4'-OH site. Two thermostable variants for this enzyme show only a single amino acid changes in their primary amino acid sequences (T130K, D80Y). It is not known how single residue replacements, which are distant from active site and monomer-monomer interface, result in various degrees of changes on thermostability of the enzyme. Thermodynamic parameters of the binary enzyme–aminoglycoside complexes, however, show highly significant differences. The data, acquired in H_2O and D_2O in this work by isothermal titration calorimetry also demonstrate that solvent reorganization upon ligand binding show large differences between the two variants. The heat capacity change (ΔCp) also show antibiotic-dependent differences between the two variants. Thus, data shown in this work suggest that thermodynamics of ligand-protein interactions and solvent effects may be among the molecular parameters that separate thermophilic proteins from simply those that are thermostable but otherwise identical to the mesophilic counterparts.

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Characterization and Relative Activity of Site-Specific DHFR-AuNP Bioconjugates: Examining the Contribution of Loop Motions to Catalysis

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Although the contribution of protein motions to enzymatic catalysis has been heavily studied both experimentally and computationally, direct experimental evidence reporting on the role of protein dynamics in catalysis is lacking. We seek to understand the dynamic motions in enzymes relating to catalysis. To interrogate this, dihydrofolate reductase (DHFR) is conjugated to gold nanoparticles (AuNPs) via site-specific bioconjugation of a cysteine residue to the AuNP. Activity of DHFR is then monitored as a function of the attachment site on the protein to the AuNP. The specific attachment sites include both the flexible FG loop and a rigid alpha helix. The resulting protein-nanoparticle conjugates are thoroughly characterized via UV/Vis spectrophotometry, Dynamic Light Scattering, and SDS-PAGE. A novel methodology for the determination of protein concentration on the AuNPs will be presented herein. Our results indicate that DHFR remains active on the AuNP surface. We also report on the location-specific turnover effects of AuNP attachment. Specifically, attachment of the FG loop of DHFR to the AuNP yields a more substantial reduction in activity in comparison to attachment of DHFR via the rigid alpha helix, demonstrating the significance of dynamics in enzyme catalysis. This work emphasizes the importance of thoroughly studying the dynamic motions of enzymes relating to catalysis and has been achieved using bioconjugation of DHFR to AuNPs, demonstrating a useful methodology for determining the role in the motions of proteins.

Directing off-pathway protein oxidation to preserve enzyme activity: at last a role for the proximal tryptophan of KatG

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Catalase-peroxidase (KatG) strongly resembles cytochrome c peroxidase, and contains many of the same active site residues, including the proximal tryptophan necessary for cytochrome c peroxidase activity. These two enzymes, though both capable of peroxidase activity, exhibit three orders of magnitude difference in catalase activity[1]. This striking difference in function given similar protein structure begs investigation, particularly of the proximal tryptophan (W321 in KatG). When *wt* KatG is reacted with H_2O_2, a catalytically essential radical centered on the KatG-unique Met-Tyr-Trp covalent adduct gives way to an exchange-coupled signal attributed to W321 at the time H_2O_2 consumption ceases. This suggests that W321 is part of a route for off-pathway electron transfer and KatG catalase inactivation. Consistent with this, a W321F KatG variant revealed higher initial rates of activity, seeming to indicate that the proximal Trp detracts from KatG catalase turnover. However, on further investigation we have observed that the increased initial rate of W321F catalase activity coincides with more rapid enzyme inactivation. In addition, W321F catalase inactivation could not be recovered by the inclusion of a peroxidatic electron donor (PxED) to the same extent as *wt* KatG, indicating that electron transfer through a route other than W321 disables the enzyme from accessing mechanisms to recover catalase activity. Turnover-dependent inactivation in conjunction with a time-dependent decay of PxED-recoverable activity suggests that KatG utilizes a hole-hopping mechanism very similar to cytochrome c peroxidase[2]. However, in KatG it serves to produce a synergistic peroxidase-based mechanism to maintain KatG's unique and robust catalase activity.

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INSIGHTS INTO A LOW PROMISCUOUS AMINOGLYCOSIDE MODIFYING ENZYME, AMINOGLYCOSIDE *N3* ACETYLTRANSFERASE-VIA

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Aminoglycoside modifying enzymes (AGMEs) are plasmid-encoded enzymes found in resistant bacteria. AGMEs covalently modify their substrates and thus render the drug ineffective for the bacteria. Aminoglycoside (AG) antibiotics are bactericidal agents used to treat various bacterial diseases like tuberculosis and meningitis. However, the clinical effectiveness of these drugs has been drastically affected by the emergence of AGMEs. More than 50 different AGMEs are known, having variable levels of substrate promiscuity. However, no correlation has been confirmatively observed between the sequence or structure of an AGME and its substrate profile. We aim to understand the molecular principles underlying this ligand selectivity by deciphering the thermodynamic, structural and dynamic properties of enzyme-ligand complexes.

Kinetic, thermodynamic and structural properties of the aminoglycoside *N3* acetyltransferase VIa (AAC-VIa) are described. Despite having significant sequence similarity to highly promiscuous acetyltransferase, AAC-VIa can modify only 5 aminoglycosides, with a ~4-fold difference in the k_{cat} values. Thermodynamic studies determined the binding of ligands to be enthalpically driven and entropically unfavorable. Unlike other AGMEs, the formation of binary and ternary complexes was accompanied by a net deprotonation of the enzyme, ligand or both. Another significant difference was observed in the structure of AAC-VIa and other AGMEs in solution. Analytical ultracentrifugation (AUC) studies showed that AAC-VIa exists in a monomer-dimer equilibrium, with more dimeric form appearing with increasing concentrations of the enzyme. Binding of ligands drive the enzyme to a more monomeric form. Also, dimer formation is achieved mainly through polar interactions. Crystal structures of different complexes of the enzyme showed that structures of apo-and ligand-bound forms were identical which suggests that, unlike other AGMEs, more rigid structure of AAC-VIa may limit the active site to accommodate only few selected aminoglycosides, hence low substrate promiscuity.

Crystal structures Phosphofructokinases family members: Structural variation based on enzyme functions

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Phosphopfructokinases (Pfk-B) is large kinase family with different enzymatic functions, phosphorylates variety of sugar/non sugar substrate molecules, crucial five carbon ribose to six carbon fructose or 2-Keto-3-deoxygluconate and also nucleosides adenosine. The entire Pfk-B family members are classified based on main "GAGD" motif at ATP binding region. The sub-families are classified based on "GG" and "NxxE" motifs at the substrate-ATP binding interface. Even though PfkB family members shares common two-three domains structural organization, they activates diverse metabolic products by phosphorylation with interesting structural and biochemical properties. Several Pfk-B family genes from pathogenic and non-pathogenic organisms were cloned, expressed, purified, crystallized and solved the structure using ***S***ingle/***M***ultiple wavelength **A**namolous **D**ispersion method (SAD/MAD) and **M**olecular **R**eplacement (MR) method. Crystal structures of several Pfk-B members shows variations at the substrate binding pocket to accommodate different molecular structural scaffolds of substrates keeping overall three 3D fold similar (PDB id: 3Q1Y, 3HIC, 3JUL, 3IE7, 3KD6, 3K5W*etc*). Also, there were significant secondary structural variations results in various oligo-merization states and domain flexibility/moments during the substrate and cofactor binding. The structural functional relationships and evolution of PfkB family members in to various sub-family levels were discussed.

The thermodynamics of metal and substrate binding to taurine/α-ketoglutarate-dependent oxygenase (TauD) from *E. coli*

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Taurine/α-ketoglutarate (αKG) dioxygenase (TauD) is a nonheme iron(II) and αKG dependent metalloenzyme, which catalyzes the hydroxylation of taurine leading to its decomposition into aminoacetaldehyde and sulfite, where sulfite is a key sulfur containing metabolite in *E. coli*. The nonheme iron(II) center in TauD is formed from two histidine side chain residues and a glutamic acid coordinating to one face of the octahedral coordination geometry. This common metal binding motif has been termed the 2-His-1-carboxylate facial triad and is found in a number of nonheme manganese, iron, and cobalt containing proteins. Here we have focused our efforts to measure the thermodynamic driving forces that lead to formation of these bioinorganic centers in biology, by studying divalent metal ion coordination to TauD using isothermal titration calorimetry. Titrations of metal complexes into the metal-free (apo) TauD and the corresponding chelation experiments were performed under anaerobic environment.

The thermodynamic terms associated with cobalt(II), iron(II), and manganese(II) binding to apoTauD were deconvoluted from complex experiments, where the pH and buffer independent binding constant (K) were measured to be 2.9×10^9, 2.4×10^7, and 9.8×10^5, respectively. (The corresponding ΔG values were calculated to be -11.2 kcal/mol, -10.1 kcal/mol, and -7.1 kcal/mol, respectively.) Interestingly the measured enthalpy changes for these binding events (ΔH) are -12.7 kcal/mol, -12.8 kcal/mol, and -13.9 kcal/mol, respectively. These data are fully consistent with the Irving-Williams series, which suggest there is increasing affinity for transition metal ions from left to right across the periodic table. However, it seems this the increasing affinity is derived from increasing favorability of both the related ΔH and ΔS terms.

Structural elucidation of 5-deoxyribulose-1-phosphate aldolase in 5-deoxyribose metabolism pathway

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Radical S-adenosylmethionine (SAM) enzymes utilize a [4Fe−4S] cluster and SAM to initiate a diverse set of radical reactions, in most of cases via generation of a 5′-deoxyadenosyl radical (dAdo•) intermediate[1]. In the catalytic cycle of radical SAM, active intermediate dAdo• abstracts a hydrogen atom and 5'-deoxyadenosine (DOA) is generated as a byproduct. The accumulation of DOA can lead to inhibitory of radical SAM enzymes themselves. The metabolism of DOA is known to proceed through 5-deoxyribose via nucleosidase. However, the fate of 5-deoxyribose is still unclear. We propose a pathway for disposal of 5-deoxyribose, which proceeds through kinase, isomerase and aldolase. Here, we describe in detail the structure of metal-dependent aldolase, catalyzing the conversion of 5-deoxyribulose-1-phosphate into DHAP and acetaldehyde. Aldolase protein crystals diffracted in space group $P4_12$, and the structure was determined using the L-fuculose-1-phosphate aldolase[2] from *Streptococcus pneumoniae* (PDB entry 4C24, 53% sequence identity) as a starting model for molecular replacement. The final model was refined to 1.55 Å resolution with one monomer per asymmetric unit. The structure of aldolase is composed of a six-stranded antiparallel β-sheets core enfolded by six α-helices of varying length. The active site of aldolase located on the surface of enzyme with a metal atom coordinated with His95, His97, His157 and Glu76. We calculated different angles in coordination, it shows a trigonal pyramidal shape, which is corresponding to the coordination of Manganese (II) ion. EPR result shows a relatively strong Manganese (II) signal in the ~3,500G range. The aldolase crystal structure revealed a novel active site architecture containing a Mn^{2+} ion. This work identifies a unique aldolase for dispatching a ubiquitous and unwanted metabolic byproduct.

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Elucidate the Biosynthesis of Nucleoside Moiety in Albomycin

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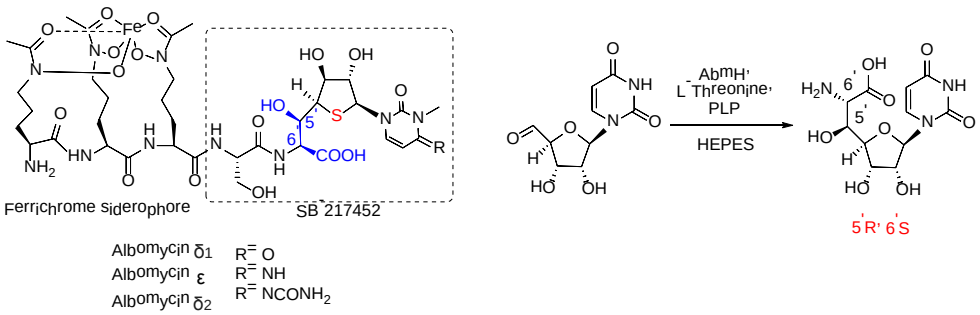
Albomycins are broad-spectrum antibiotics isolated from soil-dwelling actinomycetes. The albomycins have a minimum inhibitory concentration (MIC) as low as 10 ng/mL against *Streptococcus pneumonia.* Studies revealed that albomycins are Trojan horse antibiotics that consist of a siderphore component that is indiscriminately taken up by bacteria as an iron source. Once inside the cell, the albomycins are hydrolyzed to release a nucleoside compound SB-217452, which works as an enzyme inhibitor of bacterial seryl-tRNA synthetase.

Structurally different from other nucleoside antibiotics such as A-90289, caprazamycin, and muraymycin, the nucleoside moiety of albomycin has two features: 1) the stereo configuration of 5'-C-glycyluridine (GlyU) in albomycin is (5'R, 6'S), which is different from (5'S, 6'S) in the other nucleoside antibiotics. 2) A sulfur atom replaced the oxygen atom on the pentose ring in albomycin. Gene cluster analyzing indicated that AbmH, a homologue of LipK, is responsible for the incorporation of glycine moiety to the uridine aldehyde. LipK was functionally characterized as a L-threonine:uridine-5'-aldehyde transaldolase, which catalyzes the C-C bond-forming during the biosynthesis of the GlyU in A-90289.

Further characterization of AbmH *in vitro* found that it covalent bonded a pyridoxal-5'-phosphate as cofactor. AbmH catalyzed an aldo-type reaction to incorporate the glycine moiety on L-threonine to uridine aldehyde to form the GlyU. The product GlyU was confirmed to have (5'R, 6'S) stereo configuration, same as the structure in albomycin. Different substrates test showed that L-*allo*-threonine could also be used as a substrate in reaction.

Support for this work was provided by NIH NIAID R01 AI087849.



The Conservation of Allostery in *C. Elegans* UDP-Glucose Dehydrogenase

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The human enzyme UDP-Glucose Dehydrogenase (hUGDH) catalyzes the NAD+-dependent reaction of UDP-Glucose to UDP-Glucuronic acid[1,2]. The product is essential to glucuronidation, a detoxification pathway of drugs in the human body[3]. This process enhances the rate at which drugs are excreted, and leads to many drugs failing clinical trials. hUGDH catalysis is dependent on its hexameric structure, which is allosterically regulated by a downstream feedback inhibitor, UDP-Xylose. The binding of UDP-Xylose in the active site induces the formation of a distinct inactive conformation of the enzyme through the translation of a conserved allosteric switch[4]. Identifying ways to inhibit UGDH by studying the evolution of this switch, and the cavities it occupies, could provide ways to influence glucuronidation. Bioinformatics studies identified C. elegans UGDH (cUGDH) to be one of the more divergent versions of UGDH that still conserve the allosteric switch. Structural studies with cUGDH show that UDP-Xylose induces the same conformational change in the enzyme. Sedimentation velocity studies show that UDP-Xylose stabilizes the cUGDH hexamer in solution. These results suggest that the allosteric mechanism is preserved between the divergent enzymes. However, steady-state kinetic studies show that the affinity for UDP-Xylose in cUGDH is reduced by more than an order of magnitude. Future studies will aim at solving which structural changes in the functional allosteric switch causes a lesser affinity in cUGDH. I am currently refining the X-Ray crystallographic data of UDP-Xylose-bound cUGDH, and plan to overlay the refined structure to discover any observable differences.

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Characterization of a thermophilic N-hydroxylating monooxygenase

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Microbial N-hydroxylating monooxygenases (NMOs) catalyze the addition of a hydroxyl group to aliphatic amines (Equation 1)[1]. This reaction is a key step for some non-ribosomal peptide synthesis pathways, including the production of antibiotics[3] and small Fe-chelating compounds called siderophores[2].

$$Substrate - NH_2 + NAD(P)H + O_2 \xrightarrow{NMO,FAD} Substrate - NHOH + NAD(P)^+ + H_2O \quad (1)$$

Although the mechanism of action of certain NMOs has been studied in detail,[4] no NMO from thermophilic source has been described before. *Thermocrispum agrestre* is a thermophilic actinomycete[5] that contains a NMO, known as TheA. Multiple sequence alignment show that TheA has 49.8% identity with KtzI from *Kutsneria* sp. 744 and 37.8% identity with SidA from *Aspergillus fumigatus*, two well characterized NMOs. This work presents the characterization of *TheA*, as a model to study thermophilic NMOs. TheA was expressed in *E. coli* and successfully purified with FAD bound. Steady state kinetics was performed by following the NADPH oxidation, oxygen consumption and product formation. TheA has a higher affinity to NADPH over NADH and higher k_{cat} value with NADPH. When analysing the steady state kinetics using oxygen consumption and product formation, the preferred substrate appears to be L-ornithine. Although the enzyme is able to bind to L-lysine, it cannot catalyze the hydroxylation reaction with this substrate. This work represents the first step of the biochemical characterization of TheA and further studies such as the thermal profile, pre-steady state kinetics and structural determination must be done.

This work was supported by grants from the National Science Foundation (NSF) CHE-1506206 and MCB-1021384 and funding from Consejo Nacional para Investigaciones Científicas y Tecnológicas de Costa Rica and Ministerio de Ciencia Tecnología y Telecomunicaciones de Costa Rica.

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A conserved active site residue destabilizes WT apo-protein by 30 °C and favours metal ion binding.

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The active sites of Fe-containing SOD and Mn-containing SOD (FeSOD and MnSOD respectively) are organized around a single redox-active ion, Fe or Mn, which alternates between its 3+ and 2+ states during turnover. The proteins are homologues, and some members of the family support activity with either Fe or Mn (Fe&MnSODs), while others bind either metal ion but display activity with only one. A second-sphere Gln/His residue is the most commonly conserved difference between FeSODs and MnSODs, which supply the Gln from postion 69 or 146, respectively. Mutation of Gln69 of E. coli FeSOD to Glu or His produced large changes in the E° and catalytic activity indicating that the residue at position 69 has a large influence on the affinity for at least one of the Fe oxidation states (1). Analogous studies of MnSOD have found that replacement of Gln146 results in changes in activity and redox tuning, but the Q146E-MnSOD mutant has evaded study because it fails to bind Mn. We have compared the stability and metal ion binding of a series of mutants of the MnSOD protein wherein different amino acids occupy position 146. These studies shed light on the relationship between metal ion binding and protein stability in this series of related variants of MnSOD. In particular, Q146E-apoMn-SOD displays a melting temperature elevated over that of WT-apoMn-SOD by 30 °C, and other mutations produce intermediate behaviour. Thus the WT apoMn-SOD is less thermally stable than many of the variants. We propose that the WT protein conserves a destabilizing amino acid at position 146 as part of a strategy for favouring metal ion binding, as well as redox tuning.

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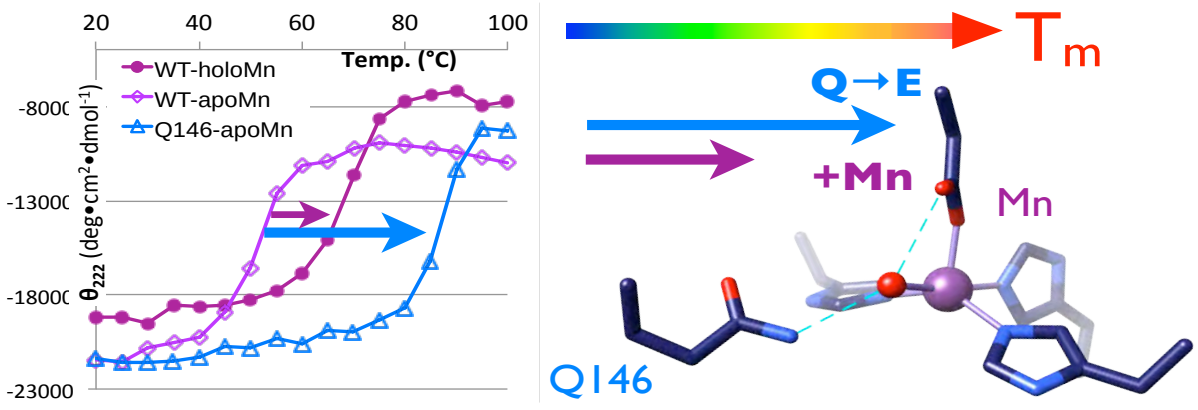

Conformational Change of 2-(2' Hydroxyphenyl)benzenesulfinate Desulfinase During Catalysis

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Increasingly stringent regulation of sulfur oxide emissions and environmental stewardship necessitates more effective fossil fuel desulfurization technologies. While traditional catalytic means of desulfurization effectively remove simple sulfur compounds, more complex thiophenic molecules remain intact; these thiophenic molecules now account for the majority of sulfur emissions from liquid transportation fuels. Biodesulfurization via enzyme catalysis has the potential for highly specific, rapid thiophenic desulfurization occurring at ambient temperature and pressure. The 4-step catabolic pathway converts dibenzothiophene (DBT), a common crude oil contaminant, into the sulfur-free molecule 2-hydroxybiphenyl (2-HBP) without the disruption of carbon-carbon bonds. 2-hydroxybiphenyl desulfinase (DszB), the rate-limiting enzyme in this biocatalytic process, is capable of selectively cleaving carbon-sulfur bonds. Accordingly, fundamental understanding of the molecular mechanisms of DszB must be developed. Based on crystallographic evidence, we hypothesize that DszB undergoes an active site conformational change associated with the catalytic mechanism. Moreover, we anticipate this conformational change is responsible, in part, for enhancing product inhibition. Rhodococcus erythropolis IGTS8 DszB was recombinantly produced and purified via Escherichia coli BW25113 to test these hypotheses. Activity and the resulting conformational change of DszB in the presence of 2-HBP were tested. The activity of recombinant DszB appears comparable to the natively expressed enzyme and is inhibited via competitive binding of the product, 2-HBP. Using circular dichroism, we observed conformational changes in DszB upon introduction of product, 2-HBP.

Study of electron bifurcation in Electron transferring flavoprotein AB systems: Mutation of bifurcating flavin binding site to simplify the system

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Electron transferring flavoproteins (Etfs) are soluble enzymes containing FAD as a cofactor for transferring electrons from dehydrogenases to the membrane bound respiratory chain. Current research demonstrates the capacity for electron bifurcation in the *Pyrobaculum aerophilum EtfAB* (*Pae* EtfAB) and *Rhodopseudomonas palustris* EtfAB (*Rpal* EtfAB) systems. Electron bifurcation has been dought a third fundamental mode of energy conservation mechanism, in which endergonic and exergonic redox reactions are combined. These ETFs have two FAD molecules, in which β FAD is the proposed site of bifurcation responsible for transfer of one electron to high potential α FAD site and the other electron to the low potential ferredoxin. By performing anaerobic spectro-electrochemical titrations reduction potentials (E^o s) of individual flavin sites are emerging. Direct conversion of oxidized flavin to fully reduced flavin without accumulation of a semiquinone intermediate is tentatively assigned to bifurcating flavin site. Another approach to simplify the multi-flavin system under study, is to mutate the β FAD site. Our mutation strategy seeks to replace existing amino acids around the β FAD with larger amino acids in order to prevent flavin binding.

The Detection of Nitric Oxide by Membrane Inlet Mass Spectrometry Provides the Basis of an Assay For Nitric Oxide Synthase

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Nitric oxide synthases (NOS, EC 1.14.13.39) catalyze the oxygen and NADPH-dependent oxidation of L-arginine to citrulline and NO through two monoxygenation reactions [1]. There are three isoforms (endothelial, eNOS; neuronal, nNOS; and inducible, iNOS) that each possess different biochemical and regulatory characteristics. These enzymes are important in understanding oxidative stress, cellular signaling, and vasodilation. While numerous methods are available for the detection of NO in biological samples and for the assay of NOS activity, these remain difficult tasks as each method has its own distinct limitations and complex considerations. Methods available to assess NOS activity include the Griess reaction [2] and the measurement of the conversion of oxyhemoglobin to methemoglobin [3]. Membrane inlet mass spectrometry (MIMS) uses a semipermeable membrane as an inlet to a mass spectrometer for the measurement of the concentration of small uncharged molecules in solution [4]. MIMS has previously been applied to the detection and measurement of NO [5,6]. In this work, we update and extend the application of MIMS to the detection of NO by using a newly commercially available instrument and probe assembly and by applying this method to the assay of NOS (in both an end point assay and a direct, continuous assay) .

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Enzymatic studies on AsFMO1, a flavin dependent monooxygenase found in garlic allicin biosynthetic pathway.

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Garlic is not only one of the most popular spices in the world, but it is also known for its wide range of therapeutic, antibacterial, antifungal, antiviral, immunostimulating, antioxidant and cholesterol lowering effects (1). These benefits originate, in part, from the sulfur-containing molecule called allicin, which is also responsible for the unique odor and flavor of garlic. These unique characteristics are part of the protective mechanism of garlic designed to drive invaders away and activated only upon tissue damage. Mechanical stress triggers the enzymatic conversion of alliin stored in the cytosol into allicin, its sulfenic acid form. This conversion is catalyzed by the vacuolar enzyme alliinase (2). While allinase has been extensively studied, the pathway for the synthesis of its precursor alliin has not been characterized. This study focuses on the protein called AsFMO1, a flavin dependent monooxygenase that convert S-allyl-L-cysteine into alliin using NADPH and oxygen. AsFMO1 was expressed in *Escherichia coli* BL21(DE3) cell line using the pVP56K plasmid. The protein was expressed as a fusion protein with Maltose Binding Protein (MBP). AsFMO1 was purified with FAD bound. An HPLC assays was developed to assess activity of AsFMO1.

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A method to titrate enzyme activity *in vivo*.

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Folate (Vitamin B9) is involved in one carbon transfer reactions required for the synthesis of DNA and amino acids. Our current understanding of the folate pathway is mostly based on *in vitro* studies, which are very different from the crowded environment in the cell. *E. coli* produces osmoprotectants during times of osmotic stress. This leads to perturbation of water activity inside the cell, and an increase in macromolecular crowding. We have shown earlier that, *in vitro*, osmolytes weaken the binding of dihydrofolate to dihydrofolate reductase in the folate pathway. We hypothesize that an increased osmolyte concentration in the cell will also prevent the functioning of other folate pathway enzymes by interaction of osmolytes with the various folate redox states. In this study, we studied the effect of osmotic stress on the folate synthesizing and metabolizing enzymes such as dihydropteroate synthase (*folP*), dihydrofolate reductase (*folA*), methylenetetrahydrofolate reductase (*metF*) and serine hydroxymethyltransferase (*glyA*) *in vivo*. Studies were done with knockout and rescued strains. Protein expression in rescued strains was limited to very low levels using a P_{tet} promoter and a protein degradation tag. We can titrate the enzyme activity in the rescued strains by osmotic stress. Osmotic stress studies have indicated that the rescued strain was unable to grow in higher osmolality conditions when compared to knockout strains. We predict this is due to an increase in osmolyte concentration *in vivo* which leads to interaction of osmolytes with folate intermediates in the pathway. This is turn decreases the efficiency of the folate pathway enzyme.

Tartronic Acid and Diethyl Ketomalonate: Inhibition and Substrate Promiscuity of Bicupin Oxalate Oxidase

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Oxalate oxidase (E.C. 1.2.3.4) catalyzes the oxygen-dependent oxidation of oxalate to carbon dioxide in a reaction that is coupled with the formation of hydrogen peroxide. Oxalate oxidase from *Ceriporiopsis subvermispora* (CsOxOx) is the first bicupin enzyme identified that catalyzes this reaction [1]. Employing a spectrophotometric assay in which H_2O_2 production is coupled to the horseradish peroxidase (HRP) catalyzed oxidation 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and a membrane inlet mass spectrometer assay [2], we characterize the reaction of CsOxOx with mesoxalate (oxopropanedioic acid), tartronic acid, and diethyl ketomalonate. Analysis of kinetic data using alternative substrates provides structure activity relationship information. Oxalate oxidase is of interest for a number of applications including pulping in the paper industry [3], as a component of enzymatic biofuel cells [4], and in pancreatic cancer cell treatment [5]. These and other potential uses motivate efforts to tailor the properties of oxalate oxidase through directed evolution and/or rational design. Understanding the degree of promiscuity (or fidelity) of CsOxOx is an important endeavor as it may provide a basis for these modifications.

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Role of Loop L1 Dynamics in *Pseudomonas aeruginosa* D-Arginine Dehydrogenase

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The conformational changes of mobile loops positioned at the entrance of active sites of enzymes often participate in catalysis to control the access to the active site, to orient catalytic residues and to shield the active site from the bulk solvent.[1, 2] Previous crystallographic data on D-arginine dehydrogenase from *Pseudomonas aeruginosa* (*Pa*DADH) showed the presence of an active site loop L1 with two different peptidyl regions including residues 45-47 located at the FAD-binding site and residues 50-56 positioned at the entrance of the active site.[3] In the two peptidyl regions of loop L1, only the S45, A46 and Y53 residues adopt major conformational changes corresponding to the open (ligand-free) and closed (product-bound) conformations.[3] In the closed conformation the side chain of Y53 prevent the exit of the product from the active site whereas the side chain of A46 swings closer to the FAD and the side chain of S45 points away from the FAD.[3] In the open conformation Y53 points away from the active, while the side chain of A46 swings away from the FAD and the side chain of S45 points closer to the FAD. The alternate conformations of the S46 and A46 residues, which are not in direct contact with the substrate, have been dubbed the Ser/Ala switch.[3]

In this study, S45 was mutated to alanine and A46 to glycine. The role of the conformational change of the Ser/Ala switch in *Pa*DADH was studied through molecular dynamics, steady-state and rapid reaction kinetics techniques. Molecular dynamics of loop L1 showed higher probabilities in the S45A and A46G variant enzymes to be in the open conformation compared to the wild-type *Pa*DADH, consistent with an exposed active site to the solvent. The flavin fluorescence intensity was ~2-fold higher in the S45A and A46G variant enzymes with respect to the wild-type *Pa*DADH, with a 9 nm bathochromic shift of the emission band. The k_{cat}/K_m values with D-arginine in both variants, were ~13-fold lower in comparison with the wild-type *Pa*DADH. Moreover, a hollowed pH-profile was observed on the k_{cat} value with D-arginine consistent with restricted proton movements in catalysis. Rapid reaction kinetic data showed no saturation with the slow substrate D-leucine in the reductive half-reaction for the variant enzymes. All taken together the data indicate that the dynamics of loop L1 is important for substrate binding and catalysis in *Pa*DADH.

Support: NSF CHE-1506518 (G.G.)

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An Investigation into the Potential Dual Role of SirC in the Alternative Heme Biosynthetic Pathway of *Methanosarcina acetivorans* C2A

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The methanogenic archaeon, *Methanosarcina acetivorans* C2A, utilizes tetrapyrroles such as heme, factor III, and coenzyme F430 for energy production. Each of these tetrapyrroles contain different metal ions (iron, cobalt, and nickel, respectively), which are generally thought to be inserted into sirohydrochlorin, a common biosynthetic intermediate of each pathway, by a unique chelatase. Interestingly, the genome of *M. acetivorans* C2A encodes only two class II chelatase (CbiX) homologs, and one precorrin 2 dehydrogenase (SirC). SirC is responsible for the production of sirohydrochlorin for each of the aforementioned tetrapyrrole biosynthetic pathways, and is homologous to the N-terminal dehydrogenase/ferrochelatase domain of the trifunctional enzyme siroheme synthase (CysG), which converts uroporphyrinogen III to siroheme. Since SirC is found within the alternative heme biosynthetic (*ahb*) gene cluster, which does not contain a chelatase homolog, it is possible that SirC is responsible for both the formation and ferrochelation of sirohydrochlorin in *M. acetivorans* C2A. Here, the ability of SirC to carry out both of these reactions, and for early-stage tetrapyrrole biosynthetic intermediates to spontaneously chelate metals, is examined.

Rate Constants from Transient Kinetic Data for Multi-Step Reversible Reactions by Exact Algebraic Solutions

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Transient kinetics gives the most direct and detailed view into enzyme action available, but quickly leads to complicated math. Data collected from transient kinetics experiments are typically a signal varying with time in the form of sums of exponentials multiplied by amplitudes. The decay of the exponentials is controlled by observed rate constants – eigenvalues for the system of differential equations (rate laws) describing the mechanism. The observed rate constants/eigenvalues are composed of the intrinsic rate constants from the mechanism. Computing the rate constants from the eigenvalues/observed rate constants is an important goal because rate constants report directly on transition state structure and stabilization – but computing rate constants is often tough, especially for multi-step reversible reaction sequences.

A classic approach to analyzing reaction pathways is to vary a substrate or ligand concentration across a wide range while maintaining pseudo-first-order conditions, and plotting the observed rate constants as a function of concentration. Approximations such as the steady-state assumption (sometimes) allow rate constants to be estimated. However, doubts linger – were the approximations valid? If not, the analysis is inherently flawed. The current state-of-the-art approach eschews individual curve-fits in favor of fitting entire sets of experimental traces simultaneously to the numerical solution of the system of differential equations describing the mechanism. In wise hands, this can be a very successful approach, and is even easy with the right (expensive) software, but is also easily abused if the software is used as a black-box without the guidance of sound kinetic judgment.

A method is presented here for algebraically computing exact values of rate constants from the k_{obs}-values obtained from classic unsophisticated curve-fitting. The analysis assumes: **1.** pseudo-first-order conditions, and **2.** k_{obs}-values are available for all phases, *i.e.*, no reaction phases can be missing. Exact equations for each rate constant can be obtained from the variation of the observed rate constants (eigenvalues) with concentration of substrate by using Vieta's formulae. Vieta's formulae relate the coefficients of a polynomial to its roots. The characteristic polynomial obtained in solving the kinetic eigenvalue problem has coefficients that are functions of rate constants, and its roots (eigenvalues) are k_{obs}-values. Vieta's formulae prescribe plots of combinations of k_{obs}-values, rather than individual k_{obs}-values, as a function of substrate/ligand concentration. These plots are lines; algebra can be used to determine the rate constants from the slopes and intercepts derived from experiments. The complexity of the algebra increases with more reaction steps in the sequence, and also as the bimolecular step is moved from the first reaction of a sequence to later steps. Examples will be presented.

STM2360 is a Novel D-Lysine Decarboxylase in *Salmonella enterica* serovar Typhimurium

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STM2360 is a gene located in a small operon of unknown function in *Salmonella enterica* serovar Typhimurium. This operon also contains homologs of amino acid transporters and alanine racemase. We have expressed the recombinant enzyme of STM2360 in *E. coli* and purified the His-tagged protein by Ni-affinity chromatography. The amino acid sequence of STM2360 shows significant homology (~30%) to diaminopimelate decarboxylase (DapDC), a Fold III pyridoxal-5'-phosphate (PLP) dependent enzyme. However, this enzyme does not exhibit DapDC activity. Diaminopimelate is a meso compound, containing both D- and L-amino acid moieties. The D-amino acid binding site of DapDC proximal to the PLP is conserved in STM2360, but the sequence of STM2360 does not exhibit the conserved arginine in the distal L-amino acid binding site. Instead, this residue is replaced by a glutamate in STM2360, suggesting that the distal site would prefer to bind a cationic ammonium group. Thus, we have found that the protein coded by STM2360 has a previously unknown catalytic activity, D-lysine decarboxylase. This is only the second known PLP-dependent decarboxylase acting on a D-stereocenter, and the first to act on a D-amino acid. We have obtained crystals of D-lysine decarboxylase and have collected a data set to 1.6 Å. It is possible that this D-lysine decarboxylase activity is part of an acid stress response element in *Salmonella*. Homologs of STM2360 with high sequence identity (>80%) are found in other common enterobacteria, including species of *Klebsiella*, *Citrobacter*, *Hafnia*, as well as representatives of the Firmicutes genus *Clostridium*.

Conformational changes in multidrug transporter DrrAB: fluorescence-based approaches to study substrate binding

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The DrrAB system forms a dedicated exporter of two anticancer antibiotics, doxorubicin (Dox) and daunorubicin (Dnr), in soil bacterium *Streptomyces peucetius*. This system belongs to the ATP binding cassette (ABC) super-family of membrane proteins. DrrA is the ATP binding subunit, which forms a complex with the integral membrane protein DrrB. This system is homologous and functionally similar to P-glycoprotein, which confers multidrug resistance (MDR) in human cancer cells. Therefore, DrrAB provides an excellent model for understanding MDR. Although DrrAB forms a dedicated transporter for Dox and Dnr, it was recently shown to export multiple MDR substrates, including Dox, Hoechst33342 and eithidium bromide (1). However, kinetics of drug and nucleotide binding to this system have not been studied. Moreover, how these two proteins communicate to transduce energy of ATP hydrolysis for drug transport is not understood. In this study, fluorescence approaches, including intrinsic tryptophan (Trp) fluorescence and extrinsic probes, were used to analyze binding kinetics and conformational changes of DrrA and DrrB. Specific high affinity binding with nucleotides, including ATP, ADP and GTP, was demonstrated using Trp fluorescence quenching. Multiple drugs were also found to bind to DrrAB in a saturable manner, which exhibited variable affinities and number of binding sites. Additionally, evidence of crosstalk between DrrA and DrrB was obtained through inter-subunit conformational changes using extrinsic fluorescent probe IAANS. These findings not only confirm that the DrrAB system can bind multiple drug substrates, but they also have the potential to elucidate mechanisms for communication between DrrA and DrrB.

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Functional characterization and inhibition of the tetracycline destructases, an emerging class of antibiotic resistance flavoenzymes

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Tetracyclines are an extensively used class of broad-spectrum antibiotics that inhibit protein synthesis by blocking tRNA from binding to the ribosome. They have played an important role in both human and veterinary medicine since their discovery in the 1940s. However, the spread of antimicrobial resistance has limited their effectiveness[1]. Understanding how resistance develops, and the mechanisms by which is operates, is key to developing new therapeutics which can regain lost activity.

The tetracycline destructases are a family of nine enzymes discovered in soil functional metagenomics selections. While they are structurally similar to TetX, a previously known tetracycline resistance enzyme, they degrade tetracyclines in a novel manner[2]. Crystal structures of three representative family members showed unique substrate binding modes. *In vitro* kinetic studies were used to identify inhibitors that rescue tetracycline activity against tetracycline destructase-expressing pathogens.[3] Our results provide a roadmap for the functional characterization of tetracycline destructases and reveal mechanistic insights for inhibitor design.

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The Hypothetical Nitronate Monooxygenase PA0660 from *Pseudomonas aeruginosa* PAO1 Encodes Instead a Diaphorase

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PA0660 from *Pseudomonas aeruginosa* PAO1 is currently classified as a hypothetical nitronate monooxygenase (NMO) but no evidence at transcript or protein level is present. Six conserved motifs were identified in the protein sequence of PA0660, which are present in more than 1000 bacterial hypothetical NMOs. This study complements the two previous studies on the improvement of the gene function prediction of hypothetical NMOs in *Pseudomonas aeruginosa* (1, 2). PA0660 was purified to high levels and the enzymatic activity was investigated. Despite its annotation as a hypothetical nitronate monooxygenase, no enzymatic activity was detected with the physiological substrate, propionate 3-nitronate, or the neutral form, 3-nitropropionic acid. Absorption spectroscopy and mass spectrometry demonstrated a tightly, non-covalently bound FMN in the active site of the enzyme.

The kinetic characterization of PA0660 showed a small NAD(P)H oxidase activity, with a turnover rate of ~0.1 s^{-1} at NAD(P)H concentrations ranging from 6.25 - 100 μM and atmospheric oxygen. This indicates that the NAD(P)H oxidase activity is likely not the primary function of PA0660. In the presence of the electron acceptor DCPIP (55 μM), the enzyme was able to turnover with NADH (3 - 540 μM) or NADPH (2 - 340 μM) with $^{app}k_{cat}$ values of 9 and 12 s^{-1}, respectively. Steady-state kinetic data provided evidence of positive cooperative behavior upon NADPH binding with a Hill coefficient value close to 2, and substrate inhibition was observed at the range of NADPH concentrations used (K_i ~ 180 μM). Additionally, in the presence of NADH the reaction obeys a Michaelian model of enzyme catalysis, and no substrate inhibition was observed. Benzoquinone was also tested as a potential oxidizing substrate. PA0660 was able to efficiently turnover with benzoquinone as the oxidizing substrate and NADH and NADPH at fixed concentrations of 100 and 60 μM, with turnover rates of 19 and 55 s^{-1}, respectively. Due to the ability of using both NADH and NADPH as electron donors, PA0660 can currently be classified as a diaphorase.

This study was supported in part by grant CHE-1506518 from the NSF

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Engineering Orthogonal Pathway of Engineered E3 Ligase RNF38 by Phage Display

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RNF38, RING finger protein 38, is a RING-type E3 ligase ligase of the ubiquitin (UB) transfer cascades in the cell. The role of RNF38 cell regulation is still unknown, but one study identified an interaction of RNF38 with p53. Ubiquitin cascades *in vivo* are complex with multiple cross-reactive E1, E2, and E3 ligases transferring ubiquitin (UB) to target substrates. Ubiquitin cascades *in vivo* are complex with multiple cross-reactive E1, E2, and E3 ligases transferring ubiquitin (UB) to target substrates. Previously our lab engineered UB, E1, and E2 enzymes to enable the specific transfer of an engineer UB (xUB) through engineered E1 and E2 (xE1 and xE2). The current project focuses on generating Ring domain libraries of RNF38 for the selection of specific xE2-xRNF38 pairs that would allow the exclusive transfer of xUB to the substrate proteins of RNF38. We have generated Ring libraries of RNF38 with 4 or 5 key residues randomized at the E2 binding site. We will display the Ring library on the surface of M13 phage and select for Ring mutants relaying xUB to xE2. After we identify functional xE2-xRNF38 pairs, we will use it to assemble an orthogonal UB transfer (OUT) cascade to identify the RNF38 substrates by proteomics.

Siderophore Production and Isolation Techniques

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Siderophores are secondary metabolites that bacteria produce and secrete to scavenge iron from iron-limiting conditions. There are many applications for siderophores, such as antibiotic delivery agents, fluorescent probes, and most commonly seen in the clinic, to treat iron-overload disease in patients due to its high iron binding affinity[1]. They are transported through the cell membrane using siderophore-binding proteins that differ in their specificity. There are currently a few ways to isolate siderophores and other secondary metabolites through column purification techniques[2]. This project aims to discover a way to isolate these siderophores produced in low quantities from bacterial cultures grown in the lab. By attaching a known siderophore binding protein, FhuD2, to a Nickel Agarose column, it is possible to flow crude culture to allow specific binding of siderophores to a column, and flowing through the rest of the culture. Depending on the siderophore of interest, the protein can be expressed, isolated, and attached to the column. Ion exchange columns can further improve the purification by separating siderophores based on charge. Three siderophores have been used to show use of this purification technique, Ferrioxamine B, Acetyl Ferrioxamine B, and Succinyl Ferrioxamine B. Through Mass Spectrometry, it was shown that in crude mixtures, these three siderophores can be isolated.

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Probing the Effects of Oligomerization and Domain Swapping on the Signal Transduction within Globin Coupled Sensors

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Globin coupled sensors (GCS) are sensory proteins used by bacteria to determine the surrounding gaseous environment. The function of a GCS is determined by the output domain of the GCS, which include phosphodiesterases, kinases, and diguanylate cyclases (DGC). Diguanylate cyclase domains produce cyclic dimeric guanosine monophosphate (c-di-GMP) from guanosine triphosphate (GTP). C-di-GMP is a bacterial secondary messenger and a major regulator of biofilm formation. *Pectobacterium carotovorum ssp. carotovorum* and *Bordetella pertussis* both contain GCS proteins (*Pcc*GCS and *Bpe*GReg, respectively) with DGC output domains. Previous works has shown that oxygen binding in the globin domain regulates the output domain, but the signaling mechanism and structure of GCSs are not well characterized. Isolated globin domains from *Pcc*GCS (*Pcc*Globin) and *Bpe*GReg (*Bpe*Globin) have been characterized to assist in elucidating these gaps. The oligomeric state of PccGlobin is dimeric while *Bpe*Globin is monomeric, indicating potential oligomer binding sites in the globin domain. As full length *Pcc*GCS and *Bpe*GReg exist as different oligomeric states (dimer-tetramer-octamer and monomer-dimer-tetramer, respectively), the globin domain appears to be a major determinant of oligomerization. The globin truncations also revealed altered oxygen dissociation kinetics, as compared to *Pcc*GCS and *Bpe*GReg. Furthermore, dimerization of the globin domain has been shown to correlates with biphasic dissociation kinetics. Site-directed mutagenesis has been used to interrogate the relative roles of distal pocket hydrogen-bonding residues in stabilizing bound O_2 and contributing to each dissociation rate. Further investigation into the dimerization of the globin domain has been accomplished using domain swapping. These studies elucidated the global effects of protein oligomerization on conformation of the heme domain, and will aid in identifying key requirement for signal transduction within the globin coupled sensor family.

In Search of Prenylated Flavin: Progress Towards Isolation of Putative Prenylated FMN

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Previously we reported the soluble expression and purification of a putative flavin prenyltransferase[1-4] (HmfG) and decarboxylase (HmfF) from *Cupriavidus basilensis*[5]. Here, we report on subsequent progress and on ongoing work to isolate and characterize a yellow component from purified HmfG. This yellow component, hypothesized to be a prenylated flavin, or prenylation intermediate has thus far eluded characterization. Attempts to purify the yellow component routinely result in loss of the yellow component, which may be oxidizing or otherwise degrading to a colorless state during purification.

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Discovery of inhibitors of UDP-galactopyranose mutase from *Aspergillus fumigatus*

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Aspergillus fumigatus is an opportunistic human pathogen and the most common causative agents of allergic bronchopulmonary aspergillosis and invasive pulmonary aspergillosis. Although there are treatments for *A. fumigatus* infections, the mortality rate among immunocompromised patients is >50%. The fungal cell wall is an essential organelle required to maintain the cell integrity. It also plays an important role in primary interactions between pathogenic fungi and their hosts since it provides adhesive properties and protection against host defense mechanisms. Galactomannan and galactofuranose are the major components of the *A. fumigatus* cell wall. The enzyme UDP-galactopyranose mutase (UGM; E.C. 5.4.99.9) is a key enzyme the biosynthesis of galactofuranose. UGM is a flavoenzyme that catalyzes the conversion of UDP-galactopyranose to UDP-galactofuranose. Deletion of UGM in *A. fumigatus* causes an attenuation of virulence in a mouse model of invasive aspergillosis. Furthermore, the absence of galactofuranose results in a thinner cell wall that correlates with an increased susceptibility to several antifungal agents (1). The absence of human homologs of UGM and its importance in infection of *A. fumigatus* and human parasites such as *Leishmania major* (2) and *Brugia malayi* (3) makes it an attractive target for drug development. In this work, we present the results of a HTS assay using the previously reported ADP-TAMRA[4] fluoresce probe with a 2320 compounds library against UGM. Hit validation was performed with a series of orthogonal assays including UPLC and DSF. Natural occurring flavonoids were identified as inhibitors of AfUGM. In order to get an insight into the mechanism of inhibition, several derivatives of were also studied.

Research reported in this publication was supported by the National Institute of General Medical Science of the National Institutes of Health under award number R01GM094469

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Biosynthesis of Non-Ribosomal Peptide Beta-Lactones by Plant-Associated Pseudomonas fluorescens

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Non-ribosomal peptide synthetases (NRPSs) are multi-domain modular biosynthetic assembly lines that polymerize amino acids into a myriad of biologically active and often structurally complex molecules, including the life-saving non-ribosomal peptide (NRP) antibiotics vancomycin, daptomycin, and penicillin. To increase structural diversity NRPSs draw from the pool of 23 proteinogenic and >500 non-proteinogenic amino acids. Terminal thioesterase (TE) domains of modular NRPSs employ diverse release strategies for off-loading thioester-tethered polymeric peptides from termination modules typically via hydrolysis, aminolysis, or cyclization to provide mature antibiotics as carboxylic acids, esters, amides, lactams, and lactones, respectively. Here we report the first enzyme-mediated formation of a highly strained β-lactone ring during TE-mediated cyclization of a β-hydroxy thioester to release the antibiotic obafluorin (Obi) from an NRPS assembly line. The Obi NRPS (ObiF) contains a type I TE domain with a rare catalytic cysteine residue that plays a direct role in β-lactone ring formation. We present a detailed genetic and biochemical characterization of the entire obi β-lactone biosynthetic gene cluster in plant-associated Pseudomonas fluorescens. Our findings validate that peptides containing β-hydroxy-amino acids can be enzymatically cyclized to strained β-lactone rings and systematically incorporated into peptide scaffolds by NRPS biosynthetic assembly lines for potential applications as antimicrobial, anticancer, antiviral, and anti-obesity agents.

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Conversion of Lignin Degradation Products to Vanillin via Laccase-Functionalized Membranes

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Conversion of a model lignin degradation fragment into a value- added product using a bio-inspired membrane is demonstrated. The enzyme laccase from *Trametes versicolor* catalyzes oxidation of lignin fragments, employing O_2 as the only other required substrate. Thus, it provides an efficient, renewable route for remediating and repurposing waste streams from cellulose extraction. We now show that it can also produce a product of commercial value, by demonstrating production of the sought-after product vanillin, upon passage of a solution of model lignin fragment through a membrane functionalized with laccase. The membrane was produced by non-covalently entangling *T. versicolor* laccase within a matrix via layer-by-layer assembly on a PVDF membrane. Passage of a solution of vanillyl alcohol through the membrane under varying conditions permitted optimization of product yield and device performance. Vanillin is an essential component of foods, fragrances and personal care products, and annual demand exceeds 15,000 tons whereas only 2,000 tons are obtainable as natural vanilla. Therefore, our demonstrated ability to produce vanillin adds value to lignin waste streams. Our demonstration of lignin remediation and valuation illustrates the possibilities accessible via the immobilization of enzymes of choice on bio-inspired membranes.

Change in the rate-limiting step in UDP-galactopyrasnoe mutase with UDP-arabinopyranose

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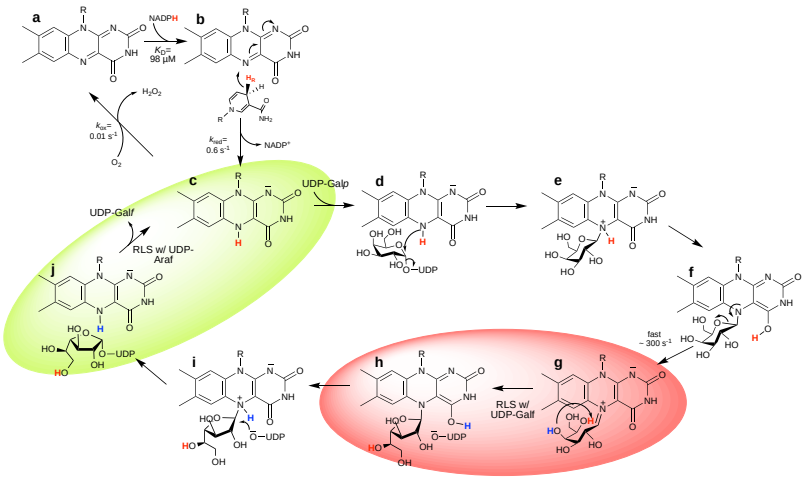
The flavoenzyme UDP-galactopyranose mutase (UGM) is a key enzyme in galactofuranose biosynthesis. UGM catalyzes the 6-to-5 ring contraction of UDP-galactopyranose to UDP-galactofuranose (1). Galactofuranose is absent in humans yet is an essential component of bacterial and fungal cell walls and a cell surface virulence factor in protozoan parasites. The UGM reaction is redox neutral, which is atypical for flavoenzymes, motivating intense examination of the chemical mechanism and structural features that tune the flavin for its unique role in catalysis. We have previously shown that the UGM reaction involves the formation of a covalent flavin-sugar intermediate (1,2,3). The reactivity of eukaryotic UGM with other substrates has not been determined. Here, we present the characterization of the reaction of UGM from *Aspergillus fumigatus* with UDP-arabinofuranose (UDP-Ara*f*) as a substrate. The UGM reaction with UDP-Ara*f* was probed using steady-state kinetics, solvent and viscosity effects, molecular dynamics simulations, mutagenesis, and x-ray crystallography. The results with UDP-Ara*f* show that the rate-limiting step of the reaction is product release, while with UDP-Gal*f* is recyclation of the ring (Scheme 1). Mutagenesis and molecular dynamics studies suggest that movement of residues in the active site limit substrate dynamics and regulate the rate-limiting step in the UGM reaction.

Supported by the NIGMS under award number R01GM094469



Scheme 1. Chemical mechanism of UDP-galactopyranose mutase. The rate-limiting step (RLS) with UDP-Gal*f* is ring contraction (shown in red). In contrast, with UDP-Ara*f* the RLS is product release (shown in green).

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Investigating the Metabolic Role of a Dioxygenase and Sulfutransferase in Bacterial MDO

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Putative CDO enzymes have been recognized in several different bacterial organisms. Bacterial enzymes typically have less than 30% amino acid sequence identity with mammalian CDO, and a Gly residue replaces the Cys residue known to play a role in crosslink formation in mammalian CDO. The bacterial CDO homologs are often divided into two subclasses recognized as CDO and MDO. Bacterial CDO has a conserved arginine residue in a similar position as Arg60 in mammalian CDO, and has alleged substrate specificity for L-cysteine. However, bacterial MDO contains a conserved glutamine residue substituted for the Arg. Several substrates including 3-mercaptopropionate, have been proposed for bacterial MDO.[1] Although 3-MPA is the most commonly proposed substrate, the environmental prevalence of 3-MPA is unclear. In various bacteria, the MDO gene is on the same operon as an annotated sulfurtransferase, but the presence of the sulfurtransferase gene has not been recognized. Sulfurtransferases and dioxygenases have been found on the same operon in other systems, and there are three proposed mechanisms in which dioxygenases utilize the product of the reaction catalyzed by sulfurtransferases. Therefore, MDO and the sulfurtransferase are metabolically linked.

The mechanistic properties of the MDO bacterial enzyme from *P. aeruginosa* (*Pa*MDO) were investigated. Results from circular dichroism revealed that the secondary structures of both wild-type CDO and *Pa*MDO were similar. Assorted thiol containing substrates were tested with *Pa*MDO, such as 3-MPA, 3-mercaptopyruvate, and 3-mercaptosuccinate. 3-Mercaptopyruvate is a common substrate for sulfurtransferases, while 3-mercaptosuccinate has been recognized as a possible substrate for MDO.[2] The k_{cat}/K_m value for *Pa*MDO with 3-MPA was approximately 2-fold lower than the catalytic efficiency for *Pa*MDO with mercaptopyruvate. However, *Pa*MDO demonstrated only nominal activity with 3-mercaptosuccinate. These studies suggest that 3-mercaptopyruvate could be a viable substrate for *Pa*MDO. Further studies will be performed to determine how the two reactions catalyzed by MDO and sulfurtransferase are coupled.

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Use of 19F NMR to probe conformational changes of arrestin protein, a group of multi-functional signaling adaptors

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In addition to their canonical function as negative regulators of G protein coupled receptors (GPCRs), arrestins, a small family of cyclic protein, serve as multi-functional adaptors by tethering several signaling components together. To elicit the appropriate cellular response, arrestins have to ensure that the right components are linked together to prevent the formation of mismatched protein complexes. In addition to more than 800 GPCRs, two non-visual arrestins can interact with dozens other protein partners. Thus, the recruitments of the signal effector proteins to arrestin should be precisely regulated. We hypothesize that binding induced conformational changes play decisive roles in regulating the recruitments of binding partners to arrestins. It is now clear that arrestin goes significant conformational movements in many regulating process. To exploit the arrestin-mediated signaling as adaptors, the structural basis of protein-protein interactions between arrestin and non-receptor binding partners is essential. The broad objective of this proposal is to explore the binding-induced conformational changes of arrestins by signaling effector proteins. The primary goal of this current proposal is developing a ^{19}F-NMR method to detect the local movements of arrestin protein. We have generated a series of single-cysteine variants of arrestin-3, in which a cysteine was re-introduced into a specific location on this protein. The active thio (-SH) functional group on cysteine is employed as a labelling site. So we are able to observe the binding-induced conformational changes of arrestin-3 upon associating with its binding partners. Here, we presenting our preliminary studies on detecting the release of C-tail from basal arrestin-3 conformation induced by inositol hexaphosphate 6 (IP-6).

Mechanistic Study on a Nitronate Monooxygenase Involving Flavosemiquinones during Catalysis

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Propionate 3-nitronate (P3N) is a natural toxin found in plants and fungi. P3N irreversibly inhibits mitochondrial succinate dehydrogenase, which leads to a variety of neurological disorders similar to Huntington's disease (1, 2). Up to date, no antidote has been discovered for P3N poisoning. Nitronate monooxygenase from *Pseudomonas aeruginosa* PAO1 (PaNMO) was the first NMO identified and characterized in bacteria. The enzyme was shown to oxidize P3N to malonic semialdehyde for energy production with FMN as cofactor (3).

Mechanistic and spectroscopic investigation on PaNMO was carried out using steady-state kinetics, pH effects, and UV-visible and time-resolved absorption spectroscopy in this study. Both neutral and anionic flavosemiquinones were observed during enzymatic turnover with P3N and dioxygen using a stopped-flow spectrophotometer equipped with a photodiode array detector. The relative amount of the neutral and anionic flavosemiquinones was dependent on pH in the PaNMO active site. A dioxygen-dependent intermediate was detected by monitoring absorbance at 300 nm during catalysis. The lifetime of the intermediate was pH-dependent. A proposed mechanism is herein presented.

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This study was supported in part by grant CHE-1506518 from the NSF (G.G.) and a Molecular Basis of Disease Fellowship from Georgia State University (S.D.).

Novel Tetracycline Inactivation Mechanisms Catalyzed by an Emerging Class of Antibiotic Resistant Flavoenzymes

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Tetracyclines are considered one of the first major antimicrobial agents to be recognized as broad spectrum antibiotics (1). The therapeutics quickly gained popularity because of their low toxicity, high efficacy, and timely discovery (2). However, the excitement surrounding the discovery of a new and highly potent natural product therapy led to widespread production and usage of the drug. As a result, tetracycline resistance mechanisms were documented within the first 5 years of clinical use (3). Through semisynthetic and total syntheses, second and third generation tetracycline derivatives with improved activity have been developed. Nonetheless, advancements in bacterial resistance mechanisms have evolved to effectively compete with synthetic progress.

Recently, a new class of tetracycline inactivating flavoenzymes known as tetracycline destructases was identified (4). Mechanistic studies of enzymatic tetracycline inactivation are needed in order to develop new strategies to manage and overcome this emerging class of resistance enzymes. We aim to determine the absolute structures of tetracycline degradation products to yield a better mechanistic understanding of destructase-mediated tetracycline inactivation. Thus far, our studies have shown that the tetracycline destructases have diverse substrate specificity and inactivate tetracyclines via unique oxidation mechanisms including monohydroxylation and Baeyer-Villiger oxidation to give a variety of degradation products. Our mechanistic studies may inspire new methods to modify tetracycline structures to evade tetracycline destructases and better control the spread of tetracycline resistance.

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Probing Heme Autoreduction of Shr-NEAT2 Domain in *Streptococcus pyogenes*

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Streptococcus pyogenes is a Gram-positive pathogenic bacterium. It belongs to group A β-hemolytic streptococcus (GAS) bacteria. This bacterium is responsible for a wide range of infections and diseases including streptococcal pharyngitis, rheumatic heart disease, impetigo, and many more diseases. For growth, GAS obtains iron from host proteins that contain heme, e.g., hemoglobin. *S. pyogenes* utilizes a direct uptake system where a donor transfers the heme to proteins expressed on the surface of the cell. Shr is one protein expressed in this system and contains two NEAT domains, Shr-NEAT1 and Shr-NEAT2 [1]. A combination of UV-visible, Raman, and magnetic circular dichroism spectroscopies were used to indicate the axial ligand set. Shr-NEAT2 is isolated as a mixture of the Fe(II) and Fe(III) forms of the protein. It is prone to autoreduction, autoreducing as the pH increases and autoxidizing as the pH decreases. We have made mutations to study its autoreduction tendencies.

This work was supported by National Institutes of Health Grants AI057877 (ZE) and AI072719−01 (KRR), and the Research Corporation (DWD). We thank Georgia State University for a Molecular Basis of Disease Fellowship for ED.

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Engineering substrate specificity of nitronate monooxygenase through docking and mutagenesis studies

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Nitronate monooxygenases (NMO; E.C. 1.13.12.16) are flavin-dependent enzymes that oxidize nitronates to aldehydes and nitrite. The physiological substrate of these enzymes, propionate 3-nitronate (P3N), is a highly toxic nitro-compound that irreversibly inhibits succinate dehydrogenase in the Krebs cycle[1]. The crystal structure of NMO from *Pseudomonas aeruginosa* PAO1 shows an (βα) 8-barrel fold, also known as TIM-barrel, which consists of eight parallel β-strands forming a central barrel, surrounded by eight α-helices. In the (βα) 8-barrel fold, active site residues are commonly located at the end of the barrel associated with the C-terminal ends of β-strands and the loops connecting these to α-helices. Due to the spatial separation of their activity and stability faces, TIM-barrels provide in general an ideal scaffold for modifying catalytic activity and engineering synthetic enzymes[2].

The protein structure analysis of NMO showed several highly conserved residues among 475 putative NMOs, i.e., Tyr^{109}, His^{133}, His^{183}, Tyr^{299}, Tyr^{303} and Trp^{325}, suggesting important roles in either substrate binding or catalysis. Flexible molecular docking of P3N in the active site of the enzyme using Auto Dock Vina revealed that three Tyr residues (Tyr^{109}, Tyr^{299}, and Tyr^{303}) in Pa-NMO might have hydrogen bonds with the carboxyl moiety of P3N. By site-directed mutagenesis, Tyr 299 was mutated to Phe and the resulting enzyme variant was purified and studied kinetically. The Y299F variant displayed significantly different kinetic parameter that was dependent on the substrate (P3N or pentyl 1-nitronate). With P3N, the $^{app}(k_{cat}/K_m)$ value for the mutated enzyme was 8-fold lower than in the wild-type enzyme. With alkyl nitronates lacking a carboxyl moiety, instead, the $^{app}(k_{cat}/K_m)$ value for pentyl 1-nitronate was 120-fold larger than in wild-type NMO. Thus, replacement of Y299 to phenylalanine in the active site of NMO altered significantly the substrate specificity of the enzyme.

This study was supported in part by grant CHE-1506518 from the NSF (G.G.).

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Structural and functional characterization of colibactin resistance gene *clbS*

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Colibactin is an uncharacterized secondary metabolite produced by certain strains of *E. coli* present in the human gut. It induces double-stranded DNA breaks in eukaryotic cells and promotes colorectal cancer and tumor formation under host inflammatory conditions.[1] The colibactin biosynthetic pathway has been studied extensively by various groups since its discovery nearly a decade ago. The biosynthetic gene cluster consists of several NRPS, PKS, hybrid NRPS/PKS, transporter and a resistance gene. The biosynthesis involves a prodrug like resistance strategy where the inactive genotoxin is transported to the periplasm, cleaved and processed to the active form. Two genes *clbM* and *clbQ* from this *pks* island have already been structurally characterized in our laboratory. The ClbM structure was recently solved and was identified as a colibactin MATE transporter.[2] Here, we present the X-ray structure of the colibactin resistance gene *clbS*. It protects the host bacteria from the genotoxic effects of colibactin. The structure was solved to 2.0 Å and reveals ClbS as a DNA-binding protein.

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Identifying the Multiple Binding Sites between Arrestin-3 and JNK3

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Arrestin is a small family of multi-functional adaptor proteins. In additional to its colonial function as negative regulators to hundreds of G protein-coupled Receptors (GPCRs), arrestins also mediate a complex second wave G protein-independent signaling network by recruiting several kinases to construct signaling cascades, but the major interaction sites of this process remain largely unknown. In this study, several computational methods are employed in an attempt to accurately predict major interaction sites between arrestin-3 and one of its binding partners, c-Jun N-terminal Kinase 3 (JNK-3). These methods utilize the Protein Frustratometer to survey the energy landscape of the protein in question to identify residues that exist in a high energy conformation, as well as the Evolutionary Trace method to identify residues that are highly conserved down the phylogenetic tree, signifying evolutionary importance. Crystal structures of both proteins are subsequently submitted to ClusPro in order to sample possible docked conformations of the two structures. Our gathered data suggests that JNK-3 favors binding on its kinase domain, with seventy-four to eighty-two percent of all docked conformations depicting binding in this location, as well as having minor sites of interaction located on the C-terminal. Arrestin-3 is shown to favor binding on a hydrophobic proline hinge region located near the N-terminal, with thirty-nine to fifty-eight percent of all docked conformations depicting binding in this area. The methods developed here can be employed to predict the binding interfaces between arrestins and other partners.

Investigation of HtaA-CR2 in Heme Uptake from *Corynebacterium diphtheriae*

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Corynebacterium diphtheriae is the causative agent of diphtheria; Iron is essential for its survival and for virulence. The pathogen can use host heme as a source of iron. Heme uptake involves an ABC hemin transporter system HmuTUV, as well as the HtaA (with two conserved heme binding domains, CR1 and CR2) and HtaB proteins. The goal of this study is initial biophysical characterization of HtaA-CR2. The protein was purified using a strep-tag affinity column. Amino acid sequence alignment suggested that there are three fully conserved amino acids that could be axial ligand(s) (Y361, H412, and Y490). The mutants of Y361A, H412A, and Y490A showed significantly low heme binding (≤ 5%). The resonance Raman data and UV-visible absorbance spectrum of HtaA-CR2 are consistent with tyrosine heme coordination. HtaA-CR2 is highly stable protein. Thermal denaturation experiments were performed in the presence of guanidinium hydrochloride (GdnHCl) (1.5 – 3 M). HtaA-CR2 unfolds very slowly; the half-life of unfolding was 5 h in the presence of 6.8 M GndHCl at 37 ^{o}C. In contrast, apo WT and the mutants unfolded very easily, indicating that the protein is highly stabilized by bound heme.

Characterization of an N-hydroxylating monooxygenase (FzmM) involved in fosfazinomycin biosynthesis in *Streptymyces* species XY332.

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Microorganisms such as *Streptomyces*, are well known for their capability to produce a variety of natural products including antibiotics, fungicides, and anti-tumor compounds (1). The biosynthesis of these compounds is typically accomplished through secondary metabolic pathways that involve novel enzymatic reactions. In the case of the biosynthesis of the antibiotic fosfazinomycin from *Streptomyces* species XY332, the formation of an N-N bond is a point of interest as this mechanism is not well-established (2). This specific pathway is unique as both nitrogen atoms are thought to originate from the amino acid L-aspartate. Previous work has alleged that the two Flavin dependent oxidoreductase enzymes identified in *Streptomyces* XY332 fosfazinomycin biosynthetic gene cluster, FzmM and FzmL, are involved in N-N bond formation (3). In particular, FzmM is involved in the first step of fosfazinomycin biosynthesis catalyzing the N-hydroxylation of L-aspartate and subsequent N-oxidation of N-hydroxy-L-aspartate to produce nitrosuccinate. The research here aims to further characterize the enzyme which was successfully expressed and purified with FAD bound. Buffers for protein stability and activity were optimized, and steady state kinetic characterization was performed by following product formation and NADPH oxidation. This work was supported by grants from the National Science Foundation (NSF) CHE-1506206 and MCB-1021384.

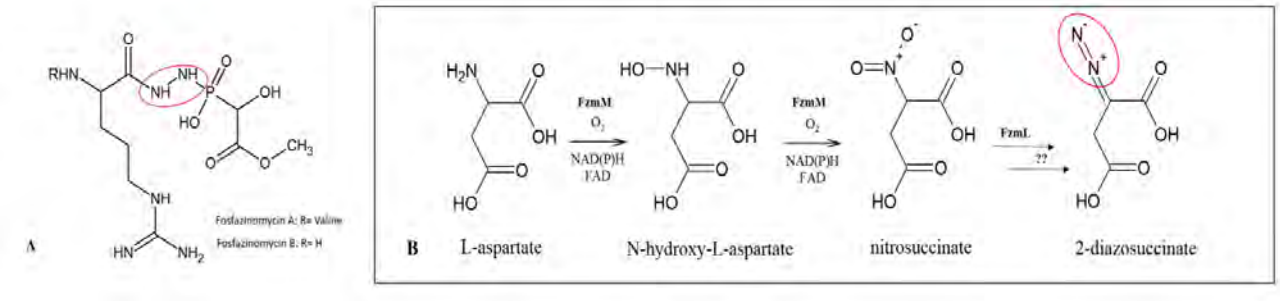

Figure 1: Structure of fosfazinomycin (A), and reactions catalyzing N-N bond formation (B) (3).

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Structural Insights into the Role of [2Fe-2S] Clusters in Bacterial Ferrochelatases

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Heme is an essential cofactor for various enzymes in organisms. It is a key compound for several biological functions such as drug and hormone metabolism and oxygen transport throughout the cell. When there is a build-up of porphyrin, the heterocyclic ring that forms the heme compound, genetic diseases known as porphyrias can occur (1). Ferrochelatase is the terminal enzyme in the heme biosynthetic pathway that inserts ferrous iron into the porphyrin ring to make the complete protoheme compound. Human ferrochelatases are known to contain an [2Fe-2S] cluster (2). Recently, several bacterial ferrochelatases have been shown to contain this cluster, as well (3,4). In addition, there are at least three different motifs that are hypothesized to coordinate the iron-sulfur cluster in these bacterial enzymes. The reason for mammalian and bacterial ferrochelatases to contain this cluster is unknown. Therefore, the goal of this project is to provide structural analysis of how the [2Fe-2S] clusters are coordinated by the various motifs in these enzymes. Techniques such as protein purification and X-ray crystallography are being actively used to produce structures of bacterial ferrochelatases and provide insight into the role of the iron-sulfur cluster. Initial crystal conditions were obtained for ferrochelatases from *Ruegeria pomeroyi*, *Caulobacter crescentus* and *Myxococcus xanthus*. Further optimization under these conditions are being conducted to obtain high quality crystals for X-ray diffraction.

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Small-molecule anti-virulence agents for the prevention of *S. mutans* biofilms

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Dental caries is considered to be a major health concern in the United States as it can result in tooth loss, infection, and in some cases, even death by sepsis. The World Health Organization reports the incidence of an alarming 42% in young children and more than 90% in adults, and caries treatment is also associated with substantial economic burden. Dental caries is an irreversible disease process- once a cavity is formed, the only remedy is to drill out the decay and fill the tooth with restorative material. So, developing drugs to prevent and control the formation of dental caries is now a high priority in American Dentistry. While dental plaque consists of more than 700 different bacterial species living in complex bacterial communities called oral biofilms, mutans streptococci, represented by *Streptococcus mutans* has been implicated as the major etiological agent in the initiation and development of dental caries. The formation of tenacious biofilms is the hallmark of *S. mutans* induced pathogenesis of dental caries. Not only can *S. mutans* form biofilms readily on the tooth surface, but this bacterium rapidly produces lactic acid from dietary sugars which lead to tooth decay. Due to the biofilms that are resistant to conventional antibiotics, current marketed therapies are not species-specific and kill pathogenic species as well as beneficial species, which are protective against the formation of pathogenic biofilms. In order to develop a species specific therapeutic agent, we have targeted the *S. mutans* glucosyltransferases (Gtfs) for our drug design. These enzymes metabolize sucrose into water insoluble and soluble glucans, which play a role in mediating irreversible attachment of *S. mutans* to the tooth and also provide an extracellular matrix, shielding the bacteria from the host immune response, and antimicrobial agents. A diverse library of small molecules identified based on the *in silico* screening against X-ray crystal structure of GtfC, one of the contributing Gtfs, was subjected to a biofilm formation inhibition assay to identify potent small molecules that inhibit *S. mutans* biofilm formation specifically. 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 concentrations.

Dissecting the roles of the enzymatic and scaffolding functions of EXO1 *in vivo*

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Activation induced cytidine deaminase (AID) generates U:G mismatches in immunoglobulin (Ig) variable-regions (V) genes during somatic hypermutation (SHM) and switch-regions (SR) during class switch recombination (CSR). Exonuclease 1 (EXO1) is essential for both processes. It has been reported that mice expressing EXO1 with the cancer associated E109K mutation (*Exo1*^{EK}) did not have the defects in SHM and CSR seen in *Exo1*^{null} mice, suggesting that the enzymatic activity of EXO1 was not required for antibody diversification. However, subsequent biochemical work revealed that the untagged full length EXO1-E109K protein retained WT exonuclease activity while and EXO1-D173A (*Exo1*^{DA}) active site mutation had negligible enzymatic activity (1, 2). We therefore examined this question by generating new mice expressing the enzymatically nonfunctional EXO1-D173A mutation (Exo1^{DA}) and comparing them to EXO1-knockout (Exo1^{null}) and WT mice. Meiosis in Exo1^{DA} and WT mice was identical while Exo1^{null} mice were sterile, suggesting that only the scaffolding role of EXO1 is essential for meiosis. Mutation accumulation in a reporter gene elsewhere in the genome was comparable between Exo1^{DA} and Exo1^{null} mice, and twice as high as the WT mice, suggesting that the enzymatic role of EXO1 is important for non-Ig canonical error free mismatch repair. Deep-sequencing of 186.2 heavy chain variable region in B cells from (4-hydroxy-3-nitrophenyl)acetyl (NP) immunized mice showed the mutation spectra of Exo1^{DA} mice was different from the Exo1^{null} mice but was not significantly different from the WT mice. This supports our previous observation in Exo1^{EK} mice that scaffolding functions of EXO1 are critical for ncMMR at the Ig variable regions of B cells, while its exonuclease activity is not required or somehow compensated for by other unknown mechanisms. In contrast, CSR efficiencies of Exo1^{DA} and Exo1^{null} mice were defective compared to WT mice, and residual switching events were slightly enriched in blunt and short SR microhomologies. This could be due to a requirement for EXO1 exonuclease activity in the resections required to create the double stranded DNA breaks. Taken together, the data suggest that EXO1 recruits other factor(s) to promote ncMMR repair in the Ig V regions during SHM, while its enzymatic role is clearly required during CSR. It will be important to identify the factor(s) that are required for the excision of AID-generated G:U mismatches in the V regions in order to elucidate how these regions are exposed to error prone repair and the introduction of A:T mutations during antibody affinity maturation.

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Ph Dependence of the Oxidative Cleavage of 2,4-Dihydroxyacetophenone (DHA) by 2,4-Dihydroxyacetophenone Dioxygenase

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Abstract

The enzyme 2,4'-dihydroxyacetophenone dioxygenase (DAD), originally identified in betaproteobacteria, catalyzes the oxidative cleavage of 2,4'-dihydroxyacetophenone (DHA) into benzoic acid and formic acid.[1,2] The nature of the cleavage has led to the proposal of a mechanism reminiscent of either the intradiol- or extradiol dioxygenases. To better characterize the components that make up the reaction, this study focuses on enzyme activity across a range of buffer pH. To determine the pH-dependence of activity, absorbance assays monitoring the conversion of DHA into benzoic acid were done at pH intervals of 0.5 from 2.5 to 10.0. Assays across the basic pH range showed both a decrease in activity and a shift in the UV spectrum of the DHA substrate. Analysis of the pH-dependence of reaction rate will be compared to the pK_a determined for the substrate to help identify the source of the pH-dependence. This information will assist in elucidating the role of pH in the DAD mechanism. The culmination of data from these experiments will be used to interpret the source of the pH-dependence.

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Acknowledgements

This project is supported by grants from the National Center for Research Resources (5 P20 RR016461) and the National Institute of General Medical Sciences (8 P20 GM103499) from the National Institutes of Health.

Distinct Differences Between Firmicute and Actinobacterial Coproheme Decarboxylases

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The coproheme decarboxylase (ChdC) enzyme catalyzes the last step of the coproporphyrin dependent heme biosynthetic pathway, which is found in Actinobacteria and Firmicutes (1, 2). To date, published data from other groups have focused on firmicute ChdC mechanism and structure (3, 4). However, amino acid sequence analysis and preliminary structural data have indicated structural differences between firmicute and actinobacterial ChdCs. ChdC decarboxylates coproheme, forming heme *b*, via an oxidative decarboxylation and requires an electron acceptor for catalysis. We have found that the electron acceptor H_2O_2, which allows activity for published firmicute ChdCs, elicits very little or no activity with actinobacterial ChdCs. Consequently, we have tested the alternate electron acceptors FMN and *Escherichia coli* ferrodoxin ($FldA_{Ec}$), which enabled activity with actinobacterial ChdC but showed very limited or no activity with the firmicute ChdC from *Bacillus subtilis*, strengthening our hypothesis.

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Major drug resistance mutation L76V redistributes van der Waals interactions at the flap-body junction of HIV-1 protease

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HIV-1 protease (PR) mutation L76V is classified as a major resistance mutation (1). L76V is associated with resistance to certain protease inhibitors (PI) but also hyper-susceptibility to others (*2*). It has been previously shown that substituting L76, an integral member of the hydrophobic core, with V76 destabilizes the protease dimer thus providing a novel mechanism of resistance (*3*). In the present research, PR with L76V (PR_{76}) was studied to further understand the molecular mechanism of resistance incurred by the single mutation. Inhibition kinetic values (Ki) were determined to measure the potency of FDA-approved antiviral drugs, lopinavir and tipranavir, as well as for investigational inhibitors. Furthermore, the structures of PR_{76} co-crystallized with each inhibitor were solved to identify any structural changes due to the mutation. For each inhibitor, PR_{76} had higher Ki values in comparison to each wild-type counterpart. Structural analyses revealed that hydrogen-bond interactions between inhibitor and PR were conserved, however, the hydrophobic core interactions pertaining 76V were different from wild-type PR. Mutation L76V may enable movement of the flexible flaps and facilitate dissociation of bound inhibitor from the active site.

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Distinct Roles for Interfacial Hydration in Site-Specific DNA Recognition by ETS-Family Transcription Factors

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The ETS family of transcription factors is a functionally heterogeneous group of gene regulators that share a structurally conserved, eponymous DNA-binding domain.(1) Unlike other ETS homologs, such as Ets-1, DNA recognition by PU.1 is highly sensitive to its osmotic environment due to excess interfacial hydration in the complex. To interrogate interfacial hydration in the two homologs, we mutated a highly conserved tyrosine residue, which is exclusively engaged in coordinating a well-defined water contact between the protein and DNA among ETS proteins, to phenylalanine. The loss of this water-mediated contact blunted the osmotic sensitivity of PU.1/DNA binding, but did not alter binding under normo-osmotic conditions, suggesting that PU.1 has evolved to maximize osmotic sensitivity. The homologous mutation in Ets-1, which was minimally sensitive to osmotic stress due to a sparsely hydrated interface, reduced DNA-binding affinity at normal osmolality but the complex became stabilized by osmotic stress. Molecular dynamics simulations of wildtype and mutant PU.1 and Ets-1 in their free and DNA-bound states, which recapitulated experimental features of the proteins, showed that abrogation of this tyrosine-mediated water contact perturbed the Ets-1/DNA complex not through disruption of interfacial hydration, but by inhibiting local dynamics induced specifically in the bound state. Thus, a configurationally identical water-mediated contact plays mechanistically distinct roles in mediating DNA recognition by structurally homologous ETS transcription factors.

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Secondary Interaction Interfaces with PCNA Control Conformational Switching of DNA Polymerase PolB from Polymerization to Editing

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Replicative DNA polymerases (Pols) frequently possess two distinct DNA processing activities – DNA synthesis (polymerization) and proofreading (3'-5' exonuclease activity). The polymerase and exonuclease reactions are performed alternately and are spatially separated in different protein domains. Thus, the growing DNA primer terminus has to undergo dynamic conformational switching between two distinct functional sites on the polymerase. Furthermore, the transition from polymerization (pol) to exonuclease (exo) mode must occur in the context of a DNA Pol holoenzyme, wherein the polymerase is physically associated with the processivity factor Proliferating Cell Nuclear Antigen (PCNA) and primer-template DNA. The mechanism of this conformational switching and the role that PCNA plays in it had remained obscure, largely due to the dynamic nature of the ternary Pol/PCNA/DNA assemblies. Here, we present computational models of the ternary assemblies for the archaeal polymerase PolB. We have combined all available structural information for the binary complexes with electron microscopy (EM) data and have refined atomistic models for the ternary PolB/PCNA/DNA assemblies in the pol and exo modes using molecular dynamics simulations. In addition to the canonical PIP-box/IDCL interface of PolB with PCNA, contact analysis of the simulation trajectories revealed new secondary binding interfaces, distinct between the pol and exo states. Using targeted molecular dynamics (TMD), we explored the conformational transition from pol to exo mode. We identified a hinge region between the thumb and palm domain of PolB that is critical for conformational switching. With the thumb domain anchored onto the PCNA surface, the neighboring palm domain executed rotational motion around the hinge, bringing the core of PolB down toward PCNA to form a new interface with the clamp. A helix from PolB containing a patch of arginine residues was involved in the binding, locking the complex in the exo-mode conformation. Together, these results provide a structural view of how the transition between the pol and exo states of PolB is coordinated through PCNA to achieve efficient proofreading.

Conformational changes of 2’-hydroxybiphenyl-2-sulfinate desulfinase

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Biodesulfurization using the 4S enzymatic pathway can effectively remove sulfur from refractory thiophenic molecules at ambient conditions. 2’-hydroxybiphenyl-2-sulfinate (HBPS) desulfinase (DszB), one of four enzymes in the 4S pathway, catalyzes the cleavage of the carbon-sulfur bond to produce 2-hydroxybiphenyl (HBP) and sulfite; this final step in the 4S pathway exhibits the slowest reaction rate, partially as a result of product inhibition. Here, we examine the effect of solvent on a large-scale conformational change, which may have implications for understanding the mechanism of product inhibition. Molecular Dynamics (MD) simulation was used to analyze how the HBPS substrate and inhibitory product, HBP, bind in DszB active site and influence protein conformational changes in two solutions: aqueous buffer and a biphasic hexane-water (0.5 vol/vol) solution. Three loops, including residues 50-60 (loop 1), 135-150 (loop 2), and 180-200 (loop 3), were identified as participating in the dominant conformational change; they are located close to active site and appear to form a “gate” that may be responsible for ligand ingress/egress. In aqueous solution, both HBPS and HBP remained bound in a stable position in the active site. HBPS-bound DszB displayed large fluctuations in a region distal to the active site (residues 220-240), and the entire protein backbone was less stable in comparison to HBP-bound DszB. Structural studies suggest the catalytic residues, Cys27, His60, and Arg70, remain close to the HBPS substrate and maintain substrate interactions; though in the HBP-bound DszB, the catalytic cysteine moved away from the active site along the simulation. In biphasic solution, HBPS-bound DszB showed improved stability in the catalytic center. Due to the high affinity of HBP for the organic phase, HBP escaped the active site to the hexane-aggregated phase at the nonpolar DszB surface region, leaving the gate open facilitating the next reaction. Umbrella sampling provided a quantitative assessment of the energetic penalty for the gate conformational change. The energy cost associated with the gate opening from the close position was 3 kcal/mol. This value was independent of bound ligand and solvent conditions.

Elucidation of the Biosynthesis Pathway for the Key Coenzyme of Methanogenesis and Anaerobic Methane Oxidation

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Methyl-coenzyme M reductase (MCR) is the key enzyme of methanogenesis and anaerobic methane oxidation (AOM). The activity of MCR is dependent on the unique nickel-containing tetrapyrrole, coenzyme F430. We used comparative genomics to identify the coenzyme F430 biosynthesis (cfb) genes and characterized the encoded enzymes. The pathway was found to involve nickelochelation of sirohydrochlorin by the first nickel-specific chelatase, amidation to form a novel tetrapyrrole, Ni-sirohydrochlorin a, c-diamide, an un-presented a 6-electron reduction/ γ -lactamization reaction by a primitive homolog of nitrogenase, and intramolecular carboxylic ring formation by a Mur ligase homolog. This study significantly advances our understanding of coenzyme F430 biosynthesis and MCR maturation, identifies new targets for inhibitors of natural greenhouse gas emissions, and sets the stage for metabolic engineering efforts utilizing MCR (*1*).

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Investigating Novel RNA and Nucleotide Metabolic Pathways in Bacterial Physiology

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Catabolism of RNA is an important process in bacterial metabolism, and regulation of nucleotide pools has been implicated in virulence processes, such as biofilm formation and motility (1,2). Employing a LC-MS/MS-based assay recently developed within the group (3), this work reports the first quantification of nucleoside 2',3'-cyclic monophosphates (2',3'-cNMPs) in *Escherichia coli*. Previous reports have linked 2',3'-cNMPs to cellular stress and damage in eukaryotic systems (4) suggesting an interesting connection with nucleotide pools. The present work demonstrates that 2',3'-cNMPs in *E. coli* arise from RNase I-catalyzed RNA degradation, presumably as part of a previously unknown nucleotide salvage pathway. Intriguingly, *E. coli* growing in a biofilm have reduced 2',3'-cNMP levels compared to planktonic bacteria. Moreover, biofilm formation and motility increase in RNase I-deficient *E. coli*, suggesting a role for RNase I and/or 2',3'-cNMPs in virulence. In addition, these nucleotides are involved in activating transcription of the bacterial rtc operon, which functions in nucleic acid repair following stress (5). To further investigate the biological functions of 2',3'-cNMPs, experiments are underway to perturb 2',3'-cNMP pools using cell-permeable analogs and a recombinant 2',3'-cyclic-nucleotide phosphodiesterase, both in the presence and absence of RNase I.

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Biochemistry as a topic of instruction in the general chemistry laboratory

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Undergraduate students enrolled in honors general chemistry laboratory are introduced to a variety of topics and techniques from inorganic, physical, organic, and analytical chemistry. Biochemistry is the only major branch of chemistry not included in freshman laboratory instruction at Auburn University. The vast majority of these students will never advance beyond organic chemistry; therefore, we implemented a new experiment so that all major disciplines of chemistry are represented to achieve an accurate portrayal of the field as a whole.

Over the course of the 2016-2017 academic year, two simplified biochemistry laboratory experiments were introduced into honors general chemistry. Maintaining the natural flow of the course, at home readings and a short pre-lab lecture was given prior to the experiments. The first experiment introduced polymerase chain reaction (PCR) to demonstrate how forensic scientist are able to replicate a single piece of DNA found at a crime scene. Replicated DNA was compared to standards and analyzed by gel electrophoresis. The second experiment introduced enzyme catalysis and function. Through spectroscopical techniques students monitored and compared the differences in product formation from a wild-type and a mutated enzyme. Surveys were administered before instruction and after experimentation to assess students' attitudes and interest in the topic of biochemistry.

How an Arginine Switch Preserves the Catalase Activity of KatG: Strategic Use of an Active Site Tryptophan for off-pathway Electron Transfer

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The catalase activity of KatG is prone to inactivaiton due to off-pathway intramolecular electron transfer. The primary route of radical transfer away from the active site appears to start at the enzyme's so-called proximal tryptophan (W321). Inclusion of a peroxidatic electron donor mitigates against the accumulation of inactive states that result from this process. All KatGs have a conformationally dynamic arginine (R418), and its position affects the electronic structure of the KatG active site. To investigate the impact of this arginine switch on off-pathway radical transfer and KatG inactivation, we produced R418N and W321F/R418N KatG and compared their properties to the wild-type enzyme. Like wild-type, the extent of O_2 production by R418N KatG indicated that enzyme inactivation occurred prior to full consumption of H_2O_2. Also similar to wild-type, R418N KatG catalase activity could be retained by inclusion of a peroxidatic electron donor such that full stoichiometric production of O_2 could be observed; however, R418N produced at least ten fold more oxidized electron donor during O_2 production. This suggested that while wild-type levels of catalase activity could be sustained with R418N, it came at the expense of a much greater extent of off-catalase electron transfer. In the absence of the proximal tryptophan ***and*** the arginine switch (i.e., W321F/R418N KatG) the extent of O_2 production was 10-fold less than R418N, suggesting greater vulnerability to peroxide-dependent inactivation. That inclusion of a peroxidatic electron donor only increased catalatic O_2 production twofold further suggested that the electron donor only had limited ability to prevent inactivation. Interestingly, W321F/R418N KatG showed peroxidase activity about two orders of magnitude greater than the wild-type enzyme, revealing an uncoupling of KatG's two major activities. Stopped-flow investigation of electron donor oxidation and the return of the enzyme's ferric state provided additional evidence of uncoupling. Our data suggest that R418 limits off-catalase electron transfer in KatG. However, even in the absence of arginine switch, radical transfer via the proximal tryptophan produces electron-donor rescue-able intermediates. In the absence of both, greater propensity toward off-catalase electron transfer is combined with a limited ability to restore the catalase-inactive intermediates that result.

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