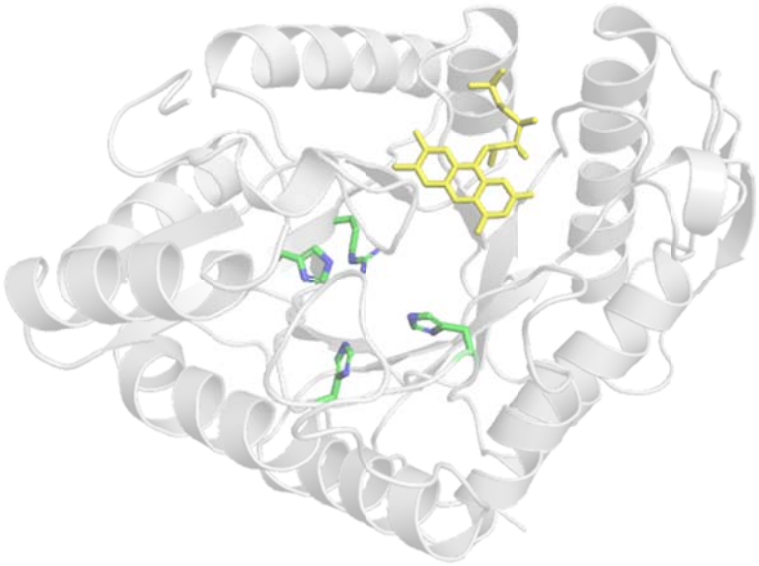
Fourth Southeast Enzyme Conference



Saturday, April 20, 2013

Georgia State University
Atlanta, GA

Urban Life Building
College of Law
140 Decatur Street
Room 220

Fourth Southeast Enzyme Conference

Saturday, April 20, 2013

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Southeast Enzyme Conference (SEC)

Meeting	Year	Program Chair	Site Chair	Site
I	2010	Giovanni Gadda	Will Lovett	GSU
II	2011	Nigel Richards	Giovanni Gadda / Will Lovett	GSU
III	2012	Robert Phillips	Giovanni Gadda / Will Lovett	GSU
IV	2013	Holly Ellis	Giovanni Gadda / Neil Renfroe / Will Lovett	GSU
V	2014			

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8:30-8:50 Ebony Miller, Clark Atlanta University, Atlanta
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11:20-11:40 Michael Duff, University of Tennessee, Knoxville
Effects of Molecular Crowding on the Binding Affinity of Dihydrofolate for Dihydrofolate Reductase

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Computational, Structural and Kinetic Evidence that Vibrio vulnificus FrsA is not a Cofactor-Independent Pyruvate Decarboxylase

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Biochemical evaluation of the decarboxylation and decarboxylation-deamination activities of plant aromatic amino acid decarboxylases

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Extreme redox tuning from the dawn of aerobic metabolism

3:30-3:40 Concluding Remarks – **Holly R. Ellis**, Auburn University, Auburn

Session 1:

John Haseltine

Chair

Investigation of Substrate Oxidation in Choline Oxidase through Further Mutagenesis of Histidine Residues[†]

Crystal Smitherman[‡] *and Giovanni Gadda*[‡§⊥]

Departments of [‡]Chemistry and [§]Biology, and [⊥]The Center for Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30302-4098

Choline oxidase (CHO, E.C. 1.1.3.17; choline-oxygen 1-oxidoreductase) catalyzes the oxidation of choline to glycine betaine through a two-step, four electron reaction with betaine aldehyde as an intermediate and oxygen as the final electron acceptor [(1)]. The first catalytic step is the removal of the hydroxyl proton from the choline substrate by a yet unidentified active site residue with a pK_a value of ~7.5 [(1)]. The conserved histidine residue located at position 466 was previously considered and mutated to alanine or aspartate [(2, 3)]. The alanine mutation revealed the importance of the histidine in the stabilization of the transition state and the overall microenvironment in the active site [(2)]. However, pH-profiles were consistent with the base still being present in the mutated enzyme. The incorporation of a negative charge with the aspartate mutation caused a loss in catalytic function and no stabilization of the flavosemiquinone [(3)].

In this study, His466 was replaced with glutamine. The rate of flavin reduction in the His466Gln mutant enzyme was measured to be 6 orders of magnitude slower than the rate measured with the wild type enzyme. The mutant enzyme was also analyzed spectroscopically using fluorescence, CD, and NMR to elucidate the overall fold of the protein in comparison with the wild type enzyme. These analyses showed that the change in the rate of flavin reduction upon mutating histidine to glutamine was not due to denatured or misfolded protein. The results presented here are consistent with His466 being the catalytic base required for the activation of choline. They also demonstrate that careful selection of the amino acid replacement is crucial when applying mutagenesis to answer mechanistic questions on enzyme function.

1. Fan, F., and Gadda, G. (2005) On the catalytic mechanism of choline oxidase, *J. Am. Chem. Soc. 127*, 2067-2074.
2. Ghanem, M., and Gadda, G. (2005) On the catalytic role of the conserved active site residue His466 of choline oxidase, *Biochemistry 44*, 893-904.
3. Ghanem, M., and Gadda, G. (2006) Effects of reversing the protein positive charge in the proximity of the flavin N(1) locus of choline oxidase, *Biochemistry 45*, 3437-3447.

[†]This work was supported in part by NSF MCB-1121695.

The use of bacterial cellulose from Acetobactor.xylinum for the application of immobilizing different compounds to enhance overall functionality.

Ebony Miller, Sharifeh Mehrabi, David Logan, and Eric Mintz

Department of Chemistry, Clark Atlanta University Atlanta, Ga 30314

Cellulose is the most abundant biopolymer on earth, recognized as the major component of plant biomass, but also a representative of microbial extracellular polymers. Bacterial cellulose (BC) belongs to specific products of primary metabolism and is mainly a protective coating. Bacterial cellulose is synthesized by bacteria belonging to the genera *Acetobacter, Rhizobium*, *Agrobacterium*, and *Sarcina*; however, its most efficient producers are Gram-negative, acetic acid bacteria *Acetobacter.xylinum* (1). This biopolymer is being cultivated for the immobilization of the biocatalyst laccase, horseradish peroxidase and the photo catalyst TiO_2, for use in water purification. Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) is an enzyme containing 4 catalytic copper atoms that catalyzes the one electron oxidation of phenolic compounds coupled with the four electron reduction of molecular oxygen (2). TiO_2 in water gets activated by UV light which produces ROS that can oxidize organic compounds. Immobilization increases the overall functionality, reusability, and stability of catalysts. Immobilization of the laccase enzyme has usually been achieved by chemically modifying the surface by tethering the enzyme, usually via gluteraldehyde. The process used in this study produces the cellulose in the presence of the bio and photo catalysis, incorporating the catalysts into the fibers of the cellulose as it grows. The stability and activity of the immobilized bio catalase laccase was measured by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or (ABTS). To measure the efficiency of the immobilized photo catalysis TiO_2, water contaminated with *Candida.albicans* was tested for inactivation after 22 h of exposure under UV light. These experiments indicate that bacterial cellulose is a good support system for catalysis and could be used as a support system for various types of catalysis without using added linking reagents to the system.

1. Prof. Dr. Eng. Stanislaw Bielecki, Dr. Eng. Alina Krystynowicz, Prof. Dr. MariannaTurkiewicz, Dr. Eng. Halina Kalinowska. Bacterial Cellulose Review.
2. Adinarayana Kunamneni, Antonio Ballesteros, Francisco J. Plou and Miguel Alcalde. Fungal laccase – a versatile enzyme for biotechnological applications. Communicating Current Research and Educational Topics and Trends in Applied Microbiology.

Kinetic Studies of α-Isopropylmalate Synthase from *Methanococcus jannashii*

Garima Kumar and Patrick A. Frantom

Department of Chemistry, The University of Alabama, Tuscaloosa, Alabama, 35487

α-Isopropylmalate synthase (IPMS) is a model enzyme for the study of allosteric mechanisms as it catalyzes the first step in leucine biosynthesis in archae, bacteria and some eukaryotes. α-Isopropylmalate synthase is subject to feedback inhibition by L-leucine through an allosteric mechanism and contains evolutionarily conserved catalytic and regulatory domains. Recent phylogenetic analysis suggests two origins of IPMS. The most characterized IPMS with regard to allostery is from *Mycobacterium tuberculosis* (*Mt*IPMS). To investigate conservation (or lack of these) of allosteric mechanisms, the allosteric properties of an IPMS predicted to have come from an alternate origin need to be compared with those determined for MtIPMS. Here, IPMS from *Methanococcus jannashii* (*Mj*IPMS), a thermophilic archae, is being studied. Kinetic parameters for MjIPMS determined at 37°C exhibited a k_{cat} = 190 ± 10 min^{-1}, K_{AcCoA} = 100 ± 10 μM and K_{KIV} = 170 ± 20 μM. Kinetic parameters determined at 55°C matched well with the values at 37°C with the exception of the k_{cat} value being 450 ± 30 min^{-1}. It was determined that *Mj*IPMS exhibits *V*-type inhibition by leucine similar to *Mt*IPMS with a K_i = 85 ± 7 nM. Also similar to *Mt*IPMS, leucine has no effect on the quaternary structure of *Mj*IPMS as determined by size-exclusion chromatography. However, *Mj*IPMS appears to be a tetramer whereas *Mt*IPMS is a dimer. This suggests allosteric mechanism is independent of quaternary structure for IPMS enzymes.

Synthesis and Evaluation of Inhibitors Selective for Tryptophan Indole-Lyase and not Tryptophan Synthase. Toward the Development of Anti-biofilm Formation.

Quang T. Do,[†] Giang T. Nguyen,[†] Victor Celis,[†] and Robert S. Phillips[†,§]

[†] Department of Chemistry, University of Georgia, Athens, Georgia 30602

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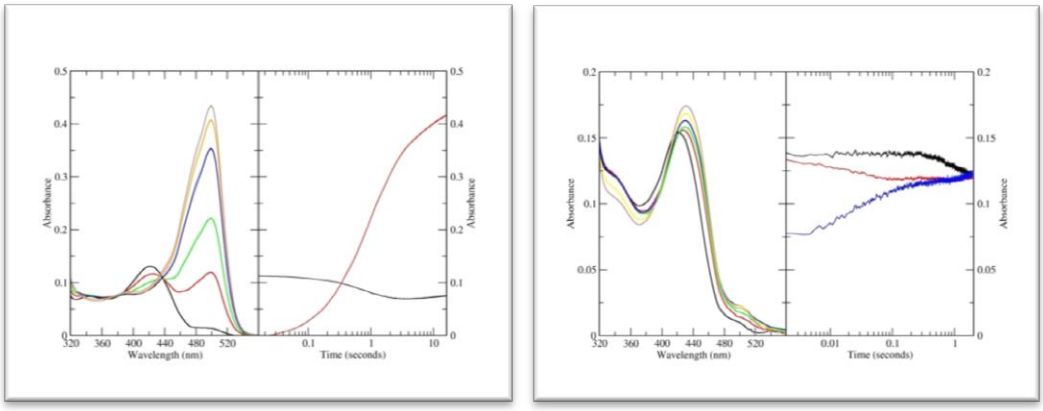

Abstract

Biofilm is a structured community of bacteria enclosed within a self-produced polysaccharide polymeric matrix that generally showed higher resistance to chemical and biological attack than in their planktonic state. Biofilm formation has a negative impact on the operability of machinery in industries, the environment, as well as our healthcare system due to bacterial infections. Recent reports suggested that tryptophan indole-lyase (TIL, tryptophanase, EC 4.1.99.1), a pyridoxal-5'-phosphate (PLP) dependent bacterial enzyme as a promising and selective target for anti-biofilm formation and antibiotic resistance. As previously suggested by our work that quinonoid is an intermediate in the physiological reaction of TIL, we anticipated that homologation of the physiological substrate, L-tryptophan (Trp), at the α-carbon would provide analogues resembling the transition state and potentially inhibits the activity of TIL. The work herein discussed the design, synthesis and evaluation of L-homotryptophan (**1a**) and L-bishomotryptophan (**1b**) as inhibitors for TIL. Our results indicated that **1a** is a moderate inhibitor of TIL with Ki = 178 μM, whereas **1b** displayed potent inhibition with Ki = 6.8 μM. Results from rapid-scanning stopped-flow experiments indicated the formation of an external aldimine and quinonoid with **1a** but only the formation of an external aldimine for **1b**, indicating their mechanistic differences in inhibition for TIL. In addition, Trp analogues evaluated as efficient inhibitors of TIL were also previously reported to display efficiency toward inhibition of Trp synthase, an enzyme widely distributed in bacteria, fungi and plants. Our results indicated that **1a** and **1b** are highly selective toward TIL than Trp synthase. We wish to report that **1b** is the first potent inhibitor with high selectivity for TIL, suitable for the approach toward development of an antibacterial treatment.

Acknowledgement: QTD would like to thank the University of Georgia for support of this work through the Innovative and Interdisciplinary Research Grant for Doctoral Students.

Session 2:

Victor Davidson

Chair

Circular Permutation of Old Yellow Enzyme: Characterization of a Complete Synthetic Library

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Departments of [‡]Chemistry and [§]Biochemistry, Emory University, Atlanta, GA 30322

Old yellow enzyme from *Saccharomyces pastorianus* (OYE1) is an FMN containing NAD(P)H dependent oxidoreductase capable of catalyzing the trans-hydrogenation reaction on a variety of activated alkenes. These enzymes offer a valuable alternative to synthetic catalysts affording chemical building blocks useful in pharmaceutical and biotech applications. Although this enzyme has been in the spotlight for its role in reduction chemistry, OYE1 could benefit from successful engineering strategies towards generating designer catalysts with enhanced activity and enantioselectivity for desired substrates. We employed circular permutation (CP) to engineer OYE1, as the technique has been shown in the past to improve catalytic activity as a result of termini relocation in loop and secondary structures near the active site environment. The OYE1 circular permuted library was directly synthesized using a whole gene synthesis strategy and assayed against several α,β- unsaturated alkene substrates utilizing the PURExpress *in vitro* transcription/translation (IVT) technology, demonstrating a novel screening approach. Library evaluation using our IVT screening platform successfully identified ~30% of the OYE1 library having better activity than wild type. The results also clearly highlight three specific sectors in the OYE1 scaffold responsible for the most dramatic alterations in catalytic activity for all substrates evaluated. In addition to the changes in substrate specificity as a result of permutation, we are also exploring the substitution of FMN with cofactor analogs in CP variants as an orthogonal engineering strategy. We have demonstrated alterations in catalytic activity as well as the catalysis of a different reaction as a result of the change in redox potential via tandem effects through both cofactor substitution and permutation.

Effects of Molecular Crowding on the Binding Affinity of Dihydrofolate for Dihydrofolate Reductase

Michael Duff,[‡] Nidhi Desai,[‡] Greyson Dickey[‡] and Elizabeth Howell[‡]

[‡]Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, TN 37994

The reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR), using NADPH as a cofactor, is an essential part of the folate cycle. The product, THF, is necessary for the synthesis of methionine, purine nucleotides, thymidylate, and other compounds. Thus, the inhibition of DHFR leads to interruption of DNA synthesis and consequently cell death, making this enzyme a crucial target in the treatment of cancer and other diseases. Previous studies examined the effects of small molecule osmolytes on the substrate interactions with two non-homologous DHFRs, *E. coli* chromosomal DHFR (EcDHFR) and R67 DHFR, with vastly different active site structures. The results indicated that DHF weakly interacts with the osmolytes in solution, shifting the binding equilibrium from DHF bound to DHFR to unbound DHF. It is hypothesized that similar weak, nonspecific interactions may also occur between cellular proteins and DHF. Weak interactions between cellular proteins and DHF would have consequences *in vivo*, where the concentration of the cellular milieu is approximately 300 g/L. Under the crowded conditions in the cell, there is a higher propensity for intermolecular interaction.

Crowding effects of macromolecules in concentrations similar to those *in vivo* were examined. Isothermal titration calorimetry (ITC) and enzyme kinetic assays were used to detect effects of molecular crowders by monitoring activity of the (DHFR)•NADPH or DHF complex and the ternary DHFR•DHF•NADPH complex in the presence of these crowders. To recreate the conditions of molecular crowding *in vivo,* the binding of the enzyme-ligand complexes in the presence of molecular weight crowding agents (lysozyme or casein) was examined. Analysis of the K_d's and K_m's indicated a correlation between increased molecular crowding in the solution and weakened binding of the DHFR•substrate complexes. These findings indicate an importance of molecular crowding on EcDHFR activity *in vivo.*

Computational, Structural and Kinetic Evidence that *Vibrio vulnificus* FrsA is not a Cofactor-Independent Pyruvate Decarboxylase

Whitney F. Kellett[‡], Elizabeth Brunk[§], Bijoy J. Desai[#], Alexander A. Fedorov[⊥], Steven C. Almo[⊥], John A. Gerlt[#], Ursula Rothlisberger[§] and Nigel G. J. Richards[‡]

[‡]Department of Chemistry and Chemical Biology, Indiana University Purdue University Indianapolis, Indianapolis, IN 46202, USA; [§]Laboratory of Computational Chemistry and Biochemistry, Ecole Polytechnique Fédérale Lausanne, CH-1015 Lausanne, Switzerland; [#]Departments of Biochemistry and Chemistry, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA; [⊥]The Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

The fermentation-respiration switch (FrsA) protein in *Vibrio vulnificus* was recently reported to catalyze the cofactor-independent decarboxylation of pyruvate (*1*). We now report QM/MM calculations that examine the energetics of C-C bond cleavage for a pyruvate molecule bound within the putative active site of FrsA. These calculations suggest that the barrier to C-C bond cleavage in the bound substrate is 28 kcal/mol (*2*), which is similar to that estimated for the uncatalyzed decarboxylation of pyruvate in water at 25 ^{o}C. In agreement with the theoretical predictions, no pyruvate decarboxylase activity was detected for recombinant FrsA protein that could be crystallized and structurally characterized. Indeed, the X-ray crystal structure, determined at 1.95 Å resolution, of the protein prepared by us was essentially identical to that reported earlier except an unknown ligand, modeled as hexanoate, was located in the putative active. These results suggest that the functional annotation of FrsA as a cofactor-independent pyruvate decarboxylase is incorrect.

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2. Kellett, W. F., Brunk. E., Desai, B. J., Fedorov, A. A., Almo, S. C., Gerlt, J. A., Rothlisberger, U., and Richards, N. G. J. (2013) Computational, structural and kinetic evidence that *Vibrio vulnificus* FrsA is not a cofactor-independent pyruvate decarboxylase, *Biochemistry 52*, 1842-1844.

Biochemical evaluation of the decarboxylation and decarboxylation-deamination activities of plant aromatic amino acid decarboxylases

Michael P. Torrens-Spence[‡], Pingyang Liu[‡], Haizhen Ding[‡], Kim Harich[‡], Glenda Gillaspy[‡], and Jianyong Li[‡]

Department of Biochemistry[‡], Engel Hall 204, Virginia Tech, Blacksburg, VA 24061, USA.

Plant aromatic amino acid decarboxylase (AAAD) enzymes are capable of catalyzing either decarboxylation or decarboxylation-deamination on various combinations of aromatic amino acid substrates. These two different activities result in the production of arylalkylamines and the formation of aromatic acetaldehydes respectively. Variations in product formation enable individual enzymes to play different physiological functions. Despite these catalytic variations, arylalkylamine and aldehyde synthesizing AAADs are indistinguishable without protein expression and characterization. In this study, extensive biochemical characterization of plant AAADs was performed to identify residues responsible for differentiating decarboxylation AAADs from aldehyde synthase AAADs. Results demonstrated that a tyrosine residue located on a catalytic loop proximal to the active site of plant AAADs is primarily responsible for dictating typical decarboxylase activity whereas a phenylalanine at the same position is primarily liable for aldehyde synthase activity. Mutagenesis of the active site phenylalanine to tyrosine in *Arabidopsis thaliana* and *Petroselinum crispum* aromatic acetaldehyde synthases primarily converts the enzymes activity from decarboxylation-deamination to decarboxylation. The mutation of the active site tyrosine to phenylalanine in the *Catharanthus roseus* and *Papaver somniferum* aromatic amino acid decarboxylases changes the enzymes decarboxylation activity to a primarily decarboxylation-deamination activity. Generation of these mutant enzymes enables the production of unusual AAAD enzyme products including indole-3-acetaldehyde, 4-hydroxyphenylacetaldehyde, and phenylethylamine. Our data indicates that the tyrosine and phenylalanine in the catalytic loop region could serve as a signature residue to reliably distinguish plant arylalkylamine and aldehyde synthesizing AAADs. Additionally, the resulting data enables further insights into the mechanistic roles of active site residues.

Session 3:

Holly Ellis

Chair

Extreme redox tuning from the dawn of aerobic metabolism

Anne-Frances Miller[§‡], Carrie Vance[§], Ting Wang[‡], Emine Yikilmaz[‡§]

Dept. of Chemistry [‡]University of Kentucky, Lexington KY and [§]The Johns Hopkins University, Baltimore MD

A crucial turning point in the chemical evolution of life was acquisition of the capacity to exploit water as the electron source for photosynthetic capture of solar energy. The success of oxygenic photosynthesis radically changed the chemical nature of most of the habitable space on earth. As arrogant heirs to these events, it is worth noting that no known extant aerobe lacks a superoxide dismutase, and most possess several, in addition to catalase(s), superoxide reductase, peroxidase(s) and additional antioxidant devices. The urgency of the need to be able to detoxify superoxide is evinced by the fact that superoxide dismutase (SOD) evolved on at least three independent occasions. The most ancient of the SODs is believed to be the Fe-dependent SOD (FeSOD) whose descendants include the FeSOD and MnSOD of *E. coli*, as well as the MnSOD of human mitochondria.(*1*) The fact that higher organisms and the organelles that produce the most superoxide now use $Mn^{3+/2+}$ as the catalytic metal ion makes excellent chemical sense given that Fe catalyzes deleterious Fenton chemistry under conditions of oxidative stress. However the very chemical property that makes $Mn^{3+/2+}$ less toxic requires that the SOD protein exert very different redox tuning in order to make $Mn^{3+/2+}$ active in disproportionation of superoxide. Our research has shown that for the *E. coli* enzymes, the protein features needed to make $Mn^{3+/2+}$ active preclude SOD activity for $Fe^{3+/2+}$. Thus, evolution has had to modify the protein of a vital enzyme without ever loosing function in order to acquire independence from Fe and tolerance to O_2. Since the major biochemical differences between $Fe^{3+/2+}$ and $Mn^{3+/2+}$ are the reduction midpoint potential of the metal ion and the Lewis acidity of coordinated water, we have sought to understand how the SOD proteins tune these properties of the active site metal ion. Based on a synergistic combination of electronic spectroscopy of the metal ion, thermodynamic characterizations of individual events in turnover, and mutagenesis to alter key protein residues, we provide a proton-mediated modulation of redox reactivity (PMRR) mechanism able to explain the half-V difference in redox tuning exerted by MnSOD protein vs. FeSOD protein,(*2, 3*) and propose a path by which the ancestral FeSOD could have acquired Mn-based catalytic activity.

1. Miller, A.-F. (2012) Superoxide dismutase: Ancient enzymes and new insights., *FEBS Lett. 586*, 585-595.
2. Miller, A.-F. (2008) Redox tuning over almost 1 V in a structurally-conserved active site: lessons from Fe-containing superoxide dismutase, *Acc. Chem. Res. 41*, 501-510.
3. Vance, C. K., and Miller, A.-F. (1998) A simple proposal that can explain the inactivity of metal-substituted superoxide dismutases., *J. Am. Chem. Soc. 120*, 461-467.

Abstracts for Poster Presentations:

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

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66. Bennett, Elizabeth
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Kinetic Analysis of D-Arginine Dehydrogenase by pH and Inhibition Effects

Jacob Ball [α], *Swathi Gannavaram* [α], *and Giovanni Gadda* [α,β,γ]
Department of [α] Chemistry, [β] Biology, [γ] The Center for Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30303

D-Arginine Dehydrogenase (DADH) was purified from *Pseudomonas aeruginosa*, a Gram-negative pathogenic bacterium linked to disease acquired in hospitalized patients.[1] *P. aeruginosa* utilizes DADH to catalyze the amine oxidation of D-arginine to iminoarginine, which decomposes in solution to form 2-keto arginine and ammonia. The products of the DADH reaction are converted to useable L-arginine by a coupled anabolic NAD(P)H-dependent L-arginine dehydrogenase.[2] The enzyme exhibits preference for substrates harboring a positively charged side chain, as well as large, hydrophobic residues to a lesser extent.[3]

The effect of pH on the steady-state kinetic parameters with D-arginine and D-lysine as substrates has been investigated here to determine the differences in the pK_a values related to the kinetic parameters in each reaction mechanism. Derivation of the kinetic rate constant equations has revealed which steps are evident in catalysis and substrate binding. L-arginine has been tested as a competitive inhibitor to determine intrinsic pK_a values corresponding to the binding of D-arginine that belong to ionizable groups on the substrate or the enzyme. The pK_a values were then assigned taking into consideration the published X-ray crystal structures of the enzyme in complex with bound products of the enzymatic reaction.[4]

1. Bennet, J.V., (1974) Nosocomial Infections Due to Pseudomonas, *J. Infect. Dis. 130*, S4-S7
2. Li, C., and Lu, C.-D. (2009) Arginine racemization by coupled catabolic and anabolic dehydrogenases, *Proc. Nati. Acad. Sci. U.S.A. 106*, 906-911.
3. Yuan, H., Fu, G., Brooks, P. T., Weber, I., and Gadda, G. (2010) Steady-state kinetic mechanism and reductive half-reaction of D-arginine dehydrogenase from *Pseudomonas aeruginosa*, *Biochemistry 49*, 9542-9550.
4. Fu, G., Yuan, H., Li, C., Lu, C. D., Gadda, G., and Weber, I. T. (2010) Conformational changes and substrate recognition in Pseudomonas aeruginosa D-arginine dehydrogenase, *Biochemistry 49*, 8535-8545.

This study was supported in part by NSF MCB-1121695 (G.G.), a GSU Molecular Basis for Disease Summer Fellowship (J.B.), and a GSU Molecular Basis for Disease Fellowship (S.G.).

The incorporation of near infrared (NIR)-functionalized probes in the development of a rapid bioanalytical technique to monitor the catalytic activity of alkanesulfonate monooxygenase using Laser Induced Fluorescence Capillary Zone Electrophoresis (LIF-CZE).

Garfield Beckford[1], Maged Henary[1], Holly Ellis[2] and Gabor Patonay[1]
[1] Georgia State University, University Plaza, Atlanta, GA, 30303
[2] Auburn University, Department of Chemistry and Biochemistry, 179 Chemistry Building, Auburn, AL, 36849

A new bioanalytical technique to detect and monitor the catalytic activity of the sulfur assimilating enzyme; alkanesulfonate monooxygenase (EC 1.14.14.5) is described for the first time. In this spectroscopic bioanalytical assay a group of fischer based *n*-butyl sulfonate substituted dyes that exhibit distinct variation in absorbance and fluorescence properties and strong binding to serum albumin was identified. In polar solvents, these soluble compounds are strongly fluorescence [1, 2], however form the less soluble aggregated species with virtual loss of fluorescence when the sulfonate groups are cleaved by the enzyme to form the corresponding straight chain aldehyde derivatives. Upon binding to the serum albumin the dyes again become fluorescent. This protein-ligand binding relationships, along with changes to the absorbance and emission of the different forms of the fluorophores are key factors in developing this protocol. This new technique promises quick, portable, sensitive, reliable and the possibility of 'on-the-spot' detection, advantages not readily realized with other commonly used techniques such as polymerase chain reaction (PCR) and Surface Plasmon Resonance (SPR). Our goal is to further develop and optimize this spectroscopic assay to detect the enzyme activity *in vitro* with the possibilities of *in vivo* application.

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Osmolyte effects on binding of ligand to FolM

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Abstract
FolM is a chromosomally encoded protein that belongs to the pteridine reductase family that reduces various dihydropterins to the tetrahydro state. Though it has high efficiency for catalyzing reduction of pteridine molecules, it also shows a low dihydrofolate reductase activity. It can reduce dihydrofolate (DHF) to tetrahydrofolate (THF) using NADPH as a cofactor and can play a role in FolA deficient strains. FolM is a tetrameric protein with four active sites. It forms the ternary complex with NADPH binding to FolM prior to DHF. It is genetically unrelated to either *E.coli* chromosomal or plasmid encoded R67 dihydrofolate reductase DHFR). It is less sensitive to methotrexate, which is an anti cancer drug, compared to chromosomal DHFR.
The binding of substrate and co-factor to chromosomal DHFR and R67 DHFR has been studied. The role of water in binding has been established by addition of small molecule osmolytes that perturb water activity. The presence of osmolytes alters binding affinities. In presence of osmolytes, NADPH binds tightly suggesting water release whereas DHF binds weakly indicating water "uptake", which is unusual. Weaker binding of DHF indicates that it may be interacting with the osmolytes and therefore, there is a competition between the osmolyte and DHFR molecules to bind to DHF. This hypothesis can be extended to other proteins from the folate metabolism pathway for e.g. FolM.
Isothermal titration calorimetry studies have shown similar osmolyte effects on binding of NADPH to FolM. Different osmolytes like betaine, DMSO, ethylene glycol and glycerol were used. The number of water molecules released or up taken was also calculated. The preliminary results indicate tighter NADPH binding to FolM in presence of betaine and DMSO whereas weaker binding in presence of ethylene glycol and glycerol.

Determining the Influence of the Metal-Binding Site in Isopropylmalate Synthase from *Mycobacterium tuberculosis*

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The enzymes isopropylmalate synthase (IPMS), homocitrate synthase (HCS), and 3-hydroxy-3-methylglutaryl-coenzyme A lyase (HMG-CoA lyase) belong to the DRE-TIM metallolyase superfamily. Isopropylmalate synthase catalyzes a condensation reaction between α-ketoisovalerate and acetyl-CoA to form isopropylmalate and CoA. IPMS requires a divalent metal cation, bound to two histidine residues and a single aspartate residue (Figure 1). The metal ion is essential for catalysis as it is proposed to polarize the α-keto group for nucleophilic attack. An adjacent metal binding site approximately 2Å away is found in the crystal structures of IPMS from *Mycobacterium tuberculosis* (MtIPMS) and HGM-CoA lyase in which the metal is liganded by an additional asparagine residue (Asn 321 in MtIPMS, shown in Figure 1). It is not clear whether this adjacent binding mode is an artifact or is required for catalysis. In HCS, the adjacent binding site is not present because the asparagine and aspartate residues are replaced with conserved isoleucine and glutamate residues, respectively. In order to determine the importance of the adjacent metal binding site for MtIPMS, site-directed mutagenesis was used to perform alanine scanning on the metal binding site residues, as well as additional variants that mimic the HCS binding site. None of the enzyme variants catalyzed the condensation reaction of MtIPMS, but rather catalyzed the hydrolysis of AcCoA, a side reaction of MtIPMS. In two of the variants (N321A and D81E) the hydrolysis is inhibited by the presence of Mg^{2+} with apparent inhibition constants similar to that determined for the WT enzyme. In addition, apparent K_i values (2-10 mM) are similar to K_{act} (3.6 ± 0.4 mM) for Mg^{2+} in the WT enzyme suggesting that the two enzyme variants do not disrupt the primary metal binding site. Thus, the N321A substitution indicates that the adjacent metal binding site is important for the condensation reaction to occur. The variant enzyme (N321I/D81E) that mimics the active site of HCS did not catalyze the condensation reaction, suggesting that these changes were not sufficient for catalysis to occur.

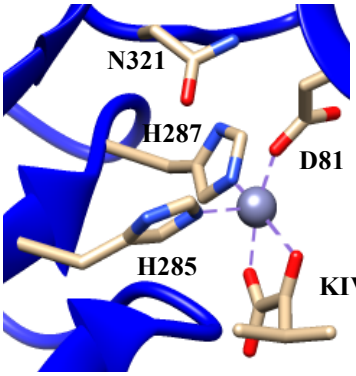

Figure 1: Metal Binding Site of MtIPMS (PDB:1SR9)

pH Profiles of D-Arginine Dehydrogenase with D-Histidine and D-Methionine

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D-Arginine dehydrogenase (DADH) is a flavin-dependent enzyme that requires FAD as a cofactor for catalysis (*1*). It is found in *Pseudomonas aeruginosa*, a common opportunistic human pathogen (*2*). DADH catalyzes the oxidation of D-amino acids to iminoacids, which are subsequently converted to α-ketoacids and ammonia in solution (*1*). Previous studies have established that the enzyme catalyzes the oxidation of all the D-amino acids, with the exception of D-glutamate, D-aspartate, and glycine (*3*). Based on the k_{cat}/K_m values at pH 8.7, the best substrates for the enzyme are D-arginine (i.e., 10^6 $M^{-1}s^{-1}$) followed by D-lysine, D-tyrosine, D-methionine, D-phenyalanine, D-histidine, and D-leucine (*3*).

It is interesting that the two most effective substrates are D-arginine and D-lysine, both of which have ionizable side chains that can carry a positive charge. Furthermore, D-histidine also has an ionizable side chain that can be positively charged, yet its k_{cat}/K_m is lower than D-tyrosine, D-methionine, and D-phenylalanine.

In this study, the pH profiles of the steady state kinetic parameters with D-histidine and D-methionine as the substrate for the enzyme were determined in order to establish the contribution of the ionizable side chain of the substrate to catalysis. These two amino acids were chosen based on their comparable k_{cat}/K_m values at pH 8.7 (i.e., 10^3-10^4 $M^{-1}s^{-1}$) (*3*). The resulting pH profiles showed differences in the number of ionizable groups participating in enzyme turnover, suggesting the importance of the correct ionization state of the side chain of the substrate for optimal enzyme activity. Steady state kinetic parameters will be closely examined alongside available x-ray crystallography structures to unveil the differences in enzyme turnover between the two substrates.

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Discovery of a New Cross-Talk between Protein Arginine Methyltransferase Isoforms

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Protein arginine methyltransferases (PRMTs) are a class of epigenetic enzymes that facilitate the transfer of methyl group from S-adenosyl-L-methionine (SAM) to the guanidino group of arginine residues of histone and a variety of non- histone substrates. The PRMT family is separated into type I, type II, and type III based on the final methylated form of the arginine residue. PRMTs have been linked to cellular processes ranging from cell movement, proliferation, regulation, and DNA repair. Abnormal methylation levels have been associated to kidney, heart and lung associated diseases such as various cancers and asthma. With the diverse range of substrates for each PRMT there is also overlap of substrate methylation leading to the question of potential cross-talk amongst the PRMTs. PRMTs can form monomethyl arginines and the final dimethylation can be performed by another PRMT member. Interestingly, PRMT1 has been shown to heterodimerize with PRMT2 to increase methylation activity. In our study, we analyzed the PRMT interactions and have found PRMT1 interacts with and can methylate PRMT6. This novel interaction shows the first evidence of PRMT methylation by another PRMT isoform and could provide a deeper understanding on how PRMTs intercommunicate with one another for establishing arginine methylation marks in the cellular milieu.

Molecular Dynamics of the T4 Bacteriophage Clamp Loader in Various ATP Loaded States

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Processivity clamps, such as eukaryotic Proliferating Cell Nuclear Antigen (PCNA), act as attachment sites for proteins involved in replication, cell-cycle control and DNA repair. These sliding clamps are comprised of three subunits forming closed ring-shaped structures around DNA. This closed shape around DNA necessitates an activated mechanism to help in the loading processes. In eukaryotic cells, this is accomplished by the action of a clamp loader assembly, a multi-protein molecular machine that uses the energy of ATP binding and hydrolysis to open and reseal PCNA around DNA (1). Replication factor C (RFC), a eukaryotic clamp loader, has been shown to assist PCNA in attaching to DNA. To further investigate this process, the T4 clamp loader system was simulated. It is desirable to model three states of activation of the clamp loader: 1) ATP loaded prior to hydrolysis 2) ADP loaded after all sites are hydrolyzed and 2) the clamp loader with all nucleic derivatives removed from the system. Using these structures, insight into the conformational changes at the interface that trigger clamp opening and closing can be investigated. The models were simulated with molecular dynamics (MD) in explicit solvent initially for 80 ns to expose the conformational dynamics of the systems. RMSD trajectory analysis shows that the systems are not convergent at 80 ns and additional free and accelerated MD (aMD) techniques were attempted to produce convergent systems that can be analyzed and used in further investigations.

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V-Type Allosteric Inhibition of Isopropylmalate Synthase from *Mycobacterium tuberculosis* is Accomplished by a Change in the Rate - Determining Step

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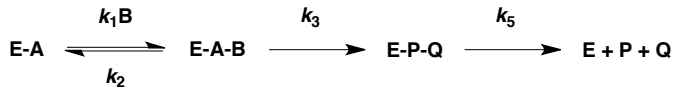
The enzyme α-isopropylmalate synthase isolated from *Mycobacterium tuberculosis* (MtIPMS) catalyzes a Claisen condensation reaction between acetyl-CoA (AcCoA) and α-ketoisovalerate (KIV) to form isopropylmalate and CoA, which is the first committed step in the biosynthesis L-leucine. This is a two-step process in which the intermediate isopropylmalyl-CoA is formed in a condensation step followed by hydrolysis. The enzyme is feedback inhibited by L-leucine. Kinetically, L-leucine acts as a *V*-type inhibitor, lowering the value for V_{max} while the K_M values for substrates remains relatively unchanged. A decrease in the value for V_{max} can be caused by a decrease in the rate of either a chemical step or the rate of product release. In order to determine if L-leucine inhibition is due to changes in the rate-determining step, rapid-reaction kinetics were determined using a QFM-400 rapid chemical quench instrument (BioLogic). In the absence of L-leucine rapid burst kinetics are observed indicating that product release is the rate-determining step. Dynafit (Biokin) was used to fit the progress curve to the mechanism shown in Scheme 1 where k_3 represents the sum of net rate constants for the two chemical steps and k_5 represents the sum or net rate constants for the release of both products. Values of 17 ± 3 s^{-1} and 2.4 ± 0.1 s^{-1} were determined for k_3 and k_5, respectively. However, when MtIPMS is incubated with 200 μM L-leucine no burst is observed and a rate of 0.4 s^{-1} is determined. This suggests that L-leucine inhibits MtIPMS by decreasing the rate of one of the chemical steps. In order to identify which of the chemical steps (condensation or hydrolysis) is affected, kinetic isotope effects were determined in the presence of L-leucine (500 μM). The presence of L-leucine increases the ^{D2O}V value from unity to 1.6 ± 0.2 suggesting that hydrolysis of the isopropylmalyl-CoA intermediate is rate-determining in the inhibited enzyme.

Scheme 1



Circular Permutation of Old Yellow Enzyme: Characterization of a Complete Synthetic Library

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Old yellow enzyme from *Saccharomyces pastorianus* (OYE1) is an FMN containing NAD(P)H dependent oxidoreductase capable of catalyzing the trans-hydrogenation reaction on a variety of activated alkenes. These enzymes offer a valuable alternative to synthetic catalysts affording chemical building blocks useful in pharmaceutical and biotech applications. Although this enzyme has been in the spotlight for its role in reduction chemistry, OYE1 could benefit from successful engineering strategies towards generating designer catalysts with enhanced activity and enantioselectivity for desired substrates. We employed circular permutation (CP) to engineer OYE1, as the technique has been shown in the past to improve catalytic activity as a result of termini relocation in loop and secondary structures near the active site environment. The OYE1 circular permuted library was directly synthesized using a whole gene synthesis strategy and assayed against several α,β- unsaturated alkene substrates utilizing the PURExpress *in vitro* transcription/translation (IVT) technology, demonstrating a novel screening approach. Library evaluation using our IVT screening platform successfully identified ~30% of the OYE1 library having better activity than wild type. The results also clearly highlight three specific sectors in the OYE1 scaffold responsible for the most dramatic alterations in catalytic activity for all substrates evaluated. In addition to the changes in substrate specificity as a result of permutation, we are also exploring the substitution of FMN with cofactor analogs in CP variants as an orthogonal engineering strategy. We have demonstrated alterations in catalytic activity as well as the catalysis of a different reaction as a result of the change in redox potential via tandem effects through both cofactor substitution and permutation.

Enzymes in the Kynurenine Pathway

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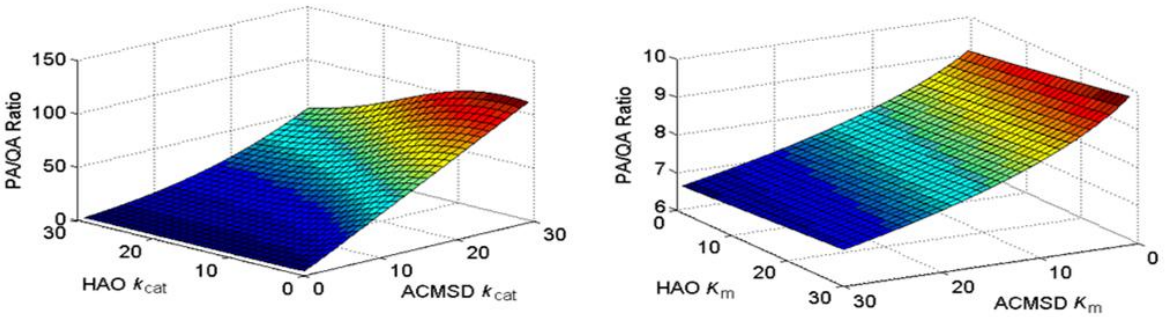
In mammals there are two separate pathways by which tryptophan can be metabolized, the serotonin and the kynurenine. Though the serotonin pathway is well studied, it accounts for a small portion of metabolized tryptophan. The bulk of tryptophan not used for protein synthesis is catabolized though the kynurenine pathway (KP), a pathway with neuroactive intermediates such as kynurenic acid and quinolinic acid (QA). The kynurenine pathway is of interest to study because it is the sole source of endogenous QA, a compound which has been shown to be an N-methyl-D-aspartate receptor agonist, and elevated levels of QA have been associated with several disease states: Alzheimer's disease, anxiety, depression, epilepsy, AIDS dementia, and Huntington's disease.

This research focuses on the activity of a unique trio of enzymes, the QA Trio, in the tryptophan kynurenine pathway: 3-hydroxyanthranilic acid 2,3-dioxygenase (HAD), α-amino-β-carboxymuconic-ε-semialdehyde decarboxylase (ACMSD), and α-amino-β-muconic-ε-semialdehyde dehydrogenase. This proposed metabolome converts 3-hydroxyanthranilic acid (3-HAA) to 2-aminomuconate (2-AM) by an oxidative ring cleavage followed by non-oxidative decarboxylation and finishing with oxidation of terminal the aldehyde to a carboxylic acid. Both intermediates in this trio, α-amino-β-carboxymuconic-ε-semialdehyde (ACMS) and α-amino-β-muconic-ε-semialdehyde (AMS), are unstable and spontaneously cyclize to form QA and picolinic acid (PA) respectively. A CE-MS method was developed to separate and quantitate the substrate and product of the first two enzymes in this trio. Computer simulations in MATLAB were employed to analyze the data obtained by MS, and preliminary works indicate a possible new feedback inhibition mechanism in the KP.



Synthesis and Evaluation of Inhibitors Selective for Tryptophan Indole-Lyase and not Tryptophan Synthase. Toward the Development of Anti-biofilm Formation.

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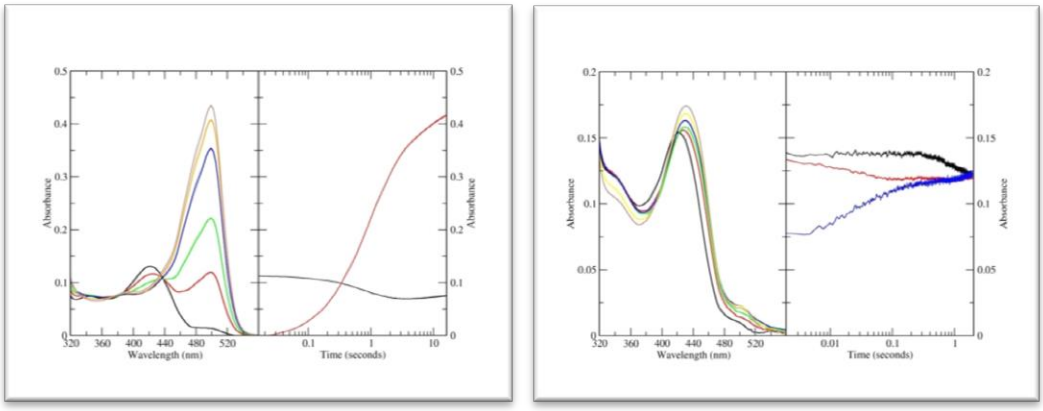

Abstract

Biofilm is a structured community of bacteria enclosed within a self-produced polysaccharide polymeric matrix that generally showed higher resistance to chemical and biological attack than in their planktonic state. Biofilm formation has a negative impact on the operability of machinery in industries, the environment, as well as our healthcare system due to bacterial infections. Recent reports suggested that tryptophan indole-lyase (TIL, tryptophanase, EC 4.1.99.1), a pyridoxal-5'-phosphate (PLP) dependent bacterial enzyme as a promising and selective target for anti-biofilm formation and antibiotic resistance. As previously suggested by our work that quinonoid is an intermediate in the physiological reaction of TIL, we anticipated that homologation of the physiological substrate, L-tryptophan (Trp), at the α-carbon would provide analogues resembling the transition state and potentially inhibits the activity of TIL. The work herein discussed the design, synthesis and evaluation of L-homotryptophan (**1a**) and L-bishomotryptophan (**1b**) as inhibitors for TIL. Our results indicated that **1a** is a moderate inhibitor of TIL with Ki = 178 μM, whereas **1b** displayed potent inhibition with Ki = 6.8 μM. Results from rapid-scanning stopped-flow experiments indicated the formation of an external aldimine and quinonoid with **1a** but only the formation of an external aldimine for **1b**, indicating their mechanistic differences in inhibition for TIL. In addition, Trp analogues evaluated as efficient inhibitors of TIL were also previously reported to display efficiency toward inhibition of Trp synthase, an enzyme widely distributed in bacteria, fungi and plants. Our results indicated that **1a** and **1b** are highly selective toward TIL than Trp synthase. We wish to report that **1b** is the first potent inhibitor with high selectivity for TIL, suitable for the approach toward development of an antibacterial treatment.

Acknowledgement: QTD would like to thank the University of Georgia for support of this work through the Innovative and Interdisciplinary Research Grant for Doctoral Students.

Investigating the Baeyer-Villiger Monooxgenase MtmOIV for the Combinatorial Biosynthesis of New Mithramycin Analogues.

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Baeyer−Villiger monooxygenases (BVMOs) have been shown to be powerful biocatalysts for synthetic organic chemistry applications and play key roles in the biosynthesis of various natural products. MtmOIV, a homodimeric FAD- and NADPH-dependent BVMO, catalyzes a key frame-modifying step of the mithramycin biosynthetic pathway, reacting with its natural substrate premithramycin B via a Baeyer−Villiger reaction. Here we report the significantly improved structure of MtmOIV and the structure of MtmOIV in complex with its substrate premithramycin B, allowing us to identify key residues important for substrate recognition and catalysis. Kinetic analyses allowed us to probe the substrate-binding pocket of MtmOIV and to discover the putative NADPH binding site. This is the first substrate-bound structure of a BVMO with this fold providing insight into substrate recognition in other important enzymes from this family. This structure provides the necessary information for broadening the specificity of MtmOIV for the generation of new anticancer agents.

Effects of Molecular Crowding on the Binding Affinity of Dihydrofolate for Dihydrofolate Reductase

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The reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR), using NADPH as a cofactor, is an essential part of the folate cycle. The product, THF, is necessary for the synthesis of methionine, purine nucleotides, thymidylate, and other compounds. Thus, the inhibition of DHFR leads to interruption of DNA synthesis and consequently cell death, making this enzyme a crucial target in the treatment of cancer and other diseases. Previous studies examined the effects of small molecule osmolytes on the substrate interactions with two non-homologous DHFRs, *E. coli* chromosomal DHFR (EcDHFR) and R67 DHFR, with vastly different active site structures. The results indicated that DHF weakly interacts with the osmolytes in solution, shifting the binding equilibrium from DHF bound to DHFR to unbound DHF. It is hypothesized that similar weak, nonspecific interactions may also occur between cellular proteins and DHF. Weak interactions between cellular proteins and DHF would have consequences *in vivo*, where the concentration of the cellular milieu is approximately 300 g/L. Under the crowded conditions in the cell, there is a higher propensity for intermolecular interaction.

Crowding effects of macromolecules in concentrations similar to those *in vivo* were examined. Isothermal titration calorimetry (ITC) and enzyme kinetic assays were used to detect effects of molecular crowders by monitoring activity of the (DHFR)•NADPH or DHF complex and the ternary DHFR•DHF•NADPH complex in the presence of these crowders. To recreate the conditions of molecular crowding *in vivo,* the binding of the enzyme-ligand complexes in the presence of molecular weight crowding agents (lysozyme or casein) was examined. Analysis of the K_d's and K_m's indicated a correlation between increased molecular crowding in the solution and weakened binding of the DHFR•substrate complexes. These findings indicate an importance of molecular crowding on EcDHFR activity *in vivo.*

Does Methylation of Arginine Modulate Biomolecule Interactions?

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Arginine is one of three positively charged amino acids found in biological systems. Electrostatic interactions are critical in facilitating a variety of protein-protein and protein-nucleic acid interactions, in addition to helping maintain protein solubility and structure stability. Arginine can also be methylated by post-translational modification enzymes to modulate its behavior. Methylation of arginine introduces a moiety that can sterically hinder binding of a ligand. In addition, such a modification may perturb the pK_a of the side chain. Because the ionic state of amino acid side chains is a key determinant of enzymatic activity, the determination of pK_a values is relevant to enzyme substrate and biomolecule interactions. We are using Nuclear Magnetic Resonance (NMR) and a modified Henderson-Hasselbalch equation to determine pK_a values of modified arginine variants. Our results reveal that methylation does not appreciably affect the pK_a value of the guanidino head group. Moreover, we show that the previously estimated pK_a value of arginine of 12.48, determined in 1930 and referred to since, is too low.

Studies on the Roles of Tyrosine 53 and 249 in D-Arginine Dehydrogenase from *Pseudomonas aeruginosa*

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D-arginine dehydrogenase (DADH) is an FAD-dependent enzyme that catalyzes the oxidation of D-amino acids to their corresponding iminoacids. Crystal structures of the enzyme in complex with either iminoarginine (Fig. 1) or iminohistidine showed that the main chain atoms of either make several hydrogen bonding and/or electrostatic interactions with residues such as His48, Tyr53, Glu87, Arg222, Tyr249, Arg305 and Gly332. In a previous study carried out on the wild type recombinant enzyme using D-leucine as substrate, the amine oxidation proceeded with the requirement of two groups with pK_as of 10.3 and 9.6 for substrate binding and its oxidation (corresponding with flavin reduction). For tight binding of the substrate the group with a pK_a of 10.3 is required to be protonated while for optimal substrate oxidation and thus flavin reduction, a group is required to be deprotonated with a pK_a of 9.6 to stabilize the transition state. We postulated either the active site Tyr249 or Tyr53 on a mobile loop as being probable candidates for the group involved in substrate binding. Tyr53 is further proposed to also stabilize the transition state for substrate oxidation.

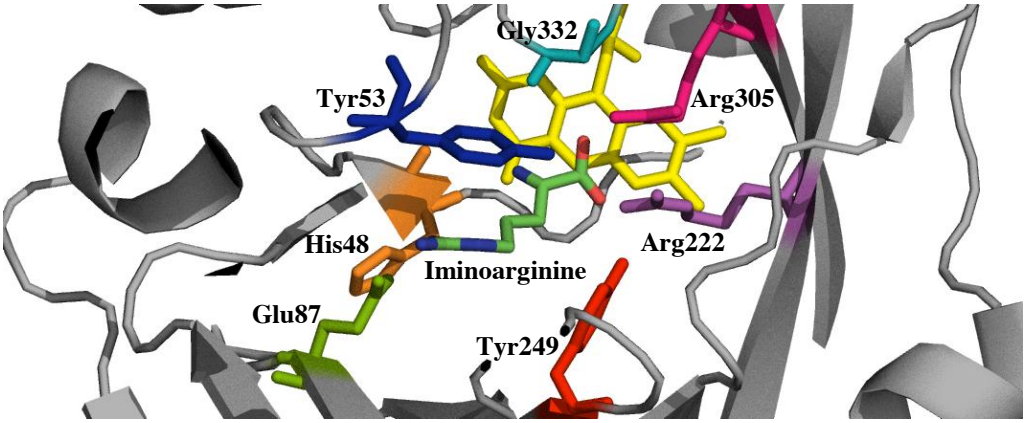

Fig 1. Crystal Structure of DADH - iminoarginine complex (3NYE).

This study investigates the contributions to substrate binding and catalysis by the active site residues Tyr249 and Tyr53, through the use of site-directed mutagenesis, UV-visible spectroscopy, pH profiles on steady state and rapid reaction kinetics, solvent viscosity effects, kinetic isotope effects as well as by multiple substrate analyses. The results will be discussed in view of the available crystal structures of the enzyme and comparisons will be made with the wild type enzyme and other enzymes catalyzing similar oxidations of amino acids or hydroxyl acids, such as pig kidney D-amino acid oxidase (Tyr224 & 228), spinach glycolate oxidase, (Tyr24 & 129) and flavocytochrome b_2 (Tyr143 & 254).

This study was supported in part by NSF MCB-1121695 (G.G.) and a GSU Molecular Basis for Disease Fellowship (S.G.).

Detection and Characterization of reaction intermediates in IspH

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The causative agents of malaria, anthrax, tuberculosis, plague, and cholera as well as plant plasmids utilize the 1- deoxy- D-xylulose-5-phosphate pathway (DOXP) for the synthesis of isoprenoid precursors. IspH, (E)-4-Hydroxy-3-methylbut-2-enyl diphosphate reductase is the ultimate enzyme that catalyzes the conversion of (E)-4-hydroxy-3- methylbut-2-enyl diphosphate (HMBPP) into the isoprenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate. Humans use another pathway, the mevalonate, hence having an in-depth understanding of the reaction mechanisms of the DOXP route and the enzymes involved can be helpful in developing potential anti-infective drugs and herbicides that will be less toxic to humans. Two methods were used to study the enzyme; electron paramagnetic resonance (EPR) spectroscopy based kinetics study, where samples were prepared by mixing IspH and dithionite with a solution containing the substrate HMBPP. The reaction was allowed to proceed for a set amount of time after which it was stopped by rapid freezing of the reaction mixture. More samples were prepared by varying the time of incubation. Two signals with g values 2.170 and 2.077 were detected in these studies. The one with 2.170 g value has already been studied with electron nuclear double resonance (ENDOR) spectroscopy and the distance of the cluster from ^{31}P and ^{2}H-C4 labeled substrate was estimated as Fe–^{31}P distance (r) to be∼7 Å and the Fe−D to be∼3.4Å. From these results we propose 4Fe-4S cluster of IspH directly binds to the hydroxyl of the substrate HMBPP. The second species will be investigated in the near future.

Binding the Core of a Holliday Junction

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Holliday junctions, a four way DNA junction formed by a crossover between two duplexes, are an essential intermediate formed during DNA recombination and double strand break repair. This intermediate structure is cleaved into its two constituent duplexes by a resolving enzyme such as T7 Endonuclease I. Holliday junctions are a major mechanism to repair double stranded DNA breaks; binding ligands to the core of the junction could prevent this enzymatic cleavage from occurring which will result in diminished DNA repair and cause apoptosis. We have developed an assay which screens for drugs that can inhibit the enzymatic resolution of a Holliday junction and we are investigating techniques to confirm the binding location of a ligand to the core of a DNA Holliday junction.

Evaluating the Mechanistic Properties of Cysteine Dioxygenase with an Altered Metal Coordination Center

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Cysteine dioxygenase (CDO) plays an essential metabolic role in maintaining appropriate cysteine levels in mammalian systems. Due to the highly reactive thiol sidechain, intracellular cysteine levels must be adequately balanced between the need for protein synthesis and the toxic effect of high cysteine concentrations. Imbalances in cysteine regulation have been associated with numerous disease processes. CDO is a mononuclear iron-containing enzyme that catalyzes the first step in L-cysteine metabolism, namely the oxidation of the sidechain thiol to form L-cysteine sulfinate. Following the production of L-cysteine sulfinate, the metabolic pathway branches leading to the formation of taurine or pyruvate and sulfite. CDO belongs to the cupin family which contains one iron center coordinated by three histidine ligands and one carboxylate ligand most commonly provided by a glutamate residue. In CDO, this carboxylate ligand has instead been replaced with a cysteine residue.

The evolutionary role for the Glu to Cys iron ligand substitution is currently unknown. To investigate the reason for this variation, Cys93 was substituted with glutamate by site-directed mutagenesis, and the kinetic and spectroscopic properties of the C93E variant were compared with wild-type CDO. Results from metal analysis revealed a percentage approaching 100% iron bound in C93E CDO compared to ~30% in the wild-type enzyme. EPR spectra showed a distinctly sharper signal, suggesting a perturbation in the coordination environment of the iron center due to the additional glutamate residue. Results from kinetic studies measuring the consumption of dioxygen with varying cysteine concentrations revealed an approximate 30-fold decrease in catalytic efficiency. Cysteine oxidation and oxygen consumption were fully coupled in the C93E CDO variant, indicating that there was no aberrant oxygen utilization with this variant. Substrate analogs were tested on both C93E and wild-type CDO, and it was discovered that there was activity when cysteamine was used as a substrate. Previous studies have reported L-cysteine as the only substrate for CDO, and if CDO can in fact oxidize cysteamine to hypotaurine, there are huge metabolic consequences. Contrary to some substrate binding models, our evidence suggests that during the course of catalysis L-cysteine coordinates the iron center through the amino and carboxyl groups leaving the thiol sidechain less sterically hindered for reaction with dioxygen.

The Power of Two: The Contribution of Arg51 and Arg239* to the Catalytic Mechanism in a Zinc-dependent Decarboxylase

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α-Amino-β-carboxymuconic-ε-semialdehyde decarboxylase (ACMSD) is proposed to be a prototypical member of a new protein subfamily in the amidohydrolase superfamily representing a novel non-hydrolytic C-C bond breaking activity.[1,2] In a previous study, we reported the structure of ACMSD from *Pseudomonas fluorescens* (*Pf*ACMSD) as a dimer.[3] Two conserved arginine residues in the putative substrate binding pocket of ACMSD, Arg51 and Arg239*, have attracted our attention. Arg239* intrudes into the active site from the neighboring protomer chain. The two arginine residues seem properly positioned to stabilize the substrate by providing ionic interactions with its two carboxyl groups. However a new structure of the human enzyme bound with a glycolic metabolic intermediate shows a monomeric structure. This observation opens a new question as to whether or not dimerization is required for ACMSD activity. This work seeks to answer this question and elucidate the roles of the Arg residues. The elution profile of *Pf*ACMSD from size exclusion chromatography shows that the protein is in equilibrium between monomer and dimer forms in solution. The specific activity of *Pf*ACMSD is seen to increase with greater stock protein concentrations. In this study, Arg51 and Arg239* were each mutated to alanine and lysine, and all four mutants were purified as soluble, inactive proteins. However, when R51A and R239A* were mixed, decarboxylation activity slowly recovered. The heterodimer hybridization experiment strongly supports that the active state of *Pf*ACMSD is its dimer form. The crystal structure of a heterodimer obtained by mixing R51A and R239A* mutants was solved to 2.0 Å resolution and will be presented at the conference. We also determined structure of human ACMSD and it is in the dimeric state. Together, these data reveal the precise role of the two arginine residues in the catalytic mechanism.

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Direct phosphorylation of riboflavin (analogs) to FMN via engineered FAD synthetases

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The preparation of flavin mononucleotide (FMN) and FMN analogs from their corresponding riboflavin form is traditionally performed in a two-step procedure, involving an initial enzymatic conversion of riboflavin to flavin adenine dinucleotide (FAD) by FAD synthetase (E.C. 2.7.7.2), followed by hydrolysis of the adenyl moiety with snake venom phosphodiesterase to yield the corresponding FMN derivative. To simplify the protocol, we have engineered the FAD synthetases from *Corynebacterium ammoniagenes* and *Thermotoga maritima* by deleting the N-terminal portion of the two-domain enzymes. The newly created biocatalysts are stable and show broad substrate specificity as demonstrated by the direct and quantitative phosphorylation of riboflavin and its analogs to the corresponding FMN form. The combination of high catalytic activity and robust heterologous overexpression in *E. coli* makes these artificial FMN synthetases useful tools for the biosynthesis of FMN cofactors.

α-Isopropylmalate synthase from *Escherichia coli* is subject to *K*-type regulation by L-leucine

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The allosteric regulation of enzymes by small molecules is a fundamental concept in biochemistry and provides a mechanism by which cells can respond to changes in environmental conditions. Isopropylmalate synthase (IPMS) catalyzes the first step in the biosynthesis of L-leucine. The enzyme catalyzes a Claisen-condensation between acetyl-CoA and α-ketoisovalerate (KIV) to form isopropylmalate and CoA. Structurally, IPMS consists of two well-conserved domains, an *N*-terminal TIM-barrel catalytic domain (Pfam id: PF00682) and a *C*-terminal regulatory domain (Pfam id: PF08502). IPMS is subject to feedback inhibition by L-leucine binding to the regulatory domain. Surprisingly, although IPMS enzymes share a conserved regulatory domain, varying mechanisms of allosteric regulation have been reported including effects on V_{max} and K_M in the absence and presence of changes to quaternary structure. Recent bioinformatics results suggest two distinct evolutionary origins for IPMS enzymes. IPMS from *Mycobacterium tuberculosis* (*Mt*IPMS) is the best-characterized representative enzyme. *Mt*IPMS is a homodimeric enzyme that exhibits no change in quaternary structure or active site architecture upon binding of L-leucine. Kinetically, L-leucine acts as a *V*-type noncompetitive inhibitor on *Mt*IPMS affecting the V_{max} variable while K_M remains relatively unchanged. IPMS from *Escherichia coli* (*Ec*IPMS) is predicted to originate from a different progenitor than *Mt*IPMS. The two enzymes share 20% identity; however, the majority of identical residues are located in the catalytic domain. The kinetic parameters for *Ec*IPMS in the absence of L-leucine were determined with a V_{max} value of 26 ± 0.6 min^{-1} and K_M values of 124 ± 9.0 μM and 10 ± 1 μM for AcCoA and KIV, respectfully. L-Leucine acts as a *K*-type competitive inhibitor versus the substrate AcCoA, with a K_i value of 24 ± 5 μM. Analytical size-exclusion chromatography of *Ec*IPMS (MW_{calc} 57,298 Da) gives peaks corresponding to 233 kDa and 114 kDa suggesting an equilibrium between tetrameric and dimeric forms. In the presence of L-leucine (100 μM), the enzyme gives a single peak with an experimental molecular weight of 128 kDa, consistent with a shift toward the dimeric form.

Computational, Structural and Kinetic Evidence that *Vibrio vulnificus* FrsA is not a Cofactor-Independent Pyruvate Decarboxylase

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The fermentation-respiration switch (FrsA) protein in *Vibrio vulnificus* was recently reported to catalyze the cofactor-independent decarboxylation of pyruvate (*1*). We now report QM/MM calculations that examine the energetics of C-C bond cleavage for a pyruvate molecule bound within the putative active site of FrsA. These calculations suggest that the barrier to C-C bond cleavage in the bound substrate is 28 kcal/mol (*2*), which is similar to that estimated for the uncatalyzed decarboxylation of pyruvate in water at 25 ^{o}C. In agreement with the theoretical predictions, no pyruvate decarboxylase activity was detected for recombinant FrsA protein that could be crystallized and structurally characterized. Indeed, the X-ray crystal structure, determined at 1.95 Å resolution, of the protein prepared by us was essentially identical to that reported earlier except an unknown ligand, modeled as hexanoate, was located in the putative active. These results suggest that the functional annotation of FrsA as a cofactor-independent pyruvate decarboxylase is incorrect.

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Base Flipping by the AlkD Glycosylase: An Unprecedented Base Excision Strategy

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The base excision repair process seeks to identify and remove single-base lesions from DNA. This process is initiated by glycosylases: enzymes that perform "base flipping" to extrude damaged nucleotides and cleave them from DNA. The recently discovered glycosylase, alkylpurine glycosylase D (AlkD) searches for and removes 3-methylAdenine and 7-methylGuanine, cytotoxic lesions caused by endogenous methylating agents, from DNA(*1*). Crystal structures of AlkD at the beginning and end of the base extrusion process indicate that, in stark contrast to all other known glycosylases, the enzyme makes no direct contacts to the lesion being acted upon and instead relies entirely on protein-DNA backbone interactions to perform base extrusion(*2*). To reveal the mechanistic details of this process, we have employed a molecular dynamics method known as the partial nudged elastic band to recreate a low-free energy path for this base flipping mechanism(*3*). This path is a good candidate for umbrella sampling calculations, which have yielded a putative Potential of Mean Force (PMF) for the reaction. This PMF indicates specific energy barriers for the AlkD base flipping process, possible intermediate stabilization strategies utilized by AlkD and theoretical insight regarding AlkD's adoption of this unprecedented base extrusion method.

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Kinetic Studies of α-Isopropylmalate Synthase from *Methanococcus jannashii*

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α-Isopropylmalate synthase (IPMS) is a model enzyme for the study of allosteric mechanisms as it catalyzes the first step in leucine biosynthesis in archae, bacteria and some eukaryotes. α-Isopropylmalate synthase is subject to feedback inhibition by L-leucine through an allosteric mechanism and contains evolutionarily conserved catalytic and regulatory domains. Recent phylogenetic analysis suggests two origins of IPMS. The most characterized IPMS with regard to allostery is from *Mycobacterium tuberculosis* (*Mt*IPMS). To investigate conservation (or lack of these) of allosteric mechanisms, the allosteric properties of an IPMS predicted to have come from an alternate origin need to be compared with those determined for MtIPMS. Here, IPMS from *Methanococcus jannashii* (*Mj*IPMS), a thermophilic archae, is being studied. Kinetic parameters for MjIPMS determined at 37°C exhibited a k_{cat} = 190 ± 10 min^{-1}, K_{AcCoA} = 100 ± 10 μM and K_{KIV} = 170 ± 20 μM. Kinetic parameters determined at 55°C matched well with the values at 37°C with the exception of the k_{cat} value being 450 ± 30 min^{-1}. It was determined that *Mj*IPMS exhibits *V*-type inhibition by leucine similar to *Mt*IPMS with a K_i = 85 ± 7 nM. Also similar to *Mt*IPMS, leucine has no effect on the quaternary structure of *Mj*IPMS as determined by size-exclusion chromatography. However, *Mj*IPMS appears to be a tetramer whereas *Mt*IPMS is a dimer. This suggests allosteric mechanism is independent of quaternary structure for IPMS enzymes.

Enzymes Responsiable of First Two Steps of 4-Nitroaniline Biodegradation by *Rhodococcus* sp. Strain JS360

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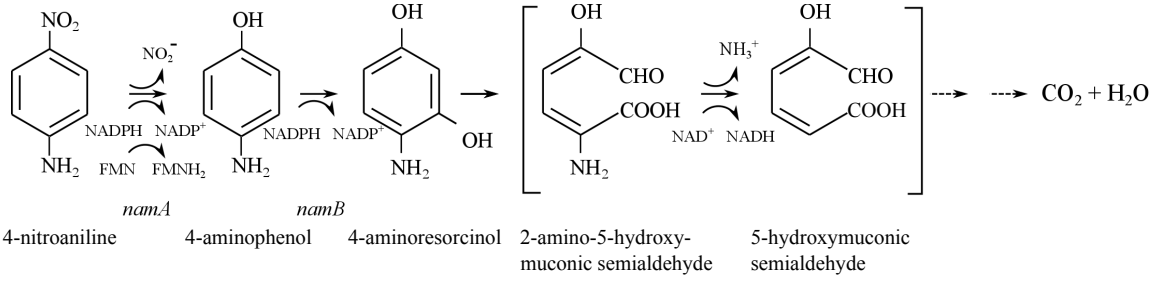
4-Nitroaniline (4NA) is a toxic compound used in dye synthesis, and even though its biodegradation has been previously reported, the mechanism is unknown. *Rhodococcus* sp. JS360 was isolated from 4NA-contaminated soil by selective enrichment. When grown on 4NA, the isolate released a stoichiometric amount of nitrite followed by less than stoichiometric ammonia release. Enzyme assays coupled with respirometry revealed that the first and second steps of 4NA degradation involve monooxygenases followed by ring cleavage prior to deamination. Annotation of the whole genome revealed candidate monooxygenases including 4NA monooxygenase (*namA*) and 4-aminophenol (4AP) monooxygenase (*namB*) that were cloned to E.*coli* and transformed 4NA to 4AP and 4AP to 4-aminoresorcinol. The 4NA biodegradation revealed a novel pathway for nitroanilines and defined two unique monooxygenase mechanisms likely to be involved in the biodegradation of similar compounds.



Structural Basis Of Mechanically Regulated Selectin Functions

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We propose to use Molecular Dynamics (MD) simulations to study the stability, the conformational changes, and the ligand dissociation of the Selectins under mechanical forces. Selectin-ligand interactions mediate the first step of a multistep adhesion and signaling cascade that recruits leukocytes to inflamed endothelium at sites of infection and injury. Malfunction of these interactions can result in a number of inflammatory and thrombotic disorders. Selectins are functionally important to the immune system that protects the body from pathogens such as bacteria and viruses and diseased tissues such as cancer. Insufficient and over-reactive immune responses lead to various diseases.

In this study, the structural mechanisms of conformational changes will be investigated in L- Selectin-ligand interactions. Cocrystals of selectin display 2 conformational states that differ in the angle formed by the principal axes of the lectin and EGF domians. The state with an small angle and the other with an obtuse angle correspond to a low and high affinity conformations, respectively. The low affinity has an open binding site whereas the high affinity has a closed one. Although models were proposed[1,2,], there exists no conclusive evidence for correlating the conformational changes between the binding site and the hinge angle to elucidate the mechanism for transmission of allostery. By means of MD simulation, we unveil the relationship between the orientation of hinge angle and the conformation change of binding pocket by applying single mutations at three critical residues located at the hinge. Our hypothesis is that these residues propagate the hinge angle motion to the binding pocket.

The groundwork of this proposal is MD simulation combined with rigorous experimental data produced in the Zhu lab. This kind of study is novel and provides a unique means for elucidating the mechanical regulation of bio-molecular interactions at the atomic resolution.

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RNPEPL1 Expression Decreases in LPS Induced Inflammation

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Arginyl aminopeptidase like-1 (RNPEPL1) is an M1 family zinc metalloprotease first characterized in 2008. RNPEPL1 contains the $HEXXH(X)_{18}E$ domain and enzymatic activity crucial to placement in the M1 family. This protease has a high affinity for Met, Glu, and Cit, as well as a broad pH range. Many human members of the M1 aminopeptidase family are well known to play various roles in inflammatory response. In this study, RNPEPL1 RNA and protein levels were analyzed during an induced inflammatory response. Bone marrow derived macrophages from FVB/NJ mice were subjected to 24 hours of LPS treatment and harvested. Microarray and western blot analysis showed that RNPEPL1 RNA and protein levels did decrease significantly in LPS induced inflammation.

Novel Small Molecule Inhibitors and Factors Affecting Cleavage of the Essential Bacterial Enzyme Peptidyl-tRNA Hydrolase 11

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Peptidyl-tRNA Hydrolase 1 (Pth1; E.C. 3.1.1.29) is an essential enzyme in bacteria that cleaves the ester bond between nucleotide and peptide of peptidyl-tRNA. Peptidyl-tRNA is generated from incomplete protein translation and the expression of minigenes. Build-up peptidyl-tRNA is toxic, presumable due to tRNA starvation. Bacterial Pth1 is highly conserved. Therefore small molecule inhibitors effective against Pth1 in one bacterial strain are likely to work against others – i.e. broad spectrum inhibition. Moreover, humans have multiple Pth and Pth-like enzymes an nod essential Pth1 homolog. Thus Pth1 inhibitors are prime candidates for new antibiotic development

While the structure has been solved from several species (1), the catalytic mechanism of Pth1 cleavage of peptide from RNA remains unknown. An essential histidine and aspartate suggest a serine protease mechanism, but no serine is in or near the substrate binding cleft. Further, cleavage is heightened at elevated pH, contrary to what is observed for chymotrypsin (2), and greatly reduced below pH = 7.4. Mg^{2+} is required for hydrolytic activity, though Ba^{2+} and Zn^{2+} can serve as substitutes with varying degrees of diminished activity.

Further insight into the catalytic mechanism can be gained from related studies. For example, we have discovered a family of small molecule Pth1 inhibitors from a combinatorial library and multiple cloudforest extracts that display Pth1 inhibition. NMR data indicate the combinatorial molecules bind in the active site, in agreement with competitive binding curves from titration experiments. *In silico* modelling points to several important interactions that are conserved among inhibitors and the natural substrate, peptidyl-tRNA. Neutron crystallography is underway to determine the orientation of conserved water molecules in the active site. Other studies focus on determining the structure of a catalytically inactive enzyme bound to peptidyl-tRNA, including the overall shape from small angle scattering and high resolution data from NMR and crystallography. NMR backbone dynamics show differences between mobility near the active site for different species, correlated to cleavage rate. Taken together, these data begin to reveal the complex mechanism of binding and catalytic cleavage for this essential and fundamental biological enzyme family.

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The use of bacterial cellulose from Acetobactor.xylinum for the application of immobilizing different compounds to enhance overall functionality.

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Cellulose is the most abundant biopolymer on earth, recognized as the major component of plant biomass, but also a representative of microbial extracellular polymers. Bacterial cellulose (BC) belongs to specific products of primary metabolism and is mainly a protective coating. Bacterial cellulose is synthesized by bacteria belonging to the genera *Acetobacter, Rhizobium*, *Agrobacterium*, and *Sarcina*; however, its most efficient producers are Gram-negative, acetic acid bacteria *Acetobacter.xylinum* (1). This biopolymer is being cultivated for the immobilization of the biocatalyst laccase, horseradish peroxidase and the photo catalyst TiO_2, for use in water purification. Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) is an enzyme containing 4 catalytic copper atoms that catalyzes the one electron oxidation of phenolic compounds coupled with the four electron reduction of molecular oxygen (2). TiO_2 in water gets activated by UV light which produces ROS that can oxidize organic compounds. Immobilization increases the overall functionality, reusability, and stability of catalysts. Immobilization of the laccase enzyme has usually been achieved by chemically modifying the surface by tethering the enzyme, usually via gluteraldehyde. The process used in this study produces the cellulose in the presence of the bio and photo catalysis, incorporating the catalysts into the fibers of the cellulose as it grows. The stability and activity of the immobilized bio catalase laccase was measured by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or (ABTS). To measure the efficiency of the immobilized photo catalysis TiO_2, water contaminated with *Candida.albicans* was tested for inactivation after 22 h of exposure under UV light. These experiments indicate that bacterial cellulose is a good support system for catalysis and could be used as a support system for various types of catalysis without using added linking reagents to the system.

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Post-translational modifications: Regulating the regulators

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Major histocompatibility class II (MHCII) molecules present extracellular antigens to CD4+T cells and play vital roles in regulating adaptive immune responses. Expression of MHCII is tightly controlled at the level of transcription and requires binding of the Class II Transactivator (CIITA) to the MHCII promoter. CIITA is also highly regulated, both at the level of transcription and posttranslational modifications. Previously, we have linked activation of CIITA to monoubiquitination (Mono Ub) on degron proximal lysine residues and have shown CIITA Mono Ub to be dependent on prior phosphorylation. Mono Ub has opposing effects on CIITA as Mono Ub CIITA is initially targeted to the MHCII promoter to drive MHCII expression and, following promoter binding, Mono Ub CIITA is polyubiquitinated and degraded by the 26S proteasome. We now identify the kinase ERK 1/2 as being responsible for CIITA phosphorylation at degron localized serine 280. Further, we show ERK 1/2 increases CIITA ubiquitination (Mono and Poly), CIITA transactivity, and leads to a decrease in CIITA half life. Further, ERK 1/2 has been identified in phosphorylation at Serine(s) 286, 288, and 293, leading to CIITA decreased transactivation. Acetylation of CIITA is mediated in part by pCAF, and pCAF has recently been observed to posses E3 ligase capabilities. While the E3 ligase for CIITA remains unknown, our data suggests pCAF is involved in CIITA ubiquitination, although whether though its acetylase or ligase activity remains unclear. Together our preliminary data support that CIITA's PTMs work together in order to form a network of signals which allows for CIITA's transient transactivation that drives MHCII expression. These data further our understanding of how CIITA is regulated by PTMs and the enzymes responsible. Our observations will yield insights into the complicated interactions existing between protein PTMs and move the field closer to cracking the "PTM code." Research supported by grants from the American Cancer Society (to S.F. Greer).

Inhibition Characteristics of a human lymphatic filarial Aspartic Proteinase Inhibitor (Aspin) and its preliminary NMR studies

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In this study, the aspartic protease inhibition characteristics of an Aspin from a filarial parasite *Brugia malayi* and its NMR structural details were investigated. The overall aim of the study is to explain the inhibition and binding properties of Aspin from it's structural point of view. Multidimensional NMR, UV Spectroscopy and Isothermal Titration Calorimetry are the experiments that have been performed to carry out this analysis to understand the structural, kinetics and binding characteristics, respectively. The human enzymes that were considered for this study are: Pepsin, Cathepsin D, Cathepsin E and Renin. The results of this analysis suggest that Bm Aspin inhibits the activities of all the four human aspartic proteases that were examined. The binding reactions were enthalpy driven as suggested by the exothermic reactions. The kinetic studies indicate that the protease inhibition by the Aspin to be competitive for Pepsin & Cathepsin-E, non-competitive for Renin and mixed for Cathepsin-D. The triple resonance NMR experiments that have been carried out on the Aspin in SDS micelles indicate the feasibility to determine the atomic structure by NMR. In addition, the binding studies of the Aspin with the human proteases have been initiated from the NMR titration studies.