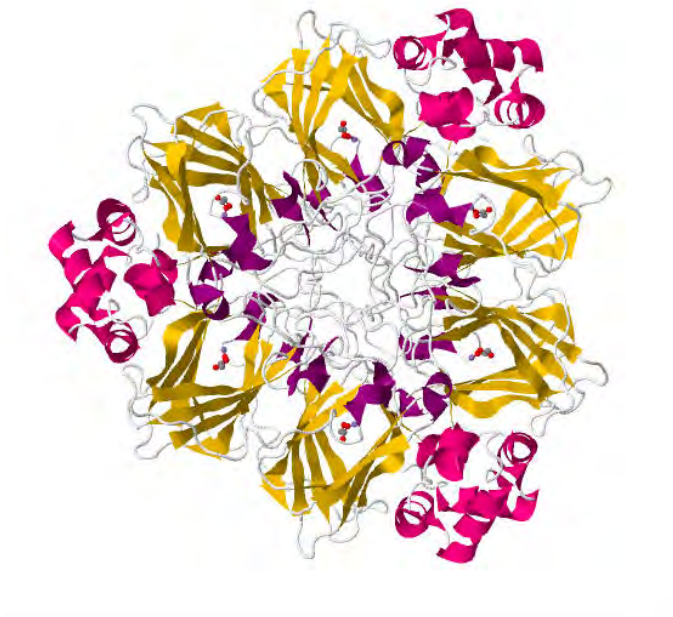
Second Southeast Enzyme Conference



Saturday, April 2, 2011

Georgia State University
Atlanta, GA

Helen M. Alderhold Learning Center
60 Luckie Street
Room 24

Second Southeast Enzyme Conference

Saturday, April 2, 2011

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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			

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Session 1 - *Chair*, **Holly Ellis**, Auburn University

8:30-9:00 Tihami Qureshi, University of Tennessee, Knoxville
"Detecting Conformational Changes In The RCL of Human PAI-1 Using An Environmentally-Sensitive Fluorescent Probe"

9:00-9:30 John Robbins, Auburn University, Auburn
"The Role of Arg226 in the Desulfonation Mechanism of the Two-Component Alkanesulfonate Monooxygenase System"

9:30-10:00 Ashley Casey, University of Alabama, Tuscaloosa
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"Luciferase-Based Chemiluminescent Measurement of Cellular Production of Nitric Oxide"

11:00-11:30 Mario Moral, University of Florida, Gainesville
"Inhibition by Nitric Oxide of Catalysis by Oxalate Decarboxylase"

11:30-12:00 Jonathan Park, Georgia Institute of Technology, Atlanta
"NAD(P)H oxidase V (NoxV) from Lactobacillus plantarum displays enhanced operational stability even in absence of reducing agents"

Location: Atrium, Alderhold Learning Center

12:00-2:00 Lunchbox and Poster Session

Session 3 - *Chair*, **Yujun George Zheng**, Georgia State University

2:00-2:30 Kednerlin Dornevil, Georgia State University, Atlanta
"A 61-Year-Old Mystery: How Peroxide Activates Ferric Tryptophan 2,3-Dioxygenase to the Ferrous State"

2:30-3:00 Andrea Pennati, Georgia State University, Atlanta
"Choline-Glycine Betaine Metabolic Pathway in Aspergillus fumigatus"

3:00-3:50 Dale Edmondson, Emory University, Atlanta
"Mechanistic Perspectives of Monoamine Oxidase Catalysis"

3:50-4:00 Concluding Remarks - **Nigel Richards**, University of Florida, Gainesville

Session 1:

Holly Ellis

Chair

Detecting Conformational Changes In The RCL of Human PAI-1 Using An Environmentally-Sensitive Fluorescent Probe. Tihami Qureshi , Cynthia B. Peterson. Department of Biochemistry, Cellular, and Molecular Biology. University of Tennessee, Knoxville

Human plasminogen activator inhibitor-1 (PAI-1) is a proinflammatory, proadhesive, antifibrinolytic *ser*ine *p*rotease *in*hibitor (serpin) that has a wide variety of physiological roles and is required for normal blood hemostasis.[1,2] PAI-1 differs from most homologous serpins in that it exists in a metastable native conformation and undergoes latency to a more stable, but inactive, conformation under physiological conditions.[3] During this conformational change, a disordered loop, termed the reactive center loop (RCL), inserts as the fourth strand into the central β-sheet A (s4A)(Fig. 1). Functionally, this structural element is important for the inhibitory activity of the serpin because it contains the peptide bond that mimics the natural substrate for target serine proteases. The activity of PAI-1 is also influenced by its interaction with the glycoprotein vitronectin (VN). Binding of VN stabilizes PAI-1 in its active conformation and increases its inhibitory lifetime by approximately one- to two-fold.[1] However, the mechanism by which VN stabilizes PAI-1 is currently not agreed upon. Earlier studies have suggested that VN binding stabilizes the active conformation of PAI-1 by sterically preventing loop insertion in the central β-sheet.[1] Yet, a recent study reports the increased incorporation of RCL-mimicking peptides to VN-bound PAI-1, contradicting the latter assessment.[5] Thus, the question of how VN stabilizes PAI-1 and whether the local dynamics of the RCL contribute to global conformational changes remains. **Our hypothesis is that VN stabilizes the active conformation of PAI-1 by affecting the conformation of RCL.** In order to test this hypothesis, single cysteine mutants were engineered along the length of the RCL in order to be labeled with an environmentally-sensitive fluorescent probe: N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) ethylene diamine (NBD). The RCL-labeled PAI-1 can subsequently be analyzed by fluorimetry in the presence of VN to detect any changes in conformation based on solvent accessibility. Thereby, the effect of VN binding on the conformation of the RCL of PAI-1 may be used to evaluate the mechanism of VN-associated PAI-1stabilizaton.

The Role of Arg226 in the Desulfonation Mechanism of the Two-Component Alkanesulfonate Monooxygenase System

John M. Robbins and Holly R. Ellis

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Two-component flavin-dependent monooxygenases are involved in various metabolic and biosynthetic processes in microorganisms. Efforts to elucidate the details governing the catalytic mechanisms of these systems continue to be an area of active investigation. The alkanesulfonate monooxygenase enzyme is found in a diverse range of bacterial organisms and utilizes free $FMNH_2$ supplied by an independent NAD(P)H dependent FMN reductase (SsuE) to alleviate periods of limited sulfur bioavailability. Catalysis by the monooxygenase enzyme results in the oxygenolytic cleavage of a carbon-sulfur bond from sulfonated substrates to yield free FMN, aldehyde, and metabolically available sulfite.[1] The SsuD reaction has been shown to be dependent on a C4a-(hydro)peroxyflavin intermediate to catalyze the desulfonation of alkanesulfonates.

Active site amino acid residues have been proposed to play a direct mechanistic role in acid-base catalysis at specific steps in the reaction pathway. Sequence and structural analyses of the monooxygenase enzyme were used to identify several conserved residues near the proposed active site with the potential to contribute to catalytic function. Variants of these amino acid residues were constructed and evaluated using different kinetic approaches including chemical rescue experiments, single turnover kinetics, photodiode array, deuterium solvent isotope effects, and pH dependence studies. The pH dependence of k_{cat} indicated SsuD requires a group with a pK_a of 6.6 ± 0.1 to be deprotonated and a second group with a pK_a of 9.5 ± 0.1 to be protonated. The results of the present study indicated an active site Arg226 plays a substantial role in catalysis as any mutation to this residue resulted in complete inactivation of the enzyme, although guanidinium rescue experiments performed with R226A SsuD recovered 1.5% of the overall activity. In single-turnover experiments at 370 nm performed at pH 8.5 mixing $FMNH_2$ in one drive syringe against SsuD and varying octanesulfonate in the other drive syringe, wild-type SsuD was shown to stabilize the C4a-(hydro)peroxyflavin intermediate while the Arg226 variants showed no accumulation of the flavin intermediate. In single turnover experiments performed at pH 7.5 mixing FMN, SsuD, SsuE in one drive syringe against NADPH and octanesulfonic acid in the other drive syringe, a flavin binding and/or transfer step from SsuE to SsuD was shown to be unaffected by the amino acid substitutions to Arg226. These combined results demonstrate Arg226 is essential in stabilizing the formation of the C4a-(hydro)peroxyflavin intermediate that is crucial to the overall SsuD catalytic mechanism.

References:

1. Eichhorn, E., Van der Ploeg, J. R., and Leisinger, T. (1999) Characterization of a two-component alkanesulfonate monooxygenase from *Escherichia coli*, *J. Biol. Chem. 274*, 26639-26646.

Allosteric Regulation of α-Isopropylmalate Synthase from *Mycobacterium tuberculosis*

Ashley K. Casey, Jordyn L. Johnson, and Patrick A. Frantom

Department of Chemistry, The University of Alabama Box 870336 Tuscaloosa, Al 35487

Allosteric regulation of proteins, where the binding of a molecule at one site affects the chemical and physical properties at a distal site, has been studied in the field of biochemistry for over forty years. Historically, allostery was described as a two-state model, where active and inactive forms of the enzyme exist through changes in structural features. However, in the past ten years, allostery is being thought of as a global change in the dynamics of a protein in the absence of a change in shape. The enzyme α-isopropylmalate synthase from *Mycobacterium tuberculosis* (*Mt*IPMS), which catalyzes the first step in the biosynthesis of L-leucine, is being studied to obtain a greater understanding of the allosteric regulation and protein dynamics of a large multidomain enzyme. *Mt*IPMS is known to display slow onset inhibition in the presence of leucine, however K_m values for both substrates are not affected. The slow onset mechanism is intriguing given that leucine binds in the regulatory domain over 50 Å from the active site (Figure 1).

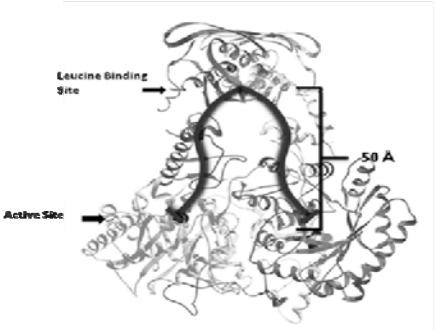


Figure 1: Crystal Structure of *Mt*IPMS (PDB: 3FIG1). The leucine binding site in the regulatory domain binds 50 Å from the active site.

Solution phase hydrogen/deuterium exchange experiments mapped changes in the protein dynamics upon leucine binding. The results identified a conserved active site helix that contains ligands essential to binding the divalent metal ion and α-KIV in the allosteric mechanism. This result led to several testable models of allosteric regulation. Site-directed mutagenesis was implemented in order to probe the active site helix residues' role in catalysis and regulation. Based upon the kinetic parameters determined, each residue plays a role in efficient catalysis. However, none of the enzyme variants affect the kinetics of leucine inhibition when compared to the wild-type enzyme. As another approach to understanding the allosteric mechanism, alternate amino acids were tested as inhibitors of the enzyme. Alternate amino acids studied thus far display non-competitive inhibition (K_i = 250-3,000 μM) with no slow-onset mechanism. These results suggest that the determinant for the slow-onset mechanism does not occur in the catalytic domain but in the regulatory domain. The ability of the divalent metal to activate catalysis in the presence of leucine was also tested. The results indicated that the concentration of the divalent metal needed for activation is not affected by the presence of leucine. Finally, we tested if leucine binding uncoupled the hydrolysis of AcCoA from the condensation reaction. NMR data shows that the addition of leucine does not uncouple the C-C bond formation and α-isopropylmalate and free CoA are seen as the only products.

Session 2:

Andreas Bommarius

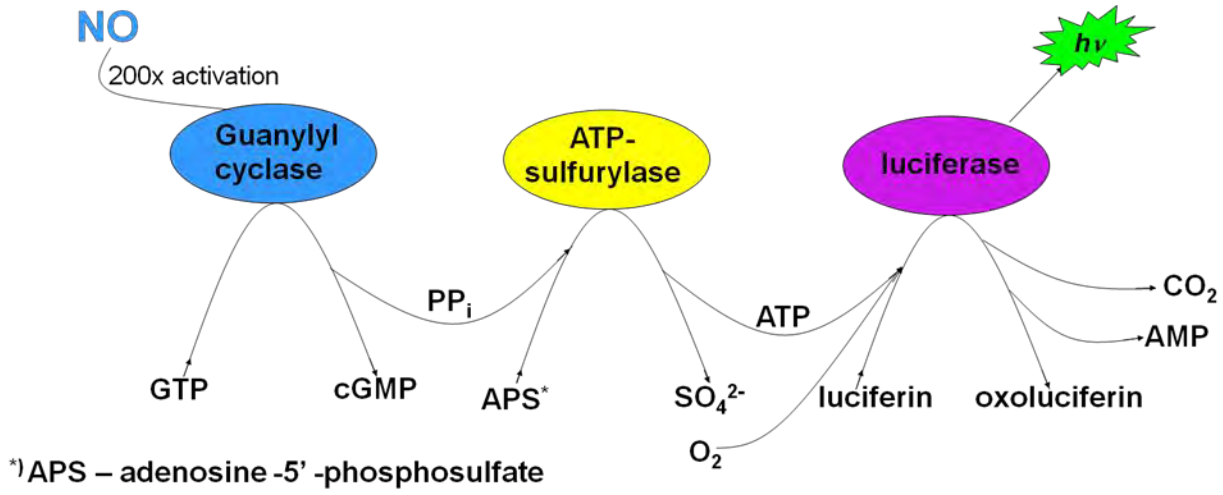
Chair

Luciferase-Based Chemiluminescent Measurement of Cellular Production of Nitric Oxide

Yakov Woldman[1], *Tim D. Eubank*[2], *Laura A. Sumner*[2], *Mikhail Gavrilin*[2], *Denis Komarov*[2] *and Valery V. Khramtsov*[2]

[1]*Valdosta State University, Valdosta, GA 31698;* [2]*Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH, 43210*

Nitric oxide (NO) is a free radical involved in many physiological processes including regulation of blood pressure, immune response, and neurotransmission. We recently suggested a highly sensitive chemiluminescence approach to measurements of NO in biological samples (*Free Rad. Biol. Med.* ***2009****, 47, 1339-45*). The approach is based on using a natural nitric oxide target, soluble guanylyl cyclase (sGC), which catalyzes the conversion of guanosine triphosphate to guanosine 3′, 5′-cyclic monophosphate and inorganic pyrophosphate. The suggested enzymatic assay uses the fact that the rate of the reaction increases by about 200 times when NO binds with sGC and, in so doing, provides a sensor for nitric oxide. Luminescence detection of the above reaction is accomplished by converting inorganic pyrophosphate into ATP with the help of ATP sulfurylase followed by light emission from the ATP-dependent luciferin–luciferase reaction.



The method allows for the measurement of NO concentrations in physiologically relevant nanomolar range and NO generation with the rates as low as 100 pM/min.

With this method, the generation of NO by cellular cultures has been measured. Murine RAW cells induced by interferon-γ (INF- γ) and lipopolysaccharide from *E. coli* (LPS) demonstrated NO production about 100 amol/min per cell. Untreated cells did not exhibit NO production beyond detection limit. NO generation measured by analysis of nitrite, the final product of NO metabolism, corresponded closely to the figures obtained for NO generation, but required much longer incubations to produce measurable amount of nitrite. Sensitivity of the method allows detecting NO generated by 10^3 cells. Addition of inhibitor of NO-synthase, N-monomethyl arginine, inhibited measured production of NO. Human cells (THP-1) treated with phorbol ester and either LPS, or INF- γ, or both, do not show NO production within detection limits. The method can be applied in situations where only a limited number of cells is available, e.g. biopsy.

Supported by NIH grant R21 HL089036 and Valdosta State University Faculty Research Grant to YYW.

Inhibition by Nitric Oxide of Catalysis by Oxalate Decarboxylase

Mario Edgar G. Moral[†], Chingkuang K. Tu[‡], Witcha Imaram[†], Alexander Angerhofer[†], David N. Silverman[‡], and Nigel G. J. Richards[†]

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[‡]*Departments of Pharmacology & Biochemistry, College of Medicine, University of Florida, Box 100267 HSC 1600 SW Archer Rd, Gainesville, FL 32610.*

Oxalate decarboxylase (OxDC) is a manganese-containing enzyme, which catalyzes the non-oxidative breakdown of monoprotonated oxalate to carbon dioxide and formate. The reaction requires dioxygen, even though it remains unclear as to its role in the overall mechanism, and where it binds in the enzyme. Studying the effects of an oxygen analogue such as nitric oxide (NO) on the catalytic reaction, may shed light on the location and role of the dioxygen in the enzyme during catalysis. However, traditional endpoint assays used to measure the kinetics of this enzyme preclude the ability to look into the progress of the reaction as the products are formed. Here we report the use of membrane inlet mass spectrometry as a continuous and real-time method of monitoring catalysis by OxDC from *B. subtilis,* by observing the accumulation of CO_2 in solution from its mass peak *m/z* 44 (or *m/z* 45 for $^{13}CO_2$). NO was generated in solution from NONOates, and complete inhibition by micromolar NO (K_i = 40 ± 10 μM) was observed. The inhibition was reversed after a time lag of several minutes by the addition of O_2. Electron paramagnetic resonance of a rapidly frozen reaction mixture showed that the multiplet splittings of Mn(II) were not perturbed by the presence NO. This suggests that although NO is an inhibitor, it does not directly bind to Mn(II) site with the smallest fine structure ($|D|$ = 1200 MHz) in the enzyme.

Acknowledgements

We thank Stephen Bornemann John Innes Centre, Norwich UK) for the provision of the plasmid construct of the polyhisitidine-tagged wild-type *B. subtilis* OxDC. This work was supported by a grant from the NIH DK061666 and NIH GM25154.

NAD(P)H oxidase V (NoxV) from *Lactobacillus plantarum* displays enhanced operational stability even in absence of reducing agents

Jonathan T. Park[a], Jun-Ichiro Hirano[a,b], Vaijayanthi Thangavel[a,c], Bettina R. Riebel[d], and Andreas S. Bommarius[a,e]*
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* Corresponding author

Active pharmaceutical ingredients (APIs) such as L-sugars and keto acids are favorably accessed through selective oxidation of sugar alcohols and amino acids, respectively, catalyzed by NAD(P)-dependent dehydrogenases. Cofactor regeneration from NAD(P)H conveniently is achieved via water-forming NAD(P)H oxidases (nox2), which only need molecular oxygen as co-substrate. Turnover-dependent overoxidation of the conserved cysteine residue in the active site of water-forming NAD(P)H oxidases is the presumed cause of the limited nox2 stability.

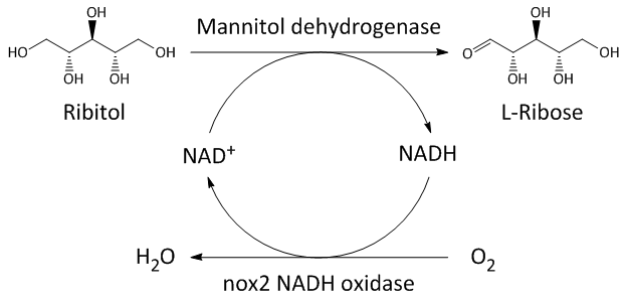

Figure 1 Schematic conversion of ribitol to L-ribose through mannitol-1-dehydrogenase from Apium graveolens complemented with NADH cofactor regeneration using nox2 NADH oxidase.

We present a novel NADH oxidase, NoxV from *Lactobacillus plantarum*, with specific activity of 167 U/mg and apparent kinetic constants at air saturation and 25°C of $k_{cat,app}$ = 212 s^{-1} and $K_{M,app}$ = 50.2 μM in the broad pH optimum from 5.5-8.0. The enzyme features a higher stability than other NAD(P)H oxidases against overoxidation, as is evidenced by a higher total turnover number, in the presence (168,000) and, most importantly, also in the absence (128,000) of exogenously added reducing agents. While the native enzyme shows exclusively activity on NADH, we engineered the cofactor binding pocket to generate variants, G178K,R and L179K,R,H that accommodate and oxidize both NADH and NADPH as substrates.

Acknowledgements
The authors would like to thank the Center for Drug Design, Development, and Delivery (CD4) for financial support (GAANN).

Session 3:

Yujun George Zheng

Chair

A 61-year Mystery Solved: How can Ferric Tryptophan 2,3-Dioxygenase be Activated to the Ferrous State by Peroxide?

Rong Fu, Rupal Gupta, Siming Wang, Jiafeng Geng, Kednerlin Dornevil, Michael P. Hendrich, and Aimin Liu
Department of Chemistry, Georgia State University, Georgia 30302
‡Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Abstract

Tryptophan 2,3-dioxygenase (TDO) is an essential enzyme in the pathway of NAD biosynthesis and important for all mammalian organisms. Here we report a biochemical and spectroscopic study of the reaction of H_2O_2 with ferric TDO. The results show that in the presence of L-Trp, the reaction with peroxide causes enzyme reactivation. In the absence of L-Trp, a previously unknown catalase-like activity is detected. Mössbauer spectroscopy has allowed for the characterization of the ferrous form of TDO generated during the reaction; and a compound ES-type ferryl intermediate generated from the catalase-like activity. The ferryl intermediate exhibits Mössbauer parameters of δ = 0.055 mm/s, ΔE_Q = 1.755 mm/s. During enzyme reactivation, an L-Trp dimer and a monooxygenated L-Trp byproducts are observed by mass spectrometry. Detection of these products has allowed us to propose a mechanism for the reactivation that goes through a possible ferryl species. Density functional theory calculations performed in this study highlight the contribution of the protein microenvironment on the structural influences of the high-valent Fe intermediate. In addition, our results indicate that one of the oxygen atoms inserted into L-Trp during TDO's dioxygenase activity is readily exchangeable with solvent in ^{18}O-labeling experiments.

Choline-Glycine Betaine Metabolic Pathway in *Aspergillus fumigatus*

Andrea Pennati[1], Karine Lambou[2], Jean-Paul Latgé[2] and Giovanni Gadda[1, 3, 4]

Departments of [1] Chemistry and [3] Biology, [4] The Center for Biotechnology and Drug Design, Georgia State University, P.O. Box 4098, Atlanta, Georgia 30302-4098
[2] Unité des Aspergillus, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France.

The genes encoding choline oxidase (*codA*) and betaine aldehyde dehydrogenase (*badh*) are clustered as a single transcriptional unit on the genome of *Aspergillus fumigatus*. Phylogenetic analysis showed that the two genes are conserved in most ascomycetes. Both proteins were heterologously expressed in *Escherichia coli* and the products purified to homogeneity. Choline oxidase is a flavin dependent enzyme that catalyzes the four electron oxidation of choline to glycine betaine with betaine aldehyde as intermediate and reduction of molecular oxygen to hydrogen peroxide. Betaine aldehyde dehydrogenase oxidizes betaine aldehyde to glycine betaine with reduction of $NAD(P)^+$ to NAD(P)H. The two enzymes are co-localized in the cytoplasm of swollen and germinated conidia and might catalyze sequential reactions that result in the synthesis of glycine betaine.

The catabolism of glycine betaine is used to provide nitrogen and/or carbon source during the conidial germination of the fungus as shown by the studies on the single and double mutants but is not involved in its virulence.

This study was supported in part by NSF-CAREER MCB-0545712 (G.G.)

Dr. Dale Edmondson

Emory University
Department of Biochemistry
Atlanta, GA

Abstracts for Poster Presentations:

The poster session will be held in the atrium of the Helen M. Alderhold Learning Center.

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65. Zhang, Han
66. Zhang, Ying

Improved Thermostability of an Amino Ester Hydrolase by Modified Structure-Guided Consensus Concept

Janna K. Blum[1], M. Daniel Ricketts[2], Thanh Ha[2] and Andreas S. Bommarius[1,2]

[1] Department of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta,GA
[2] Department of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA

An α-amino ester hydrolase (AEH, E.C. 3.1.1.43) applicable to synthesis of semi-synthetic antibiotics was cloned from the genomic DNA of *Xanthomonas campestris pv. campestris sp.* strain ATCC 33913. AEHs catalyze the synthesis and hydrolysis of α-amino β-lactam antibiotics. The enzyme was characterized for thermodynamic and kinetic parameters. The enzyme shows optimal ampicillin hydrolytic activity at 25ºC and pH 6.8, with kinetic parameters k_{cat} of 72.5 s^{-1} and K_M of 1.1 mM. The AEH enzymes have been shown to have excellent synthetic capability. However, this enzyme exhibits poor thermostability with an optimal temperature of 25°C and observed half-life of < 7 minutes at 30°C and a T_{50}^{30}, the temperature at which the half-life is 30 minute, of 27ºC.

To improve the thermostability of the AEH a modified structure-guided consensus model of seven homologous enzymes was generated along with analysis of the B-factors from the available crystal structures of the known AEH from *Xanthomonas citri*. The degree of consensus was restricted to >50% of the homologous proteins, unless the residue had an average B-factor in the top 20 of the protein, in which case then only a absolute consensus, a plurality, was required. Eighteen single variants were constructed and analyzed for T_{50}^{30}.

Of the eighteen variants, nine improved the thermostability over the wild-type, a hit rate of 50%. The single variant A275P improved the specific activity to 1.4-fold activity of the wild-type with no effect of the thermostability. In the second round, double and triple mutants were constructed, resulting in a triple mutant, A275P/N186D/V622I. A mutation that incorporated an intersubunit disulfide bridge into the triple mutant had the highest thermostability with a T_{50}^{30} of 37ºC (ΔT_{50}^{30} = 10ºC); however, the mutation had a 5-fold decrease in activity. In the third round, independent NNK saturation of two high B-factor sites, K34 and E143, resulted in our best variant, which is a quadruple mutant, E143H/A275P/N186D/V622I, that has a T_{50}^{30} of 34ºC and 1.3-fold activity compared to wild-type.

The modified structure-guided consensus method successfully led to an improved AEH with minimal library of < 200 variants screened. The mutated positions selected based on B-factor required saturation to demonstrate improved protein function, as most of the single mutations toward the consensus residue did not affect either the stability or activity.

Acknowledgements
This work was supported in part by a National Institutes of Health Grant (5R01AI064817-02) and the NSF Graduate Research Fellowship. Additionally, we would like to acknowledge funding from the Georgia Tech Presidential Undergraduate Research Assistantship (PURA) for funding of Dan Ricketts and Thanh Ha.

Pinpointing the Molecular Basis for Metal Effects on PAI-1

Joel Bucci, Larry Thompson, Jenny Yang, and Cynthia Peterson

Department of Biochemistry and Cellular and Molecular
Biology, University of Tennessee, Knoxville

Plasminogen Activator Inhibitor-1 (PAI-1) is a unique member of the serine protease inhibitor family because it possesses inherent instability at physiological conditions. PAI-1 inhibits serine proteases such as tissue type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), functioning as the principal regulator of fibrinolysis, and pericellular proteolysis, respectively. Our lab has uncovered a novel effect of transition metals on the modulation of PAI-1 stability and the fibrinolysis pathway. Here, we aim to identify the metal binding site(s) within PAI-1, and determine how metals affect PAI-1/VN interaction. To this end, we will perform a computational prediction to map potential metal coordination sites within PAI-1. In addition to the prediction, a recently developed crystal structure of PAI-1 W175F indicated an asymmetric heterodimer interface, which houses a zinc atom. We were pleased to note that the residues involved were predicted in the computational search. We plan perform site directed mutagenesis on all predicted sites, with the aim to perturb potential metal coordination sites. We will have a major focus on the binding site involved in both the prediction and the crystal structure. To detect quantifiable differences in metal binding affinity, we will employ Surface Plasmon Resonance, and stability measurements. This strategy should facilitate identification of the metal binding site(s) on PAI-1. With this information, we can make significant strides in how this metals affect PAI-1 interaction with VN, and regulation of fibrinolysis.

Structural Dynamics of Cytochrome P450 Enzymes: Implications in Drug Design

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Cytochrome P450s are a class of heme-containing enzymes that catalyze chemical reactions in various lifeforms, such as biosynthesis of steroid hormones and hydroxylated fatty acids, metabolism of an extensive variety of xenobiotic compounds, including a high percentage of drugs produced by the pharmaceutical industry. The catalytic cycle of these enzymes entail many steps during which they bind specific substrate molecules, add an oxygen atom to them in a series of oxidation and reduction reactions, and form product with high regio- and stereo-selectivity. Structural studies on several P450s have thus far revealed that they have highly conserved structural features. Given this fact, it is likely that diversity of substrate binding and catalysis observed in various P450s is regulated by intrinsic characteristics of the protein that are not observable in static representations of their structures. One such factor is the dynamic flexibility inherent in the active site and the substrate binding regions for these P450s that allows them to recognize multitude of substrates and monooxygenate them at a variety of positions. It has been observed that there is a varying degree of plasticity within these regions in all P450s and such dynamic motions have become a topic of intense research inquiry, especially in the drug metabolizing enzymes due to the potential implications in drug design. Of the techniques currently available to observe dynamic effects in proteins, Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as the primary technique to characterize dynamics on timescales spanning from picoseconds to seconds. To demonstrate the feasibility of NMR measurements for characterizing these motions in individual amino acids and to assess the degree to which they are correlated to the functional dynamics of the molecule as a whole on all relevant timescales, we have conducted various NMR dynamics experiments on a model P450 system, CYP101 from the bacteria *P. putida*, which is the most-studied representative of the P450 superfamily. For these NMR studies, CYP101 was isotopically labeled in an *E. coli* expression system and subjected to NMR studies in the form of relaxation time measurements which were then analyzed to obtain dynamic parameters reflecting motions in the picosecond-nanosecond time-scale. Similarly, chemical exchange rate measurements were carried out to obtain dynamic parameters reflecting motions on the microsecond-millisecond time-scale. These dynamic measurements were performed on CYP101 with and without the presence of its substrate, camphor, in order to compare the dynamic differences between the two forms. This comparison allowed us to identify the regions that are dynamic and the extent to which their dynamics controls camphor binding. This methodology can be extended to define the dynamic portions of other P450 enzymes, such as those involved in drug metabolism in humans and characterize the role of dynamics in binding various drugs. This will allow drug designers to demarcate the dynamic regions as sites for potential exploitation in the creation of improved, more efficient pharmaceutical products.

Acknowledgements
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Allosteric Regulation of α-Isopropylmalate Synthase from *Mycobacterium tuberculosis*

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Allosteric regulation of proteins, where the binding of a molecule at one site affects the chemical and physical properties at a distal site, has been studied in the field of biochemistry for over forty years. Historically, allostery was described as a two-state model, where active and inactive forms of the enzyme exist through changes in structural features. However, in the past ten years, allostery is being thought of as a global change in the dynamics of a protein in the absence of a change in shape. The enzyme α-isopropylmalate synthase from *Mycobacterium tuberculosis* (*Mt*IPMS), which catalyzes the first step in the biosynthesis of L-leucine, is being studied to obtain a greater understanding of the allosteric regulation and protein dynamics of a large multidomain enzyme. *Mt*IPMS is known to display slow onset inhibition in the presence of leucine, however K_m values for both substrates are not affected. The slow onset mechanism is intriguing given that leucine binds in the regulatory domain over 50 Å from the active site (Figure 1).

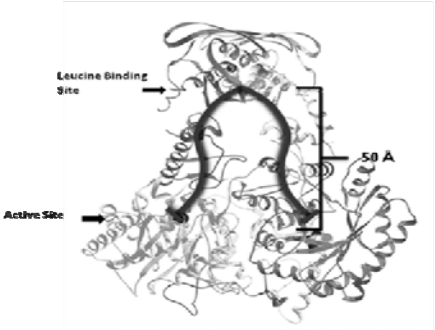

Figure 1: Crystal Structure of *Mt*IPMS (PDB: 3FIG1). The leucine binding site in the regulatory domain binds 50 Å from the active site.

Solution phase hydrogen/deuterium exchange experiments mapped changes in the protein dynamics upon leucine binding. The results identified a conserved active site helix that contains ligands essential to binding the divalent metal ion and α-KIV in the allosteric mechanism. This result led to several testable models of allosteric regulation. Site-directed mutagenesis was implemented in order to probe the active site helix residues' role in catalysis and regulation. Based upon the kinetic parameters determined, each residue plays a role in efficient catalysis. However, none of the enzyme variants affect the kinetics of leucine inhibition when compared to the wild-type enzyme. As another approach to understanding the allosteric mechanism, alternate amino acids were tested as inhibitors of the enzyme. Alternate amino acids studied thus far display non-competitive inhibition (K_i = 250-3,000 μM) with no slow-onset mechanism. These results suggest that the determinant for the slow-onset mechanism does not occur in the catalytic domain but in the regulatory domain. The ability of the divalent metal to activate catalysis in the presence of leucine was also tested. The results indicated that the concentration of the divalent metal needed for activation is not affected by the presence of leucine. Finally, we tested if leucine binding uncoupled the hydrolysis of AcCoA from the condensation reaction. NMR data shows that the addition of leucine does not uncouple the C-C bond formation and α-isopropylmalate and free CoA are seen as the only products.

Design, Synthesis, and Polymerase-catalyzed Incorporation of Click-Modified Boronic Acid-TTP Analogues

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Abstract: DNA molecules are known to be important materials in sensing, aptamer selection, nanocomputing, and construction of unique architects. The incorporation of modified nucleobases affords DNA unique properties for applications in areas that are otherwise difficult or not possible. Earlier, we have demonstrated that the boronic acid moiety can be introduced into DNA through polymerase-catalyzed reactions. In order to study whether such incorporation by polymerase is a general phenomenon, we designed and synthesized four boronic acid-modified TTP analogues. The synthesis of analogues 4 and 5 was through the use of a single dialkyne tether for both the Sonogashira coupling with thymidine and later Cu-mediated (2+3) cycloaddition for linking the boronic acid moiety. This approach is much more efficient than the previously described method, and paves the way for the preparation of a large number of boronic acid-modified TTPs with a diverse set of structural features. All analogues showed very good stability under PCR conditions and were recognized as a substrate by DNA polymerase, and thus incorporated into DNA.

Keywords: boronic acid-modified TTP analogues • glycoprotein • synthesis • incorporation • click reaction

Abstract for Second Annual Southeast Enzyme Conference

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Geobacter daltonii **Utilizes the Toluene Degradation Pathway during growth on Benzene**

The recent oil spill in the Gulf of Mexico exemplifies how substantial quantities of aromatic compounds can be introduced to the anaerobic sediment of the ocean floor. To date, bioremediation of one of these aromatics, benzene, has only been demonstrated for aerobic microorganisms. We have found that the obligate anaerobe *Geobacter Daltonii* is capable of growing on benzene as it's sole carbon and energy source. Genomic analysis reveals the presence of duplicate copies of the toluene-activating enzyme, benzyl succinate synthase (BSS). Activation of two copies of the gene for the benzylsuccinate synthase α subunit (*bssA*) during growth on benzoate, toluene, or benzene was tested using RT-PCR. The findings suggest that benzene oxidation proceeds through use of the toluene oxidation pathway in *G. daltonii.* In an attempt to characterize the similarities/differences between the gene products of these two BSS homologs, we are cloning and overexpressing both of the BSS enzyme complexes.

Characterization of Enzymes Involved in the Biodegradation of *t*-Butanol

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Methyl *t*-butyl ether (MTBE) is a gasoline additive with pollutant qualities. Bacterial strains have been isolated that can degrade MTBE. Microbiological evidence has identified a pair of enzymes (MdpJ/MdpK) that are proposed to oxidatively degrade *t*-butanol, a step in MTBE biodegradation. MdpK is predicted to activate molecular oxygen to hydroxylate *t*-butanol, while MdpJ is predicted to act as an oxidoreductase for MdpK. Both enzymes are hypothesized to have a Rieske iron-sulfur center. Our objective is to confirm the *in vitro* activity of MdpJ and characterize its biochemical properties. We have developed a soluble, isolatable form of MdpJ by creating a fusion enzyme to maltose binding protein (MBP). In trial experiments we are able to separate MdpJ from MBP through a thrombin cleavage. Initial studies completed on the MdpJ-MBP fusion indicate that one equivalent of flavin is bound to the enzyme, and the construct forms a dimer as determined through size exclusion chromatography.

An Enzyme Trio in the Kynurenine Pathway of Tryptophan Catabolism Prevents Neurotoxin Formation

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In mammals, there are two separate pathways by which tryptophan can be metabolized, into serotonin and through the Kynurenine pathway respectively. Only a small portion of ingested tryptophan is converted to serotonin and then melatonin. Over ninety-nine percent is catabolized through the Kynurenine pathway into a number of neuroactive molecules such as kynurenic acid, quinolinic acid, a NAD^+ precursor, picolinic acid or to acetyl-CoA which can enter glycolysis. The Kynurenine pathway is of interest to study because elevation all of the aforementioned neuroactive compounds is associated with several disease states: Alzheimer's disease, anxiety, depression, epilepsy, AIDS dementia, and Huntington's disease. This research will focus on the enzymatic activity of an unique enzyme trio in the tryptophan kynurenine pathway: 3-hydroxyanthranilic acid 2,3-dioxygenase (HAD), α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD), and α-amino-β-muconate-ε-semialdehyde dehydrogenase (AMDH). Both the substrate and product of ACMSD and AMDH are unstable compounds that spontaneously autocyclize. Therefore, the three enzymes must interact with each other to avoid leaking of the metabolites. This enzyme trio controls the in vivo levels of an important neurotoxin, especially in AIDS dementia complex (ADC).

Discovery of organic Inhibitors of Protein Arginine Methyltransferases

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By transferring methyl groups to designated targets of the cell, Protein Arginine Methyltransferases 1 (PRMT1) and 5 (PRMT5) regulate many normal as well as pathological processes of the body. Diseases, e.g., cancer, may arise from the enzyme's improper methylation patterns upon histone and nonhistone substrates, leading to aberrant regulation of cellular signaling pathways. Despite a growing number of determined and suggested roles for the PRMT family of enzymes in disease states, development of inhibitory compounds has been lagging behind. In the current experiment, His-tagged PRMT1 was expressed via a vector pET-28(b+), which had been incorporated into *Escherichia coli* BL21(DE3) cells, purified upon Ni-NTA beads in an affinity chromatography. His-tagged PRMT5 was expressed via a vector, pET-28a(+) with coexpression of MEP50 in *Escherichia coli* BL21(DE3) cells. The respective levels of methyltransferase activity of the PRMTs was then assessed, using the ^{14}C labeled S-adenosyl methionine and the N-terminal H4 peptide as the substrates. Activity was then quantified using liquid scintillation. Purified PRMTs, with acceptable levels of activity, were subsequently employed to determine the effectiveness of approximately twenty small molecule inhibitors, against PRMT 1 and PRMT 5 activity, respectively. Based on measured IC_{50} values, we find that several compounds have promising potential as therapeutic agents against PRMT1 and PRMT5 activity, respectively.

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Enzymatic Synthesis of L-Tryptophan by *E.*coli Whole-cells Catalysis from Indole, Glycerol, and Ammonia Acetate

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In the recent years, an increase in biodiesel production worldwide has resulted in a surplus of glycerol, originated as a by-product from biodiesel processing. It is estimated that glycerol accounts for approximately 10% by volume of the biodiesel being produced. In 2008, Europe produced an estimated $7.75e^6$ tons of biodiesel, whereas the United States on the other hand, produced an approximate $9.60e^4$ tons of biodiesel. It is part of our current interest and research effort to convert this abundant resource into a marketable commodity.

It was previously reported that glycerol can be converted into glyceraldehydes-3-phosphate by Escherichia coli under aerobic and anaerobic conditions. Glyceraldehyde-3-phosphate can further undergo glycolysis to give pyruvate as the end product. In addition, tryptophan indole-lyase (tryptophanase), a pyridoxal-5-phosphate dependent bacterial enzyme can catalyze the synthesis of L-tryptophan from indole, ammonia and pyruvate. Our initial interest was to determine whether glycerol can be converted into L-tryptophan, a more valuable material, by first converting into pyruvate, along with a supplemental source of indole and ammonium acetate.

Using *E.* coli competent cells BL21 (DE3), with plasmid pET15b:tnaA which over-expresses tryptophanase, we were able to produce L-tryptophan by supplementing the reaction medium with indole, glycerol, and ammonium acetate. The presence of L-tryptophan was detected in both thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) at 278nm. Preliminary data indicates we were able to successfully demonstrate and validate the biosynthetic pathway for tryptophan starting with glycerol using recombinant technology. At present, it is part of our ongoing effort to optimize the percent conversion for this process.

Acknowledgement
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Thermodynamics and Solvent Effects on Substrate and Cofactor Binding in *E. coli* Chromosomal Dihydrofolate Reductase

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Chromosomal dihydrofolate reductase from *E. coli* catalyzes the reduction of dihydrofolate to tetrahydrofolate using NADPH as a cofactor. The thermodynamics of ligand binding were examined using an isothermal titration calorimetry approach. Using buffers with different heats of ionization, zero to a small, fractional proton release was observed for dihydrofolate binding while a proton was released upon $NADP^+$ binding. The role of water in binding was additionally monitored using a number of different osmolytes. Binding of $NADP^+$ is accompanied by the net release of ~5-24 water molecules, with a dependence on osmolyte identity. In contrast, binding of dihydrofolate is weakened in the presence of osmolytes, consistent with "water uptake." Different effects are observed depending on osmolyte identity. The net uptake of water upon dihydrofolate binding was previously observed in the non-homologous R67 encoded dihydrofolate reductase (dfrB or type II enzyme; Chopra et al., *J. Biol. Chem. 283,* 4690-4698). As R67 dihydrofolate reductase possesses a non-homologous sequence and forms a tetrameric structure with a single active site pore, the observation of weaker DHF binding in the presence of osmolytes in both enzymes implicates co-solvent effects on free dihydrofolate. Consistent with this analysis, stopped flow experiments find betaine mostly affects DHF binding by alterations in k_{on}, while betaine mostly affects NADPH binding by changes in k_{off}. Finally, non-additive enthalpy terms when binary and ternary

cofactor binding events are compared suggest the presence of long-lived conformational transitions that are not accounted for by a simple thermodynamic cycle.

A 61-year Mystery Solved: How can Ferric Tryptophan 2,3-Dioxygenase be Activated to the Ferrous State by Peroxide?

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Abstract

Tryptophan 2,3-dioxygenase (TDO) is an essential enzyme in the pathway of NAD biosynthesis and important for all mammalian organisms. Here we report a biochemical and spectroscopic study of the reaction of H_2O_2 with ferric TDO. The results show that in the presence of L-Trp, the reaction with peroxide causes enzyme reactivation. In the absence of L-Trp, a previously unknown catalase-like activity is detected. Mössbauer spectroscopy has allowed for the characterization of the ferrous form of TDO generated during the reaction; and a compound ES-type ferryl intermediate generated from the catalase-like activity. The ferryl intermediate exhibits Mössbauer parameters of δ = 0.055 mm/s, ΔE_Q = 1.755 mm/s. During enzyme reactivation, an L-Trp dimer and a monooxygenated L-Trp byproducts are observed by mass spectrometry. Detection of these products has allowed us to propose a mechanism for the reactivation that goes through a possible ferryl species. Density functional theory calculations performed in this study highlight the contribution of the protein microenvironment on the structural influences of the high-valent Fe intermediate. In addition, our results indicate that one of the oxygen atoms inserted into L-Trp during TDO's dioxygenase activity is readily exchangeable with solvent in ^{18}O-labeling experiments.

PRMT-Substrate Interaction and Regulation

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Protein arginine methyltransferases (PRMTs) catalyze the transfer of methyl groups from S-adenosyl-L-methionine (AdoMet, SAM) to the guanidino group of arginines in histone and nonhistone protein substrates. Thus far, eleven human PRMT members have been identified at the proteomic level and categorized into two major types, type I and type II, according to substrate and product specificity. PRMT1, a predominent type I PRMT, plays a key role in transcriptional control and becomes a potential target for therapeutic treatment of cancer and cardiovascular disease. Histone H4 undergoes extensive posttranslational modifications (PTMs) at its amino-terminal tail, including methylation and acetylation, which profoundly affect the on-and-off status of gene transcription. Our data reveal that the effect of lysine acetylation on arginine methylation depends on the site of acetylation and the type of methylation. While certain acetylations present a repressive impact on PRMT-1 mediated methylation (type I methylation), lysine acetylation generally is correlated with enhanced methylation by PRMT5 (type II dimethylation). These findings provide new insights into the regulatory mechanism of Arg-3 methylation by H4 acetylation, and unravel that complex intercommunications exist between different PTM marks *in cis*. On the other hand, we found that PRMT1 dissociates from the monomethylated substrate much faster than transfer of the second methyl group, indicating its low processivity in catalysis.

A Novel Activity for Fungal Nitronate Monooxygenase: Detoxification of the Metabolic Inhibitor Propionate-3-Nitronate

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Nitronate monooxygenase (NMO; E.C. 1.13.12.16) is an FMN dependent enzyme that oxidizes alkyl nitronates to their corresponding carbonyl compounds and nitrite. While the kinetic and mechanistic properties of the fungal NMO from *Neurospora crassa* and *Williopsis saturnus* var. Mrakii have been extensively characterized, the physiological role of the enzyme is still unknown. The current study demonstrates that the enzyme oxidizes propionate-3-nitronate (P3N), the highly toxic conjugate base form of the plant metabolite 3-nitropropionate (3NPA), and provides compelling evidence that the physiological role of NMO is detoxification. Thus, the enzyme appears to play a similar role as propionate-3-nitronate oxidase from *Penicillium atrovenetum* (1) and may suggest a general defensive strategy among fungal species.

The Michaelis constants of both fungal NMOs were significantly lower with P3N as substrate than with primary alkyl nitronates. P3N was toxic to *E. coli* cells lacking NMO, but the toxicity was overcome through either expression of the recombinant gene for NMO or through addition of exogenous enzyme to the cultures. Both *W. saturnus* and *N. crassa* were able to grow in the presence of up to 20 mM 3NPA, which is within the concentration range naturally produced in the environment (*i.e.* ~17 mM *in Penicillium* (*1*)). A knockout mutant of *N. crassa* lacking the gene encoding for NMO could not grow at concentrations above 600 μM, which establishes a role of the enzyme in detoxification of the nitronate. The results indicate that NMO functions to protect the fungi against the toxicity of 3NPA and P3N.

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Role of Tyrosine 249 in substrate binding of D-Arginine Dehydrogenase from *Pseudomonas aeruginosa*

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D-Arginine dehydrogenase is a flavin dependent enzyme that has been isolated from the Gram-negative bacterium *Pseudomonas aeruginosa*, a human pathogen.[1] It catalyzes the oxidation of D-arginine to iminoarginine.[1,2] The crystallographic structures of the enzyme in complex with either iminoarginine or iminohistidine have been published to high resolutions (≤ 1.3 Å). The availability of the crystal structure in complex with the product enabled to understand the different interactions between the enzyme and the substrate. The enzyme iminoarginine complex (Figure 1) showed several hydrogen bonding and electrostatic interactions between main chain and side chain atoms of Tyr53, Glu87, Arg222, Tyr249, Arg305, Gly332 and the substrate.[2]

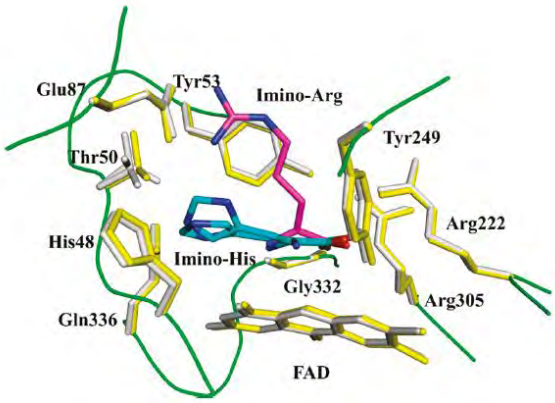

Figure 1. Crystal Structure of DADH - iminoarginine complex

Here we have focused on probing the role of Tyr249 in substrate binding. For the purpose of this study, two mutants Tyr249Phe and Tyr249Met have been obtained through site-directed mutagenesis. Rapid kinetics and kinetic isotope effects have been used to investigate the role of Tyr249. The mechanistic data will be discussed in the context of the available structures of the enzymes.

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Application of Thiol-sensitive Fluorogenic Probes for HAT Activity Assay

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ABSTRACT

Histone acetyltransferases (HATs) catalyze the acetylation of specific lysine residues in histone and nonhistone proteins. On the chromatin template, major lysine acetylation destabilizes the nucleosome structure and promotes the accessibility of transcription factors to genetic loci. Recent studies showed that acetylation is widely distributed among cellular proteins, suggestive of diverse functions of HATs in cellular pathways. Here we evaluated a series of fluorogenic compounds for the detection of the enzymatic activities of HATs. This sensitive fluorescent acetyltransferase assay works by detecting the production of CoA generated produced in the HAT reaction with sulfhydryl sensitive fluorogenic probes. Upon conjugation to the thiol group of CoA, the compounds gain enhanced quantum yields and strongly fluorescence, permitting facile quantization of HAT activities. We investigated several common thiol-sensitive fluorogenic compounds, including B-8, P-28, A433, D10251, D10253, CPM, M1378 and CME, for their abilities as HAT activity probes. Many important properties were characterized, including kinetics of reaction with thiol, interference with HAT activity, and magnitude of fluorescence enhancement. Our data suggest that CPM and CAE are excellent HAT probes owing to their fast reaction kinetics and dramatic fluorescent changes during the HAT reaction. Further, our microtiter plate measurements show that this method is suited for adaption to screening small molecule inhibitor of HATs in high-throughput format, highlighting the value of this assay strategy in new drug discovery.

Keywords: histone acetyltransferase; HAT; fluorescent probe; epigenetics; chromatin modification

Conformational Changes and Substrate Recognition in *Pseudomonas aeruginosa* D-Arginine Dehydrogenase

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Abstract: DADH catalyzes the flavin-dependent oxidative deamination of d-amino acids to the corresponding α-keto acids and ammonia. Here we report the first X-ray crystal structures of DADH at 1.06 Å resolution and its complexes with iminoarginine ($DADH_{red}$/iminoarginine) and iminohistidine ($DADH_{red}$/iminohistidine) at 1.30 Å resolution. The DADH crystal structure comprises an unliganded conformation and a product-bound conformation, which is almost identical to the $DADH_{red}$/iminoarginine crystal structure. The active site of DADH was partially occupied with iminoarginine product (30% occupancy) that interacts with Tyr53 in the minor conformation of a surface loop. This flexible loop forms an "active site lid", similar to those seen in other enzymes, and may play an essential role in substrate recognition. The guanidinium side chain of iminoarginine forms a hydrogen bond interaction with the hydroxyl of Thr50 and an ionic interaction with Glu87. In the structure of DADH in complex with iminohistidine, two alternate conformations were observed for iminohistidine where the imidazole groups formed hydrogen bond interactions with the side chains of His48 and Thr50 and either Glu87 or Gln336. The different interactions and very distinct binding modes observed for iminoarginine and iminohistidine are consistent with the 1000-fold difference in k_{cat}/K_m values for d-arginine and d-histidine. Comparison of the kinetic data for the activity of DADH on different d-amino acids and the crystal structures in complex with iminoarginine and iminohistidine establishes that this enzyme is characterized by relatively broad substrate specificity, being able to oxidize positively charged and large hydrophobic d-amino acids bound within a flask-like cavity.

Acknowledgements

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Regulation and Characterization of the *dadRAX* Locus for D-Amino Acid Catabolism in *Pseudomonas aeruginosa* PAO1

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D-Amino acids are essential components for bacterial peptidoglycan, and these natural compounds are also involved in cell wall remodeling and biofilm disassembling. In *P. aeruginosa*, the *dadAX* operon, encoding D-amino acid dehydrogenase DadA and amino acid racemase DadX, is essential for D- and L-Ala catabolism, and its expression requires a transcriptional regulator DadR. In this study, the purified recombinant DadA alone was sufficient to demonstrate the proposed enzymatic activity with very broad substrate specificity; it utilizes all D-amino acids tested as substrates except D-Glu and D-Gln. DadA also showed comparable k_{cat} and K_m values on D-Ala and several D-amino acids. The *dadRAX* knockout mutants were constructed and subjected to growth phenotype analysis on amino acids. The results revealed that utilization of L-Ala, L-Trp, D-Ala, and a specific set of D-amino acids as sole nitrogen sources was abolished in the *dadA* mutant and/or severely hampered in the *dadR* mutant while growth yield on D-amino acids was surprisingly improved in the *dadX* mutant. The *dadA* promoter was induced by several L-amino acids, most strongly by Ala, and by D-Ala only among all tested D-amino acids. Enhanced growth of the *dadX* mutant on D-amino acids is consistent to the finding that the *dadA* promoter was constitutively induced in the *dadX* mutant, where exogenous D-Ala but not L-Ala reduced the expression. Binding of DadR to the *dadA* regulatory region was demonstrated by electromobility shift assays, and the presence of L-Ala but not D-Ala increased affinity by 3-fold. The presence of multiple DadR-DNA complexes in the *dadA* regulatory region was demonstrated *in vitro*, and the formation of these nucleoprotein complexes exerted a complicated impact on promoter activation *in vivo*. In summary, the results from this study clearly demonstrate DadA as solely responsible enzyme for the proposed D-amino acid dehydrogenase activity of broad substrate specificity, the physiological functions DadRAX on catabolism of several D-amino acids, and support L-Ala as the signal molecule for induction of the *dadAX* genes through DadR binding to several putative operator sites.

Acknowledgments
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A Survey of Aspartate-Phenylalanine and Glutamate- Phenylalanine Interactions in the Protein Data Bank: Searching for Anion-π Pairs

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Protein structures are stabilized using non-covalent interactions. In addition to the traditional non-covalent interactions, newer types of interactions are suggested to be present in proteins. One such interaction, an anion-π pair, has been previously proposed where the positively charged edge of an aromatic ring interacts with an anion, forming a favorable anion-quadrupole interaction (Jackson et al., *J Phys Chem B* **2007**, *111*, 8242-8249). To study the role of anion-π interactions in stabilizing protein structure, pairwise interactions between phenylalanine (Phe) with the anionic amino acids, aspartate (Asp) or glutamate (Glu), were analyzed. Particular emphasis was focused on identification of Phe and Asp or Glu pairs separated by less than 7Å in the high resolution, non-redundant Protein Data Bank. Simplifying Phe to benzene and Asp or Glu to formate molecules facilitated *in silico* analysis of the pairs. Kitaura-Morokuma energy calculations were performed on roughly 19,000 benzene-formate pairs and the resulting energies analyzed as a function of distance and angle. Edgewise interactions typically produced strongly stabilizing interaction energies (-2 to -7.3 kcal/mol), while interactions involving the ring face resulted in weakly stabilizing to repulsive interaction energies. The strongest, most stabilizing interactions were identified as preferentially occurring in buried residues. Anion-π pairs are found throughout protein structures, in helices as well as β-strands. Numerous pairs also had nearby cation-π interactions as well as potential π-π stacking. While over a thousand structures did not contain an anion-π pair, the remaining 3134 structures contained approximately 2.6 anion-π pairs per protein, suggesting it is a reasonably common motif that could contribute to overall structural stability of a protein.

Insight into the Catalytic Mechanism: Mutagenesis and Kinetic Studies of α-Amino-β-Carboxymuconic-ε-Semialdehyde Decarboxylase (ACMSD)

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Abstract:

α-Amino-β-carboxymuconic-ε-semialdehyde decarboxylase (ACMSD) determines the partitioning of the metabolic fates in both the 2-nitrobenzoic acid degradation pathway and the kynurenine pathway. ACMSD is a zinc-dependent enzyme that catalyzes a transition metal-dependent non-oxidative decarboxylation reaction. In this work, we carried out mutagenesis, ICP metal analysis, quinone staining, EPR, fluorescence, resonance Raman analyses, steady-state kinetic and kinetic isotopic effect studies to probe the catalytic mechanism of ACMSD. A novel decarboxylase mechanism will be proposed in the presentation.

Effect of Steam Explosion on Degradability and Accessiblity of Loblolly Pine for Biofuel Applications

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Abstract

Among the various biomass pretreatment methods, steam explosion is one of the most widely used due to its cost-effective disruption of the plant structure and hence enhanced enzymatic accessibility.
Three different substrates, Avicel, phosphoric acid swollen cellulose (PASC), and SO_2 steam-exploded loblolly pine (SELP), were subjected to enzymatic hydrolysis and subsequent adsorption study. Despite the complexity of both substrate and enzyme mixtures, the SELP adsorption isotherm can be fitted to Langmuir equation ($R^2 > 0.97$). The saturation point was reached at ~6.24 mg/ml total enzyme loading. Adsorption data were also correlated with initial rates and once again showed the dependence of initial rate on initial adsorption amount.

Acknowledgement

Y.K. thanks Institute of Paper Science and Technology at Georgia Institute of Technology for financial support.

Effects of Progressive Deletion of a Unique Loop on Structure and Function of Catalase-Peroxidases

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Catalase-peroxidases (KatGs) catalyze hydrogen peroxide decomposition by two distinct mechanisms using a single active site. This active site catalyzes substantial catalase turnover even though it bears no resemblance to classical catalases. Instead, the active site is superimposable on classical peroxidases like cytochrome c peroxidases, enzymes which have little if any catalase activity. One of the unique features imparting this bifunctionality is large loop 1 (LL1). LL1 forms part of a substrate access channel to heme buried in the active site and provides the tyrosine residue in the unique methionine-tyrosine-tryptophan covalent adduct essential for catalase but not peroxidase activity. This study focuses on identifying additional roles of LL1 in the unique catalytic properties of KatGs. Variants lacking portions of LL1 were subjected to steady state and transient state kinetic analysis. All variants showed a complete loss of catalase activity but the deletion variants showed substantial increase in peroxidase activity compared to wild type and Y226F enzyme. This increase in peroxidase activity was traceable to rapid reduction of high oxidation state intermediates by exogenous electron donors, and coincident prevention of enzyme inactivation by peroxides. These results highlight the role of LL1 as a gate keeper to limit typical peroxidase activity in favor of catalase turnover. Y226F has no catalase activity and insufficient capability to use exogenous electron donors to sustain peroxidase activity.

This project was supported by National Science Foundation (MCB- 0641614).

Kinetic studies of citramalate synthase from *Leptospiro interrogans*

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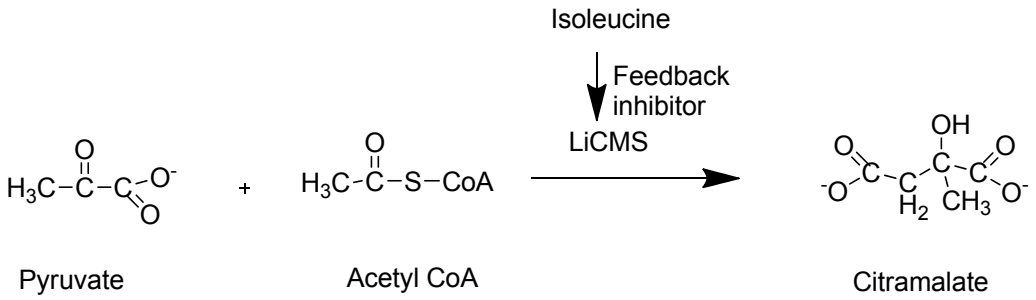
Allostery in proteins is a key regulator of cellular processes and has been an important research area for the past 40 years. We are working on the allosteric behavior of the enzyme citramalate synthase from *Leptospiro interrogans* (*Li*CMS). *Li*CMS catalyzes the first step in isoleucine synthesis, a Claisen condensation between pyruvate and acetyl-CoA, and is feedback inhibited by isoleucine.



*Li*CMS is structurally similar to *Mycobacterium tuberculosis* Isopropylmalate synthase (*Mt*IPMS), an enzyme catalyzing the first step in leucine synthesis. Both the enzymes catalyze Claisen condensation reactions and are inhibited by the final product of the pathways they catalyze. However the mechanism of inhibition in both of these enzymes is quite different as in *Li*CMS the binding of substrate is affected whereas in *Mt*IPMS the rate of chemistry of reaction is altered. A crystal structure of *Li*CMS has previously been reported along with a basic kinetic characterization of the enzyme (Peng, Z. *et al. Biochem.J.* (**2009**) 421, 133). Due to our interest in allosteric behavior of enzymes we are working on a more rigorous kinetic characterization of the enzyme. We have successfully expressed and purified a more active enzyme than reported previously. The K_m for acetyl-CoA determined in TAPS buffer at pH 8.14 is 190 μM, about 5 fold less than the previously reported value. It is also reported that monovalent metal ions affect the activity of the enzyme. Here we report that K^+ ions activate the enzyme in a positively cooperative manner.

Computational Investigation of the Mechanism of Oxalate Decarboxylate

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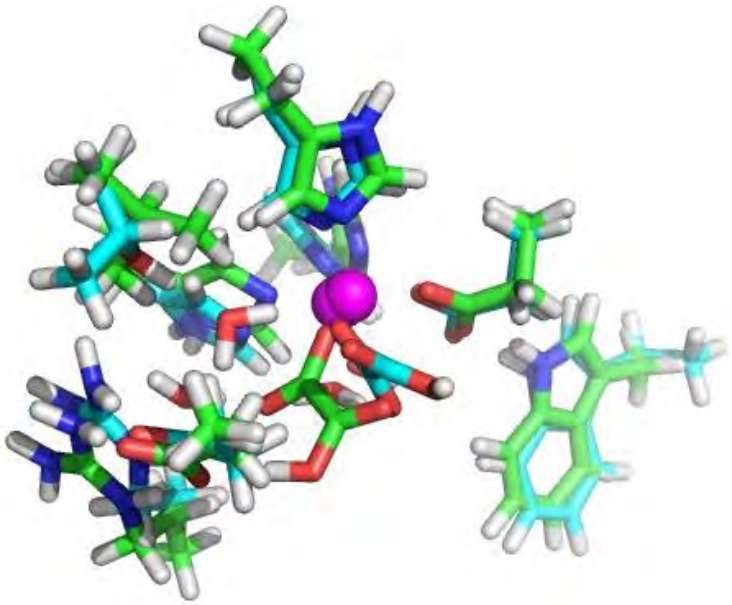
Oxalate decarboxylase is a manganese dependent enzyme that catalyzes the breakdown of oxalate to carbon dioxide and formate in the presence of oxygen. The mechanism by which this reaction occurs is not well understood, but, based on mutagenesis studies, work with heavy atom isotopes, and EPR data, a mechanism has been proposed. Computational investigation of this reaction, including using QM/MM to determine the energy profile for each step of the proposed mechanism and in a mutant, T165V, was attempted to increase our understanding of the enzyme. There is a difference of approximately eight kcal/mol for the proton transfer step between the wild type and the mutant. This poster will include details on the methods and results of this investigation and the implications of the calculations on the proposed mechanism.



Representation of the QM region for the WT enzyme (green) and the T165V enzyme (blue). The mutation appears to change the position of a water molecule near the active site.

Correlating the enzymatic activity of APE1 mutants to conformational changes in the active site: Insight from molecular dynamics

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Abstract

Base excision repair (BER) is the DNA repair pathway most commonly employed to recognize, excise, and replace incorrect or damaged bases. An essential step in BER is the action of AP endonucleases on abasic DNA substrates – a reaction whereby the abasic sites are processed to generate 3'-hydroxy termini suitable for DNA repair synthesis and ligation. There has been substantial kinetic data accumulated on active site mutants of the major human AP endonuclease APE1 which could be helpful in elucidating the mechanism of this important participant in BER. We used classical molecular dynamics to test the importance of specific residues (notably E96, D210, N212, H309, Y171, N212 and D283) in the mechanism against this ample mutagenesis data available for APE1. Our goal was to delineate the exact mechanistic role of all active site residues essential for catalysis. To this end, we performed simulations of 19 active site mutants in conjunction with new kinetics assays carried out by our collaborators. The results have been analyzed in terms of changes in the geometry for the nucleophilic attack and the availability of the nucleophile and correlated to changes in activity compared to wild type APE1. In a follow up study we plan to delineate the distinct contributions to catalysis of these residues from AIMD-QM/MM by examining the mutations' effect on the computed activation energies.

Keywords: APE1; AP sites; BER

Comparisons of Fe-Superoxide Dismutase and Fe-Substituted (Mn) Superoxide Dismutase.

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Even for enzymes that rely on bound metal cofactors for activity and integrity, the coordination shells of the protein around the metal cofactor control the chemistry. However, the effects of second sphere interactions around metal cofactors are still not well understood. The Fe- and Mn-specific superoxide dismutases (SOD) are two SODs with the same amino acid ligands and the similar structures, but different metal ion requirements for activity based on very subtle differences in the second sphere. Studying of these two SODs may address the significance of the protein surrounding the ligand sphere.

E. coli Fe-superoxide dismutase (FeSOD) and Fe-substituted Mn-superoxide dismutase [FeMn)SOD] have been overexpressed and purified from wild-type *E. coli*. SDS PAGE and the nitroblue tetrazolium (NBT) assay were used to qualitatively identify these two SODs in complex mixtures. The cytochrome C-reduction interference assay was used to measure the specific activity of the enzymes and the ferrozine assay was used to quantify total Fe. To characterize the distribution of iron among different protein species, we used the in-gel stain of Che-fu Kuo et al (1988).

Yields, specific activities, metal ion contents and spectroscopic characteristics of these two enzymes with and without 100mM potassium chloride and 40mM azide will be compared to elucidate the basis for the inactivity of Fe(Mn)SOD.

Acknowledgements: This work was supported by NIH funding (R01GM085302).
The authors thank the department of Chemistry, University of Kentucky for the support of a Kentucky Opportunity Fellowship to Xiaonan Mei.

Heavy Metal Inhibition of the *E. coli* DNA Repair Protein MutM

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MutM, also known as formamidopyrimidine DNA glycosylase (Fpg), is an enzyme that initiates base excision repair in prokaryotes such as *E. coli*. It recognizes and removes 7, 8-dihydro-8-oxoguanine (8-oxoguanine) and other DNA base modifications. MutM is a trifunctional enzyme, with glycosylase, apurinic/apyrimidinic-lyase, and 5'-terminal deoxyribose phosphate-excising activities, thus converting the DNA base damage into single-strand breaks. DNA excision activity of MutM was reported to be inhibited by heavy metals like cadmium, copper and mercury, which might be one mechanism of heavy metal carcinogenesis. The structural zinc finger motif located at the C-terminus of the enzyme is believed to be the target of heavy metals. Our research shows that heavy metals such as cadmium bind to apo-MutM at a 1:1 molar ratio, most likely at the empty zinc finger site. Through a hyperchromicity-based kinetic assay using double-stranded oligonucleotides with an 8-oxoguanine lesion as the substrate, we have determined that both apo-MutM and heavy metal-bound MutM have lost enzyme activity compared to holo-MutM with approximately 1 molar equivalent of zinc. However, because of the strength of zinc binding to MutM, heavy metals are unlikely to replace the zinc from the zinc finger under physiological conditions.

Acknowledgements

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High-Field Dynamic Nuclear Polarization based on an Endogenous Flavin Radical

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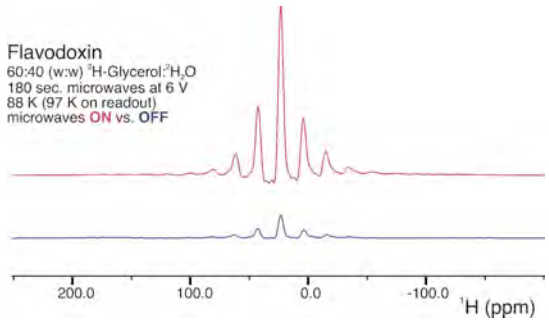
We demonstrate 15-fold enhancement of solid-state NMR signal amplitudes by dynamic nuclear polarization (DNP) from a stable naturally-occurring radical in a protein: the flavin mononucleotide (FMN) semiquinone of flavodoxin. The linewidth of flavodoxin's EPR signal indicates DNP via a solid effect mechanism. This is supported by the microwave power dependence and the field-dependent enhancement profile. Our enhancement is consistent with the 15-fold enhancement obtained based on the solid-effect and trityl radical added to 40 mM [Hu et al (2007) J. Chem. Phys 126:044512], whereas our flavodoxin radical was present at 2 mM or less. The magnitude of enhancement as well as the time constant with which it develops respond to manipulation of the isotopic composition of the sample. Deuteration of the protein to 85 % increased the T_1 and buildup time by factors of five and seven, respectively, consistent with the six-fold dilution of protons by deuterons, and thus a six-fold reduction in the average dipolar coupling between protons. Slowed dissipation of polarization can likewise explain the two-fold higher maximal enhancement than that obtained in proteated protein. By contrast, the long buildup time observed in non-glassy samples was not accompanied by a long T_1, and in this case the enhancement was greatly depressed. Thus, the utilization of endogenous radicals opens the way for strong enhancement of the NMR signals of proteins, and in conjunction with selective isotopic labeling, is anticipated to permit selective enhancement of residues nearby, and distance measurements.



^{1}H NMR signal with and without DNP enhancement.

Acknowledgements

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Inhibition by Nitric Oxide of Catalysis by Oxalate Decarboxylase

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Oxalate decarboxylase (OxDC) is a manganese-containing enzyme, which catalyzes the non-oxidative breakdown of monoprotonated oxalate to carbon dioxide and formate. The reaction requires dioxygen, even though it remains unclear as to its role in the overall mechanism, and where it binds in the enzyme. Studying the effects of an oxygen analogue such as nitric oxide (NO) on the catalytic reaction, may shed light on the location and role of the dioxygen in the enzyme during catalysis. However, traditional endpoint assays used to measure the kinetics of this enzyme preclude the ability to look into the progress of the reaction as the products are formed. Here we report the use of membrane inlet mass spectrometry as a continuous and real-time method of monitoring catalysis by OxDC from *B. subtilis,* by observing the accumulation of CO_2 in solution from its mass peak *m/z* 44 (or *m/z* 45 for $^{13}CO_2$). NO was generated in solution from NONOates, and complete inhibition by micromolar NO (K_i = 40 ± 10 μM) was observed. The inhibition was reversed after a time lag of several minutes by the addition of O_2. Electron paramagnetic resonance of a rapidly frozen reaction mixture showed that the multiplet splittings of Mn(II) were not perturbed by the presence NO. This suggests that although NO is an inhibitor, it does not directly bind to Mn(II) site with the smallest fine structure ($|D|$ = 1200 MHz) in the enzyme.

Acknowledgements

We thank Stephen Bornemann John Innes Centre, Norwich UK) for the provision of the plasmid construct of the polyhisitidine-tagged wild-type *B. subtilis* OxDC. This work was supported by a grant from the NIH DK061666 and NIH GM25154.

STIMULATION OF CATALASE ACTIVITY OF CATALASE-PEROXIDASES BY PEROXIDASE REDUCING SUBSTRATES: NEW FUNCTIONS FROM OLD SCAFFOLDS

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Catalase-peroxidases are heme-containing enzymes that catalyze the decomposition of hydrogen peroxide by two distinct mechanisms using a single active site. Despite sharing no sequence similarities with monofunctional catalases, they possess substantial catalase as well as peroxidase activities. Both functions have different pH optima (<4.0 for peroxidase activity and >6.5 for catalase activity) and the prevalence of one over the other depends on pH and availability of a reducing substrate. The prevailing mechanistic understanding of the interplay of these two functions has always been to superimpose both cycles. This conception defines both activities to be mutually competitive. As such, a peroxidase reducing substrate would be expected to inhibit catalase activity. However, we have observed a $\geq$ tenfold stimulation of catalase activity by peroxidase reducing substrates. The activation effect was pH dependent and was most prominent at low pH (pH<5) where catalase activity would normally be greatly diminished. The dominant heme intermediate in the steady state during reducing-substrate-enhanced catalase activity is one that has long been known as an inactive intermediate in monofunctional peroxidase enzymes. Our discovery suggests that the peroxidase active site in catalase-peroxidases might be utilizing peroxidase mechanistic features to support the newly acquired catalase activity. This is substantiated by the fact that very little of the peroxidase electron donor accumulates in its oxidized state. The vast majority of H_2O_2 consumption under these conditions is by electron donor-activated catalase activity. We have proposed a mechanism that accounts for the catalase activation effect and while this may have relative small or no impact on an organism like *E. coli* where there are redundant catalase systems; however, it may have substantial physiological implications for *M. tuberculosis* which uses heme-dependent catalase-peroxidase as its sole catalase-active enzyme.

ACKNOWLEDGEMENTS

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Functional and Structural Characterization of the Crosslinked and Non Crosslinked Isoforms of Cysteine Dioxygenase

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Cysteine dioxygenase (CDO) is mononuclear Fe (II)-dependent thiol dioxygenase that uses both atoms of dioxygen to oxidize the sulfhydryl group of cysteine to generate cysteine sulfinic acid. The cysteine sulfinic acid can then partition between two different metabolic pathways to form sulfate and pyruvate or taurine. Sequence analysis and structural studies classify CDO as a member of the cupin superfamily because it contains the conserved cupin motifs[1]. Most members of the cupin superfamily coordinate their active site metal in a 3-His-1-Glu metal coordination environment but CDO deviates from this paradigm in that it coordinates its active site Fe^{2+} by a facial triad of 3-His side chains. Another interesting feature of CDO is the presence of the cysteine-tyrosine crosslink located over the active site iron. Further evaluation of this crosslink indicates that it is not homogenous in the purified recombinant enzyme, and previous characterization of CDO has involved a heterogenous mixture of the crosslinked and non crosslinked isoforms. To date, the homogenous crosslinked and non crosslinked isoforms of CDO have not been characterized independently.

Results from previous studies have shown that the homogenously crosslinked isoform of CDO is catalytically inactive and the activity of CDO has been attributed to the non crosslinked form of the enzyme. Interestingly, the recombinant CDO enzyme expressed in cell lysate exists as the non crosslinked isoform, and the crosslink is generated during the course of the purification procedure. The activity of CDO in the cell lysate has been shown to be 10-fold higher than that of purified wild-type enzyme. The inactivity of the crosslinked isoform suggests that there are functional differences between the crosslinked and noncrosslinked isoforms that would lead to erroneous results when studying the heterogenous enzyme mixture. The present study focuses on generating and characterizing the two isoforms independently. The homogenously crosslinked isoform was generated by incubating CDO in the presence of excess ferrous iron, cysteine, and dioxygen. In order to obtain the noncrosslinked CDO enzyme, the protein was purified under anaerobic conditions to prevent dioxygen from initiating crosslink formation. Analysis of the two isoforms by SDS-PAGE showed a single lower band that represented the crosslinked isoform and a single upper band that represented the non crosslinked isoform. The difference in migration patterns of the two isoforms could be linked to structural perturbations of the CDO enzyme that alters the active site architecture. Each of the CDO isoforms was analyzed by circular dichroism spectroscopy to determine if there were any overall structural differences. The susceptibility of each isoform to limited tryptic digestion was evaluated and the difference in the digestion pattern was analyzed by SDS-PAGE. Mass spectrometric analysis of the digested peptides was performed to determine where the altered proteolytic sites were located relative to the protein structure. The results from these experiments have provided valuable insight into the kinetic and structural properties of the two isoforms of this unique thiol dioxygenase.

1. McCoy, J. G., Bailey, L. J., Bitto, E., Bingman, C. A., Aceti, D. J., Fox, B. G., and Phillips, G. N. J. (2006) Structure and mechanism of mouse cysteine dioxygenase, *Proc. Natl. Acad. Sci. U. S. A. 103*, 3084-3089.

Haem reductase activity of the Streptococcal Haemoproteins Receptor (Shr)

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Group A streptococcus (GAS), a β-haemolytic human pathogen, expresses a NEAT protein, Shr, which binds several haemoproteins and extracellular matrix (ECM) components. Shr is a complex, membrane-anchored protein, with a unique N-terminal domain (NTD) and two NEAT domains separated by a central leucine-rich repeat region. In this study we have analyzed the functional domains in Shr. We show that Shr obtains haem in solution and furthermore reduces the haem iron; this is the first report of haem reduction by a NEAT protein. More specifically, we demonstrate that the haem reduction is carried out mainly by the NEAT domain located in the second position from the N-terminal domain (NEAT2).

NAD(P)H oxidase V (NoxV) from *Lactobacillus plantarum* displays enhanced operational stability even in absence of reducing agents

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Active pharmaceutical ingredients (APIs) such as L-sugars and keto acids are favorably accessed through selective oxidation of sugar alcohols and amino acids, respectively, catalyzed by NAD(P)-dependent dehydrogenases. Cofactor regeneration from NAD(P)H conveniently is achieved via water-forming NAD(P)H oxidases (nox2), which only need molecular oxygen as co-substrate. Turnover-dependent overoxidation of the conserved cysteine residue in the active site of water-forming NAD(P)H oxidases is the presumed cause of the limited nox2 stability.

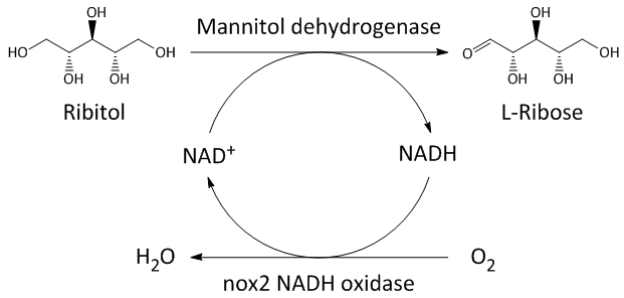

Figure 1 Schematic conversion of ribitol to L-ribose through mannitol-1-dehydrogenase from Apium graveolens complemented with NADH cofactor regeneration using nox2 NADH oxidase.

We present a novel NADH oxidase, NoxV from *Lactobacillus plantarum*, with specific activity of 167 U/mg and apparent kinetic constants at air saturation and 25°C of $k_{cat,app}$ = 212 s^{-1} and $K_{M,app}$ = 50.2 μM in the broad pH optimum from 5.5-8.0. The enzyme features a higher stability than other NAD(P)H oxidases against overoxidation, as is evidenced by a higher total turnover number, in the presence (168,000) and, most importantly, also in the absence (128,000) of exogenously added reducing agents. While the native enzyme shows exclusively activity on NADH, we engineered the cofactor binding pocket to generate variants, G178K,R and L179K,R,H that accommodate and oxidize both NADH and NADPH as substrates.

Acknowledgements
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A Selective and Efficient Fluorescent Chemoprobe for Sulfide in Aqueous Solution

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Abstract: Hydrogen sulfide is regarded as a very important gasotransmitter that plays a critical role in regulating blood pressure. However, currently there is no good method for the sensitive and quantitative detection of hydrogen sulfide. We have found that Dansyl-azide (DNS-Az) is a reduction-sensitive fluorescence chemoprobe for hydrogen sulfide in aqueous solutions, including commercial bovine serum. It is very selective for sulfide among 18 anions tested and other common reducing species. The detection limit is 1 μM in buffer/Tween and 5 μM in bovine serum with an S/N ratio of 3:1. The linear relationship obtained in bovine serum covers the reported endogenous concentration range of hydrogen sulfide. The detection was also performed in 96-well plates by using a micro-plate reader. The simplicity and ease in measurements make this agent extremely easy to use. In addition, sulfide level in the biological system is tightly regulated and can experience rapid changes in concentration. The unprecedented fast response (within seconds) by DNS-Az to sulfide allows it to be used for the detection of transient changes in sulfide levels. The probe, DNS-Az, is simple in structure, very easy to synthesize, and stable and amenable to long-term storage. This fast, selective, efficient and low-cost detection method for sulfide will be very useful in the research field of hydrogen sulfide.

Key words: Fluorescent probes · analytical methods · hydrogen sulfide · aqueous solution · azide reduction

Choline-Glycine Betaine Metabolic Pathway in *Aspergillus fumigatus*

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The genes encoding choline oxidase (*codA*) and betaine aldehyde dehydrogenase (*badh*) are clustered as a single transcriptional unit on the genome of *Aspergillus fumigatus*. Phylogenetic analysis showed that the two genes are conserved in most ascomycetes. Both proteins were heterologously expressed in *Escherichia coli* and the products purified to homogeneity. Choline oxidase is a flavin dependent enzyme that catalyzes the four electron oxidation of choline to glycine betaine with betaine aldehyde as intermediate and reduction of molecular oxygen to hydrogen peroxide. Betaine aldehyde dehydrogenase oxidizes betaine aldehyde to glycine betaine with reduction of $NAD(P)^+$ to NAD(P)H. The two enzymes are co-localized in the cytoplasm of swollen and germinated conidia and might catalyze sequential reactions that result in the synthesis of glycine betaine.

The catabolism of glycine betaine is used to provide nitrogen and/or carbon source during the conidial germination of the fungus as shown by the studies on the single and double mutants but is not involved in its virulence.

This study was supported in part by NSF-CAREER MCB-0545712 (G.G.)

Substituent Effects on the Reaction of β-Benzoylalanines with *Pseudomonas fluorescens* Kynureninase

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Kynureninase is a pyridoxal-5'-phosphate dependent enzyme that catalyzes the hydrolytic cleavage of L-kynurenine to give L-alanine and anthranilic acid. β-Benzoyl-L-alanine, the analogue of L-kynurenine lacking the aromatic amino group, was shown to a good substrate for kynureninase from *Pseudomonas fluorescens,* and the rate-determining step changes from release of the second product, L-Ala, to formation of the first product, benzoate (Gawandi, V. B., *et al*., (2004) *Biochemistry 43*, 3230-3237). In this work, a series of aryl-substituted β-benzoyl-DL-alanines was synthesized and evaluated for substrate activity with kynureninase from *P. fluorescens*. Hammett analysis of k_{cat} and k_{cat}/K_m for 4-substituted β-benzoyl-DL-alanines with electron withdrawing and electron donating substituents is nonlinear, with a concave downward curvature. This suggests that there is a change in rate determining step for benzoate formation with different substituents, from *gem*-diol formation for electron-donating substituents to C_β-C_γ bond cleavage for electron-withdrawing substituents. Rapid-scanning stopped-flow kinetic experiments demonstrated that substituents have relatively minor effects on formation of the quinonoid and 348 nm intermediates, but have a much greater effect on the formation of the aldol product from reaction of benzaldehyde with the 348 nm intermediate. Since there is a kinetic isotope effect on its formation from β,β-dideutero-β-(4-trifluoromethybenzoyl)-DL-alanine, the 348 nm intermediate is proposed to be a vinylogous amide derived from abortive β-deprotonation of the ketimine intermediate. These results provide additional evidence for a *gem*-diol intermediate in the catalytic mechanism of kynureninase.

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Detecting Conformational Changes In The RCL of Human PAI-1 Using An Environmentally-Sensitive Fluorescent Probe. Tihami Qureshi , Cynthia B. Peterson. Department of Biochemistry, Cellular, and Molecular Biology. University of Tennessee, Knoxville

Human plasminogen activator inhibitor-1 (PAI-1) is a proinflammatory, proadhesive, antifibrinolytic *ser*ine *p*rotease *in*hibitor (serpin) that has a wide variety of physiological roles and is required for normal blood hemostasis.[1,2] PAI-1 differs from most homologous serpins in that it exists in a metastable native conformation and undergoes latency to a more stable, but inactive, conformation under physiological conditions.[3] During this conformational change, a disordered loop, termed the reactive center loop (RCL), inserts as the fourth strand into the central β-sheet A (s4A)(Fig. 1). Functionally, this structural element is important for the inhibitory activity of the serpin because it contains the peptide bond that mimics the natural substrate for target serine proteases. The activity of PAI-1 is also influenced by its interaction with the glycoprotein vitronectin (VN). Binding of VN stabilizes PAI-1 in its active conformation and increases its inhibitory lifetime by approximately one- to two-fold.[1] However, the mechanism by which VN stabilizes PAI-1 is currently not agreed upon. Earlier studies have suggested that VN binding stabilizes the active conformation of PAI-1 by sterically preventing loop insertion in the central β-sheet.[1] Yet, a recent study reports the increased incorporation of RCL-mimicking peptides to VN-bound PAI-1, contradicting the latter assessment.[5] Thus, the question of how VN stabilizes PAI-1 and whether the local dynamics of the RCL contribute to global conformational changes remains. **Our hypothesis is that VN stabilizes the active conformation of PAI-1 by affecting the conformation of RCL.** In order to test this hypothesis, single cysteine mutants were engineered along the length of the RCL in order to be labeled with an environmentally-sensitive fluorescent probe: N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) ethylene diamine (NBD). The RCL-labeled PAI-1 can subsequently be analyzed by fluorimetry in the presence of VN to detect any changes in conformation based on solvent accessibility. Thereby, the effect of VN binding on the conformation of the RCL of PAI-1 may be used to evaluate the mechanism of VN-associated PAI-1stabilizaton.

Conformational fluctuations and allosteric effects during enzyme catalysis

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Enzymes are dynamic molecules. Recent evidence indicates that the protein motions at multiple time-scales play a significant role in promoting the catalytic mechanisms of enzymes. Detailed computational modeling of several enzymes including cyclophilin A and dihydrofolate reductase (DHFR) has revealed networks of protein motions that are important for catalytic activity. The reaction promoting motions are conserved as part of the enzyme fold across several species even though they share low sequence homology. It is hypothesized that these discovered networks of motions provide a mechanism for coupling of the hydration-shell and bulk solvent as well as the external region to the enzyme reaction in the active-site. To characterize this linkage, we use an integrated information theoretic and biophysical approach to analyze energy connectivity along the residues that constitute the conserved networks. Our results for DHFR indicate that in spite of low sequence homology across multiple species, the information propagation patterns between flexible regions and the active site are remarkably similar. The biophysical approach further reveals that pathways of energetic connectivity in DHFR across multiple species are also similar. The results provide insights into enzyme mechanisms as well as the mechanism of long-range control (allostery) of enzyme function.

The Role of Arg226 in the Desulfonation Mechanism of the Two-Component Alkanesulfonate Monooxygenase System

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Two-component flavin-dependent monooxygenases are involved in various metabolic and biosynthetic processes in microorganisms. Efforts to elucidate the details governing the catalytic mechanisms of these systems continue to be an area of active investigation. The alkanesulfonate monooxygenase enzyme is found in a diverse range of bacterial organisms and utilizes free $FMNH_2$ supplied by an independent NAD(P)H dependent FMN reductase (SsuE) to alleviate periods of limited sulfur bioavailability. Catalysis by the monooxygenase enzyme results in the oxygenolytic cleavage of a carbon-sulfur bond from sulfonated substrates to yield free FMN, aldehyde, and metabolically available sulfite.[1] The SsuD reaction has been shown to be dependent on a C4a-(hydro)peroxyflavin intermediate to catalyze the desulfonation of alkanesulfonates.

Active site amino acid residues have been proposed to play a direct mechanistic role in acid-base catalysis at specific steps in the reaction pathway. Sequence and structural analyses of the monooxygenase enzyme were used to identify several conserved residues near the proposed active site with the potential to contribute to catalytic function. Variants of these amino acid residues were constructed and evaluated using different kinetic approaches including chemical rescue experiments, single turnover kinetics, photodiode array, deuterium solvent isotope effects, and pH dependence studies. The pH dependence of k_{cat} indicated SsuD requires a group with a pK_a of 6.6 ± 0.1 to be deprotonated and a second group with a pK_a of 9.5 ± 0.1 to be protonated. The results of the present study indicated an active site Arg226 plays a substantial role in catalysis as any mutation to this residue resulted in complete inactivation of the enzyme, although guanidinium rescue experiments performed with R226A SsuD recovered 1.5% of the overall activity. In single-turnover experiments at 370 nm performed at pH 8.5 mixing $FMNH_2$ in one drive syringe against SsuD and varying octanesulfonate in the other drive syringe, wild-type SsuD was shown to stabilize the C4a-(hydro)peroxyflavin intermediate while the Arg226 variants showed no accumulation of the flavin intermediate. In single turnover experiments performed at pH 7.5 mixing FMN, SsuD, SsuE in one drive syringe against NADPH and octanesulfonic acid in the other drive syringe, a flavin binding and/or transfer step from SsuE to SsuD was shown to be unaffected by the amino acid substitutions to Arg226. These combined results demonstrate Arg226 is essential in stabilizing the formation of the C4a-(hydro)peroxyflavin intermediate that is crucial to the overall SsuD catalytic mechanism.

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Solvent Viscosity Effects on α-Isopropylmalate Synthase from *Mycobacterium tuberculosis*

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A better understanding of allosteric regulation mechanisms is important for the creation of industrial synthetic enzymes and for use as a possible drug target for new medical treatments. Using the enzyme α-isopropylmalate synthase (IPMS) with the inhibitor leucine as a model enzyme system, insight can be gained into allosteric mechanisms. Leucine acts as a V-type inhibitor, affecting the V_{max} value for the reaction while the values for the Michaelis constants are unchanged. This suggests that leucine binding affects the rate-limiting step in the reaction. As previously reported, lack of an observable kinetic isotope effect suggests that chemistry is not rate-limiting in the reaction. In order to determine if product release is the rate-limiting step, solvent viscosity studies have been initiated. Results concerning the effect of the microviscogens glycerol, ethylene glycol, sucrose, and trehalose on enzyme activity will be reported.

Study of Tyrosyl Radical Generated During Catalytic Turnover of Oxalate Decarboxylase.

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Oxalate decarboxylase is a manganese-containing enzyme that catalyzes the non-oxidative decarboxylation of oxalate to formate and carbon dioxide. A sharp EPR (S=1/2) signal, corresponding to a tyrosyl radical, is observed during the steady-state turnover of the enzyme. The role of this tyrosyl radical species in the decarboxylase mechanism is still not clear, and its location in the enzyme remains unknown. We have systematically mutated all the conserved tyrosine residues, and in this poster we report the successful expression and purification of these mutants along with their respective kinetic characterization. Employing further techniques in electron paramagnetic resonance (EPR) spectroscopy, we will show an analysis of this tyrosyl radical in an attempt to detect its location in the enzyme during catalytic turnover.

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Discovery of NIR Compounds for PRMT1 Inhibition

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Abstract:

Histones are subject to a host of various posttranslational modifications of proteins which play pivotal role in chromatin mediated nuclear events, such as transcription and DNA damage repair. Among different post-translational modifications, arginine methylation is catalyzed by protein arginine methyltransferases (PRMTs), which utilize the cofactor S-adenosyl-L-methionine (AdoMet, SAM) as a methyl donor to specific arginine residues in histone and nonhistone protein substrates, resulting in mono and di-methylated arginine residues and S-adenosyl-L-homocysteine (AdoHcy, SAH). PRMTs are involved in the regulation of diverse biological processes such as DNA transcription, RNA processing, DNA repair, and cell differentiation. Deregulation of PRMT1 has been linked to certain diseases such as breast cancer and leukemia, which suggests its role as a potential drug target. Potent and selective novel small molecule inhibitor of PRMT1 is of great pharmacological significance. Herein, we investigate new NIR small molecules for PRMT1 inhibition using radioactive methylation assay. From the screening one compound with IC_{50} of ≈ 4 μM was obtained. Steady-state kinetic analysis shows that theinhibitor is noncompetitive in respect to both H4-20 peptide and ^{14}C labeled AdoMet. Docking was conducted to examine the binding site of the inhibitor on PRMT1. From the closer look of the different conformation from docking study three interacting amino acid residue with the nitrogen of the inhibitor were mutated. The mutated proteins were prepared and activity study was tested in presence of inhibitor.

Examining the Activity of Sulfite Reductase through Mutations in the Hemoprotein

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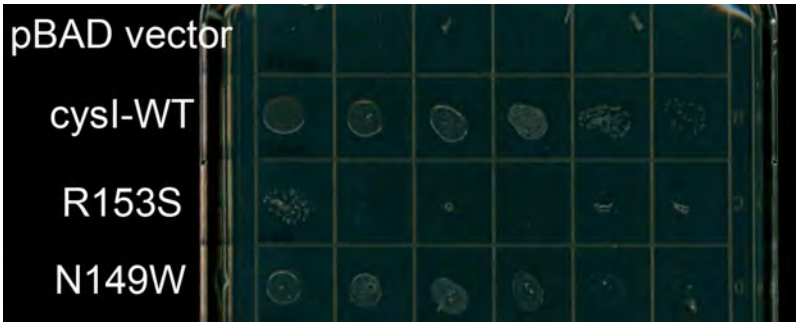
Sulfite Reductase (SiR) is an enzyme found in organisms that performs the six electron reduction of sulfite to sulfide. SiR and a homologous protein, nitrite reductase, are the only known proteins that perform a single atom reduction of six electrons. Two homologous SiRs, assimilatory SiR (aSiR) and dissimilatory SiR (dSiR), perform this reduction, but within different pathways. aSiR reduces sulfur into a S^{2-} oxidation state for incorporation into biological molecules (*i.e.* cysteine, methionine, etc.). dSiR uses sulfur for anaerobic respiration within some archea and sulfate reducing bacteria.

Through comparative structural analysis of the two homologs, aSiR and dSiR, amino acids were identified that play significant roles in the activity of SiR. Two of these residues, Asn149 and Arg153, were mutated independently to tryptophan and serine for examination into their specific role in the activity. Structural, kinetic, and qualitative methods were designed to interrogate the influence of these single amino acid changes. Understanding their role in catalysis will provide insight into the mechanism of reduction in SiR and general structure function relationships in proteins.

The effect on activity of these mutations was measured qualitatively by culturing a SiR knockout strain of *Escherichia coli* transformed with the mutant SiR expression vector in a reduced sulfur-deprived environment; in this assay, cell survival is proportional to SiR activity. Native and N149W SiRHP were fully functional in this assay, whereas the empty vector and R153S were not (Figure 1).



In addition to the complementation assay, a quantitative assay also measured the enzyme's activity using UV-vis spectroscopy. Methyl viologen (MV) was reduced in the presence of sodium dithionite, giving a spectroscopic signal at 604 nm. SiR introduced into the solution in the presence of sodium sulfite caused reduction of sulfite through the oxidation of MV. Finally we used a protease assay to test the effect of the point mutation on the stability of the N-terminal 60 amino acids, which are believed to protect the active site by interacting with an active site loop during SiR's catalytic cycle, but are not ordered in the x-ray crystal structure of aSiR.

Comparing the results of these three assays for SiRHP activity, we propose roles for Asn149 and Arg153 as part of the active site loop in the catalytic activity of SiRHP.

Is a Catalytic Base Required for Choline Activation in Choline Oxidase from *Arthrobacter globiformis*?

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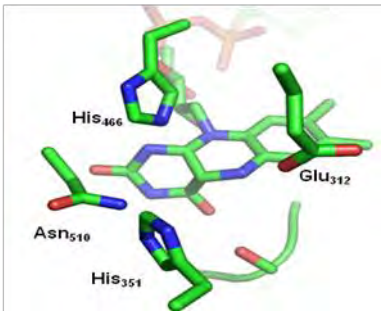
Choline oxidase (E.C. 1.1.3.17) from *Arthrobacter globiformis* has been extensively characterized *in vitro*. The enzyme catalyzes the four-electron, flavin-linked oxidation of choline to glycine betaine with the formation of the intermediate betaine aldehyde. Choline oxidation is initiated by the enzyme-catalyzed deprotonation of its hydroxyl group, which is followed by a hydride transfer reaction from the activated alkoxide to the flavin N(5) atom (*1*). To this day, the identity of the catalytic base that activates choline is unknown. Two histidines located in the active site (see figure) have been established with the determination of the crystal structure of choline oxidase to a resolution of 1.86 Å (*2*). One of the two histidines is highly conserved among flavoproteins that oxidize alcohols (e.g., His466) and we propose that it is the group that removes a proton from the hydroxyl group of the substrate.

Mutant variants of His466 and His351 were prepared and the resulting purified enzymes were investigated in their kinetic, mechanistic and structural properties. Kinetic parameters of the mutant enzymes, including double mutants, suggest that His466 may be essential for catalysis, but not His351. Indeed, the His466Q enzyme exhibited a first-order rate constant for flavin reduction that was eight orders of magnitude lower than that of the wild type. Currently, we are working on the elucidation of the crystallographic structure of the mutant enzyme in order to demonstrate that the loss of enzymatic activity is due solely to the replacement of His466 with glutamine. Mechanistic and structural data will be presented.



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[†]This work was supported in part by Grant NSF-CAREER MCB-0545712 (G.G.)

The Stroupe lab at Florida State University uses state-of-the-art structural biology techniques to study a host of biologically and chemically interesting proteins. The main focus of the lab is to understand how information moves in a eukaryotic cell from its life as genomic DNA, through the pre-mRNA intermediate, and out of the nucleus. By visualizing the chromatin and the pre-mRNA transcripts using cryo-TEM, we are determining 3D structures for these, and other, structures that are involved in the genotype to phenotype transformation.

For more information about graduate opportunities in the Stroupe lab at Florida State University, please contact Dr. Stroupe (mestroupe@bio.fsu.edu) or go to http://www.bio.fsu.edu/cmb/ (Graduate program in Cell and Molecular Biology) http://www.sb.fsu.edu/mob/ (Graduate program in Molecular Biophysics)

Designing A Novel Class of Thrombin Sensors with Fast Ratiometric Response

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Protease activation is vital to regulate biological processes and highly related with various diseases. Currently, there is a strong need to develop protease sensors with strong protein substrate specificity and quantitatively measurement of the enzymatic action in cell/vivo in real time. Here we report our achievement in designing a novel class of single EGFP-based thrombin sensors. These designed protease sensors exhibit rapid kinetic responses and large ratiometric fluorescence change.

Acknowledgments
This work was supported in part by NIH grants GM62999 and EB007268 and a Brain & Behavior (BB) seed grant to Jenny J. Yang, and a BB fellowship to Shen Tang.

Advanced Kinetic Studies of the Glycosyltransferase MshA from *Corynebacterium glutamicum*

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The glycosyltransferase MshA from *Corynebacterium glutamicum* (CgMshA) catalyzes the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine (UDP-GlcNAc) to 1-L-*myo*-inositol-1-phosphate (L-I1P) in a retaining mechanism. Available crystallographic evidence indicates a large conformational change occurring after UDP-GlcNAc binding. To further study CgMshA kinetic mechanism and the possible significance of the conformational change, a pH profile, solvent isotope effects, solvent viscosity effects and temperature dependence studies have been performed. The pH profile plots from k_{cat} and k_{cat}/K_M data suggest that there is one ionizable moiety that must be deprotonated for maximal activity. The pKa values determined from the profiles are 6.9 ± 0.1, 7.2 ± 0.1, and 6.7 ± 0.1 from the log k_{cat} and log k_{cat}/K graphs for L-I1P and UDP-GlcNAc, respectively. We propose this ionizable group is the β-phosphoryl group on the donor sugar, consistent with the substrate-assisted catalytic mechanism previously proposed. A normal solvent isotope effect of 3.3 ± 0.2 on k_{cat} measurements, along with a linear plot from a proton inventory, indicates partially rate-limiting transfer of a single proton. Control experiments indicated that solvent viscosity effects might be contributing to the solvent isotope effect. However, the viscosity corrected solvent isotope effects range from 1.8 to 2.0. Ethylene glycol and sucrose were used as microviscogens in solvent viscosity experiments. The slopes of reciprocal rate plots, k_{cat}^{η}, are 2.3 ± 0.2 and 1.0 ± 0.2 for ethylene glycol and sucrose, respectively. These values are consistent with diffusion related events being rate limiting, and the large value derived using the ethylene glycol microviscogen points to a conformational change playing a significant role in the kinetic mechanism. Consistent with a kinetically important conformational change, temperature dependence studies indicate a $\Delta H^{\ddagger}$ = 15.9 ± 1.1 kcal/mol and a significant amount of bond formation and/or breaking occurring in the transition state.

Kinetic and Spectropscopic Characterization of *Ceriporiopsis subvermispora* Bicupin Oxalate Oxidase Expressed In *Pichia pastoris*

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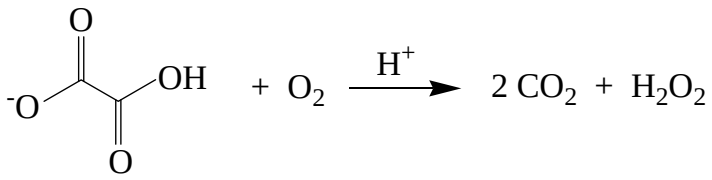
Oxalate oxidase (E.C. 1.2.3.4) catalyzes the oxygen-dependent oxidation of oxalate to carbon dioxide in a reaction that is coupled with the formation of hydrogen peroxide (Scheme below). Although there is currently no structural information available for oxalate oxidase from *Ceriporiopsis subvermispora* (CsOxOx), sequence data and homology modeling indicate that it is the first manganese-containing bicupin enzyme identified that catalyzes this reaction. Interestingly, CsOxOx shares greatest sequence homology with bicupin microbial oxalate decarboxylases (OxDC).



CsOxOx activity directly correlates with Mn content and other metals do not appear to be able to support catalysis. EPR spectra indicate that the Mn is present as Mn(II), and are consistent with the coordination environment expected from homology modeling with known X-ray crystal structures of oxalate decarboxylase from *Bacillus subtilis*. EPR spin-trapping experiments support the existence of an oxalate-derived radical species formed during turnover. We have determined that acetate and a number of other small molecule carboxylic acids are competitive inhibitors for oxalate in the CsOxOx catalyzed reaction. The pH dependence of this reaction suggests that the dominant contribution to catalysis comes from the monoprotonated form of oxalate binding to a form of the enzyme in which an active site carboxylic acid residue must be unprotonated.

Effect of Temperature on the Kinetic Isotope Effects of the Active Site Variant S101A of Choline Oxidase

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Choline oxidase catalyzes the four-electron oxidation of choline to glycine betaine with betaine aldehyde as an intermediate. FAD is reduced by the alcohol substrate and oxidized by molecular oxygen to give hydrogen peroxide. Previous studies have revealed the mechanistic, biochemical and structural details of the oxidation of choline by the enzyme (*1*). The reaction starts with the enzyme-catalyzed deprotonation of the hydroxyl group of the alcohol substrate yielding an alkoxide species. Through electrostatic and hydrogen bonding interactions with a number of active site residues, the alkoxide species positions correctly for the hydride transfer from the α-carbon of the substrate to the N(5) atom of the enzyme-bound flavin (*2*). The hydride transfer reaction occurs quantum mechanically, as previously established by temperature effects of the kinetic isotope effect (KIE) (*3*). In the X-ray crystallographic structure of the enzyme S101 locates less than 4 Å from the flavin N(5) atom, suggesting it may play a role in the reaction (*4*). The S101A variant of choline oxidase was purified in this study to elucidate the role of the serine side chain in the hydride transfer reaction.

The purification of the S101A enzyme was carried out following the same protocol previously used for the wild type enzyme. Initially, the K_m values for oxygen were determined with choline as substrate at three temperatures (7 ^oC, 25 ^oC and 40 ^oC) to be between 17 μM and 60 μM. These results were used to determine the oxygen concentrations that are required to saturate the enzyme with oxygen for the determination of the true KIE. Finally, the effect of temperature on the KIE with choline and 1,2-[^{2}H4]-choline as substrate for the S101A enzyme was investigated at fixed, saturating [oxygen], in order to probe the role of the S101 residue in the hydride transfer reaction catalyzed by the enzyme (4). The results will be presented on the poster.

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Conformational Insights of Pin1 Substrates

Hector Adam Velazquez and Donald Hamelberg

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Abstract

Pin1 is an important signaling protein in mitosis which catalyzes the *cis-trans* isomerization about proline peptide bonds when there is a phosphorylated serine or threonine preceding the proline residue. Here we investigate the configurational preferences of free Pin1 substrates and the effects of phosphorylation on the *cis-trans* isomerization of the free substrates. This was done by calculating the Ramachandran space for substrates analogues along the φ, ψ, and ω degrees of freedom and making the classic φ/ψ plots for the serine, threonine, and proline residues. In addition, ω/ψ plots were made from the ω and ψ angles of proline. The φ/ψ plots show that the population of the α_R region increases upon phosphorylation for both the serine and threonine cases at the expense of the β and α_L region. The ω/ψ plots show that a thermodynamically stable valley is formed upon phosphorylation allowing facile transition from the β to the α_R state. The angular diffusion constants were also calculated for the phosphorylated and unphosphorylated cases. For serine, the calculate diffusion constants for the unphosphorylated and phosphorylated cases are 1.0 ± 0.2 degrees^2/ps and 0.52 ± 0.07 degrees^2/ps, respectively. For threonine, the diffusion constant for the unphosphorylated and phosphorylated cases are 1.0 ± 0.2 degrees^2/ps and 0.6 ± 0.1 degrees^2/ps, respectively. This is evidence that the kinetics of the *cis-trans* isomerization are slower upon phosphorylation even though the transition is more thermodynamically favorable as depicted in the ω/ψ plots. In addition, simulations of the Pin1-substrate complex were

also performed. These simulations show that when in the *cis* conformation the phosphorylated residue prefers to be in β conformation while the *trans* state the phosphorylated residue prefers to be in the α_R region. The transition state was also modeled and it shows a combination of both the α_R and β conformations.

The differences between Mn-Superoxide Dismutase and its mutant Q146E

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The Mn-Superoxide Dismutase (MnSOD) is a manganese containing enzyme which is responsible for the following ping pong reactions:

$$Mn(3+) + O_2^{\cdot-} \rightarrow Mn(2+) + O_2$$
$$Mn(2+) + O_2^{\cdot-} + 2H^+ \rightarrow Mn(3+) + H_2O_2$$

Because of the homology between MnSOD and FeSOD, metal mis-incorporation sometimes occurs *in vivo*. Thus it is found that there is a mixture of MnSOD and Fe-substituted MnSOD (Fe(Mn)SOD) in *E.coli*. Because the Fe^{3+}/Fe^{2+} couple has a lower reduction midpoint potential than the Mn^{3+}/Mn^{2+} couple, Fe(Mn)SOD can not oxidize superoxide. We proposed to test our understanding of redox tuning in SOD by testing a mutation predicted to confer activity on Fe bound in (Mn)SOD protein. We constructed the mutant Q146E of MnSOD, in which a conserved glutamine in the second shell of the active site is replaced by glutamate. We found that the Q146E mutant behaves quite differently from the WT MnSOD. The yield of the Q146E-MnSOD was lower, 53.5mg vs. 90.3mg of WT-MnSOD from a 2L growth of *E.coli* on M9 medium. Cells overexpressing Q146E-MnSOD grew slower than cells expressing WT-MnSOD in LB medium as well. The cytochrome C assay shows that the activities of both Q146E-MnSOD and Q146E-Fe(Mn)SOD are negligible. However the low catalytic activity could be substantially a result of the low metal content. The ferrozine assay shows that the metal content of Q146E Fe(Mn)SOD is about 0.2 Fe per dimer while for the WT Fe(Mn)SOD, it is about 0.4 Fe per dimer. We excluded competition from binding of other metals such as nickel and zinc. Instead, we propose that metal ion incorporation is low due to highly favourable protein folding that occludes the active site faster than metal ion binds. At pH 7 the melting temperatures (T_ms) of apo MnSOD, holo MnSOD and apo Q146E are 55°C, 80°C and 85°C respectively, based on circular dichroism-monitored thermal melting curves. Thus, Q69E-apo-(Mn)SOD is ultra stable in the absence of bound metal ion, even though WT-(Mn)SOD is much less stable in the absence of bound metal ion than is the holoenzyme. In order to identify conditions that will permit greater incorporation of metal ions into the mutant SOD proteins, we monitored the fluorescence of Co^{2+} and compared Co^{2+} incorporation into the Q146E mutant and the WT apo-protein. The WT (Mn)SOD protein is willing to accept metal ion at 40°C (pH 7) whereas the Q146E-(Mn)SOD protein is not. However Q146E-(Mn)SOD protein can assimilate Co^{2+} at 80°C. These results are in accordance with the T_m determined from the melting curves, and point to a means of generating Q146E-Fe(Mn)SOD.

Borrowing the E.coli catalase-peroxidase C-terminal domain as a scaffold for generation of new heme-dependent catalysts

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Catalase-peroxidases (KatGs) have a two-domain structure derived from gene duplication and fusion. Each domain resembles a plant peroxidase. The N-terminal domain contains the heme-dependent, bifunctional active site. Due to a relatively small number of modifications, the C-terminal domain has lost its ability to bind heme or catalyze any discernable reaction. Sequence alignments and structural comparisons show that the "inactive" C-terminal domain retains all 10 major helices observed in the N-terminal domain and other plant peroxidase superfamily members. Indeed, the helical architecture of the C-terminal domain "active site" is indistinguishable from that of the N-terminal domain as well as the other class I plant peroxidases. Consequently, the C-terminal domain appears to provide an ideal platform for engineering a new heme-dependent catalyst. The C-terminal domain lacks an appropriate ligand to the heme iron as Arg replaces the typical proximal His ligand, and the cavity formerly occupied by heme is filled by aliphatic Met and Phe side chains. To restore heme-binding capability to $KatG^C$, site-directed and deletion mutagenesis were used to make progressively eliminate the obstacles to heme binding by our separately expressed and isolated C-terminal domain ($KatG^C$). Comparison of UV-vis and MCD spectra for $KatG^C$ and M616G/R617H double variant suggested that heme binding was restored as a result of the substitutions and that the bound heme was in a hexacoordinate, low-spin ferric state. Additional modifications produced more sharply defined UV-vis and MCD spectra very similar to those observed for the stand-alone KatG N-terminal domain ($KatG^N$). Further modifications have been made in the so-called distal cavity to reintroduce catalytic side chains observed in typical peroxidases (e.g., the distal His general base and distal Arg) based on the one of our heme-binding variants. The catalytic oxidation of peroxidase electron donors was observed as a result of introducing a distal Histidine. A second-order rate constant of $10^2\ M^{-1}\ s^{-1}$ was observed at pH 5, 25 °C, indicating the partial restoring peroxidase activity. Additional modifications will focus on reorienting and adjusting residues to optimize active site H-bonding to fully restore peroxidase activity.

Acknowledgements
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HIV-1 Protease: Structural Perspectives on Drug Resistance

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Drug resistance is a major challenge in treatment of the HIV/AIDS pandemic. We are investigating the molecular mechanisms of drug resistance to clinical inhibitors of HIV protease. This knowledge is applied in structure-guided designs for novel antiviral inhibitors with enhanced interactions with conserved regions of the protease structure. Crystal structures at high to atomic resolutions (0.84-1.5Å) demonstrate structural changes due to drug resistance mutations and diverse inhibitors or peptides. We have analyzed HIV-1 protease with ~20 different single mutations and selected combinations to reveal the molecular mechanisms of drug resistance. Drug resistance mutations show distinct effects: 1) mutations in the inhibitor binding cavity can directly alter inhibitor binding; 2) mutations at the dimer interface can alter protease stability; 3) other mutations can have indirect effects on protease activity and inhibition by altering the unliganded protease or the interactions with reaction intermediates. In order to design new drugs we have analyzed many protease complexes with clinical and investigational inhibitors. Our drug design strategy of introducing new polar interactions with inhibitors is based on early studies of the conserved pattern of protease-inhibitor hydrogen bonds. Darunavir, which was approved in 2006 for AIDS therapy, and a series of novel antiviral inhibitors have demonstrated the success of this structure-guided strategy to combat resistance. The new insights into the mechanisms of drug resistance and strategies for drug design have wide impact in many diseases. Moreover, the data provide a uniquely valuable resource for analysis of structural variation due to mutations or ligands.

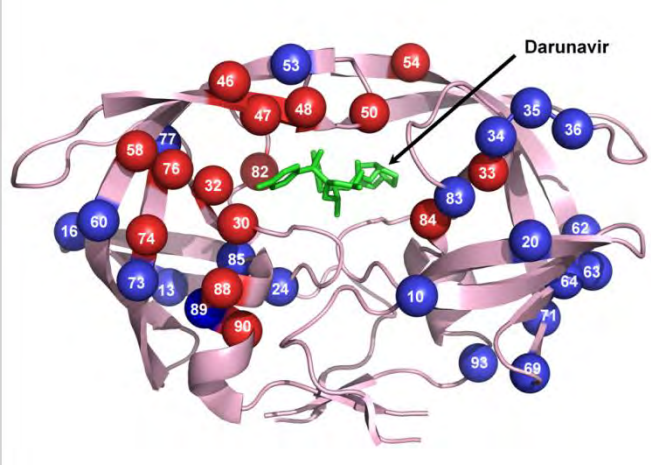

Figure: Sites of drug resistant mutations mapped on the dimer of HIV protease with bound inhibitor, darunavir.

The research was supported in part by the National Institute of Health grant GM062920 (ITW), and the Georgia State University Research Program Enhancement award (CHS).

Luciferase-Based Chemiluminescent Measurement of Cellular Production of Nitric Oxide

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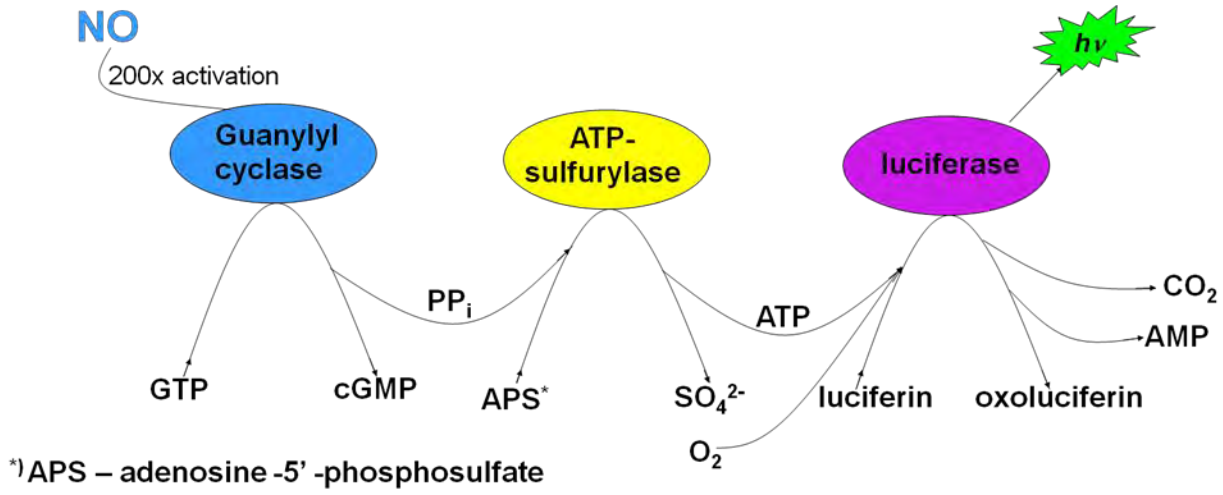
Nitric oxide (NO) is a free radical involved in many physiological processes including regulation of blood pressure, immune response, and neurotransmission. We recently suggested a highly sensitive chemiluminescence approach to measurements of NO in biological samples (*Free Rad. Biol. Med.* ***2009****, 47, 1339-45*). The approach is based on using a natural nitric oxide target, soluble guanylyl cyclase (sGC), which catalyzes the conversion of guanosine triphosphate to guanosine 3′, 5′-cyclic monophosphate and inorganic pyrophosphate. The suggested enzymatic assay uses the fact that the rate of the reaction increases by about 200 times when NO binds with sGC and, in so doing, provides a sensor for nitric oxide. Luminescence detection of the above reaction is accomplished by converting inorganic pyrophosphate into ATP with the help of ATP sulfurylase followed by light emission from the ATP-dependent luciferin–luciferase reaction.



The method allows for the measurement of NO concentrations in physiologically relevant nanomolar range and NO generation with the rates as low as 100 pM/min.

With this method, the generation of NO by cellular cultures has been measured. Murine RAW cells induced by interferon-γ (INF- γ) and lipopolysaccharide from *E. coli* (LPS) demonstrated NO production about 100 amol/min per cell. Untreated cells did not exhibit NO production beyond detection limit. NO generation measured by analysis of nitrite, the final product of NO metabolism, corresponded closely to the figures obtained for NO generation, but required much longer incubations to produce measurable amount of nitrite. Sensitivity of the method allows detecting NO generated by 10^3 cells. Addition of inhibitor of NO-synthase, N-monomethyl arginine, inhibited measured production of NO. Human cells (THP-1) treated with phorbol ester and either LPS, or INF- γ, or both, do not show NO production within detection limits. The method can be applied in situations where only a limited number of cells is available, e.g. biopsy.

Supported by NIH grant R21 HL089036 and Valdosta State University Faculty Research Grant to YYW.

Enzymatic Study and Chemical Inhibitors of Histone Acetyltransferase Tip60

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Tip60 (HIV-1 TAT-interactive protein, 60 kDa) is a key member of the MYST family of histone acetyltransferases (HATs) and plays important functions in many cellular processes and human diseases. We report here both substrate-based analog inhibitors and small molecule inhibitors for Tip60. In the first strategy, we designed, synthesized and evaluated a series of substrate-based analogs for the inhibition of Tip60. The structures of these analogs feature that coenzyme A is covalently linked to the side chain amino group of the acetyl lysine residues in the histone peptide substrates. These bisubstrate analogs exhibit stronger potency in the inhibition of Tip60 compared to the small molecules curcumin and anacardic acid. The substrate-based analog inhibitors will be useful mechanistic tools for analyzing biochemical mechanisms of Tip60, defining its functional roles in particular biological pathways, and facilitating protein crystallization and structural determination.

We also performed a virtual screening using the crystal structure of Esa1 (the yeast homolog of Tip60) and 105 small molecules were selected as potential hits. Then, the radioactive HAT assay was used for experimental screening. From the first-round screening, we focus on a series of small molecules that bear structure similarity with JWu-10 to identity lead inhibitors. We also tested the selectivity of this new class of Tip60 inhibitors. IC50 was compared to find out the structure-activity relationship. The discovery of new small molecule inhibitors provides important chemical tools for functional study of Tip60 and is of great potential to be developed into valuable anticancer agents.

Deciphering the role of glucosamine-6-phosphate in the riboswitch action of *glmS* ribozyme

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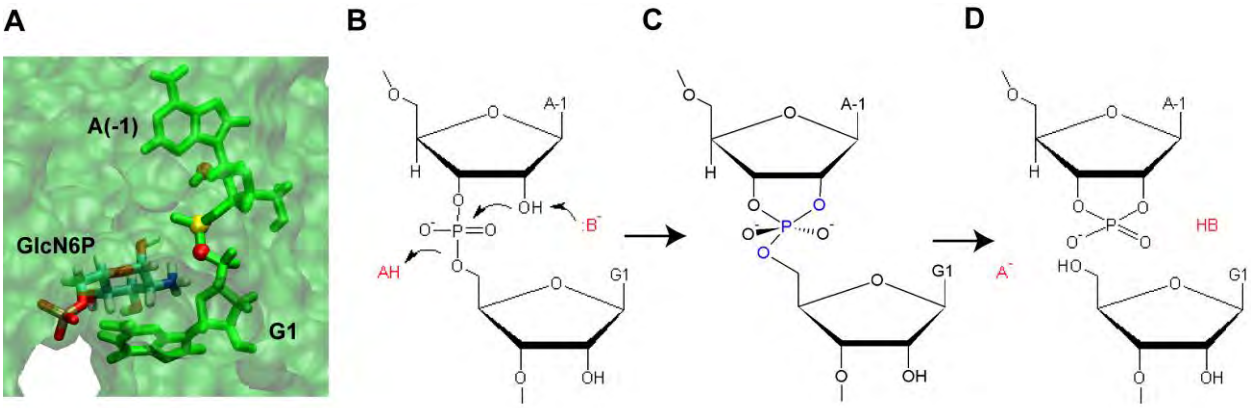
Self-cleaving catalytic RNAs (ribozymes) regulate genes in many organisms through cleavage of the backbone phosphodiester bond. The *glmS* ribozyme/riboswitch accelerates self-cleavage upon binding a metabolite, glucosamine-6-phosphate (GlcN6P), terminating protein translation in Gram-positive bacteria. Self-cleavage of *glmS* is believed to proceed via a general acid-base mechanism.



The general acid and general base are not known, and the role of the GlcN6P cofactor is even less well understood. The amine group of GlcN6P has the ability to either accept or donate a proton and could therefore potentially act as an acid or a base. We have determined the preferred protonation state of the amine group in the wild-type and an inactive G40A mutant using molecular dynamics simulations and free energy calculations. Here we show that, upon binding of GlcN6P to wild-type *glmS,* the pK_a of the amine moiety is altered by the active site environment, decreasing by ~2.2 from a solution pK_a of ~8.2 and slightly increasing to ~8.4 upon binding to the G40A inactive mutant of *glmS*. These results suggest that GlcN6P acts as a general acid in the self-cleavage of *glmS*. However, in the G40A inactive mutant of *glmS*, the results suggest that the ability of GlcN6P to easily release its proton is diminished, in addition to the possible lack of G40 as the base.

Acknowledgments

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Investigation of the Flavin Transfer Mechanism in the Two-component Alkanesulfonate Monooxygenase System

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When inorganic sulfur is limiting in the environment, bacteria are capable of utilizing alkanesulfonates as alternate sulfur sources. An FMN reductase (SsuE) and an $FMNH_2$-dependent monooxygenase (SsuD) are expressed as a two-component system to generate sulfite and aldehyde from alkanesulfonates. Typically, flavin cofactors are tightly bound to proteins through covalent or non-covalent interactions. A distinct feature of the alkanesulfonate monooxygenase system is that the flavin is utilized as a substrate instead of a bound prosthetic group. The flavin substrate is reduced by SsuE, and the reduced flavin is subsequently transferred to SsuD. Although an increasing number of bacterial flavin-dependent two-component systems have been identified, the mechanism of flavin transfer has not been fully determined [1].

The mechanism of flavin transfer between SsuE and SsuD could either occur through a dissociative or a channeling mechanism involving protein-protein interactions. Although static protein interactions between SsuE and SsuD have previously been identified, there has been no kinetic evidence to support a channeling mechanism [2]. In order to kinetically discern between a dissociative or channeling mechanism for reduced flavin transfer between SsuE and SsuD, a modified method first described by Geck and Kirsch was employed [3]. These studies rely on obtaining a transferring enzyme that is catalytically inactive, but still able to bind substrates. When inactive transferring enzyme is added to the reaction mixture containing active transferring enzyme, a decreased activity of the receiving enzyme would suggest the flavin transfer occurs through the channeling mechanism. Otherwise the transfer would occur through the dissociative mechanism. Based on the recently obtained three-dimensional structure of SsuE with flavin bound, two amino acid candidates (Arg10 and Ser13) involved in flavin coordination were targeted for substitution. Structural and kinetic studies indicated the R10A/S13A SsuE variant was catalytically inactive and the K_d value for FMN binding was 20-fold higher compared to wild-type SsuE. Therefore, R10A/S13A SsuE was selected as the inactive mutant candidate for the flavin transfer studies. The catalytic activity of SsuD in the presence of both wild-type and R10A/S13A SsuE showed a distinct decrease. This result suggests the inactive SsuE variant competes with the wild-type SsuE for the interaction site on SsuD, and those SsuD that are associated with inactive SsuE are not forming products, leading to an overall decreased catalytic activity. Therefore it is suggested that the flavin transfer occurs through a channeling mechanism in the two-component alkanesulfonate monooxygenase system.

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Mechanisms of subunit interface disruption in human PCNA and in the heterotimeric alternative sliding clamp 9-1-1

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Abstract:

The Proliferation Cell Nuclear Antigens (PCNA) represent the eukaryotic version of the sliding clamps, which are capable of providing an anchoring platform for DNA polymerases and ensure high processivity of DNA synthesis. The loading of PCNA on DNA is regarded as an activated process since it requires the opening of one of the subunit-subunit interfaces that is stabilized by a network of hydrogen bonds, salt-bridges and hydrophobic interactions. In this study, we applied Steered Molecular Dynamic (SMD) on both intact and truncated PCNAs to probe the mechanism of subunit interface disruption. From the intact homotrimer PCNA pulling run, we identified a directional breaking down mechanism. The results were extended when we utilize the truncated system to carry out multiple pulling runs and then analyzed clusters with different breaking down manners.

Designing single fluorescent protein-based caspase sensor for monitoring apoptosis in living cells

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Intracellular apoptotic signals regulated by caspase-cascade systems are closely associated with human diseases such as cancer and neurodegenerative diseases. Monitoring the activation and inhibition of caspase 3 and other caspases with fluorescence spectrum changes in living cells is essential for further understanding these processes. Here, we report progress in the development of caspase sensors based on a single fluorescent protein. These developed sensors exhibit strong enzymatic selectivity as well as high sensitivity based on observed ratiometric fluorescence changes. Additionally, our sensors can be targeted to different subcellular locations, such as the ER and mitochondria. We have further applied these sensors to monitor caspase-dependant apoptosis in different cells. Our results indicate that different inducers and drugs have diversified effects on triggering cell death pathways.

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Mechanistic Study of Tip60 catalysis

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Tip60 (HIV1-Tat interacting protein, 60kDa), a member from MYST family of histone acetyltransferase (HATs), is involved in many important cellular processes, such as gene transcription, DNA damage repair, cell cycle and apoptosis. HATs can acetylate lysine residue by transferring the acetyl group from acetyl CoA to the ε-amine of the lysine. Based on the sequence similarity, HATs can be classified into four major families: GCN5/PCAF family, MYST family (MOZ, Ybf3, Sas2 and Tip60), p300 family and RTT109. Enzyme from different family undergoes distinct catalytic mechanism. GCN5 is shown to form a ternary complex in the catalysis. P300 utilizes Theorell-Chance (hit and run) mechanism. However, the catalytic mechanism of MYST family is still not clear. In this work, we mainly focus on the acetylation of histone H4 by Tip60. Bisubstrate kinetics was used to elucidate the catalytic mechanism. Moreover, product inhibition and chemical method were used to confirm the mechanism.

The results suggest the catalysis of Tip60 undergoes ping pong mechanism. Cys 369 and Glu 403 play important roles in the reaction. Tip60 can also autoacetylate itself on lysine both in vivo and in vitro. The autoacetylation on lysine is an irreversible process. However, the acetyl group on cysteine can be removed by base, indicating that the acetyl cysteine acts as an intermediate during the catalysis.

Characterization of Xenobiotic Reductase A (XenA): Study of Active Site Residues, Substrate Spectrum and Stability

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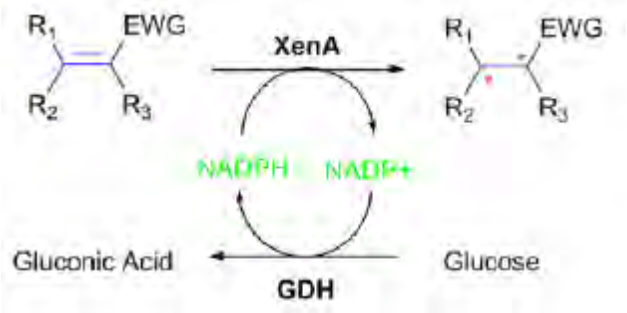
The Old Yellow Enzyme (OYE) family is a class of flavin dependent enzymes that catalyze the stereoselective reduction of activated C=C bonds at the expense of nicotinamide cofactors. This asymmetric bioreduction creates up to two new stereogenic centers and thus has gained increasing interest from industry as a new tool in chiral intermediates synthesis. Members of the OYE family were reported to catalyze the bioreduction of a variety of different substrates such as α,β-unsaturated aldehydes, ketones, imides, nitro groups, nitriles, carboxylic acids, nitro esters, and nitro aromatics.

In this work, the substrate spectrum and stereoselectivity of Xenobiotic reductase A (XenA) were characterized. The QuickChange® mutagenesis protocol was used to generate C25G, C25V, and A59V mutants. We analyzed wild type XenA (WT) and the effect of mutations on thermal and chemical stability, enzyme activity, and regio- and stereoselectivity with various α,β-unsaturated compounds and nitro compounds. XenA has broad catalytic activity and reduces various α,β-unsaturated and nitro compounds with moderate to excellent stereoselectivity. Single mutants C25G and C25V are able to reduce nitrobenzene, a non-active substrate for the wild type, to produce aniline. Total turnover is dominated by chemical rather than thermal instability.



Scheme 1 Asymmetric *trans*-bioreduction of C=C bonds coupling XenA with cofactor recycling system through half-oxidative and half-reductive reaction (EWG = electron withdrawing group, GDH = glucose dehydrogenase, * = center of chirality).

(1) Yanto, Y., Yu, H., Hall, M., and Bommarius, A.S., "*Characterization of Xenobiotic Reductase A (XenA): Study of Acive Site Residues, Substrate Spectrum and Stability",* Chem. Commun., 2010, 46, 8809-8811.

Characterization of the heme uptake pathway proteins from C*orynebacterium diphtheriae* and S*treptococcus pyogenes*.

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Abstract: Iron, a vital nutrient for most of the bacteria, is primarily bound to heme in the human host. Bacteria use heme as a source of iron and often have sophisticated heme uptake pathways. Inhibition of the heme uptake pathway may be a strategy to reduce the virulence of bacterial pathogens. The causative agent of diphtheria, *Corynebacterium diphtheria*, imports heme via an ABC uptake transporter. We are studying the HmuT protein, which is the lipoprotein component of the transporter. Two new findings are unexpected. UV-visible spectroscopy shows that this protein as isolated binds a porphyrin, rather than heme. Electrospray ionization mass spectrometry (ESI-MS) and UV-visible spectroscopy studies suggest that two tetrapyrroles are bound.

Studies on the ABC transport pathway in *Streptococcus pyogenes* have focused on SiaA, the protein homologous to HmuT. We studied alanine mutants of a number of residues in the heme binding pocket. The residues near the heme, especially the ones that have interactions with propionic acids (K61A and C58A) and axial ligands (M79A and H229A), have significant effects on heme binding. Detailed guanidinium-induced denaturation studies on C58A show that it unfolds very slowly, with changes seen for a number of days after the addition of guanidine hydrochloride.

On the Catalytic Mechanism of D-Arginine Dehydrogenase

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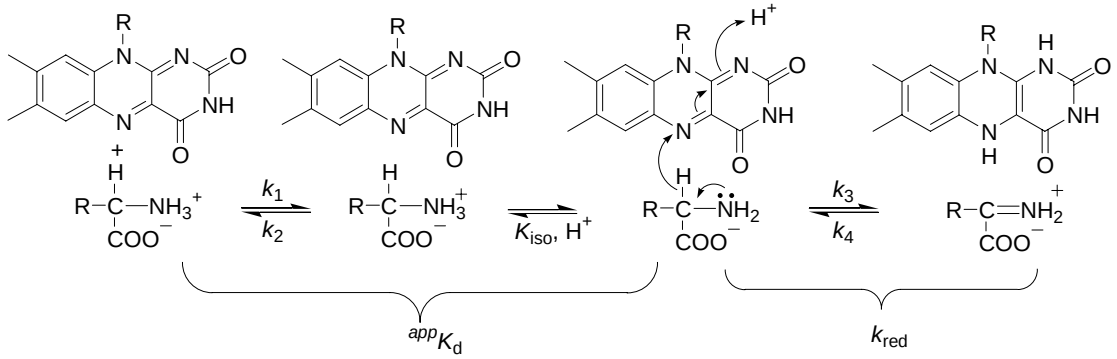
D-arginine dehydrogenase (DADH) catalyzes the oxidation of D-amino acids to the corresponding imino acids, which are non-enzymatically hydrolyzed in solution to α-keto acids and ammonia. The enzyme has broad substrate specificity, with preference for D-arginine and D-lysine. D-leucine is the slowest amongst the substrates for which steady state kinetic parameters are available.[1,2] In this study, the chemical mechanism of leucine dehydrogenation catalyzed by DADH was explored with a combination of pH, substrate, solvent and β-secondary kinetic isotope effects (KIE) and proton inventories by using rapid kinetics in which the enzyme was reduced with D-leucine in a stopped-flow spectrophotometer.

There was an unexpected solvent KIE of 2.5 and an inverse β-secondary KIE of 0.9 on the $^{app}K_d$ value. Substrate and solvent KIE on k_{red} were also observed. The data taken together allowed us to propose the mechanism for leucine oxidation of Scheme 1. Leucine oxidation proceeds through the release of a proton from the substrate α-NH_3^+ to either the solvent or a solvent exchangeable active site residue that occurs upon formation of a competent E_{ox}-S complex (K_{iso}). This is followed by a hydride transfer reaction from the C_α atom of the unprotonated, activated substrate to the flavin N5 atom that occurs concomitant with protonation of the flavin N(1) atom of the reduced cofactor (k_3). Dependence of the $^{app}K_d$ and k_{red} values on pH identified pK_a values for groups that stabilize the ground and transition states. The data will be discussed in the context of the crystallographic structures at high resolutions (<1.3 Å) of the enzyme in complex with iminoarginine or iminohistidine.[1]

Scheme 1. Proposed Mechanism for Oxidation of Leucine by DADH



This study was supported in part by NSF-CAREER MCB-0545712(G.G.).

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The Extreme C Terminus of DrrA Contains Unique Motifs Involved in Function and Assembly of the DrrAB Complex

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The present study focuses on assembly of the doxorubicin resistance protein complex DrrAB, a bacterial ATP-binding cassette (ABC) transporter with functional similarity to mammalian P-glycoprotein. DrrA is the ABC subunit and carries out ATP-binding and hydrolysis, whereas DrrB is the transmembrane subunit and forms the substrate translocation pathway. Two novel regulatory motifs, LDEVFL and CREEM, are identified in the extreme C terminus of DrrA. Disulfide cross-linking analysis showed that the CREEM motif and the region immediately upstream of CREEM participate directly in forming an interaction interface with the N-terminal cytoplasmic tail of DrrB. A series of mutations created in the LDEVFL and CREEM motifs drastically affected overall function of the DrrAB transporter. Mutations in the LDEVFL motif also significantly impaired interaction between the C terminus of DrrA and the N terminus of DrrB as well as the ability of DrrA and DrrB to co-purify, therefore suggesting that the LDEVFL motif regulates CREEM-mediated interaction between DrrA and DrrB and plays a key role in biogenesis of the DrrAB complex. Further, modeling analysis indicated that the LDEVFL motif is critical for conformational integrity of the C-terminal domain of DrrA and may be essential for the ability of DrrA to interact with DrrB as well as the overall function of the complex. This is the first report which describes the presence of an assembly domain in an ABC protein and uncovers a novel mechanism whereby the ABC component facilitates the assembly of the membrane component. Homology sequence comparisons showed the presence of the LDEVFL and CREEM motifs in close prokaryotic and eukaryotic homologs of DrrA, suggesting that these motifs may play a similar role in other homologous drug and lipid export systems.

Drug Resistance Mutation L76V Decreases the Dimer Stability and Rate of Autoprocessing of HIV-1 Protease by Reducing Internal Hydrophobic Contacts

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The mature HIV-1 protease (PR) bearing drug-resistance mutation L76V (PR_{L76V}) is significantly less stable, with >7-fold higher dimer dissociation constant (K_d) of 71 ± 24 nM and twice the sensitivity to urea denaturation (UC_{50} = 0.85 M) relative to PR. Differential scanning calorimetry showed a decrease in T_m of 12 °C for PR_{L76V} in the absence of inhibitors, and 5-7 °C in the presence of inhibitors darunavir (DRV), saquinavir (SQV) and lopinavir (LPV), relative to PR. Isothermal titration calorimetry gave a ligand dissociation constant of 0.8 nM for DRV, ~160-fold larger than that of PR, consistent with DRV resistance. Crystal structures of PR_{L76V} complexed with DRV and SQV were determined at resolutions of 1.45-1.46 Å. Compared to the corresponding PR complexes, the mutated Val76 lacks hydrophobic interactions with Asp30, Lys45, Ile47, and Thr74, and exhibits closer interactions with Val32 and Val56. The bound DRV lacks a hydrogen bond with the main chain of Asp30 in PR_{L76V} relative to PR, possibly accounting for resistance to DRV. SQV shows slightly improved polar interactions with PR_{L76V} compared to PR. Although the L76V mutation significantly slows the N-terminal autoprocessing of the precursor TFR-PR_{L76V} to give rise to the mature PR_{L76V}, the co-selected M46I mutation counteracts by enhancing this rate but renders the TFR-$PR_{M46I/L76V}$ precursor less responsive to inhibition by 6 μM LPV while retaining inhibition by SQV and DRV. The correlation of lowered stability, higher K_d and impaired autoprocessing, with reduced internal hydrophobic contacts suggests a novel molecular mechanism for drug resistance.

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