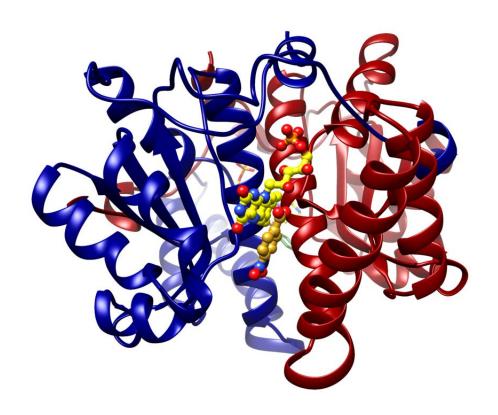
Sixth Southeast Enzyme Conference



Saturday, April 11, 2015

Georgia State University Atlanta, GA

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Sixth Southeast Enzyme Conference

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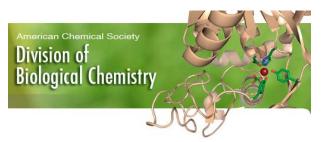














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Meeting	Year	Program Chair	Site Chair	Site
I	2010	Giovanni Gadda	Will Lovett	GSU
II	2011	Nigel Richards	Giovanni Gadda /	GSU
			Will Lovett	
III	2012	Robert Phillips	Giovanni Gadda /	GSU
			Will Lovett	
IV	2013	Holly Ellis	Giovanni Gadda /	GSU
			Neil Renfroe /	
			Will Lovett	
V	2014	Liz Howell	Giovanni Gadda /	GSU
			Will Lovett /	
			Neil Renfroe /	
			Robert Daniel	
VI	2015	Anne-Frances	Giovanni Gadda /	GSU
		Miller	Will Lovett /	
			Gwen Kenny /	
			Robert Daniel	
VII	2016			

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Schedule:

Location: Urban Life Building, Room 220:

All Talks 15 min plus Q&A up to 20 min total!

7:30-8:00 Breakfast

8:00-8:10 Welcome and Introduction – Anne-Frances Miller, University of Kentucky, Lexington

Session 1 - Chair, Ellen Moomaw, Kennesaw State University, Kennesaw

8:10-8:30 Bradley Kossmann, Georgia State University, Atlanta

Computational Discovery of Activating and Repressing States for the Liver Receptor Homologue 1 Ligand Binding Domain

8:30-8:50 Naazneen Sofeo, Iowa State University, Ames

Characterization of the Arabidopsis thaliana Acetyl-CoA Synthetase Putative Carboxylate Binding Pocket

8:50-9:10 Nicholas Keul, The University of Georgia, Athens

The Role of Intrinsic Disorder in Human UDP-Glucose Dehydrogenase

9:10-9:30 Olive Njuma, Auburn University, Auburn

Participation of the Proximal Tryptophan as a Potential Conduit for Catalase-Peroxidase Inactivation

9:30-11:00 Poster Session

Session 2 - Chair, Marcello Forconi, College of Charleston, Charleston

11:00-11:20 Heba Abdelwahab, Virginia Polytechnic Institute, Blacksburg

Drug Resistance in Nocardia farcinica: Mechanism of Rifampicin Inactivation

11:20-11:40 John Robbins, Georgia Institute of Technology, Atlanta

Unusual 8-formyl-FAD Governs Activity of Formate Oxidase (FOX) from Aspergillus oryzae

11:40-12:00 Kunhua Li, University of Florida, Gainesville

Structure and Mechanism of the Flavoprotein, FscN a Siderophore Interacting Protein from the Fuscachelin A biosynthetic Gene Cluster

12:00-12:20 Paritosh Dayal, Auburn University, Auburn

Examining the Role of Structural Dynamics for Reduced Flavin Transfer in the Alkanesulfonate Monooxygenase System

12:20-12:40 Break, Lunches Available in West Exhibit Area

12:40-1:20 Breakout Session: Lunch with NSF

Dr. Engin Serpersu, A Workshop on Grant Opportunities

1:20-2:40 Poster Session

Session 3 - Chair, Anne-Frances Miller, University of Kentucky, Lexington

2:50-3:50 J. Martin Bollinger, Pennsylvania State University, University Park

Demystifying the Chemical Magic of Non-Heme-Iron Enzymes in Natural Product Biosynthesis

3:50-4:00 Concluding Remarks – Anne-Frances Miller, University of Kentucky, Lexington

Session 1:

Ellen Moomaw Chair

Computational Discovery of Activating and Repressing states for the Liver Receptor Homologue 1 Ligand Binding Domain

Bradley Kossmann[‡], Paul Musille[#], Ivaylo Ivanov[‡] and Eric Ortlund[#]₋
Department of [‡]Chemistry, Georgia State University, Atlanta, GA 30302-4098; [#]Department of Biochemistry, Emory University, Atlanta, GA 30322

Phospholipids (PLs) are unusual signaling hormones sensed by the nuclear receptor liver receptor homologue-1 (LRH-1), which has evolved a novel allosteric pathway critical to support appropriate interaction with coregulators depending on the ligand status^(I). LRH-1 plays an important role in controlling lipid and cholesterol homeostasis and is a potential target for the treatment of metabolic and neoplastic diseases^(2, 3). While the prospect of modulating LRH-1 via small molecules is exciting, the molecular mechanism linking PL structure to transcriptional coregulator preference remains elusive. Studies show that binding to an activating PL-ligand, such as dilauroylphosphatidylcholine (DLPC) favors LRH-1s interaction with transcriptional coactivators to upregulate gene expression (4). Crystallographic and solution-based structural studies indicate that DLPC binding causes structural fluctuations outside of the canonical activation surface in an alternate activation function (AF) region, encompassing the β-sheet-H6 region of the protein. However, the mechanism by which dynamics in the alternate AF influences coregulator selectivity remains elusive. Here we study the dynamics within the LRH-1 ligand binding domain with molecular dynamics, dynamical network analysis and principal component analysis. We show that the allosteric network through LRH1's ligand binding domain encourages motions that converge on two distinct conformational clusters, one each for the activating and repressing modes of the receptor.

- 1. Musille, P. M., Pathak, M. C., Lauer, J. L., Hudson, W. H., Griffin, P. R., and Ortlund, E. A. (2012) Antidiabetic phospholipid-nuclear receptor complex reveals the mechanism for phospholipid-driven gene regulation, *Nature structural & molecular biology* 19, 532-537, S531-532.
- 2. Fayard, E., Auwerx, J., and Schoonjans, K. (2004) LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis, *Trends in cell biology 14*, 250-260.
- 3. Burridge, S. (2011) Obesity and diabetes: An antidiabetic phospholipid, *Nature reviews*. *Drug discovery 10*, 493.
- 4. Ortlund, E. A., Lee, Y., Solomon, I. H., Hager, J. M., Safi, R., Choi, Y., Guan, Z., Tripathy, A., Raetz, C. R., McDonnell, D. P., Moore, D. D., and Redinbo, M. R. (2005) Modulation of human nuclear receptor LRH-1 activity by phospholipids and SHP, *Nature structural & molecular biology* 12, 357-363.

Characterization of the *Arabidopsis thaliana* acetyl-CoA synthetase putative carboxylate binding pocket

Jason H. Hart^{1,3}, Naazneen Sofeo^{1,3}, Al Culbertson^{1,3}, Marna Y Nelson^{1,2,3}, David J. Oliver^{2,3}, Basil J. Nikolau^{1,3}

¹ Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA; ² Department of Genetics, Development and Cell Biology; ³ Engineering Research Center for Biorenewable Chemicals Iowa State University, Ames, IA 50011, US

The precursor molecule in fatty acid and polyketide biosynthesis pathways is a two carbon short chain acyl-CoA molecule. Acetyl CoA synthetase (ACS) catalyzes the activation of the acetate to acetyl-CoA by forming a thioester bond with coenzyme A while hydrolyzing ATP to AMP and pyrophosphate. The hydrophobic cavity between the N and C terminal domains of Methanothermobacter thermoautotrophicus acetyl CoA synthetase is the probable binding pocket for acetate and mutations therein affect catalytic function, enabling the enzyme to bind and activate not only acetate but also butyrate [1]. The Arabidopsis thaliana acetyl-CoA synthetase was compared to other acyl-CoA synthetases, and was computationally modeled on the available crystal structures of the Saccharomyces cerevisiae ACS1 and Salmonella enterica ACS. This allowed the identification of the residues that make up the putative carboxylate binding pocket residues. To further understand substrate selectivity and binding within the putative carboxylate binding pocket, selected residues were mutated to resemble the homologous residues in the *Pseudomonas* chlororaphis isobutyryl-CoA synthetase. We targeted four residues (Ile³²³, Thr³²⁴, Val³⁹⁹ and Trp⁴²⁷) that are proposed to form the carboxylate binding pocket, and we found that two residues, Trp⁴²⁷ and Thr³²⁴ are primarily involved in determining the carbon-chain length of acceptable carboxylate substrates. By combining two mutations (Val³⁹⁹Ala, and Trp⁴²⁷Gly) the enzyme was able to utilize butyrate with a similar catalytic efficiency as the wild-type enzyme with acetate. Additional mutations of Thr³²⁴ allowed the enzyme to utilize propionate with similar efficiency as the wild type enzyme with acetate. Therefore, these mutations created a more promiscuous acyl-CoA synthetase.

1. C. Ingram-Smith, B.I. Woods, K.S. Smith, Biochemistry 45 (2006) 11482-11490

The Role of Intrinsic Disorder in Human UDP-Glucose Dehydrogenase

Nicholas D. Keul, Renuka Kadirvelraj, Krishnadev Oruganty, Andrew M. Sidlo and Zachary A. Wood

Biochemistry & Molecular Biology, University of Georgia, Athens, GA 30602, USA

Intrinsic disorder persists with high frequency in the proteome and has been shown to have functional relevance for many enzymes^I, 2 . Human UDP- α -D-glucose-6-dehydrogenase (hUGDH) has an intrinsically disordered C-terminal tail (ID-tail) that is 30 residues in length. Here we show the ID-tail is important for the affinity of the competitive feedback inhibitor UDP-xylose. Deletion of the ID-tail (Δ C-term) reduces the affinity for UDP-xylose by 11-fold. Despite its disorder in crystal structures, fluorescence equilibrium binding studies show that unlabeled ID-tail peptide binds tightly (77 nM) to Δ C-term hUGDH. NMR analysis shows that an isotopically labeled peptide corresponding to the ID-tail is disordered in solution, but binds specifically to Δ C-term hUGDH. Finally, sedimentation velocity analysis shows that the ID-tail stabilizes the hexameric structure of hUGDH. Future studies will focus on identifying the functional unit of the ID-tail and how it interacts with the hexamer.

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- [3] Kadirvelraj, R., Custer, G. S., Keul, N. D., Sennett, N. C., Sidlo, A. M., Walsh, R. M., Jr., and Wood, Z. A. (2014) Hysteresis in human UDP-glucose dehydrogenase is due to a restrained hexameric structure that favors feedback inhibition, *Biochemistry* 53, 8043-8051.

Participation of the Proximal Tryptophan as a Potential Conduit for Catalase-Peroxidase Inactivation

Olive J. Njuma, Ian Davis*, Elizabeth N. Ndontsa, Aimin Liu*, Douglas C. Goodwin
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University, AL 36849. *Departments of Chemistry, Georgia State University, Atlanta, GA
30302-4098

Found primarily in bacteria and lower eukaryotes, catalase-peroxidases (KatGs) decompose H₂O₂ by two mechanisms. KatG bears great structural similarity to typical peroxidases like cytochrome c peroxidase. However, it is the sole member of its superfamily with appreciable catalase activity. Contrary to the prevailing paradigm which predicts mutual antagonism between KatG's two activities, we observed the stimulation of catalase activity by peroxidatic electron donors (PxEDs). A long delay between the conclusion of catalatic H₂O₂ consumption, and the return of the enzyme to it's resting (Fe^{III}) state suggested that catalase-inactive intermediates accumulated during turnover, a phenomenon likely linked to off-mechanism protein oxidation. With PxEDs included, catalatic H₂O₂ consumption and reemergence of resting enzyme occurred simultaneously, suggesting that PxEDs prevented the accumulation of inactive intermediates. We produced a KatG variant (W321F) which replaced the proximal Trp, a likely pathway for offmechanism electron transfer, with non-oxidizable Phe. This variant showed greater unassisted catalase activity than wild-type KatG and a correspondingly lesser stimulatory effect from PxEDs. For W321F KatG, the delay between the conclusion of H₂O₂ consumption and return of the Fe^{III} state was substantially shorter than observed for wild-type. Transient kinetic studies showed the same initial rates of H₂O₂ consumption by both proteins but this rate diminished more rapidly for wild-type than W321F KatG. By visible absorption and electron paramagnetic resonance (EPR) spectroscopic measurements, both wild-type and W321F KatG showed the same intermediate (Fe^{III}-O₂•[MYW]^{•+}) at 10 ms, but later, at the point of H₂O₂ depletion, a broad exchange-coupled radical was observed for wild-type but not W321F. The properties of the radical were consistent with a Fe^{IV}=O W321*+ state and would account for the subsequent accumulation of an inactive Fe^{III}-O₂. W321. and Fe^{III}-O₂. W states. W321. and W radicals were not detected in wild-type KatG when ABTS, a PxED was included. Taken together, these data suggest that PxEDs enhance KatG catalase activity by preventing the accumulation of inactive intermediates that result from off-pathway protein oxidation, and that the proximal tryptophan is part of one route for such oxidation events.

Session 2:

Marcello Forconi Chair

Drug resistance in Nocardia farcinica: Mechanism of rifampicin inactivation.

Heba Abdelwahab^{#§‡}, Pablo Sobrado^{#§⊥}

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[‡] Department of Chemistry, Damietta University, New Damietta, Egypt, 34517.

Nocardia farcinica, is a gram positive bacterium that mainly causes infectious nocardiosis of the lungs (pulmonary) or the entire body (systemic) in immunocompromised individuals. Patients can suffer pulmonary, neurological and cardiac symptoms. Antibiotics have been incorporated into a regime of long-term treatment with sulfonamides, especially in case of non-responders (1). Nocardia spp. is naturally resistant to wide varieties of antibiotics. Rifampicin monooxygenase (RifM), a Flavin dependent enzyme, catalyze the first step in rifampicin inactivation pathway. In the presence of NADPH and oxygen, RifM converts rifampicin to 2'-N-hydroxy-4-oxorifampicin (with 100 fold lower antibiotic activity) (Fig. 1) (2, 3). Here, we present the expression, purification, and characterization of the recombinant form of RifM. The rifm gene was cloned into the pET15b vector in frame for expression as N-terminal 6x Histagged protein. The protein was purified by immobilized metal ion affinity chromatography using HisTrap columns. From 60 g of cell paste, ~200 mg of purified RifM protein was obtained. The extinction coefficient at 450 nm of 10990 M⁻¹ cm⁻¹ was determined for the FAD bound to RifM. The steady-state kinetic parameters were measured by monitoring the oxygen consumption. The results show a k_{cat} value of 4.0 \pm 0.2 s^{-1} and K_m values of $125 \pm 20 \mu\text{M}$ and $10 \pm 2 \mu\text{M}$ for NADPH and rifampicin, respectively. The rate of flavin reduction and order of substrate addition were studied by stopped-flow spectrophotometry. The results are compared to the family of flavindependent monooxygenases.

Figure 1. NADPH dependent reaction catalyzed by RifM.

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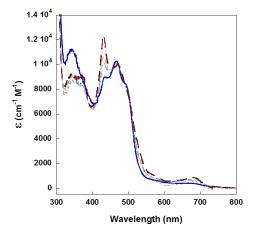
Unusual 8-formyl-FAD Governs Activity of Formate Oxidase (FOX) from Aspergillus oryzae

John M. Robbins †‡, Giovanni Gadda *||_, and Andreas S. Bommarius *†‡§

†School of Chemical and Biomolecular Engineering, †Parker H. Petit Institute for Bioengineering and
Bioscience, and §School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0100,
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Recently, formate oxidase (FOX; E.C. 1.2.3.1) from Aspergillus oryzae was identified as a member of the glucose-methanol-choline (GMC) oxidoreductase superfamily of enzymes through amino-acid sequence and three-dimensional structural analysis; FOX is the first and only reported member of the GMC enzymes capable of catalyzing the oxidation of an acid substrate. Additionally, WT FOX was shown to exhibit an unusual UV absorption spectrum that was due to a non-covalently bound 8-formyl FAD in place of the typical FAD cofactor present in most GMC oxidoreductases. Although the presence of an enzyme bound 8-formyl FMN has been reported previously as a result of site-directed mutational studies on lactate oxidase (LOX), FOX is the first reported case of 8-formyl FAD being present in a wild-type enzyme. While FAD is bound covalently through an 8α -N^{ϵ 2}-histdyl linkage has been shown to be important for catalysis in some GMC enzymes, the formation of 8-formyl-FAD in LOX has been shown previously to result in complete inactivation of the enzyme; the presence of 8-formyl-FAD in FOX was proposed to be an artifact. As a result, both the formation and potential role of the 8-formyl-FAD cofactor in formate oxidase was investigated through the use of steady-state kinetics, sitedirected mutagenesis, ICP analysis, UV and fluorescence spectrometry, LCMS, and lightexposure studies. Surprisingly, the results from these studies not only indicate that 8-formyl-FAD is present in the active form FOX but that its formation is crucial for activity.



Structure and mechanism of the flavoprotein FscN, a Siderophore Interacting Protein from the fuscachelin A biosynthetic gene cluster

Kunhua Li and Steven D. Bruner*
* Department of Chemistry, University of Florida, Gainesville, FL 32611

Iron is an essential cofactor required for a majority of organisms functioning as a global regulator for many cellular, metabolic and biosynthetic processes. Acquisition of iron is critical for microbe's survival and virulence. Microorganisms have evolved diverse strategies to import and utilize iron including the synthesis and secretion of ferric-iron specific siderophores to scavenge the environmental iron. The intracellular fate of acquired iron via the siderophore pathway is one of the least understood steps in the complex process. A common route to cellular incorporation is the single electron reduction of ferric to ferrous iron proposed to be catalyzed by specific and non-specific reducing agents. Siderophore biosynthesis gene clusters often contain one or two families of specific redox-active enzymes: the flavin-containing 'siderophoreinteracting protein' and iron/sulfur ferric siderophore reductases. Here we describe the structure and characterization of the siderophore-interacting protein, FscN from the fuscachelin1 siderophore gene cluster of T. fusca. The structure shows a flavoreductase fold with a noncovalently bound FAD cofactor along with an unexpected metal bound adjacent to the flavin site. We demonstrated that FscN is redox-active and measured the reduction and binding of ferric-fuscachelin. The presented work provides a structural basis for the activity of a siderophore-interacting protein and further insight into the complex and important process of iron utilization. Moreover, we present the structures of FscJ, a predicted type III periplasmic binding protein that is able to bind and import ferric-fuscachelin. Several X-ray structures detail ligand-free conformational changes at different pHs indicating complex interdomain flexibility of the siderophore receptors. Structure and mechanistic studies of the proteins in the T. fusca fuscachelin gene cluster provide valuable insight into the complex molecular mechanisms of siderophore recognition and transportation.

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Examining the Role of Structural Dynamics for Reduced Flavin Transfer in the Alkanesulfonate Monooxygenase System

Paritosh V. Dayal[†], Harsimran Singh[#], LauraBusenlehner[#], and Holly R. Ellis[†]
Department of Chemistry and Biochemistry, Auburn University[†], Alabama, 36849; Department of Chemistry, The
University of Alabama[#], Alabama, 35487

Alkanesulfonate monooxygenase enzymes are expressed under sulfur-limiting conditions, and catalyze the desulfonation of a wide-range of alkanesulfonate substrates. The SsuE enzyme is a NADPH-dependent FMN reductase that provides reduced flavin to the SsuD monooxygenase (1). The mechanism of reduced flavin transfer in the flavin-dependent two-component systems occurs either by free-diffusion or channeling. Previous studies have provided evidence of protein-protein interactions between SsuE and SsuD, but the identification of potential interaction sites has not been investigated (2). Current studies utilized HDX-MS to identify protected sites on SsuE and SsuD. A conserved α-helix on SsuD located near the active site cavity showed a decrease in percent deuteration when SsuE was included in the reaction. The surface of the α -helix is comprised of multiple polar amino acid residues, which may play a key role in promoting protein-protein interactions. Specific SsuD variants were generated in order to investigate the role of the α-helix in protein-protein interactions and catalysis. Variants containing substitutions at the charged residues showed a six-fold decrease in activity, while a deletion variant of SsuD lacking the α-helix showed no activity when compared to wild-type. In addition, there were no protein-protein interactions identified between SsuE and his-tagged SsuD variants in pull-down assays. Stopped-flow studies were performed to monitor the flavin reductive and oxidative half-reactions. A notable lag-phase is typically observed between flavin reduction and oxidation that was previously shown to represent flavin transfer from SsuE to SsuD. The SsuD variants showed an absence of this lag-phase due to disrupted flavin transfer. A competitive assay was devised to evaluate the mechanism of flavin transfer in the alkanesulfonate monooxygenase system (3). A variant of SsuE that interacted with SsuD but was not able to reduce FMN decreased the desulfonation activity of SsuD. The Y118A SsuE variant competes with wild-type SsuE for docking sites on SsuD, and reduced flavin transfer cannot effectively occur. These collective studies define the importance of protein-protein interactions for the efficient transfer of reduced flavin from SsuE to SsuD leading to the desulfonation of alkanesulfonates.

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Session 3:

Anne-Frances Miller Chair

Demystifying the chemical magic of non-heme-iron enzymes in natural product biosynthesis

J. Martin Bollinger, Jr.

Department of Chemistry and Department of Biochemistry and Molecular Biology, Penn State University, University Park PA 16802

Non-heme-iron (NH-Fe) enzymes activate O₂ for an array of biomedically and agriculturally important oxidation reactions. Our past decade's work has characterized iron(IV)-oxo (ferryl) complexes in several such NH-Fe enzymes. Most often, the ferryl complexes generate substrate radicals by abstracting hydrogen (H•) from aliphatic carbons, ²⁻⁶ leading to formation of new C-O, ²⁻⁴ C-Cl/Br, ⁵ or C-S bonds. ¹ Motivated by our success in rationalizing the divergent outcomes of the NH-Fe 2-(oxo)glutaratedependent aliphatic hydroxylases and halogenases, we now seek both to exploit the ferryl manifold for novel carbon-functionalization reactions⁷ and to explain the structural and mechanistic bases for several other natural reaction types, including dehydrogenation of an alcohol to epoxide, stereo-inversion of a chiral carbon, and desaturation and cleavage of C-C bonds, that are initiated by ferryl complexes in other NH-Fe enzymes. Insight obtained will inform combinatorial design of new antibiotic and anticancer drugs.

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 "Evidence for Hydrogen Abstraction from C1 of Taurine by the High-Spin 3. Fe(IV) Intermediate Detected during Oxygen Activation by Taurine:α-Keto-
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- 7.
- halogenase, SyrB2," *Proc. Natl. Acad. Sci. USA*, **2009**, 106, 17723-17728. Matthews, M.L.; Chang, W.-c.; Layne, A.P.; Miles, L.A.; Krebs, C.; Bollinger, J.M., Jr. "Direct Nitration and Azidation of Aliphatic Carbons by an Iron-dependent Halogenase," *Nat. Chem. Biol.* **2014**, *10*, 209-215. Wang, C.; Chang, W. C.; Guo, Y.; Huang, H.; Peck, S.C.; Pandelia, M.E.; Lin, G.M.; Liu, H.W.; Krebs, C.; Bollinger, J.M., Jr. "Evidence that the Fosfomycin-Producing Epoxidase, HppE, Is a Non-Heme-Iron Peroxidase," *Science* **2013**, *342*, 991-995. 8.
- Chang, W.-c.; Guo, Y.; Wang, C.; Butch, S. E.; Rosenzweig, A. C.; Boal, A. K.; Krebs, C.; Bollinger, J.M., Jr. "Mechanism of the C5 Stereoinversion Reaction in the Biosynthesis of Carbapenem Antibiotics," *Science* **2014**, *343*, 9. 1140-1143.

Abstracts for Poster Presentations:

The poster session will be held in the atrium of the Urban Life Building.

Without exceptions, the abstracts are arranged alphabetically by the last name of the first person in the list of authors.

- 1. Au, Samantha
- 2. Ball, Jacob
- 3. Bennett, Elizabeth
- 4. Bhojane, Purva P.
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- 6. Boland, Erin L.
- 7. Bullows, James
- 8. Burns, Justin L.
- 9. Lizana, Paul Cardenas
- 10. Carter, E. Kathleen
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- 12. Chen, Wei-Hung
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- 16. Duff, Michael R.
- 17. Dunkle, Jack A.
- 18. Emerson, Joseph P.
- 19. Fontaine, Benjamin M.
- 20. Forbes, Dianna L.
- 21. Fuanta, Rene
- 22. Geng, Jiafeng
- 23. Graham, Claire J.
- 24. Gross, Phillip G.
- 25. Guan, Siqi
- 26. Gumpper, Ryan
- 27. Han, Tingting
- 28. Han, Shen
- 29. Hart, Jason H.
- 30. Hu, Hao
- 31. Hudson, Will
- 32. Esaki, Shingo
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Extending the utility of the amine dehydrogenase: increased affinity and synthesis of the (S)-amine

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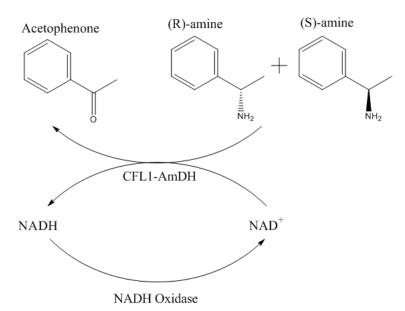
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The novel amine dehydrogenase (AmDH) has been recently developed and further characterized. Through protein engineering of the amino acid dehydrogenase scaffold, the amine dehydrogenase now catalyzes the reduction of prochiral ketones to chiral amines. 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). In addition, F-AmDH exhibited a low affinity for ammonia (K_M: 550 mM), leading the enzyme to perform in unfavorable conditions. Protein engineering of residues in the binding pocket has led to an increased affinity towards ammonia.

Previous work involving the AmDH included the development of a biphasic organic solvent system to allow for conversion of hydrophobic substrates. The F-AmDH naturally catalyzes formation of the (R)-amine. To further continue our reaction engineering work, oxidative amination in a biphasic organic solvent system has led to a successful conversion to the (S)-amine. For proof of concept, we have produced (S)-methylbenzylamine ((S)-MBA) from the racemate, catalyzed by leucine amine dehydrogenase (L-AmDH).



The Kinetic Characterization of PA1024 from *Pseudomonas aeruginosa* Establishes the Enzyme as an NADH:menadione Reductase and not a Nitronate Monooxygenase

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Due to the increasingly rapid pace of gene sequencing, the number of functional predictions for proteins far exceed experimental verification or determination of functions (1) This holds true for the nitronate monooxygenase (NMO) family of enzymes, which comprises mostly hypothetical proteins and accounts for >5000 genes in the GenBankTM. The gene product PA1024 from *Pseudomonas aeruginosa* PAO1 is an FMN-containing protein that is currently classified as a 2-nitropropane dioxygenase (2), the previous name for NMO (3). In a previous study, PA1024 was classified as NMO based on the presence of 2-nitropropane (2NP) in the crystal structure of the enzyme, gene prediction, and a qualitative enzymatic assay carried out with 2NP (3). 2NP is an alternate substrate for a limited number of NMOs (Class II), but not Class I NMO (4, 5). The physiological substrate for NMO, P3N (propionate 3-nitronate) (4, 5), was unknown at the time of the study on PA1024 and therefore was not tested as substrate for the enzyme.

In this study, we demonstrate that P3N, 2NP and other nitronates or nitroalkanes are not substrates for PA1024. The enzyme, instead, exhibits NADH oxidase and NADH:menadione reductase activities. Hence, PA1024 is not an NMO, and should be classified as an NADH:menadione reductase. PA1024 possesses seven conserved motifs that are present in over 1000 sequences from bacteria and fungi, and two higher eukaryotes. These seven motifs are different from the motifs that define class I NMOs and could be used to further improve gene prediction.

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Heme Binding in *Corynebacterium diphtheriae* HmuT: An Investigation of Conserved Residues

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Pathogenic bacteria commonly require iron as a nutritional source for survival and virulence. As a result, bacteria have developed various strategies to obtain iron from the host, commonly in the form of heme. Certain bacterial pathogens take up heme utilizing the proteins located in their cellular membranes. Many of them are becoming increasingly antibiotic resistant. Characterization of such pathogenic heme transport systems may lead to antibiotic alternatives to treating these bacterial infections.

The heme uptake pathway (hmu) of *Corynebacterium diphtheriae* is a multiprotein pathway responsible for the binding and transporting of heme into the cell. One such protein involved in this pathway, HmuT, delivers heme to the HmuUV ABC-type transporter. Sequence alignment and homology modeling studies of HmuT point to possible heme binding residues, as well as other conserved residues, utilized during heme transfer. Mutations of these potential heme axial ligands, as well as nearby residues, are performed and characterized via UV-visible, circular dichroism, magnetic circular dichroism, and resonance Raman spectroscopies. These results as a whole indicate that axial ligation, heme pocket residue interactions, and heme iron oxidation state all play a role in the mechanism of HmuT heme transfer.

Exploration of weak interactions between folate and betaine

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In vitro studies with chromosomal dihydrofolate reductase (EcDHFR) and an R-plasmid encoded dihydrofolate reductase (R67 DHFR) have shown that weak interactions between osmolytes and the substrate, dihydrofolate (DHF), decrease DHF affinity towards these enzymes. The changes in binding affinity with water activity were unique for each osmolyte, indicating preferential interactions between osmolyte and folate and its derivatives. Further characterization of these weak interactions is essential for better understanding of in vivo effects of folate and its various redox states with available functional groups inside the cell. Quantitation of weak interactions between folate and betaine was done using a vapor pressure osmometry method that gives a preferential interaction co-efficient (μ_{23}/RT); this provides a scale for measuring the preference of folate for betaine relative to water. The μ_{23}/RT values can be predicted using an accessible surface area calculation as per the Record lab (Capp et al., 2009 Biochemistry 48:10372). The predicted μ_{23}/RT value for folate, which consists of a pteridine ring linked to a pamino benzoate ring (p-ABA) and a glutamate tail, indicates that folate equally prefers water and betaine. To test this prediction, we experimentally measured the μ_{23}/RT of folate and found a folate concentration dependence of the μ_{23}/RT values. At high concentrations, folate is known to dimerize via ring stacking with the pteridine ring of one monomer stacking on the p-ABA ring of the second monomer. The glutamate tails are free in the dimer. Our osmometry results are consistent with the aromatic rings preferring to interact with betaine as previously reported. The folate μ_{23}/RT value was also found to be dependent on its protonation state. Neutral folate tends to preferentially interact with betaine whereas the anionic form excludes betaine. Thus, we find that folate dimerization and protonation states affect its interaction with betaine. These studies are important as how enzymes behave in vivo may be different than in the dilute solutions used in vitro studies. In other words, DHF "stickiness" may become significant in vivo when many molecules are present in the cytoplasm.

Can μ_{23} /RT values be used to predict osmotic stress effects on ligand binding? In some cases, yes. However, the caveat is whether all the ligand atoms are used in binding. For example, glutamate has been shown to exclude betaine and calculation of the μ_{23} /RT for polyglutamylated folates predicts an overall exclusion of betaine from the polyglutamylated folate. This should translate into tighter binding of polyglutamylated folate to DHFR. However, as only the first glutamate of folate interacts with the pore of R67 DHFR, additional glutamates minimally contribute to binding. Thus, we predict that exclusion of betaine from the additional glutamates will yield minimal effects on folate binding to R67 DHFR. To test this, we studied betaine effects on binding of folate and pteroyltetra-glutamate (PG4) to R67 DHFR. We find betaine addition weakens binding of both folate and PG4 to R67 DHFR. Thus, our findings indicate that betaine exclusion from the additional glutamates does not contribute to its binding to R67 DHFR.

An unprecedented NADPH domain conformation in Lysine Monooxygenase NbtG provides insights into uncoupling of oxygen consumption from substrate hydroxylation

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N-hydroxylating monooxygenases (NMOs) are involved in the biosynthesis of iron-chelating hydroxamate-containing siderophores that play a role in microbial virulence. These flavoenzymes catalyze the NADPH- and oxygen-dependent hydroxylation of amines, such as those found on the side chains of lysine and ornithine. In this work we report the biochemical and structural characterization of Nocardia farcinica Lys monooxygenase (NbtG), which has similar biochemical properties to mycobacterial homologs. NbtG is also active on D-Lys although it binds L-Lys with a higher affinity. Differently from the ornithine monooxygenases PvdA, SidA and KtzI, NbtG can use both NADH and NADPH and is highly uncoupled, producing more superoxide and hydrogen peroxide than hydroxylated Lys. The crystal structure of NbtG solved at 2.4 Å resolution revealed an unexpected protein conformation with a 30° rotation of the NAD(P)H domain with respect to the FAD domain that precludes binding of the nicotinamide cofactor (Fig. 1). This "occluded" structure may explain the biochemical properties of NbtG, specifically with regard to the substantial uncoupling and limited stabilization of the C4a-hydroperoxyflavin intermediate. Biological implications of these findings are discussed.

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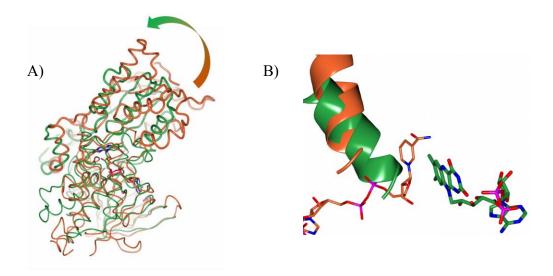


Fig. 1. Comparison of SidA (PDB code 4b64; NADP+ complex) and NbtG. (A) The large change in the orientation of the NADPH-binding domains is visualized by superimposing NbtG (green) and SidA (orange) structures using their respective FAD-binding domains (rmsd = 2.5 Å for the equivalent C α atoms). The shaded arrow outlines the additional 33° rotation, which is needed for the optimal superposition of the NADP+-binding domains (highlighted as bold worms). (B) Close-up view of the flavin site in NbtG (green) and SidA (orange). Because of the domain rotation, the nicotinamide-ribose site in NbtG is physically occluded by an α -helix (residues 215-229) of the NADP+-binding domain.

Structural complementation of the catalytic domain of Pseudomonas exotoxin A

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Exotoxin A is a major virulence factor of *Pseudomonas aeruginosa*. It is a tripartite protein with mono-ADP-ribosyltransferase activity in its C-terminal domain (known as domain III or PE3), which is structurally homologous to diphtheria toxin A and cholix toxins from Corynebacterium diphtheriae and Vibrio cholera, respectively. PE3 has strict substrate specificity for a specific diphthamide residue in eukaryotic elongation factor 2 (eEF2) in an NAD⁺-dependent manner. ADP-ribosylation of eEF2 leads to cessation of ribosomal protein synthesis followed by apoptotic cell death. We have recently engineered structural complementation into PE3 by dissecting the enzyme at an extended loop and fusing each fragment to one subunit of an antiparallel heterospecific coiled coil. *In vitro* ADP-ribosylation and protein translation assays demonstrate that the resulting fusions — supplied exogenously as genetic elements or purified protein fragments — had no significant catalytic activity or effect on protein synthesis individually, but in combination catalyzed the ADP-ribosylation of eEF2 and inhibited protein synthesis. Although complementing PE3 fragments are less efficient catalytically than intact PE3 in cell-free systems, co-expression in live cells transfected with transgenes encoding the toxin fusions inhibits protein synthesis and causes cell death comparably as intact PE3. We are currently interested in applying split PE3 in two applications: first, as a search engine for screening protease libraries for novel sequence specificities in live cells. Second, since PE3 is widely used as a cytocidal payload in receptor-targeted protein toxin conjugates, complementation of split PE3 offers a direct extension of the immunotoxin approach to generate bispecific agents that may be useful to target complex cellular phenotypes.

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Coexpression and analysis of Benzylsuccinate and M-Hydroxybenzylsuccinyl-CoA Synthases in Anaerobic Aromatic Hydrocarbon Oxidizing *Geobacter daltonii* Strain FRC-32

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Fe(III)-reducing bacterium *Geobacter daltonii* is capable of oxidizing aromatic hydrocarbons anaerobically via the radical forming enzyme benzylsuccinate synthase (Bss), a glycyl radical enzyme in the lyase enzyme superfamily. Annotation of the *Geobacter daltonii* strain FRC-32 genome revealed the presence of a homologue to Bss with 76% identity named m-hydroxybenzylsuccinyl-CoA synthase (Mbs). Using ExPASy's Prosite tool to detect motifs within the gene, an s-adenosylmethionine (SAM) site on the activating subunit of Mbs was located, suggested that the Mbs holoenzyle and its activating subunit can work in conjunction with one another to methylate benzene into toluene, after which it can continue onward via Bss in the toluene degradation pathway. Using site directed mutagenesis and cloning techniques, purification and isolation of Bss and Mbs, along with their activating subunits, is under way, after which analysis of both enzymes will proceed. The main aim of this study is to compare the catalytic activity of Bss and Mbs and to elucidate the mechanism involved in anaerobic oxidation of benzene by *G. daltonii*. We hypothesize that Mbs is utilized during benzene degradation in *G. daltonii*, methylating it to toluene using a SAM site on its activating subunit.

Probing the Role of Globin Coupled Sensors in Bacterial O2-Dependent Signaling

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Recent studies have suggested that heme proteins play a role in sensing the bacterial environment and controlling the switch between motile and sessile (biofilm) states. Biofilm formation is regulated by cyclic di-GMP levels, which are controlled by the opposing functions of diguanylate cyclases and phosphodiesterases. Globin coupled sensors are heme proteins that consist of a globin domain linked by a central domain to an output domain. Diguanylate cyclase-containing globin coupled sensors are found in a number of bacteria, including human and plant pathogens, and their diguanylate cyclase activity has been shown to be controlled by the ligation state of the heme. Current efforts to elucidate the signal transduction mechanism of these enzymes have found that cyclase activity is correlated with oligomerization state changes. In addition, recent work suggests that these sensor proteins control O2-dependent bacterial virulence. These studies provide insight into how cells can exert exquisite control over cyclase activity of globin coupled sensors and the pathways they control.

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Mechanically-regulated pathways employed by viruses to escape immune recognition: An atomistic study

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One subset of leukocytes is cytotoxic T lymphocytes (CD8+ T cells) that are capable of inducing cytolytic activity on cells presenting foreign antigen peptide. T cell receptors (TCR) expressed on CD8+ T cells interacts with peptide and class I Major Histocompatibility Complex (MHC) molecule on antigen presenting cells. TCR found on each T cell is unique in that they are specific in scanning pMHC complexes. Antigens are foreign substances that induce immune response. Antigens are composed of several antigenic determinants, known as epitopes, and the epitopes can bind to a specific TCR and/or antibody.

Viral persistence is observed in Cytomegalovirus (CMV) and Hepatitis C (HCV) infection. CMV does not pose a threat to an immune competent subject in terms of developing a life-threatening disease; however infected subject with HCV evolves to chronic state during the course of infection. The development into chronic condition is linked in part to the rise of "viral mutations" that have decreased functional effects as those of the wild type (WT) thereby escaping recognition from the immune system. Nevertheless, the true extent of TCR-pMHC interaction, its role in the molecular mechanism of antigen recognition, and the tolerance of leukocytes to viral epitopes upon infection, still remain a speculative issue, largely due to the lack of experimental approaches that could definitively address this question. TCR/pMHC interaction must also stand mechanical forces in order to prevent dissociation from external fluid flow or substrate stresses. MD simulation combined with experiments are employed to understand the mechanically-regulated pathways of TCR-pMHC interaction in the context of viral infection and epitope recognition. It will be described the molecular mechanisms by which mutant epitopes escape detection by the immune system and correlate this loss of recognition by the TCR with a characteristic mechanical behavior of TCR-pMHC response (catch-slip/slip-only bond)

This kind of study is novel and provides a unique means for elucidating the mechanical regulation of bio-molecular interactions at the atomic resolution.

Molecular basis of sliding clamp diffusion along DNA

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Whether sliding clamp diffusion along DNA is governed by simple Brownian motion or driven by internal motions of the DNA remains an elusive question. Here we investigate the DNA motion-driven diffusion hypothesis. We have employed molecular dynamics to sample the movement of three sliding clamps, each in complex with a 30mer DNA duplex. Each sampled clamp presents a unique inner pore diameter and electrostatic environment, which is thought to influence the propagation. To understand the driving force behind sliding clamp diffusion, we have quantified the protein-DNA interactions for each clamp. Additionally, we have analyzed the protein-DNA global motions that accurately describe the interplay between the internal motions of the DNA and displacement of the clamp, yielding further insight into the relationship between sliding clamp propulsion by DNA.

Investigation of key residues in α -isopropylmalate synthase from *Methanococcus jannaschii* for binding acetyl-coenzyme A

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α-Isopropylmalate synthase (IPMS) catalyzes the first step of L-leucine biosynthesis in archaea, bacteria, and some eukaryotes. The enzyme catalyzes a Claisen-like condensation between α-ketoisovalerate (KIV) and AcCoA. Surprisingly, both phylogenetic analysis and protein similarity network data suggest there are two evolutionarily distinct forms of IPMS with representatives in all three domains of life. The two forms share approximately 20% identity between members and are called IPMS1/CMS1 and IPMS2 based on clustering in a sequence similarity network. Previous work from Gerlt and co-workers suggests that enzymes with identical activities forming multiple clusters may be the result of "pseudoconvergent evolution". In these cases, the two versions of the enzyme may have evolved separately from distinct, but related, promiscuous ancestors. Distinct evolution paths were seen to result in differences in reaction stereospecificity or differentially conserved substrate selection strategies. Using IPMS from Mycobacterium tuberculosis (MtIPMS) and Methanococcus jannaschii (MjIPMS) as representatives of the IPMS2 and IPMS1/CMS1 clusters, respectively, the Frantom lab has investigated if differential substrate selection strategies resulted from pseudoconvergent evolution in the IPMS family. The two enzymes are predicted to have identical residues for selecting KIV, but residues involved in AcCoA selection have not been identified. Analysis of multiple sequence alignments containing members of both clusters indicate that in IPMS1/CMS1 sequences, K47, R94, R98, and R110 (MjIPMS numbering) are well conserved, while the corresponding residues in IPMS2 sequences are neutral. This suggests these four sites may play a significant role in MiIPMS to bind AcCoA. Alanine scanning mutagenesis was used to investigate the role of each residue in AcCoA binding in MjIPMS. R94A and K47A substitutions result in drastic effects on kinetic parameters. These two MjIPMS variants show a 20~150 fold decrease in k_{cat} value and a 100~1000 fold increase in K_{AcCoA} value, which indicates that these two site residues are critical for both binding AcCoA and catalytic activity of MjIPMS. Now the study is focused on characterizing the corresponding sites in MtIPMS.

Structural and Functional Studies of Epimerization Domains from Antibiotic Biosynthetic Pathways

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Bacteria and fungi use large multi-modular enzymes, the nonribosomal peptide synthetases (NRPSs), to produce peptides (NRPs) that have broad structural diversity and biological activity. The core catalytic domains on NRPSs are organized into modules with the essential catalytic functions necessary for the incorporation of one amino acid building block. A characteristic of NRPs is the frequent occurrence of non-proteinogenic D-amino acids in natural products. D-amino acids are introduced by the presence of an epimerization domain inserted into the core module domain structure. Although studies of epimerization domains have been reported, the substrate binding mode, chemical mechanism and organization in synthetase machinery is not clear. The non-ribosomal synthesis of the cyclic peptide antibiotic gramicidin S is accomplished by two large multifunctional NRPSs, GrsA and GrsB. GrsA consists of three domains responsible for the incorporation of D-phenylalanine into gramicidin S. We have determined the di-domain X-ray structure of the peptidyl carrier protein-epimerase fragment (PCP-E), of GrsA to 1.9Å resolution. The 561 amino acid residue construct is folded into three subdomains with the active site located at centre. The spatial arrangement of the PCP and the epimerization domain represent a catalytically relevant state of the flexible di-domain. Accordingly, the closed configuration of PCP-E interface provides insight into protein/protein recognition and substrate delivery during the assembly line process. The flexible linker in the structure assists in the movement of the PCP domain freely to accommodate with domains. The presented di-domain structure reveals novel aspects of carrier domain interactions with catalytic epimerization machinery and additionally provides insights into the mechanism of homologous peptide condensation chemistry.

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Characterization of Entamoeba histolytica acetate kinase PP_i/P_i specificity

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Acetate kinase (ACK) catalyzes the reversible phosphorylation of acetate to produce acetyl phosphate. This enzyme is nearly ubiquitous in bacteria, present in one genus of archaea, and has been identified in a few eukaryotic microbes. Typically, ACK utilizes ATP/ADP as phosphoryl donor/acceptor (equation 1), and the bacterial enzymes are fully reversible. However, the *Entamoeba histolytica* ACK (EhACK) exclusively utilizes PP_i/P_i and cannot use ATP/ADP as substrate (equation 2). This is the only known ACK with this property.

Equation 1: acetyl phosphate + ADP \leftrightarrows acetate + ATP acetate + PP acetate + PP acetate + PP.

Structural comparisons between EhACK and the well-studied *Methanosarcina* thermophila ACK (MtACK) revealed an occlusion in the entrance to the pocket that accommodates the adenosine moiety of ATP in EhACK. A $Gln^{323}Gly$ -Met³²⁴Ile EhACK variant was constructed to mimic the wider mouth of the pocket seen in MtACK. The k_{cat} in the acetate-forming direction was reduced approximately 9-fold but no activity was detected with either ADP or ATP as substrate in the appropriate direction of the reaction. However, increased inhibition by ADP and ATP versus the wild type EhACK was observed, suggesting that the variant may have increased ability to bind ADP and ATP but the active site architecture requires further optimization. Additional corresponding changes in the EhACK and MtACK active sites will be constructed and the variants will be examined for increased ability to utilize ADP/ATP and P_i/PP_i , respectively.

MauG: A Study of bis-Fe(IV) and Substrate Radicals

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Iron-dependent enzymes are prevalent in nature and participate in a wide range of biological redox activities. Frequently, high-valence iron intermediates are involved in the catalytic events of iron-dependent enzymes, especially when the activation of peroxide or molecular oxygen is involved. Building on the fundamental framework of iron-oxygen chemistry, these reactive intermediates constantly attract significant attention from the enzymology community. During the past few decades, tremendous efforts from a number of laboratories have been dedicated to the capture and characterization of these intermediates to improve mechanistic understandings. In 2008, an unprecedented bis-Fe(IV) intermediate was reported in a c-type diheme enzyme, MauG, which is involved in the maturation of a tryptophan tryptophylquinone cofactor of methylamine dehydrogenase¹. This intermediate, though chemically equivalent to the well-characterized high-valence iron intermediates, such as compound I, compound ES, and intermediate Q in methane monooxygenase, as well as the hypothetical Fe(V) species in Rieske non-heme oxygenases, is orders of magnitude more stable than these other high-valence species in the absence of its primary substrate. It has been recently discovered that the bis-Fe(IV) intermediate exhibits a unique near-IR absorption feature which has been attributed to a novel charge-resonance phenomenon². It has also been shown that this bis-Fe(IV) intermediate is capable of generating a di-radical species on its substrate protein³. This poster presentation summarizes the current knowledge of this high-valence iron intermediate and substrate radical, explores the formation and consequences of charge-resonance, investigates the chemical and thermal stability of the bis-Fe(IV) state of MauG⁴, and begins to describe the radical character of small molecule mimics of the substrate.

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Why would the Tibetan antelope eat detergents? A story of a widespread enzyme and its (probably unknown) biological function.

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The enzyme SdsA1 has been implicated in the degradation of sodium dodecyl sulfate (SDS, a common component of soaps and detergents) by the pathogenic bacterium *Pseudomonas aeruginosa*. Although SdsA1 can hydrolyze SDS and other alkyl sulfates, this secreted protein is inhibited by modest concentrations of SDS, suggesting that SDS hydrolysis may not be the physiological function of SdsA1. Further, BLAST search for SdsA1 homologs returns more than 500 proteins with at least 35% identity to SdsA1, including proteins present in eukaryotes (among others, in *Saccharomyces cerevisiae*, cucumber, *Ciona intestinalis*, owl limpet, and the Tibetan antelope), which seem highly unlikely to have evolved or acquired this protein under selective pressure to hydrolyze detergents. Thus, it is possible that all these proteins perform a yet-unknown function or, alternatively, that the mettalo-β-lactamase scaffold of SdsA1 can rapidly adapt to evolve new functions. We are currently studying the substrate preference and the effect of possible inhibitors on the SdsA1-catalyzed hydrolysis of alkyl sulfates and similar compounds to determine a plausible set of alternative substrates for SdsA1. Further, we are expressing homologous enzymes from *Saccharomyces cerevisiae* (Bds1) and *Rhodococcus ruber* (CddY) to compare their substrate specificities.

Modification of Enzymes for Use in Biosensors

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Development of sensitive and stable sensors is necessary for measuring food quality and contamination in the agricultural industries. While use of enzymes in sensors is ideal because of the selectivity and sensitivity, one major drawback is the relatively low stability of the biomolecules. Chemically modification of enzymes can be used to improve their stability. Additionally, it is hypothesized that enzyme stability can be further improved through modification with hydrophobic molecules. In this study, we examine the effects of chemical modification of enzymes with hydrophobic, aromatic molecules towards the goal of fabricating biosensors from the modified enzymes.

Glucose oxidase (GOx) and alcohol oxidase (AOx) were chemically modified with either benzoate or aniline using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling. Prior to chemical modification of GOx and AOx, the thermal stability of the unmodified enzyme was characterized by differential scanning calorimetry. Once baseline thermal characterization of the enzymes was completed, chemical modification protocols were optimized to retain maximal enzyme activity, structure and stability after modification. GOx samples contained 20-30 modified residues per monomer, yet the enzyme still retained >95% activity as measured by k_{cat} . Benzoate modified samples suffered no loss in thermal stability, while aniline modified samples had a T_m that was 8 °C greater than the unmodified enzyme. AOx contained six to eight modifications per monomer and retained 75% of its activity. Modification of AOx with benzoate was slightly destabilizing ($\Delta T_m = -4$ °C), while aniline modifications stabilized the enzyme ($\Delta T_m = +3$ °C). This research suggests that modification of acidic residues with aniline improves the stability of enzymes, which will help lead to more stable biosensors.

The Structural Basis for Recognition and Modification of the 30S Ribosomal Subunit by an Antibiotic Resistance Methyltransferase.

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Ribosomal RNA contains modifications at specific nucleotides in all organisms, some of which control resistance to antibiotics. However, the mechanisms are not well understood controlling how modification enzymes recognize their specific target nucleotide and position it within the active site. We report the crystal structure of the pathogen-derived aminoglycoside-resistance rRNA methyltransferase NpmA bound to the 30S ribosomal subunit in a pre-catalytic state (1). The structure reveals that NpmA recognizes conserved rRNA tertiary structure rather than RNA sequence. Additionally, the structure shows NpmA flips the target nucleotide from its position within the helical base stack to position it within the active site. The structure provides a general framework for investigating the mechanisms of other rRNA modification enzymes.

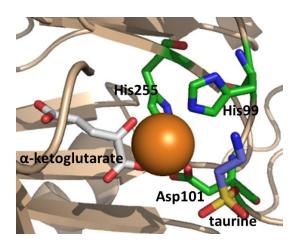
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The Thermodynamics of Iron(II) binding in α-Ketoglutarate/Taurine Dioxygenase

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α-Ketoglutarate/Taurine Dioxygenase (TauD; E.C. 1.14.11.17) is a nonheme iron enzyme that catalyzes the oxidative decarboxylation of α-ketoglutarate (αKG) to generate a high valent iron species that can hydroxylate taurine. Although this enzyme is well-studied mechanistically, the biophysics behind metal ion binding are not well understood. Here we discuss our efforts to characterize the thermodynamics behind ferrous iron binding to the 2-His-1-carboxylate facial triad in TauD using isothermal titration calorimetry (ITC) and new data collected by differential scanning calorimetry (DSC) on TauD and Fe-TauD that supports our interpretation. Iron(II) binding was measured under an anaerobic environment to yield a binding equilibrium of 2.4 (±0.1) × 10^7 (K_d = 43 nM) and a Δ G° value of -10.1 (±0.1) kcal/mol. Further analysis of the enthalpy/entropy contributions indicates iron(II) binding is enthalpically driven, where Δ H = -11.6 (±0.3) kcal/mol. Investigations into the unfavorable entropy term led to data that is consistent with water organization within the Fe-TauD structure.



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Probing the Physiological Roles of Atypical Cyclic Nucleotide Monophosphates

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Adenosine- and guanosine-3',5'-cyclic monophosphate (3',5'-cAMP, -cGMP) are well established mammalian second messengers that mediate pathways such as glycogen metabolism, vasodilation, and phototransduction (1). Reports suggest that 3',5'-cCMP may function in tumorigenesis and cell proliferation (2), but to date neither the pathway functions nor the proteins involved have been validated. The current work has quantified basal cNMP levels in rat organs (3) and aims to identify putative nucleotidyl cyclase(s) responsible for cCMP biosynthesis via tandem MS-based proteomics. Currently, cytidylate cyclase activity has been partially fractionated from mammalian organ homogenates and E. coli cell lysates. Regioisomeric nucleoside 2',3'-cyclic monophosphates (2',3'-cNMPs) also have been implicated in stress-response pathways as products of mRNA degradation. Acute mammalian organ damage generates 2',3'-cAMP and leaf wounding in Arabidopsis increases levels of 2',3'-cAMP and -cGMP (4). Free 2',3'-cNMPs also exist in bacteria (5), but potential physiological roles remain to be discovered. The current study has quantified 2',3'-cNMP levels in *E. coli* and seeks to identify the ribonucleases responsible for regulating 2',3'-cNMP concentrations. Moreover, the present work demonstrates that endoribonucleases of bacterial toxin-antitoxin systems modulate 2',3'-cNMP levels. Efforts are currently underway to explore the effects of altered 2',3'-cNMP concentrations on phenotype using a 2',3'-cyclic nucleotide phosphodiesterase and non-hydrolyzable 2',3'-cNMP analogues.

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Cysteine Dioxygenase: Not Your Ordinary Cupin

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Cysteine Dioxygenase (CDO) is an iron containing enzyme belonging to the cupin superfamily. CDO catalyzes the conversion of L-cysteine into L-cysteine sulfinic acid in the presence of dioxygen. The L-cysteine sulfinic acid intermediate is a branch point in sulfur metabolism leading to the formation of pyruvate and sulfite or taurine. A common structural feature of the cupin superfamily is the double stranded β-helix, more commonly known as the "cupin jelly roll" [1]. Several cupin enzymes contain two partially conserved motifs representing the 3-His/1-Glu coordination of the iron center. Unlike most cupin enzymes the mononuclear iron center of CDO is weakly coordinated by a 3-His motif. This traditional glutamate residue is replaced by a cysteine (Cys93) in mammalian CDO. Due to this evolutionary post-translational modification a unique thioether crosslink is able to form with a nearby tyrosine residue (Tyr157). In wild-type CDO the crosslink exists as a heterogeneous mixture of both noncrosslinked and crosslinked species. Previous studies suggest that the crosslink increases the catalytic efficiency of cysteine oxidation [2].

In order to investigate the evolutionary modifications of mammalian CDO, Cys93 was substituted with glutamate to generate the C93E CDO variant. Because C93E no longer contains cysteine it is unable to form the crosslink with tyrosine, existing only as the noncrosslinked species. There was a 10-fold decrease in the $k_{\text{cat}}/K_{\text{m}}$ value for C93E CDO compared to the wildtype enzyme with L-cysteine. The L-cysteine analogs D-cysteine and cysteamine showed similar $k_{\text{cat}}/K_{\text{m}}$ values as the L-cysteine substrate; however, the k_{cat} value for cysteamine was 13-fold higher. There was no observable activity when 3-mercaptopionic acid was used as the substrate, which suggests that the amino group of the substrate is essential for activity. Analogous to the wild-type enzyme, C93E CDO purified as two distinct chromophoric species representing different ionization states. The two chromophoric species had a similar percentage of iron bound, and showed comparable steady-state kinetic properties. The EPR spectra showed a high spin ferric signal for both species and a 2-fold higher Fe(III) spin concentration for one of the chromophoric species. The glutamate was coordinating the iron center in the three-dimensional structure of C93E CDO similar to the metal center geometry observed for the 3-His/1-Glu cupin motif. The coordination by Glu would occupy a ligand coordination site essential for catalysis that would prevent bidentate binding of L-cysteine and dioxygen to the metal center.

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Site-Directed Incorporation of Intrinsic Fluorescence in Shikimate Kinase to Evaluate Catalysis and Inhibition

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Mycobacterium tuberculosis is a prolific pathogen, and multidrug and extensively drug resistant strains are wide-spread threats to human health. However, only one new class of antitubercular agent has been approved by the FDA since the 1960s. Shikimate kinase catalyzes conversion of shikimic acid and ATP to shikimate-3-phosphate and ADP, a reaction absent from human metabolism (1). Consequently, M. tuberculosis shikimate kinase (MtSK) is potential target for new antitubercular agents. In targeting MtSK, it is important that candidate inhibitors target the shikimate-dependent rather than ATP-dependent components of the MtSK reaction mechanism because ATP mimics are likely to have broad cross-reactivity. MtSK is devoid of tryptophan (W). Analyses of amino acid sequence and SK protein structures revealed sites likely to tolerate replacement of existing amino acids with W. By site-directed mutagenesis, we introduced W in the enzyme's active site-LID domain (V116W), shikimate-binding domain (E54W), and nucleotide-binding domain (N151W). All the proteins were expressed and purified, and the correct molecular masses of each protein (accounting for the intended substitution) were confirmed using LC-ESI-MS. The secondary structure for each protein was evaluated, and all were highly similar to wild-type MtSK. The kinetic parameters of wild-type and each MtSK variant were obtained using a coupled spectrophotometric assay. With respect to ATP and shikimate concentration, each variant displayed kinetic properties highly similar to the wild-type enzyme. Each variant showed excitation and emission spectra consistent with the presence of tryptophan, though the wavelength and intensity profile of fluorescence emission was distinct across the variants due, most likely, to the differing solvent environment for each one. Our data suggest that these variants will provide a valuable method for screening inhibitors for their mechanism of interaction with MtSK.

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Bis-Fe(IV): Nature's Sniper for Long-Range Oxidation

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The biosynthesis of tryptophan tryptophylquinone, a protein-derived cofactor, involves a long-range reaction mediated by a *bis*-Fe(IV) intermediate of a di-heme enzyme, MauG. This high-valence intermediate is generated from the resting di-ferric state as a result of oxidation by H₂O₂. Recently, a unique charge-resonance (CR) phenomenon was discovered in this intermediate, and a biological, long-distance CR model was proposed. This model suggests that the chemical nature of the *bis*-Fe(IV) species is not as simple as it appears; rather, it is composed of a collection of resonance structures in a dynamic equilibrium. Here, we experimentally evaluated the proposed CR model by introducing small molecules to, and measuring the temperature dependence of, *bis*-Fe(IV). Spectroscopic evidence was presented to demonstrate that the selected compounds increase the decay rate of the *bis*-Fe(IV) species by disrupting the equilibrium of the resonance structures that constitutes the proposed CR model. The results support this new CR model and bring a fresh concept to the classical CR theory.

Upon addition of H_2O_2 to di-ferric MauG, the formation of the *bis*-Fe(IV) intermediate is observed on a millisecond time scale, implying the involvement of certain residues at the distal heme pocket in facilitating the binding of H_2O_2 and the cleavage of the O-O bond. For peroxide-utilizing enzymes, a histidine residue is typically found at the distal pocket to facilitate the binding and activation of H_2O_2 . A survey of the distal pocket of Heme_{5C} only reveals a glutamate residue, Glu113, which is 5.6 Å from the heme iron. In this poster, an EPR-centered study on the reactive intermediates of the Glu113 mutant will be presented.

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Analyzing the Roles of Histidine and Serine Residues in the Active Site of Cysteine Dioxygenase

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Cysteine dioxygenase (CDO) is a mononuclear iron-containing enzyme that belongs to the cupin super family. CDO is responsible for catalyzing the oxidation of L-cysteine to L-cysteine sulfinic acid. The iron of CDO is coordinated by three histidine residues, and a unique thioether crosslink between Tyr157 and Cys93 is adjacent to the iron center. Homogeneously crosslinked CDO shows a 10-fold increase in catalytic activity. A histidine (His155) and serine (Ser153) residue are adjacent to the crosslink, and form a putative hydrogen bonding network with Tyr157. The location of these conserved amino acids relative to the crosslink and metal center suggests they may play be involved in specific catalytic events.

In order to investigate the potential roles of His155 and Ser153 in crosslink formation or cysteine oxidation, two single variants (H155A CDO and S153A CDO) were constructed and purified and compared to that of wild-type CDO. The percentage of iron bound in both variants was comparable to that of wild-type CDO, but the mutations did not appear to alter the secondary structures when compared to wild-type. Purified wild-type and S153A CDO exist as a heterogeneous mixture of crosslinked and noncrosslinked species, while H155A CDO exists predominately in the noncrosslinked form. At increasing substrate (L-cysteine) concentrations, wild-type CDO is able to form a homogenous crosslinked species. The H155A CDO and S153A CDO variants were able to form the fully crosslinked species similar to the wild-type enzyme. The S153A CDO variant demonstrated a 4-fold decrease in the $k_{\text{cat}}/K_{\text{m}}$ value, and there was a 2-fold decrease in the $k_{\text{cat}}/K_{\text{m}}$ value for H155A CDO compared to wild-type CDO. These studies suggest that His155 and Ser153 are not directly involved in crosslink formation, but may play a role an indirect role in catalysis through stabilization of the thioether crosslink.

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Evidence of Kinetic Cooperativity in Dimeric Ketopantoate Reductase from *Staphylococcus aureus*

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Ketopantoate reductase (KPR) catalyzes the NADPH-dependent production of pantoate, an essential precursor in the biosynthesis of coenzyme A^{1,2}. Previous structural studies have been limited to E. coli KPR, a monomeric enzyme that follows a sequential ordered mechanism³. Here we report a 1.8Å resolution dimeric KPR structure from Staphylococcus aureus, with sedimentation velocity analysis showing the dimer is stable in solution. Analysis of unpublished KPR structures in the PDB reveals that a majority of KPRs are dimeric. Steady state analysis of S. aureus KPR reveals strong positive cooperativity with respect to NADPH (Hill coefficient of 2.5), while the substrate ketopantoate inhibits activity at high concentrations. These observations are consistent with a random addition mechanism wherein the initial binding of NADPH is kinetically preferred. Förster resonance energy transfer (FRET) studies of equilibrium binding of NADPH show a small degree of cooperativity between dimeric subunits (Hill coefficient of 1.2). This supports a model of kinetic cooperativity based on the preferred binding of NADPH. This model is consistent with the observations from the A181L substitution, which increases the K_m of ketopantoate 844-fold without affecting k_{cat} . The KPR_{A181L} crystal structure shows that Ser239, which is known to be involved in ketopantoate binding⁴, is displaced by the substitution. The reduction in ketopantoate binding affinity would enhance the kinetically preferred NADPH binding path, making the mechanism appear sequentially ordered and reducing the kinetic cooperativity. In fact, the KPR_{A181L} NADPH saturation curve is hyperbolic, consistent with the random addition mechanism.

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Investigation of Conformational Dynamics in *Corynebacterium Glutamicun* MshA, Retaining GT-B Glycosyltransferase, by Hydrogen Deuterium Exchange Mass Spectrometry

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Tuberculosis is a common and infectious disease caused by mycobacteria. The products of the unique biosynthetic pathways of mycobacteria have become the target of new treatments, since they are essential to the growth of mycobacteria. One class of important products are mycothiol (MSH), which are necessary for the maintenance of appropriate reducing environment in cells. MshA catalyzes the first step of MSH biosynthesis. It transfers acetylglucosamine from donor UDP-N-acetylglucosamine (UDP-GlcNAc) to acceptor myo-inositol-1-phosphate (I1P). The crystal structure of MshA has been reported and the C-terminal domain rotation of MshA on UDP-GlcNAc binding also has been identified. However, dynamic changes of MshA on substrate binding cannot be observed by crystallization. In order to understand dynamic changes of MshA on substrate binding, hydrogen deuterium exchange mass spectrometry (HDX-MS) was used to investigate its internal motions. The exchange rate of backbone amide hydrogen for deuterium is related to protein secondary structure, solvent accessibility, and backbone flexibility. The results suggest that both substrates UDP-GlcNAc and I1P can bind to C-terminal domain of MshA independently, disagreeing with the previous proposal that I1P can only bind to MshA after UDP-GlcNAc. HDX data shows that the binding of UDP-GlcNAc induces big dynamic changes of MshA, mainly in C-terminal domain and peptides around the active site. These peptides tend to have lower solvent accessibility and decreasing dynamics due to hydrogen bonding or other interactions. However, these peptides do not have big dynamic changes on I1P binding, suggesting that I1P cannot induce the formation of MshA active conformation even though it can bind to MshA independently.

Examining the role of an intruding residue in tryptophan 2,3-dioxygenase from a neighboring subunit

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Since the discovery of serotonin as a vital neurotransmitter, most of the studies has primarily focused on its production from L-Trp. However, conservative estimations of L-Trp metabolism indicate that the production of serotonin plays a small role in L-Trp catabolism, with up to 90% of tryptophan being consumed by the kynurenine pathway for the *de novo* biosynthesis of nicotinamide adenine dinucleotide (NAD). The first step of this pathway is catalyzed by tryptophan 2,3-dioxygenase (TDO) and its isozyme indoleamine 2,3-dioxygenase. This is also a committed and regulatory step of the pathway. Utilizing a heme cofactor, TDO/IDO employ dioxygen to catalyze the ring opening of the indole moiety on L-Trp. Over the past decade, TDO and IDO have garnered much attention for their roles in the immunoregulation and proliferation of various types of tumor cells. While IDO's catalytically active form is a monomer, TDO exists as a tetramer. Within TDO's active site an intruding tyrosine residue from the adjacent subunit. Using site-directed mutagenesis, kinetics, and spectroscopic analyses the role of this residue has been investigated.

A study of accumulation of mutations in plasmodius falciparum dihydrofolate reductase

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Dihydrofolate reductase (DHFR) is an enzyme that plays an essential role in the folate pathway, reducing dihydrofolate to tetrahydrofolate, which is crucial in the production of DNA, RNA, and protein. Therefore, Dihydrofolate reductase (DHFR) usually acts as the target of the antifolate drugs. Antimalarial drugs, such as pyrimethamine (Pyr), have long been used as treatment of malaria due to its important role in inhibiting dihydrofolate reductase-thymidylate synthase (DHFR-TS) in plasmodium falciparum (pf). However, mutations have emerged, such as S108N, C59R/S108N, N51I/C59R/S108N/I164L/, which have led to antimalarial resistance. S108N is considered as the origin of the subsequent multiple sites mutants with higher antimalarial resistance. To gain more insight into the binding mechanism and antifolate resistance, we applied molecular dynamics simulation on pfDHFR-TS and three popular mutants, which are complexed with Pyr and NADPH. The results indicate that mutations have caused obvious conformational changes in and out of protein binding pocket. The center of mass distance between binding pocket and Pyr is larger in quadruple mutant than that of wild type protein. Molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) calculation results also show that the binding free energy between Pyr and protein is the lowest in the wild type protein, while the binding free energy is getting higher as mutations accumulate in the mutants we studied. The results are consistent with experimental observations¹ that indicate the quadruple mutant is most resistant to Pyr relative to the wild type, single mutant and double mutant protein mentioned above.

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Measurement of HAT activity with a bioorthogonal chemical probe mediated fluorescent assay

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Histone acetylation is one of the most important post translational modifications mediated by histone acetyltransferases (HATs) and is closely related with many physiological and pathological processes. However, current assays used to measure HATs activity or screen HATs inhibitors are either bio-hazardous, inaccurate or complicated. Herein, we develop a new mix-and-read fluorescent assay to measure HAT activity by directly measuring the yield of product. MOF (MYST1/KAT8) is an important histone acetyltransferase which is able to acetylate 16th Lysine (K16) from histone H4 N-terminal tail and mutation of 317th amino acid of human MOF from Isoleucine to Alanine enables it to react with 3azidopropionyl Coenzyme A (3AZ-CoA), which is a chemically reactive Ac-CoA surrogate. After azidopropionyl group is transferred onto peptide, alkyne-Dabcyl could be linked to peptide through click reaction and Dabcyl will quench the fluorescent emission from fluorescein. With a series of measurement, we've demonstrated that there is clear correlation between fluorescence intensity change and the extent of enzymatic reaction, based on which, enzyme activity could be accurately quantified and this assay is also applicable in inhibitor screening as well.

Characterization of the *Arabidopsis thaliana* acetyl-CoA synthetase putative carboxylate binding pocket

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The precursor molecule in fatty acid and polyketide biosynthesis pathways is a two carbon short chain acyl-CoA molecule. Acetyl CoA synthetase (ACS) catalyzes the activation of the acetate to acetyl-CoA by forming a thioester bond with coenzyme A while hydrolyzing ATP to AMP and pyrophosphate. The hydrophobic cavity between the N and C terminal domains of Methanothermobacter thermoautotrophicus acetyl CoA synthetase is the probable binding pocket for acetate and mutations therein affect catalytic function, enabling the enzyme to bind and activate not only acetate but also butyrate [1]. The Arabidopsis thaliana acetyl-CoA synthetase was compared to other acyl-CoA synthetases, and was computationally modeled on the available crystal structures of the Saccharomyces cerevisiae ACS1 and Salmonella enterica ACS. This allowed the identification of the residues that make up the putative carboxylate binding pocket residues. To further understand substrate selectivity and binding within the putative carboxylate binding pocket, selected residues were mutated to resemble the homologous residues in the *Pseudomonas* chlororaphis isobutyryl-CoA synthetase. We targeted four residues (Ile³²³, Thr³²⁴, Val³⁹⁹ and Trp⁴²⁷) that are proposed to form the carboxylate binding pocket, and we found that two residues, Trp⁴²⁷ and Thr³²⁴ are primarily involved in determining the carbon-chain length of acceptable carboxylate substrates. By combining two mutations (Val³⁹⁹Ala, and Trp⁴²⁷Gly) the enzyme was able to utilize butyrate with a similar catalytic efficiency as the wild-type enzyme with acetate. Additional mutations of Thr³²⁴ allowed the enzyme to utilize propionate with similar efficiency as the wild type enzyme with acetate. Therefore, these mutations created a more promiscuous acyl-CoA synthetase.

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Exploration of Cyanine Compounds as Selective Inhibitors of Protein Arginine Methyltransferases: Synthesis and Biological Evaluation

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Protein arginine methyltransferase 1 (PRMT1) is involved in many biological activities, such as gene transcription, signal transduction and RNA processing. Overexpression of PRMT1 is related to cardiovascular diseases, kidney diseases and cancers; therefore, selective PRMT1 inhibitors serve as chemical probes to investigate the biological function of PRMT1 and drug candidates for disease treatment. Our previous work¹ found trimethine cyanine compounds that effectively inhibit PRMT1 activity. In our present study, the structure activity relationship for the cyanine compounds was systematically investigated with these features: 1) the two nitrogencontaining "head" is necessary for maintaining the activity; 2) increasing hydrophobicity generally leads to better inhibition; 3) a sulfur atom in position 3 of indolium group results in equal or better activity than dimethylmethine; 4) pentamethine as the spacer seems better than trimethine; and 5) no larger than bromine can be tolerated on the *meso*-carbon of the spacer. Among the compounds studied, a pentamethine compound, E-84 (compound 50), showed inhibition on PRMT1 at micromolar level and 6-25 fold selectivity over CARM1, PRMT5 and PRMT8. The cellular activity suggested that compound 50 permeated the cellular membrane, inhibited cellular PRMT1 activity and blocked leukemia cell proliferation. Additionally, our molecular docking study suggested compound 50 might act by occupying the cofactor binding site, which provided a roadmap to guide further optimization of this lead compound.

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Network Analysis for Evolving Allosteric Communication in 3-ketosteroid Receptor

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3-ketosteroid receptors include the androgen receptors (AG), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and progesterone receptor (PR). They belong to the nuclear hormone receptors (NRs) family that regulate gene expression through transcriptional control pathway, being descended from a common ancestor, ancestral steroid receptor 2 (AncSR2).[1] Evolution within the 3-ketosteroid receptor family led to changes protein binding affinity. It was found that they exhibit the different binding affinity to activating glucocorticoid response element ((+)GRE), and only some of them have the ability to bind to repressing glucocorticoid receptor element (nGRE).[2] We have presented dynamic network models to investigate allosteric communication for 3-Ketosteroid nuclear receptor DNA binding domain within MR and GR lineage, and discuss how the binding characteristics correlate with historical mutations that happened in the DNA binding domain from network perspective.

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Crystallographic and spectroscopic snapshots reveal a dehydrogenase in action

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Aldehydes are ubiquitous intermediates in metabolic pathways and their innate reactivity can often make them quite unstable. There are several aldehydic intermediates in the metabolic pathway for tryptophan degradation that can decay into neuroactive compounds that have been associated with numerous neurological diseases. An enzyme of this pathway, 2-aminomuconate-6-semialdehyde dehydrogenase, is responsible for 'disarming' the final aldehydic intermediate. Here we show the crystal structures of a bacterial analogue enzyme in five catalytically relevant forms: resting state, one binary and two ternary complexes, and a covalent, thioacyl intermediate. We also report the crystal structures of a tetrahedral, thiohemiacetal intermediate, a thioacyl intermediate and an NAD+-bound complex from an active site mutant. These covalent intermediates are characterized by single-crystal and solution-state electronic absorption spectroscopy. The crystal structures reveal that the substrate undergoes an E/Z isomerization at the enzyme active site before an sp3-to-sp2 transition during enzyme-mediated oxidation.

Elucidation of O₂-Dependent Stress Response Pathways in Vibrio brasiliensis

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Bacteria have developed strategies in response to environmental and physical stress. The stressosome signalling system was first identified and characterized in Gram-positive model bacterium *Bacillus subtilis*. (1) The 1.8 MDa, pseudo-icosahedra protein complex, stressosome in *B. subtilis*, is involved in the activation of the alternative RNA polymerase σ -factor, σ^B , in response to diverse environmental stresses. (2) Gene clusters encoding stressosome homologs have been found in representatives of most bacterial phyla. (3, 4) The diverse stress signals are conveyed through "partner-switching" mechanism, in which serine and threonine phosphorylation controls the interactions among proteins in the signalling pathway. Although the stressosome complex in *B. subtilis* has been extensively characterized, the signal recognized by the sensor protein, RsbR, remains inconclusive.

The $\sigma^{\rm B}$ -dependent general stress response pathways in the marine bacterium Vibrio brasiliensis, as well as the closely related pathogen V. vulnificus, has yet to be identified. In this study, we are interested in characterizing a stressosome-regulated biochemical pathway in model bacterium V. brasiliensis, in order to gain insights into mechanisms of pathogenicity of the Vibrio phyla. We have identified candidate genes (rsb genes) encoding homologs of the stressosome components, RsbR, RsbS and RsbT, from V. brasiliensis. Preliminary data has demonstrated the role of RsbT as a serine kinase in V. brasiliensis. Similar to B. subtilis, RsbT does not only have the ability to autophosphorylate itself, but can also phosphorylate RsbR, in a time-dependent manner. Subsequent experiments to characterize the kinase activity of RsbT towards RsbS, as well as the interactions among RsbR, RsbT and RsbS in stressosome complex from V. brasiliensis, are underway. In V. brasiliensis, RsbR contains of an N-terminal hemebound globin domain, which can bind O₂ and may serve as a bacterial O₂ sensing domain. The ability of bacteria to adjust to changing O₂ level suggests that signalling pathway can sense extracellular O₂ and adjust its intracellular signalling.(5) Further studies on the O₂ dependence of stress response will be performed to establish the role of RsbR as a regulator of stressosome signaling. The study of stressosome-dependent signaling pathways in V. brasiliensis will improve our understanding of the mechanism of pathogenicity of the *Vibrio* phyla.

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An Investigation into the Effect of Metal Ion in Protective Antigen and Tumor Endothelial Marker 8 Binding

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Anthrax toxin is transported into human cells after its protective antigen (PA) binds to its receptor. The receptor, tumor endothelial marker 8 (TEM8), has been identified as a potential anticancer target for its involvement in angiogenesis. This project investigates the role of the metal ion in the TEM8/PA binding mechanism to provide information relevant to anticancer therapy development. Structures of the TEM8/PA complex were produced through homology modeling, and canonical conformational ensembles of the TEM8/PA complexes involving different metal ions in TEM8 were generated through molecular dynamics simulations. Binding thermodynamics were also assessed using a Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method. In addition, the dissociation constant between TEM8 and PA in the presence of different divalent metal ions was determined experimentally via fluorescence resonance energy transfer (FRET). Both computational and experimental results indicate the metal ion in TEM8 contributes significantly to the binding affinity. Simulation shows the existence of Mg2+, Zn2+ or Ca2+ in TEM8 corresponds to a sequentially reduced affinity between TEM8 and PA. Further, computational analyses suggest the differences in TEM8/PA affinity are consistent with the behavior of closely related integrin proteins known to adopt two conformations (open and closed) correlated with different levels of activity.

Exploring the Mechanism of Acetyl-CoA Synthetase (ADP-forming) from Entamoeba histolytica

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ADP-forming acetyl-CoA synthetase (ACD; [EC 6.2.1.13]) catalyzes the conversion of acetyl-CoA to acetate to generate ATP from ADP via substrate level phosphorylation. ACD activity was first partially purified from *Entamoeba histolytica*, a protozoan parasite that causes amoebic dysentery and liver abscess in humans. This amitochondriate lacks the TCA cycle and oxidative phosphorylation; consequently, it relies on glycolysis and amino acid degradation as key pathways for ATP generation. Studies have shown that acetate and ethanol are the primary metabolites produced by *E. histolytica* during growth on glucose. Biochemical characterization of *E. histolytica* ACD suggests it may play a role in conservation of energy by extending the glycolytic pathway to produce ATP and acetate. Alternatively, ACD may also function in acetate utilization during colonization in the large intestine, as the enzyme functions well in both directions of the reaction.

The ACD reaction mechanism was first assumed to be analogous to the three-step mechanism of the well-studied succinyl-CoA synthetase (SCS) from the TCA cycle due to their high sequence similarity. Although the SCS mechanism involves a single phosphoenzyme intermediate, Brasen *et al.* (1) proposed a four-step reaction mechanism involving a second phosphoenzyme intermediate due to transfer of the phosphoryl group from a His residue on the α subunit to His residue on the β subunit before the final transfer to ADP to produce ATP.

Site-altered variants have been constructed for His²⁵² and His⁵³³, the two residues proposed to participate in phosphoryl transfer in *E. histolytica* ACD. The H252A variant is inactive in either direction, confirming the essentiality of His²⁵² and supporting its role in phosphorylation. In contrast, the His⁵³³ variants retain activity, albeit significantly reduced, suggesting this residue is important, but not essential to the mechanism. We have also examined Asp⁶⁷⁴ for which the equivalent residue in SCS has been implicated to stabilize the phosphoenzyme intermediate (2). Although each of the Asp⁶⁷⁴ variants exhibits reduced activity, conservative replacement with Glu was least deleterious. This result suggests the negative charge of Asp⁶⁷⁴ is important to catalytic activity, consistent with a role for this residue in stabilization of the phosphohistidine intermediate.

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Hydrogen deuterium exchange mass spectrometry defines SufE D74R dynamics and interaction with SufS cysteine desulfurase

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Backbone amide hydrogen deuterium exchange mass spectrometry (HDX-MS) is a powerful technique to probe protein structure, conformational dynamics required for binding, and protein-protein interactions. We use traditional HDX-MS to determine structural interactions between SufS and its partner SufE, both members of SUF system for iron sulfur (Fe-S) cluster biogenesis in Escherichia coli. SufS, a cysteine desulfurase, mobilizes persulfide from cysteine and transfers it to SufE, an accessory protein that stimulates Fe-S cluster formation. Our previous HDX-MS studies localized a region required for SufS-SufE interaction. Additionally, a crystal structure of the homologous protein complex CsdA-CsdE suggested that SufE Asp74 might form a salt bridge within SufS-SufE interaction. To test the putative interaction model site directed mutagenesis was used to create a D74R variant of SufE. Previous biophysical results from the Outten laboratory indicate that SufE D74R has a stronger binding affinity for SufS than wildtype SufE and still enhances SufS desulfurase activity. Here, we report that HDX-MS analysis of SufE D74R shows an increase in solvent accessibility and dynamics in a loop containing the active site Cys51 (residue 38-56) used to accept persulfide from SufS. These structural dynamics changes of SufE D74R may promote its interaction with SufS by shifting the SufE conformation to a "sulfur acceptor" state.

Highly Drug Resistant HIV-1 Proteases

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The virally encoded protease is an important drug target for AIDS therapy. Despite the potency of the current drugs, infections with resistant viral strains limit the long term effectiveness of therapy. Highly resistant variants of the HIV protease have been identified in clinical isolates and characterized at the molecular level by biophysical and biochemical methods. About 20 mutations are required to produce extreme resistance to multiple inhibitors with several orders of magnitude worse binding affinity. Structural analysis of protease dimers shows loss of protease interactions with the inhibitors due to mutations that expand the binding site cavity and an increased variation in the flap conformations in the absence of bound inhibitor. Studied highly resistant proteases and strategies for their inhibition are described as well as new insights into an extreme example.

Investigation of mechanism of substrate selectivity in the Claisen condensation-like family of enzymes

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The DRE-TIM metallolyase superfamily uses conserved active site architecture to catalyze a vast range of C-C bond forming and breaking reactions. One subgroup of the superfamily, the Claisen condensation-like subgroup, catalyzes the condensation of various αketo acids on acetyl-CoA. As of 2014, this subgroup contained ~ 4300 sequences with six different α-keto acid specificities reported. Determining the active site residues affecting substrate selectivity would be of immense help for better annotation of functionalities in this large number of sequences and identifying new activities. In an attempt to identify the mechanism of substrate selectivity, α-isopropylmalate synthase (MjIPMS) and citramalate synthase (MjCMS) from the organism Methanococcus jannashii were employed. MjIPMS and MiCMS catalyze the condensation of different α-ketoacids, keto-isovalerate and pyruvate on AcCoA, respectively. In order to determine the set of residues governing the substrate selection properties, comparison of the active site architecture is essential. Based on structural analysis and multiple sequence alignment of the gene sequences, differentially conserved residues potentially affecting substrate selectivity were identified. Residues F66, L92, F114 of MiIPMS and the S45, F71, V93 of MjCMS were predicted to affect substrate selectivity. To probe the role of these residues alanine scanning, along with swapping the residues to generate single, double and triple variants was performed. The results of kinetic characterization did not agree with the hypothesis of altered substrate preferences. Even the most efficient variant L92F/F114V MjIPMS (k_{cat}/K_{M} = $(11000 \pm 600 \text{ M}^{-1}\text{min}^{-1})$ did not accept pyruvate with greater efficiency than WTMjIPMS($k_{\text{cat}}/K_{\text{M}} =$ (15000 ± 180 M⁻¹min⁻¹). Similarly, all the variants of MjCMS were unable to accommodate the larger keto-acid, α-ketoisovalerate into its binding pocket as its substrate. Inability of MiCMS variants to accept α-ketoisovalerate as its substrate, and unaltered efficiency of MjIPMS in accepting pyruvate as its substrate, suggests that rational design of variants is not sufficient to understand the mechanism of substrate selectivity in the CC-like subgroup.

Regulation of *Cryptococcus neoformans* XFP1 Activity by Phosphorylation of Serine⁴⁵⁴

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Cryptococcus neoformans is an invasive opportunistic pathogen of the central nervous system and the most frequent cause of fungal meningitis resulting in more than 625,000 deaths per year worldwide. Acetate has been shown to be a major fermentation product during cryptococcal infection, but the significance of this is not yet known. We are investigating the xylulose-5phosphate/fructose-6-phosphate phosphoketolase1 (Xfp1) - acetate kinase (Ack) pathway, one of two possible pathways for acetate production in C. neoformans. Until now, the biochemical characterization of fungal XFP1 has not been reported. We have produced recombinant C. neoformans XFP1 (Cn-Xfp1) in E. coli using various expression vectors with different purification tags. However, the recombinantly produced Cn-XFP1 is soluble but lack activity. Previous proteomic studies indicate that the Xfp1 family may undergo post-translational modification for activity. Beltrao et al. (1) examined the phosphoproteome in Schizosaccharomyces pombe and identified Xfp as being phosphorylated at Ser⁴⁵⁴. This residue is completely conserved among Xfp1 sequences but is universally replaced by Gly in both bacterial and fungal Xfp2 sequences. In order to obtain pSer⁴⁵⁴ Cn-XFP1, we utilized a system developed by the Rinehart lab for producing pSer proteins in E. coli. An E. coli codon optimized gene encoding Cn-XFP1 was cloned into pCRT7-NT vector and the recombinant pSer⁴⁵⁴ Cn-XFP was produced. Addition of Tween-20, Triton X -100 and 7% glycerol in the lysis buffer improved the solubility of Cn-XFP1. The pSer⁴⁵⁴ Cn-XFP1 is active and kinetic studies indicate that the enzyme displays substrate cooperativity with fructose-6phosphate but not with inorganic phosphate. Examination of the bacterial XFP structure suggests that pSer⁴⁵⁴ from one monomer interacts with the catalytic glutamate (Glu⁴⁷⁹) residue in another monomer.

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Structural Basis for Substrate and Enantiomer Selectivity in the GRE-Dependent dehydratases

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Glycyl radical enzymes (GREs) are a diverse superfamily of enzymes that utilize radical mechanisms to catalyze difficult, but often essential, chemical reactions $^{1-3}$. In this work, we present biochemical and structural data for the GRE-dependent diol dehydratase from *Roseburia inulinivorans* (RiDD) and the glycerol dehydratase from *Clostridium butyricum* (CbGD). We demonstrate that these enzymes have very different substrate and enantiomer selectivity despite high sequence identity (48%). Specifically, the CbGD catalyzes the dehydration of glycerol and (S)-1,2-propanediol but not (R)-1,2-propanediol. In contrast, we show that the RiDD does not utilize glycerol but will utilize both (S)-1,2-propanediol and (R)-1,2-propanediol as a substrate. Interestingly, the RiDD also produces some acetone with either enantiomer of 1,2-propanediol. We also report the crystal structure of the RiDD that, when considered in light of the biochemical data, provides an explanation for the observed substrate and enantiomer selectivity as well as new insight into the radical-catalyzed dehydration reaction of both enzymes.

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In Silico Model for NaAtm1 family ATP Binding Cassette Exporter Binding Site Interactions: Insights From Molecular Dynamics Simulations

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The ATP Binding Cassette (ABC) exporters use ATP as an energy source in the export of substrates across the membrane in transition metal homeostasis and detoxification processes. Crystal structures of the NaAtm1 ABC exporter showed that the primary binding site of the cellular antioxidant thiol-containing glutathione (GSH) is located close to the inner membrane surface in a large cavity. In addition, two binding sites of glutathione derivatives GSSG were also reported (1). When combined with computationally sophisticated and rigorous free energy techniques, molecular dynamics (MD) simulations of NaAtm1 type ABC exporters can elucidate the microscopic origins of substrate specificity in the primary binding site and the second binding site mentioned above and the driving forces of biological function during the substrate translocation cycle. From our MD simulations, several specific locations in the large drug binding pocket are identified as preferred binding locations. These observations are consistent with the hypothesis that the entire internal cavity plays a role in substrates recognition, binding and transportation. MD simulations of mutants involving several residues in both the substrate and ATP binding sites indicate that those mutations change the conformation of NaAtm1 ABC exporters and consequently alter the ATPase activity.

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Investigating the Role of the π -helix in the FMN Reductase of the Alkanesulfonate Monooxygenase System

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Flavin reductases are involved in diverse reactions that involve electron transfer processes. In the alkanesulfonate monooxygenase two-component system, a NADPH-dependent FMN reductase (SsuE) provides reduced flavin to the monooxygenase enzyme (SsuD). The alkanesulfonate monooxygenase system catalyzes the desulfonation of a diverse range of alkanesulfonates when sulfur is limiting in various bacterial systems (1). The SsuE enzyme undergoes a tetramer-dimer oligomeric switch that is dependent on the presence of FMN. It is unclear how these different oligomeric states relate to catalysis and how they are regulated.

A conserved π -helix which results from the insertion of a conserved tyrosine residue in the α4-helix is present at the tetramer interface of SsuE. The Tyr118 residue is within hydrogen bonding distance with the carboxyl group of Ala78 across the tetramer interface. This hydrogen bond is proposed to be vital in maintaining the tetrameric structure of SsuE (2). Variants of Y118 SsuE were generated to investigate the role of the π -helix in SsuE. The resultant Y118A and Y118S SsuE proteins were FMN-bound while the Y118F and ΔY118 SsuE variants were flavin-free as purified. Interestingly, the Y118W SsuE had weakly-bound flavin which was lost during the purification process. The Y118 SsuE variants were able to effectively bind FMN giving comparable dissociation constants as wild-type SsuE. Each of the Y118 SsuE variants was able to reduce FMN with similar activity to that of wild-type. Although desulfonation activity was observed with the Y118F SsuE variant, there was no measurable activity in the coupled assays monitoring desulfonation by SsuD with the other Y118 variants. These results imply that Tyr118 is vital for the transfer of FMNH₂ to SsuD, but does not affect flavin reduction. In addition, the results suggest that multiple non-covalent interactions are important for the structural stability of SsuE as implicated by the Y118F SsuE desulfonation activity. These findings provide insights on the evolutionary role of the Tyr118 insertion (resulting to a π -helix) in SsuE.

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Kinetic investigation of a semi-conserved active site glutamine residue in α -isopropylmalate synthase from *Mycobacterium tuberculosis*

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The DRE-TIM metallolyase superfamily is an evolutionarily-related group of enzymes that catalyze carbon-carbon bond forming and breaking reactions via the stabilization of an enolate intermediate. One well-characterized member of this superfamily is the enzyme isopropylmalate synthase from Mycobacterium tuberculosis (MtIPMS), which catalyzes the first committed step of the leucine biosynthesis pathway by mediating the Claisen-like condensation reaction between acetyl-CoA (AcCoA) and α -ketoisovalerate (α -KIV) to form isopropylmalate. Previous structural and bioinformatic studies have demonstrated that the semi-conserved active site residue Q84 may play a role in reaction specificity for MtIPMS compared to other members of the superfamily, especially with respect to the development of aldolase-like activities (Casey, et al. (2014) Biochemistry 53, 2915). Site-directed mutagenesis was used to investigate the role of Q84 during catalysis in MtIPMS. Undergraduate students in the Fall 2014 Biochemistry Laboratory course generated four variants of MtIPMS: Q84C, Q84E, Q84H (an aldolase mimic), and Q84L. All four variants were successfully expressed and purified, and all variants retain their dimeric structure as determined by gel filtration chromatography. A kinetic investigation of the effect of each substitution on the Michaelis-Menten parameters of MtIPMS is currently underway and results of these studies will be reported.

Identification and characterization of a novel chlorophyll catabolite from the bioluminescent dinoflagellate Pyrocystis fusiformis

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Dinoflagellates are an important group of marine microorganisms and the causative agents of red tides. Certain species are photosynthetic and capable of bioluminescence induced by physical agitation. The bioluminescence reaction, which is regulated on a circadian rhythm, involves the oxidation of a luciferin substrate by the enzyme dinoflagellate luciferase. Dinoflagellate luciferin is derived from chlorophyll in an unknown pathway. A proteomic investigation is underway to elucidate the biosynthetic pathway of dinoflagellate luciferin, which may aid in the development of algicides for the remediation of coastal seawaters and the use of dinoflagellate luciferase as a reporter gene and cellular imaging agent.

Identifying Effective Quenchers to Eliminate the Substrate-Cofactor Interference in the Scintillation Proximity Assay of Histone Acetylation

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Histone acetyltransferases (HATs) mediate the transfer of an acetyl group from the cofactor, acetyl-CoA, to the ε- amino group of specific lysines in diverse protein substrates, most notably nuclear histones. HATs are classified into several main families which include GCN5/PCAF, MYST, and CBP/p300 [1]. The deregulation of HATs is connected to a number of disease states [2, 3]. Reliable and rapid biochemical assays for HATs are critical for understanding biological functions of protein acetylation, as well as for screening small-molecule inhibitors of HAT enzymes. In this report, we present a scintillation proximity assay for the measurement of HAT enzymatic activities. The acetyl donor was [3H]Ac-CoA and a biotinmodified histone peptide served as the HAT substrate. Following the HAT reaction, streptavidincoated beads were added to induce proximity of acetylated substrate to the scintillant molecules. However, we observed strong nonspecific binding between the cofactor and the histone peptide substrates, which adversely influenced the SPA performance. To prevent this problem, a set of chemical agents were evaluated to eliminate the cofactor-substrate interaction, thus providing reliable SPA readings. With optimization, the SPA showed consistent and robust performance for HAT activity measurement and HAT inhibitor evaluation. Overall, this mix-and-measure assay does not require any washing procedure, can be utilized in the microplate format, and is well suited for high-throughput screening of HAT chemical modulators.

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S. mutans GTF: A Novel Target for the Prevention of Dental Caries

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Streptococcus mutans has been recognized as the major etiological agent in the initiation and advancement of dental caries. Through the use of its extracellular glucosyltransferases (GTFs), S. mutans metabolizes sucrose into insoluble- and soluble- glucans, which play a critical role in the formation of the exopolymeric matrix of dental biofilms. Current marketed mouthwashes kill pathogenic as well as commensal bacterial species. We target S. mutans GTFs to discover agents that will selectively inhibit bacterial biofilm formation without disturbing the bacterial growth. With the help of in-silico screening and synthetic studies, we have identified low micromolar inhibitors of S. mutans GTFs and biofilm without affecting the growth of S. mutans, and other commensal streptococci (S. sanguinis and S. gordonii). We are continuing to optimize the potency of the lead compound by SAR studies to successfully identify a selective anti-biofilm agent.

Elucidation of the Heme d_1 Biosynthetic Pathway

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Pseudomonas aeruginosa is an opportunistic human pathogen that causes severe nosocomial infections and is responsible for the majority of lung infections in cystic fibrosis patients. P. aeruginosa is capable of anaerobic growth using nitrate or nitrite as a terminal electron acceptor via the denitrification pathway. In this pathway, nitrite is reduced to nitric oxide by nitrite reductase (NIR). Moreover, a functional NIR is required for the virulence of P. aeruginosa. The activity of pseudomonal NIR is dependent upon the unique cofactor heme d_1 . In P. aeruginosa, at least eight genes are required for the formation of heme d_1 from uroporphyrinogen III, the last common intermediate in the biosynthesis of all tetrapyrroles, though the exact roles each of the encoded enzymes play are unknown. Siroheme has been identified as an intermediate in the heme d_1 biosynthetic pathway, and is formed from uroporphyrinogen III by the action of the multifunctional enzyme, CysG. However, certain denitrifying bacteria, such as Paracoccus denitrificans, lack a homolog of cysG. An investigation using a combination of in vitro and in vivo methods is currently underway to elucidate the heme d_1 biosynthetic pathway in these organisms.

Phe-448 in Tyrosine Phenol-lyase is Involved in Catalysis by Creating Ground-state Strain

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Tyrosine phenol-lyase (TPL) catalyzes the reversible elimination of phenol from L-tyrosine. The mechanism has been proposed to involve acid/base catalysis with Tyr-71 acting as an acid to transfer a proton to the leaving group, and Arg-381, as a possible base to deprotonate the phenol, assisting in the proton transfer. Previous crystal structures have shown that the aromatic ring of the bound 3-F-L-tyrosine substrate is bent out of plane with the C_{β} - C_{γ} bond by about 20° (1). This strain is in the direction that the aromatic ring must move to reach the transition state for elimination. Conserved residues Phe-448 and Phe-449 act as wedges to force the substrate strain by steric contact (Figure 1). In the strained complex, H-bonds can form to Arg-381 and Thr-124. We have probed the role of Phe-448 in the TPL mechanism by site-directed mutagenesis to alanine and leucine. F448A and F448L TPL have near wild-type activity with S-(o-nitrophenyl)-L-cysteine and S-ethyl-L-cysteine, but only about 0.1-0.01% activity for elimination of phenol from L-tyrosine. Stopped-flow kinetic experiments show that the formation of external aldimine and quinonoid intermediates is not affected by the mutations. Thus, these mutations of Phe-448 specifically affect the step of elimination of phenol from L-tyrosine. These results support our previous proposal that substrate strain contributes to the catalytic mechanism of TPL.

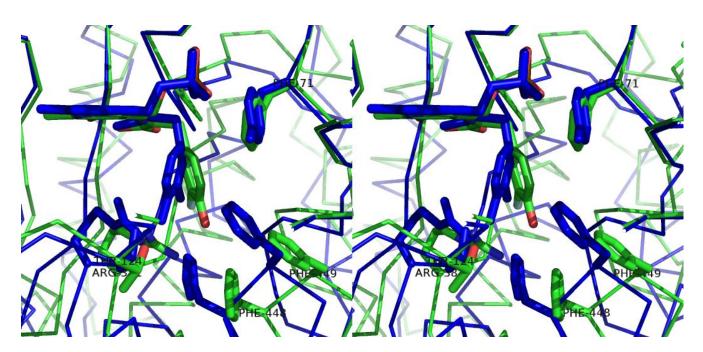


Figure 1. Overlay of open (green) and closed (blue) quinonoid intermediates of TPL complexed with 3-F-L-tyrosine.

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H Transfer Coupled to e Transfer in Nitroreductase

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Nitroreductase from Enterobacter cloacae (NR) is a member of a large family of homologues represented in all branches of the tree of life. NR has distinguished itself on the basis the diverse sizes and chemical types of substrates it is able to reduce (1). This might be an evolved characteristic suiting NR for a role in metabolism of diverse occasional toxins. While there are numerous studies of determinants of substrate specificity, we know less about mechanisms by which enzymes can be inclusive. We find that NR reduces para-nitrobenzoic acid (*p*-NBA) via a simple mechanism limited by the chemical step in which the nitro group is reduced (2). Thus, for this substrate, NR's mechanism dispenses with gating steps that in other enzymes can enforce substrate specificity (2). Our data demonstrate that substrate reduction is accomplished by rate-contributing hydride transfer from the flavin cofactor coupled to proton transfer from solvent, but do not identify specific amino acids with a role. This is consistent with our crystal structures, which reveal a spacious solvent-exposed active site bounded by a helix that moves to accommodate binding of substrate analogs (3,4). Because it is able to reduce TNT (trinitrotoluene), herbicides and pesticides, NR has important potential utility in bioremediation.

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A Thiol-specific Modification Strategy for Semi-synthesis of SUMOylated Arrestin-3

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Arrestins are proteins that serve as almost universal regulators of G-protein-coupled receptors (GPCRs). Once bound to GPCRs, Arrestins terminate the G proteins signaling, alter and redirect signaling to a variety of different pathways, and cause internalization with intracellular trafficking of GPCRs. Although the post-translational modifications (PTM) of arrestins were reported to play essential roles in mediating these regulating functions including ubiquitination, phosphorylation, acetylation and small ubiquitin-like modification (SUMO), the molecular mechanisms for arrestin's PTM remain largely unexplored. The biggest challenges are generating homogenously modified arrestins because the enzymatic reactions are not practical, due to the low efficiency and complexity of the three step reactions. To gain insight into structural and functional features, we intend to modify arrestin-3 with SUMO protein by a thiolspecific semi-synthesis strategy. The single cysteine (containing thiol group -SH) arrestin variants (K295C and K400C) are generated on the backbone of a cysteine-less arrestin protein (A3CL) by site-direct mutagenesis. Three different approaches are employed to link SUMO protein to the thiol group on arrestin-3: (1). Disulfide-directed SUMOylation; (2). SUMOaldehyde; (3). SUMO terminal alkynes. The modification of arrestin-3 will be confirmed and evaluated by Western Blot, SDS-page, and LC-MS/MS. These semi-synthesis methods could be easily applied to ubiquitin and other ubiquitin-like modifications.

Selective Inhibitors for Protein Arginine Methyltransferases

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Chromatin remodelling regulated by histone post-translational modification is a primary epigenetic event crucial to living cells. Protein arginine methyltransferases (PRMTs) as one of the major histone modifying enzymes, are the key players in various physiological processes, including cell death, cell cycle progression and RNA processing. Type I PRMTs catalyze the formation of asymmetric dimethylarginine, type II catalyze the formation of symmetric dimethylarginine. PRMT1 is a major type I that accounts for more than 85% of total methylation in cells. PRMT5 is the pre-dominant type II PRMT. They both methylate numerous nuclear and cytoplasmic substrates; some of which are postulated to drive tumor genesis. Abnormality of PRMTs are related to many pathological conditions such as cancer. To date, many reported inhibitors have limited selectivity and potency against the PRMTs. Identifying selective and potent inhibitors of PRMTs is both innovative and significant not only because of their use as chemical tools to understand function of PRMTs in epigenetics and oncology, but because they are potential therapeutics that targets PRMT up-regulation in cancer. Here we report the identification of inhibitors with low micromolar potency for specific inhibition of PRMT1 or PRMT5, by combining molecular modelling, structure activity relationship analysis, enzyme kinetic assessment and cellular studies. Our data suggest that one leading inhibitor 2,5-bis(4amidinophenyl)furan (compound 1) targets the enzyme active site and is primarily competitive with the substrate and noncompetitive toward the cofactor. Compound 1 can effectively inhibit intracellular PRMT1 activity and blocks cell proliferation of leukemia cells. This compound was further used as a probe to study cell signalling of PRMT1. Based on the structure of compound 1, we performed ligand based virtual screening and biochemical screening and identified PRMT1 or PRMT5 inhibitors with improved potency.

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Isothermal Titration Calorimetry Uncovers a Three Carbon Substrate for *Ceriporiopsis subvermispora* Bicupin Oxalate Oxidase

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Isothermal titration calorimetry (ITC) may be used to determine the kinetic parameters of enzyme catalyzed reactions in spectrophotometrically transparent solutions [1]. We report here the use of the multiple injection method of ITC to characterize the catalytic properties of oxalate oxidase (OxOx, E.C. 1.2.3.4) from Ceriporiopsis subvermispora (CsOxOx). Oxalate oxidase is a manganese dependent enzyme that catalyzes the oxygen-dependent oxidation of oxalate to carbon dioxide in a reaction that is coupled with the formation of hydrogen peroxide. CsOxOx is the first bicupin enzyme identified that catalyzes this reaction [2]. The multiple injection ITC method of measuring OxOx activity involves continuous, real-time detection of the amount of heat generated (dQ) during catalysis, which is equal to the number of moles of product produced times the enthalpy of the reaction (dH_{app}). Determination of the kinetic parameters of a reaction using this method, therefore, requires two experiments 1) determination of the enthalpy of the reaction from the complete conversion of substrate to product, and 2) determination of the differential power effects from the continuous conversion of substrate to product. Steady-state kinetic constants using oxalate as the substrate determined by multiple injection ITC are comparable to those obtained by a continuous spectrophotometric assay in which H₂O₂ production is coupled to the horseradish peroxidase catalyzed oxidation of 2,2'-azinobis-(3ethylbenzthiazoline-6-sulphonic acid) [3] and by membrane inlet mass spectrometry [4]. Furthermore, we used multiple injection ITC to identify that mesoxalate (oxopropanedioic acid) is a substrate for the CsOxOx with a K_M comparable to that of oxalate.

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Bioinformatic Investigation of Oxalate Degrading Cupin Enzymes

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Nature's complex distribution of enzymes was formed over time by the duplication of genes and subsequent random mutation and selection. Sequence divergence results in newly evolved enzymes adapting the catalytic site of its predecessor to new strategies for reactant binding and chemical processing. Oxalate oxidase (OxOx) and oxalate decarboxylase (OxDC) share many structural and mechanistic features. Both oxalate oxidase are members of the cupin superfamily which displays a remarkable functional diversity and includes catalytically inactive seed storage proteins, auxin binding proteins, and nuclear transcription factors as well as sugarbinding metal-independent epimerases and metal-dependent enzymes possessing dioxygenase, decarboxylase, oxidase, synthase, isomerase, and other activities (1). OxDC is a manganese dependent bicupin enzyme that degrades oxalate to carbon dioxide and formate. The most characterized OxDC is that from Bacillus subtilis (2). Plant OxOx enzymes possess a single cupin domain and are, therefore, structurally characterized and classified as monocupin. OxOx from the white rot fungus Ceriporiopsis subvermispora is the first bicupin oxalate oxidase to be characterized. In this work, we use sequence similarity networks, partial order optimum likelihood determinations (3), and phylogenetic trees to explore shared catalytic strategies of OxOx and OxDC and to identify amino acids implicated in reaction specificity.

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Solvents, micelles, and random proteins: Are they different from computationally-designed Kemp eliminases?

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De-novo computational design of enzymes has the potential to produce significant advancements in our ability to treat pollutants and to cure diseases. To date, the most successful example of such design is represented by artificial Kemp eliminases, which can speed up the Kemp elimination of the aromatic substrate 5-nitrobenzisoxazole by seven orders of magnitude, relative to the same reaction in aqueous solution. These enzymes use carboxylates (Asp or Glu) in the active site as the general base. However, this reaction is also accelerated by at least the same factor by acetate in non-polar solvents, such as acetonitrile, suggesting that simply partitioning the aromatic substrate to relatively hydrophobic environment such as the interior of a protein might already provide large rate accelerations.

Herein, we used micelles and long-chain carboxylates and phosphates to determine the magnitude of rate acceleration on the Kemp elimination. We found that these simple systems accelerate the Kemp elimination by about four orders of magnitude. Whereas micelles possess a hydrophobic core, linear-free energy relationships indicate that the reaction takes place in an environment that is very similar to that of water, suggesting that the rate acceleration is due to positioning of the reactive groups, rather than non-specific medium effects. Because there are no specific interactions between the micelle and the substrate or the catalyst, our results suggest that loose positioning (rather than specific, enzyme-like one) can greatly accelerate the Kemp elimination.

In addition, we are investigating whether proteins that have an active site capable of accommodating the 5-nitrobenzisoxazole substrate (such as steroid-binding proteins) can accelerate the Kemp elimination. We found that, in addition to the already known Kemp eliminase activity of serum albumins, ketosteroid isomerase (KSI) significantly accelerates the Kemp elimination. Using site-directed mutagenesis, we are currently investigating whether KSI uses a carboxylate in the active site, or if the reaction proceeds through a different mechanism.

Oxidative Detoxification of Propionate 3-Nitronate by Nitronate Monoxygenase and Novel Detection of Enzymatic Products

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Propionate 3-nitronate (P3N) is a highly toxic nitro compound that is prevalent in legumes and fungi. P3N is an irreversible inhibitor of mitochondrial succinate dehydrogenase which leads to a variety of neurological disorders and even death at sufficiently high concentration. Cases of P3N poisoning have been documented in both domestic livestock and humans. Nitronate monooxygenase (NMO, E.C. 1.13.12.16) catalyzes the oxidative denitration of P3N through a single electron-transfer reaction with peroxynitro acid as an intermediate and oxygen as the final electron acceptor. After formation of the enzyme-substrate complex, a single electron is transferred from the nitro substrate to the FMN enzyme-bound cofactor. Oxygen reoxidizes the flavin cofactor and is incorporated into the product through two possible mechanisms that both show formation of 3-peroxy-nitropropanoate. This species further breaks down to the final product. Dependent upon which oxygen species reacts with radical P3N (either O2 or superoxide), two mechanisms have been proposed for the formation of either malonic acid and nitrite or malonic semialdehyde (MSA) and nitrate. In this study, kinetics, spectroscopic and mass spectrometry techniques were used to determine the products of the enzymatic oxidation of P3N by nitronate monooxygenase.

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Mechanistic and Structural Study of Bacterial Propionate 3-Nitronate Monooxygenase from *Pseudomonas* sp. Strain JS189

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Propionate 3-nitronate (P3N) is a natural toxin that irreversibly inhibits mitochondrial succinate dehydrogenase. P3N poisoning leads to a variety of neurological disorders and even death at sufficiently high concentrations (1). Up to date, no antidote has been discovered for P3N poisoning. *Pseudomonas* sp. strain JS189 was isolated for its capability to grow on P3N as the sole carbon and nitrogen source. Survival of this species was likely due to the presence of an FMN-dependent enzyme, propionate 3-nitronate monooxygenase (P3NMO), which was shown to oxidize P3N to malonic semialdehyde for energy production (2).

In this study, a mechanistic characterization of P3NMO was carried out using steadystate kinetics, pH effects, and UV-visible and time-resolved absorption spectroscopy to probe species that are relevant for catalysis. Varied relative amounts of neutral and anionic flavin semiquinones were observed after anaerobic reduction of P3NMO at different pH values, displaying a p K_a value of 6.6 for semiquinone ionization in the enzyme active site. The presence of both flavin semiquinone was detected also during enzyme turnover with P3N using a stoppedflow spectrophotometer equipped with a photodiode array. To gain insights on the active site, a homology model of P3NMO was built with PaNMO as the template (4Q4K; 59% sequence identity) by using Swiss-Model. A histidine residue at position 133 is optimally positioned for stabilization of the flavin semiquinones. This is consistent with pH profiles of the steady-state kinetics suggesting the presence of an ionizable group in the protonated state being required for maximal turnover of the enzyme with P3N as substrate.

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Glucosaminate 6-phosphate ammonia lyase from Salmonella typhimurium: A novel Pyridoxal-5'-phosphate dependent enzyme

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Salmonellosis is a foodborne pathogenic disease that affects many mammals, particularly humans; however, it has few treatments other than time, and the few treatments may be ineffective and prolong symptoms. Surprisingly, Salmonella enterica v. typhimurium shows a dependence on rare substrates, such as D-glucosaminic acid, for efficient colonization of the gut of animals. For this reason, we have studied the structure and metabolism of enzymes in S. typhimurium involved in D-glucosaminate metabolism, specifically the D-glucosaminate PTS permease (DgaABCD) and D-glucosaminic acid 6-phosphate ammonia lyase (DgaE). We have expressed and purified DgaE, and determined its properties. Sequence alignments show that DgaE is a member of the aminotransferase superfamily. The oligomeric structure of DgaE is proposed to be a dimer from gel filtration results. DgaE activity is easily followed by a coupled spectrophotometric assay, including 2-keto-3-deoxygluconate aldolase (DgaF) and lactate dehydrogenase, with NADH, at 340 nm. We have screened for possible inhibitors of DgaE. Amino acid analogues such as D-serine, and phosphorylated carbohydrate analogues such as αglycerophosphate were found to be inhibitors. In addition, we have found that inorganic phosphate and potassium ions are inhibitors of DgaE. The only way to determine the three dimensional structure of DgaE is through protein crystallography. Hence, we have recently obtained crystals of DgaE. A better understanding of the structure and mechanism of DgaE may open the way to new medicinal treatments for Salmonellosis.

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The Heme Scavenger Cell Surface Protein HtaA-CR2 in Corynebacterium diphtheriae

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Corynebacterium diphtheriae is the causative agent of diphtheria; it requires iron to survive and for virulence. Host heme can serve 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. The holo and apo forms of the protein were separated by using hydrophobic interaction columns. HtaA-CR2 forms a dimer in solution. Increased ionic strength of the buffer, extend of heme loading and the presence of a reducing agent do not change the oligomerization state. The protein thermally unfolds only in the presence of GndHCl. Raman spectroscopy indicated that the heme axial ligands in HtaA-CR2 might be a tyrosine and a histidine. According to the sequence alignment, two tyrosine and one histidine residues are fully conserved (Y361, H412 and Y490). The Y361A, H412A and Y490A proteins were constructed by site directed mutagenesis. Raman spectroscopy and thermal unfolding will be used to evaluate the mutant proteins.

Steady-State Kinetics of an Active Site Variant of D-Arginine Dehydrogenase with Serine 45 Mutated to an Alanine

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D-Arginine can be oxidized to iminoarginine by D-arginine dehydrogenase (DADH) from *Pseudomonas aeruginosa*. The enzyme uses FAD as cofactor and does not react with molecular oxygen in catalysis. Therefore, phenazine methosulfate (PMS) was used as electron acceptor *in vitro*. In the active site of the enzyme, S45 sits atop the flavin cofactor with the hydroxyl group in proximity of the C4a atom of the flavin. In the corresponding position in the active site of D-amino acid oxidase, which instead reacts with oxygen in catalysis, there is an alanine residue. Consequently, we have replaced S45 in D-arginine dehydrogenase with alanine in an attempt to confer oxygen reactivity to the enzyme.

The S45A variant of D-arginine dehydrogenase was purified to high levels. Its steady-state kinetics were investigated with arginine or leucine as substrate and PMS or oxygen as electron acceptor. In the absence of PMS, no oxidation of arginine or leucine was observed, consistent with lack of reactivity of the enzyme with oxygen in turnover. Thus, the mutation alone does not confer the ability to the enzyme to react with oxygen. At pH 9.0, the k_{cat}/K_m value of the S45A variant enzyme was $3.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, which is 10-fold smaller than that of the wild-type enzyme. In contrast, the k_{cat} value was 1.2 folds greater than wild-type DADH with a value of 204 s^{-1} . These results suggest that the lack of a hydroxyl group on the side chain of residue 45 did not alter significantly the catalytic properties of the enzyme with the physiological substrate. In contrast, the k_{cat}/K_m and k_{cat} values with leucine as substrate were 200 M⁻¹s⁻¹ and 0.2 s⁻¹, respectively, with the S45A enzyme and $3,600 \text{ M}^{-1}\text{s}^{-1}$ and 17 s^{-1} with the wild-type enzyme. This may be due to a suboptimal orientation of the D-leucine substrate in the active site of the enzyme per the effect of the mutation.

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Effects of Temperature and Pressure on the Enzyme Activity of Tryptophan Synthase

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Tryptophan Synthase is a pyridoxal 5'-phosphate dependent enzyme that catalyzes the reaction of indole-3-glycerol phosphate and L-serine to form L-tryptophan in bacteria, plants, and fungi. While the effects of different factors such as temperature, pressure and cations on catalytic properties of tryptophan synthase extracted from Salmonella typhimurium (StTrpSyn) have been extensively investigated (1,2), our interest has been turned to Photobacterium profundum, living in a deep sea environment, whose tryptophan synthase (PpTrpSyn) is predicted to have different properties. We have studied the relationships between the enzyme activity of PpTrpSyn and factors of temperature and pressure in the presence of different cations (Na⁺, K⁺) separately and compared to these of StTrpSyn. Some thermodynamic parameters have been determined. The activation energy (Eact) is lower at high temperature than low temperature which suggests that high temperature converted the enzyme from an "open" conformation to a "closed" one. Besides, the activation volume (ΔV^{\ddagger}) in pressure-activity experiment of PpTrpSyn is around half lower than StTrpSyn. As we expected, PpTrpSyn has less tolerance for high temperatures but higher tolerance with high pressure than StTrpSyn, in accordance with the environment in which P. profundum lives. The catalyticαβconformation change within the enzyme maybe tuned to the environmental conditions by changes in the amino acids at the subunit interface.

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The HIV-1 protease L76V mutation is a double-edged sword

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Untreated infection by the Human Immunodeficiency Virus (HIV) causes AIDS, with a death toll of over 39 million lives. The virus is a global threat to public health since over 35 million people are infected with HIV worldwide. The cocktail of anti-HIV drugs used in Highly Active Antiretroviral Therapy (HAART) may halt viral replication and deter disease progression into AIDS. However, the virus has an inherently high rate of mutation, which allows for the rapid evolution of drug resistant variants. One key drug target is the virally-encoded protease, which is essential for the maturation step in viral replication. Resistance-associated mutations occur in the active site, the flaps, the dimer interface, or the hydrophobic core of the protease. Here, the effect of the substitution of leucine 76 with valine within the hydrophobic core is analyzed by X-ray crystallography. Mutation L76V is associated with reduced dimer stability, delayed autoprocessing, and resistance to certain drugs while having increased susceptibility towards others (1). Previous structural analyses suggested that the L76V mutant is resistant to Darunavir but more susceptible to Saguinavir due to alterations in inhibitor-protease polar interactions at the active site as well as changes in the network of hydrophobic interactions surrounding residue 76 (1). The present study focuses on another set of inhibitors; while the L76V protease is resistant to Lopinavir, Tipranavir may be more potent against the mutant. Although the mutation does not alter interactions between the two inhibitors and residues at the active site of the protease, a change in van der Waals interactions surrounding residue 76 is observed. Many hydrophobic contacts are lost with nearby residues, while new ones are made with two neighboring valines. This analysis will help in the design of novel inhibitors for resistant virus.

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QM/MM Simulations of DFP and (S)-Sarin Hydrolysis by DFPase: Implications for Engineering Bioscavengers

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Organophosphorus (OP) nerve agents such as (S)-sarin are among the most highly toxic compounds that have been synthesized. Engineering enzymes that catalyze the hydrolysis of nerve agents ("bioscavengers") is an emerging prophylactic approach to diminishing their toxic effects. Although its native function is not known, disopropyl fluorophosphatase (DFPase) from Loligo vulgaris catalyzes the hydrolysis of OP compounds. Here, we investigate the mechanisms of diisopropylfluorophosphate (DFP) and (S)-sarin hydrolysis by DFPase with quantitativelyaccurate quantum mechanical/molecular mechanical (QM/MM) umbrella sampling simulations. We find that the mechanism for hydrolysis of DFP involves nucleophilic attack by Asp229 on phosphorus to form a pentavalent intermediate. P—F bond dissociation then yields a phosphoacyl enzyme intermediate in the rate-limiting step. The simulations suggest that a water molecule, coordinated to the catalytic Ca²⁺, donates a proton to Asp121 and then attacks the tetrahedral phosphoacyl intermediate to liberate the diisopropylphosphate product. In contrast, the calculated free energy barrier for hydrolysis of (S)-sarin by the same mechanism is highly unfavorable, primarily due to the instability of the pentavalent phosphoenzyme species. Instead, simulations suggest that hydrolysis of (S)-sarin proceeds by a mechanism in which Asp229 could activate an intervening water molecule for nucleophilic attack on the substrate. These findings may lead to improved strategies for engineering DFPase and related six-bladed β-propeller folds for more efficient degradation of OP compounds [1].

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Development of A Photoswitchable Diamidine Compound for Protein Arginine Methyltransferase 1

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The protein arginine methylation is an arginine modification process which adopts *S*-adenosyl-L-methionine (SAM or AdoMet) as a methyl donor to transfer one or two methyl groups to guanidino nitrogen of arginine residues. It is catalyzed by protein arginine methyltransferase (PRMTs) and PRMT1 is the predominant type of PRMTs (>85%) in vivo. To decipher the biological roles of PRMT1 and its potential in human diseases, it is highly desirable to develop chemical probes specific to PRMT1 with minor disturbance to others. Previously we have reported two PRMT1 selective inhibitors (DB75 and stilbamidine). Based on these compounds, here we designed, synthesized and characterized a new azo compound, namely azastilbamidine. The cis/trans conversion of this compound as a function of light illumination is supposed to be capable of switching on/off PRMT1 inhibition. This compound may provide a useful pharmacological tool for easily optical inhibition of PRMT1, and it may also be useful to discriminate different functionalities between PRMT isoforms.

Effect of osmotic stress on enzymes of the folate pathway

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Folate (Vitamin B9) is involved in one carbon transfer reactions required for the synthesis of important macromolecules such as DNA and amino acids. Since humans cannot synthesize folate, malfunctioning of the enzymes involved in folate metabolism can lead to various diseases. In addition, enzymes in the folate metabolic pathway are potential drug targets in bacteria. Current understanding of the folate pathway is mostly based on in vitro studies, which are very different from the crowded environment that exists in the cell. *E.coli* is known to produce osmoprotectants, such as glycine betaine, 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 of the folate pathway. We hypothesize that increased osmolyte concentration in the cell will also prevent the functioning of the folate pathway enzymes by interaction of osmolytes with the folate pathway intermediates. Here we studied the effect of osmolytes on the enzymes Methylenetetrahydrofolate reductase (metF) and serine hydroxymethyltransferase (glyA) required for methionine and glycine synthesis respectively in E. coli. Studies were done with knockout and rescue strains for metF and glyA. The knockout mutants were restored to prototrophy by addition of folate end products while the enzyme was produced under tetracycline control in rescue strains. Osmotic stress studies indicated that the rescue strain grew under lower osmolality conditions as compared to knockout strains. We predict that this is due to an increase in osmolyte concentration in vivo which leads to interaction of osmolytes with folate intermediates in the pathway.

Characterization of heme metabolism in Streptococcus pyogenes

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Abstract

Group A streptococcus (GAS) is a Gram-positive human pathogen that causes infections of skin and mucosal membranes, resulting in mild to severe manifestations. Heme is the major iron source for GAS during infection. While heme uptake and breakdown are essential for pathogenesis, the mechanism of heme catabolism in GAS remains unknown. Here we describe an iron-regulated gene in GAS, which we named *hupZ* for <u>Heme Utilization Protein Z</u>. We constructed and characterized a *hupZ* mutant in which the *hupZ* gene was inactivated. In excess, heme is bacteriostatic and damages the cell envelope in GAS. Growth analysis revealed that the *hupZ* mutant is hypersensitive to heme and is severely attenuated for growth in blood. Here, we describe biochemical and spectroscopic analyses of the HupZ protein. In summary, we have identified a protein in GAS that is important for GAS growth in blood and heme tolerance. To the best of our knowledge, this is the first protein among *Streptococcus* species with such activity.

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