25th Enzyme Mechanisms Conference

January 4-8, 2017
St. Pete Beach, Florida

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25th Enzyme Mechanisms Conference

Don CeSar Hotel, St. Pete Beach, Florida
January 4-7, 2017

PROGRAM

WEDNESDAY, JANUARY 4TH

3:00-7:00 pm	Registration – Ballroom Foyer
7:00-9:00 pm	Welcome Reception – King Charles Room
	All Lecture Sessions will be held in the Grand Ballroom Poster sessions will be in South Terrace, Buena Vista, Del Prado and 5th Floor Foyer

THURSDAY, JANUARY 5TH

7:30-11:00 am	Late Registration
7:15-8:15 am	Continental Breakfast – King Charles Room
8:15-8:30 am	Welcome – **Richard B. Silverman,** Northwestern University
8:30 am-12:20 pm	**Session 1: Enzymes Important to Drug Design** **Chair: Haitao (Mark) Ji**, Moffitt Cancer Center and Research Institute
8:30-9:05 am	**Ronald T. Raines**, University of Wisconsin-Madison *Ribonuclease A: from k_{cat}/K_M to the Clinic*
9:05-9:10 am	Q & A
9:10-9:45 am	**Robert A. Copeland**, Epizyme, Inc. *Tazemetostat, A First-in-Class Inhibitor of EZH2: From Bench to Bedside to Bench*
9:45-9:50 am	Q & A
9:50-10:20 am	Coffee Break
10:20-10:55 am	**Kenneth A. Johnson**, University of Texas at Austin *Allosteric Inhibition of Hepatitis C Viral RNA-dependent RNA Polymerase*
10:55-11:00 am	Q & A

11:00-11:35 am **David S. Weinstein**, Bristol-Myers Squibb
Pseudokinase Domain of the Janus Kinase (JAK) TYK2: A Novel Target for the Treatment of Immune-Mediated Inflammatory Diseases
11:35-11:40 am Q & A

11:40 am-12:15 pm **Jeanne A. Hardy**, University of Massachusetts Amherst
Native Allosteric Regulation of Caspase-6
12:15 pm-12:20 pm Q & A

Lunch (not provided)

12:20-3:00 pm Free time

3:00-5:30 pm Poster Session 1 (Thursday posters, P01-P60; cash bar)

7:30-10:10 pm **Session 2: Heme- and Nonheme Iron Enzymology**
Chair: Ping Li, Kansas State University

7:30-8:05 pm **Thomas L. Poulos**, University of California, Irvine
Structural Biology of Redox Partner Binding: Simple and Complicated
8:05-8:10 pm Q & A

8:10-8:45 pm **F. Peter Guengerich**, Vanderbilt University
Mechanisms of C-C Cleavage Reactions by Cytochrome P450s in Steroid Biosynthesis
8:45-8:50 pm Q & A

8:50-9:25 pm **Lana Saleh**, New England Biolabs
Insight into DNA 5-Methylcytosine Oxidation by the Mouse Ten-Eleven Translocation 2 (mTET2) Enzyme
9:25-9:30 pm Q & A

9:30-10:05 pm **John D. Lipscomb**, University of Minnesota
Arylamine to Nitro Oxygenation by CmlI: How to Catalyze a 6-Electron Oxidation with a 2-Electron Oxidant
10:05-10:10 pm Q & A

FRIDAY, JANUARY 6TH

7:30-8:30 am Continental Breakfast

8:30 am-12:20 pm **Session 3: Covalent Modification of Enzymes**
Chair: Thomas M. Makris, University of South Carolina

8:30-9:05 am **Michael Z. Hoemann**, AbbVie
Bruton's Tyrosine Kinase (BTK) – Considerations for the Profiling and Design of Irreversible Covalent Inhibitors
9:05-9:10 am Q & A

9:10-9:45 am	**Walter Fast**, University of Texas at Austin *Two Deaths of Dimethylarginine Dimethylaminohydrolase*
9:45-9:50 am	Q & A
9:50-10:20 am	Coffee Break
	Session 4: Enzymes Involved in Signaling **Chair: Andrew J. Andrews**, Fox Chase Cancer Center
10:20-10:55 am	**Shahriar Mobashery**, University of Notre Dame *Nexus Among Cell-Wall Turnover, Antibiotic Resistance and Virulence in Pseudomonas aeruginosa*
10:55-11:00 am	Q & A
11:00-11:35 am	**Philip Cole**, Johns Hopkins University *Mechanistic Insights Into PTEN Regulation*
11:35-11:40 am	Q & A
11:40-12:15 pm	**Ruma Banerjee**, University of Michigan Medical School *Enzymology of Hydrogen Sulfide Signaling*
12:15-12:20 pm	Q & A
	Lunch (not provided)
12:20-3:00 pm	Free time
3:00-5:30 pm	Poster Session 2 (Friday posters, P61-P119; cash bar)
7:30-10:10 pm	**Session 5: Founder's Award, Radical Chemistry, and More** **Chair: Edwin Antony**, Marquette University
7:30-7:35 pm	Presentation of Founder's Award, Richard B. Silverman
7:35-8:00 pm	**Founder's Award Lecture** **James K. Christenson**, University of Minnesota *β-Lactones from β-Hydroxy Acids: a New and Widespread Reaction Catalyzed by an ATP Dependent Ligase/Synthetase Superfamily Enzyme*
8:00-8:05 pm	Q & A
8:05-8:40 pm	**Joan B. Broderick**, Montana State University *Mechanism & Control in Radical SAM Enzymes*
8:40-8:45 pm	Q & A
8:45-9:20 pm	**Catherine L. Drennan**, Massachusetts Institute of Technology *Shake, Rattle, & Roll: Capturing Snapshots of Ribonucleotide Reductase in Action*
9:20-9:25 pm	Q & A
9:25-9:40 pm	**Richard B. Silverman** *Tribute to Myron Bender and in Memorium*

9:40-9:52 pm	**Poster talk 1** **Sumedh Joshi**, Texas A & M University *Mechanistic Studies of a Unique Radical SAM Enzyme, MqnE*
9:52-9:55 pm	Q & A
9:55-10:07 pm	**Poster talk 2** **Zhengan Zhang**, University of Illinois, Urbana-Champaign *In vitro Reconstitution and Substrate Specificity of the Biosynthesis of the Core Scaffold of the Thiopeptide Thiomuracin*
10:07-10:10 pm	Q & A

SATURDAY, JANUARY 7TH

7:30-8:30 am	Continental Breakfast
8:30 am-12:20 pm	**Session 6: Enzymes Involved in Biosynthetic Pathways** **Chair: Songon An**, University of Maryland, Baltimore County
8:30-9:05 am	**David H. Sherman**, University of Michigan *Decoding a New Class of Indole Alkaloid Cyclases in the Stigonematales Cyanobacteria*
9:05-9:10 am	Q & A
9:10-9:45 am	**Hung-wen (Ben) Liu**, University of Texas at Austin *Mechanistic Studies of the Cyclization Reactions in the Biosynthesis of Spinosyn*
9:45-9:50 am	Q & A
9:50-10:20 am	Coffee Break
10:20-10:55 am	**Robert P. Hausinger**, Michigan State University *Synthesis of the Ni-Pincer Cofactor of Lactate Racemase*
10:55-11:00 am	Q & A
11:00-11:35 am	**Craig A. Townsend**, Johns Hopkins University *Thermodynamic Problems and Solutions in β-Lactam Antibiotic Biosynthesis*
11:35-11:40 am	Q & A
11:40-12:15 pm	**Tobias J. Erb**, Max Planck Institute *CETCH Me If You Can: A Synthetic Pathway for the Fixation of Carbon Dioxide*
12:15-12:20 pm	Q & A
12:20-1:20 pm	Lunch (Box lunch provided, King Charles Room)
1:20-5:00 pm	**Session 7: Coenzyme-dependent Enzymes** **Chair: Nicholas R. Silvaggi**, University of Wisconsin-Milwaukee

1:20-1:55 pm	**Paul F. Fitzpatrick**, Texas A&M University *Structural Basis for Regulation in the Aromatic Amino Acid Hydroxylases*
1:55-2:00 pm	Q & A
2:00-2:35 pm	**Steven Rokita**, Johns Hopkins University *Reductive Dehalogenation Mediated by a Flavoprotein*
2:35-2:40 pm	Q & A
2:40-3:15 pm	**Neil Marsh**, University of Michigan *Prenyl-Flavin: a New Cofactor for Catalyzing Aromatic Decarboxylation Reactions*
3:15-3:20 pm	Q & A
3:20-3:50 pm	Refreshments
3:50-4:25 pm	**Amnon Kohen**, University of Iowa *An Unprecedented Mechanism of Nucleotide Methylation by Flavin dependent Thymidylate Synthase*
4:25-4:30 pm	Q & A
4:30-5:05 pm	**Michael Toney**, University of California, Davis *Reaction Specificity in Pyridoxal Phosphate and Chorismate Enzymes*
5:05-5:10	Q & A
6:00-7:00 pm	Cocktails (cash bar) – South Terrace (5th floor)
7:00-10:00 pm	Conference Banquet – King Charles Room (5th floor)

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SPEAKER ABSTRACTS

Session 1: Enzymes Important to Drug Design

O1. Ribonuclease A: from k_{cat}/K_M to the Clinic
Ronald T. Raines

O2. Tazemetostat, A First-in-Class Inhibitor of EZH2: From Bench to Bedside to Bench
Robert A. Copeland

O3. Allosteric Inhibition of Hepatitis C Viral RNA-Dependent RNA Polymerase
Kenneth A. Johnson, Jiawen Li, Daniel Deredge, Patrick L. Wintrode and Serdal Kirmizialtin

O4. Pseudokinase Domain of the Janus Kinase (JAK) TYK2: A Novel Target for the Treatment of Immune-Mediated Inflammatory Diseases
Ryan M. Moslin, Stephen T. Wrobleski, Shuqun Lin, Yanlei Zhang, John S. Tokarski, Javed Khan, Jodi K. Muckelbauer, John S. Sack, Huadong Sun, Manoj Chiney, Paul A. Elzinga, Nelly Aranibar, Anjaneya Chimalakonda, Joann Strnad, Yuval Blat, Charu Chaudhry, Kathleen M. Gillooly, Kim W. McIntyre, Percy H. Carter, Louis J. Lombardo, John E. Macor, James R. Burke, and David S. Weinstein

O5. Native Allosteric Regulation of Caspase-6
Kevin Dagbay, Ishan Soni, Eric Okerberg, Jennie Green, Tyzoon Nomanbhoy, John W. Kozarich and Jeanne A. Hardy

Session 2: Heme- and Nonheme Iron Enzymology

O6. Structural Biology of Redox Partner Binding: Simple and Complicated
Thomas L. Poulos, Scott Hollingworth, Dipanwita Batabyal and Georges Chreifi

O7. Mechanisms of C-C Cleavage Reactions by Cytochrome P450s in Steroid Biosynthesis
F. Peter Guengerich, Francis K. Yoshimoto, Eric Gonzalez, and Michael J. Reddish

O8. Insight into DNA 5-Methylcytosine Oxidation by the Mouse Ten-Eleven Translocation 2 (mTET2) Enzyme
Esta Tamanaha, An Li, Janani Sridar, and Lana Saleh

O9. Arylamine to Nitro Oxygenation by CmlI: How to Catalyze a 6-Electron Oxidation with a 2-Electron Oxidant
Anna J. Komor, Cory J. Knoot, Andrew J. Jasniewski, Brent S. Rivard, Lawrence Que, Jr., and John D. Lipscomb

Session 3: Covalent Modification of Enzymes

O10. Bruton's Tyrosine Kinase (BTK) - Considerations for the Profiling and Design of Irreversible Covalent Inhibitors
Michael Z. Hoemann

O11. Two Deaths of Dimethylarginine Dimethylaminohydrolase
Walter Fast

Session 4: Enzymes Involved in Signaling

O12. Nexus Among Cell-Wall Turnover, Antibiotic Resistance and Virulence in *Pseudomonas aeruginosa*
Shahriar Mobashery

O13. Mechanistic Insights Into PTEN Regulation
Philip Cole

O14. Enzymology of Hydrogen Sulfide Signaling
Ruma Banerjee

Session 5: Founder's Award, Radical Chemistry, and More

O15. β-Lactones from β-Hydroxy Acids: a New and Widespread Reaction Catalyzed by an ATP Dependent Ligase/Synthetase Superfamily Enzyme
James K. Christenson, Jack E. Richman, Matthew R. Jensen, Jennifer Y. Neufeld, Carrie M. Wilmot, and Lawrence P. Wackett

O16. Mechanism & Control in Radical SAM Enzymes
Masaki Horitani, Krista A. Shisler, Amanda Byer, Rachel Hutcheson, Kaitlin S. Duschene, Amy Marts, William E. Broderick, Brian M. Hoffman, Joan B. Broderick

O17. Shake, Rattle, & Roll: Capturing Snapshots of Ribonucleotide Reductase in Action
Christina M Zimanyi, Percival Yang-Ting Chen, Gyunghoon Kang, Michael A Funk, Catherine L Drennan

O18. Mechanistic Studies of a Unique Radical SAM Enzyme, MqnE
Sumedh Joshi, Dmytro Fedoseyenko, Nilkamal Mahanta, Derek Gagnon, Brett Hirsch, Vern Schramm, R. David Britt, Tadhg P. Begley

O19. In Vitro Reconstitution and Substrate Specificity of the Biosynthesis of the Core Scaffold of the Thiopeptide Thiomuracin
Zhengan Zhang, Graham A. Hudson, Nilkamal Mahanta, Jonathan I. Tietz, Douglas A. Mitchell and Wilfred A. van der Donk

Session 6: Enzymes Involved in Biosynthetic Pathways

O20. Decoding a New Class of Indole Alkaloid Cyclases in the Stigonematales Cyanobacteria
Shasha Li[2], Andrew N. Lowell, Sean A. Newmister, Fengan Yu, Robert M. Williams, and David H. Sherman

O21. Mechanistic Studies of the Cyclization Reactions in the Biosynthesis of Spinosyn
Hung-wen Liu

O22. Synthesis of the Ni-Pincer Cofactor of Lactate Racemase
Matthias Fellner, Benoît Desguin, Jian Hu, and Robert P. Hausinger

O23. Thermodynamic Problems and Solutions in β-Lactam Antibiotic Biosynthesis
R.-F. Li, R. A. Oliver, D. H. Long, D. R. Marous, N. M. Gaudelli and C. A. Townsend

O24. CETCH Me if You Can: A Synthetic Pathway for the Fixation of Carbon Dioxide
Thomas Schwander and Tobias J. Erb

Session 7: Coenzyme-dependent Enzymes

O25. Structural Basis for Regulation in the Aromatic Amino Acid Hydroxylases
Paul F. Fitzpatrick

O26. Reductive Dehalogenation Mediated by a Flavoprotein
Abhishek Phatarphekar, Jimin Hu, Arnab Mukherjee, Qu Su, Petrina Boucher, and Steven Rokita

O27. Prenyl-Flavin: a New Cofactor for Catalyzing Aromatic Decarboxylation Reactions
E. Neil G. Marsh, Kyle L. Ferguson and Nattapol Arunrattanamook

O28. An Unprecedented Mechanism of Nucleotide Methylation by Flavin Dependent Thymidylate Synthase
Mishanina, T.V., Yu, L., Karunaratne, K., Mondal, D., Corcoran, J.M., Choi, M.A., Kohen, A.

O29. Reaction Specificity in Pyridoxal Phosphate and Chorismate Enzymes
Michael Toney

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THURSDAY POSTER ABSTRACTS

P1. Structure-Based Drug Design (SBDD), Synthesis and Evaluation of Peptides and Non-β-Lactam Inhibitors of Y-49 β-Lactamase from *Mycobacterium tuberculosis*
Janet Gonzalez, Manfred Philipp, and Cristina Clement

P2. The Bacillithiol Biosynthesis Enzymes: A Structural and Functional Analysis
Kelsey R. Winchell, Andrew J. VanDuinen, Aaron M. Rosenberg, Emily A. David, Christopher J. Royer, and Dr. Paul D. Cook

P3. The Transition State of D-Alanine: D-Alanine Ligase as a Potential Drug Target for Treatment of Tuberculosis
Patrick L. Fernandez and Andrew S. Murkin

P4. Crystallographic Studies of Human Acetylcholinesterase Reactivation by Oximes
Oksana Gerlits, Mikolai Fajer, Xiaolin Cheng, Donald Blumenthal, Palmer Taylor, Zoran Radić, Andrey Kovalevsky

P5. Trehalose-6-Phosphate Phosphatase Structure and Inhibitor Design
Christine M. Harvey, Chunliang Liu, Patrick Mariano, Kim Janda, Daniel Globisch, Debra Dunaway-Mariano, and Karen N. Allen

P6. Antibacterial Drug Discovery: Rationally Designed Inhibitors of MEP Synthase
Amanda Haymond, Chinchu Johny, Claire Johnson, Emily R. Jackson, Geraldine San Jose, Dr. Cynthia Dowd, Dr. Robin Couch

P7. A Tethered-Rheostat Model for the Intramolecular Activation of the Anti-Cancer Target USP7/HAUSP by Its HAUSP Ubiquitin-Like Domains 4 and 5
Nicole M. Hjortland, Anuj U. Patel, and Andrew D. Mesecar

P8. Utilizing a Minimally-Perturbing Fluorophore to Gain Mechanistic Insight into a Pro-Mutagenic Reaction in Bacteria
Zachary M. Hostetler[3], Itthipol Sungweinwong, Matthew J. Culyba, E. James Petersson, Rahul M. Kohli

P9. Unavailing the Molecular Foundations Behind the Resistance Profile of Cancerous Thymidylate Synthase
Zahidul Islam, Ilya Gurevic, Muhammad Saeed, William Marquardt, Sobia Saeed, Janet Finer-Moore, Robert Stroud and Amnon Kohen

P10. Rational Design of Selective Small-Molecule Inhibitors for the *β*-Catenin/T-Cell Factor Protein–Protein Interaction
Haitao (Mark) Ji

P11. Locking Chemoselectivity in the Cytochrome P450 OleT
Jose A. Amaya, Cooper D. Rutland, Nicholas Leschinsky and Thomas M. Makris

P12. Mechanistic Studies of a Bifunctional α-Ketoglutarate Dependent non-Heme Iron Enzyme, AsqJ
Wei-chen Chang, Justin Lee, Jikun Li, Justin Dicks, and Yisong Guo

P13. Mechanistic Insights Into Bacterial Dye-Decolorizing Peroxidases
Ruben Shrestha, Chao Chen, Likai Song, Brian V. Geisbrecht, and Ping Li

P14. Structural and Mechanistic Insight into the Phosphorylation-Dependent Autoinhibition of PTEN
Daniel R. Dempsey, Zan Chen, Stefani N. Thomas, Dawn Hayward, David M. Bolduc, Philip A. Cole

P15. Enzymology of H_2S Oxidation in Nanodiscs
Ruma Banerjee, Aaron P. Landry

P16. Mechanistic Studies of a Unique Radical SAM Enzyme, MqnE
Sumedh Joshi, Dmytro Fedoseyenko, Nilkamal Mahanta, Derek Gagnon, Brett Hirsch, Vern Schramm, R. David Britt, Tadhg P. Begley

P17. Structure of a Key Intermediate in the Reaction Catalyzed by the Dual Specificity Methylase RlmN
Erica L. Schwalm, Tyler L. Grove, Amie K. Boal, and Squire J. Booker

P18. Regulatory Metabolic Complex for Glucose Metabolism in Living Cells: the Glucosome
Casey L. Kohnhorst, Minjoung Kyoung, Miji Jeon, Danielle L. Schmitt, Erin L. Kennedy, Julio Ramirez, Syrena M. Bracey, Bao Tran Luu, Sarah J. Russell, and Songon An

P19. Targeting Aerobactin Biosynthesis in Hypervirulent *Klebsiella pneumoniae*
Daniel C. Bailey, Eric J. Drake, Thomas A. Russo, MD, and Andrew M. Gulick, PhD

P20. Fungal Metabolomics for Large-Scale Analysis of Secondary Metabolism and Biosynthesis
Kenneth D. Clevenger, Jin Woo Bok, Rosa Ye, Galen P. Miley, Thomas Velk, Cynthia Chen, KaHoua Yang, Peng Gao, Matthew Lamprecht, Paul M. Thomas, M. N. Islam, Chengcang C. Wu, Nancy P. Keller, Neil L. Kelleher

P21. Ipdab, a Key Virulence Determinant in *Mycobacterium tuberculosis,* is a Cholesterol Ring-Cleaving Hydrolase
A. Crowe, N. Watanabe, L. Worrall, S. Workman, J. Rogalski, N. Strynadka, and L.D. Eltis

P22. Enzymes for Decorating the Heterocyclic Aromatic Ring of Phenazine Antibiotics from *Lysobacter antibioticus*
Jiasong Jiang, Yangyang Zhao, Stephen Wright, Liangcheng Du

P23. Crystal Structures of the *E. coli* Dihydrodipicolinate Synthase in Complex with Pyruvate and Acetopyruvate
Christian D. Fleming, William E. Karsten, Leonard Thomas, Lilian Chooback

P24. Enzyme Function Discovery as a Foundation for Exploring Novel Secondary Metabolites
Swapnil V. Ghodge, Kristen A. Biernat, Matthew R. Redinbo, and Albert A. Bowers

P25. The Characterization of the *ydj* Operon, a Carbohydrate Catabolic Pathway Found in *E. coli*
Frank M. Raushel, Jim Thoden, Hazel Holden, and Jamison P. Huddleston

P26. Phytoene Desaturase: an FAD-dependent Plant Carotenoid Biosynthetic Enzyme
Brian K. Barr, Cassandra A. Cairns, Ruben Ferreira de Carvalho, Najuma S. Babirye and Faith O. Osinaga

P27. NMR Crystallography of a Carbanionic Intermediate in Tryptophan Synthase: Chemical Structure, Tautomerization, and Reaction Specificity
Bethany G. Caulkins, Robert P. Young, Michael F. Dunn, and Leonard J. Mueller

P28. Extended Hydrogen Bonding Network Influences Electronic Modulation of Pyridoxal 5'-Phosphate in Aspartate Aminotransferase
Steven Dajnowicz, Jerry M. Parks, Xiche Hu, Andrey Y. Kovalevsky, Timothy C. Mueser

P29. Structural Features of Two-Component FMN-Dependent Reductases that Provide a Functional Advantage
Jonathan M. Musila and Holly R. Ellis

P30. Characterization of a New FAD: NAD(P)H-Quinone Oxidoreductase in *Pseudomonas aeruginosa* Strain PAO1
Elias Flores and Giovanni Gadda

P31. Active Site Hydrogen Bonding Promotes Oxidative Decarboxylation by Cytochrome P450 OleT
Job L. Grant, Jose A. Amaya, Julia C. Bian, Cooper D. Rutland, Thomas M. Makris

P32. Renalase: Emerging Details of an Expanding Family of Enzymes
Joseph V. Roman, Brett A. Beaupre, Graham R. Moran, Matthew R. Hoag

P33. Structural Insights into the 6-OH-FAD in the Y249F Variant of D-Arginine Dehydrogenase from *Pseudomonas aeruginosa*
Archana Iyer, Renata A.G. Reis, Swathi Gannavaram, Alexander M. Spring, Markus W. Germann, Johnson Agniswamy, Irene T. Weber and Giovanni Gadda

P34. Kinetic and Structural Studies on the Acetolactate Synthase from *Klebsiella pneumoniae*
Alexander J. Latta, Catherine Njeri, Forest H. Andrews, and Michael J. McLeish

P35. Divergent Mechanisms of Allosteric Regulation of Pyruvate Carboxylase by Acetyl Coenzyme A
Yumeng Liu, Martin St. Maurice

P36. Reaction Rates and Intermediates in Ser26 Variants of Benzoylformate Decarboxylase
Michael J. McLeish, NiLen Lian, Sui Tial Tial, Elly Mawi and Bruce D. Ray

P37. Enzyme and Substrate Pieces of Glycerol-3-Phosphate Dehydrogenase: Reconstruction of a Robust Hydride-Transfer Catalyst
Archie C. Reyes, Tina L. Amyes and John P. Richard

P38. Overcoming an Optimization Plateau in the Directed Evolution of Highly Efficient Nerve Agent Bioscavengers
Moshe Goldsmith, Nidhi Aggarwal, Yacov Ashani, Halim Jubran, Per Jr. Greisen, Sergey Ovchinnikov, Haim Leader, David Baker, Joel L. Sussman, Adi Goldenzweig, Sarel J. Fleishman and Dan S. Tawfik

P39. Mechanistic Studies of Engineered Ascorbate Peroxidase, a Genetically Encoded Cell Imaging Probe
Alice Y. Ting, Stephanie S. Lam, Jeffrey D. Martell

P40. Pathways of Acetylation by Rtt109-Vps75
Yin-Ming Kuo, Ryan Henry, and Andrew J. Andrews

P41. Asymmetry and Cooperativity in Nitrogenase
Edwin Antony

P42. Discovery and Characterization of Fused 4-OT: Insights into the Sequence-Structure-Function Relationships and Evolutionary History of the Tautomerase Superfamily
Bert-Jan Baas, Jake LeVieux, Rebecca Davidson, Patricia C. Babbitt, Christian P. Whitman

P43. Rapid Timescale Binding Analysis of T4 DNA Ligase DNA-Binding
Robert J. Bauer, Thomas J. Jurkiw, Thomas C. Evans Jr. and Gregory J. S. Lohman

P44. Glycosylase Activity on Nucleosome Core Particles with Varied Lesion Positioning
Katharina Bilotti, Sarah Delaney

P45. Asymmetry in Substrate Reduction by the Nitrogenase-like Dark Operative Protochlorophyllide Oxidoreductase (DPOR) Complex
Elliot I. Corless, Mark S. Soffe, Karamatullah Danyal, Robert Kitelinger, Sofia Origanti Lance C. Seefeldt and Edwin Antony

P46. Inhibition of Bacterial HMM DD-Peptidases by Peptidyl Thioesters
Kinjal Dave and Rex F. Pratt

P47. Weak Interactions between Typical Crowders and the Ligands of DHFR on Decreased Enzyme Activity
Michael R. Duff Jr., Nidhi Desai, Michael Craig,[1] Greyson Dickey, Ayza Taimur and Elizabeth E. Howell

P48. On the Generality of Extensive Orbital Alignments in Enzyme/Ligand Complexes
Abigail Fortier and John Haseltine

P49. Loop-Swapped Chimeras and Tryptophan Mutants of the Protein Tyrosine Phosphatases Yoph and PTP1B Investigate the Connection Between WPD-Loop Motion and Catalysis
Gwen Moise, Teisha Richan, Yalemi Morales, Sean J. Johnson, Timothy Carradonna, J. Patrick Loria and Alvan C. Hengge

P50. Structural Dynamics of SufS Cysteine Desulfurase Persulfide Intermediate
Dokyong Kim, Harsimran Singh, Yuyuan Dai, Guanchao Dong, Laura S. Busenlehner,F. Wayne Outten, and Patrick A. Frantom

P51. Nucleoside Analogue Triphosphates Allosterically Regulate Human Ribonucleotide Reductase and Identify Chemical Determinants that Drive Substrate Specificity
Andrew J. Knappenberger, Md. Faiz Ahmad, Rajesh Viswanathan, Chris G. Dealwis, and Michael E. Harris

P52. Enzyme Evolution
Priyanka Singh, An Vandemeulebroucke, Jiayue Li, Donald Hilvert and Amnon Kohen

P53. QM/MM Simulations of DFP and (*S*)-Sarin Hydrolysis by DFPase: Implications for Engineering Bioscavengers
Troy Wymore, Sara Tweedy and Charles L. Brooks III

P54. Transient Kinetics of Protein Arginine Methyltransferase 1 (PRMT1)
Hao Hu, Kun Qian, Hui Xu, You Feng, Y. George Zheng

P55. Novel Non-Hydroxamate Inhibition of Histone Deacetylase 8
Kelsey Diffley, George Murphy III, Carol Fierke

P56. Ghrelin Acylation by Ghrelin *O*-Acyltransferase: Investigation of Ghrelin Recognition During Hormone Processing and Small Molecule Inhibitor Development
Kayleigh R. McGovern-Gooch, Elizabeth R. Cleverdon, Maria B. Campana, Nivedita S. Mahajani, John D. Chisholm, and James L. Hougland

P57. IMP Dehydrogenase Forms Filaments in Response to Dysregulation of Guanine Nucleotide Homeostasis
S. John Calise and Edward K.L. Chan

P58. Novel Probes for Nucleobase Transporters
Ai Tran, Marci Wood, Ryota Yokose, Jarrod French, Yana Cen

P59. Mechanistic and Structural Investigation of Protein-Only Ribonuclease P
Kipchumba J Kaitany , Bradley P Klemm, Nancy Wu, Xin Liu, Michael J Howard, and Carol A Fierke

P60. Rational Approach to Discovery of Isocitrate Lyase Inhibitor
Margaret Moynihan, Andrew Murkin, Thomas D. Meek, Truc V. Pham

FRIDAY POSTER ABSTRACTS

P61. Synthetic Polyketide Enzymology: Platform for Biosynthesis of Antimicrobial Polyketides
Maybelle Kho Go, Jantana Wongsantichon, Vivian Wing Ngar Cheung, Jeng Yeong Chow, Robert C. Robinson, and Wen Shan Yew

P62. Mechanisms of Proton Relay and Product Release by Class A β-Lactamase at Ultrahigh Resolution
Eric M. Lewandowski, Kathryn G. Lethbridge, Ruslan Sanishvili, Joanna Skiba, Konrad Kowalski, and Yu Chen

P63. Studying Proton Transfer in the Mechanism and Inhibition of Serine β-Lactamase Acylation
Yu Chen, Ruslan Sanishvili, Orville Pemberton

P64. Peroxiredoxin Catalysis at Atomic Resolution
Arden Perkins, Derek Parsonage, Kimberly J. Nelson, O. Maduka Ogba, Paul Ha-Yeon Cheong, Leslie B. Poole, P. Andrew Karplus

P65. Synthetic Enzymology and its Applications in Bioremediation
Rashmi Rajasabhai, Yu Kai, Maybelle Go and Wen Shan Yew

P66. Propionate-3-nitronate is a Covalent, Irreversible MtICL Inhibitor
Andrew Murkin and Sneha Ray

P67. Lipidation of NDM-1 Converts Antibiotic Resistance from a "Public Good" to a "Private Good"
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O1

Ribonuclease A: from k_{cat}/K_M to the Clinic

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The vast majority of extant drugs are small molecules that block the flow of biochemical information. Enzymes also have the potential to disrupt this flow. For example, ribonuclease A, which spawned the first enzymatic reaction mechanism[1] and seminal advances in protein chemistry, is an efficient catalyst of RNA cleavage. We have discovered that this highly cationic enzyme naturally enters the cytosol of mammalian cells. By enabling ribonuclease A to evade a cytosolic inhibitor protein (Figure 1), we have endowed it with the ability to cleave cellular RNA and thereby kill human cells.[2,3] A variety of chemical and biological tools, especially "fluorogenic labels", have enabled us to reveal the kinetic mechanism by which ribonucleases exert that cytotoxic activity.[4] These ribonucleases have a marked preference for killing cancerous cells due to a nanomolar affinity for Globo H, a cell-surface glycan that is a human cancer antigen.[5] An inhibitor-evading variant of the human homolog of ribonuclease A is in a Phase I clinical trial as a cancer chemotherapeutic agent. To date, 57 patients have been treated with this enzyme at the University of Texas MD Anderson Cancer Center and the University of Wisconsin Carbone Cancer Center. Eleven of these patients achieved stable disease. Thus, decades of research on ribonuclease A are now poised to yield a drug. [This work is supported by grant R01 CA073808 (NIH).]

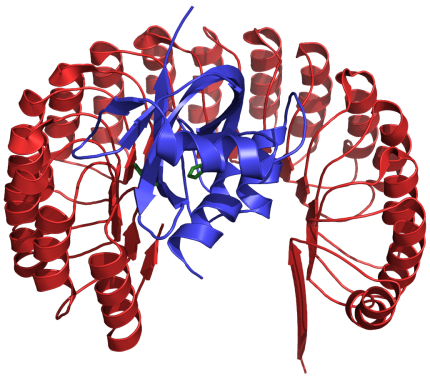

Figure 1. Structure of human ribonuclease (blue) bound to its natural inhibitor protein (red). This complex (K_d = 10^{-16} M) is the tightest known between biomolecules.[6]

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O2

Tazemetostat, A First-in-Class Inhibitor of EZH2: From Bench to Bedside to Bench

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The protein methyltransferases (PMTs) constitute a class of enzymes that catalyze the methylation of lysine or arginine residues on histones and other proteins. The enzyme EZH2 provides a representative example of altered PMTs that act as genetic drivers of specific human cancers. Point mutations of EZH2 are found in a subset of non-Hodgkins lymphoma patients; the enzymatic activity of both wild type and mutant EZH2 are required for pathogenesis in these patients. Also, deletion of the INI1 or SMARCA4 subunit of the SWI/SNF chromatin-remodeling complex occur in a number of cancer types. For example, INI1 is deleted in nearly all malignant rhabdoid tumors (MRTs), a cancer found mainly in children that carries a particularly poor prognosis. Similarly, the SMARCA4 subunit of SWI/SNF is deleted, for example, in malignant rhabdoid tumor of the ovary (MRTO, also referred to as small cell carcinoma of the ovary hypercalcemic type), an aggressive cancer affecting young women. An antagonistic relationship has been demonstrated between the biochemical action on chromatin of the SWI/SNF complex and EZH2 that is relieved in MRTs due to the INI1 deletion. We have shown that INI1-deficient MRT and SMARCA4-deficient MRTO are selectively killed by EZH2 inhibition in culture and in mouse xenograft models. Drug discovery efforts have yielded a potent, selective inhibitor of EZH2, tazemetostat (EPZ-6438), that has now transitioned into phase 2 clinical trials. This inhibitor affects the appropriate histone methyl marks in cells, leads to selective cell killing that is dependent on genetic lesions associated with EZH2 activity and effects tumor growth inhibition in xenograft models. Combining tazemetostat with other treatment modalities for non-Hodgkins lymphoma results in dramatic synergy of anti-proliferative activity in preclinical models. Results of preclinical and phase 1 clinical studies of tazemetostat will be presented.

O3

Allosteric Inhibition of Hepatitis C Viral RNA-Dependent RNA Polymerase

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Replication of the Hepatitis C viral genome is catalyzed by the NS5B RNA-dependent RNA polymerase, which is a major target of antiviral drugs currently in the clinic. Prior studies established that initiation of RNA replication could be facilitated by starting with a dinucleotide (pGG). Here we establish conditions for efficient initiation from GTP to form the dinucleotide and subsequent intermediates leading to highly processive elongation, and we examine the effects of four classes of nonnucleoside inhibitors on each step of the reaction. We show that palm site inhibitors block initiation starting from GTP but not when starting from pGG. In addition we show that nonnucleoside inhibitors binding to thumb site-2 (NNI2) lead to the accumulation of abortive intermediates 3-5 nucleotides in length. Our kinetic analysis shows that NNI2 do not significantly block initiation or elongation of RNA synthesis; rather they block the transition from initiation to elongation, which is thought to proceed with significant structural rearrangement of the enzyme-RNA complex including displacement of the β-loop from the active site. Direct measurement in single turnover kinetic studies show that pyrophosphate release is faster than the chemistry step, which appears to be rate-limiting during processive synthesis. These results reveal important new details to define the steps involved in initiation and elongation during viral RNA replication, establish the allosteric mechanisms by which NNI2 inhibitors act, and point the way to the design of more effective allosteric inhibitors that exploit this new information. Analysis by hydrogen/deuterium exchange kinetics reveals a significant increase in rigidity at the active site after binding of the inhibitors to the enzyme surface, supporting a model for allosteric inhibition. Molecular dynamics simulations reveal likely structural intermediates in the transition from the initiation to the elongation mode involving the movement of the thumb β-loop out of the active site.

O4

Pseudokinase Domain of the Janus Kinase (JAK) TYK2: A Novel Target for the Treatment of Immune-Mediated Inflammatory Diseases

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Tyrosine kinase 2 (TYK2) is a member of the non-receptor tyrosine kinase family of Janus kinases (JAK) and is required for the signaling of type-1 interferon, IL-12, and IL-23 cytokines. As these pro-inflammatory cytokines have been shown to contribute to autoimmune disease pathogenesis, and have been clinically validated with biologic agents targeted against them, a selective small molecule TYK2 inhibitor offers great promise for the treatment of diseases in which these cytokines are involved. The hallmark feature of the JAK enzymes is the presence of a pseudokinase (JH2) domain adjacent to the catalytic (JH1) domain, which serves to regulate kinase function. A phenotypic screen of an annotated collection of kinase inhibitors allowed for the identification of high affinity ligands of the TYK2 JH2 domain which stabilize its suppressive role, thereby inhibiting cytokine-mediate receptor activation of the kinase.[1] Guided by crystallographic structural information, highly potent and selective ligands for the JH2 domain have been identified. Lead optimization has resulted in the identification of BMS-986165, a molecule which displays efficacy in pharmacodynamic and autoimmune disease models and which has advanced to clinical trials.

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O5

Native Allosteric Regulation of Caspase-6

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Caspases are a class of the cysteine proteases for which the most prominent cellular role is execution of apoptotic programmed cell death. Activation of the apoptotic caspase zymogens (procaspases) leads rapidly and irreversibly to death, so caspases are tightly regulated via a number of mechanisms including zymogen activation, oligomerization, binding of regulatory proteins such as the IAP family, zinc binding, nitrosylation, ubiquitylation and phosphorylation by a series of kinases. The focus of our work has been to elucidate the molecular mechanisms of these regulatory processes so that that they can be exploited therapeutically. More than a decade ago we discovered a cavity in the executioner apoptotic caspases capable of binding non-natural small molecules, which can then function as allosteric inhibitors[a]. The size and shape of this cavity suggests that natively-occurring small molecules may bind and regulate caspases by a similar mechanism. Prior to now, although a number of groups have sought to identify such molecules, no native small molecule regulators have been observed. Interrogating human cell lysates with the ActivX ATP Probe, which features an amine-reactive nucleotide analog for covalent protein attachment and desthiobiotin to allow isolation of ATP-binding proteins, we identified the procaspase-6 zymogen as strongly reacting. ATP binds to and inhibits procaspase-6 from autoactivation, suggesting that cellular ATP levels may be important for regulating caspase-6 activity by interactions at the dimer interface cavity. In addition a number of other nucleotides impact caspase-6 function by binding to the cavity at the dimer interface. The binding affinities for the interactions of several nucleotides suggest that their interactions with caspase-6 should be relevant under particular cellular conditions. Together, these data suggest molecular regulatory intersections between metabolic process and caspase activity.

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O6

Structural Biology of Redox Partner Binding: Simple and Complicated

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While the yeast cytochrome c peroxidase-cytc system has been a paradigm for biological electron transfer (ET) reactions, a homologous system from *Leishmania major* (LmP-cytc) is somewhat simpler thereby enabling a more in depth understanding of kinetics and dynamics. The LmP-cytc system exhibits fairly straightforward enzyme kinetics which reveals a kinetic solvent isotope effect that very likely reflects the importance of proton coupled electron transfer (PCET) in the rate limiting intramolecular ET reaction between the redox active Trp and heme iron. The dominance of electrostatic interactions at the LmP-cytc interface and the lack of significant structural changes upon complex formation has enabled detailed Brownian and molecular dynamics techniques to be used to study formation and discociation of the complex. These results support the so-called "bind and crawl" mechanism wherein the two proteins rapidly form a complex and then undergo a 2-dimensional "crawl" until the optimal energetically favorable complex required for rapid ET is reached. In sharp contrast, the interaction between P450cam and its ferredoxin redox partner, Pdx, requires complicated structural changes needed to trigger the PCET machinery required for O_2 activation. A comparison between these two systems provides a deeper understanding on the diversity and dynamics of redox partner interactions.

O7

Mechanisms of C-C Cleavage Reactions by Cytochrome P450s in Steroid Biosynthesis

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Cytochrome P450 (P450) enzymes catalyze four major C-C cleavage reactions in mammalian steroid metabolism, as well as similar reactions with drugs and other chemicals. These have been the subject of much interest for several decades, in terms of their mechanisms, and changes in these activities have major implications in both endocrine effects and disease treatment. A major issue with these enzymes is whether the "usual" oxidant, a 'Compound I' species (formally FeO^{3+}), is the oxidant or if its precursor (ferric peroxide, formally FeO_2^-) is.

P450 19A1 is the steroid aromatase, which converts androgens to estrogens in a 3-step (6-electron) process, which we determined to have a distributive mechanism.[1] Repetition of early $^{18}O_2$ labeling studies used recombinant human P450 19A1, a new derivatization method, and high-resolution mass spectrometry led to the conclusion that the product formic acid could have only been derived from a Compound I pathway.[2] The alternate products 19-CO_2H testosterone and androstenedione were identified and also attributed to a Compound I attack on C19. Human P450 19A1 and the three porcine P450 19A enzymes have selectivity for forming estrogens (beginning with C1 H abstraction), 19 CO_2H products (C19 oxidation), and 2*b*-hydroxylation.

P450 17A1 catalyzes two oxidation reactions, 17*a*-hydroxylation of progesterone and pregnenolone, and the subsequent cleavage of the 17,20 C-C bond ('lyase' reaction). $^{18}O_2$-studies are consistent with either a Compound I or FeO_2^- mechanism, and studies with the 'oxygen surrogate' iodosylbenzene indicate that a Compound I intermediate can be involved in the lyase reaction, as well as the 16- and 6*b*-hydroxylation of the 17*a*-OH steroids that we identified.[3] The results can be interpreted to mean that either (a) Compound I does all of the observed reactions or (b) that there are contributions of both Compound I and the ferric peroxide to the lyase reaction but the Compound I does the hydroxylation. In fish, the related P450 17A2 does only the hydroxylation reaction but only trace lyase chemistry, but our crystal structures show very little difference in the active sites.[4] Human P450 17A1 has a rather distributive kinetic mechanism in catalyzing the 17*a*-hydroxylation and lyase steps, i.e. 17*a*-OH products dissociate and reassociate.

P450 11A1 is a mitochondrial P450 that catalyzes the oxidation of cholesterol (and related precursors)[5] to key intermediate pregnenolone in a 3-step (6-electron) reaction. Evidence for a Compound I mechanism comes from ENDOR/EPR studies by others, with formation of product in good yield.[6] Using our ^{18}O results from work with P450 17A1,[3] we were able to trap the carbonyl products of P450 11A1 in an $^{18}O_2$ study with the intermediate 21,22-dihydroxycholesterol and establish that the reaction proceeds via nucleophilic attack of one or both hydroxyls on Compound I, as opposed to alternate –OH hydrogen abstraction mechanisms.[7]

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O8

Insight into DNA 5-Methylcytosine Oxidation by the Mouse Ten-Eleven Translocation 2 (mTET2) Enzyme

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The ten-eleven translocation 2 enzyme from mouse (mTET2) is an iron(II)/alpha-ketoglutarate(aKG)-dependent dioxygenase, which catalyses the iterative oxidation of 5-methylcytidine on DNA to 5-hydroxymethylcytidine (5hmC), 5-formylcytidine (5fC), and 5-carboxycytidine (5caC). In each of these three steps, mTET2 uses its Fe(II) cofactor to activate oxygen for the oxidation of the main base substrate and decarboxylation of the aKG co-substrate to succinate. A dual role for the mammalian TET 1-3 enzymes has been reported in the literature. On one hand, these enzymes result in a "stalling TET modification" forming "stable" 5hmC at certain genomic sites. "Stable" 5hmC plays a role as an epigenetic marker similarly to 5mC. On other genomic sites, TETs effect a "processive TET modification" allowing for the formation of 5fC and 5caC. On most genomic sites, these two bases are intermediates in active demethylation *via* the TET/thymine DNA glycosylase/base excision repair pathway. In this study, we employ kinetic tools to reveal the intrinsic properties of mTET2 that contribute to its mode of function *in vivo*. We also provide evidence that the catalytic function of mTET2 as well as its local occupancy on the genome account for its observed physiological functions.

O9

Arylamine to Nitro Oxygenation by CmlI: How to Catalyze a 6-Electron Oxidation with a 2-Electron Oxidant

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Chloramphenicol is synthesized by *Streptomyces venezuelae* from L-*para*-aminophenylalanine by employing a nonribosomal peptide synthetase (NRPS, CmlP) and associated tailoring enzymes. Two of the tailoring enzymes, CmlA and CmlI, incorporate nonheme dinuclear iron clusters to catalyze steps in the process. These are the archetypal enzymes for the application of the diiron cofactor in NRPS-mediated biosynthetic pathways. The final step in the pathway, catalyzed by CmlI, is the 6-electron oxidation of an aryl-amine precursor to the aryl-nitro moiety of the active antibiotic. Incorporation of ^{18}O into the nitro group shows that both oxygens originate from molecular oxygen. Since the known diiron cluster-containing oxygenases catalyze at most 2-electron oxidation reactions, the observed 6-electron oxidation must involve multiple steps. Transient kinetic studies have identified a diiron(III)-peroxo intermediate as the reactive species, in contrast to the high valent Fe(IV)-oxo species usually encountered in this enzyme class. The remarkable stability of the diiron-peroxo intermediate ($t_{1/2}$ = 3 h at 4 °C) has allowed detailed studies of the 6-electron oxidation pathway. In this presentation, X-ray crystallography is combined with X-ray absorption, resonance Raman, and Mössbauer spectroscopies, as well as kinetic and analytical studies to describe a novel mechanism for the oxidation reaction. Models for the generation, structure, and regulation of the diiron-peroxo species will be presented. The unique characteristic of the diiron-peroxo intermediate to participate in both electrophilic and nucleophilic chemistry appears to be displayed in the CmlI-mediated reactions, providing insight into a new catalytic dimension for the versatile diiron cluster.

Supported by GM118030 (to J.D.L.) and NIH GM38767 (to L.Q.)

O10

Bruton's Tyrosine Kinase (BTK) - Considerations for the Profiling and Design of Irreversible Covalent Inhibitors

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Bruton's tyrosine kinase (BTK) has become an important target for both oncology and immunology diseases. Ibrutinib (Imbruvica™), a covalent irreversible inhibitor of BTK, is approved for mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL) and Waldenströms macroglobulinemia (WM).[1,2] In addition, a number of compounds have progressed to Phase II clinical trials for oncology and immunology indications (acalabrutinib, HM-71224, G-0853 and PRN-1008). For irreversible covalent inhibition, there are two steps to the mechanism, 1) the molecule binds to the protein in a reversible manner, referred to as K_i and 2) an irreversible reaction between an electrophile and a nucleophilic residue, such as a cysteine, to form a covalent bond, referred to as K_{inact}. For ABBV-105, the electrophile is an acrylamide and the residue for covalent modification is Cysteine 481, which is located within the ATP binding pocket of BTK.[3] The resulting covalent adduct silences the kinase signaling pathway until such a time as new protein can be synthesized. This can have advantages over reversible inhibition as with a very short time period of exposure to the inhibitor, one could achieve prolonged blockade of signaling in vivo. The approach to designing an optimal irreversible BTK inhibitor is very different than the approach used for reversible inhibitor programs. This talk will highlight those differences in terms of time-dependent inhibition, understanding the importance of K_i and K_{inact} and which assays were critical for the screening funnel for the program. In addition, this talk will highlight how the data we generated was utilized to design for compounds with optimal profiles for preclinical safety studies.

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O11

Two Deaths of Dimethylarginine Dimethylaminohydrolase

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Enzymes in the pentein superfamily catalyze the transformation of guanidines by catalyzing hydrolysis to ureas (hydrolase), hydrolysis to amines (dihydrolases) or transfer of an amidino group to acceptor substrates (amidinotransferases). These diverse reactions all proceed through a similar covalent intermediate, an S-alkylpseudothiouronium adduct with the active-site Cys. The enzyme dimethylarginine dimethylaminohydrolase (DDAH) exemplifies pentein hydrolases. Human DDAH1 functions as a regulator of nitric oxide production by catabolizing asymmetric N^{ω},N^{ω}-dimethyl-L-arginine, an endogenous inhibitor of nitric oxide synthases and a biomarker for cardiovascular diseases. The physiological functions of microbial DDAH isoforms are less well defined. During a study of active-site residues, the T165L variant of *P. aeruginosa* DDAH was covalently inactivated by both endogenous and synthetic substrates with a partition ratio of approximately 80 turnovers per inactivation event. Substrate inactivation was studied using steady-state kinetics, substrate analogs, product analysis, mass spectrometry and X-ray crystallography, and proposed to result from formation of a 1 carbon-containing S-alkylthiocarbamate adduct through a mechanism that echoes aspects of the related dihydrolase and amidinotransferase mechanisms. A potential application for substrate-inactivated enzymes is discussed and represents the first death in the title. The second is more premeditated and describes the mechanism of DDAH inactivation by a 4-halopyridine discovered during a screen of fragment-sized inhibitors. DDAH1 is a demonstrated inhibitor target for septic shock and idiopathic pulmonary fibrosis. Neutral 4-halopyridines are not very reactive to biological nucleophiles, but DDAH catalyzes modification of the active-site Cys by stabilizing the more electrophilic pyridinium form of the inactivator through interaction with a neighboring Asp residue. The requirement for catalysis, but not by the normal mechanism, categorizes these compounds as quiescent affinity labels of DDAH. Small molecule models are used to rank the electrophilicity of halopyridines with electrophiles more commonly used for protein modification and the correlation of increasing pK_a with increased reactivity and decreased selectivity demonstrates tradeoffs common among covalent inhibitors. However, the requirement for catalysis, not just high effective molarity, imparts additional selectivity to halopyridine inactivators. Proteomic screens indicate that this strategy may be more widely applicable to covalent inhibition of other targets.

O12

Nexus Among Cell-Wall Turnover, Antibiotic Resistance and Virulence in *Pseudomonas aeruginosa*

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Members of *Enterobacteriaceae* and *Pseudomonas aeruginosa* have the ability to sense damage inflicted to their cell wall by b-lactam antibiotics. The process involves chemical signaling, which will be a subject of my presentation. A primary mechanism for this sensing and signalling involves the events of cell-wall recycling. The cell-wall is degraded for recycling and then the cell wall is synthesized de novo for the repair function. The recycling events get initiated by the functions of a family of 11 lytic transglycosylases, which generate the signalling factors that influence transcriptional events in the cytoplasm. The structures and mechanisms of these enzymes and those of the early cytoplasmic steps of recycling have been the subject of study in my lab, which I will disclose in my presentation.

O13

Mechanistic Insights Into PTEN Regulation

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PTEN is a PIP3 (phosphtatidyl inositol triphosphate) phosphatase and a well-established tumor suppressor gene. PTEN is itself phosphorylated on a cluster of four C-terminal Ser/Thr residues, and this appears to negatively influence PTEN's membrane binding activity in cells. We will describe our efforts involving protein semisynthesis to characterize the detailed functions of these Ser/Thr phosphorylations on PTEN's structure and function. In addition, we will discuss aspects of how PTEN is targeted for destruction by a ubiquitin E3 ligase, WWP2, and several layers of regulation of this process. Various aspects of our findings may point to new therapeutic directions to re-activate PTEN in cancer.

O14

Enzymology of Hydrogen Sulfide Signaling

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Despite the excitement about the varied physiological effects mediated by H_2S and the consequent profusion of literature on H_2S biology, there is a large gap in our understanding of how cells maintain very low steady-state levels of H_2S and amplify the signal as needed (*1*). Three enzymes in the sulfur network are important for its biogenesis. Two catalyze well-described non-H_2S producing reactions in the transsulfuration pathway and also synthesize cysteine persulfide from cystine (*2*), raising questions about how the decision between these competing reactions is made in the cell. The pathway for H_2S oxidation resides in the mitochondrion where the enzymes successively oxidize sulfide to sulfate. While sulfate is innocuous, a number of the intermediates in the sulfide oxidation pathway are reactive and their role in sulfide-based signaling remains to be assessed (*3*). We have recently discovered a noncanonical sulfide oxidation pathway (*4, 5*) and the challenging heme-dependent oxidation chemistry will be discussed.

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O15

β-Lactones from β-Hydroxy Acids: a New and Widespread Reaction Catalyzed by an ATP Dependent Ligase/Synthetase Superfamily Enzyme

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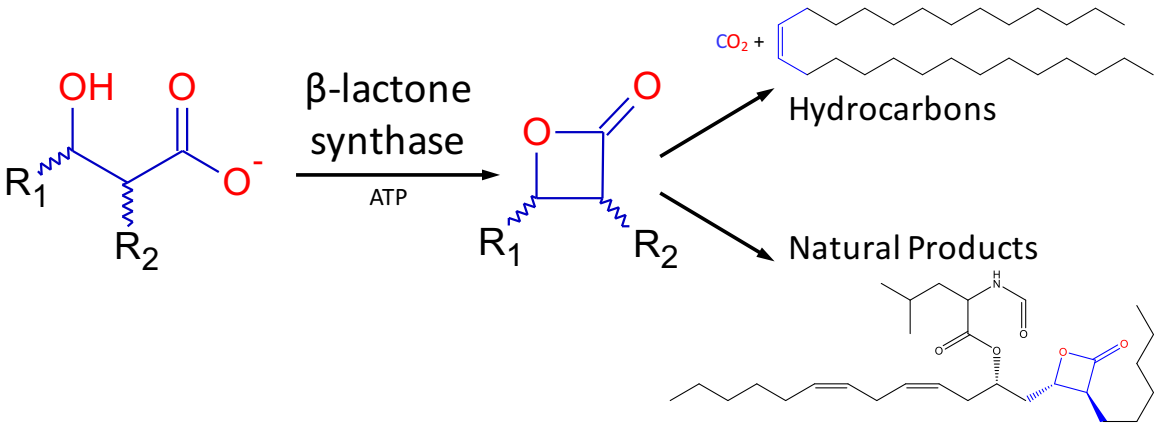
Natural enzymes within a given superfamily may catalyze distinct reactions and participate in completely different metabolic pathways within a cell. While studying the enzymatic mechanisms of hydrocarbon biosynthesis by the bacterium *Xanthomonas campestris,* we discovered a novel enzyme denoted here as β-lactone synthase. A mechanism is advanced in which the β-lactone synthase uses ATP to activate the carboxylic acid group of the substrate with AMP. The β-hydroxyl group can then attack the activated carbonyl carbon to displace AMP and form the β-lactone ring. Preliminary evidence further suggests the β-lactone is then processed by another novel enzyme, β-lactone decarboxylase, that produces carbon dioxide and a long-chain olefin that is targeted to the membrane. Bioinformatic analyses have revealed that the β-lactone synthase and other enzymes participating in hydrocarbon biosynthesis show high sequence identity to proteins encoded by known β-lactone-producing natural product gene clusters. These studies have uncovered common chemical mechanisms and evolutionary connectivity between cell membrane and cell protection chemicals within bacteria.



O16

Mechanism & Control in Radical SAM Enzymes

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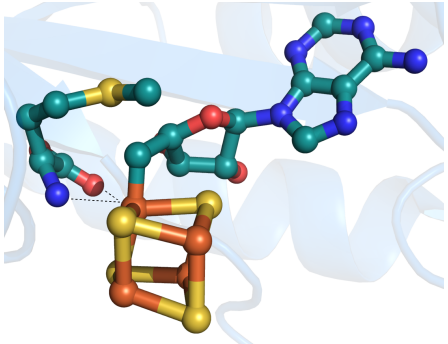
Radical *S*-adenosyl-L-methionine (SAM) enzymes catalyze diverse radical reactions in key pathways throughout all kingdoms of life. Although the ultimate reactions are disparate, they are unified by a common initiation mechanism thought to involve iron-sulfur cluster-mediated reductive cleavage of SAM to produce a 5'-deoxyadenosyl (5'-dAdo•) radical intermediate; the 5'-dAdo• intermediate abstracts a hydrogen atom from substrate to initiate subsequent chemistry. In order to probe the stepwise mechanism of this radical initiation process, we have used chemical and spectroscopic methods to trap and characterize intermediate states. Use of the SAM analog *S*-3',4'-anhydroadenosyl-L-methionine (anSAM) allows production of the allylically stabilized anhydroadenosyl radical intermediate in the active site of lysine 2,3-aminomutase (LAM).[1] We have used electron-nuclear double resonance spectroscopy of this stabilized radical complex to reveal how the active site exerts van der Waals control of the reacting radical, allowing only the most minimal movements as the anhydroadenosyl radical is guided to the substrate hydrogen. In this way, LAM tames the 5'-dAdo• radical, preventing it from carrying out harmful side reactions: this 'free radical' in lysine 2,3-aminomutase is never 'free'.[2] As a complement to using the anSAM analog, we have examined the reaction of SAM with the enzyme pyruvate formate-lyase activating enzyme (PFL-AE) and its substrate pyruvate formate-lyase (PFL) using rapid freeze-quench coupled to electron paramagnetic resonance (EPR) and electron-nuclear double resonance (ENDOR) spectroscopies. PFL-AE catalyzes the conversion of PFL to its catalytically active state containing a stable glycyl radical at G734. EPR and ENDOR analyses of samples quenched during the PFL-AE reaction reveal formation of a catalytically competent intermediate in which the 5'-C of a 5'-deoxyadenosyl moiety is directly bonded to the unique iron of the [4Fe-4S] cluster of PFL-AE.[3] The surprising discovery of this organometallic intermediate leads to fundamental questions regarding the mechanism by which the intermediate forms, the functional significance of this intermediate, and whether this intermediate is common to all radical SAM enzymes.



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O17

Shake, Rattle, & Roll: Capturing Snapshots of Ribonucleotide Reductase in Action

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Ribonucleotide reductases (RNRs) convert ribonucleotides to deoxyribonucleotides, a reaction that is essential for DNA biosynthesis and repair. These enzymes reduce all four ribonucleotide substrates, with specificity regulated by the binding of an effector to a distal allosteric site. In all characterized RNRs, the binding of effector dATP alters the active site to select for pyrimidine over purine nucleotides, whereas effectors dGTP and TTP select for substrates AD(T)P and GD(T)P, respectively. This presentation will describe our recent efforts to determine the molecular basis of specificity regulation for the prototypic class Ia *E. coli* RNR. In short, we have determined crystal structures of *E. coli* class Ia RNR with all four substrate/specificity effector-pairs bound (CDP/dATP, UDP/dATP, ADP/dGTP, GDP/TTP) that reveal the conformational rearrangements responsible for this remarkable allostery. These structures delineate how RNR 'reads' the base of each effector and communicates substrate preference to the active site by forming differential hydrogen bonds, and thereby maintaining the proper balance of deoxynucleotides in the cell.

O18

Mechanistic Studies of a Unique Radical SAM Enzyme, MqnE

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Menaquinone (Vitamin K) is an essential cofactor for humans which plays an important role in the blood clotting cascade and bone morphogenesis. Recently, a new menaquinone biosynthetic pathway was discovered that uses chemistry very different from that used in the well-characterized *E. coli* pathway.[1] We discovered a missing enzyme viz. Aminofutalosine Synthase (MqnE) in the pathway and showed its *in-vitro* reconstitution.[2] MqnE is the first radical SAM enzyme which catalyzes the addition of the 5'deoxyadenosyl radical to the substrate instead of H atom abstraction. MqnE is proposed to catalyze energetically challenging radical 1,2 O→C transposition reaction through an O-neophyl rearrangement (Figure 1). We have designed several substrate analogs to block specific downstream steps in the proposed MqnE reaction mechanism (Figure 1). Characterization of shunt products generated in the MqnE reaction with these substrate analogs have substantiated the presence of the proposed radical intermediates. Furthermore, we have used continuous-wave and pulsed EPR techniques to provide direct spectroscopic evidence for the captodative radical intermediate (3). Additionally, we have demonstrated *in-vivo* inhibition studies against *H. pylori* to explore MqnE as a potential drug target. These mechanistic studies have helped to elucidate the complex rearrangement chemistry catalyzed by the enzyme MqnE in the Vitamin K biosynthesis.

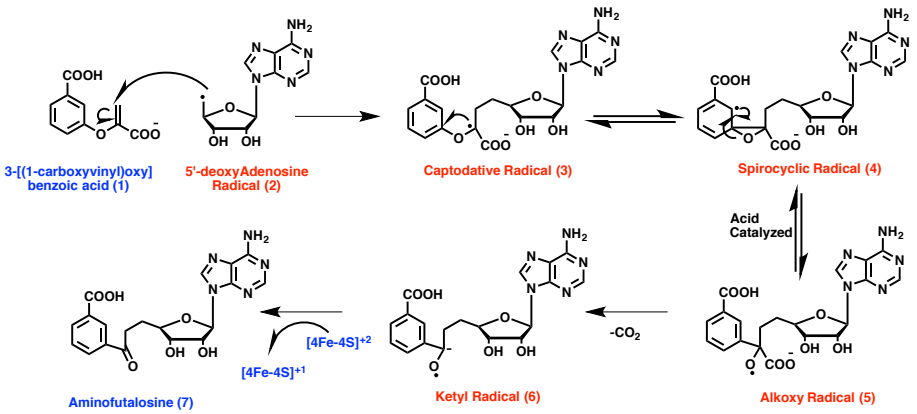

Figure 1: Mechanistic proposal for MqnE

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In Vitro Reconstitution and Substrate Specificity of the Biosynthesis of the Core Scaffold of the Thiopeptide Thiomuracin

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Thiopeptides are potent antibiotics that inhibit protein synthesis. They are made by a remarkable post-translational modification process that transforms a linear ribosomal peptide into a polycyclic structure. Herein, we report the in vitro biosynthesis of the core scaffold of the thiopeptide thiomuracin, the first of such example for this class of peptides, as well as the further determination of substrate specificity and biosynthetic timing of the biosynthetic enzymes. We show that cyclodehydration precedes dehydration, and that dehydration is catalyzed by two proteins in a $tRNA^{Glu}$-dependent manner to generate four alkenes. Then two of these alkenes undergo a formal [4+2] cycloaddition to form a tri-thiazole-substituted pyridine macrocycle. We show the order of thiazole and alkene formation, reveal the minimal structural changes necessary to render TbtA a substrate for dehydration, the parts of the TbtA peptide that are recognized by the various enzymes, and identify important residues of the enzyme TbtD for catalysis of a formal [4+2] cycloaddition process.

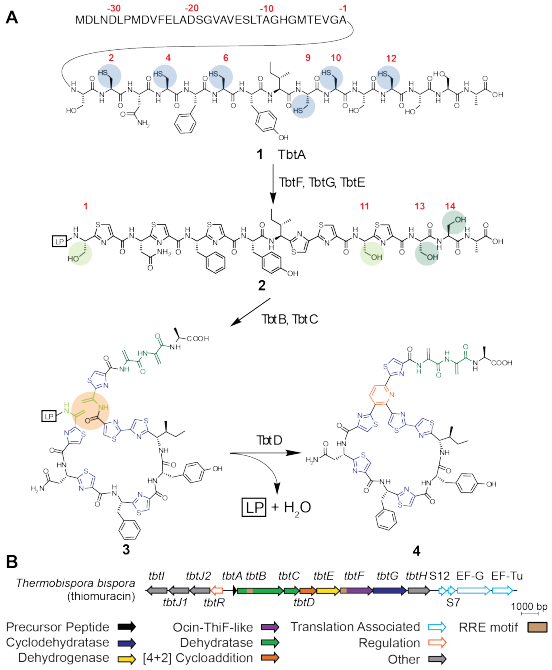

Figure 1. (**A**) Biosynthetic route to the thiomuracin core scaffold. (**B**) Gene cluster for the biosynthesis of thiomuracin.

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Decoding a New Class of Indole Alkaloid Cyclases in the Stigonematales Cyanobacteria

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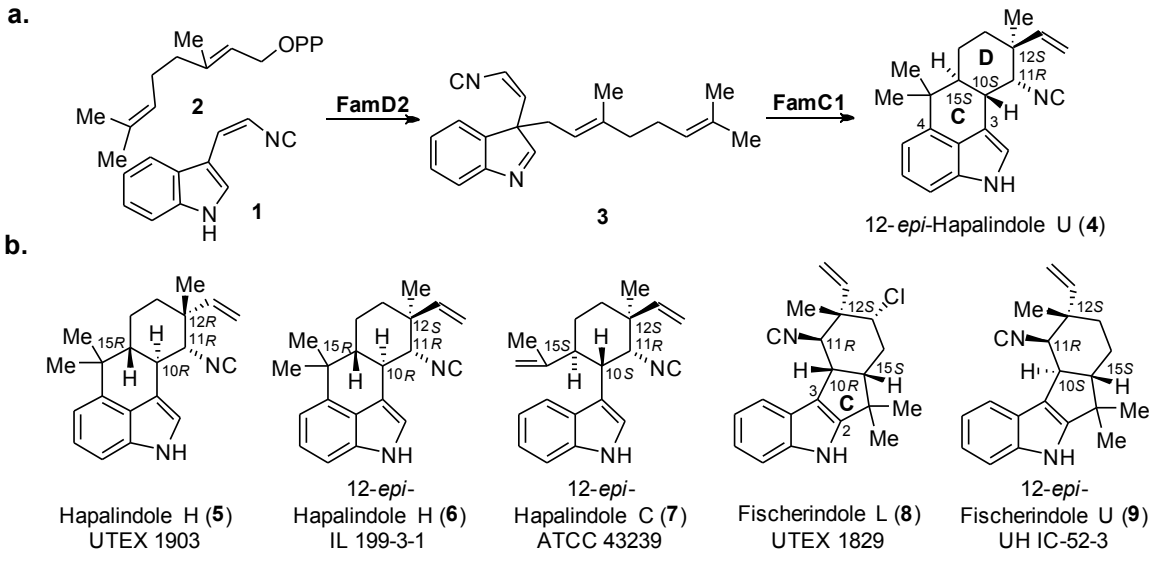
The formation of C-C bonds in an enantioselective fashion to create complex polycyclic scaffolds in the hapalindole/fischerindole type alkaloids represents a compelling and urgent challenge in microbial biosynthesis. Here we determine the biochemical basis for tri-/tetracyclic core formation in these secondary metabolites, involving a new class of cyclases that catalyze a complex cyclization cascade.



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Li, S., Lowell, A. N., Yu, F., Newmister, S. A., Williams, R. M. and Sherman, D. H. 2017. Decoding cyclase-dependent assembly of hapalindole and fischerindole alkaloids. Submitted

O21

Mechanistic Studies of the Cyclization Reactions in the Biosynthesis of Spinosyn

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Spinosyns are polyketide-derived macrolides produced by *Saccharopolyspora spinosa*. They exhibit excellent insecticidal activity with low mammalian toxicity and little environmental impact. Structurally, the spinosyns consist of a 22-member macrolactone ring fused to a perhydro-*as*-indacene core scaffold. In addition, they are glycosylated with tri-*O*-methylrhamnose and a highly deoxygenated forosamine. The aglycone portion of the spinosyns is unusual among polyketide-derived secondary metabolites due to the presence of three intramolecular carbon-carbon bonds that constitute the *as*-indacene core. The unusual nature of the spinosyn aglycone suggests an intriguing biosynthetic pathway, and recent progress in understanding the enzyme-catalyzed reactions responsible for the construction of its complex tetracyclic core will be presented.

O22

Synthesis of the Ni-Pincer Cofactor of Lactate Racemase

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Lactic acid racemization is involved in lactate metabolism and cell wall assembly of many microorganisms. Lactate racemase, LarA, of *Lactobacillus plantarum* harbors a tethered (SCS)Ni(II) pincer complex derived from niacin (1). Synthesis of the enzyme-bound cofactor requires three accessory proteins, LarB, LarC, and LarE, which are widely distributed in microorganisms (2). The structures of the accessory proteins are unknown, but their functions have been partially uncovered (3). LarB is a carboxylase/hydrolase of nicotinic acid adenine dinucleotide, forming pyridinium-3,5-dicarboxylate mononucleotide (P2CMN). LarE is a new member of the PP-loop superfamily and catalyzes sacrificial sulfur insertion into P2CMN forming pyridinium-2,5-dithiocarboxylate mononucleotide, a process requiring Mg·ATP and involving an enzyme-substrate adduct. LarC is a nickel-binding protein that donates the metal to the cofactor. Here I focus on analysis of the x-ray crystal structure of LarE apoprotein and its complexes with AMP, ATP, and CoA. In addition, heavy metal binding was observed at a 3-fold symmetry site in the quaternary structure of LarE, and structures with Mn, Fe, Ni and Zn were obtained. These studies offer insights into the intricate process for assembling the (SCS)Ni(II) pincer cofactor.

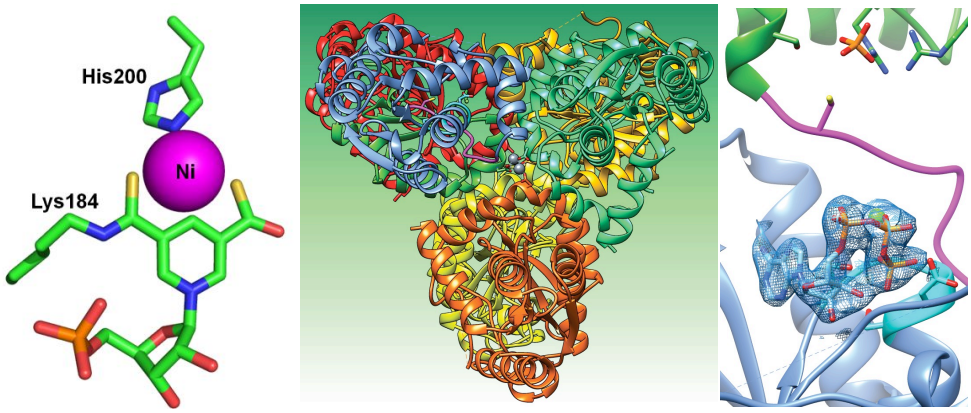

Figure 1. Structures of the (SCS)Ni(II) pincer in LarA, LarE, and a closeup of the LarE·Mg·ATP complex.

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Thermodynamic Problems and Solutions in β-Lactam Antibiotic Biosynthesis

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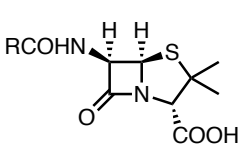
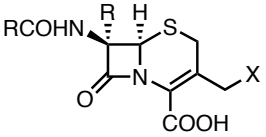
The serine hydrolases are perhaps the classic example of convergent protein evolution. All modern members of this large class of enzymes can be assigned to one of at least 13 "clans", each descended from a single ancestor. The D,D-transpeptidases (penicillin-binding proteins, PBPs) that carry out critical, late cross linking steps in bacterial cell wall biosynthesis all belong to a single clan, as do the three families of seryl β-lactamases associated with antibiotic resistance. The active site geometries and hydrolytic mechanism held in common among these enzymes is matched by naturally-occurring inhibitors/inactivators — the β-lactam antibiotics, which comprise >60% of the antimicrobials used in human medicine.

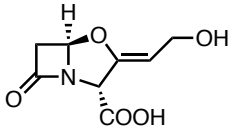
Five distinct structural classes of β-lactam antibiotics are known illustrating, as will be discussed, four evolutionarily and remarkably different strategies for their biosynthesis. Recent observations on monocyclic β-lactam formation in nocardicin A and the monobactam sulfazecin (below) have revealed unprecedented catalytic functions in non-ribosomal peptide synthetase (NRPS) enzymes that lie at the heart their biosyntheses.



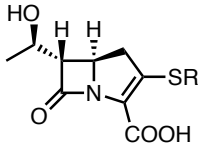
Penams



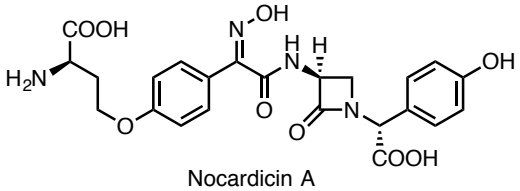
Cephems (R = H)
Cephamycins (R = OCH_3)



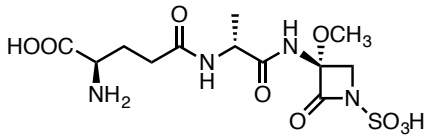
Clavulanic Acid



Carbapenems



Nocardicin A



Sulfazecin

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CETCH Me if You Can:
A Synthetic Pathway for the Fixation of Carbon Dioxide

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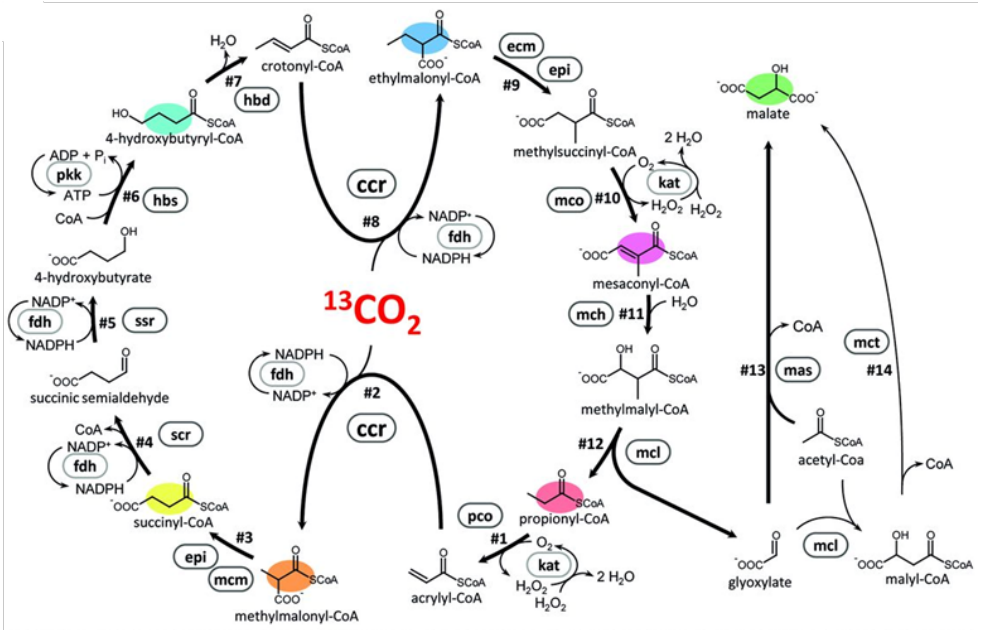
Carbon dioxide (CO_2) is a potent greenhouse gas that is a critical factor in global warming. At the same time atmospheric CO_2 is a cheap and readily carbon source. Yet, synthetic chemistry lacks suitable catalysts to functionalize the CO_2-molecule, emphasizing the need to understand and exploit the CO_2-mechanisms offered by Nature.

In our talk we will (1) discuss the evolution and limitation of naturally existing CO_2-fixing enzymes and pathways. We will (2) present strategies for the engineering and design of artificial CO_2-fixation reactions and pathways (*Peter et al. 2015*), and (3) outline how these artificial pathways can be realized and further optimized to create metabolic CO_2-fixation modules (*Schwander et al. 2016*).

An example for such synthetic CO_2-fixation modules is the CETCH cycle. The CETCH cycle is an *in vitro*-reaction network of 17 enzymes that was established with enzymes originating from nine different organisms of all three domains of life and optimized in several rounds by enzyme engineering and metabolic proofreading. In its version 5.4, the CETCH cycle converts CO_2 into organic molecules at a rate of 5 nanomoles of CO_2 per minute per milligram of protein. This is slightly faster than the Calvin cycle under comparable conditions and notably at 20% less energy per CO_2 fixed.



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O25

Structural Basis for Regulation in the Aromatic Amino Acid Hydroxylases

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The non-heme iron-containing enzymes tyrosine hydroxylase and phenylalanine hydroxylase catalyze the hydroxylation of the aromatic side chains of their respective substrates. Phenylalanine hydroxylase initiates the catabolism of excess phenylalanine in the diet; a deficiency in the enzyme results in the disease phenylketonuria. Tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of the catecholamine neurotransmitters dopamine, norepinephrine, and epinephrine. The sequences of their N-terminal regulatory domains differ significantly, consistent with divergent regulatory mechanisms. Tyrosine hydroxylase is regulated by a balance between feedback inhibition and phosphorylation, while phenylalanine hydroxylase is allosterically regulated by phenylalanine. Using a combination of structural approaches we have determined the structural basis for the regulation of the two enzymes, showing that a common structural fold underlies discrete regulatory mechanisms.

O26

Reductive Dehalogenation Mediated by a Flavoprotein

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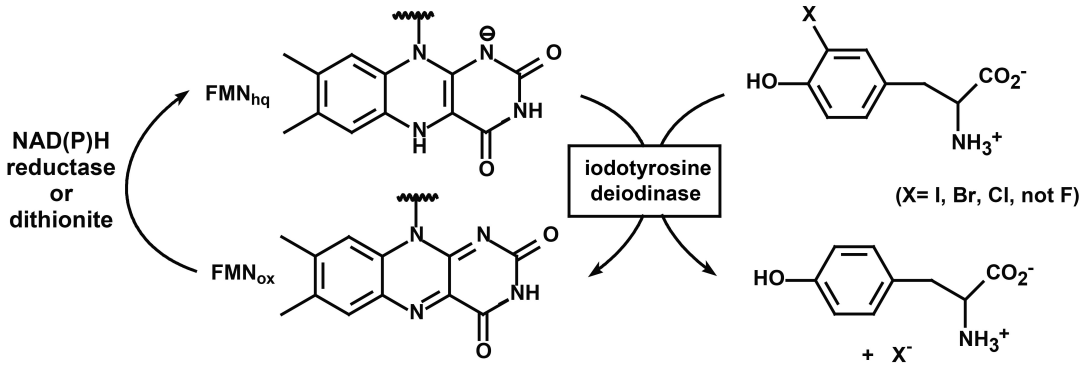
The flavoprotein iodotyrosine deiodinase (IYD) was first identified by its ability to salvage iodide from iodotyrosine, a byproduct of thyroid hormone biosynthesis in mammals.[1] However, its function in vivo is likely broader than its name implies since IYD also dehalogenates bromo- and chlorotyrosines [2] that are generated from leukocyte activation. This enzyme is unusual for both its cofactor dependence and strategy for dehalogenation. Typically, haloaromatics are subject to oxidative transformation by heme-dependent enzymes in mammals but IYD instead promotes a reductive process using flavin mononucleotide (FMN). Mechanistic and structural studies on IYD now focus on its distribution, catalysis and origin in nature. Crystallographic data revealed signature residues that have been used to identify IYD in the genomes of all metazoa and some bacteria.[3] Although the physiological relevance of halotyrosines has not yet been established in *Drosophila*, its IYD homolog exhibits a substrate specificity equivalent to that of the mammalian enzyme.[4] IYD is a member of the nitro-FMN reductase structural superfamily, but unlike most enzymes in this group, IYD acts by promoting single electron transfer steps.[5] Coordination between the zwitterion of halotyrosine, the pyrimidine ring of FMN and the protein's active site lid is necessary to stabilize the one electron reduced FMN semiquinone. Loss of any one of these features is sufficient to suppress dehalogenation. In contrast, the vestigial ability of IYD to act as a nitroreductase is promoted by destabilization of the FMN semiquinone.[6]



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O27

Prenyl-Flavin: a New Cofactor for Catalyzing Aromatic Decarboxylation Reactions

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Decarboxylation reactions are typically associated with very high activation energies and thus Nature has evolved enzymes that use a wide variety of mechanistic strategies to catalyze decarboxylations. The latest addition to this diverse family of enzymes is ferulic acid decarboxylase, which was recently shown to use a novel flavin mononucleotide (FMN)-derived cofactor to catalyze the decarboxylation of a range of substituted phenylacrylic acids. Homologous enzymes also appear to be involved in bacterial ubiqinone biosynthesis. FMN is converted from a redox cofactor to an electron sink capable of facilitating decarboxylation reactions by addition of a six-membered ring, derived from isoprene, that bridges N5 and C6 of the tricyclic isoalloxazine system. This talk will present recent experiments aimed at elucidating the mechanism of the decarboxylation reactions catalyzed by this novel cofactor.

O28

An Unprecedented Mechanism of Nucleotide Methylation by Flavin Dependent Thymidylate Synthase

Mishanina, T.V., Yu, L., Karunaratne, K., Mondal, D., Corcoran, J.M., Choi, M.A., Kohen, A.

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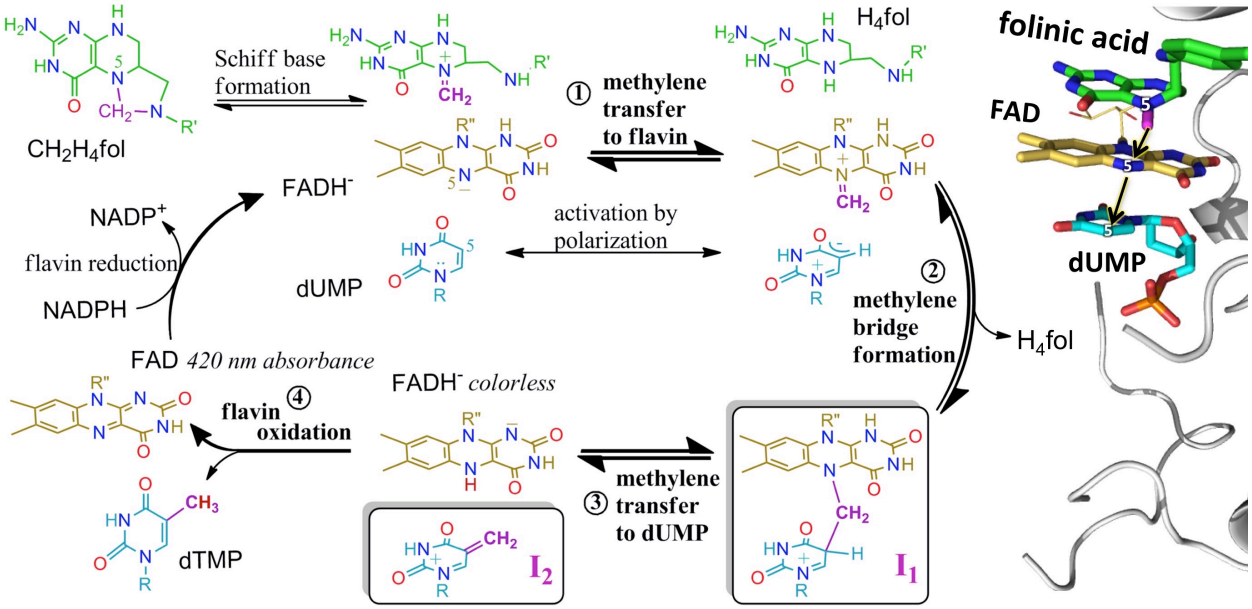
In several human pathogens, *thyX*-encoded flavin-dependent thymidylate synthase (FDTS) catalyzes the last step in the biosynthesis of thymidylate, one of the four DNA nucleotides. *ThyX* is absent in humans, rendering FDTS an attractive antibiotic target; however, the lack of mechanistic understanding prohibits mechanism-based drug design. I will present the trapping and characterization of two consecutive intermediates, which together with previous crystal structures[1] indicate that the enzyme's reduced flavin relays a methylene from the folate carrier to the nucleotide acceptor.[2] Furthermore, these results corroborate an unprecedented activation of the nucleotide that involves no covalent modification but only electrostatic polarization by the enzyme's active site.[3] These findings indicate a mechanism that is very different from thymidylate biosynthesis in humans, underscoring the promise of FDTS as an antibiotic target.



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O29

Reaction Specificity in Pyridoxal Phosphate and Chorismate Enzymes

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Pyridoxal phosphate (PLP) dependent enzymes catalyze a large number of different types of reactions, generally by stabilizing carbanionic intermediates. The variety of reactions catalyzed and the mechanistic commonalities raise the question of how reaction specificity is controlled in this large family of enzymes. A variety of evidence from different laboratories will be discussed to present our current state of understanding. Stereoelectronic effects play a major role in determining which bond to Ca of the external aldimine intermediate, common to all PLP enzymes, is initially broken to form the carbanionic quinonoid intermediate. The fate of the quinonoid intermediate, the second major branchpoint for reaction specificity, is strongly influenced by the protonation state of the cofactor. For a given reaction type, active site interactions with PLP are conserved. These reaction-specific interactions control cofactor protonation, and thereby the stability of the quinonoid intermediate and its distribution of electron density among the reactive carbons.

Structure-Based Drug Design (SBDD), Synthesis and Evaluation of Peptides and Non-β-Lactam Inhibitors of Y-49 β-Lactamase from *Mycobacterium tuberculosis*

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Pathogen resistance to β-lactam antibiotics is spreading. One of the most effective resistance mechanisms involves the production of β-lactamases that hydrolyze β-lactam antibiotics. More than 840 β-lactamases are known to date. One of the most effective approaches to overcome the resistance to β-lactam antibiotics involves the discovery of new *non β-lactam* scaffolds, inhibitors of β-lactamases. In the search for new β-lactamase inhibitors, we employed ZINCPharmer to develop 3-D pharmacophores that can be further used to search the ZINC database of commercial compounds. In addition, we report a structure-based design approach for the discovery of potential tetrapeptides inhibitors of Y-49 enzyme, a class A beta-lactamase, from *Mycobacterium tuberculosis*. The tetrapeptide scaffold was derived from the original sequence RRGHYY which was found to inhibit class A *Bacillus anthracis* Bla1, (K_i = 42 μM) and class A TEM-1 β-lactamase, (K_i = 136 μM) (Huang W et al., *Protein Eng Des Sel* 16:853-860). *In silico* docking experiments using *Autodock Vina* and the protein target 3M6B.pdb lead to the discovery of tetrapeptides 2HN-R-X-H-Y-CONH2, where X was varied with all 20 natural L- and -D-amino acids. Our initial structure-activity relationship (SAR) studies established that acidic and basic amino acids (such as Asp, Lys and Arg) and small neutral like Gly occupying the X-position (P2) would favor a lower uM inhibitory constant (Ki). As such, the tetrapeptide RRHY had Ki of 5.1 μM while the tetrapeptides RDHY and RGHY had Ki of 6.3 μM and 5.5 μM respectively. We propose a new tetrapeptide derived pharmacophore which could be used for further designing of linear and cyclic peptides with D- and unnatural amino acids with improved anti-β lactamase activity.

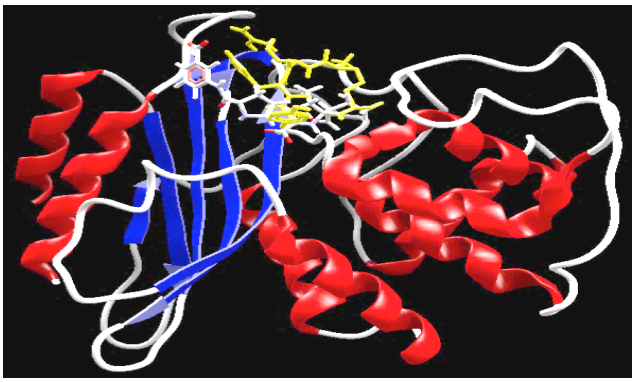

Figure 1: *Poseview* of RRHY tetrapeptide (yellow) in the active site of the3M6B; overlaid over 1RG ligand (CPK color) (docking: Autodock/Vina).

P2

The Bacillithiol Biosynthesis Enzymes: A Structural and Functional Analysis

Kelsey R. Winchell, Andrew J. VanDuinen, Aaron M. Rosenberg, Emily A. David, Christopher J. Royer, and Dr. Paul D. Cook

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Bacillithiol is a low molecular weight thiol produced by many pathogenic gram-positive bacteria such as *Bacillus anthracis* and *Staphylococcus aureus*. The compound is involved in the maintenance of redox homeostasis, detoxification of xenobiotic agents, and resistance to the FDA-approved antibiotic fosfomycin. It is produced via a pathway utilizing the enzymes BshA (a glycosyltransferase), BshB (a zinc-dependent deacetylase), and BshC (a cysteine ligase). Here we will discuss the ligand-bound X-ray crystallographic structures of BshA and BshC that our lab has recently determined. Our product-bound BshA structures corroborate the S_Ni-like mechanism that it and other GT-B retaining glycosyltransferases are hypothesized to utilize. Our BshC structure reveals that it is a unique enzyme that is distinct in several ways from any other cysteine ligase characterized to date. The overall structures and bound ligands give insight into the function of these enzymes and may provide routes for potential therapies to circumvent fosfomycin resistance mechanisms.

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The Transition State of D-Alanine: D-Alanine Ligase as a Potential Drug Target for Treatment of Tuberculosis

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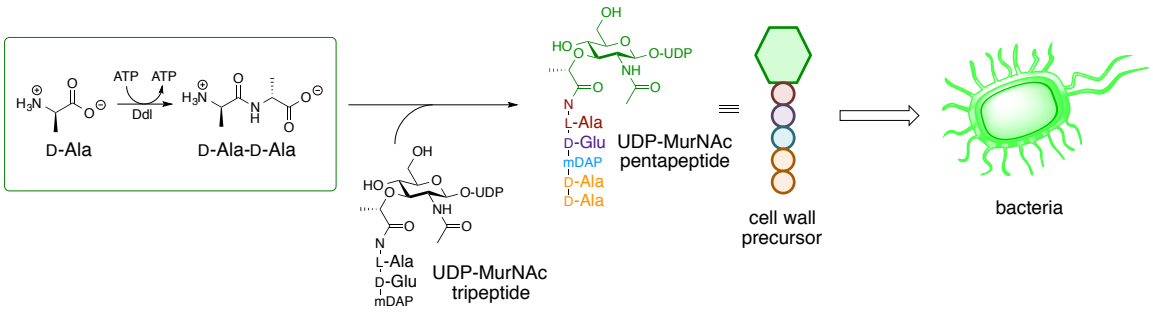
Multidrug-resistant infections present an escalating problem that demands exploration of novel targets. Inhibiting an enzyme unique to the pathogen is one strategy to thwart its proliferation in its host. One such target in the case of tuberculosis, caused by *Mycobacterium tuberculosis*, is the enzyme D-alanine:D-alanine ligase (Ddl), which is involved in constructing the peptidoglycan layer that is an essential component of the organism's cell wall. We aim to inhibit this enzyme by developing transition state analogs, a powerful class of inhibitors that mimic the transition state, which is held tightly by the enzyme. To aid in analog design, a model of the transition state can be obtained through kinetic isotope effect (KIE) measurements. Here, we present the synthesis of ^{13}C-labeled D-Ala substrates and their use in measuring ^{13}C KIEs for Ddl by NMR spectroscopy. These results will be used in concert with ^{2}H, ^{15}N, and ^{18}O KIEs to be measured in future studies to establish the enzymatic transition state.



P4

Crystallographic Studies of Human Acetylcholinesterase Reactivation by Oximes

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Human acetylcholinesterase (hAChE; EC 3.1.1.7) is responsible for degrading neurotransmitter acetylcholine at synapses of the nervous system. Organophosphate (OP) nerve agents and pesticides inactivate hAChE through chemical modifications of the enzyme's active center leading to a life-threatening increase of acetylcholine (ACh) concentration. Exposure to OPs may be fatal if not treated. The current generation of oxime antidotes is not highly efficient; the rates of nucleophilic reactivation are far slower than the catalytic rate of ACh hydrolysis. Insights into the molecular structures of *Torpedo* and mammalian AChEs reveal the underlying limitations in enhancing reactivation rates, but limited in their ability to resolve positions of hydrogens important for understanding proton transfer in reactivation of OP-inhibited AChEs. The only experimental method that can provide this information is macromolecular neutron crystallography.

In preparation for the neutron diffraction studies we have obtained several new X-ray structures of native and VX-phosphonylated hAChE in complex with oxime reactivators, RS2-170B and RS-194B, at 2.15-2.45 Å resolution range. In these complexes the enzyme forms crystals in previously unseen unit cell (a=b=124.3, c=129.1 Å; space group $P3_1$) amenable to neutron crystallography. Ternary structures with RS2-170B reveal that the oxime binds in two conformations to non-modified and OP-conjugated active center suggesting that conformational changes can happen inside the active center cavity for this reactivator. In one conformation the oxime moiety extends towards the catalytic Ser203 and could represent reactive orientation of the oxime. Conversely, in our structures RS-194B binds with the oximate pointing away from Ser203 and towards the entrance to the gorge in the non-modified active center and the entire molecule is pushed out of the active center to bind in the peripheral site in the VX-modified structure.

In addition, we have determined the first room temperature (RT) structures of hAChE:BW284c51 complex at 3.2 Å and hAChE:RS2-170B at 2.8 Å. Detailed analysis of interactions between the reactivator and the enzyme in both 100K and room-temperature structures will be presented. (Supported by Grant 1U01NS083451 by the COUNTERACT program from NINDS).

P5

Trehalose-6-Phosphate Phosphatase Structure and Inhibitor Design

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Trehalose is a sugar synthesized by many bacteria, fungi, plants and invertebrates. Trehalose is synthesized in a two-step process involving trehalose-6-phosphate (T6P) synthase, which catalyzes the conversion of glucose-6-phosphate and uridine-diphosphate-glucose into T6P followed by T6P phosphatase (T6PP), which catalyzes the dephosphorylation of the phosphosugar to form trehalose. Without T6PP, the build-up of T6P and absence of trehalose decreases the viability and virulence of fungi, bacteria and nematodes[1,2,3]. Therefore, T6PP has become a popular target for therapeutic intervention in diseases such as tuberculosis and lymphatic filariasis caused by infectious agents. Here, we present the 1.89 Å structure of *Salmonella typhimurium* (St) T6PP. The x-ray crystallographic structure of *apo* St T6PP structure shows features seen in the haloalkanoic acid dehalogenase superfamily (HADSF) type II members[4], with a core Rossmann-like fold (residues 1 - 95, 175 - 246) and an α/β fold cap domain (residues 96 - 174). Overlaying a recently published structure of T6P-bound fungal T6PP[5] with St T6PP shows in addition to the four core motifs that bind the phosphoryl group in HADSF members, the cap residues Glu123, His132, and Glu167 are involved in binding the substrate. Unlike most unliganded HADSF structures, the St T6PP structure without cofactor or bound substrate has a closed conformation of the cap domain with respect to the core domain. Therefore, this crystal form may be amenable to soaking inhibitors into the active site. Inhibitor-bound structures will help rationally design inhibitors to increase specificity and potency. In our laboratories, inhibitors of T6PP from several organisms have been uncovered by screening of an FDA approved drug library. These include Closantel and Cephalosporin C. Future efforts will explore incorporation of additional binding interactions from the Closantel and Cephalosporin C scaffolds to create a highly specific inhibitor for T6PP. This work was funded by R21-AI103484 to KNA and DD-M.

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P6

Antibacterial Drug Discovery: Rationally Designed Inhibitors of MEP Synthase

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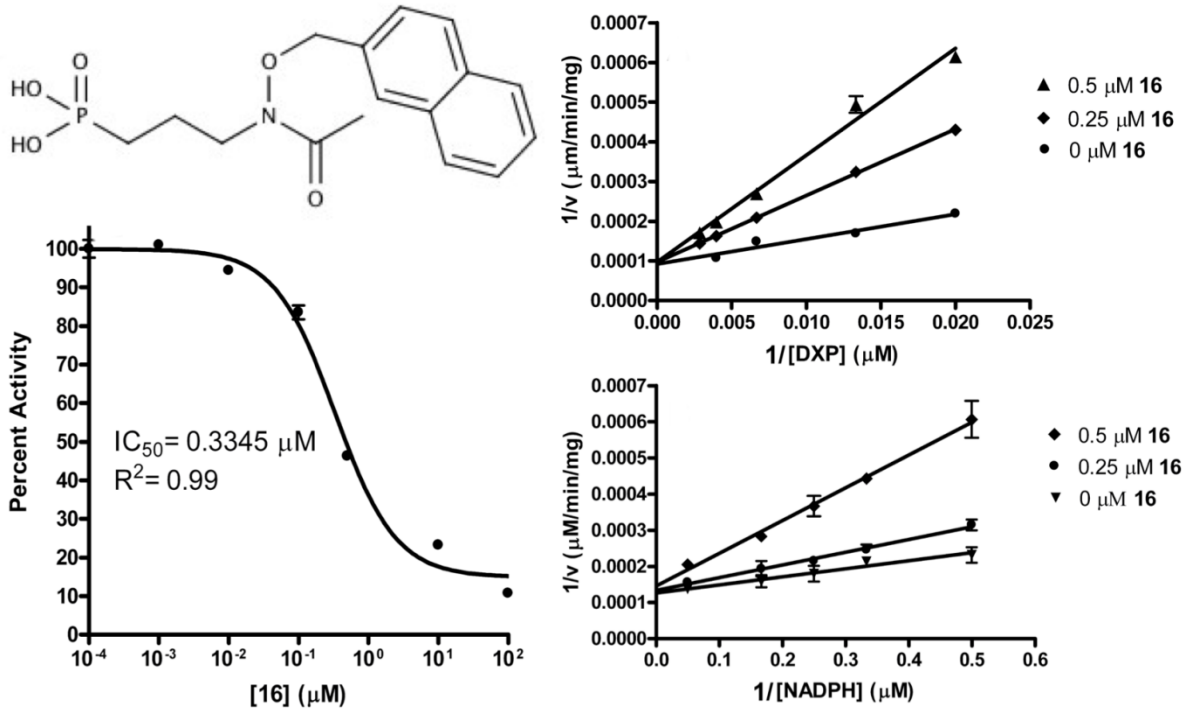
Antibiotic resistance is a rapidly growing problem in healthcare with many frontline antibiotics no longer effective against several pathogens. Additionally, the ease by which antibiotic resistance can be deliberately engineered into biothreat agents further underscores the need for novel antibiotics. To address this need, we are utilizing a target-based approach to drug discovery, screening purified *Yersinia pestis* MEP synthase against a rationally designed library of small molecules. Our best hit to date has a nanomolar IC_{50} and is effective against bacterial cultures. Mechanism of action plots suggest that it binds via a "flip and lock" mechanism, where one moiety of the small molecule binds in the cofactor site, causing a conformational change in the enzyme. This conformational change allows the enzyme to "lock" on the second moiety of the inhibitor which binds in the substrate active site.



A Tethered-Rheostat Model for the Intramolecular Activation of the Anti-Cancer Target USP7/HAUSP by Its HAUSP Ubiquitin-Like Domains 4 and 5

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Ubiquitin Specific Protease 7 (USP7/HAUSP) is a well-studied deubiqutinating enzyme that regulates many critical signaling pathways in the cell, including regulation of the tumor suppressor p53/MDM2 pathway. The isolated catalytic domain of USP7 has 30-fold lower activity than the full-length enzyme. Previous structural and kinetic studies of USP7 have attributed this lack of activity to the catalytic triad being misaligned. Additional structures have shown that to achieve full activity, the USP7/HAUSP Ubiquitin-like (HUBL) domains 4 and 5 must be present. Previous reports have also suggested that HUBL domains 1-3 are simply scaffolds for protein-protein interactions with USP7 binding partners, such as the allosteric activator guanosine 5'-monophosphate synthase. Here, the kinetic differences between the activation of the isolated catalytic domain of USP7 ($USP7_{CD}$) *in trans* by HUBL domains 1-5 and activation by HUBL domains 4-5 were evaluated and kinetic and biochemical characterization of these observed differences revealed the importance of HUBL domains 1-3 in the intramolecular activation of USP7. A new model for USP7 regulation is proposed where the HUBL domains 1-3 act as a rheostat, regulating the level of activation USP7 experiences both internally as well as by allosteric activator guanosine 5'-monophosphate synthase.

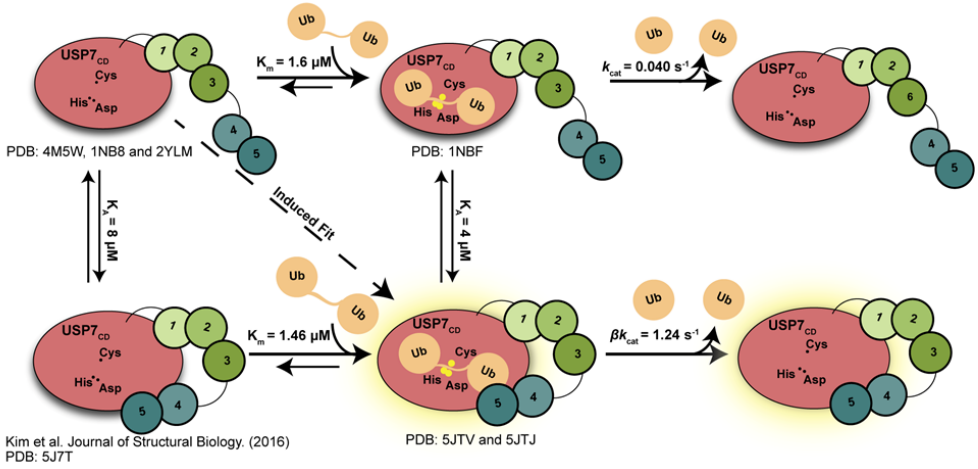

Figure 1. Developed mechanism model of USP7, a compilation of the kinetic data presented within this work and the solved X-ray crystal structures of USP7.

Utilizing a Minimally-Perturbing Fluorophore to Gain Mechanistic Insight into a Pro-Mutagenic Reaction in Bacteria

Zachary M. Hostetler[1,3], Itthipol Sungweinwong[2], Matthew J. Culyba[3], E. James Petersson[2], Rahul M. Kohli[1,3]

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The SOS stress response promotes bacterial survival and adaptation to many genotoxic insults, including antibiotics.[1] The crucial regulatory step for activation of the SOS response is LexA repressor auto-proteolysis, a reaction that abolishes LexA-mediated SOS gene repression. Whereas LexA contains all the necessary residues for intramolecular peptide bond hydrolysis, interaction of LexA with a sensor of DNA damage, RecA*, stimulates this reaction. Although RecA* can turnover multiple LexA molecules, its role in promoting auto-proteolysis is poorly understood. We aim to elucidate the mechanism of RecA*-mediated LexA auto-proteolysis by identifying the rate-determining step in the auto-proteolysis reaction pathway and determining how RecA* affects the kinetics of this step. Towards this end, we have developed a strategy for fluorescently-labeling LexA with acridone (Fig. 1), a minimally-perturbing fluorescent unnatural amino acid.[2] Here, we report on progress in generating fluorescent, acridone-labeled LexA proteins that can report on the equilibrium constants and rates of individual steps in the auto-proteolysis reaction. Ultimately, we expect to fit kinetic data for each individual step to a global reaction model to establish a mechanistic role of RecA* in LexA auto-proteolysis. Our insight into this reaction will inform efforts to inhibit LexA auto-proteolysis, potentially offering a therapeutic tool for preventing SOS-dependent acquired antibiotic resistance in bacteria.

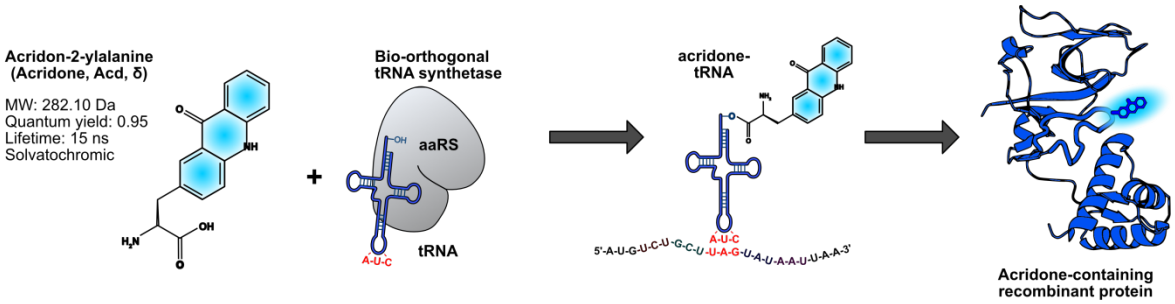

Figure 1: Scheme for site-specific, *in vivo* incorporation of acridone into recombinant protein, using an approach based on amber stop codon suppression.[3]

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P9

Unavailing the Molecular Foundations Behind the Resistance Profile of Cancerous Thymidylate Synthase

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The production of a de novo DNA base, thymidylate, is catalyzed by the enzyme thymidylate synthase (TSase), making it an important protein in DNA metabolism. The activity of this enzyme is crucial to maintain the thymidylate pool required for proper DNA replication, and disruption of the activity of TSase causes cells' thymine-less death. As a result, TSase is one of prime targets for chemotherapeutic drugs for many types of cancers, including colorectal and ovarian cancers. Substrate analog 5-Fluoro-2'-deoxyuridine-5'-monophospate (5F-dUMP) – derived from the prodrug 5-Fluorouracil (5-FU) – is a FDA-approved drug in the treatment of cancers that targets TSase.

Development of resistance to drugs is a major problem in fighting diseases. Many cancerous cell lines such as mouse neuroblastoma,[1] gastrointestinal tumor,[2] hepatoma,[3] human leukemic,[4] and human colorectal tumor (HCT)[5] cells were reported to be resistant to the cancer drug 5-FU. Some of those cell lines, when treated with 5-FU, were found to have an elevated TSase level sufficient to compensate for the amount of enzyme-inhibitor complex, appearing to show resistance to the inhibitor. However, a few cancer cells such as human leukemic[4] and HCT[5] cells did not have an increased TSase level, but still exhibited resistance to 5-FU. Analysis of mRNA and cDNA of one of those cell lines (HCT) revealed a variation of the TSase gene, which corresponds to a change of Y33 to H in the TSase protein sequence.[6] The mutant Y33H was the first reported instance of naturally occurring variation in TSase in a cancerous cell line. Here we aim to obtain molecular insights into the resistance profile of the mutant Y33H. We expressed and purified both WT and the mutant Y33H in *E. coli* as a host system. Our inhibition studies with both WT and Y33H indicate that the mutant is less sensitive than the WT to the inhibitor 5F-dUMP, establishing the recombinant mutant enzyme is also resistant to the nucleotide analogue. We thoroughly investigated both WT and the mutant protein. Our kinetic analyses revealed that, despite having no physical contact between the residue at the mutation site and substrates, the mutation has influences in the chemical steps occurring in the active site. Structurally very similar to WT, the mutant protein seems to have altered protein dynamics that could be attributed to its distinguishing properties. Obtaining the molecular mechanisms behind the drug-resistant profile of some cancerous cells caused by a variation in an enzyme would have an impact in the development of drugs aimed at exploiting the distinct properties of the mutant.

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P10

Rational Design of Selective Small-Molecule Inhibitors for the β-Catenin/T-Cell Factor Protein–Protein Interaction

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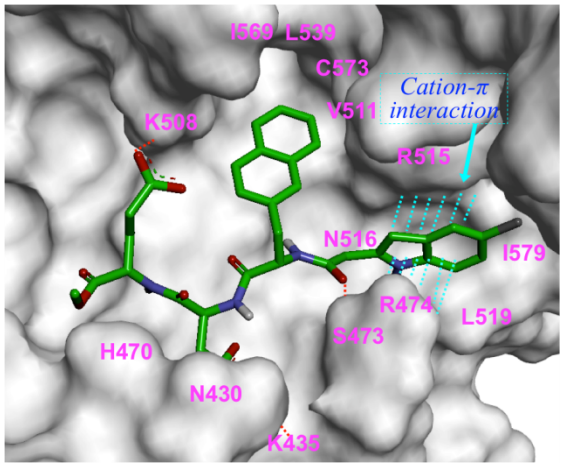
The aberrant formation of the β-catenin/T-cell factor (Tcf) complex in the canonical Wnt signaling pathway has been recognized as the major driving force for many cancers. Crystallographic and biochemical analyses reveal that the binding mode of Tcf, cadherin and adenomatous polyposis coli (APC) with β-catenin is identical. The design of small-molecule inhibitors that selectively disrupt the β-catenin/Tcf complex, while leaving the β-catenin/cadherin and β-catenin/APC interactions unaffected, is critical but challenging. Using a combination of biochemical characterization, structure-based inhibitor design, and chemical synthesis, we successfully discovered potent and selective small-molecule inhibitors for the β-catenin/Tcf over β-catenin/cadherin and β-catenin/APC interactions. The binding mode of new inhibitors was characterized by site-directed mutagenesis and structure-activity relationship studies. The cell-based studies demonstrated that new inhibitors exhibited cell-based selectivity for β-catenin/Tcf over β-catenin/cadherin and β-catenin/APC interactions, significantly attenuated canonical Wnt signaling in cancer cells, and suppressed the growth of Wnt/β-catenin-dependent cancer cells.



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P11

Locking Chemoselectivity in the Cytochrome P450 OleT

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Intense interest has focused on the development of enzymes as next-generation catalysts for the production of molecules with industrial interest. OleT, a member of the cytochrome P450 CYP152 family is an excellent candidate for the generation of advanced biofuels.

OleT catalyzes the hydrogen peroxide-dependent decarboxylation of C_n fatty acids to produce C_{n-1} terminal alkenes. Despite the ability of OleT to produce long chain alkenes with nearly exclusive chemoselectivity, its reactivity towards shorter chain length fatty acids results in undesired levels of hydroxylated side-products, which limits its catalytic potential. Our recent study of an OleT homolog has suggested that the control of substrate coordination modes may have a key role in promoting the alkene-forming pathway[1]. To further understand the importance of substrate positioning in OleT, we have utilized fluorescent and EPR spin-labeled fatty acid substrates as mechanistic probes. Product profiles, UV/Vis and EPR spectroscopy, and transient kinetics show that substrate constriction is necessary for efficient alkene production. However, this same feature compromises the rate of product release. The importance of amino acids distal from the active-site in regulating regioselective C-H abstraction and product release is shown in mutagenesis studies.

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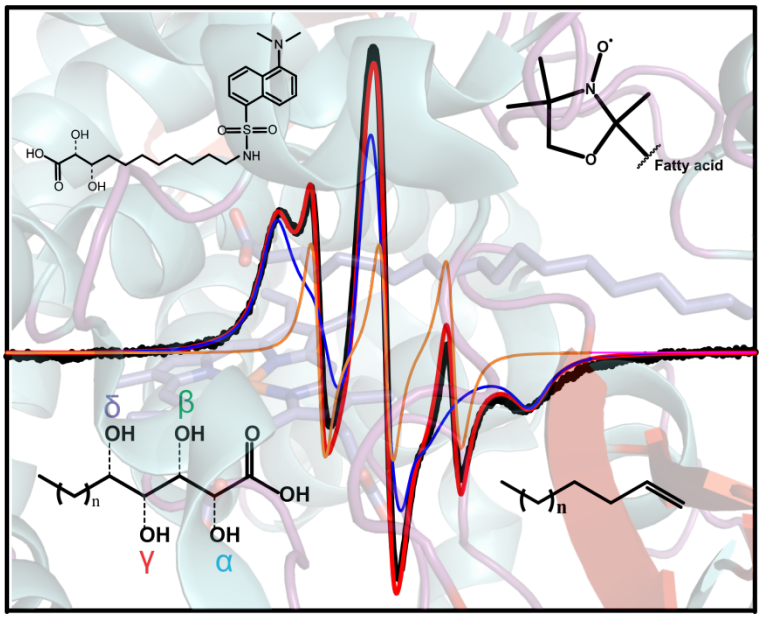

Mechanistic Studies of a Bifunctional α-Ketoglutarate Dependent non-Heme Iron Enzyme, AsqJ

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α-ketoglutarate (α-KG) dependent non-heme mononuclear iron (NHM-Fe) enzymes catalyze a broad array of chemical transformations, ranging from classical hydroxylation and dealkylation, to desaturation, epoxidation, halogenation, epimerization, endoperoxidation, as well as ring expansion/contraction reactions. In recent years, detailed mechanistic studies on this family of enzymes have revealed an emerging paradigm where diverse reactivity is likely shaped by three major factors: (1) the electronic structures of the reactive iron intermediates; (2) the presence of polar or redox-active residues in the secondary coordination sphere of the iron; and/or (3) the electronic properties of the enzyme substrates. To futher explore the interplay among these three factors in directing the reaction outcomes of α-KG NHM-Fe enzymes, we have carried out mechanistic studies on a bifunctional α-KG NHM-Fe enzyme, AsqJ, using a combined methods including transient kinetics, Mössbauer spectroscopy and liquid chromatography mass spectrometry. AsqJ is a newly discovered enzyme that is involved in the formation of 4'-methoxy-viridicatin, the core molecular scaffold for pharmaceutically attractive quinolone alkaloids. AsqJ catalyzes fascinating stepwise oxidations starting from desaturating 4'-methoxy-cyclopeptin to 4'-methoxy-dehydrocyclopeptin, which is then epoxidized to 4'-methoxy-cyclopenin at newly formed double bond moiety. Finally, the formation of 4'-methoxy-viridicatin is achieved via rearrangement/elimination. Here, we will present our recent results on discovering the reactive intermediates in both desaturation and epoxidation reactions catalyzed by AsqJ, on identifying the rate determinant steps of these two reactions under single turnover condition, and on revealing the effect of mechanistic probes utilized in directing the reaction outcomes. Finally, the mechanistic implications of these results to the understanding of multifunctionality of AsqJ will be discussed.

4'-Methoxycyclopeptin 4'-Methoxydehydrocyclopenin 4'-Methoxycyclopenin 4'-Methoxyviridicatin

Mechanistic Insights Into Bacterial Dye-Decolorizing Peroxidases

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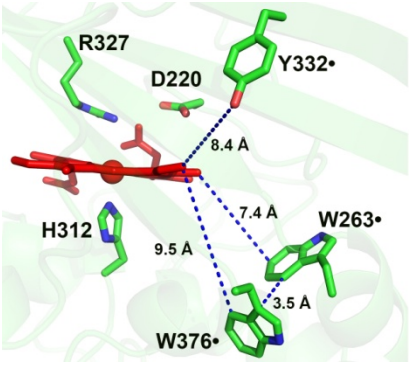
Dye-decolorizing peroxidases (DyPs) comprise a new family of heme peroxidases, which have received much attention due to their potential applications in lignin degradation and biofuel production from biomass. A new DyP from *Thermomonospora curvata* (*Tc*DyP) was identified and characterized, which is among the most active DyPs reported so far. While distal residues D220 and R327 are essential for compound I formation, proximal H312 is found important for compound II reduction. Crystal structure of *Tc*DyP at 1.75 Å suggests that small (hydroquinone, HQ) and bulky (Reactive Blue 19, RB19) substrates are oxidized in heme center and on protein surface, respectively. Transient kinetics of HQ oxidation showed that reduction of compound II to resting state is a rate-limiting step, which resolves the controversial about the aspartate mutants of A-type DyPs. RB19 is oxidized on protein surface *via* protein radicals, which was demonstrated by sigmoidal steady-state kinetics and formation of compound II-like product observed with stopped-flow spectroscopy and EPR. Among 7 tryptophan and 3 tyrosines present in matured *Tc*DyP consisting of 376 residues, W263, W376, and Y332 form surface-exposed radicals, in which only W263 also serves as a surface-exposed oxidation site for bulky substrates. SDS-PAGE and SEC demonstrated that W376 represents an off-pathway destination for electron transfer, resulting in protein crosslinks. Mutation of W376 improves compound I stability and overall catalytic efficiency toward RB19. While Y332 is highly conserved across all four DyP subclasses, its catalytic importance is minimal due to extremely small solvent-accessible areas. Understanding molecular mechanism of bacterial DyPs will allow us to engineer them for improved ligninolytic activities and other biotechnological applications.



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P14

Structural and Mechanistic Insight into the Phosphorylation-Dependent Autoinhibition of PTEN

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Phosphatase and tensin homolog (PTEN) is a tumor suppressor that negatively regulates the PI3K/AKT signaling pathway by catalyzing the hydrolysis of the 3'-phosphate of phosphatidyl inositol 3,4,5-triphosphate (PIP3). Unabated accumulation of PIP3 at the plasma membrane by loss of PTEN function leads to aberrant cellular changes in proliferation, growth, survival, and protein synthesis: a common observation in many tumors. A major checkpoint to this process depends on PTEN's lipid phosphatase function, which is regulated by a cluster of phosphorylation sites located on the C-terminal tail at positions 380, 382, 383, and 385. These modifications serve to inhibit its enzymatic function and reduce its plasma membrane binding by driving a conformational change from an open to closed state (1). The structural basis and mechanism for the phosphorylation dependent autoinhibition is not well defined; therefore, for the purposes of providing a molecular understanding of PTEN autoinhibition, we employed expressed protein ligation (EPL) to install stoichiometric and site-specific phosphorylation at these residues (2,3). We executed photocrosslinking and mutagenesis studies that mapped tail interactions with both the C2 and catalytic domain in the closed conformation. These data provide insight into the structural and mechanistic role for the phosphorylated C-terminal tail of PTEN and may contribute to the rational design of new therapies for cancer.

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P15

Enzymology of H_2S Oxidation in Nanodiscs

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The mitochondrial sulfide oxidation pathway is critical for regulating levels of hydrogen sulfide (H_2S), which plays critical signaling roles in mammals leading to cardioprotection, neuroprotection, and anti-inflammatory responses in the gastrointestinal system. The first and committed step of this pathway is catalyzed by sulfide-quinone oxidoreductase (SQR), a membrane-bound, flavin-dependent disulfide reductase. SQR converts H_2S to an enzyme-bound cysteine persulfide and subsequently transfers the sulfane sulfur to a small molecule acceptor (Fig 1). Concomitant with the latter step, the cysteine disulfide reforms in the active site while the electrons from sulfide oxidation are relayed to the FAD cofactor, to coenzyme Q_{10}, and thereon to complex III in the electron transport chain, thus coupling sulfide oxidation to mitochondrial energy metabolism. While the pivotal role of SQR in H_2S clearance makes it an attractive therapeutic target in diseases attributed to dysregulated H_2S levels, key features of the SQR reaction mechanism are unclear and studies have been hindered by the limited solubility of this membrane-bound enzyme. In the present study, we have elucidated the enzymology of human SQR in a membrane environment by incorporating it into nanodiscs. Kinetic analyses of the nanodisc-bound SQR reveal enhanced activity with several small-molecule acceptors compared to the detergent-solubilized form of the enzyme, while stopped-flow studies further elucidated the pre-steady state catalytic mechanism and the kinetics of FAD-mediated quinone reduction. These results provide new insight into the role of SQR in H_2S metabolism and establish a foundation for therapeutic targeting of this enzyme.

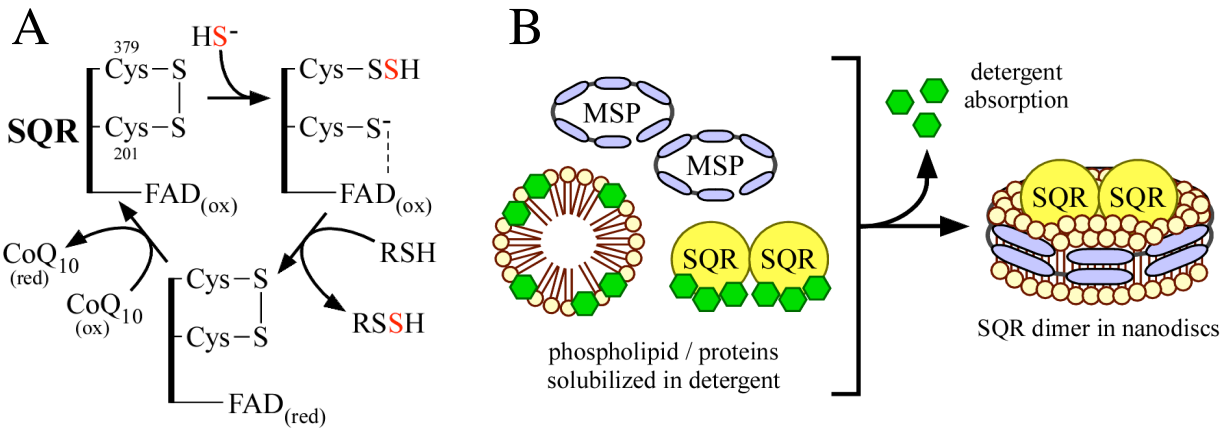

Figure 1. *A*, postulated SQR reaction mechanism, with transfer of sulfane sulfur to a generic small molecule (RSH). *B*, scheme for incorporation of detergent-solubilized SQR into nanodiscs.

Mechanistic Studies of a Unique Radical SAM Enzyme, MqnE

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Menaquinone (Vitamin K) is an essential cofactor for humans which plays an important role in the blood clotting cascade and bone morphogenesis. Recently, a new menaquinone biosynthetic pathway was discovered that uses chemistry very different from that used in the well-characterized *E. coli* pathway.[1] We discovered a missing enzyme viz. Aminofutalosine Synthase (MqnE) in the pathway and showed its *in-vitro* reconstitution.[2] MqnE is the first radical SAM enzyme which catalyzes the addition of the 5'deoxyadenosyl radical to the substrate instead of H atom abstraction. MqnE is proposed to catalyze energetically challenging radical 1,2 O→C transposition reaction through an O-neophyl rearrangement (Figure 1). We have designed several substrate analogs to block specific downstream steps in the proposed MqnE reaction mechanism (Figure 1). Characterization of shunt products generated in the MqnE reaction with these substrate analogs have substantiated the presence of the proposed radical intermediates. Furthermore, we have used continuous-wave and pulsed EPR techniques to provide direct spectroscopic evidence for the captodative radical intermediate (3). Additionally, we have demonstrated *in-vivo* inhibition studies against *H. pylori* to explore MqnE as a potential drug target. These mechanistic studies have helped to elucidate the complex rearrangement chemistry catalyzed by the enzyme MqnE in the Vitamin K biosynthesis.

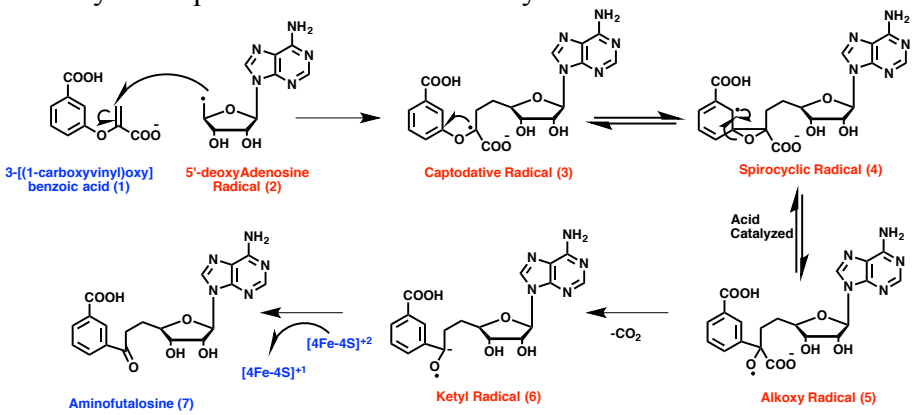

Figure 1: Mechanistic proposal for MqnE

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P17

Structure of a Key Intermediate in the Reaction Catalyzed by the Dual Specificity Methylase RlmN

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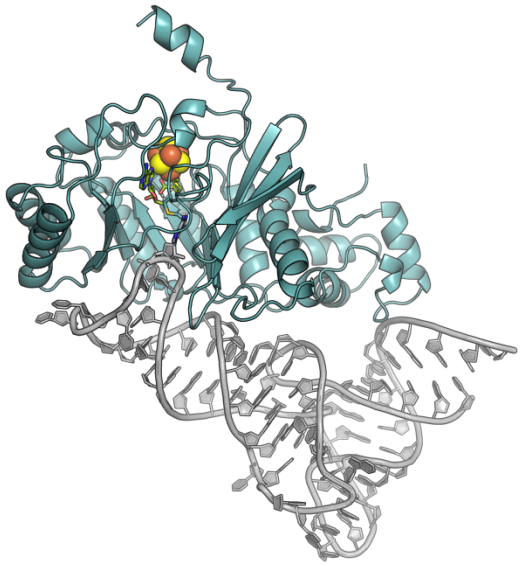
Both rRNA and tRNA are highly modified, contributing to their structural and functional integrity. RlmN is a dual specificity RNA methylase that modifies C2 of adenosine 2503 (A2053) in 23S ribosomal RNA and C2 of adenosine 37 (A37) is several *Escherichia coli* tRNAs, representing one of only two enzymes known to act on both rRNA and tRNA. Cfr, a related methylase, modifies C8 of A2503, conferring resistance to more than five classes of antibiotics. As members of the radical *S*-adenosylmethionine superfamily of enzymes, these two methylases employ a unique radical mechanism to catalyze these difficult transformations. While a structure of RlmN was previously reported, it lacked the RNA substrate. A Cys_{118}→Ala substitution in RlmN results in the capture of a covalent intermediate in which the protein is cross-linked to the RNA substrate at C2 of the target adenosine. The 2.4 Å resolution structure of this key intermediate formed using a $tRNA^{Glu}$ substrate was solved (1). This structure represents the first structure of a dual specificity methylase in complex with the entire RNA substrate. Along with mutagenesis and kinetic studies, the structure provides key insight into the mode of substrate recognition and unresolved mechanistic details of this complex reaction.



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P18

Regulatory Metabolic Complex for Glucose Metabolism in Living Cells: the Glucosome

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Sequential metabolic enzymes in glucose metabolism (i.e. glycolysis and gluconeogenesis) have long been hypothesized to form multienzyme complexes that regulate glucose-derived carbon flux in living cells. However, it has been challenging to directly observe these proposed complexes and their functional roles in various living systems. In this work, we have primarily used quantitative live-cell imaging to investigate the spatial organization of metabolic enzymes participating in glucose metabolism in living human cells. We provide multiple lines of compelling evidence that human liver-type phosphofructokinase 1, which catalyzes a bottleneck step of glycolysis, forms various sizes of cytosolic clusters in human cervical, breast, and pancreatic cancer cells, independent of the enzyme expression levels and the tagging methods of fluorescent probes. We also reveal that these cytosolic clusters colocalize with other rate-limiting enzymes involved in both glycolysis and gluconeogenesis. Subsequent biophysical measurements of a direct protein-protein interaction and diffusion coefficients of the participating enzymes strongly support the formation of multienzyme complexes in living cells. Furthermore, quantitative analysis of the differences in cluster size distributions between normal (Hs578Bst) and malignant (Hs578T) human breast tissue cell lines indicate the spatial alterations of glucose metabolism in cancer cells. Most importantly, the direction of glucose flux between the pentose phosphate pathway, serine biosynthesis and glycolysis is spatially controlled by the multienzyme complexes in a cluster size-dependent manner. Collectively, we identify a functionally active, multienzyme metabolic complex for human glucose metabolism in living cells, namely the "glucosome," which allow a cell to differentially regulate the directions of glucose-derived carbon flux in human cells.

Note that the work presented here is under revision for *Nature Chemical Biology*.

P19

Targeting Aerobactin Biosynthesis in Hypervirulent *Klebsiella pneumoniae*

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Since it was initially described in the mid-1980s in the Asian Pacific Rim, a hypervirulent pathotype of *Klebsiella pneumoniae* (hvKP) has since disseminated throughout the globe. In contrast to classical strains of *Klebsiella pneumoniae* (cKP), hvKP is able to cause serious life-threatening infections in previously healthy individuals in the community. There is fear amongst professionals in the medical community that convergence of this hypervirulent pathotype with the increasingly problematic strains of drug-resistant KP could lead to the evolution of a true "superbug" that would likely require novel therapeutics to combat. Recent work has demonstrated that the enhanced virulence of hvKP is significantly mediated by overproduction of the siderophore aerobactin. Siderophores are small molecule iron-chelators that allow bacteria to acquire sufficient quantities of this vital nutrient in the severely iron-limited host environment. We hypothesize that inhibition of aerobactin biosynthesis could be a viable therapeutic target that could yield a novel "anti-virulence" treatment for infections with hvKP and other pathogenic bacteria that rely on this siderophore. Toward targeting aerobactin biosynthesis for inhibition, we aim to lay the ground work by structurally and functionally characterizing the enzymes required to synthesize aerobactin, with specific emphasis on the synthetases IucA and IucC. Herein, we present a structural analysis of the synthetase IucA using X-ray crystallography and small-angle X-ray scattering (SAXS). In addition, we report on the apparent steady-stake kinetics and the substrate specificity of IucA. Finally, we describe the development of a biochemical assay to be employed in high-throughput screening for small-molecule inhibitors of IucA, as well as our initial screening results.

P20

Fungal Metabolomics for Large-Scale Analysis of Secondary Metabolism and Biosynthesis

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It is estimated there are as many as 5.1 million fungal species on earth, however viable genetic tools exist for only a handful and many cannot be readily cultured in the lab, preventing study of their huge biosynthetic repertoires. We report a scalable metabolomic profiling technology using untargeted liquid chromatography-mass spectrometry (LC-MS) with ultrahigh mass accuracy to systematically identify the secondary metabolite (SM) products of heterologously expressed fungal biosynthetic gene clusters (BGCs). This platform uses fungal artificial chromosomes (FACs) to capture full-length BGCs derived from *Aspergillus terreus*, *A. aculeatus*, and *A. wentii*. Host *A. nidulans* strains transformed with FACs are then screened by LC-MS, and FAC-encoded metabolites are identified. This "FAC-MS" platform is used to analyze a FAC encoding three distinct BGCs producing a novel lipopeptide, a sesterterpenoid, and the orphan benzodiazepine benzomalvin A. Genetic deletants of benzomalvin A backbone and tailoring genes within the FAC allow us to propose the first biosynthetic model for this secondary metabolite. Importantly, these deletions can be carried out using established *E. coli* genetic tools, eliminating the need to do genetics in the fungus from which the BGC was derived and opening a huge range of fungi to this type of analysis in the future. Benzomalvin A is biosynthesized by a 3 enzyme system: BenX, BenY, and BenZ. BenX is a tailoring enzyme which installs an N- methyl group on the molecule. BenY and BenZ work together to incorporate 2 anthranilate monomers and a phenylalanine monomer, to form a linear tripeptide which is hydrolytically released through cyclization by BenY-C_T. The resulting 11-member macrocyclic intermediate then undergoes transannulation catalyzed by BenY-C_T to form the benzodiazepine core. In the future, the FAC-MS pipeline will be used to facilitate direct enzymological investigation of the biosynthesis of benzomalvin A, as well as newly discovered metabolites.

Ipdab, a Key Virulence Determinant in *Mycobacterium tuberculosis*, is a Cholesterol Ring-Cleaving Hydrolase

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IpdAB, a key virulence factor in *Mycobacterium tuberculosis*, has been proposed to catalyze the hydrolysis of steroid Ring C in cholesterol catabolism. In phylogenetic analyses, IpdAB homologs from steroid-degrading bacteria formed a clade of CoA transferases (CoTs). In a coupled assay with the FadA6 thiolase, IpdAB transformed the cholesterol-derived catabolite, (*R*)-2-(2-carboxyethyl)-3-methyl-6-oxocyclohex-1-ene-1-carboxyl-CoA (COCHEA-CoA) and CoASH to 4-methyl-5-oxo-octanedioyl-CoA (MOODA-CoA) and acetyl-CoA with high specificity (k_{cat}/K_M = 2±0.7 × 10^5 $M^{-1}s^{-1}$). Contrary to characterized CoTs, IpdAB exhibited no activity towards small CoA thioesters. FadA6 acted on β-keto-thioester substrates. NMR characterization of MOODA-CoA determined the location of the CoA moiety to be consistent with IpdAB hydrolyzing COCHEA-CoA to a β-keto-thioester which undergoes thiolysis by FadA6. IpdAB lacks the catalytic glutamate residue that is conserved in the β-subunit of characterized CoTs, but Glu105 is conserved in the α-subunit IpdAB homologs. A crystal structure of the IpdAB:COCHEA-CoA complex, solved to 1.8 Å, contained a hydroxylated intermediate (Fig 1A). $Glu105^A$ was positioned 2.1 Å away from the hydroxylation site, consistent with its role as a catalytic base. The $E105^AA$ variant of IpdAB was catalytically inactive but, upon titration with COCHEA-CoA (K_D = 0.4±0.2 μM), accumulated a yellow-colored species (λ_{max} = 310 nm) typical of β-keto enolates. In the presence of D_2O, IpdAB catalyzed the deuteration of COCHEA-CoA adjacent to the hydroxylation site at rates consistent with k_{cat}. Based on these data and additional IpdAB variants, we propose a retro-Claisen condensation-like mechanism for the IpdAB-mediated hydrolysis of COCHEA-CoA (Fig 1B).

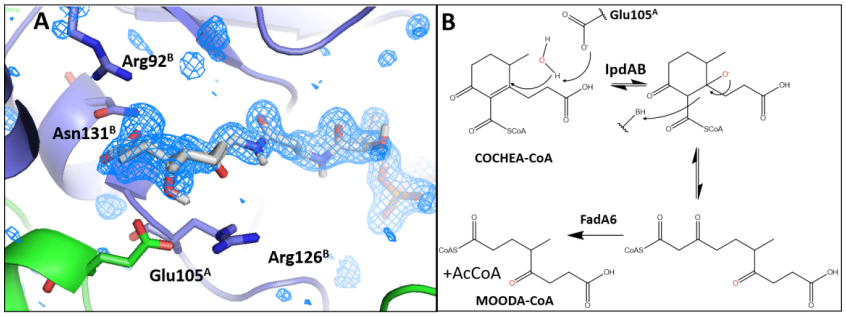

Figure 1. (A) Structure of IpdAB:COCHEA-CoA complex. (B) Proposed mechanism of ring C opening.

P22

Enzymes for Decorating the Heterocyclic Aromatic Ring of Phenazine Antibiotics from *Lysobacter antibioticus*

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Many heterocyclic aromatic *N*-oxides have potent biological activities, but the mechanism for biosynthesis of these compounds is often unclear. We recently isolated six antibacterial compounds from *Lysobacter antibioticus* OH13 and determined their structures to be phenazines, including the aromatic *N,N*-dioxide myxin, a well-known antibiotic. With the goal of understanding the multiple *N*-oxidations and *O*-methylations found in these compounds, we sequenced the OH13 genome, identified a 10-gene cluster (*LaPhz*), and showed that *LaPhz* genes were required for phenazine biosynthesis. LaPhzNO1 in the cluster was homologous to genes encoding cyclohexanone monooxygenases, flavoproteins catalyzing a Baeyer-Villiger type oxidation. Mutation of *LaPhzNO1* abolished production of all *N*-oxides, while production of two non-oxide phenazines markedly increased. We expressed LaPhzNO1 in *E. coli* and demonstrated that the enzyme is an NADPH-dependent, flavin *N*-monooxygenase. We further showed that LaPhzNO1 together with LaPhzS, another flavoprotein encoded in the gene cluster, converted phenazine 1,6- dicarboxylic acid (PDC) to 1,6-dihydroxyphenazine *N*5,*N*10-dioxide. LaPhzNO1 thus represents the first experimentally characterized *N*-monooxygenase in the biosynthesis of heterocyclic aromatic natural products. In addition, we expressed *LaPhzM* in *E. coli* and showed that the purified enzyme was able to catalyze both *O*-methylation and *N*-methylation of phenazine, using *S*-adenosylmethionine as co-substrate. These results establish the enzymatic mechanisms for phenazine ring decorations, which could lead to hundreds of phenazine natural products.

P23

Crystal Structures of the *E. coli* Dihydrodipicolinate Synthase in Complex with Pyruvate and Acetopyruvate

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The enzyme dihydrodipicolinate synthase (DHDPS) catalyzes the first committed step in the biosynthesis of L-lysine and diaminopimelae in vascular plants and bacteria. DHDPS catalyzes the condensation of pyruvate and L-aspartate-β-semialdehyde to form 2, 3 dihydrodipicolinate and is feedback inhibited by L-lysine at an allosteric site. The kinetic mechanism for DHDPS is ping pong with pyruvate binding prior to L-aspartate-β-semialdehyde. After formation of a Schiff base between pyruvate and Lys161, loss of a proton from the β-methyl group of pyruvate forms an enamine intermediate at higher pH values. Kinetic studies indicate that the allosteric inhibitor, L-lysine, binds preferentially to the E:enamine complex. Several crystal structures of *Escherichia coli* DHDPS in complex with pyruvate or acetopyruvate, with and without L-lysine bound to the allosteric site, have been solved. The structure with pyruvate bound shows pyruvate is covalently attached to Lys161. In the complex the ε-amino group of Lys161 is within hydrogen-bonding distance of Tyr133 that is thought to be involved in generation of the E:pyruvate Schiff base intermediate. Acetopyruvate, a slow-binding inhibitor of DHDPS, is also shown to be covalently attached to Lys161 in the structure with enzyme. The acetopyruate structure indicates the α-carboxyl group is rotated about 90 degrees relative to the position of the α-carboxyl group in the E;pyruvate structure. The oxygen of the aceto-group occupies a position similar to the one normally occupied by one of the α-carboxyl oxygens with pyruvate bound. A crystal structure with L-lysine bound to the E:acetopyruvate complex indicates L-lysine is bound at the allosteric site in a similar manner as L-lysine binds to apo-enzyme. However, the structure with L-lysine bound to the E:pyruvate complex indicates L-lysine is bound differently compared to how L-lysine is bound to apo-enzyme. Also, the allosteric site is more closed and may account for the greater affinity of L-lysine for the E:pyruvate complex.

Enzyme Function Discovery as a Foundation for Exploring Novel Secondary Metabolites

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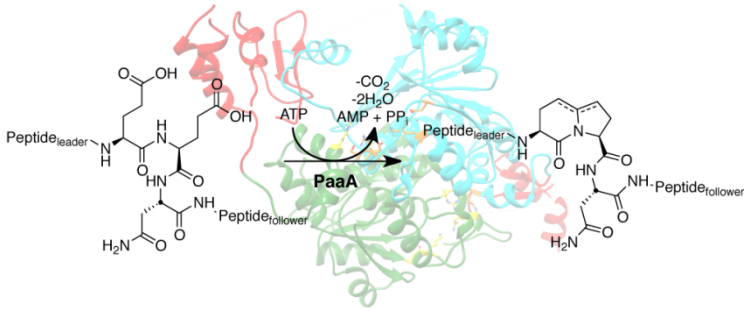
New antibiotics are urgently needed to tackle the rising menace of drug-resistant pathogens. At present, the most extensive and convenient resource available is the sequenced genomes of microorganisms. We intend to uncover novel natural products from microbial genomes by using a systematic strategy based on biochemical characterization of enzymes involved in antibiotic biosynthesis, and protein sequence homology to guide the selection of candidate enzymes from previously uncharacterized biosynthetic pathways.

We have probed the enzymatic reaction catalyzed by PaaA, an enzyme involved in the biosynthesis of the antibiotic Pantocin A. Pantocin A belongs to the ribosomally synthesized and post-translationally modified peptides (RiPP) class of antibiotics, and is produced by the organism *Pantoea agglomerans*. Biosynthesis of RiPP metabolites involves enzymatic recognition and post-translational modification of a nascent precursor peptide encoded in the genome of the organism. The biosynthesis of Pantocin A involves activation, cyclization, and oxidative decarboxylation of a Glu-Glu-Asn motif (called core peptide) located close to the center of the 30-residue precursor peptide PaaP, followed by peptide cleavage by unidentified proteases. Our study revealed that PaaA catalyzes the activation, elimination and decarboxylation using a single active site, to install a fused heterocycle on the peptide. We probed the roles of the leader and follower sequences flanking the core peptide in the substrate PaaP. The leader sequence was found to be critical for PaaP recognition by PaaA while the follower peptide was found to be crucial for the decarboxylation reaction, but not activation and elimination. PaaA is composed of two domains: an N-terminal RiPP precursor peptide recognition element (RRE) and a C-terminal E1-like adenylation domain, which binds ATP at the active site. Enzymes homologous to PaaA were found to generate distinct functional groups, viz. succinimide, phosphoramidate, thioacid and thioacyl persulfide, using a similar strategy of activation of a carboxylic functional group using ATP, followed by nucleophilic substitution. A sequence similarity network diagram of E1-like enzymes similar to PaaA revealed several candidate enzymes of unknown function that possess both domains found in PaaA, laying a foundation for exploring the biosynthesis of several potentially different molecular scaffolds.



P25

The Characterization of the *ydj* Operon, a Carbohydrate Catabolic Pathway Found in *E. coli*

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Recent evidence has shown that certain bacterial species residing in the human gut contribute to a variety disease states such as inflammatory bowel disorders (e.g. Crohn's disease and ulcerative colitis), colorectal cancers, diabetes, obesity, and cardiovascular disease. Significant resources have been invested to understand the symbiotic relationship between humans and the bacteria that make up the human microbiome and their effects on human health. Certain species of the gut microbiome have evolved the ability to process alternative carbohydrates as an energy source. This provides these bacterial species a selective advantage over other species, allowing it to outcompete the other species. This is one mechanism, known as colonization resistance, to defend against the colonization of exogenous pathogens. On the other hand, certain carbohydrate metabolites have been shown to enhance the virulence of harmful bacteria.

To increase our knowledge about the metabolic capabilities of bacterial species of the human intestinal microbiome, a computational method was developed to locate and identity novel metabolic pathways in the available genomes. Our initial method focused on biochemical pathways predicted to be involved in carbohydrate degradation. The initial search in *E. coli* yielded four potential novel gene clusters. One gene cluster of interest is comprised of eight genes containing five enzymes. These five enzymes have been subjected to extensive bioinformatic analyses (sequence similarity networks and homology modeling). They all share low sequence identity with enzymes of known function in their respectively families. All five enzymes have been cloned, purified, and screened for activity using a library of carbohydrates. All five enzymes have measured activity, and have been analyzed, in detail, with the products identified using ^{1}H NMR spectroscopy. However, the resulting activities do not form a coherent metabolic pathway. All of the enzymes are currently undergoing crystal structure screens. One crystal structure of has been solved by our collaborators, the Holden group at UW-Madison. The results and implication of observed activities are discussed and a potential novel catabolic carbohydrate pathway is proposed.

Phytoene Desaturase: an FAD-dependent Plant Carotenoid Biosynthetic Enzyme

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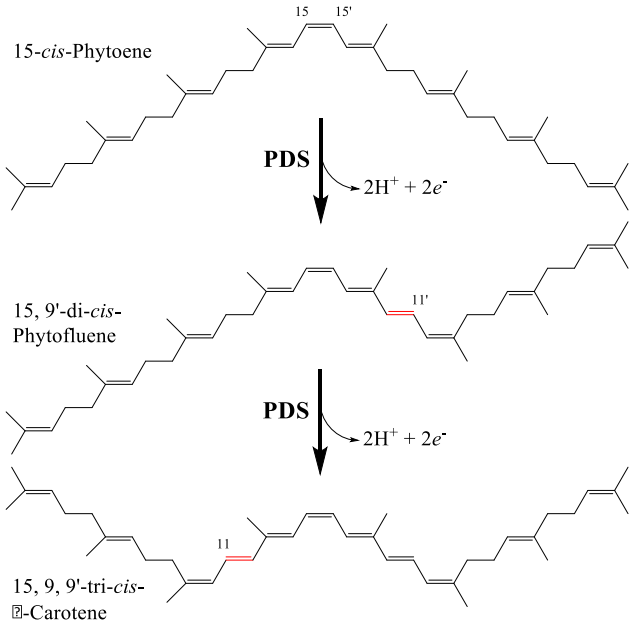
Phytoene Desaturase (PDS) is a key enzyme in the early stages of the carotenoid biosynthetic pathway in cyanobacteria, green algae and plants.[1] PDS catalyzes two consecutive $2e^-$ oxidations, as illustrated in Figure 1. PDS has been shown to contain bound FAD [2] and to have both gene sequence and mechanistic similarities to enzymes such as monoamine oxidase and protoporphyrinogen oxidoreductase. Details of the mechanism remain to be determined; one proposal is hydrogen abstraction from C_{11} and flipping of the C_9-C_{10} double bond from *trans* to *cis*, followed by hydrogen abstraction from C_{12} and radical recombination.

There is interest in bioengineering green microalgae to produce carotenoid co-products (nutraceuticals) that would improve the economic prospects of these organisms for biofuels production. For these reasons we have begun to characterize PDS from *Chlorella vulgaris* UTEX 395. Currently our lab is expressing and purifying a His-tagged form of PDS, and developing new assay procedures that will facilitate mechanistic analysis of the enzyme.

Figure 1. The reactions catalyzed by phytoene desaturase



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NMR Crystallography of a Carbanionic Intermediate in Tryptophan Synthase: Chemical Structure, Tautomerization, and Reaction Specificity

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Carbanionic intermediates play a central role in the catalytic transformations of amino acids performed by pyridoxal-5'-phosphate (PLP)-dependent enzymes. Here we make use of NMR crystallography – the synergistic combination of solid-state nuclear magnetic resonance, X-ray crystallography, and computational chemistry – to interrogate a carbanionic/quinonoid intermediate analogue in the β-subunit active site of the PLP-requiring enzyme tryptophan synthase. The solid-state NMR chemical shifts of the PLP pyridine ring nitrogen and additional sites, coupled with first principles computational models, allow a detailed model of protonation states for ionizable groups on the cofactor, substrates, and nearby catalytic residues to be established. Most significantly, we find that a deprotonated pyridine ring nitrogen on PLP precludes formation of a true quinonoid species and that there is an equilibrium between the phenolic and protonated Schiff base tautomeric forms of this intermediate. Natural bond orbital analysis indicates that the latter builds up negative charge at the substrate C^{α} and positive charge at C4' of the cofactor, consistent with its role as the catalytic tautomer. These findings support the hypothesis that the specificity for β-elimination/replacement vs. transamination is dictated in part by the protonation states of ionizable groups on PLP and the reacting substrates and underscore the essential role that NMR crystallography can play in characterizing both chemical structure and dynamics within functioning enzyme active sites.

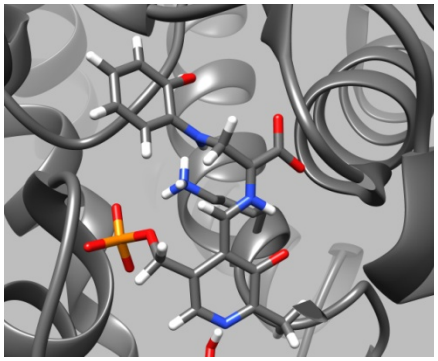

Figure 1. Protonation states and hydrogen bonding interactions for the protonated Schiff base tautomer of the carbanionic intermediate revealed by NMR crystallography in the tryptophan synthase β-subunit active site.

P28

Extended Hydrogen Bonding Network Influences Electronic Modulation of Pyridoxal 5'-Phosphate in Aspartate Aminotransferase

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Pyridoxal 5'-phosphate (PLP) is a ubiquitous, multifunctional cofactor used by a variety of enzymes to catalyze a number of chemical reactions involved in amino acid metabolism. The various active site local environments of PLP-dependent enzymes are thought to modulate the electronics of PLP in order to optimize a preferred chemical reaction. An extended hydrogen bond is coupled to the pyridinyl nitrogen of PLP in Aspartate Aminotransferase, which influences the electrophilicity of PLP. The hydrogen bonding network consists of residues D222, H143, T139, H189, and structural water molecules. Perturbing this microenvironment near the pyridinyl nitrogen effects the rate of catalysis in single-mutant variants D222T and H143L, and double-mutant variants H143L:H189L and H143F:H189L. Through steady-state kinetics, high-resolution X-ray crystal structures and quantum chemical calculations, we demonstrate that this intricate hydrogen bonding network influences the protonation state of the pyridine nitrogen of PLP. The electronic delocalization effect of PLP, which enhances catalysis, is induced by protonating the pyridinyl nitrogen to form a pyridinium cation. From quantum chemical calculations, it is apparent that D222 directly increases the pK_a of the pyridine nitrogen and stabilizes the pyridinium cation. Additionally, H143 and H189 indirectly increases the pK_a of the pyridine nitrogen, but more significantly influence the position of the proton that resides between D222 and the pyridine nitrogen. The hydrogen bonding network provides a microenvironment that increases the acidity of D222, stabilizing the proton on PLP and, thus, activating the enzyme.

P29

Structural Features of Two-Component FMN-Dependent Reductases that Provide a Functional Advantage

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The SsuE enzyme belongs to the NAD(P)H:FMN reductase family based on structural and functional similarities. While several enzymes in this family are canonical flavoproteins, a subclass of this family are FMN-dependent reductases that are associated with a specific monooxygenase enzyme.[1] The subclass of NAD(P)H:FMN reductases contains a secondary structural element referred to as a π-helix generated by the insertion of an amino acid into the conserved α-helix present in canonical flavoproteins.[2] All members of the subclass contain a π-helix located at the tetramer interface. The identification of π-helices as discrete secondary structural elements is often overlooked in the analysis of protein three-dimensional structure. An extensive analysis of protein structures has determined that 15% of protein structures contain this secondary structural element.[2] Generally, π-helices are characterized by a single residue insertion into an α-helices to provide an evolutionary advantage through enhancement or gain of protein function. The π-helices are conserved within proteins that have related functions and have been used as markers for evaluating evolutionary relationships as well as identifying unique functions associated with protein families.

The π-helix of SsuE is formed by the insertion of Tyr118 in an α-helix located at the tetramer interface of SsuE. Substitution of Tyr118 to Ala (Y118A SsuE) transformed flavin-free SsuE into a flavin-bound form. In addition, the π-helix had been converted to an α-helix in the Y118A SsuE variant similar to the canonical NAD(P)H:FMN reductases. Extracted flavin from Y118A had a mass of 457 amu similar to that of an FMN cofactor, suggesting that the Y118A SsuE variant had retained flavin specificity. Reactivity between the reduced flavin with oxygen was slow in NADPH oxidase assays, but Y118A SsuE supported electron transfer to ferricyanide. The Y118A SsuE FMN cofactor was reduced with approximately one equiv of NADPH in anaerobic titration experiments, and the flavin remained bound following reduction. There was no measurable sulfite product in coupled assays with the Y118A SsuE variant and the monooxygenase partner SsuD, further demonstrating that flavin transfer was no longer supported. Although Y118A SsuE was unable to effectively transfer flavin to SsuD, protein-protein interactions between Y118A SsuE and SsuD were still maintained. The results from these studies suggest that the π-helix enables SsuE to effectively utilize flavin as a substrate in the two-component FMN-dependent monooxygenase systems.

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P30

Characterization of a New FAD: NAD(P)H-Quinone Oxidoreductase in *Pseudomonas aeruginosa* Strain PAO1

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The product of the gene *pa1225* in *Pseudomonas aeruginosa* PAO1 is currently annotated as a putative NAD(P)H-quinone oxidoreductase. This annotation is based on a 29% sequence identity to human NAD(P)H dehydrogenase, quinone 2 [NQO2; PDB: 5LBT, E.C. 1.10.5.1]. PA1225 appears to contain FAD, based on the fluorescence emission of the purified enzyme and confirmed using MALDI. Interestingly, PA1225 is repressed 89-fold in the presence of the LysR regulator PA4203, which also represses the gene for nitronate monooxygenase (NMO). The latter enzyme catalyzes the oxidation of the mitochondrial toxin propionate 3-nitronate (1), thereby acting in detoxification. Thus, PA1225 may be a drug target against *P. aeruginosa,* which is an opportunistic gram-negative bacterium that exhibits multi-antibiotic resistance and is able to thrive in water, immunocompromised humans, and hospital settings (2).

To elucidate whether PA1225 is indeed an NAD(P)H-quinone oxidoreductase, the *pa1225* gene was amplified by PCR from the genomic DNA of *P. aeruginosa* PAO1 and ligated into vector pET20(b)+. The resulting recombinant plasmid was used to transform *Escherichia coli* strain Rosetta(DE3)pLysS to express PA1225. Initial purification of the recombinant enzyme using classical protein chromatographic approaches revealed that the FAD cofactor readily dissociates from the holoprotein. In agreement, an homology model of PA1225 using NQO2 as a template revealed a solvent exposed cofactor. Two approaches aimed at increasing the purification yield of holo-PA1225 for structural and mechanistic studies are currently underway. First, by purifying the enzyme in complex with an active site ligand to prevent the dissociation of the cofactor during protein purification. Second, by engineering a recombinant gene for PA1225 that carries a His-tag to expedite protein purification. The results of this study will be presented.

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

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P31

Active Site Hydrogen Bonding Promotes Oxidative Decarboxylation by Cytochrome P450 OleT

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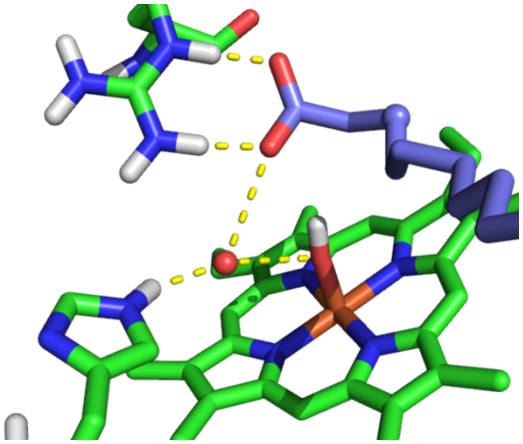
P450 OleT has been the subject of a great deal of interest due to its ability to transform abundant fatty acids into fungible fuels in the form of 1-alkenes via a carbon-carbon scission reaction. Previous work has demonstrated that OleT initiates this chemistry via hydrogen abstraction by an Fe^{4+}=oxo pi cation radical intermediate (Compound-I),[1] yielding an Fe^{4+}-OH species (Compound-II).[2] The similarity of OleT catalysis to reaction coordinates common to many metal-containing monoxygenases is peculiar as it seemingly abrogates an oxygen rebound step. Furthermore, the observed Compound-II intermediate is over six orders of magnitude more stable than what is typically postulated for P450s. It has been posited that an active site residue (His 85) may play a role in directing the reaction coordinate toward decarboxylation. [3] The importance of the His 85 residue has been investigated by site-directed mutagenesis, steady-state and transient kinetics studies. Hydrogen bonding from the His 85 residue is important for both the efficient activation of hydrogen peroxide, and in combination with substrate fatty acid, promoting decarboxylation. Furthermore, pH and kinetic solvent isotope effect (KSIE) studies suggest that the stable Compound-II intermediate decays via a proton coupled electron transfer process that is mediated by His 85. This step is likely responsible for the removal of a substrate electron that is required to produce 1-alkenes from a fatty acid.



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P32

Renalase: Emerging Details of an Expanding Family of Enzymes

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Human renalase is a flavoenzyme discovered in 2005 that was originally claimed to modulate blood pressure and heart rate by oxidizing catecholamines in blood. This assignment of activity has since been demonstrated to be erroneous, and the enzyme has instead been shown to oxidize toxic isomers of NAD(P)H. More recently, our lab identified an isozyme in Pseudomonads, making it the first example of a renalase identified outside of the Animalia. This enzyme has proven much more apt to crystallize than the human enzyme, and has afforded high-resolution crystal structures with the reaction product, NAD+, and substrate analogues, NAD(P)H, bound in the active site. Transient-state kinetic studies of both enzymes have revealed distinct substrate specificities: stark preferences for the 2DH isomers over the 6DH isomers, and also for the NADH isomers over the NADPH isomers, are noted for the bacterial enzyme while the human enzyme shows similar specificities among all four substrate molecules. Recent ligand-bound crystal structures of the bacterial enzyme show interactions between the nicotinamide moieties of the substrates and the protein binding-site that align the nicotinamide 2' position over the N5 of the flavin. Additionally, the charged 2' phosphate moiety of NADPH is shown to form no interactions favorable to binding with the protein, and instead protrudes into solvent. Results from additional kinetic experiments suggest that bonds to the proximal phosphate are also responsible for positioning the nicotinamide moiety of the substrates against the flavin for catalysis. Our lab has also recently identified a putative renalase in *Nicotiana sylvestris* that would be the first example of a renalase in Plantae. This enzyme bears high structural similarities to the human enzyme, and ligand-bound crystal structures of this enzyme may inform upon the binding interactions and specificities of the human enzyme, but, inexplicably, does not catalyze the renalase reaction.

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

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

Here we report the X-ray crystal structure of the green Y249F variant of PaDADH at 1.29 Å resolution. The results showed that the cofactor in the active site of the enzyme is a 6-OH-FAD, as expected, with an extra covalent linkage on the N(5) atom with the product keto-arginine. Other than the modified flavin, the overall structure of the green mutant enzyme is fundamentally identical to that of the wild-type enzyme with a notable difference being loop L1 that controls substrate access to the active site being present in a unique conformation. The presence of 6-OH-FAD in the Y249F variant of PaDADH demonstrates that there is an intrinsic proclivity for 6-hydroxylation of flavin when the microenvironment around the cofactor is modified by removing the hydroxyl group on the side chain Y249 [1][2]. The mechanism of formation of the 6-OH-FAD is currently under investigation.

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Kinetic and Structural Studies on the Acetolactate Synthase from *Klebsiella pneumoniae*

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In *Klebsiella pneumoniae* the formation of *S*-acetolactate is catalyzed by an acetolactate synthase (*Kp*ALS) and by an acetohydroxyacid synthase (*Kp*AHAS), both thiamine diphosphate (ThDP)-dependent enzymes. In both enzymes the reaction occurs in 4 stages: (i) formation of lactylThDP followed by (ii) decarboxylation to provide a reactive carbanion/enamine. Subsequently the enamine (iii) carries out a nucleophilic attack on a second molecule of pyruvate forming an acetolactyl-ThDP intermediate which then (iv) breaks down and releases *S*-acetolactate. That said, there are some differences. For example, ALS has a pH optimum around 6, whereas AHAS operates best around pH 8. Further, only AHAS is can synthesize *S*-acetohydroxybutyrate.

In order to understand the evolution of these differences, we are carrying out detailed structure and mechanistic studies on both enzymes. Here we will focus on *Kp*ALS which was expressed and purified as the N-terminally his-tagged variant. Initial kinetic studies showed the enzyme to be particularly sensitive to buffer conditions including inhibition by phosphate and activation by acetate. In an attempt to rationalize these observations, *Kp*ALS crystals were grown and soaked with pyruvate and the substrate analogue, β-fluoropyruvate, prior to being flash frozen. Previous structures of *Kp*ALS soaked with pyruvate show an unusual tricyclic hydroxyethyl-ThDP intermediate bound in the active site,[1] an intermediate not seen in the acetolactate synthase from *Bacillus subtilis*[2] or, indeed, in any other ThDP-dependent enzyme. Our study confirmed the presence of the tricyclic form of ThDP. However the trapped intermediate represents a different step in the reaction pathway as the acetolactyl-ThDP intermediate is observed rather than the hydroxyethyl-ThDP intermediate. Conversely, the crystals soaked with β-fluoropyruvate also show evidence of a trapped intermediate, but not the tricyclic form of ThDP.

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Divergent Mechanisms of Allosteric Regulation of Pyruvate Carboxylase by Acetyl Coenzyme A

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Pyruvate carboxylase is a biotin-dependent enzyme, composed of multiple functional domains on a single polypeptide chain, including the biotin carboxylase domain, the carboxyltransferase domain, the biotin carboxyl carrier protein domain and the allosteric domain. Acetyl coenzyme A is an allosteric activator of pyruvate carboxylase from a wide variety of species. However, the sensitivity to acetyl coenzyme A activation varies depending on the organism from which the enzyme was cloned or isolated. We are investigating the divergent allosteric activation of pyruvate carboxylase from two organisms, *Rhizobium etli* (*Re*PC) and *Aspergillus nidulans* (*An*PC). The maximum activity of *Re*PC is accelerated ~10-fold by acetyl coenzyme A, while *An*PC is **insensitive** to acetyl coenzyme A activation, despite binding acetyl coenzyme A. Previous studies have structurally and kinetically characterized *Re*PC in the presence and absence of acetyl coenzyme A. In order to investigate the divergent mechanisms of allosteric activation in *An*PC and *Re*PC, we have structurally and kinetically characterized the contributions of acetyl coenzyme A to catalytic turnover in *An*PC. We have observed a highly divergent response of *An*PC and *Re*PC to acetyl coenzyme A activation in both the overall pyruvate carboxylation reaction and in the half-reactions catalyzed at the individual catalytic domains. The binding of acetyl coenzyme A to *Re*PC at the allosteric domain is primarily mediated by two conserved arginine residues. We generated mutations at the equivalent arginine residues in *An*PC and determined that the K_a for acetyl coenzyme A was significantly increased in both mutations, indicating that acetyl coenzyme A most likely binds at the same binding site in *An*PC as it does in *Re*PC. To further clarify the mechanism of acetyl coenzyme A activation in pyruvate carboxylase, we investigated the catalytic turnover of hybrid tetramers composed of several combinations of inactivating mutations. Our data reveals that, in *Re*PC, acetyl coenzyme A constrains the translocation of the biotin carboxyl carrier protein domain, limiting it to sample only two productive motions during catalytic turnover. However, in *An*PC, acetyl coenzyme A does not alter the range of motions sampled by the biotin carboxyl carrier protein domain during catalysis. Thus, while acetyl coenzyme A binds at the same binding site in both *An*PC and *Re*PC, the divergent response of *An*PC and *Re*PC to acetyl coenzyme A activation arises, in part, from different innate carrier domain movements during catalytic turnover.

Reaction Rates and Intermediates in Ser26 Variants of Benzoylformate Decarboxylase

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Benzoylformate decarboxylase (BFDC), a thiamine diphosphate (ThDP)-dependent enzyme, catalyzes decarboxylation of benzoylformate to benzaldehyde and CO_2. For some time we have been studying the role of the ionizable residues in the mechanism of BFDC which proceeds through at least four individual chemical steps. Initially we saw that S26A, H70A and H281A variants had significantly reduced k_{cat}/K_m values and proposed a role for these residues based on stopped-flow studies with a slow substrate.[1] We were surprised when later saturation mutagenesis studies on those same residues indicated that those proposals were not necessarily accurate, and that many mutations had much less effect than had been predicted.[2]

We have now obtained X-ray structures for many of those variants but, ultimately, the structures are uninformative. Recently, a rapid-quench NMR method has been used to measure the ratios of intermediates in the reactions of several ThDP-dependent enzymes.[3] The method permits calculation of rate constants for the formation of the first intermediate, in this case mandelylThDP, its subsequent decarboxylation, as well as the combined breakdown of the enamine and product release. We have used the method to examine the effect of a variety of Ser26 mutations on the individual catalytic steps. Initially the Ser26 variants were expressed and purified and characterized using steady-state kinetics. Subsequently, the enzymes were mixed with benzoylformate and the mixture quenched with acid to trap the reaction intermediates. NMR spectroscopy was used to identify and quantitate individual intermediates. Rate constants for the formation of these intermediates were then determined and compared to those of the wild-type enzyme. Here we report those results and discuss their implications for the role of Ser26 in the BFDC reaction mechanism.

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Enzyme and Substrate Pieces of Glycerol-3-Phosphate Dehydrogenase: Reconstruction of a Robust Hydride-Transfer Catalyst

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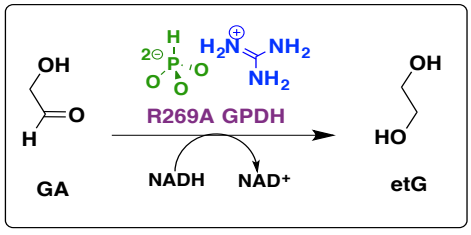
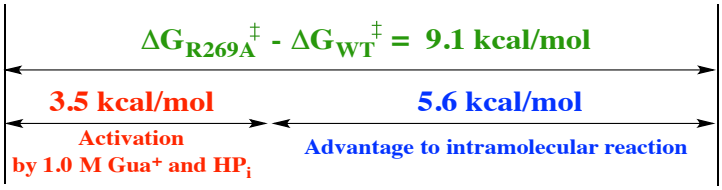
The enormous potential for the stabilization of the transition states for enzyme-catalyzed polar reactions by ion-pairing interactions between the bound ligand and protein catalyst is not widely appreciated. The stabilization of the transition state for human liver glycerol-3-phosphate dehydrogenase- (*Hs*GPDH) catalyzed reduction of dihydroxyacetone phosphate (DHAP) due to the action of the phosphodianion of substrate DHAP and the cationic side chain of R269 is at least 12.4 kcal/mol, and possibly as large as 17 kcal/mol. These strong ionic interactions enable the unprecedented efficient self-assembly of R269A *Hs*GPDH and the appropriate enzyme and substrate pieces into a robust catalyst of the reduction of truncated substrate glycolaldehyde (GA) (Scheme 1). The R269A[1] mutant of *Hs*GPDH shows no detectable activity toward reduction of GA to ethylene glycol (etG), and there is no detectable activation for this reaction by 30 mM phosphite dianion (HPO_3^{2-}) or guanidinium cation (Gua^+). However, the assembly of R269A (*Hs*GPDH), HPO_3^{2-}, Gua^+ into a catalyst of the reduction of GA, and a fourth-order reaction rate constant $k_{cat}/K_{GA}K_XK_{Gua}$ was observed. The dianions FPO_3^{2-} and SO_4^{2-} substitute for HPO_3^{2-} in this assembly. This self-assembly is favored by the strong interaction between the HPO_3^{2-} and Gua^+ pieces at the enzyme active site. The advantage for connection of *Hs*GPDH (R269A + Gua^+) and substrate pieces (GA + HPO_3^{2-}) pieces, $(\Delta G^{\ddagger}_S)_{E+Gua+GA+HPi}$= 5.6 kcal/mol (Scheme 2), is nearly equal to the sum of the advantage to connection of the substrate pieces, $(\Delta G^{\ddagger}_S)_{GA+HPi}$ = 3.3 kcal/mol, for wildtype *Hs*GPDH-catalyzed reaction of GA + HPO_3^{2-}, and for connection of the enzyme pieces, $(\Delta G^{\ddagger}_S)_{E+Gua}$ = 2.4 kcal/mol, for Gua^+ activation of the R269A *Hs*GPDH-catalyzed reaction of DHAP.[2] This shows that the R269A mutation has little or no effect on enzyme activation by HPO_3^{2-}. These results are compared with results from earlier studies on the activation of wildtype and mutant forms of triosephosphate isomerase (TIM) and orotidine-5′-monophosphate decarboxylase (OMPDC).

Scheme 1



Scheme 2



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P38

Overcoming an Optimization Plateau in the Directed Evolution of Highly Efficient Nerve Agent Bioscavengers

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Improving an enzyme's initially low catalytic efficiency with a new target substrate by an order of magnitude or two may require only a few rounds of mutagenesis and screening or selection. However, subsequent rounds of optimization tend to yield decreasing degrees of improvement (diminishing returns) eventually leading to an optimization plateau. We aimed to optimize the catalytic efficiency of bacterial phosphotriesterse (PTE) towards V-type nerve agents. Previously, we improved the catalytic efficiency of wild-type PTE towards the nerve agent VX by 500 fold, to a catalytic efficiency (k_{cat}/K_M) of $5x10^6$ M^{-1} min^{-1}. However, *in vivo* detoxification demands an enzyme with a catalytic efficiency of $\geq 10^7$ M^{-1} min^{-1}. Here, following 8 additional rounds of directed evolution and the computational design of a stabilized variant, we evolved PTE variants that detoxify VX with a $k_{cat}/K_M \geq 5 \times 10^7$ M^{-1} min^{-1} and Russian VX (RVX) with a $k_{cat}/K_M \geq 10^7$ M^{-1} min^{-1}. These final 10-fold improvements were the most time consuming and laborious, as most libraries yielded either minor or no improvements. Stabilizing the evolving enzyme, and avoiding substrate tradeoffs, enabled us to obtain further improvements beyond the optimization plateau and evolve PTE variants that were overall improved by >5000 fold with VX and by >17,000 fold with RVX. The resulting variants also hydrolyze G-type nerve agents with high efficiency, and can thus serve as candidates for broad-spectrum nerve-agent prophylaxis and post-exposure therapy using low enzyme doses.

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Mechanistic Studies of Engineered Ascorbate Peroxidase, a Genetically Encoded Cell Imaging Probe

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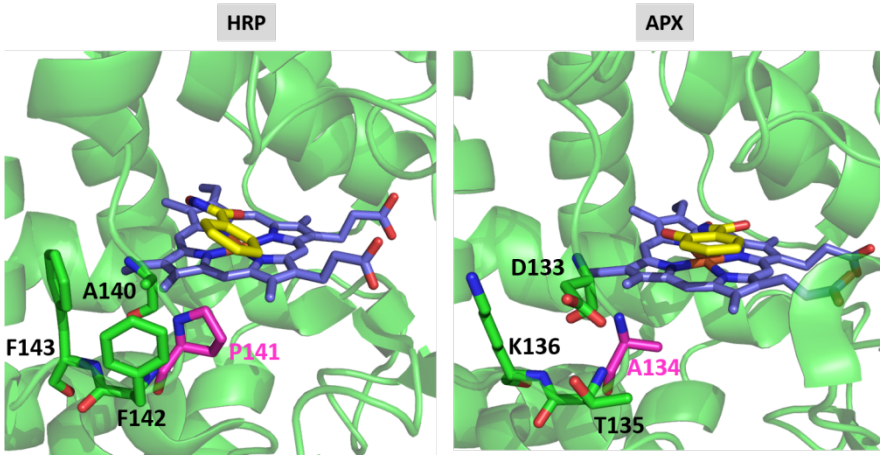
Horseradish peroxidase (HRP) is an extremely sensitive and versatile reporter enzyme that oxidizes a broad array of phenol and aniline substrates in an H_2O_2–dependent manner, allowing it to generate fluorescence, chemiluminescence, colorimetric signal, or contrast for electron microscopy. However, HRP has limited utility as a genetic imaging tag because it fails to become active in most cellular compartments. Ascorbate peroxidase (APX) is a closely related heme peroxidase that is active in all cellular compartments, but fails to produce robust imaging signal because it does not recognize HRP-like substrates. By re-engineering the active site of APX to resemble that of HRP, we developed enhanced ascorbate peroxidase (APEX) and applied it for several types of cellular imaging, including electron microscopy. Although APEX recognizes HRP-like substrates, it is far less sensitive than HRP. Using directed evolution, we developed APEX2, a single mutant of APEX that is much more sensitive in cell imaging assays. We discovered that the superior sensitivity of APEX2 arises from its enhanced resistance to H_2O_2–induced inhibition and improved heme incorporation within cells. We determined that APEX suffers from reversible inhibition by H_2O_2, and the resistance of APEX2 to this inhibitory pathway is most likely due to enhanced kinetics in a critical step of the catalytic cycle.



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P40

Pathways of Acetylation by Rtt109-Vps75

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Histone acetylation is one of the main mechanisms to regulate the accessibility of chromatin, which controls gene regulation and DNA repair in eukaryotic cells. Our previous studies have demonstrated that quantitative mass spectrometry based methods can determine the steady-state kinetic parameters of acetylation for different histone complexes catalyzed by Rtt109, a *Saccharomyces cerevisiae*, lysine acetyltransferase (KAT). Not only histone complexes but pre-exisiting histone marks have also shown the ability to alter the specificity of Rtt109.

In the present study, we carried out enzymatic assays to investigate how the pre-exising histone acetylation mark(s) affects Rtt109 specificity for histone H3/H4 tetramer by using targeted MS proteomics. We produced singly acetylated recombinant histones to examine how a single acetylation mark: H3K9, H3K14, H3K23, H3K27, or H3K56 impacts the specificity of Rtt109-Vps75. While H3K9ac and H3K23ac (two original primary sites) have little impact on selectivity, H3K27ac can divert the acetylation preference of Rtt109-Vps75 to H3K56. In addition, all of these single acetylation marks can inhibit Rtt109-Vps75 acetylation with different extents; that is, H3K56ac > H3K14ac > H3K27ac ≅ H3K9ac ≅ H3K23ac. Interestingly, the double acetylation mark (H3K9ac and H3K23ac) also shows the selectivity change to H3K56, which is similar to what H3K27ac mark does, whereas the inhibition ability is the least among all the examined acetylation marks. We also used isotopically labeled histones to understand how the presents of pre-acetylated histones can alter the selectivity by feedback inhibition. These data demonstrate that feedback inhibition is also selective suppressing specific pathways of acetylation. Together, these results provide insight into how pre-existing histone acetylation influences KAT specificity and inhibits the acetylation reaction. We are working to combine this data into a working pathway of acetylation by Rtt109-Vps75.

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Asymmetry and Cooperativity in Nitrogenase

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Nitrogenase catalyzes the reduction of dinitrogen to ammonia, representing the largest input of N into the biogeochemical nitrogen cycle. This difficult reaction involves the delivery of electrons from the Fe protein component to the catalytic MoFe protein component in a process that involves hydrolysis of two ATP per electron delivered by the Fe protein. Eight such rounds of eletron transfer are required for the reduction of dinitrogen.The MoFe protein contains two catalytic halves, each of which binds a Fe protein.

Although the mechanism nitrogenase has been investigated for over four decades, two key questions remain: 1) When is ATP used in the reaction and what catalytic steps are coupled to ATP binding, hydrolysis and product release steps? 2) Why is nitrogenase arranged as two catalytic halves?

We have shown that ATP is hydroyzed post electron transfer[1] and the slow step in the chemistry is phosphate release.[2] Hence the energy from ATP binding is used to form the enzyme complex, ATP hydrolysis is used to drive conformational events after electron transfer and Pi release is used as a switch to signal the end of one cycle of events. We have also demonstrated that the two halves exhibit negative cooperativity: electron transfer events from Fe protein in one half exerts an influence across the entire complex that partially suppresses electron transfer in the other half.[3] Conformational gating controls electron delivery from the Fe protein to the MoFe protein within each half of the complex.

Our work thus presents a novel paradigm for nitrogenase function and the substrate reduction events in both catalytic halves should not be considered as independent events. These mechanistic steps are also conserved in the related nitrogenase-like proteins that function in the chlorophyll biosynthetic pathway.

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P42

Discovery and Characterization of Fused 4-OT: Insights into the Sequence-Structure-Function Relationships and Evolutionary History of the Tautomerase Superfamily

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Fused 4-oxalocrotonate tautomerase (f4-OT) from *Burkholderia lata* is the representative member of a group of 91 homologous proteins that were discovered using Sequence Similarity Networks of the Tautomerase Superfamily (TSF). These f4-OTs were found to be closely related to the main 4-OT-subgroup within the TSF. However, f4-OTs are composed of about twice the number of residues as 4-OTs, ~127 versus ~62. The 3D crystal structure of f4-OT from *B. lata* was solved (1.78Å resolution), which revealed the signature β-α-β-fold of the TSF. Two β-α-β-units of ~58 residues were found to be joined together by a short loop, and stacked in an antiparallel fashion, like that of the short, 62-residue monomers of canonical 4-OT (can4-OT) from *Pseudomonas putida* mt-2 (RMSD: 0.9Å). f4-OT has an N-terminal proline residue buried in a putative active site pocket, similar to can4-OT. Inhibition and labelling studies with 2-oxo-3-pentynoate suggested that Pro-1 in f4-OT is unprotonated at physiological pH, and can thus act as a general base. In can4-OTs, which are generally found in metabolic degradation pathways for aromatic hydrocarbons, Pro-1 acts as a general base in the enol-keto tautomerization of a pathway intermediate. The genomic context of the f4-OTs suggests a similar physiological function. Indeed, f4-OT was found to be a highly efficient tautomerase toward 2-hydroxymuconate (2-HM, $k_{cat}/K_m = 1.5 \times 10^6$ M^{-1} s^{-1}), the physiological substrate of can4-OT. In addition, phenylenolpyruvate (PP), the signature substrate for the Macrophage Migration Inhibitory Factor subgroup within the TSF, was identified as a substrate as well ($k_{cat}/K_m = 5.7 \times 10^5$ M^{-1} s^{-1}). Soaking experiments have resulted in 3D crystal structures of f4-OT in complex with the products of the 2-HM- and PP-reactions, which is the first time this has been achieved for any member of the TSF. Mutational and mechanistic studies inspired by these findings are ongoing. Lastly, f4-OT may provide a glimpse into the early evolutionary history of the TSF. An interesting feature of f4-OT is that it still retains a 'Pro-1' at the start of its second β-α-β-unit. The diversification of function displayed by the present-day TSF appears to be linked to the fusion of two β-α-β-units into a single polypeptide. f4-OTs may thus allow us to investigate how structural diversification may have contributed to mechanistic diversification within the TSF.

Rapid Timescale Binding Analysis of T4 DNA Ligase DNA-Binding

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DNA ligases, essential to both *in vivo* genome integrity and *in vitro* molecular biology, catalyze phosphodiester bond formation between adjacent 3'-OH and 5'-phosphorylated termini in dsDNA. This reaction requires enzyme self-adenylylation, using ATP or NAD^+ as a cofactor, and AMP release concomitant with phosphodiester bond formation. Here, we present the first fast-timescale binding kinetics of T4 DNA ligase to both nicked substrate DNA (nDNA) and product-equivalent non-nicked dsDNA, as well as binding and release kinetics of AMP. The described assays utilized a fluorescein-dT labeled DNA substrate as a reporter for ligase·DNA interactions via stopped-flow fluorescence spectroscopy. The analysis revealed that binding to nDNA by the active adenylylated ligase occurs in two steps, an initial rapid association equilibrium followed by a transition to a second bound state prior to catalysis, confirming prior proposals of transient binding followed by closing to a productive ligase-nDNA complex. However, we also observe that the two-step binding-closing mechanism is not limited to only substrate binding by adenylylated ligase. These observations demonstrate the mechanistic underpinnings of competitive inhibition by rapid binding of non-substrate DNA, and of substrate inhibition by blocking of the self-adenylation reaction through nick binding by deadenylylated ligase. Based on this analysis, we additionally propose the true rate-determining step in turnover to be a conformational change corresponding to the opening of the enzyme after phosphodiester bond formation, permitting rapid dissociation of products.

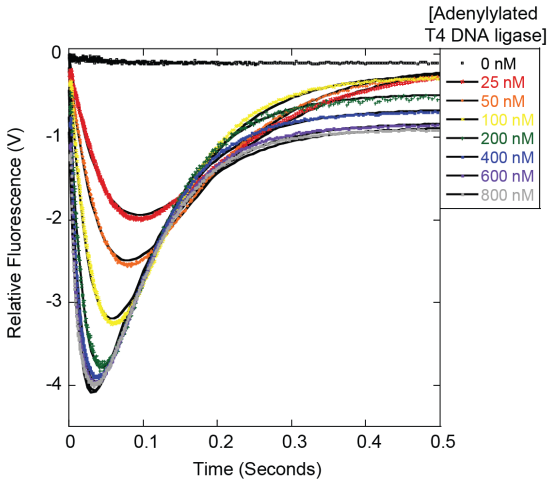

Figure 1: Stopped-flow fluorescence analysis of binding and sealing of a 24mer internally FAM-dT labeled nicked substrate by adenylylated T4 DNA Ligase

P44

Glycosylase Activity on Nucleosome Core Particles with Varied Lesion Positioning

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If left unrepaired, damage to genomic DNA causes mutations and can be cytotoxic. In particular, oxidative damage via reactive oxygen species produces the common DNA base lesion 8-oxo-7,8-dihydroguanine (8-oxoG), which is repaired via the base excision repair pathway. The first step in base excision repair is the recognition and removal of the 8-oxoG lesion by a glycosylase enzyme. In humans, this enzyme is Oxoguanine glycosylase 1 (hOGG1) and in bacteria the enzyme is Formamidopyrimidine glycosylase (Fpg). To date, most studies on these enzymes have used short DNA substrates. However, cellular DNA is packaged as chromatin, with short 145-mer units of DNA wrapped around histone protein octamers. Therefore, a model system that more closely represents the biological environment of cellular DNA is the nucleosome core particle, which consists of a 145mer DNA strand wrapped around a single histone octamer bundle. We hypothesize that the nucleosome environment could present challenges to enzymatic catalysis such as steric obstruction of the lesion or electrostatic interference of the histone tails. In this study, we use enzyme kinetics to investigate the ability of hOGG1 and Fpg to remove oxidized DNA lesions in the context of nucleosome core particle. Lesions with varied rotational position (for example, out toward solution or in toward the histone core) and translational position (relative to the dyad axis of symmetry) are investigated using single turnover kinetic techniques.

P45

Asymmetry in Substrate Reduction by the Nitrogenase-like Dark Operative Protochlorophyllide Oxidoreductase (DPOR) Complex

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Chlorophyll is the light harvesting molecule present in all photosynthetic autotrophs. In the absence of light, the penultimate step in chlorophyll synthesis is catalyzed by the multiprotein Dark Operative protochlorophyllide Oxidoreductase (DPOR) enzyme complex. DPOR stereo-specifically reduces the C17-C18 double bond of protochlorophyllide (Pchlide) to yield chlorophyllide (Chlide). This reduction requires 2 cycles of electron transfer and uses 4 ATP molecules. The DPOR complex is composed of an electron donor (L-protein) and the catalytic electron acceptor (NB-protein). The L-protein is a homodimer with a bipartite 4Fe-4S cluster and also contains two sites for ATP binding and hydrolysis. In addition, the DPOR complex is composed of two identical catalytic halves arranged as mirror-images.

In the presence of ATP, the NB-protein forms a transient complex with the L-protein which initiates the sequence of electron transfer associated events leading to substrate reduction. How ATP is used to coordinate the multi-electron reduction reaction is poorly understood. To explore the precise function of each ATP molecule in the reaction we have generated a linked-L protein dimer carrying mutations in only one or both of the ATP binding sites. A perturbation of a single ATP binding site renders the complex inactive for substrate reduction. Current and future work will be aimed at identifying the mechanistic steps that are affected when the ATP-binding sites are mutated.

P46

Inhibition of Bacterial HMM DD-Peptidases by Peptidyl Thioesters

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Bacterial DD-peptidases (PBPs) are the enzymes that catalyze cross-linking of the bacterial cell wall. Hence, their natural substrate should be a part of peptidoglycan structure. The substrate specificity of these enzymes, however, is not yet completely understood. To study the reactivity and substrate specificity of *E. coli* membrane-bound DD-peptidases, a thioester substrate analog, phenylacetyl glycyl-D-thiolactic acid (PGT) was used. The reaction of PGT with membrane-bound DD-peptidases has been studied using the fluorescent probe Bocillin Fl. The reaction of high molecular mass membrane-bound PBPs of *E. coli* (PBPs 1ab, 2 and 3) was found not to go to completion. Similar results with solubilized PBP2 (sPBP2) were obtained. Native-PAGE gel data suggested more than one form of PBP is present in solutions of sPBP2. For both the solubilized and membrane-bound DD-peptidases, data were therefore fitted by the Kintek program to a scheme incorporating protein dimers for *E. coli* membrane-bound PBP1ab, 2 and 3 and sPBP2. The results show how the reactivity of PBPs can be affected by protein protein interactions.

P47

Weak Interactions between Typical Crowders and the Ligands of DHFR on Decreased Enzyme Activity

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

Effects on ligand binding and enzyme activity by crowding macromolecules at concentrations similar to those *in vivo* were examined. Isothermal titration calorimetry (ITC) and enzyme kinetic assays were used to detect effects of molecular crowders by monitoring activity of the (DHFR)-NADPH or DHF complex and the ternary DHFR-DHF-NADPH complex in the presence of these crowders. To recreate the conditions of molecular crowding *in vivo,* the binding of the enzyme-ligand complexes in the presence of molecular weight crowding agents was examined. Analysis of the K_d's and K_m's indicated a correlation between increased molecular crowding in the solution and weakened binding of the ligands to two structurally unrelated DHFRs. Additionally, molecular weight crowders also altered the k_{cat} of DHFRs. These findings indicate an importance of weak, transient interactions between molecular crowding and DHFR ligands.

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

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Using published crystal structures, we argued previously for the presence of extensive orbital alignments within and between key strands of serine protease/protein inhibitor complexes. We also discussed their possible roles in transition-state polarization as well as kinetic evidence of through-strand electronics in a non-enzymatic acyl-transfer reaction (1). The details and context of such alignments in the trypsin and subtilisin systems present no obvious reason that the alignments should be unique to those systems, or to serine proteases, or to proteases in general. If such orbital pathways facilitate charge redistributions in suitable strand conformations, and if they help to manage charge distribution in rate-determining steps, and if they emerge simply through mutation and natural selection, then one might expect extensive orbital alignments to evolve in almost any enzyme system. This poster will review key aspects of our earlier work and present ongoing analyses of the HIV-1 protease system and the HEW lysozyme system. Figure 1 shows some extensive orbital alignments in a glycosylated HEW lysozyme mutant.

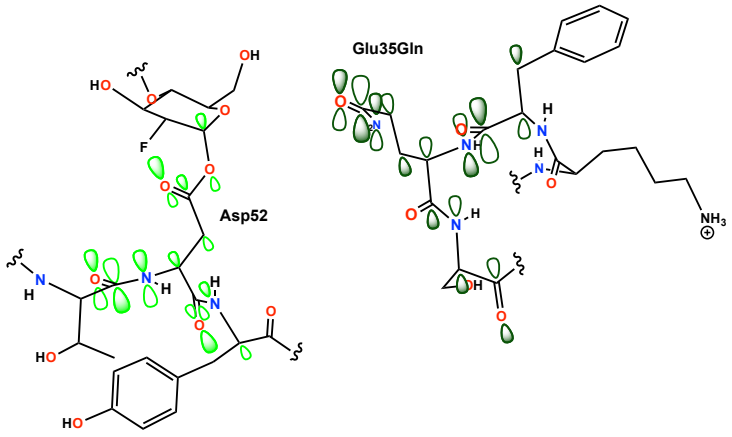

Figure 1. Extensive orbital alignments in the active site of a glycosylated HEW lysozyme mutant, from a crystal structure (PDB 1H6M) by Withers et al. (2).

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Loop-Swapped Chimeras and Tryptophan Mutants of the Protein Tyrosine Phosphatases Yoph and PTP1B Investigate the Connection Between WPD-Loop Motion and Catalysis

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Protein tyrosine phosphatases (PTPs) cleave their substrates via a ping-pong mechanism with a phosphocysteine intermediate. Despite highly superimposable active sites and the same mechanism and transition state, catalytic rates vary by three orders of magnitude within the PTP family. PTPs share a structural feature of a small dynamic loop, the WPD-loop, which contains a catalytically important general acid. Previous work showed that rate difference between two well-studied PTPs, PTP1B and YopH, are correlated with different rates of motion of their WPD-loops.(1) PTP1B is a human protein tyrosine phosphatase that dephosphorylates the insulin receptor, among other physiological roles. YopH is an essential virulence factor in *Yersinia*. Two approaches are being used to investigate factors that affect loop dynamics and the connection to catalysis. In one, chimeras have been made in which the WPD-loop from PTP1B is swapped into the analogous region of YopH. Another approach makes conservative mutations to the conserved tryptophan residue in the WPD-loop. In PTPs, the indole side chain of this residue slides within a hydrophobic pocket as the WPD loop moves. Kinetic results show that both alterations affect the rate-determining, second chemical step more than the rapid first chemical step of phosphocysteine formation. X-ray crystal structures, kinetic analysis, stopped-flow studies and kinetic isotope effects reveal consequences on protein structure and chemistry in the loop-exchanged chimeras and tryptophan mutants.

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P50

Structural Dynamics of SufS Cysteine Desulfurase Persulfide Intermediate

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Fe-S clusters are essential cofactors required for cellular functions. Biogenesis of Fe-S clusters in *Escherichia coli* under oxidative stress and iron depletion is carried out by the Suf system. Proteins involved in Suf pathway work together to mobilize sulfide and iron, form Fe-S clusters, and regulate the assembly pathway by protein-protein interactions. The *E. coli* cysteine desulfurase SufS and its accessory protein SufE work together to mobilize persulfide from L-cysteine and transfer it to the scaffold protein SufB as part of the $SufBC_2D$ complex. The overall SufS reaction occurs in two half reactions: (1) transfer of sulfide from cysteine to form an enzyme bound persulfide intermediate followed by (2) transfer of the persulfide to the accessory protein SufE. The x-ray crystallographic structures of homodimeric SufS with and without C364 persulfide have previously been reported and show static structural changes at the active site. Here, $SufS_{apo}$ was treated with excess cysteine to create the persulfide intermediate $SufS_{per}$ and amide hydrogen deuterium exchange mass spectrometry (HDX-MS) is used to investigate dynamic changes that occur upon persulfuration of SufS. HDX-MS analysis shows that conformational changes occur at the SufS dimer interface (peptides 88-100 and 243-255) in addition to changes predicted by the $SufS_{per}$ structure. Superposition of a structure containing a PLP-bound product analog indicates changes in the interactions of R92, E96, and E250 in persufurated SufS. These structural changes are nicely correlated with HDX-MS results and provide a possible mechanism for active site communication.

P51

Nucleoside Analogue Triphosphates Allosterically Regulate Human Ribonucleotide Reductase and Identify Chemical Determinants that Drive Substrate Specificity

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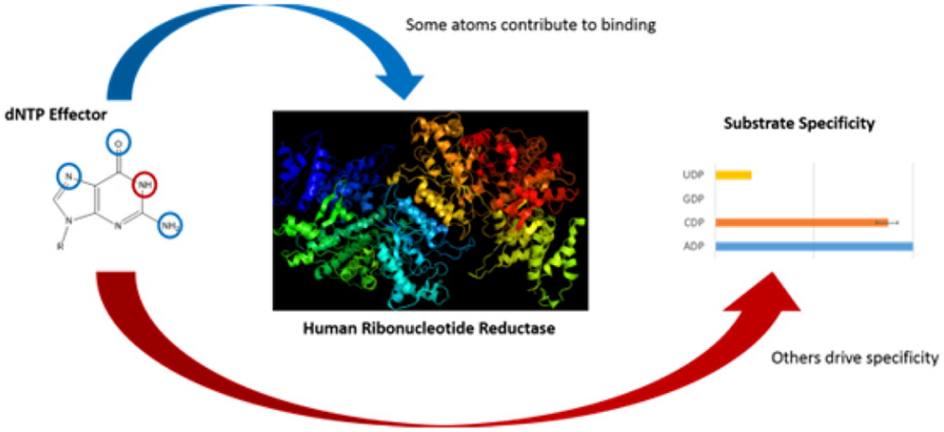
Ribonucleotide reductase (RR) enables organisms to grow and reproduce by providing the deoxynucleotides necessary for DNA replication. Human RR can reduce any of the four nucleoside diphosphates at the 2′ position, and binding of dNTP effectors (ATP/dATP, dGTP, dTTP) modulates substrate specificity at an allosteric site near the active site. This binding is known to change the conformation of a short loop (loop 2) that bridges the allosteric site and the active site. Although the relationship between effector binding and enzymatic specificity is well-established, the specific interactions through which RR recognizes the effector and changes the conformation of loop 2 remain untested. Here, we systematically interrogate the contribution of each effector functional group using a panel of dNTP analogues and multiple substrate kinetic assays. We find that the 3, 4, and 5 groups of the dTTP nucleobase all make contributions to specificity, but the N1 group of the purine effectors is the sole determinant of specificity between them. We then confirm a key prediction from crystal structures by showing that interactions with amino acid residue D287 in loop 2 are essential for perturbing loop 2's conformational space. The current study sheds light on a key mechanism by which organisms generate balanced dNTP pools for timely and accurate DNA replication and repair.



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Enzyme Evolution

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The molecular basis of enzyme evolution is underexplored, which limits our ability to understand the merge of drug-resistance and to rationally engineer biocatalysts for new functions. To gain a better understanding of how enzyme catalyzed covalent bond activation evolves, we trace the physical nature of the chemical step along the evolution of a primitive circularly permuted dihydrofolate reductase (cpDHFR).[1] Our hypothesis is that as a primitive enzyme evolves, the active site of the enzyme will better reorganize to achieve an ideal transition state for the chemical step, in conjugation with improvement in its catalytic-efficiency (k_{cat}/K_M). To investigate these hypotheses, we constructed a primitive enzyme, in which the chemical step is far from ideal, and followed that chemical step (C-H→C hydride transfer) as the enzyme evolves. We evolved the primitive variant by directed evolution and followed the chemical step through measurements of temperature dependence of intrinsic kinetic isotope effects (KIE).[2-4] KIEs and their activation parameters were measured to examine the evolution of the chemical step from a poor transition-state ensemble in the primitive enzyme to an accurate transition-state ensemble in mature variants. Indeed, the cpDHFR appears to be quite primitive by two parameters: (i) its k_{cat}/K_M is slower by 5 orders of magnitude than E. coli DHFR and 7 orders of magnitude slower than human enzyme; and (ii) it presented a steep temperature dependence of its intrinsic KIEs (reflecting poorly organized transition state). After three generations of accelerated evolution, both k_{cat}/K_M and the transition state of the chemical step improved, hence corroborating our hypothesis. Interestingly, in the first two generations, the chemical step (reflected in ΔEa, Fig. 1) improved faster than the evolutionary-pressure related to catalytic-efficiency (k_{cat}/K_M), as will be discussed in more detail at the presentation. The findings further indicate that mutations along evolution occur both in and far from the active site. The active site mutations could be potentially predicted by rational design. However, the remote mutations along evolution are beyond the current predictive power, and would benefit from empiric findings as presented here. This study sheds light on an interesting biological question and would impact our understanding of the evolution of drug resistance in pathogens and cancer, as well as enzyme engineering.

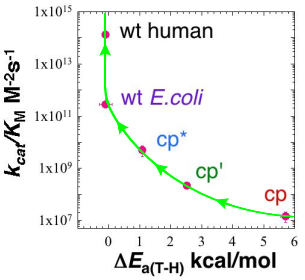

Figure 1. Observed relations between the evolving enzyme's catalytic efficiency (k_{cat}/K_M) and the accuracy of the transition-state of its chemical step (reflected in the temperature dependence of intrinsic KIEs, ΔEa).

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

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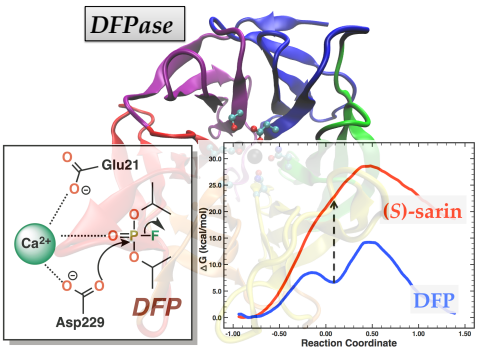
Organophosphorus (OP) nerve agents such as (*S*)-sarin are among the most highly toxic compounds that have been synthesized. Engineering enzymes that catalyze the hydrolysis of nerve agents ("bioscavengers") is an emerging prophylactic approach to diminishing their toxic effects. Although its native function is not known, diisopropyl fluorophosphatase (DFPase) from *Loligo vulgaris* catalyzes the hydrolysis of OP compounds. Here, we investigate the mechanisms of diisopropylfluorophosphate (DFP) and (*S*)-sarin hydrolysis by DFPase with quantitatively-accurate quantum mechanical/molecular mechanical (QM/MM) umbrella sampling simulations. We find that the mechanism for hydrolysis of DFP involves nucleophilic attack by Asp229 on phosphorus to form a pentavalent intermediate. P—F bond dissociation then yields a phosphoacyl enzyme intermediate in the rate-limiting step. The simulations suggest that a water molecule, coordinated to the catalytic Ca^{2+}, donates a proton to Asp121 and then attacks the tetrahedral phosphoacyl intermediate to liberate the diisopropylphosphate product. In contrast, the calculated free energy barrier for hydrolysis of (*S*)-sarin by the same mechanism is highly unfavorable, primarily due to the instability of the pentavalent phosphoenzyme species. Instead, simulations demonstrate that hydrolysis of (*S*)-sarin proceeds by a mechanism in which Asp229 activates an intervening water molecule for nucleophilic attack on the substrate. These findings may lead to improved strategies for engineering DFPase and related six-bladed β-propeller folds for more efficient degradation of OP compounds [1].



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Transient Kinetics of Protein Arginine Methyltransferase 1 (PRMT1)

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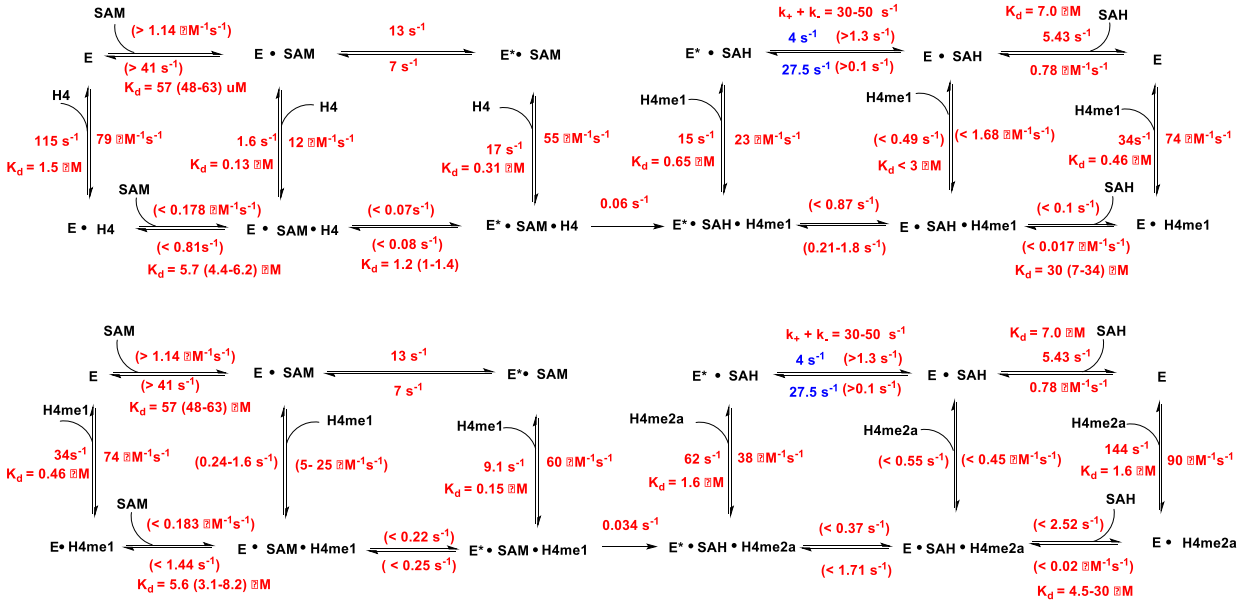
Protein arginine methyltransferases (PRMTs) catalyze the transfer of the methyl group from S-adenosyl-L-methionine (AdoMet) to the guanidino group of arginine residues in protein substrates, resulting in mono and di-methylarginine residues. Protein arginine methylation is an important posttranslational modification mark regulating epigenetics and many other cellular pathways. We sought to resolve significant kinetic steps of PRMT1 catalysis by combining steady-state and transient kinetics techniques. We have constructed a novel turnover model which reveals critical information about the ternary complex formation and methyl transfer process. Methyl transfer was found to be the rate limiting step. Significantly, the catalysis is found to follow a unique mechanism in which PRMT1 is able to randomly bind AdoMet or peptide substrate to form binary complex but follows a kinetically preferred (ordered) pathway to form the ternary complex. The delineation of PRMT1 transient kinetic mechanism provides new insights to understand biological function of arginine methylation and to design potent PRMT inhibitors.



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P55

Novel Non-Hydroxamate Inhibition of Histone Deacetylase 8

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Histone deacetylases (HDACs) have become an attractive target in cancer therapies. There are currently four FDA approved HDAC inhibitors. Presently, inhibitor design is based on targeting the divalent metal present in the active site of HDACs through functionalization around a hydroxamic acid warhead. However, the hydroxamate moiety has poor pharmacokinetics and these inhibitors have high toxicity. Using a fragment-based metalloenzyme inhibitor library focused on non-hydroxamate compounds[1], we have identified compounds with novel functional groups which inhibit HDAC8. A subset of these compounds additionally showed inhibition that was dependent on the HDAC8 bound metal (Fe(II) vs Zn(II)). While the fragments show promise for further development into HDAC inhibitors, the mechanism of inhibition needs to be determined. There are three likely mechanisms by which the compounds are inhibiting HDAC8: (1) chelating metal in a manner similar to EDTA; (2) stripping metal directly from the active site; or (3) acting as competitive inhibitors by binding in the active site. The time dependence of inhibition provides insight in the mechanism of inhibition since metal dissociation from HDAC8 is slow. These studies identify inhibitors that function as chelators and competitive inhibitors.

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P56

Ghrelin Acylation by Ghrelin *O*-Acyltransferase: Investigation of Ghrelin Recognition During Hormone Processing and Small Molecule Inhibitor Development

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Ghrelin is a peptide hormone involved in appetite stimulation, regulation of glucose homeostasis, and a range of other physiological and neurological pathways.[1] Ghrelin requires an unique posttranslational modification, octanoylation of a serine side chain, to bind its cognate receptor and activate signaling.[2] The enzyme that catalyzes this modification, ghrelin *O*-acyltransferase (GOAT), was identified in 2008 as a member of the membrane-bound *O*-acyltransferase (MBOAT) enzyme superfamily.[3] Ghrelin and GOAT are receiving increased interest as potential targets for treatment of obesity, diabetes, appetite dysregulation, and other diseases connected to ghrelin signaling. As ghrelin is the only identified substrate of GOAT, blocking ghrelin acylation using GOAT inhibitors potentially offers a specific and targeted therapeutic avenue to treat conditions impacted by ghrelin activity.

Our research focuses on understanding how ghrelin is recognized and modified by human GOAT (hGOAT), and through structure-activity analysis of both peptide substrates and inhibitors we have identified multiple functional groups involved in substrate recognition by hGOAT.[4] Using biochemical and structural methods, we aim to identify key domains within hGOAT responsible for substrate binding and catalysis. Locating the active site and catalytic residues within hGOAT is essential for understanding this unique chemical modification within the ghrelin maturation pathway. In related studies, screening of a library of small molecules has identified new small molecule inhibitors of GOAT, with several of these compounds exhibiting potent activity in cell-based ghrelin acylation assays. Structure-activity analysis of these new inhibitors provides insight into the potential mechanism for GOAT-catalyzed ghrelin acylation, laying a foundation for design of mechanism-based hGOAT inhibitors. By chemically defining the mechanism by which GOAT modifies ghrelin to its biologically active form, we are advancing the design of potent hGOAT inhibitors and moving towards the exploitation of ghrelin signaling for therapeutic intervention in diabetes and other diseases.

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P57

IMP Dehydrogenase Forms Filaments in Response to Dysregulation of Guanine Nucleotide Homeostasis

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'Rod and ring' structures (RRs) are conserved, non-membrane-bound, polymeric filament-like structures (see Fig. 1) composed, in part, of inosine 5′-monophosphate dehydrogenase (IMPDH), a key enzyme in *de novo* guanine nucleotide biosynthesis. Early studies showed that rod (~3-10 μm in length) and ring (~2-5 μm in diameter) formation can be induced in cultured mammalian cells by IMPDH inhibitors, such as ribavirin or mycophenolic acid, or by glutamine analogues, like DON (6-diazo-5-oxo-L-norleucine) or acivicin. These inhibitors can induce RR formation in all cell types examined to date, and typically cause several RR structures to form within the same cell, with rods being more common than rings. Interestingly, rings are more prevalent in undifferentiated mouse embryonic stem cells, where >90% of cells contain RRs under normal culture conditions (without drugs), suggesting that RRs function in rapidly proliferating cells. Cytidine triphosphate synthase, which catalyzes the ATP-dependent amination of UTP to CTP using glutamine as a nitrogen donor, can also localize to IMPDH-based RRs or form independent filamentous structures after DON treatment, even within the same cell. Later studies showed that glutamine deprivation or treatment with methionine sulfoximine, a glutamine synthetase inhibitor, promote RR assembly. When human HeLa or Hep3B cells are deprived of serine, RRs form in a similar manner. We have traced these effects to serine hydroxymethyltransferase-2 (SHMT2) and dihydrofolate reductase (DHFR), pivotal enzymes in one-carbon metabolism and nucleotide biosynthesis. DHFR inhibition by the anticancer and immunosuppressant drug methotrexate or siRNA-mediated knockdown of DHFR or SHMT2 lead to increased RR formation. RRs assemble when guanine nucleotide synthesis is inhibited, and disassemble after the addition of guanine nucleotide precursors, suggesting that RR formation acts as an adaptive homeostatic mechanism in response to guanine nucleotide deficiency. Assembling dynamic enzyme polymers like RRs might be a way to regulate metabolism, enhance proliferation, or control the stability and subcellular localization of IMPDH and other RR components that have yet to be determined.

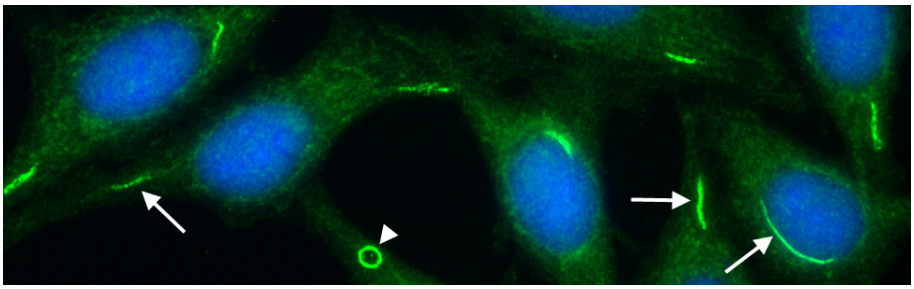

Figure 1. Human HEp-2 cells showing both rod (arrows) and ring (arrowhead) structures. Nuclei are counterstained with DAPI (blue)

Novel Probes for Nucleobase Transporters

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The transport of purine and pyrimidine nucleobases is central to nucleotide metabolism and essential for the delivery of numerous therapeutics, yet the proteins and mechanisms that govern this process are poorly understood, particularly in humans. Nearly all organisms express several classes of proteins that selectively transport nucleobases, both actively and passively, into and out of cells. In humans, however, no specific nucleobase transporters have yet been identified. The only known human proteins that accept nucleobases as substrates do so with low affinity and by an unknown mechanism. In addition, the observed nucleobase transport kinetics in human cells cannot be accounted for by this single class of transporters. The identification and characterization of nucleobase transporters has been significantly hindered by the lack of specific and accurate tools. Thus, there is a critical need to develop tools for the study of nucleobase transport and to use these tools to determine the identity, substrate specificity and mechanisms of human transport proteins involved in this process.

In this project, we will take a highly integrative chemical biology approach to capture and charaterize unknown nucleobase transporters and receptors in humans. We have developed a series of bifunctional nucleobase analogs that are effective in tagging and isolating potential nucleobase transporters. Novel biochemical assays will also be developed to enable the facile and accurate characterization of transport kinetics and substrate specificity. We expect these new tools to significantly expand the scope of our current capabilities for drug discovery and development of nucleotide antimetabolites

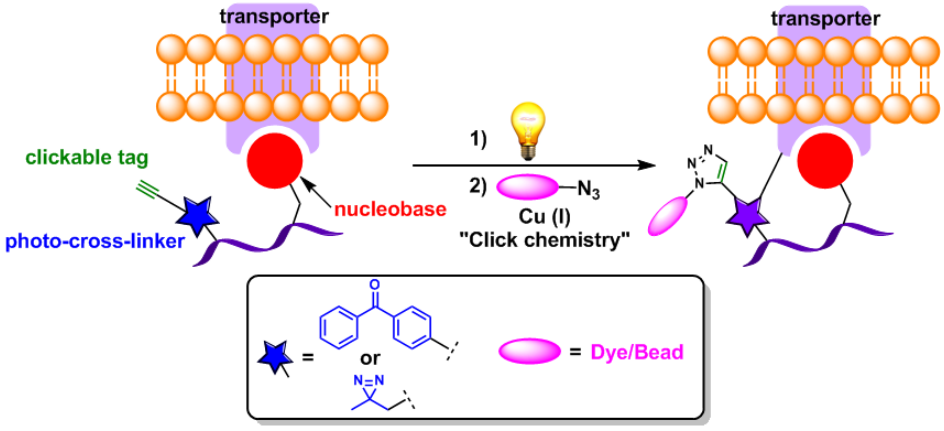

Figure 1. Schematic representation of the labeling of transporters using bifunctional probes.

P59

Mechanistic and Structural Investigation of Protein-Only Ribonuclease P

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Ribonuclease P (RNase P) is an essential endonuclease responsible for the removal of the extraneous 5' sequence of precursor transfer RNA (tRNA). Human mitochondrial RNase P (Hu MtRNase P) is a protein-only RNase P, responsible for catalyzing the 5' end processing of the 22 mitochondrial encoded tRNAs. Hu MtRNase P is a protein complex composed of three subunits: MRPP1, an S-adenosyl-methionine dependent methyltransferase; MRPP2, a dehydrogenase/reductase; and MRPP3, a metallonuclease. The MRPP3 subunit of Hu MtRNase P is an ortholog of the protein-only mitochondrial RNase P enzyme, PRORP1, found in *A. thaliana*. Both MRPP3 and PRORP1 contain a Nedd4-Bp1, YacP (NYN) metallonuclease domain, and are believed to share a putative catalytic mechanism utilizing a divalent metal-activated water nucleophile. However, unlike PRORP1, MRPP3 differs in its requirement of two additional proteins (MRPP1 and 2), questioning the roles both MRPP1 and MRPP2. Here I present a structural and mechanistic comparison of the human and plant PRORP through protein-RNA footprinting and kinetic analysis, concentrating on the roles MRPP1 and MRPP2 play in Hu MtRNase P catalysis.

Rational Approach to Discovery of Isocitrate Lyase Inhibitor

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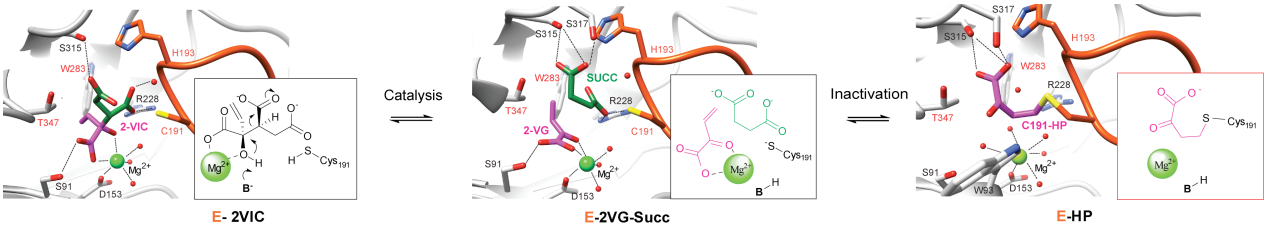
Isocitrate lyase (ICL) catalyzes a retro-aldol cleavage of isocitrate into succinate and glyoxylate which circumvents two steps involving loss of carbon dioxide in the tricarboxylic acid cycle. This alternative pathway is known as "glyoxylate shunt" and found in bacteria, fungi and plants, but not in mammals. Isocitrate lyase has been shown to be essential in the persistent infection of *Mycobacterium tuberculosis,* and thereby constitutes a target for chemotherapy for the treatment of tuberculosis. However, in the past decade, high-throughput screening of large small-molecule compound libraries has apparently yielded no chemical starting points for the development of lead or drug candidate compounds that could provide new therapeutics for the treatment of tuberculosis. Accordingly, we have initiated a rational design approach for the development of novel inhibitors of *Mycobacterium tuberculosis* isocitrate lyase. Here, we report 2-vinyl-D-isocitrate (2-VIC) as the first known mechanism-based inactivator of *Mtb* ICL1 and ICL2 which undergoes the enzyme-catalyzed, retro-aldol cleavage, yielding succinate and a Michael accepter, 2-vinylglyoxylate; the later inactivates the enzyme via its reaction with ICL active-site Cys. Analysis of a complex of ICL1:2-VIC by electrospray ionization mass spectrometry and x-ray crystallography confirmed the formation of a covalent S-homopyruvoyl adduct of the active-site Cys191. 2-VIC displayed kinetic properties consistent with covalent, mechanism-based inactivation of ICL1 and ICL2 with high efficiency (partitioning ratio < 1) and comparable to that of benzothiazinone, a potent anti-mycobacterial agent acting as a mechanism-based inactivator of DprE.



P61

Synthetic Polyketide Enzymology: Platform for Biosynthesis of Antimicrobial Polyketides

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We applied enzymological principles in synthetic biology and describe its use as an enabling platform in synthetic biology for the purposeful production of compounds of biomedical and commercial importance. In particular, we demonstrated the use of synthetic polyketide enzymology as a means to develop lead polyketide based compounds for antimicrobial therapeutics, as exemplified by the modular coupling of acid:CoA ligases to type III polyketide synthases in the biosynthesis and development of polyketide-based biochemicals. Using wild-type and rationally designed mutants of a type III polyketide synthase isolated from Oryza sativa (OsPKS), we produced a chemically diverse library of novel polyketides and identified two bioactive antimicrobials, 4-hydroxy-6-[(1E)-2-(4-hydroxyphenyl)ethenyl]-2H-pyran-2-one (bisnoryangonin) and 3,6,7-trihydroxy-2-(4-methoxybenzyl)-4H-1-benzopyran-4,5,8-trione (26OH), respectively, from a screen against a collection of Gram-positive and Gram-negative bacteria. The purification, crystallization, and structural resolution of recombinant OsPKS at 1.93 Å resolution are also reported. Using the described route of synthetic polyketide enzymology, a library of OsPKS mutants was generated as an additional means to increase the diversity of the polyketide product library. We expect the utility of synthetic enzymology to be extended to other classes of biomolecules and translated to various purposeful functions as the field of synthetic biology progresses.

P62

Mechanisms of Proton Relay and Product Release by Class A β-Lactamase at Ultrahigh Resolution

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Class A β-lactamases hydrolyze β-lactam compounds, and are one of the main resistance mechanisms against β-lactam antibiotics. Despite numerous studies over many years, key questions remain about the acylation general acid catalysis involved in protonating the substrate ring nitrogen, with Lys73 and Lys234 alternatively hypothesized to be the ultimate proton source to reprotonate Ser130, the proposed general acid. A 0.85 Å resolution crystal structure of CTXM-14 E166A mutant complexed with a decarboxylated product of a ruthenocene-conjugated penicillin allowed us to capture the hydrogen bonding network following the protonation of the leaving group and, for the first time, unambiguously show that the ring nitrogen donates a proton to Ser130, which in turn donates a proton to Lys73. These results, together with previous findings showing Lys73's function as the general base to activate Ser70, demonstrate the critical role this residue plays in the proton relay network during catalysis. Meanwhile, a 1.30 Å resolution complex structure with the original hydrolyzed product, the first such structure with an intact serine residue, reveals previously unseen conformational changes of key catalytic residues such as Ser70 and Lys73 during the release of the product. In addition, in both product complexes, Ser70 and Lys73 do not form a favorable hydrogen bond, in contrast to the short hydrogen bond (~2.5 Å) with a delocalized hydrogen found in a previous non-covalent complex. These observations demonstrate the influence of small molecule ligands on the hydrogen-bonding network of the catalytic residues and consequently, the catalytic process.

P63

Studying Proton Transfer in the Mechanism and Inhibition of Serine β-Lactamase Acylation

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Beta-lactam antibiotics (e.g. penicillins) are the most widely used antibiotics in the treatment of a diverse range of bacterial infections; however, the production of beta-lactam degrading enzymes known as beta-lactamases threatens their clinical use. A major breakthrough in combating this resistance mechanism was the development of a broad-spectrum beta-lactamase inhibitor known as avibactam. Avibactam forms a covalent bond with the catalytic serine of class A beta-lactamases. Key questions remain about why this bond is stable against hydrolysis because the catalytic water and Glu166, the general base, appear to be intact in the complex and capable of attacking the covalent linkage with the inhibitor. Here we present a preliminary 0.83 Å resolution crystal structure of the class A beta-lactamase, CTX-M-14, with avibactam, showing a unique active site protonation state trapped by the compound including a neutral Glu166 and a neutral Lys73. This structure suggests that the unique side chain of avibactam stabilizes a neutral Lys73, preventing it from extracting a proton from Glu166. Subsequently, Glu166 is not able to serve as the general base to activate the catalytic water for the hydrolysis reaction. Together with our previous structures, we now have the complete picture of the proton transfer process during the acylation reaction, from apo (0.79 Å), non-covalent complex mimicking pre-covalent Michaelis complex (0.89 Å), acylation transition state analog complex (0.85 Å) to the current complex showing the state immediately after the transition state. In addition to tracking the movement of a proton from the catalytic serine to the substrate through four key steps, these structures illustrate how ligand/substrate binding can change the protonation states of catalytic residues, how the general base can stabilize the transition state through sharing a proton equally with the catalytic serine with a low-barrier hydrogen bond, and how inhibition can be achieved by affecting residue pK_a and hindering the proton transfer process.

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Peroxiredoxin Catalysis at Atomic Resolution

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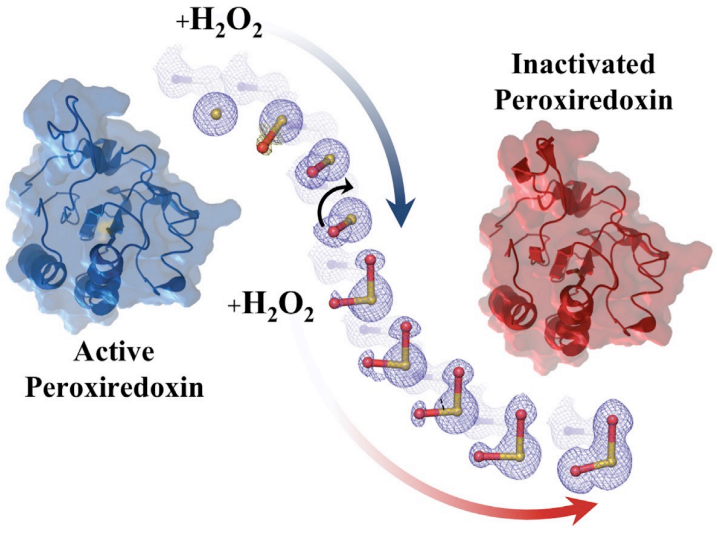
Peroxiredoxins (Prxs) are ubiquitous cysteine-based peroxidases that guard cells against oxidative damage, are virulence factors for pathogens, and are involved in eukaryotic redox regulatory pathways. We have analyzed catalytically active crystals to capture atomic resolution snapshots of a PrxQ subfamily enzyme (from *Xanthomonas campestris*) proceeding through thiolate, sulfenate, and sulfinate species. These analyses provide structures of unprecedented accuracy for seeding theoretical studies, and reveal conformational intermediates giving insight into the reaction pathway. Based on a highly non-standard geometry seen for the sulfenate intermediate, we infer that the sulfenate formation itself can strongly promote local unfolding of the active site to enhance productive catalysis. Further, these structures reveal that preventing local unfolding, in this case via crystal contacts, results in facile hyperoxidative inactivation even for Prxs normally resistant to such inactivation. This supports previous proposals that conformation-specific inhibitors may be useful for achieving selective inhibition of Prxs that are drug targets.



Perkins, A., Parsonage, D., Nelson, K. J., Ogba, O. M., Cheong, P. H., Poole, L. B., and Karplus, P. A. (2016) Peroxiredoxin Catalysis at Atomic Resolution, Structure 24, 1668-1678

P65

Synthetic Enzymology and its Applications in Bioremediation

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With the rise in consumerism, electronic waste is a large and growing problem in society today. Due to the high concentration of gold present in this waste, there is interest in the treatment and recovery of these precious metals. Current treatment methods such as chemical cyanidation and leaching have proven to be environmentally unsustainable. Thus, environmentally friendly, sustainable alternatives of recovering gold are being sought after. One potential solution lies in the use of metal reductases like mercuric reductase (MerA). MerA catalyses the reduction of cytosolic mercuric ions (Hg^{2+}) to ground state elemental mercury (Hg^{0}).

The enzyme has shown substrate promiscuity with respect to gold and thus, in the search for an efficient "gold reductase", MerA may serve as a potential candidate. The Au^{3+} reducing capabilities of MerA have been characterized and a combination of semi rational and directed evolution approaches are being undertaken to improve gold reduction capabilities. A sequence similarity network (SSN) using Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST) has also yielded other enzyme targets for gold reduction capabilities.

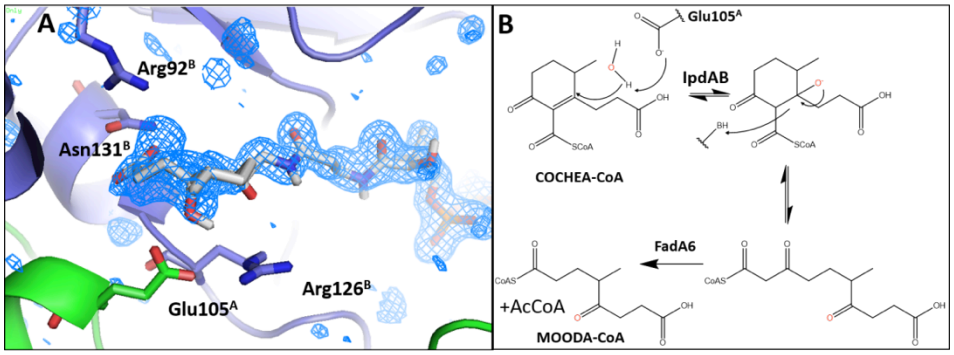

Figure 1. (A) Structure of IpdAB:COCHEA-CoA complex. (B) Proposed mechanism of ring C openin

Propionate-3-nitronate is a Covalent, Irreversible MtICL Inhibitor

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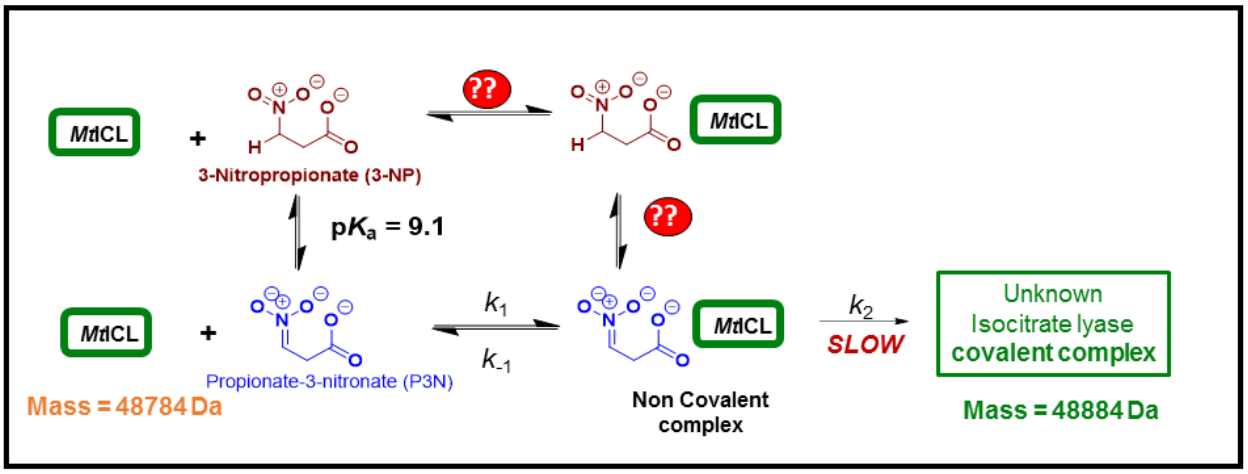
Mycobacterium tuberculosis, the causative organism for tuberculosis, is responsible for the highest mortality rate among bacterial pathogens. Persistent *M. tuberculosis* infections depend on the glyoxylate shunt, a carbon-conserving bypass in the tricarboxylate cycle consisting of isocitrate lyase (ICL) and malate synthase. Since these enzymes are absent in humans but essential to mycobacteria, they are potential drug targets. ICL catalyzes the reversible retro-aldol cleavage of isocitrate into succinate and glyoxylate. 3-Nitropropionate (3-NP) is an analog of succinate that demonstrates slow-onset inhibition of the enzyme, presumably via its conjugate base form, propionate-3-nitronate (P3N). P3N, prepared from 3-NP at pH 13, was found to inhibit the enzyme 70 times faster than 3-NP at pH 7.5. Considering that 0.025% of 3-NP (pK_a = 9.1) exists as P3N at this pH, a comparable rate of inhibition was found when the data were replotted against [P3N], strongly supporting P3N as the inhibitory form. Through jump-dilution kinetics, we revealed that P3N is an irreversible inhibitor. Electrospray ionization mass spectrometry showed a time-dependent increase of 100 Da in the mass of ICL following incubation with 3-NP or P3N (119 Da for neutral form), which was accelerated in the presence of glyoxylate. The identity of the resulting covalent complex and the mechanism of its formation are the subject of ongoing investigation. Additionally, whether 3-NP is converted to P3N strictly when free in solution or also by enzyme-assisted deprotonation is currently being probed.



P67

Lipidation of NDM-1 Converts Antibiotic Resistance from a "Public Good" to a "Private Good"

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New **D**elhi **M**etallo-β-lactamase (NDM) is a rapidly-spreading antibiotic resistance threat that renders almost an entire class of antibiotics useless. The catalytic properties of NDM-1 and homologous metallo-β-lactamases (MBLs) are similar. However, NDM-1 is expressed with an unusual type II lipidation signal sequence that results in the post-translational modification of Cys26 that anchors the enzyme to the inner leaflet of the outer membrane of Gram-negative host bacteria. To our knowledge, there are no other MBLs and very limited serine- β-lactamases with a similar lipidation sequence, and the functional impact of lipidation on antibiotic resistance is not well defined. During purification procedures, we noticed a robust β-lactamase activity in culture supernatant after pelleting cells that expressed an N-terminal deletion mutant (Δ35 NDM-1), but this activity was absent in cultures expressing the full-length enzyme. This observation led to our hypothesis that a β-lactamase lacking lipidation provides resistance to neighboring bacteria (a "public good"), but that lipidation sequesters the enzyme and only provides resistance to the host bacterium (a "private good"). This hypothesis was tested using trans-well culture plate in which the NDM-1 expressing strain is cultured in the cup and a "bystander" strain that lacks a β-lactamase is cultured in the neighboring well, with the two compartments separated by a membrane that allows passage of solvent, proteins, and outer membrane vesicles, but not bacteria. Despite similar expression levels for enzyme variants, full-length NDM-1 does not protect neighboring cells against β-lactam treatment, but the Δ35 NDM-1 variant, which lacks the lipidation site, can effectively provide resistance to bystander cells. Additional studies to better characterize this effect are underway, but this proof-of-principle experiment illustrates that NDM-1 lipidation can impact how well antibiotic resistance is shared with bystander bacteria. This finding has potential implications for β-lactam therapy in which NDM-1 carrying strains compete with other pathogenic bacteria and with commercial bacteria that normally provide colonization resistance.

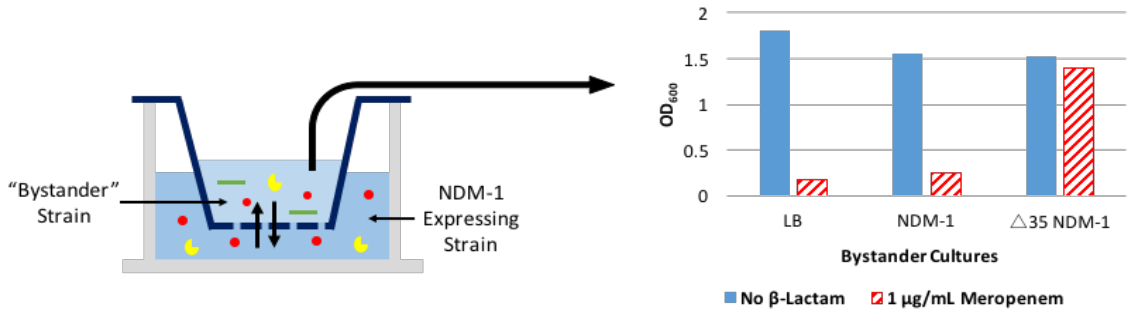

Figure 1. Bystander cell growth in respect to LB, NDM-1, and Δ35 NDM-1 co-cultures from the trans-well plate setup.

Antibiotic Resistance Evolved Via Inactivation of a Ribosomal RNA Methylating Enzyme

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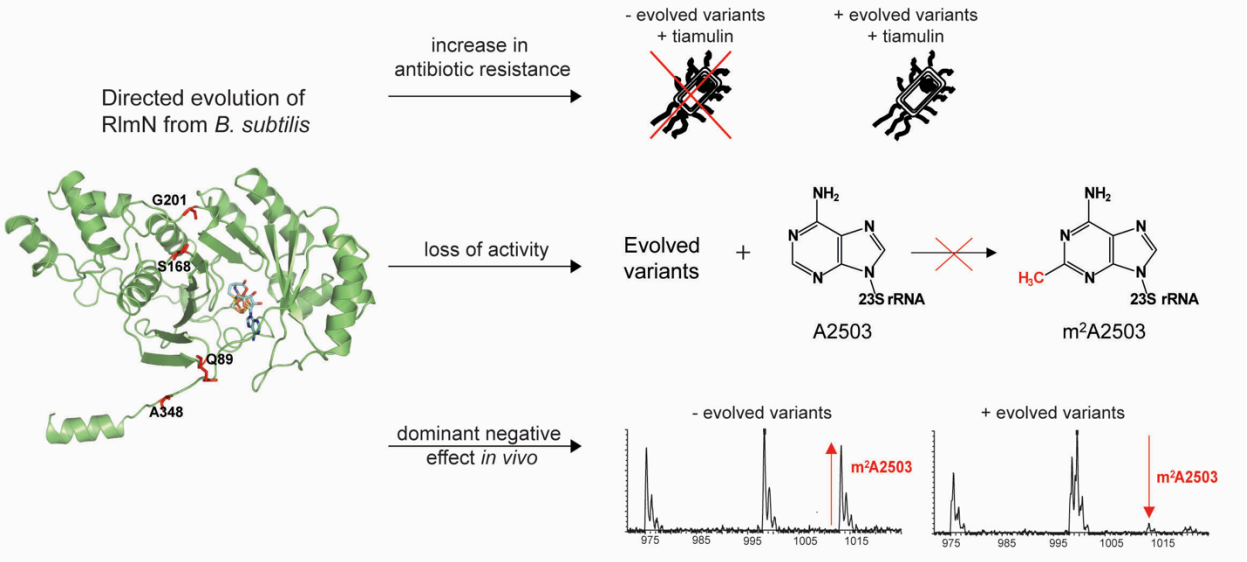
Modifications of the bacterial ribosome regulate the function of the ribosome and modulate its susceptibility to antibiotics. By modifying a highly conserved adenosine A2503 in 23S rRNA, methylating enzyme Cfr confers resistance to eight classes of ribosome-targeting antibiotics. The same nucleotide is also methylated by RlmN, an enzyme widely distributed among bacteria. While RlmN modifies C2, Cfr modifies the C8 position of A2503. Shared nucleotide substrate and phylogenetic relationship between RlmN and Cfr prompt us to investigate evolutionary origin of antibiotic resistance in this enzyme family. Using directed evolution of RlmN under antibiotic selection, we obtained RlmN variants that mediate increased antibiotic resistance. Surprisingly, these variants confer resistance not through the Cfr-like C8 methylation, but via a loss of the RlmN-like C2 methylation of A2503. Recent detection of RlmN inactivating mutations in clinical resistance isolates suggests that the mechanism used by the evolved variants is also important in a clinical setting. Additionally, as further indicated by phylogenetic analysis, it appears that Cfr did not diverge from the RlmN family but from another distinct family of predicted radical SAM methylating enzymes whose function remains unknown.



P69

Novel Human Indoleamine 2,3-Dioxygenase Inhibitors Form a Long-Lived Complex with the Enzyme

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Human indoleamine 2,3-dioxygenase 1 (hIDO1) catalyses the conversion of L-tryptophan (L-Trp) to *N*-formylkynurenine through a haem and O_2-dependent oxidation process.[1] hIDO1 is recognised as a central regulator of immune responses in a broad variety of physiological and pathological settings and is an attractive target in cancer.[2] In search of novel IDO inhibitors, we identified a series of 4-amino-1,2,3-triazoles as potent hIDO1 inhibitors. We applied a broad range of techniques including kinetics, UV spectroscopy and structural biology to build a detailed understanding of ligand interactions, kinetics and mechanism of action. We demonstrate that the potency of an exemplar molecule relies on 1) the interaction of the triazole with the haem iron, 2) the preferential binding of the compound to the ferrous form of the enzyme, 3) the slow establishment of the compound-enzyme complex and 4) the generation of a long-lived, tight complex between the 4-amino-1,2,3-triazole and hIDO1. The generation of this tight-binding complex translates to exceptional potency in cellular IDO assays, making them attractive starting points for potential future development.

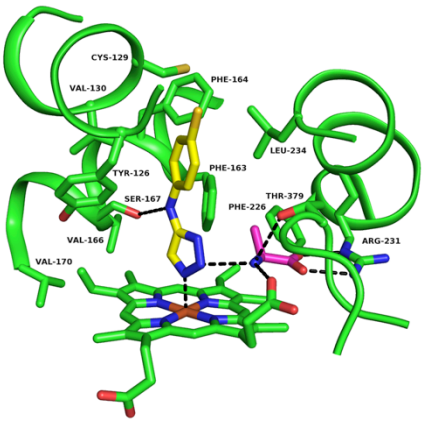

Figure 1. Co-complex structure of hIDO1 with a 4-amino-1,2,3-triazole showing the interaction of the compound with the haem iron.

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Mechanistic Studies and Inhibitor Design Toward Cruzain, a Noncanonical Cysteine Protease from *Trypanosoma cruzi*

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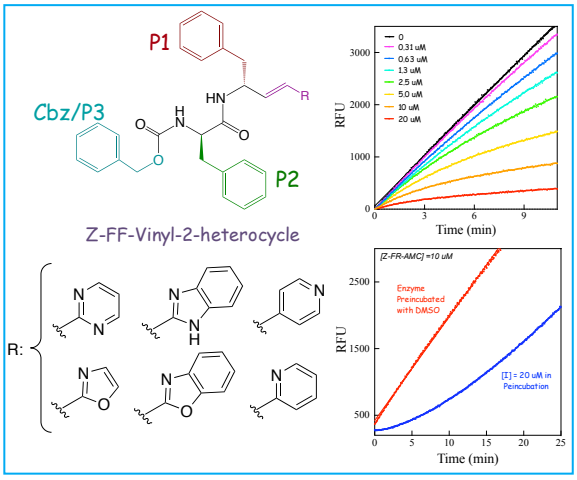
Cruzain is the major cysteine protease expressed by *Trypanosoma cruzi*, the causative agent for Chagas disease. Currently, the most efficient inhibitor towards cruzain is K11777[1] (Mu-Phe-Hph-VSPh) that has a vinyl-sulfone warhead in place of the scissile peptide bond. This compound effects irreversible inhibition via electrophilic addition to the eponymous active-site cysteine sidechain. We sought to develop other types of electrophilic warheads that could be "tuned" in terms of reactivity with the active-site cysteine by installing vinyl heterocycles as potential Michael acceptors onto a peptide backbone. These compounds inhibit cruzain in a reversible, time-dependent fashion, which are the hallmarks for slow-onset inhibition. Through global fitting the time-dependent inhibition data we were able to determine the inhibition constants for both the loose and tight complexes, as well as the k_{off} for ligand-target dissociation. In order to facilitate our effort in developing new inhibitors against this target, we also sought to have a better understanding of the enzyme mechanism. To this end, we have conducted pH-rate profiles, solvent isotope effects, and pre-steady state kinetics studies for cruzain. Normal solvent isotope effects were observed in both k_{cat} (1.8 ± 0.1) and k_{cat}/K_m (1.3 ± 0.2) for a variety of substrates. The pH-rate profiles indicate that the free form of the enzyme contains a neutral cysteine and histidine, which is more consistent with a "substrate-assisted" mechanism. The pre-steady state kinetics indicate that deacylation is the rate-limiting step in catalysis.



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P71

Catalytic strategy for Biofuel Synthesis by the Cytochrome P450 OleT

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A dwindling global energy supply, environmental concerns over rising atmospheric levels of carbon dioxide (CO_2), and the volatility of fuel costs have served as compelling impetus to explore renewable alternatives to petroleum-based fuels. OleT is a recently discovered cytochrome P450 that catalyzes the hydrogen peroxide-dependent metabolism of C_n chain-length fatty acids to synthesize C_{n-1} 1-alkenes. The decarboxylation reaction provides a route for the production of drop-in hydrocarbon fuels from an abundant natural resource. This transformation is highly unusual for a P450, which typically utilize an iron(IV)−oxo intermediate known as compound I for the insertion of oxygen into organic substrates. A basis for OleT enzymatic reprogramming is provided in transient kinetic studies, and reveal that the OleT ferryl species performs C-H bond abstraction, and forms a hyperstable iron(IV)-OH species (Compound II) that does not readily recombine with the incipient substrate radical. The direct observation of this intermediate, normally fleeting in hydroxylases, provides a rationale for the carbon−carbon scission reaction catalyzed by OleT. The contributions of the secondary coordination sphere and substrate binding modes for mediating the branchpoint between conventional oxygen rebound chemistry and C-C scission will be discussed.

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P72

Binding and Regulation of a Nonribosomal Peptide Synthetase-Modifying P450

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Nonribosomal peptides often contain amino acids with chemical moieties that not found in ribosomally-synthesized proteins, which often confer powerful pharmacological activities. (1) Construction of these complex chemical architectures is achieved through the coordinated action of nonribosomal peptide synthetases (NRPSs) and accessory tailoring enzymes. Tailoring enzymes catalyze a plethora of reactions on the maturing peptide, often while it is tethered to the NRPS. The highly discriminating nature of tailoring enzymes, combined with the modularity of the NRPS assembly process, enables a specific amino acid, and in many cases a singular C-H bond, to be selectively modified. Despite tremendous potential for natural product diversification or targeted synthesis, the principle molecular determinants which govern tailoring enzyme specificity, and the extent to which NRPS binding regulates tailoring enzyme catalysis, have yet to be fully clarified. The biosynthetic pathway of the potent antifungal nikkomycin presents an attractive model system for analyzing these interactions, as it is comprised of a relatively simple didomain NRPS (NikP1) which activates L-histidine, and a cytochrome P450 (NikQ) that beta-hydroxylates this appended amino acid. (2) Electron paramagnetic resonance spectroscopy and analytical size exclusion chromatography reveal that NikQ only binds NikP1 in defined post-translationally modified states. Redox titrations and transient kinetic studies indicate that NikP1-binding does not substantially regulate the early catalytic steps of NikQ, a trait that is highly atypical for most P450-substrate interactions. (3) Proximal mutants of NikQ have also been utilized to probe the importance of this region in the altered electronic properties of the heme-iron.

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P73

Deorphanization of Human Cytochrome P450 4X1 Reveals Substrates Bearing Tetrahydroisoquinoline Backbones

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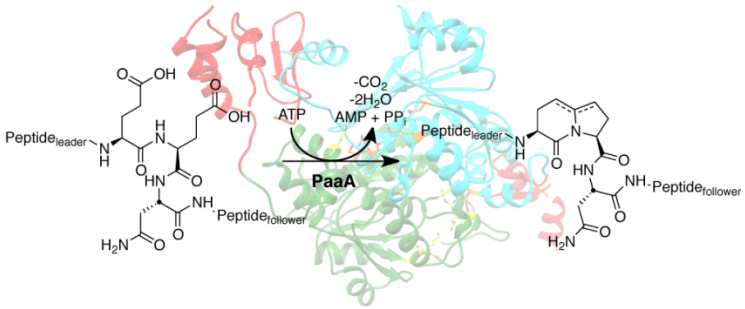
Human cytochrome P450 4X1 is an orphan P450 enzyme whose endogenous substrate is not well characterized. An untargeted metabolomics based approach was used to identify compounds bearing tetrahydroisoquinoline backbones (e.g. salsolinol and higenamine) as a new class of substrates for this enzyme. The structures of the products, characterized by high-resolution mass spectrometry, suggest a cascade reaction sequence that involves the cleavage of a carbon-carbon bond.



P74

A Cell Lysate Ubiquitin Probe-based Platform for the Identification of Small Molecule Deubiquitinase Inhibitors

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Ubiquitination is an important and reversible post-translational modification that controls a large number of cellular signaling pathways in humans. Protein ubiquitination is modulated by a class of enzymes known as the deubiquitinases (DUBs). Close to 100 DUBs have been identified in humans and are implicated in a number of human diseases including different types of cancer, neurodegenerative diseases, viral infections, and inflammatory diseases, thus making DUBs promising new targets for drug development. Currently deubiquitinase assays, particularly those amenable for high-throughput screening, have been largely limited to the minimal substrates (such as Ub-AMC) and DUBs that can be purified recombinantly. This can be problematic because a number of DUBs are large multi-domain proteins, known to associate with accessory proteins, and/or contain important post-translational modifications making them difficult to purify recombinantly. Additionally, assays that can be run in a cellular environment may lead to more potent and specific inhibitors. Here, we report the first ubiquitin-probe based AlphaLISA DUB assay that is amenable for cell lysates and high throughput screening. We highlight the power and utility of the assay using Ubiquitin C-terminal hydrolase-1 (UCHL1) as a test case, and have identified several small molecules that display inhibition towards UCHL1.

Free Radical Mediated Carbon Extension During the Biosynthesis of Peptidyl Nucleoside Antifungal Antibiotics

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The fungal cell wall is a target for many naturally occurring antifungal molecules that exhibit potent and selective activities. Understanding the biosynthesis of these antifungals is important for their future development into clinically useful molecules. Here, I will report our recent findings in the biosynthesis of the peptidylnucleoside (PN) class of antifungals (1). PNs are structurally characterized by aminohexuronic acid (AHA), a C5'-extended nucleoside essential for antifungal activity. AHA is biosynthesized from uridine 5'-monophosphate (UMP) by an unknown mechanism. To identify the AHA biosynthesis enzymes, putative C5' oxidizing enzymes were heterologously expressed and functionally characterized. An enzyme (PolH) that belongs to the radical *S*-adenosyl-L-methionine (SAM) superfamily was found to be responsible for the formation of the key cyclic nucleoside biosynthetic intermediate, octosyl acid 5'-phosphate (OAP). Mechanistic characterization of PolH revealed that the catalysis involves a unique free radical mechanism with SAM as a radical initiator, and a redox active Cys residue to control the stereospecificity of the radical reaction (Fig. 1). Moreover, our bioinformatics analysis revealed >10 PolH homologs in operons that are likely involved in biosynthesis of related antifungals. Our results together suggest the existence of previously unappreciated abundance of antifungal nucleoside natural products.

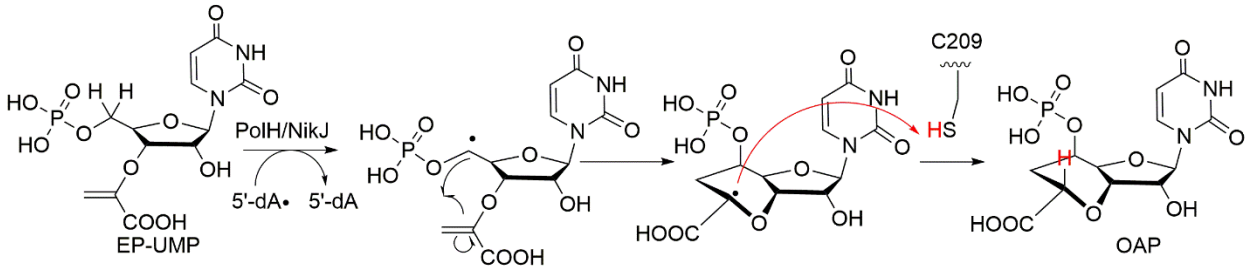

Figure 1: Proposed radical based mechanism for PolH/NikJ.

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Iterative Methyl Group Alkylation by a Cobalamin-Dependent Radical SAM Enzyme in Cystobactamid Biosynthesis

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Natural products with branched alkoxy groups have been used widely in the development of bioactive compounds. However, the biosynthesis of branched alkoxy groups like the unique *t*-butyl group is still poorly understood. The cystobactamid are a novel class of isopropyl substituted antibacterial compounds produced by myxobacteria.[1] In the gene cluster of cystobactamid biosynthesis, CysS is assigned as a cobalamin dependent radical S-adenosyl-L-methionine (SAM) enzyme, which is proposed to catalyze the iterative methylation on the 3-methoxy-4-aminobenzoic acid moieties of cystobactamid. In this study, we have reconstituted CysS *in vitro* and showed that it can perform sequential methylations of a substrate methyl ether to form ethyl, isopropyl, *t*-butyl and *sec*-butyl substituted products. These cobalamin dependent methylations also produce 5′-deoxyadenosine and S-adenosylhomocysteine in equal amounts. Isotope labeling experiments and substrate analog study suggested that the methyl group from SAM is first transferred to cobalamin followed by the attack of the substrate radical to give methylated products. To obtain multiple methylations, substrate must bear a pantetheinyl side chain, indicating the peptidyl-carrier-protein as CysS native substrates. Here, we show that CysS is able to convert a methyl group to a *t*-butyl group by radical mechanism, which expands the range of reactions that can be catalyzed by radical SAM enzymes.

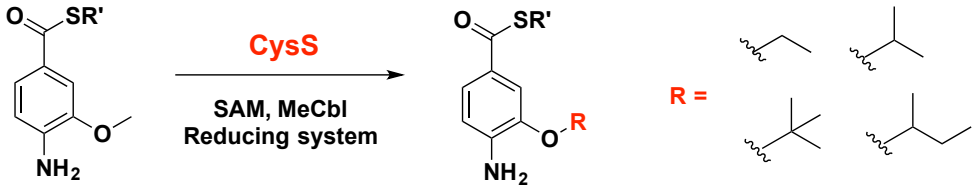

Figure 1. CysS catalyzed iterative methylations

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β-Lactones from β-hydroxy Acids: a New and Widespread Reaction Catalyzed by an ATP Dependent Ligase/Synthetase Superfamily Enzyme

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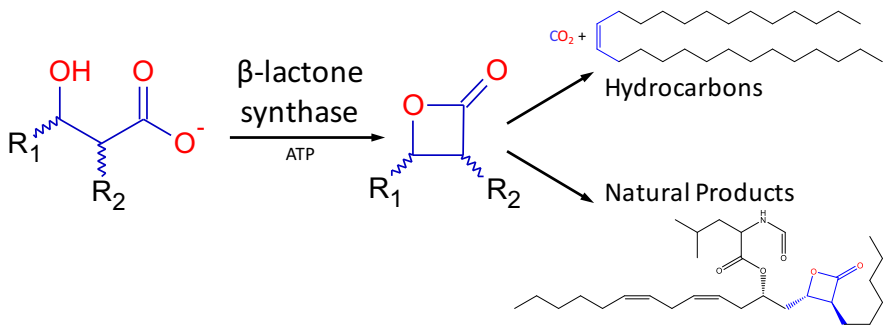
Natural enzymes within a given superfamily may catalyze distinct reactions and participate in completely different metabolic pathways within a cell. While studying the enzymatic mechanisms of hydrocarbon biosynthesis by the bacterium *Xanthomonas campestris,* we discovered a novel enzyme denoted here as β-lactone synthase. A mechanism is advanced in which the β-lactone synthase uses ATP to activate the carboxylic acid group of the substrate with AMP. The β-hydroxyl group can then attack the activated carbonyl carbon to displace AMP and form the β-lactone ring. Preliminary evidence further suggests the β-lactone is then processed by another novel enzyme, β-lactone decarboxylase, that produces carbon dioxide and a long-chain olefin that is targeted to the membrane. Bioinformatic analyses have revealed that the β-lactone synthase and other enzymes participating in hydrocarbon biosynthesis show high sequence identity to proteins encoded by known β-lactone-producing natural product gene clusters. These studies have uncovered common chemical mechanisms and evolutionary connectivity between cell membrane and cell protection chemicals within bacteria.



P78

Discovery and Characterization of Bicereucin, an Unusual D-Amino Acid-Containing Mixed Two-Component Lantibiotic

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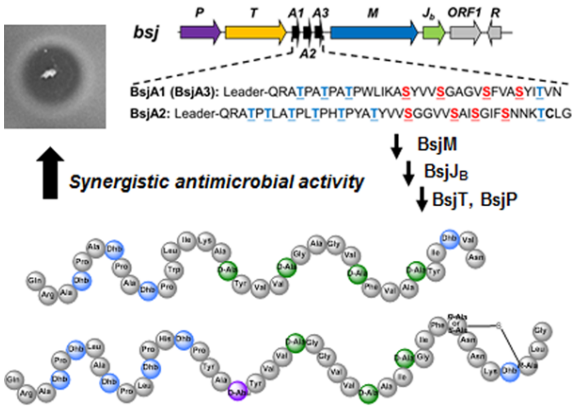
Lantibiotics are a group of ribosomally synthesized and post-translationally modified peptides (RiPPs) exhibiting antimicrobial activity.[1] They are characterized by the presence of the thioether-containing bisamino acids lanthionine and methyllanthionine. They are formed by dehydration of Ser and Thr residues, followed by intramolecular Michael-type addition of Cys thiols to the resulting dehydroalanine (Dha) or dehydrobutyrine (Dhb) residues.[2-5] Here, we report a two-component lantibiotic from *Bacillus cereus* SJ1 with unusual structural features that we named bicereucin. Unlike all previous two-component lantibiotics, only one of the two peptides of bicereucin contains a lanthionine. The second peptide lacks any cysteines but contains several D-amino acids. These are installed by the dehydrogenase $BsjJ_B$, the activity of which was successfully reconstituted *in vitro*. The proteolytic removal of the leader peptide was also performed *in vitro*. Bicereucin displayed synergistic antimicrobial activities against Gram-positive strains including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* as well as hemolytic activity. To illustrate the utility of the enzymes, an analog of the D-amino acid containing opioid dermorphin[6] was successfully produced in *E. coli* by employing the dehydratase BsjM and the dehydrogenase $NpnJ_A$.[7]



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P79

Developing Anti-Aging Therapeutics *via* Synthetic Alkaloid Enzymology

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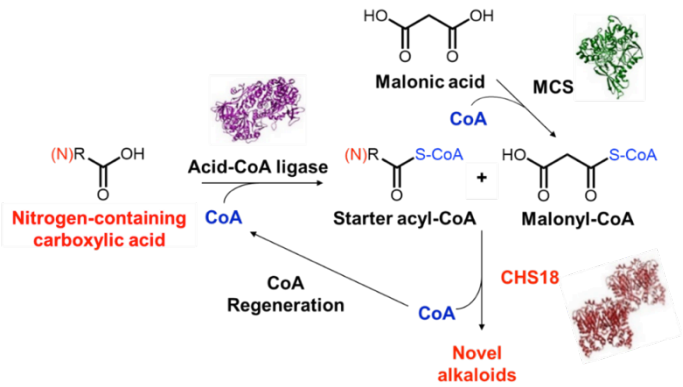
An intervention that consistently extends maximal life span is caloric restriction (CR), but its application in humans is not practical. A more feasible approach is to develop a CR mimetic that targets biochemical pathways affected by CR, and thus achieve life span extension. Alkaloids are the most structurally diverse secondary metabolites produced by microorganisms and plants, providing an untapped potential for the creation of a bioactive compound library for anti-aging therapeutic development.

Several studies have demonstrated the feasibility of performing precursor-directed combinatorial biosynthesis using promiscuous type III polyketide synthases (PKSs) to generate novel alkaloids. This highlights an unconventional and simpler method to generate alkaloids by repurposing type III PKSs for alkaloid biosynthesis. By establishing a combinatorial biosynthetic route in *Escherichia coli* and exploring the substrate promiscuity of a mutant PKS from alfalfa, many potential anti-aging alkaloids can be biosynthesized. In this approach, novel acyl-CoA precursors including nitrogen-containing precursors generated by various promiscuous acid-CoA ligases will be delivered to the mutant PKS, and the alkaloids thus generated will be introduced to *Caenorhabditis elegans* when they feed on the engineered *E. coli*. A molecular screening platform where compound libraries are screened in *C. elegans*-based life span assays can thus be developed. This study highlights the utility of synthetic enzymology in anti-aging studies, and its potential in the development of novel anti-aging nutraceuticals.



P80

Investigating "Stuffed" Domains of NRPS Assembly Lines: PchF and PchE of Pyochelin Biosynthesis

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Nonribosomal peptide synthetases (NRPSs) are one approach used by microbes to generate bioactive peptides. These bioactive peptides are not only used as secondary metabolites (toxins, pigments, siderophores – iron scavenging molecules), but have found their way into the clinic as antibiotics, anticancer drugs, and immunosuppressants. To elicit their unique bioactivity, these peptides must be tailored. Natural product chemists, metabolic engineers, and researchers in biochemistry and biotechnology work to exploit the biosynthesis of these secondary metabolites in order to generate new compounds for clinical use. The long term goal of this project is to understand the structure-function relationships of epimerases and methyltransferases that are incorporated into these assembly lines. Structural biology and mechanistic enzymology can provide novel insight and assist natural product investigations, protein engineering projects, antimicrobial development, and other therapeutic design. Currently there is no adenylation-tailoring "stuffed" didomain NRPS structures, and limited biochemical characterization exists. This project concentrates on the adenylation-epimerase didomain of PchE and the adenylation-methyltransferase didomain of PchF in the biosynthetic pathway of the siderophore, pyochelin. Initial work includes substrate analogue synthesis and the establishment of adenylation, epimerization, and methyltransferase assays.

P81

Characterization of a New γ-Glutamylase-Dependent Pathway for Ethanolamine Catabolism

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Uncharacterized bacterial enzymes currently number in the tens of millions, and this number is growing exponentially. Large-scale sequence-based strategies are required to efficiently assign functional annotations to these sequences with unknown functions. This research illustrates the utility of a multi-disciplinary approach driven by both the ligand specificity of a solute binding protein component of a transport system and high-throughput bioinformatics, as we report the discovery and characterization of a new pathway for catabolism of the metabolite ethanolamine. Ethanolamine is abundant in nature as a component of phosphatidylethanolamine in the cell membranes of eukaryotes and bacteria. Many species of bacteria derive carbon and/or nitrogen from ethanolamine via the well-characterized ethanolamine utilization (EUT) pathway. Here, we report functional and phylogenomic characterization of a new γ-glutamylase dependent pathway for ethanolamine catabolism (termed EOA) in bacteria. We use in vitro enzymatic activity and in vivo microbiological characterization guided by large-scale analyses of sequence-function relationships and genome context to delineate a four-enzyme pathway, the first step of which is ATP-dependent γ-glutamylation of ethanolamine, followed by two aliphatic carbon oxidations and hydrolysis of the γ-glutamyl amide bond to produce glycine, a source of cellular nitrogen, and regenerate L-glutamate. Analysis of the taxonomic distribution of the EOA pathway revealed that it is found almost exclusively in Proteobacteria and Actinobacteria. Interestingly, the pathways in these phyla utilize non-homologous isofunctional enzymes for catalysis of the second step, oxidation of γ-glutamyl-ethanolamide, and the final step, hydrolysis of γ-glutamyl-glycine. In total, the characterization of this pathway enabled the assignment of function to enzymes and proteins from at least ten different Pfam families encoded in the genomes of nearly 400 distinct bacterial species.

Transport System Solute Binding Protein (SBP) Guided Discovery of Novel Metabolic Pathways

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Given the exponential increase in genomic data, enhanced strategies are demanded for functional annotation and metabolism discovery. Solute binding proteins (SBPs) recognize the first reactant in a metabolic pathway, thereby constraining the regions of chemical space and the chemistries that must be considered for pathway reconstruction. In many cases, the transporter genes are colocated or coregulated with genes encoding the enzymes responsible for catabolism of the transported molecule. Herein, we illustrate the utility of an integrated "genomic enzymology" strategy that combines high-throughput ligand screening results for SBPs together with the synergetic analysis of sequence similarity network (SSN) and genome neighborhood network (GNN) to delineate novel enzymes in novel metabolic pathways for D-apiose/D-apionate. The experimental screening of ABC SBPs (Pfam family PF13407) identified ligand specificities of three orthologous SBPs (UniProt ID: Q2JZQ5, B1G898, and A6VKQ8) for D-apiose ($\Delta T_m > 5$ ^{o}C). SSNs and GNNs for the ABC SBPs (PF13407) sub-cluster that three D-apiose DSF hits located were used to identify the enzyme components of two distinct pathways that degrade D-apiose/D-apionate metabolites. We experimentally determined that **1)** conserved genome neighborhoods encoded members of PGDH_C family, members of DUF1537 family and members of RuBisCO superfamily participate in pathways for the conversion of D-apionate into glycolic acid and glycerate 3-phosphate; or conversion of furanose D-apiose into D-erythrose 4-phosphate; **2)** conserved genome neighborhoods encoded members of FGGY carbohydrate kinase and members of transketolase participate in a pathway for the conversion of furanose D-apiose into dihydroxyacetone phosphate and D-fructose 6-phosphate. Meanwhile, SSN and GNN for PGDH_C family (Pfam family PF16896) were used to identify the enzyme components of the third distinct pathway that degrade D-apionate metabolite into D-erythrose 4-phosphate which accept D-frucotose 6-phosphate to release D-sedoheptulose 7-phosphate and D-glyceraldehyde 3-phosphate. Significantly, the pathways involve several unusual reactions and enzymes. In particular, members of RuBisCO superfamily catalyze decarboxylation or trans-carboxylation reactions. Members of PGDH_C family catalyze the oxidative isomerization of D-apionate. The physiological importance of these pathways was demonstrated *in vivo* by phenotypic and genetic analyses. The integrated "genomic enzymology" strategy, as demonstrated here, is a powerful tool for assigning enzymatic functions and elucidating metabolic pathways.

In Vitro Reconstitution and Substrate Specificity of the Biosynthesis of the Core Scaffold of the Thiopeptide Thiomuracin

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Thiopeptides are potent antibiotics that inhibit protein synthesis. They are made by a remarkable post-translational modification process that transforms a linear ribosomal peptide into a polycyclic structure. Herein, we report the in vitro biosynthesis of the core scaffold of the thiopeptide thiomuracin, the first of such example for this class of peptides, as well as the further determination of substrate specificity and biosynthetic timing of the biosynthetic enzymes. We show that cyclodehydration precedes dehydration, and that dehydration is catalyzed by two proteins in a $tRNA^{Glu}$-dependent manner to generate four alkenes. Then two of these alkenes undergo a formal [4+2] cycloaddition to form a tri-thiazole-substituted pyridine macrocycle. We show the order of thiazole and alkene formation, reveal the minimal structural changes necessary to render TbtA a substrate for dehydration, the parts of the TbtA peptide that are recognized by the various enzymes, and identify important residues of the enzyme TbtD for catalysis of a formal [4+2] cycloaddition process.

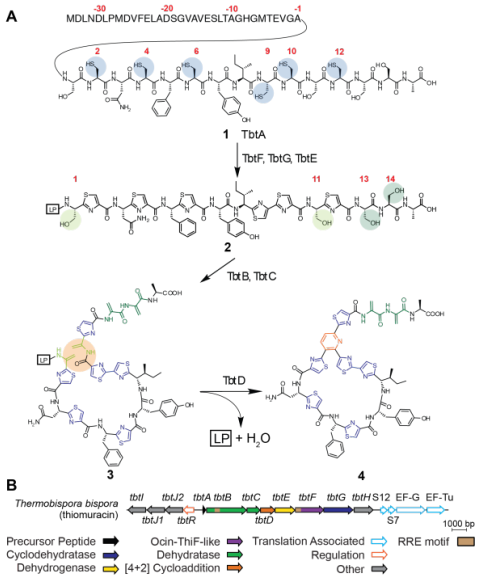

Figure 1. (**A**) Biosynthetic route to the thiomuracin core scaffold. (**B**) Gene cluster for the biosynthesis of thiomuracin.

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P84

Contrasting Reactivity of Enzymic and Nonenzymic Thiamin Intermediates and the Reality of Albery-Knowles Energetics

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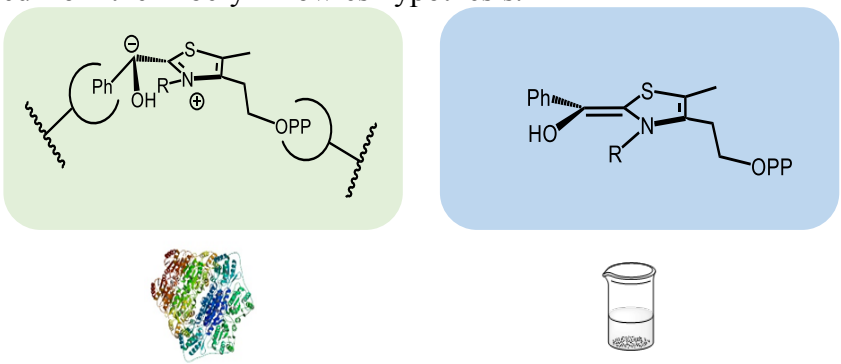
Albery and Knowles proposed that an important component of an enzyme's catalytic efficiency includes the preservation of the energetic state of reactive intermediates[1]. In contrast, reactions in solution cannot achieve similar efficiencies due to unavoidable energetic dissipation. Tittmann and coworkers have provided crystallographic evidence that an enzyme-bound intermediate derived from thiamin diphosphate (ThDP) maintains a tetrahedral carbanionic structure rather than the expected delocalized and more stable alternative found in nonenzymic models.[2] Benzoylformate decarboxylase is a ThDP enzyme that promotes reaction of analogues of its natural substrate. In particular, Kozarich and coworkers demonstrated that the p-bromomethyl analogue of benzoylformate undergoes an elimination reaction that produces toluic acid as a result of elimination and hydrolysis, while the p-chloromethyl analogue produces p-chloro-methylbenzaldehyde without elimination.[3] This differential reactivity pattern suggests that the enzyme is able to trap the intermediate in the chloro-substituted case by a protonation step that outcompetes elimination. We prepared the synthetic intermediates of the native substrate and the two substituted species. While the native substrate undergoes rapid decarboxylation and subsequent protonation, both halogenated species undergo the elimination reaction after decarboxylation to give toluic acid and thiamin. The enzymic reaction with the chloromethyl substrate establishes that the enzyme can preserve the carbanion and trap it in a rapid process whereas the nonenzymic reaction shows that elimination is intrinsically more rapid, occurring from the delocalized intermediate. The reaction of the bromomethyl derivative shows that the elimination process is intrinsically faster than protonation in both cases. Thus, the selective enzymic stabilization of the carbanionic form follows the rigorous and otherwise unexpected prediction derived from the Albery-Knowles hypothesis.



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P85

Novel Thymidylate Synthase Intermediate as an Inhibitor Blueprint

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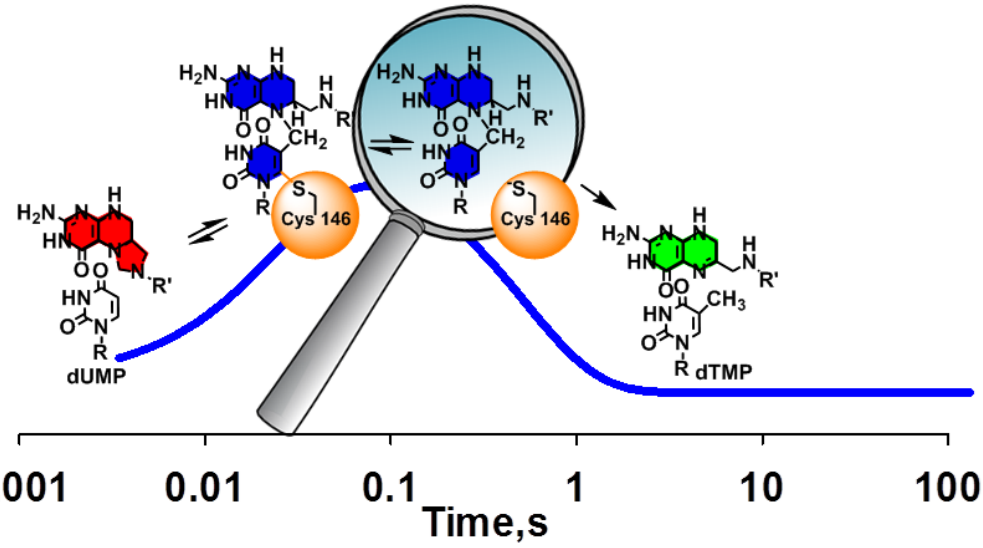
Thymidylate Synthase (TSase) is one of the prime anticancer targets as it generates the sole *de novo* source of thymidylate. Fluoropyrimidines and antifolates inhibiting TSase pathways are not only components of the most widely used therapeutic regimens, but also highly toxic agents leading to acquired resistance in tumor cells. The demand for novel inhibitor classes inspired our recent mechanistic investigation of this enzyme. TSase catalyzes reductive methylation of 2′-deoxyuridine 5′-monophosphate (dUMP) with a co-substrate 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4Fol) as a methylene and a hydride donor. The traditional view of this multistep reaction involves a sequence of intermediates covalently bound to the active site cysteine,[1] where this bond dissociation occurs only upon a product release. In contrast, recent computational studies predict lability of this bond and generation of the transient non-covalent bi-substrate intermediate.[2,3] In a critical test of this prediction we synthesized the anticipated intermediate and demonstrated its chemical and kinetic competence.[4] In addition, our new striking findings support formation of this intermediate during the natural course of TSase catalyzed reaction. Furthermore, the potential of this novel entity as a drug lead is examined by tests of its stable analog. In summary, our work provides an unconventional perspective towards chemical mechanism and drug targeting of TSase.



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New Cofactor Supports α,β-Unsaturated Acid Decarboxylation via 1,3-Dipolar Cycloaddition

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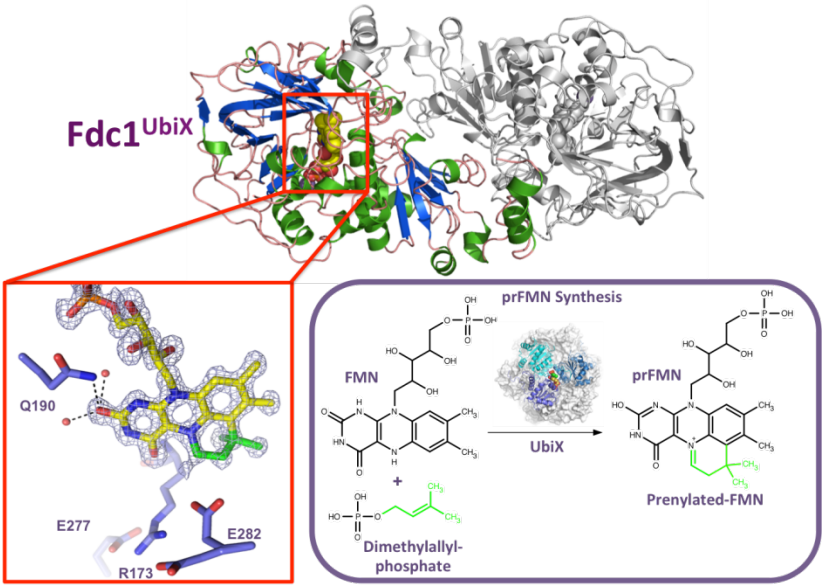
The Fdc1/UbiX decarboxylase system has been reported to decarboxylate aromatic and unsaturated aliphatic enoic acids to produce aromatic hydrocarbons and aliphatic α-olefins respectively, compounds that are of great interest in biofuel and biorefinery applications. However, the mechanism of this reaction, cofactor requirements and the roles of each component have remained enigmatic. Using atomic resolution crystallography coupled with a range of spectroscopic and biochemical methods, we show that the Fdc1 is solely responsible for the decarboxylase activity (1), and that it requires a novel cofactor, prenylated FMN (prFMN) which is synthesized by the UbiX enzyme (2). The addition of the prenyl-group to the N5 and C6 positions of the flavin isoalloxazine ring system furnishes prFMN with a fourth, non-aromatic ring and provides the cofactor with azomethine ylide character, a well known 1,3-dipole routinely used in 1,3-dipolar cycloaddition reactions by organic synthesis. A series of structures of Fdc1 in complex with substrates, products or inhibitors provide evidence that 1,3-dipolar cycloaddition supports reversible decarboxylation in this enzyme.



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Structural Evidence for Ground State Destabilization in Pyridoxal-5'-Phosphate Dependent Enzymes: The Tryptophan Indole-lyase-Oxindolylalanine Complex

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Tryptophan indole-lyase (TIL) is a bacterial enzyme that catalyzes the reversible formation of indole and ammonium pyruvate from L-tryptophan. Indole produced by TIL is a signaling molecule that regulates a number of physiological processes in *E. coli*, including biofilm formation, plasmid stability, and antibiotic resistance. Oxindolyl-L-alanine (OIA) is a potent inhibitor of TIL, with a K_i value of about 5 μM, and crystals of TIL from *Proteus vulgaris* were shown previously to form a stable quinonoid complex upon soaking with OIA (1). We have now determined the structure of the complex of *P. vulgaris* TIL with OIA at 2.15 Å. The ligand is present at ~100% occupancy and forms a quinonoid complex with the pyridoxal-5'-phosphate (PLP) cofactor. A hydrogen bond forms between the NH of the inhibitor and Asp-133. The small domain moves ~7 Å to close the active site, allowing His-458 to donate a hydrogen bond to Asp-133. This then brings Phe-37 and Phe-459 into close steric contact with the aromatic ring of OIA. Mutation of this Phe to Ala results in a 500-fold decrease in k_{cat} and k_{cat}/K_m for L-Trp, with much less effect on the reaction of other β-elimination substrates. A model of the L-Trp quinonoid complex with PLP shows that there would be a severe clash of Phe-459 and the benzene ring of the substrate, which would result in bending of the substrate aromatic ring out of plane with the C_β-C_γ bond, allowing formation of the hydrogen bond of the indole NH with Asp-133. This bending of the aromatic ring out of plane moves the substrate upward on the reaction coordinate toward the transition state, thus reducing the activation energy and accelerating the enzymatic reaction.

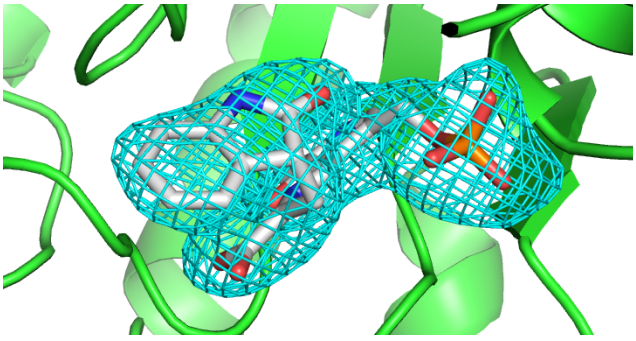

Figure 1. Sim-omit map at 4 σ of oxindolyl-L-alanine bound in the active site of *P. vulgaris* TIL.

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Alcohol Dehydrogenases: Zinc Coordination and Catalysis

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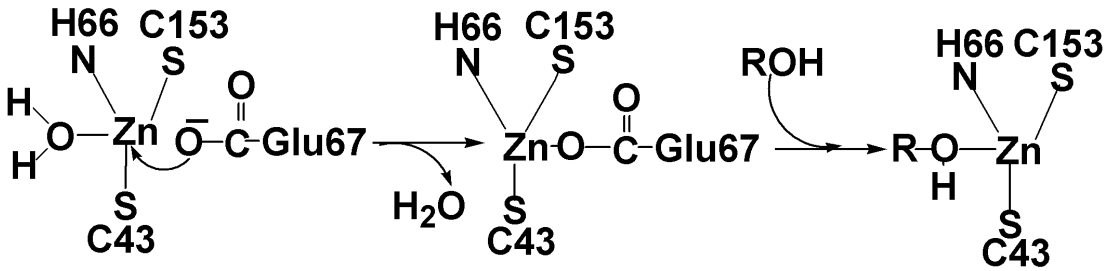
Alcohol dehydrogenases (ADH) catalyze the reversible oxidation of alcohols to aldehydes by an ordered mechanism with the coenzyme NAD^+ binding first, followed by binding of the alcohol, proton and hydride transfer, release of aldehyde, and dissociation of NADH. The oxygen of the alcohol is bound to a zinc in a tetrahedral coordination with two cysteine sulfurs and one histidine imidazole in the liver and yeast alcohol dehydrogenases. The zinc acts as a Lewis acid and stabilizes the intermediate alcoholate, which forms a low-barrier hydrogen bond with a nearby Ser or Thr hydroxyl group, which is connected by a proton relay system to His-51 that acts as a base. In an analogue of the ternary product complex with NADH, the oxygen of a formamide also binds to the zinc. The catalytic zinc of the apo-enzyme (no bound coenzyme) of horse liver ADH binds a water in place of the alcohol. When the horse liver enzyme binds NAD^+, the pK of a group (apparently the zinc-bound water) decreases from 9.5 to 7.3, and a proton is released (at pH 8.0) concurrently with a conformational change that closes up the active site and facilitates binding of substrate analogues. The question arises, then, what is the mechanism for the exchange of the zinc-bound water (or hydroxide) with the substrate? An intermediate penta-coordinated zinc with adjacent oxygens from a water and the substrate was proposed for the enzyme-coenzyme-substrate complex, but the evidence is not convincing; X-ray structures of relevant complexes have no such water. Nevertheless, such a penta-coordinated zinc could be a transient species, as the horse apo-enzyme forms stable penta-coordinated complexes with 2,2'-bipyridine and 1,10-phenanthroline. It is noteworthy that the zinc in the yeast apo-enzyme has an inverted configuration where the carboxylate of Glu-67 is in tetrahedral coordination with the two cysteines and the histidine. Thus, an alternative mechanism could involve a double displacement where the carboxyl group of the nearby glutamate residue displaces the water, inverting the configuration of the zinc, and then the substrate oxygen displaces the carboxyl group. Such a mechanism is supported by structures that have alternative positions for the zinc in some ADH complexes and by studies that show catalytic efficiency for alcohol oxidation is decreased when the glutamate residue is substituted with neutral amino acids. Computational studies of the horse enzyme also suggest that the glutamate can move to coordinate to the zinc. A role for Glu-68 (Glu-67 in yeast ADH) in the exchange of ligands on the catalytic zinc seems most reasonable. (Supported by NIH grant AA00279 and NIH training grant T32 GM008365)



P89

BsTrmFO: A Curious Methyltransferase from *Bacillus subtilis*

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BsTrmFO is a bacillus subtilis derived tRNA U-54 methyltransferase. It is proposed to methylate the uridine base in the T-arm of tRNA at position 54 to make thymidine, a highly conserved postransciptional modification in all kingdoms of life. The enzyme has a non-covalently bound flavin adenine dinucleotide (FAD) cofactor, requires nicotinamide adenine dinucleotide phosphate (NADPH) as a source of hydride, and methylenetetrahydrofolate (MeTHF) as a carbon source. TrmFO is believed to proceed via a novel version of thymidylate synthase chemistry, where the nucleotide base is activated by Michael addition with an active site cysteine. MeTHF supplies a methylene group, tetrahydrofolate is released as a product and NADPH acts to reduce the methylene. The aim of our lab is try and elucidate the mechanism of TrmFO utilizing spectrophotometric techniques, made possible by the reporting power of FAD, MTHF and NADPH. To date we have developed a reliable and reproducible expression and purification technique. Interestingly the enzyme elutes off of the cobalt affinity column with a proportion of the enzyme in an air stable flavin blue semiquinone form, that slowly oxidized over the course of a day. We have also verified the binding constants for some of the reactants and products of the reaction. This was done using either rapid mixing stopped flow, or an anaerobic titration. The redox potential was measured using a dye of known redox potential and a xanthine/xanthine oxidase reducing system. We have not yet been able to definitively show turnover for this enzyme either through mass spectrometry, or using the spectrophotometric techniques available to us. The goal of this project will be to find evidence for each observable step of the chemistry as the enzyme forms the product from the substrates.

An Analysis of the Effect of Side-Chain Interactions on the Efficiency of Glycerol-3-Phosphate Dehydrogenase Catalyzed Hydride Transfer*

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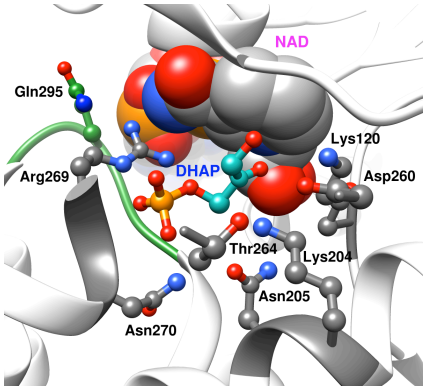
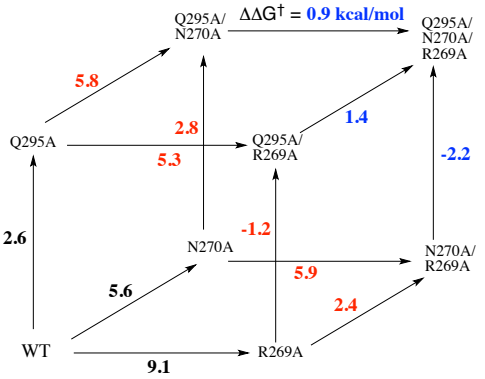
Three residues function in the activation of glycerol-3-phosphate dehydrogenase (GPDH) for catalysis of reduction of the whole substrate dihydroxyacetone phosphate (DHAP) by NADH (Figure on the left): the guanidinium cation of R269 forms a strong ion-pair to the substrate phosphodianion;[1] the amide nitrogen of N270 forms a hydrogen bond to the substrate phosphodianion and lies at one end of a string of hydrogen bonded side chains (N270, T264, N205, K204, D260 and K120)[2] that bridge the substrate phosphodianion and the carbonyl of DHAP; the amide side chain of Q295 is part of the flexible loop (L292–L297) that closes over the phosphodianion of enzyme-bound DHAP and forms a hydrogen bond to the cationic side chain of R269. The role of these side chains, and their intra side chain interactions, in reduction of the whole substrate DHAP and in phosphite dianion (HPO_3^{2-}) activation of reduction of the substrate piece glycolaldehyde (GA) catalyzed by GPDH was examined by determining the effects of all combinations of single, double and triple R269A, N270A and Q295A mutations on the kinetic parameters for these reactions. The energetic effects of these mutations on the activation barrier that governs the value of $(k_{cat}/K_m)_{DHAP}$ are illustrated in the Figure on the right. Two mutations result in increases in the activation barrier to $(k_{cat}/K_m)_{DHAP}$ for reduction of whole substrate DHAP, but in decreases in the barrier to $(k_{cat}/K_m)_{GA}$ for reduction of the truncated substrate GA. These data provide support for several conclusions, and form the basis for speculative hypotheses about the mechanism for enzyme activation by dianions.





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For NAD$^+$-dependent Glycoside Hydrolases Is There a Bona Fide Ketone Intermediate?

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Due to the importance of sugars in living systems, enzymes that remove carbohydrates (glycosidases or glycoside hydrolases) are important in many biological events. Most glycoside hydrolase (GH) families use a combination of general acid catalysis and either nucleophilic (retaining GHs, product has the same stereochemistry as the substrate) or general base (inverting GHs) catalysis. GH4 glycoside hydrolases were the first family shown to require NAD$^+$ for catalytic activity. This cofactor plays key catalytic roles in this atypical glycosidase mechanism, which involves elimination and redox steps.

Based on previous kinetic isotope effect (KIE) measurements, which involved separate rate measurements, it was proposed that this mechanism involves two sequential steps that are both partially rate-limiting, i.e., oxidation at C-3 and the subsequent C-2 deprotonation step (Fig. 1).[1] By use of the Cleland isotope effect on KIE methodology[2], Chakladar et al. suggested that hydride and proton transfer processes may be concerted.[3] The Bennet group has developed an NMR methodology for the measurement of competitive kinetic isotope effects on enzyme-catalyzed reactions.[4] As part of a continuing effort to characterize the mechanism for GH4 galactosidase-catalyzed hydrolyses several isotopically labelled fluorophenyl α-D-galactopyranosides have been targeted. The synthesis of these compounds and the measurement of KIEs on a GH4 family enzyme will be discussed.

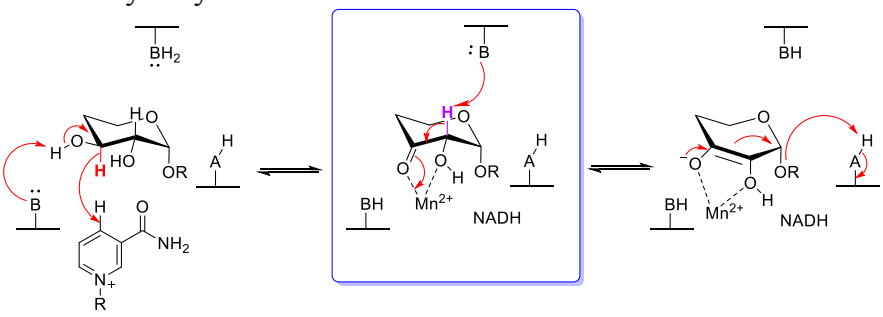

Figure 1. Proposed partially rate-limiting steps in GH4 mechanism.

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P92

Streptomyces wadayamensis MppP: a Novel PLP-Dependent L-Arginine Hydroxylase in L-Enduracididine Biosynthesis

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The nonproteinogenic amino acid L-enduracididine (L-End) is a critical component of the mannopeptimycins, a family of cyclic glycopeptide antibiotics with potent activities against drug-resistant pathogens like MRSA. Creating derivatives of mannopeptimycin and other L-End-containing natural products is hampered by the limited availability of L-End. We are investigating the L-End biosynthetic pathway in order to develop an efficient enzymatic or chemo-enzymatic route to produce this unusual amino acid. Recently, we found that MppP is a PLP-dependent L-Arg hydroxylase in L-End biosynthesis in Streptomyces wadayamensis. It reacts with L-Arg and dioxygen to yield two products, 2-ketoarginine and 4-hydroxy-2-ketoarginine in a ratio of ~1.7:1. Surprisingly, 1 equivalent of H_2O_2 is produced for each equivalent of O_2 consumed. In addition, the ratio of O_2 consumption to L-Arg consumption was ~1.4:1, suggesting that one product requires 2 equivalents of O_2, and the other only 1 equivalent. We have determined the structures of MppP in four states: the internal aldimine, the external aldimine with the substrate, L-Arg, the product complex with 4-hydroxy-2-ketoarginine, and the product complex with 2-ketoarginine. According to the structures, the N-terminal helix is disordered in the internal aldimine and covers the active site only when the substrate is bound. A glutamate residue in the N-terminal helix, E15, makes a hydrogen bonding interaction with the carboxylate of the substrate, L-Arg. This observation prompted us to make three N-terminal variants of SwMppP: E15A, E15Q, and the truncation mutant, SwMppP23-276. Steady state kinetics showed that the two point mutants had no effect on kcat or $K_{M,L\text{-}Arg}$. The truncation mutant, however, showed an approximately 10-fold increase in K_M and a 3-fold decrease in k_{cat}, which reduced the pseudo-second order rate constant by almost 25-fold. Interestingly, although the steady state kinetics (as measured by dioxygen consumption) are indistinguishable for the E15A and E15Q variants, the E15A variant does not produce 4-hydroxy-2-ketoarginine, only the abortive product 2-ketoarginine. Likewise, the truncation mutant also produced only 2-ketoarginine. Our structural and kinetic characterization of the wild-type and variant forms of SwMppP have allowed us to propose a revised model where the oxygen incorporated in the hydroxy-arginine product is derived from water rather than from dioxygen.

Structural and Mechanistic Studies of 6-Hydroxynicotinate 3-Monooxygenase: A Decarboxylative-Hydroxylase in Bacterial Nicotinate Catabolism

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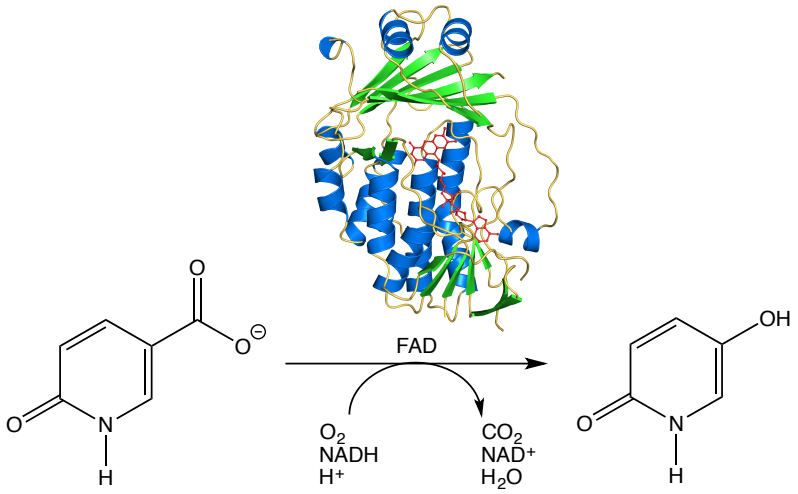
Nicotinate (vitamin B_3) can serve as a source of carbon and energy for a number of bacteria and its catabolic pathway is considered a model system for understanding the biodegradation of *N*-heterocyclic compounds in the environment. The genes coding for the enzymes of aerobic nicotinate degradation in several bacteria have recently been identified and provide opportunities to elucidate the molecular mechanisms. The crystal structure of 6-hydroxynicotinate 3-monooxygenase, a flavin-dependent enzyme that catalyzes an early step in nicotinate catabolism, has been determined at 2.1 A resolution.[1] Results from kinetic isotope effects, pH dependences of k_{cat} and k_{cat}/K_M, stopped flow analysis of the rate of O_2 incorporation, and the consequences to catalysis of removing putative active site residues suggest two potential mechanisms for this novel decarboxylative-hydroxylase.



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P94

Phosphate Release as a Probe for ATP Binding and Cleavage in Pyruvate Carboxylase

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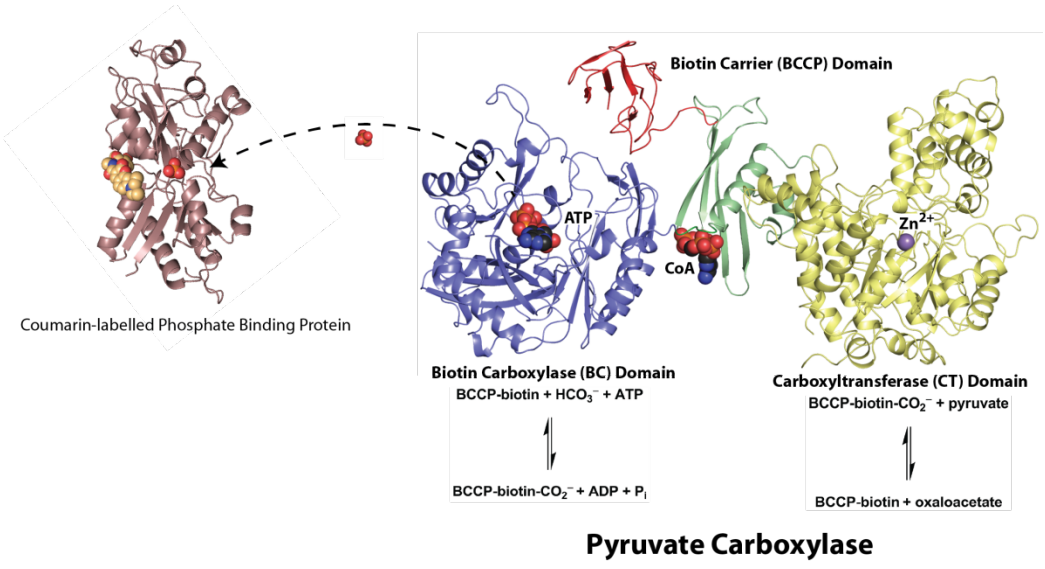
Pyruvate carboxylase (PC) is a multifunctional, biotin-dependent enzyme that catalyzes the production of oxaloacetate through the ATP-dependent carboxylation of pyruvate. The reaction proceeds in two distinct half-reactions catalyzed in two separate active sites: the biotin carboxylase (BC) domain catalyzes the bicarbonate-dependent carboxylation of the covalently attached biotin cofactor with the concomitant cleavage of Mg-ATP to Mg-ADP and P_i. The carboxylated biotin cofactor, covalently tethered to the biotin carboxyl carrier protein (BCCP) domain, subsequently travels ~60-80Å to the carboxyltransferase (CT) domain, where the carboxyl group is transferred from carboxybiotin to pyruvate to generate oxaloacetate. We have employed a long-standing fluorescence-based assay using a coumarin-modified *Escherichia coli* phosphate binding protein (1) to follow the release of phosphate from PC under both pre-steady state and steady state conditions. This assay affords substantially higher resolution than previous pre-steady state studies of ATP cleavage in PC and reveals that both the allosteric activator, acetyl-CoA, and the CT domain substrate, pyruvate, significantly alter the rate of phosphate release, but not the rate of ATP binding. We discuss these findings in relation to the current description of allosteric regulation and catalysis in PC.



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The Putative Reduced Flavin-Dependent Methanesulfinate Monooxygenase MsuC

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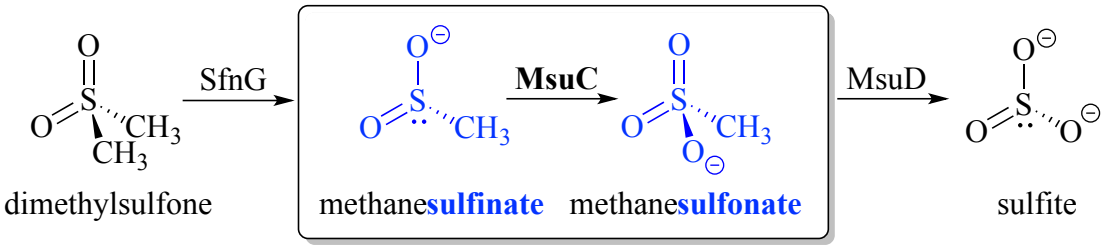
Bacteria that thrive in aerobic soil environments produce regulatory, transport, and metabolic proteins to assimilate sulfur from readily available organosulfur compounds for biosynthesis. Dimethylsulfone ($(CH_3)_2SO_2$ or $DMSO_2$) has been proposed as an intermediate in the assimilation of sulfur from dimethylsulfide-utilizing bacteria. Recently, the metabolic pathway from $DMSO_2$ to sulfite was proposed (Scheme 1), providing for the first time a physiological role for methanesulfinate, the biochemical product of $DMSO_2$ monooxygenase SfnG (1). It has also been shown that MsuD desulfonates methanesulfonate, yielding sulfite (2). Both SfnG and MsuD are two-component $FMNH_2$-dependent enzymes that function in the presence of the NADH-dependent flavin reductase MsuE. The gene *msuE* is a member of the three-gene operon *msuEDC* encoding proteins involved in sulfur assimilation, but the physiological function of *msuC* is currently unknown. The *msuC* gene is annotated to encode a putative dehydrogenase, therefore we hypothesized that MsuC might be involved in the oxidation of methanesulfinate to methanesulfonate and, thus, complete the sulfur assimilation pathway from $DMSO_2$. The *msuC* gene from *Pseudomonas fluorescens* Pf0-1 was cloned, and the protein was synthesized in *Escherichia coli* and purified. The enzyme MsuC functions as a flavoprotein monooxygenase and converts methanesulfinate to methanesulfonate in the presence of FMN, NADH, and MsuE, as evidenced by nuclear magnetic resonance spectroscopy.



Scheme 1. The proposed sulfur assimilation pathway from $DMSO_2$ to sulfite. The enzymes SfnG, MsuC, and MsuD are reduced flavin-dependent monooxygenases that utilize O_2 and $FMNH_2$ as substrates; $FMNH_2$ is provided by the NADH-dependent flavin reductase MsuE.

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P96

Development of Genetic Tookit and Synthetic Circuity in *Chromobacterium violaceum*

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Chrombacterium violaceum is a Gram-negative cyanogenic bacterium with the potential to replace current industrial cyanidation processes. However, the lack of genetic tools has severely limited the study and use of *C. violaceum* for synthetic biology. We have developed a set of genetic toolkit consisting of 2 compatible origin of replications, 3 suitable antibiotic resistance cassettes, 4 different inducible promoters systems of total 10 000 fold dynamic range of gene expression and 6 constitutive promoters with 1000 fold dynamic range of gene expression in *C. violaceum*. This is the first report of replicating vectors in *C. violaceum* and these genetic parts characterization will pave the way for genetic engineering and synthetic biology applications in *C. violaceum.*

P97

Labeling of Enzymes Using 1,3-Dipolar Cycloaddition to Facilitate Their Functional Studies

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Labeling of enzymes with small chemical probes such as fluorescent functional groups and NMR active fluorine-containing groups is a great tool to facilitate the study of functions and mechanisms of enzymes. Despite achievements such as the azide-alkyne cycloaddition reaction (1) for site-selective enzyme modifications, it remains challenging to develop labeling reagents which can work effectively under physiological conditions without affecting the common functional groups in enzymes. We will present a strategy of first genetically incorporating an unnatural amino acid, N^{ε}-acryloyl-L-lysine (2), into enzymes and then selectively labeling the acryloyl group with nitrone molecules via 1,3-dipolar cycloaddition reaction to introduce chemical probes. We consider such strategy to be a nice addition to the tool box for studying enzyme mechanisms.

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P98

Biochemical and Biophysical Aspects of a *Campylobacter* Phospho-Glycosyltransferase

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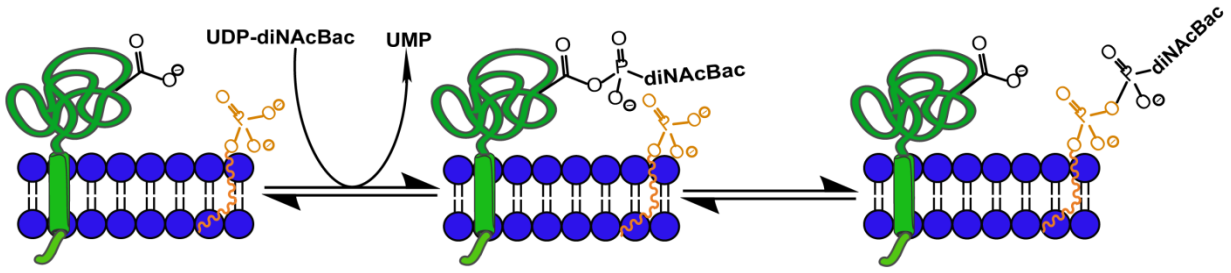
Phosphoglycosyltransferases (PGTs) are families of integral membrane proteins with diverse architectures. These enzymes function as the "gatekeepers" in essential biological pathways found in all three domains of life. PGTs catalyze the initial step in many glycoconjugate assembly pathways including those leading to peptidoglycan, N-linked glycoproteins and lipopolysaccharide O-antigen. In spite of considerable efforts, the characterization and mechanistic understanding of these important enzymes are extremely challenging in part due to the difficulties associated with the expression, purification and stability of integral membrane proteins and in part due to the limited availability of convenient enzyme activity assays.

Herein, we present studies on PglC from *Campylobacter*, which is a representative member of the extensive bitopic PGT family. PglC initiates the membrane-associated N-linked protein glycosylation pathway (pgl) in *Campylobacter*. PglC has one predicted transmembrane domain and the enzyme transfers a phospho-sugar from a soluble nucleotide-diphospho-sugar donor substrate to a membrane-anchored undecaprenyl phosphate acceptor thus generating the first membrane-bound intermediate in the pgl pathway. The kinetics and the mechanism of action of PglC were explored for the first time by employing a newly-developed luminescence-based UMP-Glo assay together with the traditional radioactivity-based assay. Based on several complementary experimental approaches PglC was determined to follow a ping-pong mechanism, which involves formation of a covalent phospho-sugar enzyme intermediate. This experimentally-validated mechanism of PglC is unprecedented among the known members of the family of PGT enzymes.

Further, extensive optimization of the expression and purification of PglC was also carried out in order to obtain PglC in high yield, purity and homogeneity for crystallographic analysis. The purified protein has now afforded reproducible crystals that have yielded full diffraction datasets. Efforts are currently ongoing to solve the structure of the protein.



P99

Real-Time Analysis of Conformational Control in Electron Transfer Reactions of Nitric Oxide Synthase (NOS)

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Mammalian nitric oxide synthases (NOS) produce the signalling molecule nitric oxide (NO) *via* a series of complex electron transfer reactions which are inferred to be controlled by calmodulin binding and protein domain dynamics. Electrons, originating from NADPH, flow through the FAD and FMN cofactors in the reductase domain, to the heme-oxygenase domain where NO is generated. Despite a wealth of structural, single molecule and ensemble spectroscopic approaches demonstrating the importance of NOS domain dynamics, motions have not been correlated experimentally with mechanistic steps in the NOS reaction cycle. In this study we have used a site directed fluorophore labelling method to decorate the NOS binding partner CaM with extrinsic Förster Resonance Energy Transfer (FRET) 'donor' and 'acceptor' molecules. We show that NOS bound CaM undergoes conformational changes which are kinetically coupled to key steps in NOS flavin reduction. Moreover, by making use of a chemically 'inert' flavin analog (5-deazaflavin mononucleotide) and isotopically labelled nicotinamide coenzymes we have assigned mechanistic steps in NOS flavin reduction as well as identify conformational driving forces (coenzyme binding/reaction chemistry) for redox-linked NOS-bound CaM dynamics.

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LigY: Metal-Dependent *meta*-Cleavage Hydrolase

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Sphingomonas paucimobilis SYK-6 uses a *meta*-cleavage pathway to catabolize lignin-derived biphenyl. In this pathway, LigY has been proposed to catalyze the hydrolysis of 4,11-dicarboxy-8-hydroxy-9-methoxy-2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (DCHM-HOPDA), a *meta*-cleavage product (MCP), to 5-carboxyvanillate (5CVA) and 4-carboxy-2-hydroxypenta-2,4-dienoate (CHPD) (1). Herein, we identified CHPD as the dienoate hydrolysis product, which is consistent with the proposed hydrolysis reaction by LigY. Sequence analysis determined that LigY belongs to the metal-dependent amidohydrolase superfamily (COG2159), unlike previously described MCP hydrolases, which use a Ser-His-Asp catalytic triad and belong to the α/β hydrolase superfamily. The specific activity of heterologously produced LigY correlated linearly with its Zn content and was abolished upon incubation with EDTA and *o*-phenanthroline. Metal titration of apo-LigY failed to generate a saturation curve suggesting a weak metal affinity atypical of metalloenzyme. Enzyme preparations with a single Zn per monomer were obtained when the culture media was supplemented with $ZnSO_4$ and had k_{cat} and k_{cat}/K_m values of 9.50 ± 0.07 s^{-1} and 25.3 ± 0.8 × 10^6 $M^{-1}s^{-1}$, respectively. A LigY crystal structure determined to a resolution of 1.8 Å revealed a single active site Zn^{2+} per protomer coordinated by His6, His8, His179 and Glu282, similar to what is observed in α-amino-β-carboxymuconic-ε-semialdehyde decarboxylase (ACMSD), another amidohydrolase (2). In solution, the protein forms a hexameric complex that can be replicated *in-crystallo* through symmetry. The substitution of conserved residues Glu282 or Arg234 reduced k_{cat} by 2 orders of magnitude. By contrast, substitution of conserved His-223 only lowered the k_{cat} by 50%. We propose a mechanism that proceeds via a gem-diol intermediate, in contrast to the acyl-enzyme intermediate reported in Ser-dependent MCP hydrolases, and expands the known range of reactions catalyzed by the amidohydrolase superfamily.

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P101

Immobilization Study of Recombinant beta-Xylosidase XynB2 from *Geobacillus stearothermophilus* on Chitosan-Chitin Support

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Enzyme immobilization is preferred for industrial and biotechnological purposes as it presents several advantages, such as stability enhancement, reusability and generally an activity increase. This study aims to immobilize XynB2, a xylan degrading recombinant enzyme from *Geobacillus stearothermophilus*, onto a chitosan-chitin polysaccharide support. The effect of parameters like pH and temperature were studied to optimize the immobilization process. The optimum pH for binding was found to be 7, performed at 75 °C. Optimal activity values for the non-immobilized enzyme were pH 6.5 and 65 °C. Enzyme amounts used for immobilization ranged 193 ± 6 µg. Finally, at the optimized conditions assay of immobilized and the free XynB2 enzyme was carried out. Even though no increase in the specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) of immobilized enzyme was observed when compared to the free enzyme, the total activity ($\mu mol \cdot min^{-1}$) remained unchanged. Biotechnological characterization showed that the reusability of the enzyme after 7 cycles retained 60% of its original activity. These results corroborate the biotechnical and economic advantages of enzyme immobilization for a variety of industrial applications. Yet, other supports must be further tested in order to obtain an effective immobilization process.

P102

Investigating HDAC8 Substrates from Test Tube to Cell

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Acetylation is an important post-translational modification found in a wide-variety of processes throughout the cell, most notably on histones during chromatin remodeling. Misregulation of acetylation is implicated in disease, making acetylation writers (lysine acetyltransferases-HATs or KATs) and erasers (acetyllysine deacetylases-HDACs or AcKDACs) attractive targets for inhibitor development. HDAC8, a class I, metal-dependent, human deacetylase, is the best structurally and biochemically understood deacetylase. However, our knowledge of HDAC8 substrates in the cell is limited. To date, the best-validated cellular substrate of HDAC8 is the structural maintenance of chromosomes protein SMC-3. Our lab and our collaborators are combining computational modeling, biochemical techniques and HDAC8-specific inhibitor studies in cell culture to identify the cellular substrates of this important enzyme. Currently, we are studying a number of potential in vivo HDAC8 substrates including histone H3, HDAC2 and isocitrate dehydrogenase 1 (IDH1). Histones tails are well-documented substrates of deacetylases, and we have purified and assayed singly acetylated histone H3, within contexts ranging from short peptides to full-length nucleosome. Acetylated K75 of HDAC2 is the best-scored aceytyllysine substrate from our computational model. We are currently working to purify and assay acetylated HDAC2 as well as identify changes in acetylation of HDAC2 upon treatment with an HDAC8-specific inhibitor in human cells. A protein-on-chip-based experiment showed HDAC8-catalyzed deacetylation of IDH1 suggesting it as an in vivo substrate. IDH1 has been purified, and in vitro assays demonstrate that HDAC8 catalyzes deacetylation of this protein. We are now working to assay changes in IDH1 acetylation in mammalian cell culture upon treatment with an HDAC8-specific inhibitor. Together, these efforts allow us to develop robust methods to identify HDAC8 cellular substrates and lead us closer to elucidating the function of HDAC8 in the cell.

P103

Determining Histone Deacetylase 8 Substrates Using Non-Natural Amino Acids

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The histone deacetylase family is comprised of 18 isozymes that catalyze the removal of an acetyl group from the ε-position of a lysine residue. Lysine (de)acetylation is a post-translational modification that affects over 3600 different macromolecules and has been implicated in various types of cancers and neurodegenerative diseases. To elucidate and identify HDAC substrates, we have incorporated the photo reactive, non-natural amino acid – p-benzonyl-4-phenylalanine (pBpa) – into HDAC8, a structurally well characterized isozyme of this family of enzymes with a largely unknown *in vivo* substrate pool. Using photo crosslinking and proteomics will allow us to identify both putative substrates and binding partners of HDAC8 and enhance the understanding of how this enzyme is regulated *in vivo*. In addition, these experiments will validate this approach as a feasible system to probe other HDAC isozymes specificities.

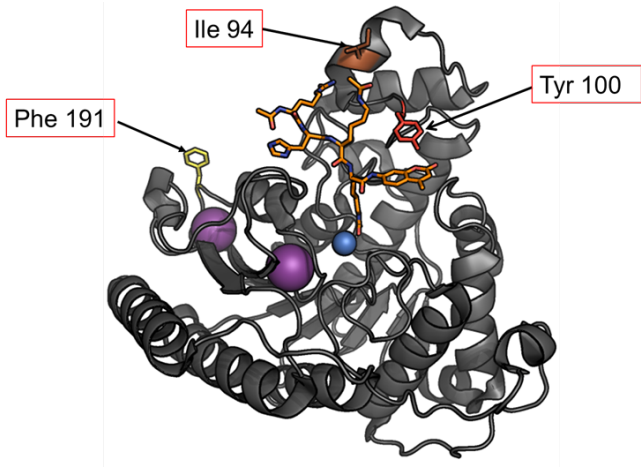

Figure 1. Non-natural amino acid substitutions for HDAC8 crosslinking. Mutations cover areas close to the active site (I94 and Y100) and far away from the active site (F191)

P104

N-Acyltransferases: Mechanism and Metabolism

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Fatty acid amides represent a broad family of bioactive lipids, **R**-CO-NH-**Y**. The structural simplicity of these compounds belies a wealth of diversity amongst this lipid family because the **R**-group is derived from fatty acids (**R**-COOH) and the **Y**-group is derived from biogenic amines (H_2N-**Y**). We have proposed that fatty acid amides are produced, *in vivo*, by a reaction between an acyl-CoA and an amine: CoA-S-CO-**R** + H_2N-**Y** → **R**-CO-NH-**Y** + CoA-SH. The enzyme catalyzing this reaction would be an *N*-acyltransferase, most likely, a member of the GCN5 superfamily of enzymes. We have expressed and characterized a number of *N*-acyltransferases in *E. coli*, including mouse glycine *N*-acyltransferase (GLYAT), four arylalkylamine *N*-acyltransferases (AANATs) from *Drosophila melanogaster*, and two AANATs from *Bombyx mori*. Our substrate specificity studies from this panel of enzymes reveal that a few of the enzymes exhibit promiscuous selectivity for acyl-CoA and/or amine substrates and a few exhibit narrow substrate specificities. Notably, we identified an AANAT in *D. melanogaster* (AANATL2) and *B. mori* (iAANAT0) that will accept long-chain acyl-CoA substrates to generate fatty acid amides. Metabolic studies in *D. melanogaster* provide strong evidence that AANATL2 does have a role, *in vivo*, in the cellular production of long-chain *N*-acylserotonins and *N*-acyldopamines. We have also identified an *N*-acyltransferase in *D. melanogaster* that catalyzes the acetylation of agmatine and polyamines and solved the high-resolution structure of this enzyme, which we named polyamine *N*-acetyltransferase (PNAT). Studies of the kinetic and chemical mechanism point to similarities amongst the *N*-acyltransferases we have investigated: the kinetic mechanism is ordered with the acyl-CoA binding first. All show an enzymic base, usually an active site glutamate, that is likely responsible for the deprotonation of the amine substrate to facilitate nucleophilic attack at the carbonyl of the acyl-CoA thioester. We have accumulated a significant portfolio of data, using a variety of techniques (site-directed mutagenesis, direct binding measurements, and protein NMR) that point towards catalytically important conformational changes in the *N*-acyltransferases. The motion associated with the conformational changes seem linked to amine substrate binding and CoA-SH release. ITC binding data for one of the *B. mori N*-acyltransferases, *Bm*-iAANAT3, shows that the amine substrate binds tightly to the enzyme only after acetyl-CoA binds. In sum, our work on set of *N*-acyltransferases has led to new insights regarding the relationship between structure and function in these enzymes, the discovery of enzymes that are, most likely, responsible for the formation of fatty acid amides *in vivo*, and the identification of previously unknown metabolites. Recent publications on this project are below (with others pending):

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P105

Experimentally Dissecting the Origins of Peroxiredoxin Catalysis

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Peroxiredoxins are ubiquitous, cysteine-based peroxidases that play critical roles in oxidant defense and regulation of signal transduction. Their highly conserved active site architecture activates the cysteine and peroxide, yet the precise roles of conserved residues remain poorly defined. Here conserved residues were mutated in a model decameric bacterial peroxiredoxin, *Salmonella typhimurium* AhpC: ten variants were studied, employing multiple complementary approaches to assess the effects on catalytic activities, pKa of the active site cysteine, structural features, and oligomeric status.

Three active site proximal mutations of AhpC, T43V, R119A and E49Q, lowered catalytic efficiency with hydrogen peroxide by 4 or 5 orders of magnitude, but did not affect reactivity toward their reductant, AhpF. pKa values of the peroxidatic cysteine were also shifted up by 1 to 1.3 pH units for these and a decamer disruption mutant, T77I. All mutants (except for decamer stabilizing T77V) exhibited destabilized decamers in reduced form and most in oxidized form. Surprisingly, AhpF more efficiently reduced these stabilized decamers. Crystal structures of most mutants showed alterations in stability or arrangement of the active site loop, but in T43S alterations were restricted to approximately equal occupancy of two side chain conformations.

Conclusion: Structural perturbations and associated decamer destabilization of many mutants accounted for many functional defects; clearly the arginine side chain must be properly situated for efficient catalysis.

P106

Making Sense of Isotope Effects and Free Energy Relationships in Enzymatic Phosphoryl Transfer

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A reaction's transition state (TS) structure plays a critical role in determining reactivity and has important implications for the design of catalysts, drugs, and other applications. Here we present recent results on TS structure in alkaline phosphatase using hybrid Quantum Mechanics/Molecular Mechanics simulations. We find that minor perturbations to the substrate have major effects on TS structure and the way the enzyme stabilizes the TS. (1) Substrates with poor leaving groups (LGs) have little cleavage of the phosphorus-LG bond at the TS while substrates with good LGs have substantial cleavage of that bond. Furthermore, an active site Zn^{2+} stabilizes the LG at the TSs with substantial bond cleavage, but active site water molecules stabilize the LG of those with less cleavage. The results predict non-linear free energy relationships for a single rate-determining step, and substantial differences in kinetic isotope effects (KIEs) for different substrates; both trends were observed in previous experimental studies, although the original interpretations of those experiments differed from the present model. To thoroughly test the model against available experiments, we conducted an extensive analysis of KIEs for this system. (2) The results show how the complex active site of a metalloenzyme has led to difficulties in interpretation of experimental results, but that the model we propose is consistent with those experiments. We will also address the question of how well TS analogue inhibitors for this system truly mimic TSs that vary between similar substrates. (3) Overall, our results demonstrate the considerable plasticity of both TS structure and how enzymes stabilize TS structures. Furthermore, our results emphasize that perturbations to reactivity that probe TS structure experimentally (i.e., substituent effects) may substantially perturb the TS they aim to probe.

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P107

Characterization of a Specific Fluorogenic Peptide Substrate and an Active Site-Directed Inhibitor to the Mitochondrial ATP-Dependent Protease Clpxp

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The goal of this work is to generate specific substrates and inhibitors for the human ATP-dependent protease known as ClpXP (Caseinolytic Protease complex containing subunits X and P) so that these materials can be used as chemical biology tools to investigate the physiological functions of this enzyme. The ClpXP complex is found in the matrix of mitochondria and is speculated to play a role in mitochondrial protein quality control. Currently, physiological substrates of human ClpXP are not well-known. Therefore, knowing metabolic changes that up-regulate ClpXP and the physiological protein substrates will provide insights into the physiological functions of this relatively understudied mammalian protease. Based on the cleavage profile of a specific peptide sequence found in an endogenous substrate of mitochondrial ClpXP, a fluorogenic peptide substrate and a mechanism-based active site label that functions as a specific inhibitor have been generated. This poster reports the characterizations of the peptide substrate and inhibitor as activity probes of human ClpXP and discusses their potentials as chemical biology tools in cell culture studies.
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P108

Experimental Validation of AcKDAC Substrates Identified Using a Chip-Based Proteomics Method

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Acetylation of protein lysine residues is a reversible post-translational modification occurring ubiquitously in eukaryotic cells. Regulation and maintenance of the acetylome is required for a myriad of physiological processes, and aberrant acetylation is associated with disease states including cancers, inflammation, and the X-linked disorder Cornelia de Lange Syndrome. Removal of this modification is catalyzed by the acetyllysine deacetylases (AcKDACs), which include 11 metal dependent isozymes. A major hurdle in developing targeted AcKDAC inhibitor-based therapies for these diseases is the lack of a comprehensive understanding of the substrate selectivity of individual AcKDAC isozymes. To address this issue, we developed a chip-based proteomics method to screen the substrate selectivity of individual isozymes. Using this technique, we identified 44 and 25 potential substrates for AcKDAC8 and AcKDAC11, respectively. These substrates were then further validated using peptide mimics to measure AcKDAC-catalyzed deacetylation with both an enzyme-coupled assay and mass spectrometry. We observe deacetylase activity of these peptide substrates with both AcKDAC isozymes and determined rate constants that ranged over three orders of magnitude. Based on these results, we expressed and purified a putative AcKDAC8 substrate, isocitrate dehydrogenase 1 (IDH1). We utilized a non-natural amino acid incorporation strategy to express IDH1 with site-specific biologically relevant acetyllysine residues and found that acetylation significantly decreased IDH1 catalytic activity. Finally, we showed that AcKDAC8 catalyzes deacetylation of these full-length protein substrates *in vitro* with high efficiency.

'Negative Catalysis' by a Conserved Threonine Prevents Self-Intoxication of Enoyl-Thioester Reductases

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Enzymes are highly specific biocatalysts, yet they can perform unwanted side reactions. Here we investigated the factors that direct catalysis in the enoyl-thioester reductase Etr1p. We show that a single conserved threonine is essential in suppressing the formation of a side product (C4 adduct) that otherwise acts as a high-affinity inhibitor of the enzyme. Substitution of this threonine with an isosteric valine increases side product formation by more than six orders of magnitude, while decreasing turnover frequency by only one order of magnitude. Our results show that promoting wanted reactions ('positive catalysis') and suppressing unwanted side reactions ('negative catalysis') are independently operating principles at the active site of Etr1p, and that the active suppression of side reactions is highly conserved in the family of medium chain dehydrogenases /reductases (MDR). Our discovery emphasizes that the active destabilization of competing transition states is an important factor during catalysis, which has implications for the understanding and the de novo-design of enzymes.

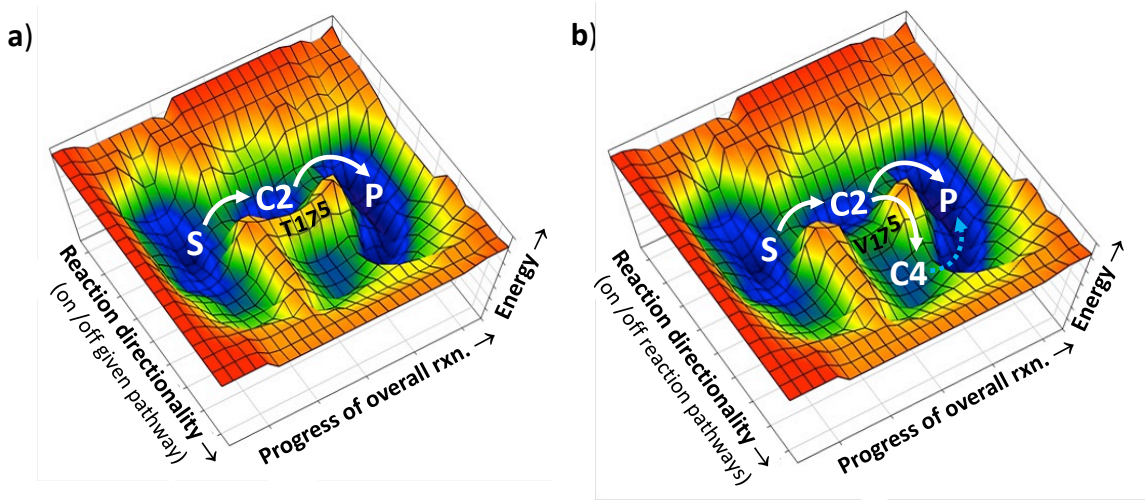

Figure 1 Hypothetical landscapes of Etr1p WT (a) and the T175V variant (b). Tyrosine 175 suppresses the unwanted production of the toxic C4 inhibitor. Mutation of this tyrosine to an isosteric valine lowers the energetic barrier to form the C4 adduct leading to increased inhibitor formation and self-intoxication of the enzyme.

P110

CTAB Zymography for the Analysis of Aspartic Proteases from Marine Sponge *Amphimedon erina*

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Electrophoresis under denaturing conditions in the presence of SDS is a standard method for the enzyme scientist. Nevertheless, there are special situations where this method may originate non-optimal results. SDS may cause protein aggregation or precipitation, and additionally some migrate abnormally, depending on the type of protein. However, SDS -an anionic detergent- may be substituted by CTAB (cetyltrimethylammonium bromide) -a cationic detergent- in order to solubilize and electrophorize proteins. CTAB electrophoresis allows the separation of proteins based on molecular weight and can be carried out at neutral or acidic pH. This work describes the development of a CTAB zymography method to analyze aspartic proteases from marine sponge *Amphimedon erina*, which present an abnormal high R_f value when run in SDS-PAGE. The special feature of using CTAB is that it binds proteins, making them positively charged and thus migrating in the opposite direction, compared to SDS-PAGE. CTAB-PAGE not necessarily results as a more complicated method compared to SDS-PAGE, though polymerization of acidic gels could somehow be considered more bothersome than regular basic gels. Here, we have enhanced the cationic electrophoresis by copolymerizing gelatin in the gel matrix in order to develop a cationic zymography method. Zymography enables the detection of enzymatic activity, under non-reducing conditions, directly on the gel. Yet, enzyme activity may be inhibited by the presence of the detergent. In this regard, after the run, the gel is washed with Triton X-100, in order to remove the detergent and allowing renaturalization of the enzyme. Afterwards, the gel is placed in an activation buffer, allowing the enzyme to catalyze the hydrolysis reaction of the substrate. Finally the gel is stained with Coomassie Brilliant Blue (CBB) in order to visualize the enzyme activity as translucid bands under a deep blue background. In this study, aspartate protease activity present in *Amphimedon erina* was 84 ± 1 nmol/min, at 280 nm employing bovine serum albumin (BSA) as substrate. The electrophoretic profile was evaluated by SDS-PAGE and CTAB-PAGE. The latter allowed separation of acidic proteins more efficiently and with reproducible results. Aspartate protease activity was confirmed employing inhibitors: 1 mM PMSF, 1 mM iodoacetamide, 10 mM EDTA, 1 mM pepstatin A, 10 mM leupeptin, 1% CTAB, 1% SDS. By using CTAB-PAGE a shift of the active band containing the enzyme activity from R_f 0.38 to 0.56 was detected, compared to SDS-PAGE results. Further studies should be performed to test the linear relationship between R_f and MW values in CTAB-PAGE, by using specific protein standards with acidic properties. It is suggested to incorporate CTAB-PAGE as an obligatory parallel analysis to SDS-PAGE when analyzing aspartate proteases.

P111

Evolutionary Drivers of Thermoadaptation in Enzyme Catalysis

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With early life likely to have existed in a hot environment, enzymes had to cope with an inherent drop in catalytic speed caused by lowered temperature. Here we characterize the molecular mechanisms underlying thermo-adaptation of enzyme catalysis in adenylate kinase using ancestral sequence reconstruction spanning 3 billion years of evolution. We show that evolution solved the enzyme's key kinetic obstacle – how to maintain catalytic speed on a cooler Earth – by exploiting transition-state heat capacity. Tracing the evolution of enzyme activity and stability from the hot-start towards modern hyperthermophilic, mesophilic and psychrophilic organisms illustrates active pressure versus passive drift in evolution on a molecular level, refutes the debated activity/stability tradeoff, and suggests that the catalytic speed of adenylate kinase is an evolutionary driver for organismal fitness.

P112

Synthesis of Dimerization Inhibitors of Neuronal Nitric Oxide Synthase

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Preclinical studies have established that overproduction of nitric oxide (NO), a soluble second messenger that is produced by neuronal nitric oxide synthase (nNOS), is linked to neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases[1-4]. Therefore, successful inhibition of nNOS could benefit patients that suffer from these diseases. However, there are three isoforms of nitric oxide synthase along with nNOS that all take part in a wide variety of biological pathways. Early development efforts with non-selective NOS inhibitors were found to interfere with too many critical biological pathways, resulting in high toxicity in animal studies. Therefore, finding a selective nNOS compound is critical for clinical success. This project specifically employs the strategy of inhibiting the dimerization of nNOS, so that the enzyme is prevented from entering its active homodimer form. Screening of compounds from the Silverman lab has identified a potential lead that has some nNOS selectivity and dimerization inhibition. GOLD docking simulation of this compound revealed two moieties: (1) heme-binding imidazolyl moiety that attaches the molecule to the heme pocket on the oxygenase region of nNOS and (2) "tail" moiety that selectively interacts with nearby nNOS specific residues causing the monomer nNOS to change confirmation, disrupting the dimer interface. Current efforts in the project involve optimizing each of the moieties in order to improve selectivity and increase potency of the compound.

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P113

Optimizing, Understanding, and Exploiting Allosteric Inhibition of Dihydrodipicolinate Synthase

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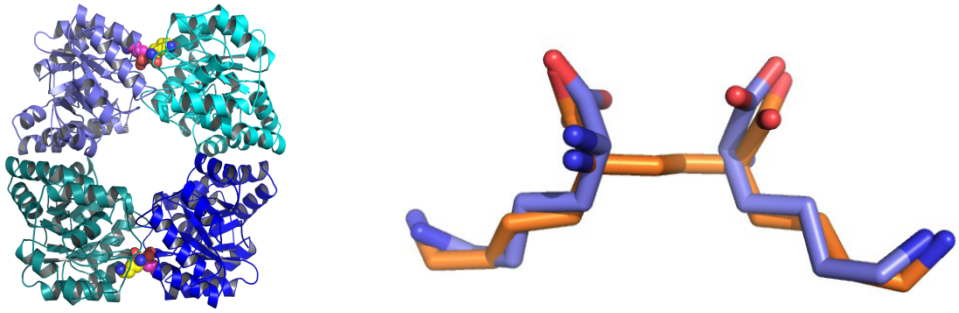
Dihydrodipicolinate synthase (DHDPS) is a Schiff base-dependent aldolase that catalyzes the reaction of pyruvate and aspartate β-semialdehyde (ASA), the first committed step in the diaminopimelate pathway by which lysine is synthesized in most bacteria and plants. This enzyme is allosterically regulated by lysine. Kinetics[1] has shown the inhibition to be cooperative, high-resolution X-ray structures have detailed the allosteric site of binding,[2] and proton-deuterium exchange experiments[3] show that the inhibitor decreases protein dynamics necessary for catalysis. We have designed molecules that mimic the binding of two lysine molecules, resulting in the most potent DHDPS inhibitor reported (K_i = 200 nM), which we named "bislysine".[4] Moreover, allosteric site mutations that negate lysine binding have little effect on bislysine potency. We have now developed a fluorescence-based method for detecting the binding of non-fluorescent inhibitors with which we can screen new inhibitor candidates.



Left: *Campylobacter jejuni* DHDPS bound to lysine (PDB 4LY8)
Right: Overlay of DHDPS-bound lysine, orange, and DHDPS-bound bislysine, blue (PDB 5F1V)

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P114

A Versatile Method to Determine the Cellular Bioavailability of Small-Molecule Protein–Protein Interaction Inhibitors

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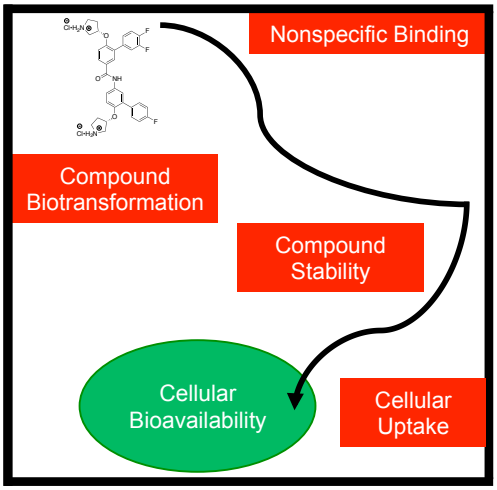
The determination of the cellular bioavailability of small-molecule inhibitors is a critical step for the interpretation of cell-based data and guides further inhibitor optimization. Traditionally, cell permeability has been the primary mechanism used to explain low cellular bioavailability of small-molecule inhibitors in cellular assays. However, other mechanisms such as cellular uptake, compound biotransformation, compound stability, and nonspecific binding also affect the cell-based activity of small-molecule inhibitors. To characterize the effect of these mechanisms, a HPLC/MS-based protocol was developed to determine the cellular bioavailability of small-molecule protein–protein interaction (PPI) inhibitors. This protocol allows the characterization of various properties for the inhibitors including the extra- and intracellular stability, dose- and time-dependent intracellular concentrations, the cell permeability, and the nonspecific binding with cell culture plates, extracellular matrices, and the cell membrane. Bromodomain and β-Catenin/B-cell lymphoma 9 PPI inhibitors were used to examine this protocol, and their cellular bioavailability in cancer cells was disclosed. Two techniques described in the protocol can also be used to study the relationship between inhibitor functional groups and substructures, and the binding with plasticware, extracellular matrices, and the nonspecific binding with the cell membrane.



P115

Profiling Cohesive-End Ligation Fidelity by Pacific Biosciences Single Molecule Real-Time Sequencing

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DNA ligases, key enzymes in molecular biology and synthetic biology, catalyze the joining of single strand breaks in duplex DNA (nicks) as well as the joining of multiple dsDNA fragments. Ligation fidelity has important consequences for the yield, accurate assembly, and maximum number of fragments that can be assembled concurrently in synthetic biology applications such as Golden Gate cloning. In this study, we have applied Pacific Biosciences single-molecule sequencing technology to develop a new method to characterize the fidelity of ligation when joining DNA fragments with short 5′-overhangs. Pools of substrates containing all possible three or four base overhang sequences were ligated with T4 DNA ligase, and the ligation products were read directly by sequencing individual molecules. This work has allowed us to determine the overall mismatch ligation frequency, the mismatch frequency for each individual subsequence, and to characterize the particular mismatches that have a high propensity for ligation. This methodology is easily adaptable to other dsDNA substrate structures, and provides a method to screen for ligases or buffer conditions that provide higher fidelity ligation reaction outcomes. The information generated by this study will allow for optimization of DNA fragment assembly protocols, both by the development of high fidelity ligases and by the avoidance of non-complementary overhang pairs prone to misligation.

P116

Site-Specifically Labeling Multi-Protein Complexes Using Non-Canonical Amino Acids

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Replication Protein A (RPA) is a single-strand DNA (ssDNA) binding protein that protects ssDNA in the cell from nucleolytic attacks and plays an integral role in maintaining genomic integrity. RPA is a heterotrimeric complex consisting of three distinct subunits - RPA 70, RPA32 and RPA14, and binds tightly to ssDNA (K_d~10^{-9}-10^{-10} M) through oligonucleotide/oligosaccharide-binding (OB) fold domains and prevents the formation of secondary structures on DNA. RPA plays an important role in the homologous recombination (HR) pathway by mediating several protein-protein interactions and hence controls how the events of DNA replication, recombination, and repair are initiated, terminated and coordinated.

For many cellular activities the double stranded DNA is unwound yielding ssDNA which is immediately coated by RPA. For other proteins to gain access to this ssDNA, RPA must first be displaced and when the events are completed, RPA must rebind to protect the DNA. How are these events coordinated and how are the activities of RPA regulated? To uncover RPA dynamics on ssDNA, we have developed an assay that monitors the kinetics of RPA binding and dissociation on ssDNA using site-specific incorporation of non-canonical amino acids (ncAAs). We have engineered the 4-azidophenylalanine (4AZP) ncAA in the RPA32 subunit within the context of the three subunit complex. Using click chemistry, Alexa dyes were tethered onto RPA yielding a fluorescently labeled RPA complex (RPA^f). RPA^f displays a robust change in fluorescence upon binding to ssDNA. The RPA^f probe allows the measurement of RPA binding and dissociation when multi-protein reactions are performed in vitro enabling bulk and single molecule methodologies. The labeling strategy is also applicable to most other multiprotein complexes. Studies on defining the activity of RPA on ssDNA in presence of various pro and anti- HR mediator proteins will be described.

High-Temperature Single-Molecule Kinetic Analysis of Thermophilic Archaeal MCM Helicases

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Chromosomal DNA helicases play an essential role in all domains of life by unwinding duplex DNA ahead of the replication fork during genome replication. The minichromosome maintenance (MCM) complex is the replicative helicase responsible for unwinding DNA during archaeal and eukaryal genome replication. To mimic long helicase unwinding events in the cell, a high-temperature single-molecule assay was designed to quantitatively measure DNA unwinding kinetics, including unwinding rate and processivity of three archaeal helicases from *Thermococcus* sp. 9°N and *Methanothermobacter thermautotrophicus*. These studies revealed that *Thermococcus* sp. 9°N MCM2 unwinds DNA at a rate of 43 ± 5 bp s^{-1} with a processivity of 4,700 ± 350 bp, *Thermococcus* sp. 9°N MCM3 unwinds at a rate of 165 ± 10 bp s^{-1} with a processivity of 4,500 ± 220 bp and *Methanothermobacter thermautotrophicus* MCM unwinds at 53 ± 8 bp s^{-1} with a processivity of 3,900 ± 480 bp. Similar unwinding rates and processivities suggest a unified unwinding mechanism despite evolutionary divergence. The development of the high-temperature single-molecule assays described here will also enable a comprehensive understanding of hyperthermophilic archaeal genome replication.

P118

Activation of Human Mitochondrial RNase P by Methyltransferase Subcomplex

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Ribonuclease P (RNase P) is essential for catalyzing cleavage of the 5' end leader from precursor tRNA (pre-tRNA). In many organisms, RNase P is a ribonucleoprotein complex with a catalytic RNA subunit. An exception to this composition is human mitochondrial RNase P (mtRNase P), which is composed of three protein subunits: MRPP1, 2 and 3. MRPP3 is the catalytic subunit containing the active site that catalyzes pre-tRNA hydrolysis. This enzyme belongs to a new class of metallonucleases and requires MRPP1 and MRPP2 for function. MRPP1 and MRPP2 form an active subcomplex that catalyzes methylation at position 9 (m^1R9) of mitochondrial tRNAs. To understand the requirements for MRPP3 activation, fluorescence anisotropy binding and transient kinetic measurements were performed demonstrating that the MRPP1•MRPP2 subcomplex enhances catalytic activity of MRPP3 by 10,000-fold and apparent substrate affinity by 200-fold. In addition, in vitro pull-down results indicate that MRPP3 forms a stable complex with MRPP1 and MRPP2 only in the presence of pre-tRNA, suggesting that MRPP3 may recognize the MRPP1•MRPP2-bound pre-tRNA or that pre-tRNA induces structural rearrangements that increase the binding affinity between MRPP1, 2, and 3. We propose a new model of substrate recognition for human mtRNase P that highlights the importance of MRPP1•MRPP2 activity on the processing of mitochondrial tRNA transcripts.

P119

Solvent Dynamics and Conformational Sub-States in Enzyme Catalysis

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Cytosolic proteins work in cellular environments markedly different from highly dilute aqueous solutions typically used in laboratory experiments. Past investigations have indicated that the surrounding solvent drives protein dynamics and therefore could impact enzyme mechanisms. We have used a combination of stop flow kinetic measurements, X-ray crystallography, quasi-elastic neutron scattering studies and computer simulations to investigate the connection between solvent and enzyme dynamics and its interplay with enzyme mechanism. In particular, the *Escherichia coli* enzyme dihydrofolate reductase (DHFR) shows that with increasing concentrations of isopropanol in solvent the pH-independent $k_{hydride}$ rate decreases more than two fold; the enzyme structure shows no noticeable differences but the dynamical motions are suppressed. More interestingly our investigations shows that the altered motions of DHFR cause significant changes in the enzyme's ability to access its functionally relevant conformational sub-states. The enzyme, in the presence of isopropanol, makes less frequent and shorter visits to the conformational sub-states relevant for achieving the transition state, explaining the observed decrease in $k_{hydride}$. Evidence regarding the role of conformational sub-states in enzyme mechanisms from other enzymes (including cyclophilin A and lipase B) will also be presented.

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CHEERS! EVENTS AT LOEWS DON CESAR HOTEL

RESTAURANT SUGGESTIONS

Inside the Loews Don Cesar Hotel

Maritana Grille – Lobby Level - Fine Dining (Dinner only)
***Private Dining Room for 12*

Sea Porch Cafe – Garden Level - Casual Dining (Breakfast, Lunch & Dinner)
***Private Dining Room for 20*

Beachcomber Grill – Poolside Outdoor Dining
Open daily from 11:00 AM – Sunset

Rowe Bar – *Garden Level* – Cool Cocktails and Coastal Cuisine
Opens daily at 11:00 AM

Pass-a-Grille Beach (south of hotel and approx 5-10 minute transfer)

The Hurricane Restaurant
807 Gulf Way
Pass-a-Grille, FL 33706
727-360-9558
www.thehurricane.com
***Private Dining Area*

Brass Monkey
709 Gulf Way, #1
St. Pete Beach, FL 33706
727-367-7620
www.thebrassmonkey.net

Gennaro's Ristorante Italiano
2004 Pass-a-Grille Way
St. Pete Beach, FL 33706
727-360-0563
www.gennarositaliano.com

Sea Critter's Cafe
2007 Pass-a-Grille Way
St. Pete Beach, FL 33706
727-360-3706
www.seacritterscafe.com
***Private Dining Area for 30*

The Wharf
2001 Pass-a-Grille Way
St. Pete Beach, FL 33706
727-367-9469
www.wharfrestaurant.org

Beth Clark - Cheers! Events at Loews Don Cesar Hotel
Office (727) 363-2127 – Cell (727) 455-2860 - beth@cheersevents.com

St. Pete Beach (north of hotel and approx 5-10 minute transfer)

Castile at Hotel Zamora
3701 Gulf Blvd.
St. Pete Beach, FL 33706
727-456-8660
www.thehotelzamora.com/dining
***Private Dining Room for 25*

Blue Fugu Japanese Steakhouse
4615 Gulf Boulevard
St. Pete Beach, FL 33706
727-367-6762
www.bluefugu.com

Selene Restaurant
4945 Gulf Boulevard
St. Pete Beach, FL 33706
727-317-2064
www.selenerestaurant.com
***Private Dining Room for 40*

Seared 1200 Chophouse
5007 Gulf Boulevard
St. Pete Beach, FL 33706
www.1200chophouse.com

Madfish
5200 Gulf Boulevard
St. Pete Beach, FL 33706
727-360-9200
www.madfishonline.com
***Private Dining Area or Buy-out Available*

Snappers Sea Grill
5895 Gulf Blvd.
St. Pete Beach, FL 33706
727-367-3550
www.snappersseagrill.com

Sola Bistro & Wine Bar
6700 Gulf Boulevard
St. Pete Beach, FL 33706
727-360-7500

Chill Restaurant & Bar
357 Corey Avenue
St. Pete Beach, FL 33706
727-360-2445
www.chillstpetebeach.com

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Additional Beach Area Options (approx 15-25 minute transfer)

Middle Grounds Grill
10925 Gulf Boulevard
Treasure Island, FL 33706
727-360-4253
www.middlegroundsgrill.com
***Private Dining Room for 35*

Salt Rock Grill
19325 Gulf Boulevard
Indian Shores, FL 33785
727-593-7625
www.saltrockgrill.com
***Private Dining Room for 45*

Sundial Center in Downtown St. Petersburg (approx 15-20 minute transfer)

Locale Market
179 – 2nd Avenue N
St. Petersburg, FL 33701
727-523-6300
www.localegourmetmarket.com

Ruth's Chris Steak House
131 – 2nd Avenue N
St. Petersburg, FL 33701
727-821-4139
www.ruthschris.com
***Private Dining Rooms – Sundial for 40 or Bayfront for 55*

Sea Salt
183 – 2nd Avenue N
St. Petersburg, FL 33701
727-873-7964
www.seasaltstpete.com
***Chef's Table/Private Dining experience available*

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Downtown St. Petersburg (approx 15-20 minute transfer)

Bella Brava
204 Beach Drive NE
St. Petersburg, FL 33701
727-895-5515
www.bellabrava.com
***Semi-private Dining Room for 25*

Parkshore Grill
300 Beach Drive NE
St. Petersburg, FL 33701
727-896-9463
www.parkshoregrill.com
***Private Dining Room "The Wine Cellar" for up to 70*

400 Beach Seafood & Tap House
400 Beach Drive NE
St. Petersburg, FL 33701
727-896-2400
www.400beachseafood.com
***Private Dining Rooms for up to 60*

Rococo Steak
655 – 2nd Avenue S
St. Petersburg, FL 33701
727-822-0999
www.rococosteak.com
***Private Dining Rooms for up to 150*

Ceviche Tapas Bar & Restaurant
10 Beach Drive
St. Petersburg, FL 33701
727-209-2299
www.ceviche.com

Moon Under Water
332 Beach Drive NE
St. Petersburg, FL 33701
727-896-6160
www.themoonunderwater.com

Red Mesa Cantina
128 - 3rd Street S
St. Petersburg, FL 33701
727-896-8226
www.redmesacantina.com

Cassis American Brasserie
170 Beach Drive NE
St. Petersburg, FL 33701
727-827-2927
www.cassisab.com

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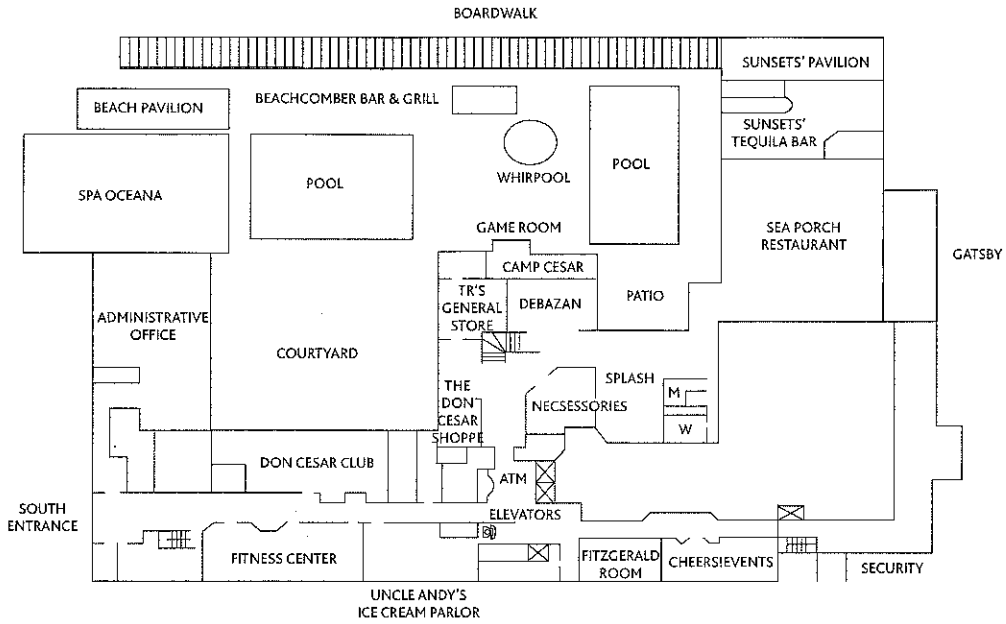
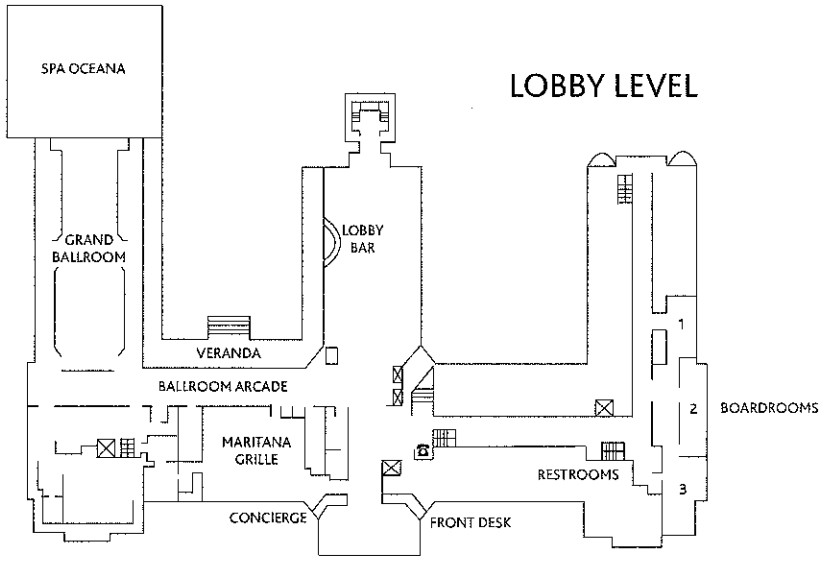
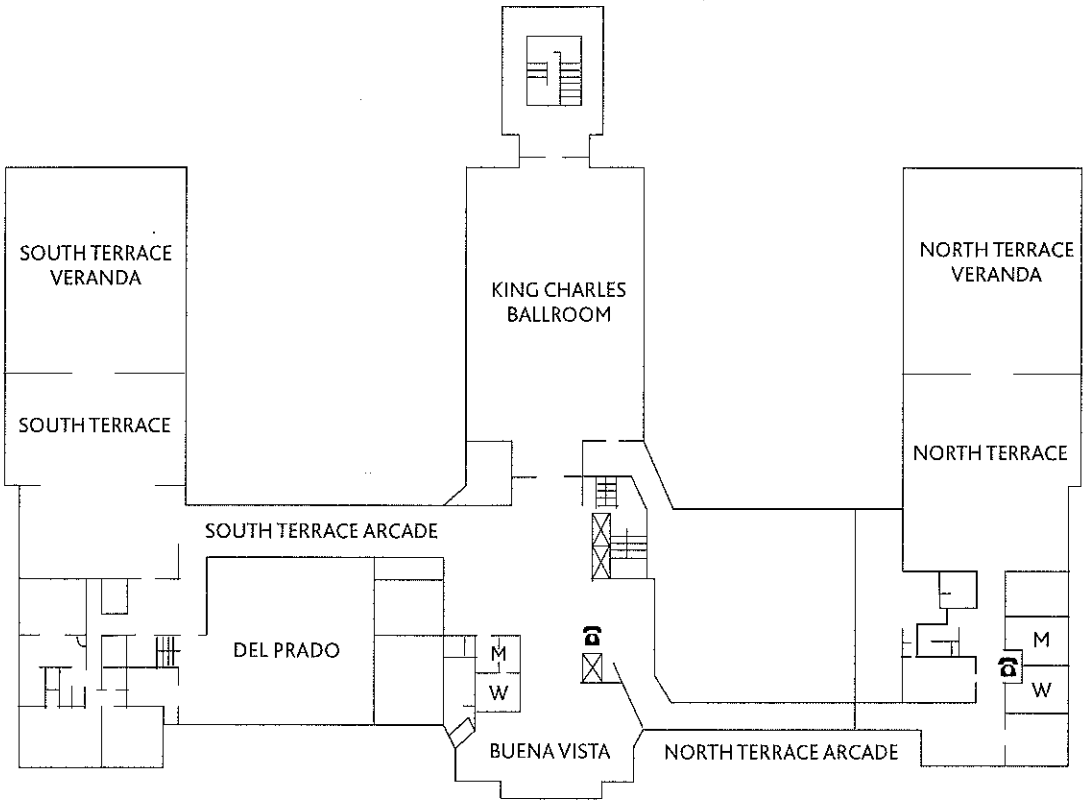
GARDEN LEVEL



LOBBY LEVEL




SOUTH TERRACE VERANDA
SOUTH TERRACE
KING CHARLES BALLROOM
NORTH TERRACE VERANDA
NORTH TERRACE
SOUTH TERRACE ARCADE
DEL PRADO
M
W
BUENA VISTA
NORTH TERRACE ARCADE
M
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FIFTH FLOOR