The 29th Enzyme Mechanisms Conference

January 4th – 8th, 2026
Omni La Costa Resort
Carlsbad, CA

Andrew Gulick and Tim Wencewicz
co-chairs

Contents

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We are also very grateful to multiple *Anonymous Conferees* for their generous support.

The Enzyme Mechanisms Conference

The Enzyme Mechanisms Conferences have brought together academic and industrial scientists to discuss new ideas at the forefront of mechanistic enzymology. The goal is to foster collegial interactions among chemists and biochemists who seek to understand the chemical basis for enzymatic catalysis and regulation of enzyme action and those who apply that knowledge for practical applications.

The conference has been held biennially since it was founded in 1969 by Tom Bruice, Bill Jencks, and Myron Bender. Over the past 55 years, the conference has provided an outstanding forum for the presentation and discussion of the most exciting advances in our understanding of the mechanisms of enzyme action and the application of this knowledge to synthetic chemistry, pharmaceutical design and agriculture.

The community of the Enzyme Mechanisms Conference includes people who are citizens of and reside in nations from around the world, are members of cultures and religions that represent all possibilities on Earth, are born to families both traditional and non-traditional, and have the full range of physical abilities, gender expression, sexual orientation and age. We use this diversity to provide a wide range of perspectives for the work of our common passion: determining how enzymes catalyze reactions and using that information for the betterment of life and the sustainability of our planet. We promote collegial academic debate in a welcoming environment and do not tolerate discrimination.

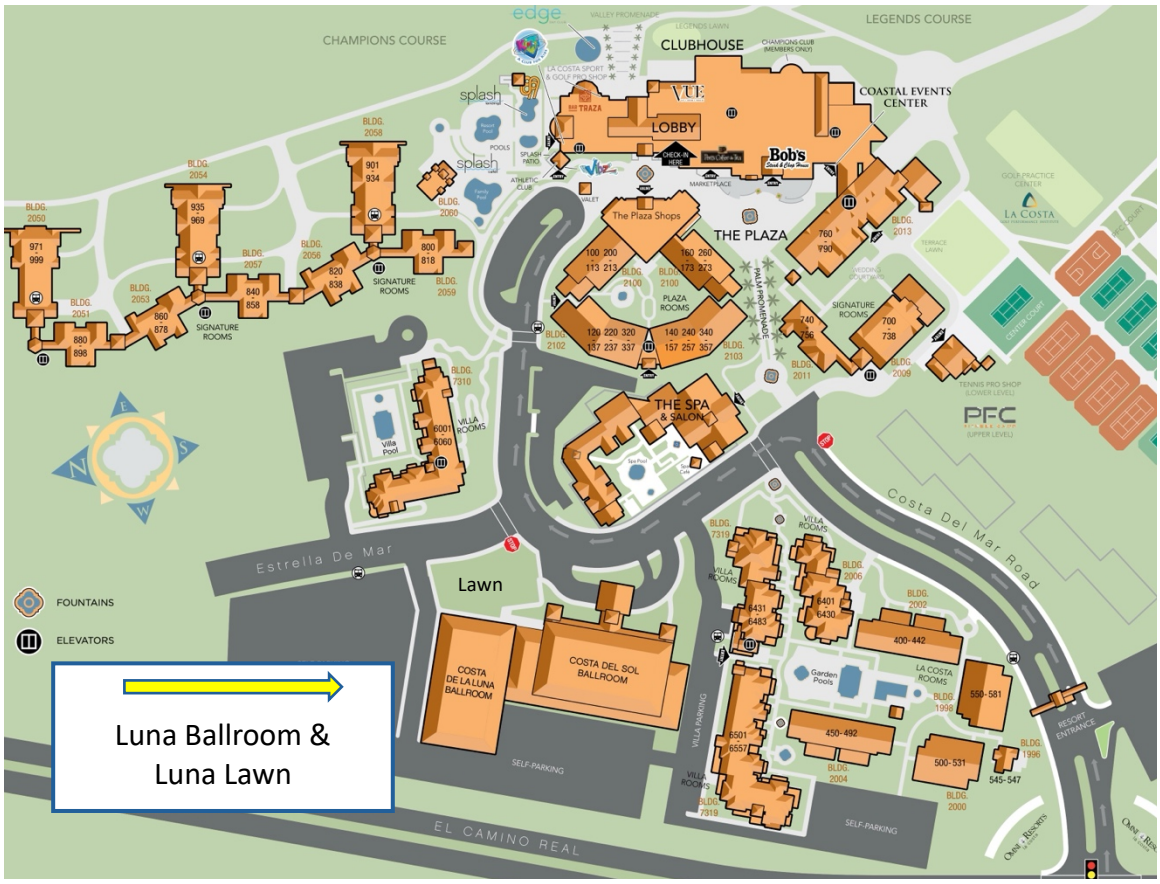
Past Enzyme Mechanisms Conferences

Year	Destination	Chair(s)
2026	Carlsbad, CA	Andrew Gulick & Tim Wencewicz
2024	Naples, FL	Audrey Lamb & Graham Moran
2022	Tucson, AZ	Wilfred van der Donk
2019	New Orleans, LA	Vahe Bandarian
2017	St. Pete Beach, FL	Richard Silverman
2015	Galveston, TX	Ken & JoAnn Johnson
2013	Coronado, CA	Thomas Meek
2011	St. Pete Beach, FL	John Richard & Tina Amyes
2009	Tucson, AZ	Karen Allen
2007	St. Pete Beach, FL	Chris Whitman
2005	Asilomar, CA	JoAnne Stubbe
2003	Galveston, TX	Frank Raushel
2001	Marco Island, FL	Vern Schramm
1999	Napa, CA	Richard Armstrong
1997	Naples, FL	John Kozarich
1995	Scottsdale, AZ	Dale Poulter
1993	Key Largo, FL	John Gerlt
1991	San Diego, CA	Joe Villafranca
1989	St. Petersburg, FL	Paul Bartlett
1987	Asilomar, CA	Tony Fink
1985	Tarpon Springs, FL	Gene Cordes
1983	Asilomar, CA	Judith Klinman
1981	Sanibel Island, FL	Perry Frey
1979	La Jolla, CA	George Kenyon
1977	Tucson, AZ	Joe Coleman
1975	San Juan, PR	Al Mildvan
1973	Los Angeles, CA	Paul Boyer
1971	Santa Barbara, CA	Tom Bruice
1969	New Orleans, LA	Bill Jencks

Enzyme Mechanisms Conference Resort Details

Omni La Costa Resort, Carlsbad CA
January 4-8, 2026

Registration: Registration will be held Sunday 1/4 from 2:30-6:00pm in the lobby of the Luna Ballroom. If you are unable to obtain your registration material during this time, please locate conference chairs (Andrew Gulick or Tim Wencewicz) for assistance.



Resort Map. All scientific and social events will be held in the Costa De La Luna Ballroom (Luna Ballroom) and adjoining Lawn.

Conferees and registered guests are kindly asked to wear their name tags at all times while attending the scientific sessions, meals, and social events.

Opening Reception: The conference Welcome and Opening Reception will be held on Sunday January 4 from 6:00 pm to 8:00 pm on the Luna Lawn. Conferees and Registered guests are welcome to attend.

Drink Tickets: At registration, conference attendees will receive drink tickets good for soft drinks, wine, or beer that can be used during the social events.

Scientific Program

Presentations will be held in the Luna Ballroom 4-7.

Poster Sessions will be held in Luna Ballroom 1-3. Posters can be hung Sunday evening and must be removed Wed at 5:00pm. Presenters of ODD Numbered posters should be present at their poster during Monday's poster session. Presenters of EVEN Numbered posters should be present at their poster during Tuesday's poster session.

Meals

The Omni La Costa has numerous restaurants and coffee shops, as well as the "Market Place" where food and items can be purchased. Downtown Carlsbad Village and the waterfront provide additional eating options via a short drive or rideshare.

Sunday. Opening reception will contain multiple reception stations with small plates of salads, proteins, sliders, and tacos, as well as cash bar. Drink tickets can be used for purchase of soft drinks, beer, or wine.

Monday. Attendees are on their own for breakfast and dinner. A lunch will be provided after session 2. The afternoon poster session will provide assorted bars as snacks. The evening poster "social session" will be held on the Luna Lawn and Luna Ballroom poster room and will contain small food stations.

Tuesday. Attendees are on their own for breakfast and dinner. A lunch will be provided after session 5. The afternoon poster session will provide assorted bars as snacks. The evening poster "social session" will be held on the Luna Lawn and Luna Ballroom poster room and will contain small food stations.

Wednesday. Attendees are on their own for breakfast. A lunch will be provided after session 8. A buffet dinner is provided at the closing banquet (Luna Lawn)

Attendees and Guests are welcome at all meals and social events

29th Enzyme Mechanisms Conference Scientific Outline

Sunday January 4, 2026

2:30 – 6:00 pm	Registration
6:00 – 9:00 pm	Opening Reception, Luna Lawn

Monday January 5, 2026

8:30 – 10:15 am	Session 1, Luna Ballroom, *Enzymes and Health*
10:15 – 10:45 am	Coffee Break
10:45 – 12:45 pm	Session 2, Luna Ballroom, *Natural Products I*
12:30 – 2:00 pm	Lunch
2:00 – 4:00 pm	Poster Session, Luna Ballroom
4:00 – 6:00 pm	Session 3, Luna Ballroom, *Enzymology I*
7:30 – 9:00 pm	Social Session, Luna Lawn

Tuesday January 6, 2026

8:30 – 10:00 am	Session 4, Luna Ballroom, *Natural Products II*
10:00 – 10:30 am	Coffee Break
10:30 – 12:30 pm	Session 5, Luna Ballroom, *Enzymology II*
12:30 – 2:00 pm	Lunch
2:00 – 4:00 pm	Poster Session
4:00 – 6:00 pm	Session 6, Luna Ballroom, *Biosynthesis*

Wednesday January 7, 2026

8:00 – 10:00 am	Session 7, Luna Ballroom, *Chemical Biology*
10:00 – 10:30 am	Coffee Break
10:30 – 12:30 pm	Session 8, Luna Ballroom, *Metalloenzymology*
12:30 – 2:00 pm	Lunch
3:00 – 5:00 pm	Session 9, Luna Ballroom, *Protein Engineering*
6:00 – 9:00 pm	Closing Banquet

Detailed Scientific Program

Sunday January 4, 2026

2:30 – 5:30 pm	Registration
6:00 – 8:00 pm	Opening Reception, Luna Lawn

Monday January 5, 2026

8:30 – 10:15 am	Session 1, Luna Ballroom, *Enzymes and Health*
	Session Chair, **Marcus Hartmann**, Max Planck Society
	8:30 **Tim Wencewicz and Andrew Gulick** Opening Welcome
	8:45 **Luiz Pedro Carvalho** (UF Scripps Institute) Discovery of a Novel Amino Acid Decarboxylase in Nature.
	9:15 **Christina M. Woo** (Harvard University) Post-Translational Regulation of Glutamine Synthetase through Enzymes and E3 Ligases.
	9:45 **Qingan Sun** (Bayer Crop Science) Selective Inhibition of Fungal IMPDH by a Novel Macrocyclic Peptide Targeting a Non-Canonical Allosteric Site.
10:15 – 10:45 am	Coffee Break
10:45 – 12:45 pm	Session 2, Luna Ballroom, *Natural Products I*
	Session Chair, **Christal Sohl**, San Diego State University
	10:45 **Kou-San Ju** (The Ohio State University) Biosynthetic Diversification of Phosphonopeptide Natural Products.
	11:15 **Bradley S. Moore** (Scripps Institute of Oceanography) Enzyme Discovery from the Seashore to the Deep Ocean.
	11:45 **Louise K. Charkoudian** (Haverford College) Exploring the Inner Workings of Type II Polyketide Synthases for Expanded Access to Biosynthetic Products.
	12:15 **Trainee Flash Talks** (1 - 5) *Three-minute presentations*
	Aodhan Beattie, Kuldeep Jangid, Ioannis Kipouros, Chang Liu, and Collin Merrick

12:45 – 2:00 pm Lunch (Provided)

2:00 – 4:00 pm Poster Session, Luna Ballroom

4:00 – 6:00 pm Session 3, Luna Ballroom, *Enzymology I*

Session Chair, **Ken Roberts**, University of South Carolina-Aiken

4:00 **Graham R. Moran** (Loyola University) A Descriptive Analysis of Transient-state Observations for Thioredoxin Glutathione Reductase from *Schistosoma mansoni*.

4:30 **Wen Zhu** (Florida State University) Dissecting the Conformational Assembly of Human Asparagine Synthetase.

5:00 **Patrick A. Frantom** (University of Alabama) Mechanisms of Protected Persulfide Transfer in the Suf Pathway for Bacterial Fe-S Cluster Assembly.

5:30 **Trainee Flash Talks** (6 - 11) *Three-minute presentations*

Mariah Avila, Gil Namkoong, Jacob Pedigo, Kyosuke Shishikura, Jitendra Singh, and Nemanja Vuksanovic

7:30 – 9:00 pm Social Session, Luna Lawn

Tuesday January 6, 2026

8:30 – 10:00 am Session 4, Luna Ballroom, *Natural Products II*

Session Chair, **Dillon Cogan**, University of Southern California

8:30 **T. Martin Schmeing** (McGill University) Megaenzyme Mechanisms

9:00 **Michael D. Burkart** (UCSD) High-Resolution Cryo-EM of Crosslinked NRPS and PKS Megasynthase Assembly Lines

9:30 **Alison Butler** (UCSB) Siderophores in Stereo: Chirality in Microbial Iron Acquisition.

10:00 – 10:30 am Coffee Break

10:30 – 12:30 pm Session 5, Luna Ballroom, *Enzymology II*

Session Chair, **Zhihao Zhuang**, University of Delaware

10:30 **Audrey L. Lamb** (UTSA) How to Make Riboflavin (in At Least Nine Complicated Enzymatic Steps).

11:00 **Robert P. Hausinger** (Michigan State University) Biosynthesis and Utilization of Nickel-Pincer Nucleotide Cofactors.

	11:30	**Pablo Sobrado** (Missouri University of Science and Technology) Hydroxylation of Amines by Flavin-Dependent Enzymes.
	12:00	**John Richard** (University at Buffalo) Substrate-Driven Protein Conformational Changes in Catalysis by Nature's Most Proficient Enzymes.
12:30 – 2:00 pm	Lunch (Provided)	
2:00 – 4:00 pm	Poster Session	
4:00 – 6:00 pm	Session 6, Luna Ballroom, *Biosynthesis*	
	Session Chair, **Truc Pham**, Atavistik Bio	
	4:00	**Elizabeth Sattely** (Stanford University and HHMI) Chemistry and Biology of Taxol Biosynthesis.
	4:30	**Joseph M. Jez** (Washington University) Molecular Controls in Plan Hormone Signaling.
	5:00	**Lona M. Alkhalaf** (University of Warwick) Structural Basis for [1,3]-Phosphate Shift in Bacterial Hormone Biosynthesis.
	5:30	**Kristin Osika *Founders' Award Winner*** (University of Pennsylvania) Structural Snapshots of Catalysis in a Bifunctional Terpene Cyclase.
7:30 – 9:00 pm	Social Session, Luna Lawn	

Wednesday January 7, 2026

8:30 – 10:00 am	Session 7, Luna Ballroom, *Chemical Biology*	
	Session Chair, **Brianne Dudiak**, Bristol Myers Squibb	
	8:00	**David J. Pagliarini** (HHMI and Washington University) New enzymes in hydroxylipid catabolism.
	8:30	**Benjamin F. Cravatt** (The Scripps Research Institute) Activity-Based Protein Profiling – Target and Ligand Discovery on a Global Scale.
	9:00	**Chihui An** (Merck & Co., Inc) Biocatalytic Synthesis of Non-Natural Peptides.

9:30 **Dhara D. Shah** (Arizona State University) A Sulfotransferase from a Gut Microbe Acts on Diverse Phenolic Sulfate Compounds, Including Acetaminophen Sulfate.

10:00 – 10:30 am Coffee Break

10:30 – 12:30 pm Session 8, Luna Ballroom, *Metalloenzymology*

Session Chair, **Adrian Jinich** (University of California San Diego)

10:30 **Amie K. Boal** (Pennsylvania State University) The Periodic Table of Ribonucleotide Reductases.

11:00 **Katherine S. Ryan** (University of British Columbia) Heme-dependent Enzymes in Nitrogen-Nitrogen Bond-Formation.

11:30 **Catherine L. Drennan** (Massachusetts Institute of Technology and HHMI) The Hard Work of Making Deoxyribonucleotides.

12:00 **Squire J. Booker** (University of Pennsylvania, PSU, and HHMI) Structure, Mechanism, and Annotation of Cobalamin-Dependent Radical SAM Methylases.

12:30 – 2:00 pm Lunch (Provided)

3:00 – 5:30 pm Session 9, Luna Ballroom, *Protein Engineering*

Session Chair, **Seokhee Kim**, Seoul National University

3:00 **Monica Neugebauer** (University of Wisconsin) Discovery and characterization of new families of nickel-dependent enzymes.

3:30 **Kelly M. Zatopek** (New England Biolabs) A NUDIX Jack-of-all-Trades: Structural and Functional Analysis of Archaeal Nudα.

4:00 **Allison S. Walker** (Vanderbilt University) Artificial Intelligence for Genome Mining and Engineering Biosynthesis.

4:30 **Yang Yang** (UCSB) Design and Development of New Radical Enzymology.

5:00 **Tim Wencewicz and Andrew Gulick**
Closing Announcements

6:00 – 9:00 pm Closing Banquet

Thursday January 8, 2026

Departure

Speaker Abstracts

Monday January 5

- Luiz Pedro Carvalho
- Christina M. Woo
- Qingan Sun
- Kou-San Ju
- Bradley Moore
- Louise Charkoudian
- Graham Moran
- Wen Zhu
- Patrick Frantom

Tuesday January 6

- Martin Schmeing
- Michael Burkart
- Alison Butler
- Audrey Lamb
- Robert Hausinger
- Pablo Sobrado
- John Richard
- Elizabeth Sattely
- Joe Jez
- Lona Alkhalaf
- Kristin Osika

Wednesday January 7

- Dave Pagliarini
- Ben Cravatt
- Chihui An
- Dhara Shah
- Amie Boal
- Katerine Ryan
- Catherine Drennan
- Squire Booker
- Monica Neugebauer
- Kelly Zatopek
- Allison Walker
- Yang Yang

Discovery of a Novel Amino Acid Decarboxylase in Nature

Luiz Pedro Carvalho[%,&]

[%] Mycobacterial Metabolism and Antibiotic Research Laboratory, The Francis Crick Institute, London, United Kingdom.

[&] Department of Chemistry, The Herbert Wertheim UF Scripps Institute, Jupiter, United States

The rapid expansion of genome sequencing has outpaced our ability to accurately assign gene function, with annotation biases often reinforcing incorrect assumptions across large enzyme families. We identify an unexpected enzymatic activity within a protein long presumed to belong to the lysine-ornithine-arginine (KOR) decarboxylase superfamily. Although Rv2531c is under strong purifying selection in Mycobacterium tuberculosis and annotated among more than 26,000 KOR-related sequences, it does not act on canonical KOR substrates. Through a combination of bioinformatics, microbiology, metabolomics, and enzymology, we reveal that Rv2531c defines a previously unrecognized subfamily of PLP-dependent enzymes with distinctive structural and kinetic properties, including an additional domain, hysteresis, and marked cooperativity. These features set it apart from well-studied enterobacterial decarboxylases and broaden the functional landscape of this enzyme superfamily, suggesting that many of its members may catalyze diverse, overlooked reactions central to microbial metabolism and evolution.

Post-translational regulation of glutamine synthetase through enzymes and E3 ligases

Christina M. Woo

Department of Chemistry and Chemical Biology, Harvard University

The E3 ligase substrate adapter cereblon (CRBN), the primary target of clinical agents thalidomide and lenalidomide, recognizes endogenous substrates bearing the C-terminal cyclic imide modification. Although C-terminal cyclic imides can form spontaneously, an enzyme that regulates their formation and thereby promotes a biological pathway connecting substrates to CRBN is unknown. Here, we report that protein carboxymethyltransferase (PCMT1) promotes formation of C-terminal cyclic imides on C-terminal asparagine residues of CRBN substrates. PCMT1 and CRBN co-regulate the levels of metabolic enzymes such as glutamine synthetase (GLUL) and inorganic pyrophosphatase 1 (PPA1) in vitro, in cells, and in vivo, and this regulation is associated with the proepileptic phenotype of CRBN knockout mouse models. The discovery of an enzyme that regulates CRBN substrates through the C-terminal cyclic imide reveals a previously unknown biological pathway that is perturbed by thalidomide derivatives and provides a biochemical basis for the connection between multiple biological processes and CRBN.

Selective Inhibition of Fungal IMPDH by a Novel Macrocyclic Peptide Targeting a Non-Canonical Allosteric Site

Qingan Sun[1], David Korasick[1], Yoann Huet[2], Joerg Freigang[3], Sasha Kweskin[1], Zhenzhen Qiao[1], Phillip Hussey[1], Jason Meyer[1], Rachel Baltz1[2], Erina Nakajima[§], Naohiro Taniguchi[§], Takeshi Asakawa[§], Kyosuke Ueda[§], Makoto Jitsuoka[§], Takanori Aoki[§], Tatsuya Niimi[§], Yui Hirata[§], Junichi Nishikawa[§] and Patrick C. Reid[§]

[1]Bayer Crop Science, Chesterfield, MO, USA

[2]Bayer Crop Science, Lyon, France

[3]Bayer Crop Science, Monheim, Germany

[§] PeptiDream Inc., Kawasaki, Japan

Inosine-5'-monophosphate dehydrogenase (IMPDH) is a critical enzyme in guanine nucleotide biosynthesis and a promising anti-fungal target. However, the development of selective small-molecule inhibitors has been hindered by the high structural conservation between fungal and human IMPDH homologs. In a collaborative effort between Bayer and PeptiDream, we employed PeptiDream's proprietary Peptide Discovery Platform System (PDPS) to identify novel peptide-based inhibitors with enhanced selectivity. Here, we report the discovery and characterization of macrocyclic peptides that potently and selectively inhibit fungal IMPDH. One of them demonstrates remarkable specificity, not only discriminating against human IMPDH but also exhibiting selectivity among different fungal IMPDH orthologs in both enzyme inhibition assays and surface plasmon resonance (SPR). Cryo-electron microscopy (cryo-EM) structural analysis reveals that the peptide binds to a previously uncharacterized allosteric site distinct from the active site, providing a structural basis for its unique selectivity profile. These findings establish the macrocyclic peptide as a promising lead for the development of next-generation antifungal agents and highlight the potential of peptide modalities to overcome selectivity challenges in drug discovery.

Biosynthetic diversification of phosphonopeptide natural products

Kou-San Ju[1,2]

[1]*Department of Microbiology, The Ohio State University, Columbus, OH, USA*

[2]*Division of Medicinal Chemistry & Pharmacognosy, The Ohio State University, Columbus, Ohio, USA*

Phosphonate natural products are potent inhibitors that have been commercialized throughout medicine, agriculture, and biotechnology. Genome mining has emerged as a powerful approach to discover new phosphonates, doubling the chemical repertoire of this family of natural products within the last decade. However, accurate de-novo prediction of phosphonates and their inhibitory properties from uncharacterized biosynthetic gene clusters remains a significant challenge due to our limited understanding of their metabolism. This presentation expands upon this knowledge by discussing the complete biosynthetic pathways for the phosphonoalamides,[1-2] a group of antimicrobial phosphonopeptides recently isolated from bacteria. We illustrate ancestral connections to primary metabolism and highlight the flexibility of amino acid ligases in accepting combinations of natural, non-canonical, and phosphonate substrates. The broad distribution of these pathways and enzymes across microbial genomes suggest these to be a conserved mechanism for phosphonopeptide biosynthesis and diversification.

References

1. Cui, J.J.; Zhang, Y.; Ju, K.S. *Angew . Chem. Int. Ed. Engl.* **2024**, *63*, e202405052.
2. Cui, J.; Ju, K.S. *ACS Chem Biol.* **2024**, *19*, 1506-1514.

Enzyme discovery from the seashore to the deep ocean

Bradley S. Moore

Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA

Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA, USA

The untapped biotechnological potential of the ocean is finally within reach. For too long, promising bioactive compounds and materials have been discovered from ocean life yet have fallen short due to inadequate supply challenges. New multi-omic and growth-coupled bioproduction strategies offer new hope in a biosustainable future in marine compound discovery and development. This presentation will focus on efforts in the Moore lab to discover and apply biosynthetic enzymes from diverse marine organisms in our efforts to biosustainably produce bioactive compounds from algae and cnidarians, and broad-spectrum, color-changing pigments from cephalopods as promising new examples.

References

1. T. S. Steele; I. Burkhardt; M. L. Moore; T. de Rond; H. K. Bone; K. Barry; V. M. Bunting; J. Grimwood; L.H. Handley; S. Rajasekar; J. Talag; T. P. Michael; B. S. Moore. Biosynthesis of haloterpenoids in red algae via microbial-like type I terpene synthases. *ACS Chem. Biol.* **2024**, *19*, 185-192.

2. T. R. Fallon; V. V. Shende; I. H. Wierzbicki; A. L. Pendleton; N. F. Watervoot; R. P. Auber; D. J. Gonzalez; J. H. Wisecaver; B. S. Moore. Giant polyketide synthase enzymes in the biosynthesis of giant marine polyether toxins. *Science* **2024**, *385*, 671-678.

3. I. Burkhardt; T. de Rond; P. Y.-T. Chen; B. S. Moore. Ancient plant-like terpene biosynthesis in corals. *Nat. Chem. Biol.* **2022**, *18*, 664-669.

4. N. E. Grayson; P. D. Scesa; M. L. Moore; J.-B. Ledoux; J. Gomez-Garrido; T. Alioto; T. P. Michael; I. Burkhardt; E. W. Schmidt; B. S. Moore. A widespread metabolic gene cluster family in metazoans. *Nat. Chem. Biol.* **2025**, *21*, 1509-1518.

5. L. B. Bushin; T. B. Alter; M. V.G. Alván-Vargas; L. Dürr; E. C. Olson; M. J. Avila; Ò. Puiggené, T. Kim; L. F. Deravi; A. M. Feist; P. I. Nikel; B. S. Moore. Growth-coupled microbial biosynthesis of the animal pigment xanthommatin. *Nat. Biotechnol., accepted.*

Exploring the inner workings of type II polyketide synthases for expanded access to biosynthetic products

Louise K. Charkoudian

[#]*Department of Chemistry, Haverford College*

Microorganisms produce structurally complex and diverse molecules with a range of medicinally relevant properties. Our undergraduate research team is interested in understanding and harnessing this remarkable biochemical feat to gain sustainable access to molecules that can better human health. We are particularly interested in developing innovative strategies for studying the two essential components of any type II polyketide synthase: acyl carrier proteins (ACPs) and ketosynthase - chain length factors (KS-CLFs). In this talk, I will share how we used inferred evolutionary history to identify previously unexplored type II polyketide synthase biosynthetic gene clusters as a reservoir for ACPs and KS-CLFs with unique properties. I will also present how we have expanded access to ACPs in their active "*holo*" form by discovering a new phosphopantetheinyl transferase (PPTase), evaluating the compatibility of a diverse library of ACPs with a set of PPTases, and unveiling important ACP-PPTase interactions to inform strategic engineering efforts. Throughout the discovery process, we have embraced unexpected results to develop novel methodologies that enable us to study important, transient interactions of biosynthetic complexes. Finally, I will connect how this work has laid a foundation for our ongoing efforts to reconstitute the biosynthesis of type II polyketides *in vitro* to obtain 'new-to-nature' type II polyketides via combinatorial biosynthesis. We hope our research— which spans organic chemistry, biochemistry and chemical education—will be of interest to a broad audience, and we welcome opportunities for collaboration with the Enzyme Mechanisms community.

A Descriptive Analysis of Transient-state Observations for Thioredoxin Glutathione Reductase from *Schistosoma mansoni*

Madison M. Smith, Tyler B. Alt, and Graham R Moran.

Department of Chemistry and Biochemistry, Loyola University Chicago, Chicago, IL 60660.

Thioredoxin/glutathione reductase from *Schistosoma mansoni* (SmTGR) is a multifunctional enzyme that catalyzes the reduction of glutathione (GSSG) and thioredoxin, as well as the deglutathionylation of peptide and non-peptide substrates. SmTGR structurally resembles known glutathione reductases (GR) and thioredoxin reductases (TrxR) but with an appended N-terminal domain that has a typical glutaredoxin (Grx) fold. Despite structural homology with known GRs, the site of glutathione reduction has frequently been reported as the Grx domain, based primarily on aerobic, steady-state kinetic measurements and x-ray crystallography. We have mapped chemical sequences that occur in TGR using U597C variant as a background facsimile of the WT enzyme. Functional assignment of five residues implicated to be involved in the chemistry catalyzed have reasonably explicitly revealed much of the underlying chemistry. These variants include residues participating in NADPH binding, thiol activation by deprotonation and one cysteine in each of the three proposed functional disulfides within this enzyme. The data for each variant is clear and in four of the five defines the role of the parent residue in catalysis. The analysis methods employed utilize three charge transfer absorption transitions that accumulate and decay in sequence in the reductive half-reaction. In addition, we present an anaerobic characterization of a series of SmTGR variants designed to establish the site of GSSG reduction. We propose that the cysteine pair most proximal to the FAD, Cys154/Cys159, equivalent to the site of GSSG reduction in GRs is the site of GSSG reduction. Anaerobic steady-state analysis of U597C, U597S, U597C+C31S, and I592STOP SmTGR demonstrate that the Grx domain is not involved in the catalytic reduction of GSSG, as redox silencing of the C-terminus results in no modulation of the observed turnover number (~0.025 s^{-1}) and redox silencing of the Grx domain results in an increased observed turnover number (~0.08 s^{-1}). Transient-state single turnover analysis of these variants corroborates this, as the slowest rate observed titrates hyperbolically with GSSG concentration and approaches a limit that coincides with the respective steady-state turnover number for each variant. Numerical integration fitting of the transient state data can only account for the observed trends when competitive binding of the C-terminus is included, indicating that the partitioning of electrons to either substrate occurs at the Cys154/Cys159 disulfide rather than the previously proposed Cys596/Sec597 sulfide/selenide. Paradoxically, truncating the C-terminus at Ile592 results in a loss of GR activity, indicating a crucial non-redox role for the C-terminus in catalysis.

Dissecting the conformational assembly of human asparagine synthetase

Wen Zhu[#]

[#]*Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL, USA*

Human asparagine synthetase (ASNS), a bidomain enzyme, is essential to the integrated stress response, enabling cell survival under amino acid deprivation.[1] Mutations in ASNS cause asparagine synthetase deficiency (ASNSD), a devastating neurological disorder characterized by impaired brain development.[2] Strikingly, most disease-associated variants do not occur at catalytic residues, pointing instead to defects in conformational dynamics critical for ASNS activity.[3,4] In this presentation, I will discuss our recent progress in dissecting the conformational assembly of ASNS using enzyme kinetics, cryo-electron microscopy, and molecular dynamics simulations. Understanding how catalytic activity is regulated in ASNS and its disease-associated variants will provide insights that inform new strategies for small-molecule inhibitor and activator design.

References

(1) Radadiya, A.; Zhu, W.; Coricello, A.; Alcaro, S.; Richards, N. G. J. Improving the Treatment of Acute Lymphoblastic Leukemia. *Biochemistry* **2020**, *59* (35), 3193–3200.

(2) Zhu, W.; Nardone, A. J.; Pearce, L. A. Advances in Human Glutamine-Hydrolyzing Synthetases and Their Therapeutic Potential. *Front. Chem. Biol.* **2024**, *3*, 1410435.

(3) Zhu, W.; Radadiya, A.; Bisson, C.; Wenzel, S.; Nordin, B. E.; Martínez-Márquez, F.; Imasaki, T.; Sedelnikova, S. E.; Coricello, A.; Baumann, P.; Berry, A. H.; Nomanbhoy, T. K.; Kozarich, J. W.; Jin, Y.; Rice, D. W.; Takagi, Y.; Richards, N. G. J. High-Resolution Crystal Structure of Human Asparagine Synthetase Enables Analysis of Inhibitor Binding and Selectivity. *Commun. Biol.* **2019**, *2* (1), 345.

(4) Coricello, A.; Nardone, A. J.; Lupia, A.; Gratteri, C.; Vos, M.; Chaptal, V.; Alcaro, S.; Zhu, W.; Takagi, Y.; Richards, N. G. J. 3D Variability Analysis Reveals a Hidden Conformational Change Controlling Ammonia Transport in Human Asparagine Synthetase. *Nat. Commun.* **2024**, *15* (1), 10538.

Mechanisms of Protected Persulfide Transfer in the Suf Pathway for Bacterial Fe-S Cluster Assembly

Patrick A. Frantom

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Iron-sulfur (Fe-S) clusters are essential metallocofactors in all organisms, and their assembly and trafficking are highly regulated due to the reactivity of free iron and sulfide. In bacteria, the multi-protein Suf pathway is a widespread Fe-S cluster assembly system and represents an underexploited target for antibacterial therapeutics. The pathway is initiated by the PLP-dependent cysteine desulfurase SufS, which cleaves L-cysteine to produce L-alanine and a covalent persulfide intermediate (Cys_{364}-S-SH in *E. coli*). Transfer of this protected intermediate requires an accessory transpersulfurase, SufE, to accept the persulfide and deliver it to the $SufBC_2D$ scaffold complex for cluster assembly. Despite the reactivity of persulfide intermediates, sulfur trafficking through the Suf pathway is resistant to exogenous oxidants and reductants. Biochemical, biophysical, and structural studies support a "reaction-based" mechanism in which formation of catalytic intermediates triggers conformational changes on both SufS and SufE that govern persulfide transfer. This mechanism defines molecular determinants for initial interaction, close approach, and initial dissociation steps in persulfide transfer from SufS to SufE and distinguishes the Suf system from other desulfurase/transpersulfurase pairs. A subsequent persulfide transfer step occurs between SufE and the SufB subunit of the $SufBC_2D$ scaffold complex, where persulfide delivery is antagonistic with the ATPase activity of the SufC subunits. These findings support an ordered sequence for scaffold reactions and suggest that "reaction-based" conformational gating mechanisms provide a general solution for protecting reactive persulfide intermediates during Fe-S cluster assembly.

Megaenzyme Mechanisms

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Secondary metabolite-producing megaenzymes biosynthesize many clinically important natural products, from early modern medicines (penicillin, bacitracin) to current blockbuster drugs (Cubicin, vancomycin) and newly-approved therapeutics (Rezafungin). Nonribosomal peptide synthetases (NRPSs), one important class of these megaenzymes, act like true macromolecular machines, having modular assembly-line logic, a complex catalytic cycle, moving parts and many active sites. Their products have a vast diversity of bioactivities because NRPSs cab use more than 500 monomer substrates as building blocks, and because they can catalyze co-synthetic modification of their peptide intermediates. I will present our latest structural and functional analyses of NRPS systems, including snapshots of the synthetase responsible for making the antibiotic linear gramicidin. These studies provide insight into the superdomain and supermodular architecture, conformational changes and mechanisms of catalysis NRPSs use to synthesize their important bio-active products. They also have important implications for successful bioengineering endeavours aimed at generation of new-to-nature therapeutics.

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High-Resolution Cryo-EM of Crosslinked NRPS and PKS Megasynthase Assembly Lines

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The intrinsic flexibility of non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs)—large, multi-domain megasynthase assembly lines—presents a major challenge to resolving their dynamic catalytic states. We employed dual site-selective covalent crosslinking combined with single particle cryo-EM to conformationally constrain these systems and visualize transient carrier protein interactions across both modular and iterative megasynthases. For the modular NRPS TycA-M1–TycB-M2,[1] tetrazine click chemistry trapped the intermodular condensation complex (PCP1–C2–PCP2) between the two modules at 2.97 Å resolution. This high-resolution structure defined the native communication (COM) domain in a helix-up orientation utilizing hydrophobic and polar contacts, including a critical K1019–D1078 salt bridge to recruit the acceptor PCP2. Comparison with a 2.12 Å X-ray structure of the PCP1–E1 domain revealed the carrier protein's processive movement, defining a 45 Å shuttling trajectory and 180° rotation required for PCP transition from the E1 (epimerization) to the C2 (condensation) domain, ensuring stereochemical fidelity of the D-Phe product. The structure also captured the downstream adenylation domain (A2) in an active, Pro-AMP liganded state, indicating that substrate priming occurs concurrently with chain extension to enhance pathway throughput. In the iterative Type I PKS,[2] mycocerosic acid synthase (MAS) (a 448 kDa homodimer from *M. tuberculosis*), stabilization via a C16- α-bromoamide probe yielded four distinct catalytic states (A–D) resolved down to 3.22 Å. These structures reveal higher-order asymmetric dynamics involving the tilting and twisting of the modifying compartment (DH-ΨKR-ER-KR) relative to the KS core. Complex A captured synchronous ACP=KS crosslinking, while Complex D revealed an asymmetric state (ACP crosslinked to DH and KS, respectively), supporting dual-chamber processing. Structural analysis of the KS domain (Complex B) identified key residues suggesting a KS gating mechanism for substrate selectivity. Conversely, DH activity (Complex C) relies on domain rotation upon ACP binding to expose the active site, suggesting rotational control for dehydration, unlike fixed Type II gating mechanisms. The structural rigidification imparted by dual site-selective crosslinking provides robust tool to achieve molecular detail for how these assembly lines achieve efficient, vectorial biosynthesis through defined protein-protein and protein-substrate interactions.

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Siderophores in Stereo: Chirality in Microbial Iron Acquisition

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Many microorganisms acquire iron using siderophore ligands with catecholate, hydroxamate, α-hydroxycarboxylate, and diazeniumdiolate coordinating groups. Through mining microbial genomes, specific classes of siderophores can be identified based on the enzymes catalyzing the Fe(III)-binding groups. In the large class of peptidic siderophores, b-hydroxyaspartate is often present as an Fe(III)-binding ligand. Biosynthesis of b-hydroxyaspartate is intriguing in regards to the stereoselective control of hydroxylation catalyzed by non heme iron enzymes. The origin of the b-hydroxyaspartate diastereomers in these siderophores is revealed in the microbial genomes.[1] A new class of tris catechol siderophores in marine and pathogenic microbes is based on a variation of enterobactin, adding a combinatoric suite of D- and L- cationic amino acids. Variation in amino acid chirality directs stereochemistry at the Fe(III) site, which in turn profoundly affects microbial growth.[2] Most recently a new class of siderophores containing a Fe(III)-binding diazeniumdiolate ligand has been discovered. The biosynthesis of the amino acid graminine, which harbors the diazeniumdiolate, is catalyzed by other iron enzymes.[3] While microbial genome mining encompasses microbes from all environments, marine microbes are found to be prominent in our investigations into the discovery, biosynthesis and reactivity of new b-hydroxyaspartate, catecholate, and diazeniumdiolate siderophores. These and further developments on the bioinorganic chemistry of these siderophores will be presented.

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How to make riboflavin
(in at least nine complicated enzymatic steps)

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Riboflavin is required for all life, and is converted to flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), cofactors required for diverse and essential biological reactions including oxidative phosphorylation, photosynthesis, DNA repair and in response to oxidative stress. Riboflavin biosynthesis requires enzymatic activities that are unparalleled in nature. Six enzymes and at least nine enzymatic transformations are required to generate riboflavin from ribulose 5-phosphate, GTP and NADPH.[1] However, many of the chemical mechanisms prescribed to the enzymes have remained largely hypothetical for decades. We will provide an update on our progress in generating experimental evidence for the hypothetical enzyme intermediates.[2-4] Interestingly, many of the experimental approaches take advantage of extraordinarily slow turnover numbers of the enzymes. We will also provide evidence for our hypothesis that the enzymes do not work in isolation, but are instead assembled into molecular machine that we name the "riboflavinator."

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Biosynthesis and Utilization of Nickel-Pincer Nucleotide Cofactors

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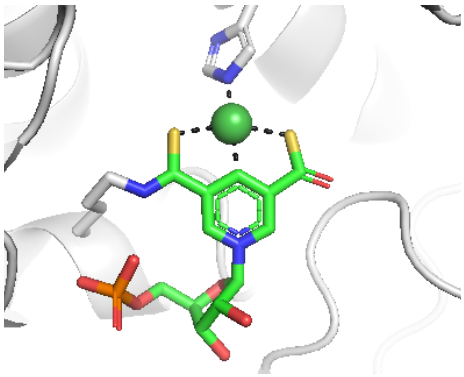

Fig. 1. NPN cofactor of lactate

Nickel-pincer nucleotide (NPN) cofactors, first identified in lactate racemase from *Lactiplantibacillus plantarum* (Fig. 1),[1] are organometallic molecules with a square-planar nickel atom that is tri-coordinated by a modified pyridinium nucleotide, forming a C-Ni and two S-Ni bonds. Within enzyme active sites, the nickel is additionally bound by a histidyl residue and in some cases the NPN cofactor is covalently tethered to a lysyl group. The cofactor is synthesized by a three-step process. LarB catalyzes C5-carboxylation and phosphoanhydride hydrolysis of nicotinic acid adenine dinucleotide to produce a dicarboxylated pyridine mononucleotide (P2CMN).[2,3] LarE catalyzes an ATP-dependent sulfur insertion reaction that converts P2CMN into a species with two thiocarboxylic acids (P2TMN).[4] LarC is a CTP-dependent nickel insertase or cyclometallase that transforms P2TMN into the nickel-pincer mononucleotide (NPMN).[5,6] In some NPN-enzymes, the cofactor is further modified to generate a nickel-pincer adenine dinucleotide (NPAD).[7] The NPN cofactors are used to catalyze proton-coupled hydride-transfer reactions of selected racemases, epimerases, and other enzymes.[7-9] Recent developments in understanding the biosynthesis and utilization of the NPN cofactor will be described.

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Hydroxylation of Amines by Flavin-Dependent Enzymes

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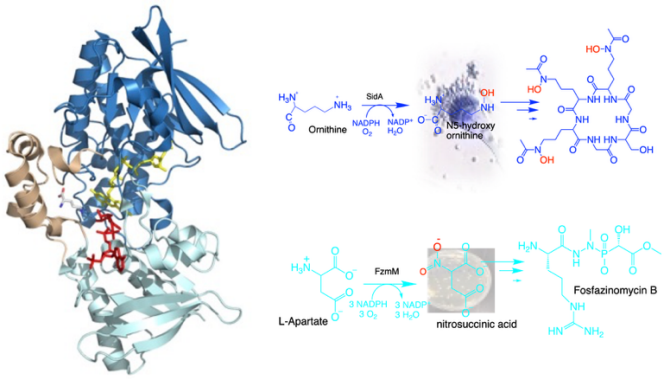
Flavin-dependent enzymes serve as versatile catalysts involved in microbial virulence, plant metabolism, and the biosynthesis of medicinal natural products[1,2]. Our research group has made significant contributions to elucidating the mechanisms of action of several flavin-dependent enzymes within these domains. This presentation will focus on the mechanistic and structural studies of a flavin-dependent nitrogen monooxygenase (NMO) involved in the formation of functional groups such as hydroxamate, nitrone, and nitro groups. These groups are essential for the activity of siderophores and natural products with antifungal and antibacterial properties. The seminar will highlight our work using biochemical, structural, and computational approaches to elucidate the role of cofactor interactions, substrate, and protein dynamics in intermediate stabilization, and substrate binding and release[3,4,5,6]. Experimental data demonstrating the formation of an enzyme–intermediate complex that enables consecutive rounds of oxidation will also be presented[7].



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Substrate-Driven Protein Conformational Changes in Catalysis by Nature's Most Proficient Enzymes

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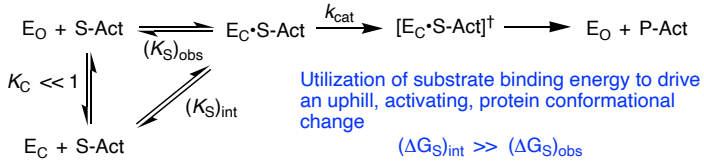
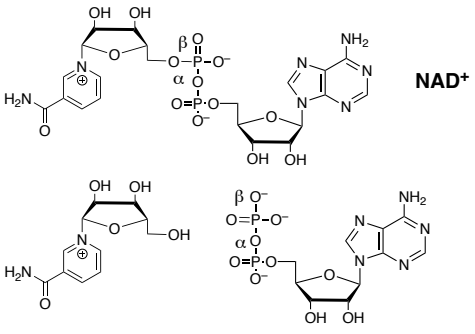
Many of Nature's most proficient enzyme catalysts exist in ground states that are inactive because the catalytic side chains are not properly positioned to stabilize the transition state of the catalyzed reaction. Rather, these side chains are positioned to interact with the substrate and undergo protein conformational changes that generate the catalytically active enzyme. Substrate-assisted protein conformational changes were first proposed to occur by Daniel Koshland: his rationale was criticized but without development of an alternative explanation for their wide-spread occurrence. Induced fit conformational changes are observed when: (i) The expression of the full transition state binding energy at the enzyme Michaelis complex would strongly favor effectively irreversible substrate binding. The substate binding energy is used instead to drive a protein conformational change. (ii) There is a large advantage for catalysis at a protein cage that favors development of optimal interactions between the protein catalyst and the reaction transition state. It is important to recognize that interactions between proteins and nonreacting substrate fragments often activate enzymes for catalysis, rather than to treat these fragments as spectators for thermoneutral conversion of the inactive enzyme $\mathbf{E_O}$ to the active form $\mathbf{E_C}$. A comparison of floppy and entropically rich structures for $\mathbf{E_O}$ with rigid and ordered structures for $\mathbf{E_C}$ suggests that their interconversion is uphill thermodynamically ($K_C << 1$, Scheme 1). Nonreacting substrate pieces provide binding energy to drive uphill conformational changes that activate enzymes for catalysis of reactions at the second substrate piece. This has been documented in experiments where phosphite dianion substitutes for the phosphate group of alkyl monophosphates to activate enzymes for catalysis of proton transfer, hydride transfer and decarboxylation reactions.[1] The results of recent studies of enzyme-catalyzed hydride transfer to NAD+ will be presented (Scheme 2). Activation is characterized for hydride transfer reactions catalyzed by glycerol phosphate dehydrogenase (GPDH), formate dehydrogenase (FDH) and phosphite dehydrogenase (PTDH), in studies to determine the kinetic parameters for unactivated, AMP- and ADP-activated enzyme-catalyzed hydride transfer to the truncated nicotinamide riboside (NR) cofactor "piece". The results show that activation of GPDH and FDH involves protein interactions with the a-ADP phosphate of NAD+, while activation of PTDH involves protein interactions with the b-ADP phosphate (Scheme 2). The X-ray crystal structures for ternary NAD+ complexes of substrate with GPDH, FDH and PTDH provide a rationale for these observed differences in activator reactivity for the different enzymes.

Scheme 1. Substrate Induced-Fit Model for Enzyme Activation



Scheme 2. The Whole NAD⁺ Cofactor and the NR Hydride Donor + ADP Activator "Pieces".



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Chemistry and Biology of Taxol Biosynthesis

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My lab is fascinated by the chemistry of plants and their role in human health and the environment. The goal of our research is to determine how plant-derived molecules are made and what they do, and to develop new strategies to accelerate the discovery process. Put simply, our work connects genes to molecules to functions in plants. We focus on pathways and molecules that can contribute to three aspects of sustainability: 1) Metabolites that plants have evolved to cope with environmental stress, which will enable crops to thrive with less energy and chemical inputs, especially in a changing climate. 2) Small molecules from medicinal plants, the pathways for which will ease access to medicines that are a staple for the treatment of disease. 3) The chemical components of dietary plants and their biosynthetic pathways, which will help us map the interplay of diet, human health, and preventive medicine. In this talk I will share our efforts to map and utilize pathways to complex taxanes of value in the clinic and, more broadly, to understand the logic of natural product biosynthesis across the plant kingdom.

Molecular Controls in Plant Hormone Signaling

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Plants respond to developmental cues and abiotic and biotic stresses by controlling both the level and activity of phytohormones. As part of that control, GRETCHEN HAGEN 3 (GH3) acyl acid amido synthetases catalyze the ATP-dependent conjugation of phytohormones with amino acids. Traditionally, GH3 proteins are associated with synthesis of the bioactive jasmonate hormone (+)-7- isojasmonoyl-L-isoleucine (JA-Ile) and inactivation of the major auxin hormone indole-3-acetic acid (IAA). Recent studies broaden the roles of GH3 proteins to include the modification of other auxins; inactivation of auxinic herbicides; and the missing step in the isochorismate pathway for the biosynthesis of the pathogen-response signal salicylic acid (SA). The GH3 protein family is an example of how a highly adaptable three-dimensional scaffold is used for the evolution of promiscuous activity across an enzyme family for modulation of diverse plant signaling molecules.

Structural basis for [1,3]-phosphate shift in bacterial hormone biosynthesis

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Phosphate groups are essential for living organisms and play essential roles in both primary and secondary metabolism. There are two types of enzyme known to process phosphate containing molecules, those that catalyse dephosphorylation (phosphatases) and phosphonate transfer (phosphomutases)[1]. However, to date there are no known examples of enzymes that catalyse transfer of a phosphate group (phosphamutase). This lecture will discuss elucidation of a key step in the biosynthesis of bacterial hormones 2-alkyl-4-hydroxyl-3-methylbutenolides (AHMBs)[2,3], and the glutarimide antibiotic gladiostatin[4], which involves a unique phosphate migration reaction catalysed by a first-in-class phosphamutase. Through a combination of sequence analysis, X-ray crystallography, substrate docking, molecular dynamics simulations, and site-directed mutagenesis, we propose a catalytic mechanism involving a conserved Asp residue, which acts as a general base / acid, and flipping in the active site of a zwitterionic intermediate, which enables phosphate migration via an elimination-addition mechanism.

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Structural Snapshots of Catalysis in a Bifunctional Terpene Cyclase

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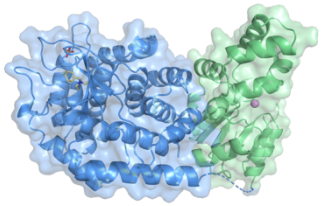
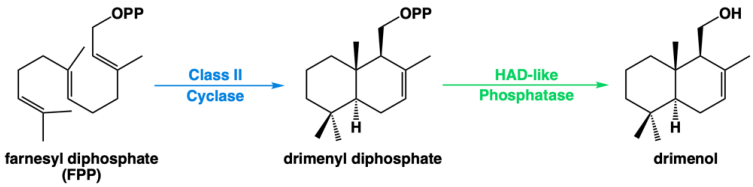
Terpenes represent the largest and most structurally diverse class of natural products, encompassing over 100,000 compounds that serve as antimicrobials, fragrances, biofuels, and more.[1] The biosynthesis of these compounds encompasses some of the most complex chemical reactions in nature, requiring the catalytic activity of terpene cyclases: enzymes which transform linear isoprenoid precursors into complex, often-polycyclic terpene structures, using their active sites as molecular scaffolds to direct the formation of a specific terpene.[2] Drimenol synthase from *Aquimarina spongiae* (AsDMS) is a highly unusual, bifunctional terpene synthase that integrates two distinct, sequential isoprenoid processing activities within a single polypeptide chain.[3] AsDMS catalyzes the cyclization of farnesyl diphosphate (FPP) to form drimenyl diphosphate, which then undergoes enzyme-catalyzed hydrolysis to yield drimenol, a sesquiterpene alcohol with antifungal and anticancer properties. Here, we report the X-ray crystal structures of AsDMS and its complex with a sesquiterpene thiol, which are the first of a terpene cyclase-phosphatase.[4] We also report structures of an inactivated AsDMS mutant, D33A-D323A AsDMS, including a complex with its native substrate, FPP. The AsDMS structure exhibits a didomain architecture consisting of a terpene cyclase β domain and a haloacid dehalogenase (HAD)-like phosphatase domain; surprisingly, AsDMS has two distinct active sites located on opposite sides of the protein. Mechanistic studies show that dephosphorylation of the drimenyl diphosphate intermediate proceeds through stepwise hydrolysis, and the hydroxyl oxygen of drimenol originates from the prenyl oxygen of FPP, rather than a water molecule from bulk solution. These experiments support a revised mechanism for this enzyme, one which updates the initial mechanism proposed by another group.[3] Surprisingly, AsDMS exhibits unprecedented substrate promiscuity, converting the substrate mimic farnesyl-*S*-thiolodiphosphate into cyclic and linear products. Ultimately, structural and mechanistic insights gained from AsDMS illustrate the functional diversity of terpene biosynthetic enzymes and provide a foundation for engineering "designer cyclase" assemblies capable of generating new terpenoid products.





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New enzymes in hydroxylipid catabolism

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Fatty acid β-oxidation (FAO) is a central catabolic pathway with broad implications for organismal health. However, various fatty acids are largely incompatible with standard FAO machinery until they are modified by other enzymes. Included among these are the 4-hydroxy acids (4-HAs)—fatty acids hydroxylated at the 4 (gamma) position—which can be provided from dietary intake, lipid peroxidation, and certain drugs of abuse. I will discuss our published[1] and ongoing work revealling that two atypical and poorly characterized acyl-CoA dehydrogenases (ACADs), ACAD10 and ACAD11, drive 4-HA catabolism in mice. Unlike other ACADs, ACAD10 and ACAD11 feature kinase domains N-terminal to their ACAD domains that phosphorylate the 4-OH position as a requisite step in the conversion of 4-hydroxyacyl-CoAs into 2-enoyl-CoAs—conventional FAO intermediates. Our ACAD11 cryo-EM structure and molecular modeling reveal a unique binding pocket capable of accommodating this phosphorylated intermediate. We further show that ACAD10 is mitochondrial and necessary for catabolizing shorter-chain 4-HAs, whereas ACAD11 is peroxisomal and enables longer-chain 4-HA catabolism. Mice lacking ACAD11 accumulate4-HAs in their plasma and females are susceptible to body weight and fat gain, concurrent with decreased adipocyte differentiation and adipokine expression. Collectively, this work defines ACAD10 and ACAD11 as the primary gatekeepers of mammalian 4-HA catabolism and sets the stage for broader investigations into the ramifications of aberrant 4-HA metabolism in human health and disease.

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Activity-based protein profiling – target and ligand discovery on a global scale

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Advances in DNA sequencing have greatly increased our understanding of the genetic basis of human disease. However, many of human genes encode proteins that remain uncharacterized and lack selective small-molecule probes. To address these problems, we have introduced activity-based protein profiling (ABPP), a chemical proteomic technology that globally profiles the functional state and small molecule interactions of proteins in native biological systems. In this lecture, I will describe the application of ABPP to generate covalent small molecule interaction (or ligandability) maps of human cells and how this information can guide the discovery of first-in-class chemical probes and drug candidates for disease-relevant proteins. Key themes will include: 1) the importance of assaying proteins in endogenous environments to realize their full small molecule interaction potential; 2) the capacity of covalent chemistry coupled with ABPP to extend the druggability of the proteome to reach historically challenging target families like adaptor/scaffolding proteins and DNA/RNA-binding proteins; and 3) the remarkably diverse ways that allosteric small molecules can regulate enzyme function in cells, including the trapping of enzymes on substrate complexes to promote neo-agonistic outcomes.

BIOCATALYTIC SYNTHESIS OF NON-NATURAL PEPTIDES

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The emergence of new therapeutic modalities requires the development of complementary tools for their efficient syntheses. Non-natural peptides have gained attention in the pharmaceutical industry due to their high selectivity, efficacy, and safety profiles. However, their widespread application has been hindered by the high costs of synthesis and the unique chemistries involved. Enzymes present a promising solution to supplement existing chemical approaches, as they offer high specificity and operate under mild and environmentally safer reaction conditions. Herein, we describe novel enzymatic strategies for the synthesis of non-natural peptides without the need for protecting group manipulations. Through the use of engineered enzymes and process development, we successfully synthesized a tricyclic peptide, opening up new possibilities for the development of novel peptide-based drugs.

A Sulfotransferase from a Gut Microbe Acts on Diverse Phenolic Sulfate Compounds, Including Acetaminophen Sulfate

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Sulfonation is one of the two main phase II detoxification pathways in eukaryotes that transforms non-polar compounds into hydrophilic metabolites[1]. Sulfotransferases catalyze these reactions by transferring a sulfo group from a donor to an acceptor molecule. Human cytosolic sulfotransferases use only 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a donor to sulfonate a variety of chemicals[2]. Less understood are microbial aryl-sulfate sulfotransferases (ASSTs), which catalyze sulfo transfer reactions, without utilizing PAPS as a donor[3]. Currently, the identity of physiological sulfo donor substrates remains unknown and sulfo acceptor substrates are underexplored. With this study, we aim to understand the potential contribution of a gut microbial enzyme to sulfonation chemistry by uncovering substrate preferences. Here, we show that a sulfotransferase (BvASST) from the prevalent gut microbe *Bacteroides vulgatus* is a versatile catalyst that utilizes a wide range of phenolic molecules as substrates that are commonly encountered by the host. With this action, it has the ability to modulate concentrations of donor phenolic sulfates like acetaminophen sulfate, dopamine sulfate, p-coumaric acid sulfate, indoxyl sulfate, and p-cresol sulfate. Moreover, we see a large adaptability in the acceptor preferences with the evidence of sulfonation for many biologically relevant phenolic molecules including p-coumaric acid, p-cresol, dopamine, acetaminophen, tyramine, and 4-ethylphenol. These results suggest that such gut microbial enzymes may impact the detoxification of a variety of phenolic molecules in the host, which were previously thought to be solely detoxified via human sulfotransferases.

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The periodic table of ribonucleotide reductases

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All organisms depend on the action of a ribonucleotide reductase (RNR) to provide the substrates for DNA replication and repair. This central function offers potential for the development of new RNR inhibitors that could serve as potent antibiotics. All class I RNRs share a common nucleotide reduction mechanism, initiated in the catalytic (alpha) subunit by a cysteine thiyl radical that abstracts an H-atom from the substrate. Class I RNRs use diverse inorganic chemistry in a separate beta subunit to generate the thiyl radical transiently on each turnover. My research group seeks to identify diversity in class I RNR metallocofactor compositions, structures, and assembly mechanisms, with the eventual goal of targeted inhibition of these pathways in bacterial pathogens. We focus on discovery and characterization of new enzyme subclasses with the goal of gaining more detailed understanding of both cofactor assembly mechanisms and the roles of subclass-specific structural features.

Heme-dependent Enzymes in Nitrogen-Nitrogen Bond-Formation

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Natural products contain diverse chemical features that are assembled by unique biosynthetic enzymes. Among natural products, molecules containing a nitrogen-nitrogen (N−N) bond include molecules with unique functional groups like hydrazines, N-nitrosos, and pyrazoles. My group has focused recently on understanding the assembly of N−N-bond-containing molecules. In this talk, I will discuss our recent discoveries in pathway discovery, enzyme characterization, and structural biology. In particular, I will highlight the role of the heme cofactor in two unrelated enzymes that both form nitrogen-nitrogen bonds. Our results help us understand how N−N-bonds are formed in the context of natural products biosynthesis and pave the way for applications in biocatalysis and targeted isolation of novel natural products.

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THE HARD WORK OF MAKING DEOXYRIBONUCLEOTIDES

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Ribonucleotide reductase (RNR) is a hard-working enzyme. Unlike other enzymes that convert one substrate to one product, RNR converts all four ribonucleotides to the corresponding deoxyribonucleotides. If RNR makes too many or too few deoxyribonucleotides, it can be toxic to the cell. If RNR makes the wrong ratio of deoxyribonucleotides, it can be toxic to the cell. Therefore, RNR is subject to multiple modes of allosteric regulation. Additionally, each round of RNR turnover requires generation of a radical species in the enzyme active site, with the source of the radical dependent on the RNR class. In this presentation, Drennan will describe her lab's work investigating allosteric regulation of substrate specificity in RNRs, i.e. how nucleotide effectors alter the preference of the RNR for each of its substrates.

Structure, Mechanism, and Annotation of Cobalamin-Dependent Radical SAM Methylases

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Cobalamin (Cbl)-dependent radical S-adenosylmethionine (SAM) methylases (RSMs) catalyze challenging methylation reactions at unactivated carbon and phosphinate phosphorus centers in a variety of small molecules and proteins. These enzymes utilize two distinct molecules of SAM: one to methylate cob(I)alamin, forming methylcobalamin (MeCbl), and the other to generate a 5'-deoxyadenosyl radical (5'-dA•). The radical abstracts a hydrogen atom from the substrate, generating a substrate radical that then engages in an S_H2-type attack on the methyl group of MeCbl. This process induces homolytic cleavage of the Co–C bond in MeCbl, yielding the methylated product.

In this lecture, I will present structural and mechanistic insights supporting this unified catalytic model, highlighting how the enzyme orchestrates these two chemically distinct SAM-dependent transformations within a single active site. I will also discuss ongoing efforts to address a major challenge in radical SAM enzymology: the functional annotation of this vast, mechanistically diverse enzyme superfamily.

Discovery and characterization of new families of nickel-dependent enzymes

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Nickel-dependent enzymes catalyze incredibly challenging transformations. However, few have been discovered to date, limiting our understanding of the role of nickel in Nature. We developed a bioinformatic pipeline to discover a new familiy of nickel enzymes that is widespread in Nature, but structurally distinct from previously reported nickel enzymes. We biochemically characterized a founding member, NphT, and discovered that it catalyzes intermolecular hydride transfer on organic substrates. We solved a 1.3 Å crystal structure of NphT bound to nickel and interrogated its mechanism via structure-guided mutagenesis. This work reveals an entirely new enzymatic scaffold that can harness nickel, expands the known nickel-catalyzed transformations to include intermolecular hydride transfer, and establishes a pipeline for the discovery of new families of nickel-dependent enzymes.

A NUDIX Jack-of-All-Trades: Structural and Functional Analysis of Archaeal Nudα

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NUDIX hydrolases are a diverse superfamily of enzymes essential to cellular metabolism, catalyzing the hydrolysis of NUDIX (nucleoside diphosphate linked to moiety X) substrates.[1] By doing so, they regulate various biochemical processes, including nucleotide pool homeostasis, detoxification of harmful metabolites, and mRNA maintenance. These enzymes are characterized by the conserved NUDIX box motif (Gx_5Ex_5[UA]xREx_2EExGU)) which contains two highly conserved glutamate residues (highlighted in red) required for the Mg^{2+} dependent catalytic activity. Interestingly, NUDIX enzymes are less represented in archaea compared to bacteria and eukaryotes, implying less dependence on NUDIX functions or greater substrate ambiguity. *Thermococcus kodakarensis*, a hyperthermophilic archaeon, has a single NUDIX enzyme encoded by the gene *TK2284.* Previous biochemical characterization of TK2284 with NDP analogs demonstrated high catalytic efficiency towards ADP-ribose, but the substrate preferences to other NUDIX substrates, including NTP analogs and mRNA caps remain elusive.[2] Here, we report a comprehensive functional and structural characterization of TK2284, we termed Nuda. Functional characterization of Nuda utilizing malachite green screening, competitive binding, U-HPLC kinetics, and mass spectrometry shows activity on a variety of damaged purine-containing molecules, with the highest preference towards ADP- Ribose, 8oxoATP and ADP-ribose capped RNAs. In parallel, structural characterization of apo or 8oxoATP bound to Nudα , using native mass spectrometry and X-ray crystallography, reveals Nuda adopts a novel homotetrameric structure not previously structurally observed by any NUDIX hydrolase. Distinct to the *Thermococcales*, Nuda harbors an extended N- terminus, which intertwine into a unique fold within each homodimer. Co-crystallization of Nuda with 8oxoATP reveals two of the four active sites are occupied and the substrate recognition pocket created by the NUDIX box motif and N-terminal extensions contain two critical aromatic residues which sandwich the purine moiety and define Nuda's substrate preference. This work suggests the sole *T. kodakarensis* NUDIX enzyme, Nuda, acts as a jack- of-all-trades hydrolyzing a diverse range of NUDIX substrates, expands our understanding of the mechanisms employed by archaeal organisms to cleanse and regulate their metabolites and nucleic acids, and further contributes to our knowledge of the diversity and evolution of the vast NUDIX superfamily.

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Artificial Intelligence for Genome Mining and Engineering Biosynthesis

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Natural products play an important role in drug discovery. However, they are often discovered serendipitously and there is a lack of tools that enable prioritization of organisms that are likely to produce active and structurally novel compounds. In addition, natural products often need to be modified in order to be developed as therapeutics, but total synthesis of natural products is challenging. My lab aims to develop Artificial Intelligence (AI)-guided genome mining techniques to enable the discovery of novel natural products with therapeutically-relevant bioactivities. Specifically, we have developed an AI method for predicting natural product bioactivity[1] and applied it to guide natural product isolation.[2] We are also developing AI methods for prioritizing BGCs by the novelty and structural properties of the compounds they produce. My lab has also been working on applying Statistical Coupling Analysis (SCA) to enzyme substrate prediction and engineering, including enzymes responsible for natural product biosynthesis.

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Design and Development of New Radical Enzymology

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Radical enzymology explores how enzymes harness highly reactive radical intermediates to catalyze chemically challenging transformations with remarkable efficiency and selectivity. Combining synthetic chemistry, enzymology and protein engineering, our group advanced novel radical enzymology which were not previously known in nature, including those which were unknown in both organic chemistry and biochemistry. First, by capitalizing on the innate redox properties of first-row transition-metal cofactors, we repurposed and evolved natural metalloproteins to catalyze unnatural radical reactions in a stereocontrolled fashion. Through a metalloenzyme-catalyzed (pseudo)halogen transfer mechanism (XAT, X = F, Cl, Br, I, N3, SCN and OCN), a range of radical C–C, C–F, C–N and C–S bond forming reactions proceeded with excellent total turnover numbers (up to 20,000) and outstanding stereocontrol. Second, by merging visible light photoredox catalysis and biocatalysis, we advanced a novel mode of pyridoxal radical biocatalysis which is new to both chemistry and biology. Cooperative photobiocatalysis allowed us to repurpose structurally and functionally diverse pyridoxal phosphate (PLP)-dependent enzymes as radical enzymes, leading to novel radical PLP enzymology. Pyridoxal radical biocatalysis provides stereoselective and protecting-group-free access to numerous useful non-canonical amino acids, including those bearing a stereochemical triad and/or tetrasubstituted stereocenters which remained difficult to prepare by other chemical and biocatalytic means. The ability to perform diversity-oriented synthesis combinatorially through previously elusive biocatalytic C–C bond formation marks a synthetically valuable advance, paving the way for broader adaptation and application of biocatalysis in medicinal chemistry. Furthermore, we demonstrate that the exploitation of biocatalyst-photocatalyst synergy affords a new paradigm to design and develop a range of stereoselective intermolecular radical reactions using new mechanisms.

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Poster Abstracts

Presenters of Odd Numbered posters should be present at their poster during the Monday afternoon poster session. Presenters of Even Numbered posters should be present at their poster during the Tuesday afternoon poster session.

1. Adak, Sanjoy
2. Albekioni, Elene
3. Alt, Tyler
4. Avila, Mariah
5. Aziz, Alaa
6. Azizollahi, Hamid
7. Balutowski, Adam
8. Bartl, Michael
9. Beattie, Aodhan
10. Bhandari, Dipendra
11. Blankenship, Sara
12. Bohne, Annkathrin
13. Cha, Lide
14. Cui, Jiayuan
15. Drexler, Lukas
16. Dudiak, Brianne
17. Eskandari, Roozbeh
18. Fisk, Mercedes
19. Garay, Erika
20. Georgiades, John
21. Gonzalez, David
22. Groetecke, Emma
23. Groom, Daniel
24. Guha, Arkajit
25. Hoffmann, Katherine
26. Hoffpauir, Zoe
27. Hubert, Felix
28. Hyde, Darius
29. Jangid, Kuldeep
30. Jew, Kristen
31. Keable, Stephen
32. Kim, Seokhee
33. Kipouros, Ioannis
34. Kovalevsky, Andrey
35. Koweek, Rebecca
36. Kramer, Lydia
37. Lara, Darwin
38. Le, Aaron
39. Lee, Kyung Hyeon
40. Lee, Kyung Hyeon
41. Liu, Chang
42. Liu, Chengli
43. Liu, Xin
44. Liu, Yujue
45. Lohman, Jeremy
46. Mamasakhlisi, Nino
47. Manje, Dasplang
48. Marko, Ingrid
49. Martell, Jeffrey
50. Martinie, Ryan
51. Maurya, Ravi
52. McDonald, Drew
53. McFarlane, Jeff
54. Mealka, Matthew
55. Meek, Thomas
56. Meneely, Kathy
57. Merrick, Collin
58. Mizutani, Taku
59. Moafian, Zeinab
60. Munan, Subrata
61. Murakami, Mario
62. Murkin, Andrew
63. Osika, Kristin
64. Namkoong, Gil
65. Palfey, Bruce
66. Papa, Johanna
67. Patel, Ketan
68. Pearce, Lucciano
69. Pham, Truc
70. Pedigo, Jacob
71. Phillips, Robert
72. Poole, Leslie
73. Rajendran, Chellammal
74. Roberts, Kenneth
75. Sakon, Joshua
76. Schleissner, Pamela
77. Shimada, Kazuki
78. Shishikura, Kyosuke
79. Simke, William
80. Singh, Jitendra
81. Smith, Corine
82. Stafford, Veronica
83. Troycke, Philip
84. Vuksanovic, Nemanja
85. Vartak, Aniket
86. Webb, Bradley
87. Whittall, Dominic
88. Zanphorlin, Leticia
89. Zhou Tianhui (Hina)
90. Zielinski, Kara

note that some entries are purposefully non-alphabetical to accommodate Flash Talks

From an eagle-killing cyanotoxin to skatole: A tale of two diiron biosynthetic enzymes

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The involvement of non-heme dinuclear iron cluster-containing enzymes in natural product biosynthetic pathways has been increasingly recognized in recent years. During the biosynthesis of the 'eagle-killing' neurotoxin, aetokthonotoxin, AetD transforms the 2-aminopropionate portion of 5,7-dibromo-L-tryptophan to a nitrile using remarkable oxidative rearrangement chemistry.[1] Employing a combination of structural, biochemical, and biophysical techniques, we characterized AetD as a non-heme diiron enzyme that belongs to the emerging heme oxygenase-like dimetal oxidase (HDO) superfamily.[2] High-resolution crystal structures of AetD, together with the identification of catalytically relevant products, provide mechanistic insights into how AetD affords this unique transformation. Subsequently, a search for AetD homologs in genome databases led to the discovery of a cyanobacterium-derived oxygen-dependent skatole synthase that singlehandedly converts tryptophan to skatole.[3] This is in stark contrast to the previously known skatole-forming enzyme, indoleacetate decarboxylase, which is strictly oxygen-sensitive. Our detailed biochemical analyses have traced the fate of the individual atoms within the 2-aminopropionate portion of L-tryptophan, revealing bicarbonate and cyanide as co-products of the reaction. Furthermore, stopped-flow experiments revealed the formation of a μ-peroxodiiron (III) intermediate during enzyme turnover. Overall, the characterization of these two diiron enzymes offers alternative pathways for nitrile and skatole biosynthesis and expands the functional repertoire of the HDO superfamily.

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Kinetic and Structural Characterization of Isocitrate Dehydrogenase 1

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Isocitrate dehydrogenases (IDHs) are the most mutated metabolic enzymes in cancer. Wild-type (WT) IDH1 and IDH2 facilitate the reversible oxidative decarboxylation of isocitrate (ICT) to α-ketoglutarate (αKG). Heterozygous mutations in IDH1 and IDH2 drive tumorigenesis through acquiring the ability to catalyze reduction of αKG to oncometabolite D-2-hydroxyglutarate (D2HG).[1] These reactions rely on specific structural features, such as dimerization, for catalysis. Three species of dimers are theoretically possible in cells harboring heterozygous IDH1 mutations: WT/WT, mutant/mutant, WT/mutant dimers. The products of the WT-catalyzed conventional reaction are the substrates for mutant-catalyzed neomorphic reaction. Due to this bifunctional activity, the heterodimeric IDH1 may act as a major source of oncometabolite, potentially due to substrate channeling or increased local substrate concentrations. Extensive research has focused on inhibiting D2HG overproduction using selective allosteric inhibitors for IDH1 and IDH2. They typically bind to the regulatory domain formed by two helices from both monomers, but secondary point mutations within this domain often drive therapeutic resistance. IDH1 and IDH2 share high sequence and structural similarity, except within the regulatory domain, which appears more rigid in IDH2. In contrast, the homologous region in IDH1 interchanges between unfolded in open and folded in closed, catalytically competent states.[2] To address the limited structural and kinetic information, we first tested the expression of WT and mutant IDH1 and used mass photometry to assess the formation and relative abundance of the dimer species. Our results indicated no evidence of monomer exchange in solution and demonstrated that all three dimer species form when WT and mutant proteins are co-expressed. After we established a reliable method to generate, visualize, and quantify the different dimer species of IDH1 in solution, we sought to understand how inhibitors interact with both oncometabolite-producing hetero- and homodimers. Then we extended our interest to understanding the local conformational differences between IDH1 and IDH2. We used hydrogen/deuterium-exchange mass spectrometry (HDX-MS) to visualize the local changes in solvent accessibility that accompany the conformational change from the open (inactive) to closed (active) enzyme conformations. We designed chimeric proteins by swapping the regulatory domains between IDH1 and IDH2, and identified the distinct structural flexibility between WT IDH1 and the IDH2-domain-swapped IDH1. Our ongoing work investigates whether this prominent difference in the stabilization of the regulatory domain is driving the selectivity of allosteric inhibitors. Together, this work provides structural and kinetic insight necessary to elucidate how to improve strategies for inhibiting oncometabolite overproduction by mutant IDH enzymes.

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Transient State Analysis of NQO1 and Direct Evidence for Inhibition by Warfarin

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NADH:Quinone Oxidoreductase 1 (NQO1) is a cysosolic quinone reductase that utilizes an FAD cofactor to catalyze the two electron reduction of a wide range of quinone substrates using electrons derived from NAD(P)H. The reductive half reaction of NQO1 was characterized using anaerobic transient state kinetic methods. Two distinct populations of enzyme were observed to catalyze this reaction with different binding affinities and rate constants, suggesting some degree of heterogeneity. Nonetheless, the dominant fraction of enzyme (~85%) binds NADH relatively weakly, 63 µM, and oxidizes the NADH rapidly, at 1330 s^{-1}. The minor fraction of enzyme (~15%) binds NADH considerably more tightly, 2 µM, but catalyzes the chemistry at 15 s^{-1}. Under steady state turnover conditions, the reaction is rate limited by the release of the ubiquinol product, occurring at ~400 s^{-1}, suggesting that the minor slow-reducing fraction of NQO1 is not contributing to the turnover number. Warfarin, a commonly prescribed anticoagulant medication, was found to inhibit NQO1 at known efficacious concentrations (~2 µM). Direct binding measurements reveal that warfarin binds, with a dissociation constant of ~1 µM for the NQO1•warfarin complex. Using transient state kinetic analyses, it was elucidated that warfarin competitively inhibits the reductive half reaction. As NQO1 is one of the major vitamin K reductases *in vivo*, it is plausible that NQO1 inhibition plays a major role in suppressing the coagulation cascade by preventing vitamin K cycling.

Biosynthetic Elucidation of Lodopyridone A: Discovery of a Novel Flexible Tryptophan 2,3-Dioxygenase Enzyme

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L-kynurenine is an important metabolite that is converted to NAD+ via the mammalian de novo NAD+ biosynthesis pathway[1]. It has been investigated for its role in a range of neurodegenerative diseases[2]. Functionalized kynurenine scaffolds, derived from the essential amino acid L-tryptophan, are seen in a variety of alkaloid natural products that demonstrate bioactivity. The tryptophan 2,3-dioxygenase (TDO) enzyme, responsible for catalyzing the transformation of L-tryptophan to N'-formyl-kynurenine, has been the target of recent protein engineering efforts[3,4] for use in biocatalysis applications, especially with halogenated tryptophan derivatives. Despite this interest, only one biochemically characterized TDO, Tar13, has been shown to natively act on a halogenated tryptophan substrate[5]. Here we identify the biosynthetic gene cluster responsible for the assembly of the chlorinated actinobacterial molecule lodopyridone A[6] and characterize the TDO from this cluster, which demonstrates unanticipated substrate flexibility. For the first time, we show the use of growth-coupling, which links the production of the pathway product to host survival, in an engineered *Pseudomonas* auxotroph strain, PUMA, to evolve these TDO enzymes. This work serves to expand our understanding of TDO specificity and act as a basis for future biocatalysis of halogenated and chemically functionalized kynurenine scaffolds.

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Integrating Enzyme Kinetics and NMR Spectroscopy to Elucidate the Catalytic Mechanism of F_{420}-depedent Sugar-6-phosphate Dehydrogenase from *Cryptosporangium arvum*

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F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD) catalyzes the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconolactone (6PG). Recent phylogenetic analyses have identified a new subclass of these enzymes, F_{420}-dependent sugar-6-phosphate dehydrogenases (FSDs), which act on a broader range of 6-phosphate sugars, including fructose-6-phosphate (F6P) and mannose-6-phosphate (M6P). One such enzyme from *Cryptosporangium arvum* (*Cryar*-FGD) was characterized using binding assays and kinetic analyses, nuclear magnetic resonance (NMR), and mass spectrometry. Results showed strong binding affinities for all substrates. Steady-state kinetic analysis revealed that G6P has the highest catalytic efficiency, with a $k_{cat(app)}$ of 6.4 ± 0.2 s^{-1}, compared to 1.4 ± 0.1 s^{-1} for F6P and 0.32 ± 0.02 s^{-1} for M6P. Pre steady-state spectral features for the G6P reaction resembled those of *Mycobacterium tuberculosis* FGD. While the F6P reaction displayed distinct spectral features, F_{420} reduction was still observed. In contrast, spectra for the M6P reaction were markedly different from those of G6P and F6P. Across all substrates, no catalytic intermediates were detected, and hydride transfer was not rate-limiting. As with G6P, the reaction with F6P also produced 6PG. Notably, NMR data showed that F6P was isomerized to G6P, suggesting isomerase activity. In contrast, M6P induced only spectral shifts, with no evidence of isomerization or 6PG formation. However, mass spectrometry confirmed oxidized products for all three sugars, each with a mass of 299.0 ± 0.1. Collectively, these findings reveal that *Cryar*-FGD exhibits both dehydrogenase and isomerase activity, uncovering a newly identified dual enzymatic function and establishing its role as a multifunctional enzyme.

Enzymatic modification of poly-N-substitued glycines (peptoids) to form thioether (poly) macrocyclic structures

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Poly-N-substituted glycines ("peptoids") have earned a reputation as widely used building blocks in chemical biology and peptide-based drug design. Incorporation of peptoid residues is straightforward via solid-phase peptide synthesis (SPPS), but generation of constrained or macrocyclic structures utilizing peptoid residues is synthetically challenging. Radical S-adenosyl-L-methionine (rSAM) enzymes exhibit broad substrate tolerance, especially in the context of ribosomally synthesized and post-translationally modified peptides (RiPPs). These enzymes are known for forging complex covalent linkages across diverse molecular frameworks; however, there has been limited exploration of how this class of enzymes can modify non-peptidic backbones. We show that PapB, a radical SAM maturase, can generate thioether bonds between a cysteine residue and carbon atoms directly on peptoid sidechainsxx. Here we present NMR data indicating chemoselectivity to the position α to the carboxylate in several peptoid backbones. This evidence indicates that PapB can be used as a biocatalytic tool for post-synthetic peptoid editing.

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Understanding Branched Non-Ribosomal Peptides Biosynthesis: Mechanistic Insights from Fimsbactin A Highlights Cy Domains Role in Scaffold Diversification

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Branch-point syntheses in nonribosomal peptide assembly are rare but useful strategies to generate tripodal peptides with advantageous hexadentate iron-chelating capabilities, as seen in siderophores. However, the chemical logic underlying the peptide branching by nonribosomal peptide synthetase (NRPS) often remains complex and elusive. Here, we untangled the unusual branching mechanism of fimsbactin A biosynthesis through a combination of bioinformatics, site-directed mutagenesis, *in vitro* reconstitution, molecular modeling, and molecular dynamics simulation. Our findings clarify the roles of the fimsbactin NRPS enzymes, uncovering catalytically redundant domains and identifying the multifunctional nature of the FbsF cyclization (Cy) domain. We demonstrate the dynamic interplay between l-serine and 2,3-dihydroxybenzoic acid derived dipeptides, partitioning between amide and ester forms via a 1,2-*N*-to-*O*-acyl shift orchestrated by the noncanonical, multichannel FbsF Cy domain. The branching event occurs in a secondary condensation event facilitated by this Cy domain with two dipeptidyl intermediates, which generates a branched tetrapeptide thioester. Finally, the terminal condensation domain of FbsG recruits a soluble nucleophile to release the final product. This study advances our understanding of the intricate biosynthetic pathways and chemical logic employed by NRPSs, shedding light on the mechanisms underlying the synthesis of complex branched peptides.[1]

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Sequence-Driven Manipulation of Chemoselectivity and Regulation in Anthranilate Synthase

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Paralogous enzymes catalyze the turnover of chorismic acid (CA) on route to an array of aromatic compounds. Examples for amination reactions include the formation of anthranilic acid (AA) by AA synthase (AS) and aminodeoxychorismic acid (ADC) by ADC synthase (ADCS).[1] Both comprise complexes of a glutaminase and a CA-aminating synthase. These complexes exhibit distinct similarities in fold, catalytic mechanism, and binding of tryptophan, which serves as allosteric inhibitor only in AS but not ADCS.[2] A subset of ADCS, dubbed ADCS^{PIAGT}, implies a particularly close relationship to AS, as the central active site motif and the first reaction step are identical.[3] However, molecular details causing the differences between AS and ADCS with respect to chemoselectivity and regulation by tryptophan are not fully understood.

A sequence similarity network of the synthase subunits confirmed the supposed high resemblance of AS and ADCS^{PIAGT}, in line with the similar catalytic mechanism and active site motif. A pair of AS and ADCS^{PIAGT} from the thermophile *Thermincola potens* revealed a particularly high sequence identity and hence, was chosen for detailed analysis. Conserved, partially co-varying residues characteristic of either AS or ADCS^{PIAGT} were grouped into regions connecting the active site with the tryptophan binding site or regions surrounding the active site. AS chimeras with individually exchanged sets of residues were constructed that displayed tryptophan in- or malsensitivity, and promiscuous ADC formation, respectively. Notably, the introduction of residues associated with tryptophan insensitivity into a chimera also enhanced promiscuous ADC formation.

This functional interconversion along with the general high similarity support a close relationship between AS and ADCS^{PIAGT} and suggest a bridging role of ADCS^{PIAGT} towards the emergence of mechanistically more proficient ADCS^{PIKGT}. Our AS-based chimera with altered chemoselectivity and tryptophan regulation might imply an entanglement of these features and, moreover, contributes to the understanding of the determinants of chemoselectivity in MST enzymes.

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Convergent Evolution of Germacrene Lactone Biosynthesis in Plants and Animals

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Germacrene lactones are molecules commonly isolated from flowering plants, with some having well-characterized functions as deterrents of arthropod herbivory[1]. Similarly, a large diversity of germacrene lactones have been isolated from corals, with anti-feedant properties in marine arthropods, prompting us to investigate differences or similarities in plant and animal germacrene lactone biosynthesis[2]. Recent work has shown that terpene cyclases, along with the co-clustered oxidative enzymes necessary for the biosynthesis of terpenoids, are present in the chromosomal DNA of soft coral animals, enabling biosynthetic studies on these terpenoids[3,4]. In this work, we identified a conserved gene cluster family, widespread amongst one of the 2 soft coral orders, responsible for the biosynthesis of germacrene lactones in corals across 7 different genomes. We developed *Pichia pastoris* as a host for enzyme discovery from these organisms, demonstrating the novel full reconstitution of a coral biosynthetic gene cluster from local San Diego gorgonian *Leptogorgia chilensis*. Partial reconstitution and scale-up of pathway intermediates enabled us to propose a biosynthetic scheme for the formation of these compounds, demonstrating a different route than germacrene lactone biosynthesis in plants.

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Structural and Enzymatic Insights into SARS-CoV-2 Main Protease Natural Variant Exhibiting Resistance to Nirmatrelvir and Ensitrelvir

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SARS-CoV-2 main protease (MPro) is a key viral enzyme responsible for processing the polyproteins required for viral replication, and has emerged as a validated target for antiviral drug development. In this study, we characterize a naturally occurring double mutant of MPro, D48Y/ΔP168, located in the substrate-binding subsites S2 and S4. Remarkably, this mutant maintains robust catalytic activity and efficient N-terminal autoprocessing, a prerequisite for dimer formation and enzymatic maturation, yet exhibits significant resistance to clinical inhibitors, including a three-fold decrease in binding affinity for nirmatrelvir and a five-fold decrease for ensitrelvir, as measured by isothermal titration calorimetry. High-resolution room-temperature X-ray crystallography, coupled with molecular dynamics simulations and B-factor analysis, revealed that the D48Y/ΔP168 variant maintains a structural fold comparable to the wild-type enzyme but adopts a more open and dynamic active site, particularly in the S2 helix, S5 loop, and helical domain. These dynamic features correlate with increased dimerization affinity (~40-fold tighter than wild type) and altered hydrogen bonding at the dimer interface. Despite increased dimer stability, thermal unfolding studies show reduced thermal tolerance by ~8 °C, suggesting a trade-off between conformational flexibility and structural robustness. Structural analyses of MPro-inhibitor complexes reveal that while the mutant still forms covalent adducts with Cys145, as seen with GC373 and nirmatrelvir, the altered dynamics and disrupted hydrophobic interactions within the binding pocket compromise binding efficiency. Our findings demonstrate that natural mutations can modulate the conformational landscape of MPro to preserve catalytic function while undermining drug binding, providing critical insight into resistance mechanisms. These results highlight the importance of considering protein dynamics and structural plasticity in the design of next-generation SARS-CoV-2 MPro inhibitors that are resilient to emerging resistance mutations.

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Structural and Mechanistic Evidence Identifies C387 as the Catalytic Proton Donor in DOKDC

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D-Ornithine/D-lysine decarboxylase (DOKDC) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme from *Salmonella typhimurium* that catalyzes decarboxylation of D-ornithine and D-lysine to produce putrescine and cadaverine, respectively[1]. It is a dimer with PLP bound at the dimer interface and is stereospecific for D-amino acids, proceeding via an S_E2 mechanism with inversion of configuration at the substrate's Cα [2]. Like other PLP-dependent decarboxylases, DOKDC requires protonation at the Cα after decarboxylation; however, the identity of this proton donor has remained unknown. Prior structural observations suggested a cysteine residue, C387, positioned ~4 Å from the substrate Cα, as a possible catalytic proton donor[2].

Substitution of C387 with serine or alanine drastically decreases catalytic efficiency (k_{cat}/K_m) as shown by the DNBS assay. The catalytic efficiency of WT with D-lysine was $6.0x10^2$ $M^{-1}s^{-1}$ compared to 14 $M^{-1}s^{-1}$ for C387S and 210 $M^{-1}s^{-1}$ for C387A. Similar decreases were observed for D-ornithine. D-arginine, which was not previously shown to be a substrate, demonstrates low activity with WT with decreased activity in the variants. The decreased activity is due to an increase in the rate of the transamination side reaction. X-ray crystallographic analysis of C387S showed that PLP had been converted to PMP, and the DNBS assay showed that transamination occurred in approximately 5-15% of turnovers, depending on the substrate.

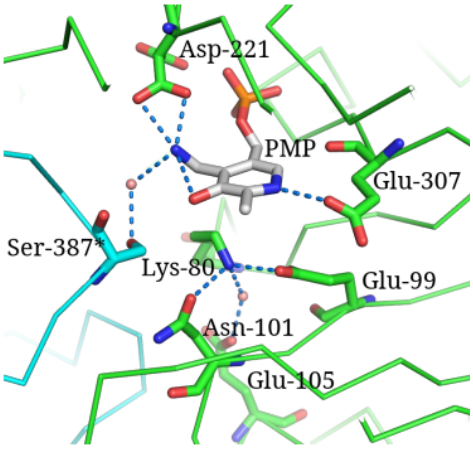

Figure 1. View of the binding contacts of PMP with the active site of C387S DOKDC. Possible hydrogen bonds are shown in blue.

Stereochemical analysis by proton NMR further supports C387's role as the proton donor. WT DOKDC protonates the product from the backside relative to the departing CO_2. The product in C387A and C387S is protonated with retention of stereochemistry. This shows that the proton is transferred from the opposite face, which is consistent with protonation by a water molecule in the active site in the absence of cysteine. This structural, kinetic, and stereochemical underscores the role of C387 in normal proton transfer.

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Distal Peptide Elongation by a Protease-Like Ligase and Two Distinct Carrier Proteins

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Closthioamide (CTA) is a unique polythioamide antibiotic from the anaerobe *Ruminiclostridium cellulolyticum*.[1,2] Unlike classical non-ribosomal peptide synthetases (NRPSs), with modular adenylation and condensation domains, CTA biosynthesis uses a non-canonical, non-modular system.[3,4] Central to this pathway is CtaG, a papain-like ligase that catalyzes amide bond formation between two distinct peptidyl carrier proteins (PCPs): CtaH, carrying para-hydroxybenzoic acid (PHBA), and CtaE, carrying a tri-β-alanine ($(\beta\text{-Ala})_3$) chain.[5]

CtaG catalyzes amide bond formation through a thiol-thioester exchange with an enzyme-bound intermediate, using a single substrate tunnel for selective, directional transfer between two distinct peptidyl carrier proteins (PCPs). This enables distal peptide elongation, in contrast to the proximal extension typical of canonical NRPS systems, and instead resembles solid-phase peptide synthesis.

Structural elucidation of CtaG revealed a bipartite architecture with a catalytic triad (Cys, His, Asp) and an aromatic substrate-binding pocket. The enzyme exhibits strict substrate specificity, acting only on PCP-bound intermediates and assigning non-interchangeable donor (CtaH) and acceptor (CtaE) roles to the carrier proteins. Importantly, CtaE binds to CtaG only in the CtaG:PHBA complex, ensuring that the acceptor PCP (CtaE) engages with the ligase exclusively at the correct stage of the catalytic cycle. This sequential binding enforces directionality and fidelity in the distal transfer of PHBA from CtaH to CtaE.

Genome mining identified related CtaG-like enzymes in the biosynthetic pathways of petrobactin, butirosin, and methylolanthanin, revealing a broader class of ribosome-independent peptide ligases. These insights expand the diversity of known peptide bond formation mechanisms and create new opportunities for engineering bioactive natural products.

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Identification and Characterization of MNIO Proteins in a Ribosomal Peptide Pathway

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Multinuclear non-heme iron dependent oxidative enzymes (MNIOs) are an emerging class of metalloenzymes. Although this protein family comprises more than 14,000 members, only a handful have been functionally characterized.[1] MNIOs are strongly associated with the tailoring of ribosomally synthesized and post-translationally modified peptides (RiPPs). They catalyze a wide array of novel oxidative modifications to construct unusual peptide scaffolds including oxazolones and thioamides[2], thiooxazoles[3], and C-terminal alpha-keto acids[4]. These unique transformations highlight the strong potential of the MNIO protein family as a repository of untapped enzyme chemistries.

Through a genome mining campaign of MNIO-modifying RiPPs, we uncovered a novel RiPP biosynthetic gene cluster (BGC) from *Peribacillus*. This BGC harbours multiple non-heme iron enzymes, including two copies of MNIOs. The sequence of precursor peptides shows no conservation to those from characterized MNIO-containing BGCs. Through heterologous reconstitution of the BGC in *E. coli*, we managed to elucidate the structure of the final product, as well as assigning the function of enzymes encoded within the BGC. Furthermore, establishing *in vitro* reactivities of the modifying enzymes enabled probing of the enzymatic mechanisms. These new reactivities of MNIOs further expand the chemical space of this enzyme family, while also providing new insights into catalysis and regulation of MNIO proteins.

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A comprehensive mechanistic investigation of Gms reveals its enzyme promiscuity

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Archaea generate glycerol dialkyl glycerol tetraether (GDGT) membrane-spanning lipids that allow them to thrive at extremely high temperatures, salinities, and low and high pH values.[1] Among other products, GDGTs can be modified to give glycerol monoalkyl glycerol tetraethers (GMGT) by forming a C(sp^3)-C(sp^3) crosslink between the two biphytanyl chains, a reaction catalyzed by GMGT synthase (Gms).[2,3] It was generally believed that GDGT is Gms' only substrate, and that its reaction mechanism is similar to that for the previously characterized tetraether synthase (Tes).[4] In this study, we report the characterization of a Gms from *Methanothermococcus okinawensis* (MoGms). We find that, in contrast to earlier predictions, MoGms uses both GDGT and the GDGT precursor, archaeol (AG), as substrates, affording a range of products. In addition to the expected crosslinks between the two hydrocarbon chains of substrates, we captured multiple sulfur-containing species, believed to be intermediates en route to various products, and identified the location of the sulfur atoms. By aligning observed intermediate species to their suspected reaction products, we identified multiple pathways by which MoGms can catalyze the formation of GMGT. Moreover, the retention time differences between Gms and Tes-exclusive products suggest the formation of various stereoisomers. To link *in vitro* observations to physiological relevance, heterologous expression of MoGms in *Δtes Thermococcus kodakarensis* was tested at 60°C, resulting in GTGT production. Our work provides new mechanistic insight into Gms catalysis and expands our understanding of archaeal lipid modification as an adaptive strategy under stress.

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On the Origin of Substrate Scope and Promiscuity in Enzymes from the Amidohydrolase Superfamily

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Enzyme catalysis is characterized by the enormous acceleration of chemical reactions and a high specificity for the respective substrates. However, numerous studies have shown that many enzymes can also use substrates other than the ones for which they have been evolutionarily optimized, albeit with lower efficiency.[1–3] Such minor promiscuous side activities can often be improved significantly by only a few mutations and hence constitute promising starting points for the generation of novel enzymes in the context of natural evolution or enzyme engineering approaches.[1,2,4] Therefore, identifying and rationalizing the scope of accessible substrates of a given enzyme would advance our knowledge of enzyme catalysis and would also be important for the use of enzymes in industrial biocatalysis.[5,6] However, even highly advanced automatic functional annotation algorithms are often unable to reliably predict the native substrate of an enzyme, let alone the identification of additional natural or even anthropogenic substrates.[7]
Here, we present a novel concept to explain and predict substrate promiscuity for members of the amidohydrolase superfamily (AHS) by analyzing specific features of the underlying enzymatic reaction mechanism. Comprehensive *in vitro* and *in silico* analyses of two AHS classes revealed that catalysis proceeds either via 1,4 or 1,6 nucleophilic conjugate addition thereby shaping substrate scope in these enzymes. Notably, these characteristics result in an inverted enantioselectivity for fleeting chiral intermediates, which are transient chiral species on the reaction pathway from an achiral substrate to an achiral product. Moreover, we demonstrate that catalysis focuses on conserved core structures rather than on all moieties of a given substrate, which increases the degree of promiscuity and evolvability in these enzymes. Using site-directed mutagenesis, we showed that an enzyme specialized in a specific nucleophilic conjugate addition can both readily adapt to secondary substrates and accommodate novel substrates by few amino acid exchanges. Hence, our study provides a framework to define enzymatic substrate scopes, reveals mechanistic principles that underly enzyme promiscuity, and demonstrates that enantioselectivity can arise in a catalyst even when both, substrates and products, are entirely achiral.

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Mechanistic Insights Enable the Development of a Novel Covalent Inhibitor of TDP2

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Tyrosyl DNA phosphodiesterase-2 (TDP2) is a DNA damage repair enzyme that resolves cytotoxic topoisomerase II (TOP2) cleavage complexes. Genetic studies have demonstrated that TDP2 depletion synergizes with TOP2 poisons such as the widely used cancer therapeutic etoposide, suggesting that TDP2 inhibition could broaden the therapeutic window of these agents. To identify a small molecule inhibitor of TDP2, we conducted a high-throughput biochemical screening campaign and discovered a micromolar inhibitor with a unique vinyl-nitrouracil pharmacophore. Two orthogonal biochemical assays – a novel Echo-MS assay and a fluorescence kinetics assay – were developed and implemented to drive structure-activity relationship (SAR) studies and elucidate inhibition kinetics. Both assays independently characterized the HTS hit and its analogues as time-dependent TDP2 inhibitors. Subsequent intact mass spectrometry and x-ray crystallography studies identified a novel covalent inhibition mechanism in which the lead chemotype forms an adduct with residue C311 of TDP2. Comparisons of wild-type and C311S TDP2 constructs in the two biochemical assays demonstrated SAR progression from compounds selective for wild-type TDP2 to those active against both constructs, indicating successful optimization of binding interactions beyond covalent modification. Collectively, these findings characterized the mechanistic basis of TDP2 inhibition for a novel covalent chemotype and drove the development of the most potent TDP2 inhibitor reported to date.

Oxocarbenium-ion Character of Human DNPH1 Guides Transition State Analogs Design

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Human 2'-Deoxynucleoside 5'-Phosphate N-Hydrolase 1 (DNPH1) controls the level of hydroxymethyl deoxyuridine monophosphate (hmdUMP), and its action attenuates the level of incorporation of the modified nucleotide into DNA. DNPH1 catalyzes a relatively fast hydrolytic depyrimidination (k_{cat} = 0.33 s^{-1}) of 5-hydroxymethyl deoxyuridine, yielding hmUracil (hmU) and 2-deoxyribose-5-phosphtae (2dR5R). The reaction is catalyzed by a double-displacement mechanism with a covalent intermediate. Analysis of the transition state of the first chemical step using multiple kinetic isotope effect (KIE) measurements revealed a dissociative mechanism with a higher energetic barrier to form 2-deoxyribocation followed by nucleophilic attack by glutamate residue (E104). Consequently, a relatively faster nucleophilic attack of a water molecule on the glycosyl-enzyme covalent intermediate releases 2dR5P. An investigation through density functional theory to match the intrinsic KIEs suggests that the bond distance at the transition state between E104 and the anomeric carbon (rC-O) is 2.3. These results confirm a dissociative $D_N^{\ddagger}*A_N$ transition state with complete hmU loss and partially associative nucleophilic glutamate residue (E104). The transition state model predicts that deoxyribose adopts a 3'-exo puckering during nucleophilic attack. The information on reaction mechanisms helped us to design inhibitors with an inhibition constant lower than 50 nM.

Uncovering the Biosynthesis of Nocardichelin, A Novel NRPS-NIS Hybrid Siderophore

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In low iron conditions, many microbes rely on the creation, secretion, and reception of siderophores to scavenge iron from the environment which can be exploited for many biomedical applications such as trojan horse antibiotic delivery. Typically, siderophores are created by Non-Ribosomal Peptide Synthetases (NRPSs), hybrid NRPS/polyketide synthases, or NRPS independent siderophore (NIS) synthetases. Nocardia carnea breaks from the typical biosynthesis of siderophores by combining an NRPS module with an NIS synthetase to create an unusual siderophore called nocardichelin. Nocardichelin is made up of 3 main components salicyloxazolone (Sal-Ox), N-hydroxy-N-succinylcadaverine (HSC) and N-hydroxy-N-tetradec-2-enoylcadaverine (HTC) in which the latter moiety adds a fatty acyl tail, and the former 2 moieties chelate iron. Through our identification of the full nocardichelin biosynthetic gene cluster (BGC), we find several uncharacterized adaptations in enzymes such as: an acyltransferase (NcdE-ATx) appended to the C-terminus of the NRPS protein (NcdE), an NIS synthetase (NcdF) which may incorporate substrates beyond HSC, a stand-alone condensation domain which may incorporate small molecule acceptor amines rather than an acceptor carrier protein, a succinylase (NcdG) which may provide novel intermediates or recycle known intermediates and a separate path to building the fatty acyl tail that becomes attached through NcdE-ATx or a separate transferase (NcdM). We present the full nocardichelin BGC, our proposed pathway with partial pathway reconstitution, biochemical analyses, and 2 published structures to begin to uncover the natural product synthesis of nocardichelin which will also inform on homologous enzymes and pathways.

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Structure- and ML-guided discovery of improved galactitol dehydrogenases for D-tagatose biocatalysis

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D-Tagatose is a high-value, low-calorie functional sweetener that can be produced via an oxidoreductive biocatalytic pathway in which galactitol dehydrogenase (GDHs) catalyzes the oxidation of galactitol. Among the GDHs identified to date, smoS from *Rhizobium meliloti* has proven the most efficient for redox-based D-tagatose production[1]. To discover enzymes with higher catalytic efficiency, we applied a structure-guided discovery pipeline centered on smoS as the reference model[2], combining protein language model (PLM) embeddings, AlphaFold structural similarity filtering, and sequence similarity network (SSN) clustering. This stepwise screening approach reduced an initial set of 3,977 structurally consistent candidates to 20 sequences selected for experimental validation.

Genes encoding GDH candidates were heterologously expressed in *Escherichia coli*, purified, and evaluated for activity using substrates employed in carbon-utilization assays, followed by kinetic characterization with Galactitol. Four enzymes exhibited higher activity than smoS, including three from various Pseudomonas strains. In contrast, the GDH from Pseudomonas sp. 44 R 15 showed the lowest catalytic efficiency and a marked loss of activity below pH 9.0.

Overall, our results demonstrate that machine-learning-assisted structural mining combined with targeted biochemical characterization is an effective strategy for discovering superior GDHs for rare-sugar biocatalysis.

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Deciphering the Mechanism of the Dual Lysine-to-Tryptophan Crosslinking Radical SAM Enzyme WgkB

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Radical SAM enzyme-modified ribosomal peptides (RaS-RiPPs) are a major source of structural novelty and exquisite biological activity among natural products. Many of these molecules feature crosslinks between unactivated aliphatic and aromatic carbon centers installed via one-electron chemistry catalyzed by diverse radical SAM enzymes. One such natural product is tryglysin A, produced by *Streptococcus ferus* as a highly potent narrow-spectrum antibiotic against other Streptococci.[1] Tryglysin A features two C–C bonds between lysine and tryptophan residues forming a unique tetrahydro[5,6]benzindole tricyclic system. These two crosslinks, within the uniformly conserved WGK motif near the C-terminus of the precursor peptide WgkA, are installed by the SPASM domain-containing radical SAM enzyme WgkB in partnership with the *trans*-acting RiPP recognition element WgkC.[2] Over the course of the reaction, no singly crosslinked intermediates have been detected, suggesting a tightly controlled mechanism prime for detailed study. To gain insights into the mechanism of WgkBC, we performed mass spectrometry-based isotope tracing experiments, which revealed the site of H-atom abstraction by the 5′-deoxyadenosyl radical. Interestingly, these experiments also showed that the lysine-Hα undergoes a rearrangement and is retained on the lysine sidechain. We are currently working to determine the final destination of this H-atom through isotope tracing via ^{1}H and ^{2}H NMR. Additional experiments probing the stoichiometry of 5′-deoxyadenosine formation versus product formation, kinetic isotope studies, and freeze-quench EPR experiments to trap radical intermediates over the course of the reaction are in progress to refine our mechanistic understanding of the remarkable transformamtion catlayzed by WgkBC.

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Understanding Tamdy Nairovirus OTU Structural and Functional Implications for Predicting Future Outbreaks of Human Infecting Nairoviruses

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Tick-borne (-) ssRNA nairoviruses have shown to impact public health at a global scale. This is highlighted by the NIH prototype Crimean Congo hemorrhagic nairovirus that is endemic in wide swaths of Africa, Asia, and Europe. Beyond the often-fatal CCHFV, several other nairoviruses have been shown to cause varying degrees of febrile illness in humans. This includes newly emergent viruses within the Tamdy subgroup. Newly discovered members comprise Songling , Yezo , and Tacheng-Tick Viruses, isolated from patients with history of tick bites across China and Japan. Concerning for the U.S., the Pacific Coast tick nairovirus was identified within a tick species known to transmit human disease. Unique to other genera within the Bunyaviridae family, nairoviruses possess an extended L protein that encodes for a viral homologue from the ovarian tumor protease (OTU) superfamily. OTUs have also recently appeared in other human viruses closely related to Tamdy nairoviruses. such Beiji norwavirus. Functionally, OTUs can possess deubiquitinating and species-specific deISGylating activity key to a viral immune evasion strategy that is likely species specific[1]. Because of its involvement in pathogenesis of the virus, these viral OTUs are considered a virulence factor. Here, we investigated the deubiquitinase (DUB) and species specific deISGylation specificities of the virulence factor OTU protease domain originating from these new species. Using a myriad of ubiquitin substrates, the DUB activities of OTUs from respective OTUs were determined. Additionally, using mix of fluorescence and BLI assays, the preferences of these proteases for certain species interferon gene produce 15 was determined. X-ray crystallography revealed distinct structural divergence and molecular insight into the previously uncharacterized species‘ OTUs. These results provide a path forward in accurate prediction of DUB and desISGylase species- specific activity among current and yet to emerge nairoviruses.

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Elucidating the Function of Acyl-CoA Dehydrogenase 11 in the Metabolism of Gamma-Hydroxy Fatty Acids

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Fatty acids govern a host of cellular functions, including metabolic signaling, gene expression, and, crucially, energy production through beta oxidation. While mitochondrial beta oxidation is the major pathway for fatty acid catabolism, more unique fatty acid substrates are incompatible with this process, necessitating the involvement of highly specialized enzymes for their processing. Among the species incompatible with beta oxidation are gamma-hydroxy fatty acids (gamma HAs), whose oxidation at carbons 1 and 4 differentiates them from the 1,3 oxidized scaffold produced by beta oxidation. Recent work[1] has implicated two acyl-CoA dehydrogenases (ACADs) of unknown mechanism in the metabolism of gamma-hydroxy fatty acids, including peroxisomal enzyme ACAD11. This flavoenzyme differs from other members of the ACAD family in that it contains an N-terminal kinase domain, suggesting that gamma HAs require phosphorylation for their dehydrogenation pathway to proceed. Additionally, ACAD11 requires no terminal electron acceptors for enzyme turnover, suggesting a unique "rebound" functionality for FAD. Drawing from these insights, I seek to determine the mechanism of ACAD11 using high-resolution mass spectroscopy (HRMS)- based deuterium tracing and stopped-flow kinetic analysis of fluorinated gamma HA substrates. Characterization of ACAD11 and its role in the catabolism of gamma HAs provides an avenue for greater understanding of HAs, including their cellular roles and implications in disease.

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Transition State Analysis of the human arginine-specific ADP-ribosyltransferase 1

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The ADP-ribosylation of various protein residues is a versatile, reversible, post-translational modification involved in the dynamic regulation of numerous cellular processes.[1-3] Human ADP-ribosyltransferase 1 (*hs*ART1, EC: 2.4.2.31) is a membrane-associated, GPI-anchored, mono-ADP-ribosyltransferase selective for mono-ADP ribosylation (MARylation) of L-arginine residues.[4] Dysregulation of *hs*ART1 activity has been shown to permit immune cell evasion in non-small cell lung cancer (NSCLC) through elevated MARylation at R125 of purinergic receptor P2X7R in P2X7R-positive T-cells, resulting in NAD^+-induced cell death (NICD) of tumor-penetrating immune cells.[5,6] With *hs*ART1 emerging as a checkpoint target in select cancers, there is a need to develop tight-binding small-molecule inhibitors. The transition state for the MARylation of P2X7R peptide will be established from kinetic isotope effect (KIE) measurements of ^{3}H-, ^{14}C-, ^{18}O-, and ^{15}N-labeled NAD^+ substrates. Quantum mechanical calculations of the reaction coordinate, mapped with experimental KIEs, are used to identify a transition state (TS) geometry. *hs*ART1 may differ from the previously characterized ADP-ribosylating cholera toxin, where transition state analysis for exclusively promiscuous NAD^+ hydrolysis indicated a highly dissociative, asymmetric, concerted mechanism with minimal contributions from the incoming nucleophile and weak contributions from the leaving group nicotinamide (NAM). *hs*ART1 is formally an N-robosyltransferase, and this class of enzymes is known to be characterized by a pronounced oxacarbenium ion character of the ribose ring.[7-9] Analogues of NAD^+ with ribocationic mimics are candidates as transition state analogues. This is the first L-arginine-specific ADP-ribosylation TS to be characterized and will guide the rational design of tight-binding TS analogs. [10]

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The common homocystinuria-associated P1173L variant of human methionine synthase impairs reductive methylation

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Vitamin B_{12} (or cobalamin) is an essential nutrient that is obtained from the diet and is needed by only two enzymes in humans: cytoplasmic methionine synthase (MTR) and mitochondrial methylmalonyl CoA mutase (MMUT). Patient mutations in the MTR gene lead to homocystinuria, which is characterized by elevated homocysteine and associated with aggressive cardiovascular diseases. Our study reports the first biochemical characterization of full-length wild-type (WT) human MTR and the most prevalent homocystinuria-causing P1173L clinical variant, which has the potential to inform therapeutic approaches for treating the disease.

MTR is a 140 KDa multi-domain protein that converts 5-methyltetrahydrofolate (CH_3-H_4F) and homocysteine to H_4F and methionine in a B_{12} dependent reaction. MTR cycles between methylcobalamin (MeCbl) and cob(I)alamin states during catalysis and is oxidatively inactivated once every 2000 turnovers via formation of cob(II)alamin. Methionine synthase reductase (MTRR) reactivates MTR, using NADPH as an electron donor to reduce cob(II)alamin to cob(I)alamin that is followed by methylation to MeCbl with S-adenosylmethionine (AdoMet). Studies on human MTR have been hampered for the past two decades due to difficulties in isolating the recombinant full-length protein. We found that the B_{12} chaperone, MMADHC, forms a stable inter-protein complex with MTR and their co-expression enables isolation of highly pure full-length MTR in yields sufficient for biochemical studies. Full-length MTR has a specific activity of 2.5 $\pm$ 0.4 μmol min^{-1} mg^{-1} and K_M values of 3 $\pm$ 2 μM and 108 $\pm$ 18 μM for homocysteine and CH_3-H_4F, respectively. P1173L MTR has a higher K_M for AdoMet (8 $\pm$ 2 μM) compared to the wild-type enzyme (K_M = 0.19 $\pm$ 0.13 μM) and decreased specific activity (0.08 $\pm$ 0.007 μmol min^{-1} mg^{-1}) in the presence of MTRR/NADPH. The reductive methylation reaction in P1173L MTR is 30-fold slower (k_{obs} = 0.003 $\pm$ 0.002 s^{-1}) than in the wild-type enzyme (k_{obs} = 0.1 $\pm$ 0.01 s^{-1}). EPR and kinetic analyses revealed that complex formation with MTRR, which limits the reductive methylation reaction in wild-type MTR, is unaffected in P1173L MTR, pointing to a switch in the rate-limiting step. Pre-steady state kinetic studies revealed pleiotropic impacts of the P1173L mutation, with electron transfer from MTRR to cob(II)alamin being slowed down ~4000 fold. Finally, our study demonstrated that physiologically relevant small-molecule electron donors, like dihydrolipoic acid, might have therapeutic potential to circumvent the penalties associated with P1173L MTR.

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Mechanism of an NIS Synthetase: DesD from *S. coelicolor*

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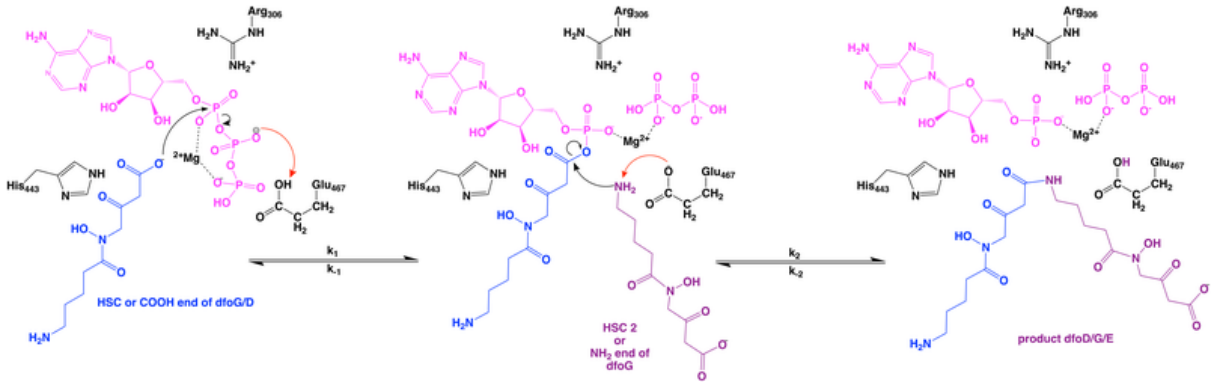
The superfamily of adenylating enzymes includes two subfamilies of siderophore synthetases. One of them, the NIS Synthetases, is an excellent target for structure-based antibiotic drug design due to its novel structure and function, and association with virulence. The NIS synthetases are understudied, but are excellent drug targets due to their novel structure and chemistry, critical role in iron acquisition, and increasing association with virulence in bacteria. NIS Synthetases are group V adenylating enzymes, a designation that offers an intriguing comparative mechanism to explore as we seek to determine the NIS Synthetase atomic level mechanism. However, several notable features exist in NIS synthetases that suggest there will be unique aspects to the mechanism that are worth exploring, such as the lack of large conformational changes, variations in catalytic residues observed, and a tricky binding site that permits iterative (in some cases) and plural positions for substrates.



Scheme 1: Proposed two-step mechanism for NIS synthetases. Step 1 involves catalytic residue Arg306 (black) coordinating the critical alpha phosphate of the ATP (magenta) and stabilizing the developing oxyanion transition state. Attack by the succinyl group of HSC (blue) on the alpha phosphate releases phosphate strain and byproduct pyrophosphate into a positively charged pocket. Catalytic residue Glu467 donates a proton to the pyrophosphate, and in step 2 coordinates the amine functional group of the second HSC molecule, abstracting a proton from the HSC amine during the bond-formation event. His443 has a significant role in a likely bidentate contact to the first HSC molecule succinyl group and adenylate intermediate – catalytically this may enhance the carbonyl electrophilicity enabling step 2.

The desferrioxamine siderophores (dfoD, G, E and B, shown above in scheme 1) are all made by *Streptomyces* bacteria through the desferrioxamine A, B, C and D pathway (DesABCD). DesD, a type C NIS synthetase, iteratively catalyzes the last three bonds made in this pathway to sequentially create all of the known siderophores in *S. coelicolor*, including the macrocyclized dfoE, a circular trimer of N-hydroxy, N-succinyl cadaverine (HSC). DesD has become a model for NIS Synthetases and several kinetic assays and crystal structure models have been published. We are exploring the roles of a proposed catalytic triad, pyrophosphate retention, and stoichiometry of metal ions for DesD and adjusted an adenylation-based 2-step and 2-proton dependend mechanism to reflect our findings. We will test this hypothesis using ITC-based pH profiles, thermodynamic binding studies and point variants of the enzyme and the work with be conducted exclusively with undergraduate researchers at our respective universities.

Hire ME: malic enzymes catalyze my transition to independence

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Malic enzyme (ME) catalyzes the oxidative decarboxylation of L-malate to pyruvate using $NAD(P)^+$ as co-substrate. MEs are widely distributed in nature, with humans possessing three distinct paralogues that are each implicated in largely unique subsets of cancer (1-5). The malic enzyme of *Mycobacterium tuberculosis* (MEZ) generates NADPH for the production of fatty acids. Knocking out MEZ in *M. tuberculosis* changes cell wall lipid composition such that tuberculosis can no longer infiltrate macrophages (6), making MEZ a drug target for the development of novel antibiotics. Despite the disease relevance of malic enzymes, the mechanisms of regulation and an physiological quaternary structure have not yet been well-described. Indeed, there is only sparse evidence for a kinetic mechanism, determined in the 1970s using the pigeon liver homologue (7). Moreover, human ME's, which purify as tetramers, have recently been shown to exhibit a monomer to dimer to equilibrium *in vivo* (8). Therefore, the goals of this work will be to provide a full pre-steady state analysis of the three human and one tubercular malic enzymes, especially in the context of differing allosteric regulation, and determine the physiologically relevant quaternary structure. By elucidating the key differences between ME homologues, specific features may be exploited therapeutically to treat distinct disease states.

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Exploring the Remarkable Non-Native Oxidative Capabilities of Marine Bacterial Flavoproteins

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Berberine bridge enzyme–like (BBE-like) flavoprotein oxidases play a central role in the biosynthesis of structurally diverse and pharmacologically important natural products, making them attractive candidates for biocatalytic applications, particularly for the generation of privileged heterocyclic scaffolds such as cannabinoids and tetrahydroisoquinoline alkaloids. However, the broader development of many BBE-like enzymes as useful biocatalysts has been limited by an incomplete understanding of their mechanisms and functional diversity.

The most extensively studied members of this family are the plant cannabinoid cyclases, which are proposed to catalyze Diels–Alder or 6π-electrocyclizations of terpene side chains via highly reactive ortho-quinone methide intermediates.[1] However, these enzymes and their closely related homologs exhibit low biocatalytic activity, restricted substrate scope, and poor expression in bacterial systems, thereby constraining their potential for broader biocatalytic applications. Other members of the BBE-like family, such as reticuline oxidase ecBBE, have more defined mechanisms, but the enzymes themselves have strict substrate requirements for generating the desired C-C bond forming oxidation.[2]

To overcome these limitations, we biochemically characterized two related bacterial BBE-like flavoproteins, Tcz9 and Clz9, from marine actinomycetes. These enzymes display a remarkably broad substrate scope and catalyze multiple classes of oxidative transformations observed across the BBE-like family. Guided by high-resolution crystal structures with bound substrates, we demonstrate that Tcz9 employs a previously uncharacterized oxidative strategy that enables efficient conversion of substrate analogs traditionally considered incompatible with canonical BBE-like enzymes.[3]

Using a combination of structural analysis, protein engineering, and mechanism-based chemical probes, we have begun to elucidate the molecular features that underlie the exceptional catalytic flexibility of Tcz9. Together, these findings reveal new mechanistic paradigms within the BBE-like enzyme family and expand the scope of oxidative transformations accessible through biocatalysis.

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Investigating the Role of IDH2 K112 in Catalysis

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Isocitrate dehydrogenase 2 (IDH2) is a mitochondrial enzyme that plays a central role in cellular metabolism by catalyzing the conversion of isocitrate to alpha-ketoglutarate. Interest in IDH enzymes, particularly IDH1 and IDH2, has grown because amplification and certain point mutations within these enzymes are known to contribute to tumor development. However, difficulties in producing and purifying human IDH2 have limited our knowledge of its catalytic mechanism. The conservation of key residues across these enzymes suggests that evolutionary pressure has maintained essential catalytic residues across species, even as IDH enzymes diverged in their cellular localization and physiological context. One such conserved residue is a lysine (K72 in IDH1, K100 in bacterial IDH, and K112 in IDH2) previously implicated in catalysis in bacterial IDH via its ability to support hydride transfer through its ionizable features. Specifically, since NADP+ and isocitrate appear to bind in a way that stable the ground state energy, it has been shown that the. The electronic features of the bacterial IDH K100 can help IDH2 K112 may help stabilize the transition state, effectively decreasing the energy barrier of hydride transfer (1). Here, I focus on IDH2 K112, as the role of this conserved residue is unknown in human IDH2. I hypothesize it can contribute to an inductive effect, playing a role in helping catalysis via a mechanism similar to that seen in bacterial IDH. I will test this by mutating this residue and determining the effect on the enzyme's function. I used site-directed mutagenesis to introduce four IDH2 variants at this position (K112M, K112Q, K112R, and K112N) to evaluate how substitutions with structurally similar amino acids—differing in charge, polarity, and reactivity—affect catalytic function. I then began expression and purification of the resulting proteins and performed steady-state kinetic analysis to measure the enzymatic activity of each mutant. After performing kinetic trials on each of the mutations, results show a dramatic decrease in activity, compared to that of WT IDH2, with K112R retaining slight activity. This suggests that the role of K112 depends not only on its positive charge but also on its structural features. By introducing additional mutations, such as histidine and tyrosine, I can further explore how this residue mediates interactions. Thus, I will dissect which factors are critical to the K112 residue and the overall performance of the IDH2 enzyme. Ultimately, these insights into IDH2 catalysis help us understand similarities and differences in IDH catalysis among and within species, and may help support the development of therapeutic inhibitors targeting this enzyme.

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Structure-Guided Engineering Of An Aromatic Ring–Hydroxylating Dioxygenase For Broad-Spectrum Phthalate Degradation

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Phthalates such as isophthalate, phthalate, and terephthalate are widespread environmental pollutants with significant health and ecological impacts. *Comamonas testosteroni* KF1 initiates isophthalate degradation through a specialized two-component enzyme system composed of isophthalate dioxygenase (IPDO) and its cognate reductase, isophthalate dioxygenase reductase (IPDR). Despite its environmental significance, the lack of structural insights into IPDO has hindered efforts to rationally redesign, optimize, and harness its chemistry. Here, we report the first crystal structures of substrate-free IPDO and its complex with isophthalate, revealing unique structural features that underpin its substrate specificity[1]. Unlike related oxygenases, phthalate dioxygenase ($\alpha_3\alpha_3$) and terephthalate dioxygenase ($\alpha_3\beta_3$), IPDO adopts a trimer (α_3) architecture, with distinct active site residues tailored to isophthalate binding. The comparative structural analysis identified steric and electrostatic constraints—particularly involving residue V178—that preclude the binding of ortho- or para-substituted substrates. Leveraging these structural insights, we engineered IPDO variants with broadened substrate specificity. Notably, the V178A and F249H substitutions enabled the enzyme to degrade three regioisomers of phthalate (phthalate, isophthalate, terephthalate) without diminishing its native activity against isophthalate. The catalytic turnover (k_{cat}) of V178A/F249H double mutant was found to be 4.8 ± 0.3, 4.9 ± 0.2 and 4.0 ± 0.2 s^{-1} for isophthalate, terephthalate and phthalate, respectively, demonstrating comparable catalytic efficiency for all three substrates. Overall, this work advances our understanding of the molecular mechanisms involved in isophthalate dihydroxylation and elucidates a rational engineering approach to expand the catalytic repertoire of IPDO for biotechnological and environmental applications.

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Structural basis for *in silico* inhibitor design targeting MqnE

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Broad-spectrum antibiotics remain the primary treatment for bacterial infections; however, they often disrupt the gut microbiome, causing side effects that can reduce patient adherence. To circumvent these issues, new, narrow-spectrum antibiotics must be developed that leverage pathogen-specific metabolic pathways. *Helicobacter pylori* (*H. pylori*), a causative agent of stomach ulcers and gastric cancer, relies on a key metabolic route ideal for narrow-spectrum therapeutic targeting. Menaquinone (MK), essential for bacterial respiration, can be synthesized through either the canonical pathway or the futalosine pathway, with *H. pylori* relying exclusively on the latter. Enzymes within this pathway have shown potential for development of narrow-spectrum antibiotics.[1-3] Central to this pathway is the enzyme aminofutalosine synthase (MqnE), which catalyzes the formation of aminofutaosine (AF) from didehydrochorismate (DHC) and *S*-adenosyl-$_L$-methionine (SAM). Here, we report the x-ray crystal structure of *Campylobacter jejuni* MqnE bound to its native substrates DHC and SAM, as well as structures in complex with DHC analogues. We also perform catalysis-in-crystals, which allows us to trap both an intermediate and product state using the inhibitor 2F-DHC. Leveraging these structural insights alongside *in silico* small-molecule screening, we identified non-native inhibitors that target MqnE in *H. pylori*. Collectively, these findings advance structure-guided development of narrow-spectrum antibiotics targeting the futalosine pathway and provide a foundation for selective therapeutic strategies against resistance *H. pylori* strains.

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High-Resolution Neutron Diffraction of Metalloproteins: Instrumentation and Methodological Advances at ORNL

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It is estimated that over one third of all proteins require metals for their biological roles, and nearly fifty percent of all proteins contain at least one metal ion.[1,2,3] Structural understanding of metalloproteins is therefore of great importance to understanding the catalytic mechanisms of a large portion of the known proteome of any organism. Techniques such as Cryo-EM and X-ray crystallography use ionizing radiation to obtain atomic structural data from enzymes, but measuring samples with these methods may alter the oxidation states of redox active metal sites. Hydrogen bonding and water networks that are essential for tuning reactivity are also generally not observable with these techniques. Neutron crystallography overcomes both limitations by allowing for detailed structural information of hydrogen atom positions without altering oxidations states or introducing radiation induced damage. The Neutron Scattering Division at ORNL is strategically investing in the development of advanced anaerobic sample preparation and handling capabilities, alongside the implementation of next-generation neutron diffraction methodologies. Notably, the incorporation of Dynamic Nuclear Polarization (DNP) is poised to circumvent traditional requirements for isotopic deuteration and large single-crystal growth, thereby enabling high-sensitivity structural studies of complex systems under non-destructive conditions.

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Mechanism of peptide macrocyclization in graspetide biosynthesis

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Graspetides are a subclass of ribosomally synthesized and post-translationally modified peptides (RiPPs), distinguished by unique side chain-to-side chain macrolactone or macrolactam bridges. In graspetide biosynthesis, ATP-grasp macrocyclases initiate macrocyclization by phosphorylating the carboxyl side chain of an Asp or Glu residue. This is followed by a nucleophilic attack from the hydroxyl or amine side chain of Thr, Ser, or Lys residue, forming ester or amide crosslinks. Although several crystal structures of ATP-grasp macrocyclases have been reported, the mechanism by which these enzymes mediate the macrocyclization and coordinate the two-step reaction remains largely unclear. Here, through structural and biochemical analyses, we present the molecular details of the macrocyclization reaction catalyzed by the ATP-grasp macrocyclase PsnB. Several co-crystal structures of PsnB bound to its substrates capture distinct stages of the macrocyclization reaction, during which the core peptide undergoes conformational changes. We have shown that highly conserved residues and sequence motifs are critical for substrate recognition, inducing core conformation shifts, and efficiently coordinating the two-step reaction pathway. Collectively, this study provides mechanistic insight into the macrocyclization reaction in graspetide biosynthesis.

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Discovery and Structural Basis of a Promiscuous Biotin Halogenase

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The Fe(II)/αKG-dependent halogenases use O_2 and free halide anions to generate a reactive chloro-Fe(IV)-oxo intermediate that homolytically cleaves specific substrate C(sp^3)-H bonds followed by controlled radical rebound to generate halogenated products.[1] These enzymes have attracted considerable interest for both elucidating their unique reaction mechanism and developing new biocatalysts for selective C–H functionalization. Yet, nearly two decades after the discovery of the first radical halogenase, SyrB2,[2,3] only a handful of new families of radical halogenases have been identified (e.g., BesD, Adev, WelO5, Dah)—most exhibiting narrow substrate scopes and/or dependence on carrier proteins within complex PKS/NRPS assemblies. The discovery of free-standing halogenases with broad substrate promiscuity therefore remains a longstanding challenge in the field.

In recent work,[4] we developed a mechanism-guided bioinformatic strategy to mine large sequence and structure databases for new radical halogenases, leading to the identification and characterization of BtnX, a radical halogenase that converts biotin (vitamin B_7) to 2*S*-chlorobiotin. In its native context, BtnX appears to participate in the algicidal activity of a marine bacterium that deprives algae of essential vitamins. Remarkably, BtnX exhibits substrate promiscuity that is unprecedented among radical halogenases, catalyzing halogenation of diverse molecules including free and substituted fatty acids, bile acids, fluorescent dyes, amino acids, and peptides. In addition to chlorination, BtnX can also transfer non-native anions (Br^-, N_3^-) to catalyze bromination and azidation reactions, enabling a wide range of downstream modifications and access to diverse C(sp^3) stereocenters. The 1.2 Å crystal structure of BtnX bound to Fe(II), chloride, αKG, and biotin reveals how its unique protein architecture enables both its regio/stereoselective halogenation reactivity and its substrate promiscuity. Overall, the discovery of BtnX expands the biocatalytic toolbox for C–H functionalization while its structural characterization offers new fundamental insights into the structure-function relationships and reaction mechanism of radical halogenases.

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Mechanistic studies of bacterial and human serine hydroxymethyltransferase using neutrons

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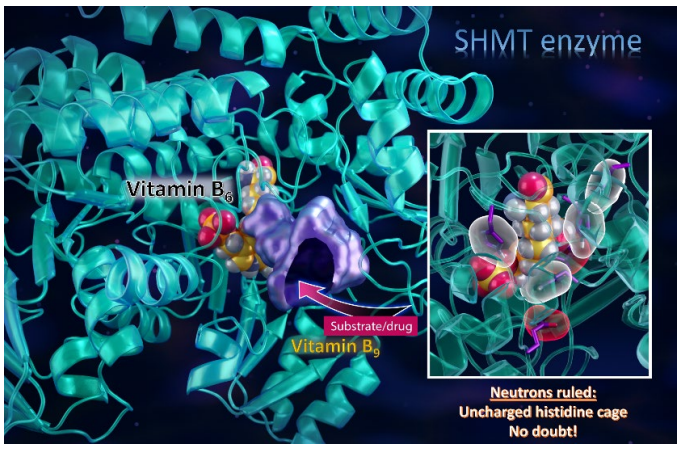
Serine hydroxymethyltransferase (SHMT) is a key enzyme in the one-carbon (1C) metabolic pathway, utilizing the vitamin B_6 derivative pyridoxal-5′-phosphate (PLP) and vitamin B_9 derivative tetrahydrofolate (THF) coenzymes to create building blocks for the downstream biosynthesis of DNA, RNA, and other essential biomolecules. Many types of cancer reprogram metabolic pathways for more efficient growth utilizing SHMT, which makes the enzyme an important drug target for the design and development of anticancer therapeutics. In pursuit of elucidating the catalytic mechanism of SHMT to aid in the design of SHMT-specific inhibitors, we are using room-temperature neutron crystallography to directly determine the protonation states in a model enzyme *Thermus thermophilus* SHMT (*Tth*SHMT). Crucially, the bacterial and human mitochondrial SHMT2 (hSHMT2) enzymes exhibit conserved active sites. We report five neutron structures of *Tth*SHMT, capturing the enzyme at various stages of its catalytic process [1,2]. We obtained the neutron structures of *Tth*SHMT in the *apo*-form that lacks the PLP coenzyme entirely, the holoenzyme with PLP in the internal aldimine form, a *pre*-Michaelis complex with L-Ser bound at the peripheral binding site, a complex with folinic acid, and a ternary complex containing PLP-Gly external aldimine and 5-methyl-THF. Nuclear density maps revealed the positions of hydrogen atoms and provided the ability to accurately assign the protonation states. Accurate mapping of the active site protonation states together with the structural information gained from the ternary complexes allow us to suggest an essential role of the gating loop conformational changes in the SHMT function and to propose Glu53 as the universal acid-base catalyst in THF-dependent catalysis. By direct observation of the locations of hydrogen atoms and tracking substrate positions, our study provides unique atomic-level understanding of the SHMT active site that will be employed to advance the design of anticancer drugs targeting hSHMT2.



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Revealing structure and activity in the large family of phosphoglycosyl transferases

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Antibiotic resistance of bacteria is a widespread threat to human health. Pathogenic bacteria such as *Campylobacter jejuni* and *Salmonella enterica* interact with their humans hosts through surface-displayed glycoconjugates, which are synthesized by membrane-associated enzymes.[1] Monotopic phosphoglycosyl transferases (monoPGTs) catalyze the first membrane-committed step in prokaryotic glycoconjugate biosynthesis, making them prime targets for inhibition. The most abundant family, the large monoPGTs (~15,600 family members), comprise a catalytic domain, a transmembrane helical bundle, and a putative regulatory domain.[2] The specificity of only a few enzymes in this family has been experimentally validated, and there is only a single structure available.[3] We report the kinetic characterization of the large monoPGTs *E. coli* WcaJ, *S. enterica* WbaP, and *T. thermophilus* WbaP purified in stable styrene maleic acid lipid nanoparticles (SMALPs), revealing their donor sugar preference. In addition, we developed a methodology to exchange SMALP-purified enzymes into detergent micelles to expand the repertoire of biophysical methodologies accessible. Additionally, using the SMALP purified sample, we apply cryo-EM for structure determination of WcaJ, a large monoPGT from the capsular polysaccharide pathway of *E. coli* colonic acid biosynthesis, further defining the dimerization interface in this family. The interface enables domain stabilization via a beta-hairpin swap in WcaJ. Ongoing studies are focused on reducing the flexibility of the catalytic domain for cryo-EM analysis to identify structural determinants of substrate specificity. Ultimately, knowledge of determinants of sugar donor specificity in the large monoPGT family will provide essential criteria for the development of therapeutics for bacterial-born diseases.

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Understanding the roles of mobile loops in the unique DXPS mechanism

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The mechanistically unique 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), a thiamin diphosphate (ThDP)-dependent enzyme, sits at a bacterial metabolic branch point. Additionally, DXPS facilitates pathogen adaptation and is absent from humans, making the enzyme an attractive antibacterial target.[1] DXPS catalyzes the formation of DXP from the decarboxylative condensation of pyruvate and subsequent carboligation of D-glyceraldehyde 3-phosphate (D-GAP).[2] Traditional DXPS inhibitors leverage the enzyme's unique ligand gated mechanism by targeting the active site to prevent product formation.[3] Previous work demonstrated the highly dynamic nature of DXPS while recent molecular simulations observed coordinated motion between two flexible loops outside of the active site.[4,5] However, little is understood how these mobile loops support the unique DXPS mechanism. Our work demonstrates that conserved aromatic residues within the 'spork' mobile loop (*Ec*287-314) are necessary to pin the closed conformation of DXPS for the effective placement of catalytic residue H299 within the active site for LThDP formation and persistence, thus connecting enzyme dynamics to mechanism.[6] Recent studies assumed a second mobile 'truncation' loop (*Ec*198-241) was mechanistically irrelevant due to a lack of sequence conservation; however, our work indicates this region supports negative cooperativity between active sites in the predicted half-of-sites reactivity of DXPS. This observation hints at potential regulatory roles of the truncation loop *in vivo*. These studies expand our understanding of the mechanistic roles of these flexible loops which is important to understand DXPS functions in bacteria and to guide novel inhibition strategies.

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Investigation of YqcI/YcgG Mechanism through DcsA

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Structure activity relationship studies have shown that installing a hydroxyl group on arginine at the N^e position significantly increases bioactivity, a motif found across multiple natural product scaffolds.[1,2,3,4] In the calcium-dependent lipopeptide antibiotic ambocidin, removal of this hydroxyl group leads to an 8 to 16-fold increase in the minimal inhibitory concentration.[1] Beyond ambocidin, nitrogen-hydroxylated arginine also appears as a key biosynthetic intermediate in pathways leading to valuable natural products such as chalkophomycin and D-cycloserine.[4,5] In the chalkophomycin pathway, the enzyme ChmN transforms arginine into dihydroxyarginine, enabling formation of the essential diazeniumdiolate motif.[4] By contrast, in the D-cycloserine pathway, DcsA converts arginine into ω-hydroxy-arginine, which is subsequently processed by a deiminase to release hydroxy-urea, a precursor to the final antibiotic scaffold.[5]

The biochemical value of site-specific nitrogen hydroxylation on the guanidinium group is clear, but achieving control over the regiospecificity of these enzymes requires structural insight. Currently, no structures exist for any YqcI/YcgG family proteins. Therefore, we propose to determine the crystal structures of DcsA and ChmN, two enzymes that act on the same substrate yet hydroxylate distinct nitrogen atoms. Structural comparison of their active sites will allow us to identify residues responsible for regiospecificity and guide targeted mutagenesis to tune or reprogram their activity.

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Kinetic Characterization of Cancer-Associated Mutants of Isocitrate dehydrogenase 2

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Isocitrate dehydrogenase 2 (IDH2) is a $NADP^+$-dependent enzyme that catalyzes the oxidative decarboxylation of isocitrate (ICT) to α-ketoglutarate (αKG). As a member of the isocitrate dehydrogenase (IDH) enzyme family, it plays a key role in cellular metabolic processes such as biosynthesis, epigenetic regulation, and redox homeostasis[1]. Mutations in IDH1 and IDH2 are drivers of many types of cancer, including 80% of low-grade gliomas and secondary glioblastomas. Mutations in IDH1/2 gain a neomorphic activity where it converts α-ketoglutarate (αKG) to D-2-hydroxyglutarate (D2HG) This can help drive tumorigenesis as high levels of D2HG competitively inhibits α-KG-dependent enzymes, leading to abnormal histone and DNA methylation patterns[3]. The most common cancer-driving mutations in IDH2 are R140Q, R172K, which are found in around 12% of patients with acute myeloid leukemia [4]. Therefore, these mutation points in IDH2 are therapeutic targets for treatment in cancer patients. Yet, mechanistic studies of mutations in IDH2 are limited compared to IDH1, most likely due to the challenging expression and purification of IDH2. Here, we show an optimized approach to express IDH2 in *Escherichia coli (E. coli*). We successfully expressed and purified high levels of IDH2, allowing us to characterize the kinetic rates of common IDH2 mutants found in cancer patients using Michaelis-Menten kinetics. We measured the catalytic efficiency of IDH2 wild-type and of mutant IDH2 (R140Q, R140L, R172K, R172S), finding differences In rates among the mutants and buffer preference. This approach will help our understanding of IDH2 enzymatic activity and its role in oncogenesis, providing valuable insights for the development of strategies targeting IDH2 mutations and the design of inhibitors.

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Characterization of FK506 binding protein (FKBP) FkpA by Peptidyl-prolyl Isomerase Enzyme Assay

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The ESKAPEE pathogens, including *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter species,* and *E. coli,* are a group of pathogenic bacteria of particular concern to global health because of their role in hospital-acquired infections and aptitude to evade antibiotic countermeasures.[1-4] We have ongoing studies on several targets from ESKAPEE pathogens, including peptidyl-prolyl-cis/trans-isomerase (PPIase) involved in protein biogenesis. Bacterial PPIases are associated with virulence and function as chaperones and foldases to process diverse protein-protein interactions during bacterial growth and infection.[5-6] To probe bacterial PPIases as a possible target for antibacterial development, we generated a computational homology model of a *K. pneumoniae* PPIase using the structure of a human PPIase bound to the drug FK506. FK506 is an FDA-approved drug that inhibits calcineurin and PPIase at two specific regions of its macrolide structure.[5-7] Based on the superposition of the two structures, we identified a compound, CT-AP1, that only bound to the PPIase but did not bind to calcineurin. We hypothesize that optimization of CT-AP1 by targeting bacterial PPIases using structure-based drug design strategies will yield more potent and novel antimicrobials against MDR gram-negative bacteria. We aim to discover and develop novel therapeutic interventions for the treatment of ESKAPEE infections using an iterative structure-based drug design approach. We coupled in silico drug screening and structural biology in parallel with the assessment of small molecules for antibacterial activity in biofilms, and inhibition profile by PPIase activity with a chymotrypsin-coupled assay.

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Substrate-dependent mechanisms of leukotriene A_4 hydrolase and immune modulation in a *Klebsiella pneumoniae* lung infection model

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The leukotriene A_4 hydrolase (LTA4H) is a unique zinc metalloenzyme with opposing bi-functional activities.[1,2] The epoxy hydrolase (EH) activity of LTA4H is associated with LTB4-mediated inflammation, while the aminopeptidase (AP) activity of the enzyme was shown to promote resolution of pulmonary inflammation by clearance of the chemotactic tripeptide PGP.[3,4] The complex bi-functional biology of LTA4H AP plays a significant role in neutrophil modulation after *Klebsiella pneumoniae* (KP) infection by showing that activated LTA4H AP results in increased survival after KP infection. We carried out enzyme kinetic assays using different amino acids containing p-nitroanilide as a reporter group. We demonstrated both a ligand and substrate-dependent mechanism of LTA4H AP activity. Three different substrates were tested to demonstrate the substrate-dependent mechanism of LTA4H AP activity in the presence of 4-methoxydiphenylmethane (4MDM) and a series of 4MDM analogs.[5,6] To better understand the kinetic mechanisms of AP activity, we determined the X-ray crystal structures of LTA4H bound to small molecule modulators. In conclusion, our study revealed that a peptidase therapeutic target can interact with its substrate and ligand in complex biochemical mechanisms, which raises important considerations for ligand design in order to explain some of the unpredictable outcomes observed in therapeutic discovery targeting LTA4H.

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Understanding the Next-level Diversity in an Emerging Superfamily of Bimetallic Oxygenases

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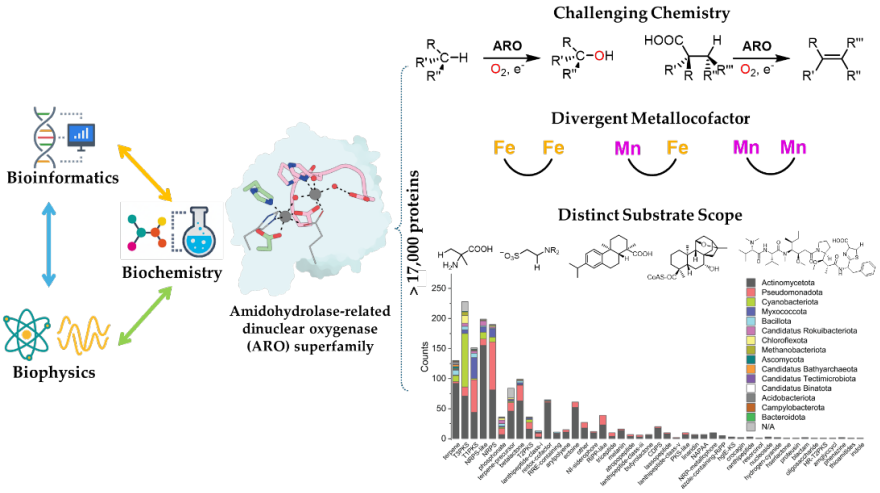
Almost half of all enzymes require metallocofactors for their functions.[1] Metalloenzyme superfamilies are typically defined by their protein scaffolds and active sites. Owing to the high tunability of protein structures, members of a single superfamily can catalyze diverse reactions with the same metallocofactor. The emerging amidohydrolase-related dinuclear oxygenases (ARO), display further versatility by utilizing divergent metallocofactors, and catalyzing diverse challenging reactions like oxidative C-H bond activations.[2–4] Here, we show bioinformatic analyses that expand the ARO superfamily to include over 17,000 unique proteins. Through the integration of structural, spectroscopic and electrochemical analysis of representative proteins and a bioinformatic methodology that identifies key secondary- and tertiary-sphere residues, we predicted the metal preference for ~90% of reported ARO sequences. These annotations were validated via the characterization of multiple new AROs, including those invoked in oxidative steps of bioactive natural products. Our study not only provides much needed tools for the investigation of metalloenzymes but also highlights the role of AROs in myriad biological processes, including possible association with over 1,600 biosynthetic gene clusters. They have great potential in protein engineering and biocatalysis.



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C-terminal Dimeric Interfaces Control Asynchronous Polyketide Elongation During Rifamycin Antibiotic Biosynthesis

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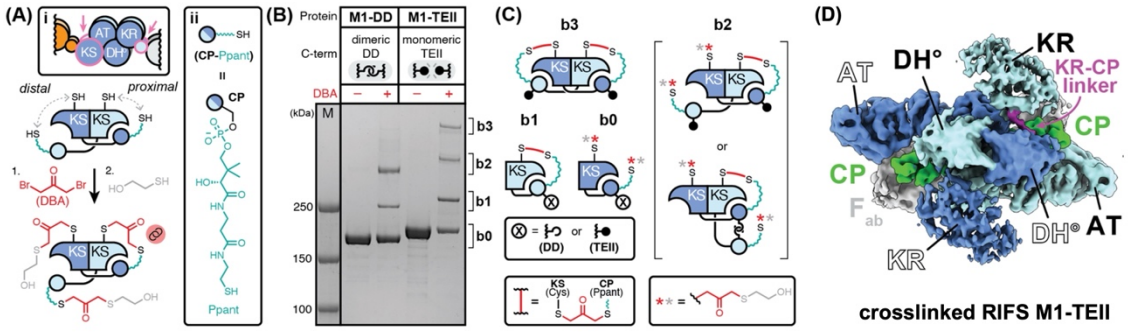
Abstract: Modular polyketide synthases (PKSs) are multifunctional enzymes to synthesize antibiotics as erythromycin and rifamycin. The rifamycin synthetase (RIFS) from *Amycolatopsis mediterranei* is a hybrid assembly line comprising an N-terminal nonribosomal peptide synthetase loading module and a decamodular PKS to generate a complex precursor to rifamycin B. While the biosynthetic functions are known for each domain of RIFS, structural and biochemical analyses of this system from purified components are relatively scarce. Here, we examine the biosynthetic mechanism of RIFS through complementary crosslinking, kinetic, and structural analyses of its first PKS module (M1). Thiol-selective crosslinking of M1 provided a plausible molecular basis for previously observed asymmetry with respect to ketosynthase (KS)–substrate carrier protein (CP) interactions during chain elongation[1,2]. Our data suggest that C-terminal dimeric interfaces—which are ubiquitous in bacterial PKSs—force adjacent CP domains to co-migrate between two equivalent KS active site chambers. Cryogenic electron microscopy analysis of M1 further supported this observation while uncovering the unique architecture of this PKS module. Kinetic analysis of M1 indicated that although removal of C-terminal dimeric interfaces supported 2-fold greater KS-CP interactions, it did not increase the partial product occupancy of the homodimeric protein. Our findings cast light on molecular details of natural antibiotic biosynthesis that will aid in the design of megasynth(et)ases with untold product structures and biological activities.



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Discovery of small molecule inhibitors to target dsRNA sensing pathway for autoimmune diseases

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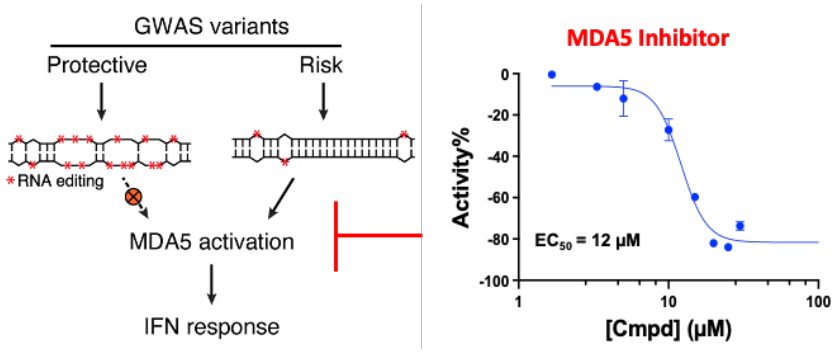
MDA5 is a type of innate immune receptor that senses pathogenic molecules through pattern recognition and triggers immune response to defend against pathogenic infection. However, aberrant sensing of "self" molecules can cause unwanted autoimmunity. Cellular "self" dsRNAs need to be edited by the ADAR1 enzyme to prevent dsRNA sensing by MDA5[1]. Human genetics data from GWAS (genome-wide association study) and RNA editing QTL (quantitative trait loci) analyses strongly link the ADAR1-dsRNA-MDA5 axis to autoimmune and inflammatory diseases, such as lupus, psoriasis, type 1 diabetes, and coronary artery disease[2]. In this work, we aim to discover small molecule inhibitors of MDA5. We have developed cell-based reporter assays and screened a small molecule library of ~200,000 compounds, from which we identified ~100 hits. Using *in vitro* biochemical validation assays such as MDA5 filament-formation, SPR (surface plasmon resonance), and specificity against MDA5 homolog RIG-I, we identified top 23 hits. We are optimizing selected top hits through structure-activity relationship iterations. This work will yield tool compounds to both study the MDA5 pathway and potentially treat MDA5-related autoimmune diseases.



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Cryo-EM Structures of the Full-Length USP9X in Complex With Activity-Based Diubiquitin Probes Reveal Mechanism of Ubiquitin Chain Recognition

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Ubiquitin-specific protease 9, X-linked (USP9X) is a key deubiquitinase involved in cell apoptosis, migration, and stem cell renewal, with its dysregulation linked to neurodegeneration and cancer. However, the structural basis for full-length (FL) USP9X function and its recognition of ubiquitin chains remain poorly understood. Here, we employed a semisynthetic approach[1] to generate active-site cysteine-targeting diubiquitin (diUb) probes with defined linkages. Using these probes, we determined the cryo-electron microscopy (cryo-EM) structures of the FL USP9X in complex with K48- and K33-linked diUb probes. Our structural and biochemical characterization revealed the ubiquitin-chain binding mode of FL USP9X. Notably, our cryo-EM structures unveiled a putative S1' ubiquitin-binding site near the unique β-hairpin insertion in the catalytic domain (CD), previously reported in our USP9X CD crystal structure[2]. Our new cryo-EM structures also identified new interactions between the S1 ubiquitin and the N-terminal region of FL USP9X, which likely explains its enhanced catalytic efficiency compared to the isolated CD. Together, these findings illustrate how FL USP9X differentially engages S1 and S1' moieties to achieve ubiquitin chain linkage-specific recognition. Moreover, our study highlights the power of semisynthetic, linkage-defined, activity-based ubiquitin probes in elucidating the structure and mechanism of deubiquitinases.

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Cryo-EM Structure of the E. coli Acetyl-CoA Carboxylase Complex, a Tube-like Filament and Reaction Chamber

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The *Escherichia coli* heteromeric acetyl-CoA carboxylase (ACC) has four subunits which are assumed to form an elusive catalytic complex and are involved in allosteric and transcriptional regulation. In essence, ACC is two enzymes, a subunit that houses the cofactor biotin, comprising a mini metabolic pathway. The *E. coli* ACC represents almost all ACCs from pathogenic bacteria making it a key antibiotic development target to fight growing antibiotic resistance. Furthermore, it is a model for cyanobacterial and plant plastid ACCs as biofuel engineering targets. Our cryo-EM structure reveals the catalytic *E. coli* ACC complex surprisingly forms tubes rather than dispersed particles. In addition, we discover key protein-protein interactions underpinning efficient catalysis and how transcriptional regulatory roles are masked during catalysis. The enzyme active sites are sequestered on the inside of the tubes along with the carboxy biotin carrier protein and all of the domains implicated in transcriptional regulation. Discovery and structural characterization of the active complex sets the stage to further uncover individual catalytic states using substrate analogs.[1]

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Structural characterization of human wild-type IDH2 and insights into acetylation-dependency on catalytic efficiency

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Wild-type (WT) isocitrate dehydrogenase 2 (IDH2) plays a key metabolic role in cancer, yet research has largely focused on mutant IDHs (mIDH) that generate the oncometabolite D-2-hydroxyglutarate (D2HG) [1]. Multiple studies have demonstrated that WT IDH2 drives reductive carboxylation and promotes tumor growth and treatment resistance [2;3]. In parallel, studies also show that WT IDH2 is regulated by acetylation, although the mechanistic consequences remain unclear [4]. Despite its significance, human WT IDH2 remains a relatively understudied enzyme. The absence of a human WT IDH2 structure and limited kinetic and regulatory insights can hinder rational drug design. To address this gap, I used spectroscopic tools and X-ray crystallography to investigate the kinetic and structural properties of WT IDH2 and its acetylation mimic. I conducted steady-state kinetics of the WT enzyme in both the forward and reverse reaction, and found that the efficiency of the conventional forward reaction is ~100-fold higher than that of the reverse reaction. To explore the structural basis for this kinetic disparity, I solved three crystal structures of human WT IDH2 in the closed conformation: two bound to forward-reaction substrates (ICT, $NADP^+$, Mn^{2+}/Mg^{2+}) and one bound to reverse-reaction substrates (αKG-NADPH, Ca^{2+}). This represents the first structures of human WT IDH2 to date. After the kinetic and structural characterization of WT IDH2, I extended my investigation to acetylation-mimic variant. The K413Q mutation in IDH2, previously examined in both human and mouse enzymes, showed a ~2-fold decrease in k_{cat} in the forward reaction [4;5]. Moreover, $NADP^+$ titration indicated that there is a ~9-fold increase in K_M, indicating that acetylation mimic mutant likely interferes with $NADP^+$ binding. To better understand the molecular interactions underlying this reduced activity, I solved the first crystal structure of the IDH2 K413Q acetylation mimic. Overall, this study helps elucidate the mechanistic insights and structural dynamics of both WT IDH2 and its acetylation-mimic form. These findings have the potential to provide a foundation that can ultimately aid in the development of WT IDH2-selective inhibitors and chemical probes.

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Electrostatic Control of Catalytic Bias in NAD(P)H Dehydrogenases: Structural and Kinetic Insights into Hydride Transfer

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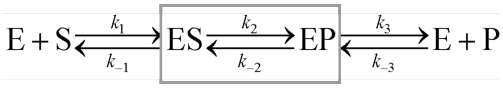
Catalytic bias refers to the propensity of a reversible catalyst (enzyme) to exert **disproportionate** rate accelerations in one direction over the other under non-equilibrium conditions.[1] Considering the reaction catalyzed by enzyme E in **Scheme 1**, at steady state conditions, **catalytic bias (ζ)** can be defined as the ratio of the respective rate constants and maximum velocities of the forward (f) and backward (b) reactions, $\boldsymbol{\zeta} = \frac{v^{(f)}}{v^{(b)}}$. Catalytic bias can also be manifested in the ratio of the transient microscopic rate constants for individual steps in the catalytic pathway *(k_1/k_{-1}, k_2/k_{-2}, and k_3/k_{-3})* Additionally, bias is likely to be more pronounced in the rate constant that is most rate-limiting. Limited studies have investigated catalytic bias, and its mechanistic determinants remain elusive, primarily due to challenges in developing precise kinetic methods and models for reversible enzyme-catalyzed reactions. Alcohol dehydrogenase (ADH) belongs to a large family of NADH-dependent dehydrogenases that catalyze the reversible oxidation of alcohols, involving a hydride transfer catalytic step.[2] Horse liver alcohol dehydrogenase is an 80kDa homodimer zinc-dependent NAD(P)H dehydrogenase, which catalyzes either alcohol oxidation or aldehyde/ketone reduction via an ordered Bi-Bi mechanism.[3]ADH is a well-studied enzyme, and disproportionate on-enzyme equilibrium rate constants have been observed in ADH variants, making it a suitable model to probe bias. We **hypothesize** that the relative stabilization or destabilization of the enzyme's equilibrium intermediates near the coenzyme or alcohol/aldehyde substrates' binding sites during catalysis influences catalytic bias. Our study aims to investigate catalytic bias in horse liver ADH using site-directed mutagenesis, steady-state, and transient kinetic methods. Understanding catalytic bias in NADH-dependent systems is significant, given their role in hydride transfer and central metabolic pathways.[3] This poster reports progress on a kinetic model and structural studies to analyze catalytic bias. We have observed a 10-fold bias toward aldehyde reduction in the native protein. However, the V292S ADH variant enhances bias toward aldehyde reduction by 54-fold compared to the native enzyme. Despite observed kinetic changes and an increased bias toward aldehyde reduction, the donor–acceptor distance for hydride transfer remained unchanged, suggesting no significant structural changes. Our findings also validate the suitability of the native ADH protein and variants for a comparative analysis to probe catalytic bias.



Reaction Scheme 1

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Inhibition of SARS-CoV-2 RNA Replication by Nucleoside Analogs

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SARS-CoV-2 continues to evolve, highlighting the need for nucleoside analogs that outperform current therapeutics. Remdesivir, an FDA-approved inhibitor, has limited use because of its toxicity. As coronavirus outbreaks persist and vaccination rates decline, developing safer, more effective inhibitors of the viral replication machinery is critical. We used pre-steady state kinetics to measure the rates of incorporation of FDA-approved or newly designed nucleoside analogs by the RNA-dependent RNA polymerase (RdRp). Because the coronavirus exonuclease complex can remove incorporated analogs, we also quantified excision rates. Through this approach, we identified 1'-cyano cytidine (CNC)[1] and 4'thiouridine (TU)[2] as strong candidates for future clinical development. Each nucleoside exhibits a distinct and previously uncharacterized mechanism for inhibiting SARS-CoV-2 RNA synthesis. Overall, our results show the best antivirals combine efficient incorporation with resistance to excision.

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Ultrahigh-Throughput Directed Evolution of Enzymes for Abiotic Reactions

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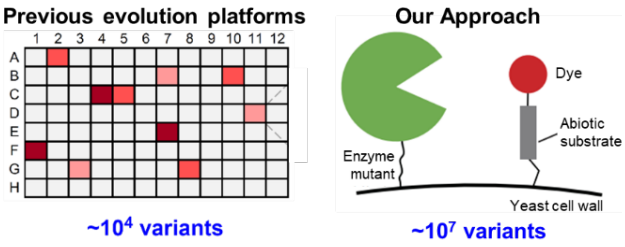
Abstract. Enzymes are promising catalysts for a variety of applications, but these applications often require enzymes to act on abiotic substrates that they did not evolve to recognize. We have developed chemogenetic platforms for ultrahigh-throughput evolution of enzymes to rapidly enhance their activity in abiotic contexts, and we have applied the resulting enzymes for waste plastics recycling, chemical biology, and synthetic methodology.[1–4] Our platforms increase the throughput of enzyme evolution toward abiotic substrates by 3 orders of magnitude, enabling the identification of pairs of beneficial mutations, including ones distal from the active site.



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Investigations in the Biosynthesis of Pantocin A

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Pantocin A is a ribosomally synthesized, post-translationally modified peptide (RiPP) natural product first isolated from *Pantoea agglomerans*.[1] The biosynthetic gene cluster that produces pantocin A contains genes coding for a precursor peptide (PaaP), two putative biosynthetic enzymes (PaaA and PaaB), and an efflux pump (PaaC).[2] Previous investigations relying heavily on mass spectroscopic data have suggested that PaaA catalyzes an ATP-dependent double dehydration/ decarboxylation of two adjacent glutamate residues in PaaP to generate the bicyclic core of pantocin A.[3] However, the report of PaaA activity lacked a full structural assignment of the PaaA product, a presumed intermediate in the formation of pantocin A. Using synthetically generated PaaP and heterologously produced PaaA, we have generated the trypsin-digested PaaA product at sufficient scale for multi-dimensional NMR analysis. Full structural assignment based on these data resolves the structural ambiguity in previous reports. Moreover, our findings lend support the revised mechanism proposed by Fleming et al.[4] and specifically to glutamate 16 as the site of initial activation by PaaA.

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S-adenosylmethionine's Dual Reactivity in B_{12}-Dependent Radical SAM Methylases

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Cobalamin-dependent Radical S-adenosylmethionine (SAM) Methylases (RSMs)[1,2] require both a [4Fe-4S] cluster and a cobalamin (Cbl) cofactor to methylate inert atoms such as sp^3- and sp^2-hybridized carbons and phosphinate phosphorus. During catalysis, these enzymes utilize two molecules of SAM in a ping-pong mechanism. One SAM molecule transfers a methyl group to cob(I)alamin via a polar S_N2 mechanism, producing methylcobalamin (MeCbl), which acts as an intermediate methyl donor. The second SAM molecule undergoes reductive cleavage at the Fe-S cluster to generate a 5′-deoxyadenosyl radical that abstracts a hydrogen from the substrate, except in the case of TsrM[3]. This process generates a substrate radical that attacks MeCbl, yielding a methylated product and a Co(II)alamin intermediate. How a single active site manages this dual SAM reactivity remains unknown, as the structural and mechanistic basis of MeCbl formation is not well understood. Using crystallography-informed molecular dynamics simulations and stopped-flow studies with specific SAM analogs, we identified a SAM-binding pocket required for MeCbl formation in the Cbl-dependent RSM Mmp10[4,5]. We also demonstrated that two tyrosines, Y23 and Y115, are part of a gating mechanism where the Cbl state functions as a molecular switch that regulates SAM's conformational change for radical and polar reactivity. This mechanism shows how protein structure influences SAM reactivity by modifying a few local interactions rather than by creating separate binding sites. This understanding simplifies the mechanistic view of Cbl-dependent RSMs, which may aid in exploiting them for the late-stage functionalization of medicinally relevant molecules.

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Biochemical deconstruction of the DNMT3A1-histone code interface

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Histone tail modifications are tightly correlated with transcriptional changes, often through altered nucleosomal DNA methylation. However, the underlying mechanisms remain poorly understood. The *de novo* DNA methyltransferase DNMT3A1 plays a central role by engaging histone H3 tails, the nucleosome acidic patch, ubiquitinated H2A, and linker DNA. We investigated these multivalent interactions using protein–protein binding assays, methylation activity and Km analyses, and Nanopore sequencing of engineered nucleosomes with tailored linkers. DNMT3A1 binding requires contributions from multiple contacts: linker DNA engagement, PWWP and ADD interactions with H3 tails, ubiquitin recognition domain (UDR) binding to H2A and DNA, and acidic patch binding. These interactions form an avidity-driven complex, complicating attribution of affinity to individual domains. Indeed, Km and binding assays revealed insensitivity to disruption of single contacts, while isolated domains (PWWP, ADD, UDR) showed pronounced responsiveness to histone modifications. A competition assay using Nanopore sequencing revealed that H3K4, H3K36, and acidic patch interactions strongly promote selective binding and linker DNA methylation, with the UDR–acidic patch dominating. In contrast, H3K27 modifications and EZH2/Prc2 showed no effect. Surprisingly, DNMT3A1 extended methylation up to 80 bp beyond the nucleosome core. These findings highlight avidity as a key principle governing DNMT3A1 nucleosome recognition and DNA methylation specificity.

Mechanisms of alternative polyamine biosynthesis in human gut microbes

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Polyamines, such as spermidine, are ubiquitous and essential substrate and regulatory molecules that modulate cell proliferation through a variety of mechanisms. Many microbes encode an alternative polyamine biosynthetic pathway that employs carboxyspermidine dehydrogenase and carboxyspermidine decarboxylase in the production of spermidine. This pathway is prevalent among human gut commensals and is an important contributor to the systemic spermidine pool in humans. To characterize these enzymes, we have chosen representative homologs from two major phyla, *Bacteroides fragilis* and *Clostridium leptum*. In both, the biosynthetic path was predicted to use putrescine as the initiating diamine in the production of spermidine. In this scenario, the dehydrogenase performs a reductive condensation of aspartate semi-aldehyde and putrescine[1]. The decarboxylase then removes the carboxylate group forming spermidine. However, recent evidence supports agmatine, not putrescine, as the preferred initiating substrate[2] (Ostlund and McFarlane unpublished). Therefore, a reordering of the biosynthetic pathway is necessary wherein the guanidino group is hydrolyzed after the formation of aminopropylagmatine by the dehydrogenase/ decarboxylase pair. Using NADPH oxidation assays in the steady-state and transient-state we have characterized substrate specificity and are examining the kinetic mechanism for the dehydrogenase homologs. A structural analysis of the dehydrogenases using X-ray crystallography and small angle X-ray scattering reveals a catalytic cycle with dynamic domain and loop movements. The activity of the decarboxylase has been investigated by mass spectrometry and transient-state stopped-flow spectrometry. X-ray crystal structures suggest key amino acid residues that distinguish substrates such as carboxyaminopropylagmatine and carboxyspermidine[3]. This work is advancing our mechanistic understanding of an alternative polyamine biosynthetic pathway that is widespread in nature and dominant in some microbial niches.

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IDH1 Heterodimer: Active Site Remodeling Revealed by X-ray Crystallography

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Human Isocitrate Dehydrogenase 1 (IDH1) is a cytosolic, $NADP^+$-dependent enzyme that catalyzes the reversible oxidative decarboxylation of isocitrate (ICT) to α-ketoglutarate (αKG), a reaction that also requires divalent metal cofactors. Oncogenic mutations in IDH1-most commonly affecting the active-site arginine at position 132-confer a neomorphic activity in which αKG is reduced to the oncometabolite D-2-hydroxyglutarate (D2HG), while the enzyme's conventional activity is lost.[1] Because dimerization is essential for catalysis, cells expressing mutant IDH1 can form three dimer species: WT/WT homodimers, WT/Mutant heterodimers, and Mutant/Mutant homodimers. The product generated by the wild-type enzyme serves as the substrate for the mutant's neomorphic reaction. Although substantial effort has gone into characterizing IDH1 mutations, most studies focus on mutant homodimers. However, due to mutant homodimers lacking conventional catalytic activity, heterodimers are believed to be the primary contributors to D2HG production in cancer cells.[2] Structural studies of WT and mutant homodimers have revealed distinct conformational rearrangements within active-site residues involved in catalysis.

Given that heterodimers contain one wild-type and one mutant subunit, they are expected to adopt unique structural features. Yet no structures of IDH1 heterodimers bound to their preferred substrates have been published. To address this gap, we used X-ray crystallography to investigate potential structural signatures specific to heterodimers. To prevent electron density averaging, several constructs of IDH1 were designed with the goal of biasing crystal packing to ensure each subunit could be resolved independently, as the only difference between the two subunits is a single point mutation within the active site.

Here, we report the first set of IDH1 WT/R132H heterodimer structures bound to substrates. In the initial structure, both monomers coordinate an α-ketoglutarate molecule within their respective active sites. Both subunits adopt a closed, catalytically primed conformation. The WT monomer displays the characteristic "seatbelt" arrangement over the NADPH cofactor, whereas the R132H monomer shows a slightly more open active site, with residue Y139 oriented inward toward the C3 carbon of α-ketoglutarate. With the novel rearrangement of residues in the active sites with substrates bound, our goal is to obtain heterodimer structures bound to drugs to further elucidate the structural changes that take place, alongside those that confer resistance to drugs.

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Irreversible Human Cathepsin L Inhibitors are Potent Anti SARS CoV-2 Agents

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Human Cathepsin L (hCatL) is a ubiquitously-expressed endosomal cysteine protease (CP) associated with inflammation and cancer. We recently demonstrated that hCatL is a critical factor for SARS-CoV-2 entry into mammalian host cells. In this study, we have develo0ped a series of apparently-irreversible tripeptide inactivators of hCatL which contain 3-pyridylalanine (3-Pyr-Ala), oxazol-5-yl alanine (5-Ox-Ala), and 2-pyridonyl alanine (2-Pyrd-Ala) and other amino acids at the P_1 position, adjacent to either an α,β-unsaturated ester or α,β-unsaturated sulfone, both of which are electrophilic warheads. The most potent inhibition vs. hCatL was achieved for inhibitors that contained 4-methoxyindole, benzyloxycarbonyl, or N-methylpiperizine groups at the P_3 position, Leu or Phe at the P_2 position, and 3-Pyr-Ala or 5-Ox-Ala groups at the P_1 position of the peptidomimetic scaffold (K_i^* = 0.4 - 35 nM). **VK-168** and **VK-261** comprised the most potent hCatL inhibitors with respective apparent tight-binding inhibition constants of K_i^* = 0.58 nM and K_i^* = 38 nM, and with anti-CoV-2 activities of EC_{50} = 120 nM (Vero-E6 cells), 78 nM (A549/ACE2) and EC_{50} = 310 nM (Vero-E6 cells), 120 nM (A549/ACE2) respectively. Other potent inhibitors **VK-166** (K_i^* = 2.6 nM), **VK-80** (K_i^* = 6.4 nM), and **VK-172** (K_i^* = 3.2 nM) blocked SARS-CoV-2 infection with $EC_{50} < 156$ nM in Vero-E6 cells and $EC_{50} \leq 230$ nM in A549/ACE2 cells. Preincubation of hCatL with inhibitors (0.5 - 12 hrs), followed by dilution with addition of saturating levels of substrate demonstrated that inactivation of CatL was irreversible.

Coming full circle: Kinetic modeling of the pericyclic reactions of isochorismate-pyruvate lyase and chorismate mutase

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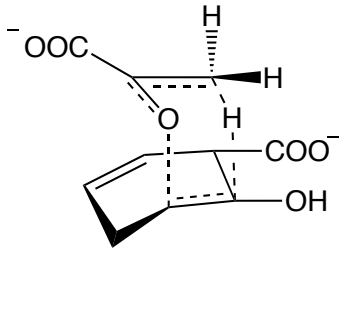
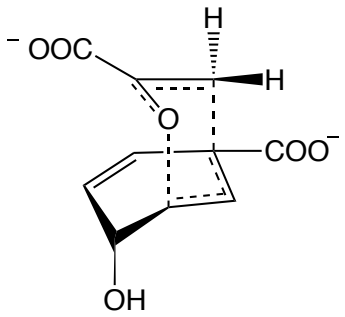
The isochorismate-pyruvate lyase from *Pseudomonas aeruginosa* (PchB) catalyzes a pericyclic reaction, a [1,5]-sigmatropic shift transfering a hydrogen from C2 to C9, producing salicylate and pyruvate from isochorismate for ultimate incorporation of the salicylate into the siderophore pyochelin[1]. PchB has structural homology to the *Escherchia coli* chorismate mutase (EcCM), which produces prephenate from chorismate for aromatic amino acid production using a distinct pericyclic reaction, a [3,3]-sigmatropic shift that transfers the pyruvate tail from the C3 ether linkage to a C1-C9 linkage[2]. These enzymes demonstrate adventitious activity, performing both pericyclic reactions, albeit at considerably lower catalytic efficiency for the non-physiological reaction in their respective biosynthetic pathways[3,4]. While the catalytic cycle has been studied by ^{2}H NMR[5], kinetic isotope effects[2], and computation modeling[6], a global model for catalysis has not been developed. Using a combination of substrate and product binding measurements with steady state and pre-steady state kinetics, the full isochorismate-pyruvate lyase and chorismate mutase kinetic mechanisms have been modeled for PchB and EcCM.



Isochorismate-pyruvate lyase transition state



Chorismate mutase transition state

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A Multifaceted Inhibitor Approach to Mapping the Catalytic Trajectory of an Iterative Siderophore Synthetase, DesD

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Under iron limiting conditions, bacteria synthesize low molecular weight ferric ion chelators, siderophores, to scavenge this crucial secondary metabolite. Siderophore biosynthesis is performed by assembly line like nonribosomal peptide synthetases (NRPS) which shuttle reactive intermediates between enzyme modules in an ordered manner via a phosphopantathienyl arm or NRPS independent siderophore (NIS) synthetases which act as standalone enzymes relying on diffusional transfer of intermediates. NIS synthetases have been historically understudied compared to their NRPS counterparts and as such much is still unknown about substrate binding/release, structural dynamics, and selectivity of product formation. A particularly interesting NIS synthetase is DesD produced by Streptomycess spp. DesD catalyzes the dimerization, trimerization, and macrocyclization of its universal monomer N^1-hydroxy-N^1-succinyl cadaverine (HSC) to form the siderophore desferrioxamine E (DFOE). A linear heterotrimer can also be biosynthesized by incorporation of N^1-hydroxy-N^1-acetyl cadaverine (HAC) on the C-terminus of an HSC-HSC homodimer to form the siderophore desferrioxamine B (DFOB). The iterative nature of this adenylation/condensation cycle poses several mechanistic questions when the vast differences in substrate sizes are taken into consideration. How does an enzyme active site accommodate substrates of such different sizes and what factors result in selective formation of DFOE and DFOB? Previous efforts utilizing an acyl sulfamoyl adenosine (AMS) inhibitor of HSC provided initial structural snapshots of DesD's adenylation site and heavy isotope feeding studies coupled with MS/MS have yielded crucial information about the order of monomer incorporation in heterotrimers.[1,2] This work expands upon these initial results through the synthesis and biochemical evaluation of additional AMS inhibitors, HSC-HSC-AMS and HSC-HSC-HSC-AMS, and a suite of transition state mimics designed to resemble the tetrahedral intermediate of the addition-elimination reaction. Both of these inhibitor types are tight binders of the enzyme active site with the AMS group resembling the AMP group of the natural reactive intermediate while the transition state mimics can serve as adenylation substrates. These rationally designed inhibitors have provided a comprehensive view of substrate binding in the adenylation reaction as well as a novel perspective on the catalytic trajectory of the incoming nucleophile in the condensation reaction.

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Norstatine scaffold formation by decarboxylative C-C bond formation catalyzed by a PLP dependent enzyme LhnE

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Norstatine (α-Hydroxy-β-amino acids) exhibit potent protease-inhibitory activity by mimicking the transition state of aspartate-protease–catalyzed peptide bond hydrolysis. Despite the presence of these motifs in multiple bioactive natural products, the enzymatic logic underlying their biosynthesis remains poorly understood. In this study, we elucidate the biosynthetic pathway of leuhistin, an α-alkylated norstatine with aminopeptidase M inhibitory activity produced by *Bacillus laterosporus* BMI156-14F1[1,2]. Genome-mining identified lhnD and lhnE as essential genes required for leuhistin assembly. Using purified recombinant proteins, we reconstituted the pathway in vitro and established that LhnD catalyzes the transamination of L-histidine to generate imidazole-5-yl pyruvate, whereas LhnE mediates a decarboxylative C–C bond-forming reaction between L-leucine and imidazole-5-yl pyruvate to yield leuhistin. Although α-oxoamine synthase (AOS) family enzymes are known to catalyze decarboxylative C–C bond formation via initial Cα-deprotonation followed by decarboxylation, isotope-labeling experiments using D_2O and deuterium-labelled L-leucine revealed that LhnE promotes C–C bond formation without proton exchange at the α-position. This identifies LhnE as an enzyme operating through a mechanistically distinct mode of decarboxylative C–C coupling. Furthermore, LhnE displayed broad substrate tolerance, enabling the synthesis of diverse natural and non-natural α-hydroxy-β-amino acids from amino acids and α-keto acids. Together, these findings uncover a previously unknown biocatalytic strategy for α-hydroxy-β-amino acid formation and establish LhnE as a promising biocatalyst for generating pharmacophores relevant to protease inhibitor development.

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Identification of Tau Deubiquitinase via Thioether-Mediated Ubiquitin Probes and Quantitative Proteomics

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Tau is a microtubule-associated protein essential for maintaining neuronal structure and function. Misfolded and aggregated Tau is implicated in the pathogenesis of various neurodegenerative diseases, particularly Alzheimer's disease (AD). Ubiquitinated Tau species have been identified in insoluble aggregates isolated from postmortem AD brain tissue, suggesting their potential contribution to disease progression. However, the mechanism underlying Tau deubiquitination remains poorly understood. Identifying deubiquitinases (DUBs) that regulate Tau deubiquitination provides valuable opportunities for developing new inhibitors and therapeutic strategies to control disease progression. Using a semisynthetic approach[1], we generated ubiquitinated Tau (Ub-Tau) that contains a Michael-acceptor warhead as an activity-based DUB probe to capture DUBs in the neuroblastoma SH-SY5Y cell line. We were able to capture several DUBs *i.e.* USP11, USP7 and UCHL1 that were previously reported to deubiquitinate Tau[2]. We also identified USP5 as a prominent candidate for regulating Tau ubiquitination and turnover. We further showed that USP5 stabilizes Tau in iHEK cells expressing a mutant P301L Tau that is prone to filament formation. We also investigated the ubiquitin–proteasome system and lysosomal autophagy pathway and their contribution to the Tau clearance in this cell model.

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Biochemical characterization of a flavodiiron protein from bird parasite *Histomonas meleagridis*: superoxide as a reaction intermediate

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Abstract. *Histomonas meleagridis* is a parasitic protozoan responsible for histomoniasis (blackhead disease) in many avian species, including domestic chickens and turkeys, and represents a major concern in poultry health.[1] Although traditionally considered an anaerobe, *H. meleagridis* can withstand transient exposure to oxygen (O_2), yet the mechanisms that enable it to tolerate fluctuating O_2 levels remain poorly understood. Genome analysis of *H. meleagridis* revealed the presence of two flavodiiron proteins (FDPs), classified as types A and F, which are known in other organisms to catalyze the reduction of O_2 to water.[2] The class F enzyme (*Hm*FDPF) displays the characteristic domain architecture of similar proteins, incorporating an N-terminal FDP core, a central rubredoxin-like domain, and a C-terminal NADH:rubredoxin oxidoreductase domain.[3] In our work, we provide a detailed biochemical investigation of *Hm*FDPF to elucidate how its multiple redox centers coordinate NADH-dependent O_2 reduction. Our results indicate that superoxide forms transiently during turnover (highlighting the challenges of capturing short-lived intermediates), yet only minimal hydrogen peroxide (H_2O_2) is released.[4] We also find that *Hm*FDPF lacks NADH:H_2O_2 oxidoreductase activity and is rapidly inactivated by H_2O_2. Overall, these findings enhance our understanding of how microaerophilic parasites regulate redox balance and limit O_2 in their surroundings, offering potential avenues for antiparasitic drug development by targeting FDP-mediated redox vulnerabilities and inducing controlled oxidative stress in *H. meleagridis* and related pathogens.

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A disulfide redox switch in glycoside hydrolases: a new regulatory mechanism in carbohydrate-active enzymes

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This study uncovers a previously unrecognized *redox-switch mechanism* that regulates the catalytic function of a glycoside hydrolase (GH2) through reversible disulfide bond formation. The enzyme, CapGH2b, originates from the uncultured bacterium '*Candidatus* Capybacter oxireducens', identified in Brazilian biodiversity metagenomes. Biochemical assays revealed that CapGH2b activity is reversibly controlled by its redox state: oxidation inactivates the enzyme, while reduction restores full activity. Structural analyses combining X-ray crystallography and high-resolution cryo-electron microscopy revealed that oxidation triggers an *order-to-disorder transition* in key active-site loops, whereas reduction restores their ordered configuration, positioning catalytic residues for classical Koshland-type retaining hydrolysis[1]. Mass spectrometry and mutagenesis confirmed the formation of an intramolecular disulfide bond between C427 (in the catalytic interface) and C517 (in the regulatory loop), which disrupts substrate recognition by sterically hindering binding. The C427A/C517A mutant, incapable of disulfide formation, exhibited constitutive activity and resistance to redox modulation, demonstrating the regulatory role of this cysteine[2]. Molecular dynamics simulations further revealed that disulfide bond formation displaces catalytic residues and increases active-site flexibility[3], an essential feature for the reversible inhibition mechanism. The C517A mutant reveals that the mechanism operates as a protective molecular feature, preventing irreversible oxidation of the catalytically essential cysteine C427[4] and maintaining enzymatic integrity under oxidative stress. Overall, CapGH2b exemplifies a new class of redox-responsive glycoside hydrolases, revealing an adaptive strategy by which microorganisms fine-tune carbohydrate metabolism in fluctuating oxidative environments[5], a finding with implications for both microbial physiology and biotechnological enzyme engineering. This discovery provides the first evidence of a *disulfide-mediated redox switch* in a carbohydrate-active enzyme, expanding the conceptual framework of redox-regulated catalysis beyond signaling or metabolic proteins[6].

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Unique Isomerase Mechanism Suggests Strategies for Inhibitor Design

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Methionine, an essential amino acid in bacteria and eukarya, is recycled in the methionine salvage pathway, which is essential to methionine auxotrophs and has been linked to cancer and other diseases. The conversion of 5-methylthioribose 1-phosphate (MTR1P) to 5-methylthioribulose 1-phosphate (MTRu1P) is a reaction included in this pathway and is catalyzed by the enzyme MTR1P isomerase (MtnA). ^{2}H and ^{13}C kinetic isotope effects (KIEs) are most consistent with an elimination–tautomerization sequence in this unprecedented reaction.[1] Interestingly, our results support a stepwise E1-like mechanism featuring ring opening to give a stabilized oxocarbenium ion intermediate, which is then deprotonated in the rate-limiting step to form a *cis*-enediol intermediate prior to tautomerization. QM/MM calculations successfully identified a transition state whose theoretical KIEs agree well with the experimental values. In addition, an inverse solvent KIE was measured for k_{cat}/K_M, providing evidence for Cys160 serving as the mechanistic base responsible for shuttling the proton between C-2 and C-1. Considering that there are no known inhibitors of MtnA, our findings provide insight into the development of small molecules that exploit its mechanism.

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Structural Snapshots of Catalysis in a Bifunctional Terpene Cyclase

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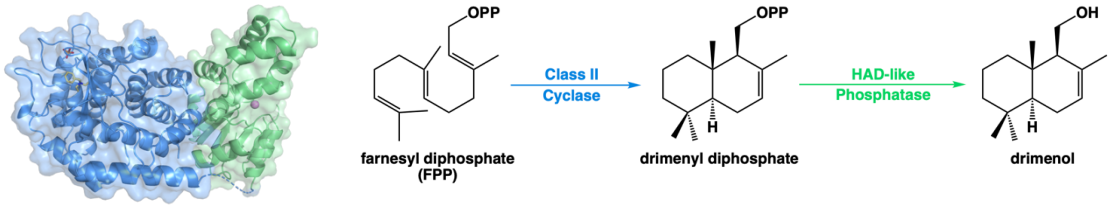
Terpenes represent the largest and most structurally diverse class of natural products, encompassing over 100,000 compounds that serve as antimicrobials, fragrances, biofuels, and more.[1] The biosynthesis of these compounds encompasses some of the most complex chemical reactions in nature, requiring the catalytic activity of terpene cyclases: enzymes which transform linear isoprenoid precursors into complex, often-polycyclic terpene structures, using their active sites as molecular scaffolds to direct the formation of a specific terpene.[2] Drimenol synthase from *Aquimarina spongiae* (AsDMS) is a highly unusual, bifunctional terpene synthase that integrates two distinct, sequential isoprenoid processing activities within a single polypeptide chain.[3] AsDMS catalyzes the cyclization of farnesyl diphosphate (FPP) to form drimenyl diphosphate, which then undergoes enzyme-catalyzed hydrolysis to yield drimenol, a sesquiterpene alcohol with antifungal and anticancer properties. Here, we report the X-ray crystal structures of AsDMS and its complex with a sesquiterpene thiol, which are the first of a terpene cyclase-phosphatase.[4] We also report structures of an inactivated AsDMS mutant, D33A-D323A AsDMS, including a complex with its native substrate, FPP. The AsDMS structure exhibits a didomain architecture consisting of a terpene cyclase β domain and a haloacid dehalogenase (HAD)-like phosphatase domain; surprisingly, AsDMS has two distinct active sites located on opposite sides of the protein. Mechanistic studies show that dephosphorylation of the drimenyl diphosphate intermediate proceeds through stepwise hydrolysis, and the hydroxyl oxygen of drimenol originates from the prenyl oxygen of FPP, rather than a water molecule from bulk solution. These experiments support a revised mechanism for this enzyme, one which updates the initial mechanism proposed by another group.[3] Surprisingly, AsDMS exhibits unprecedented substrate promiscuity, converting the substrate mimic farnesyl-*S*-thiolodiphosphate into cyclic and linear products. Ultimately, structural and mechanistic insights gained from AsDMS illustrate the functional diversity of terpene biosynthetic enzymes and provide a foundation for engineering "designer cyclase" assemblies capable of generating new terpenoid products.



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Glycyl Radical Enzyme and Its Activating Enzyme Fusion Enables Anaerobic Pyruvate Metabolism in Diatoms

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Glycyl radical enzymes (GREs) catalyze chemically challenging reactions by utilizing a protein-based radical that is post-translationally installed by a cognate GRE-activating enzyme (GRE-AE) encoded nearby in the genome.[1] GREs often play critical roles in anaerobic microbial metabolism. One of the most well-studied examples is pyruvate formate-lyase (PFL), which is responsible for the anaerobic conversion of pyruvate to acetyl-CoA and formate.[2] Due to the importance of this reaction in central metabolism, PFLs are frequently found in diverse facultative and obligate anaerobes but are uncommon in organisms that normally live in oxic environments,[3] as both activated PFL and its AE are oxygen sensitive. Using bioinformatic analysis of protein sequences in the GRE superfamily,[4] we identified a distinct phylogenetic clade that is found exclusively in diatoms and consists of fusions of GRE and GRE-AE domains. *In vitro* biochemical characterization of a fusion protein from the diatom *Fistulifera solaris*, including EPR spectroscopy, activity assays, and site-directed mutagenesis studies, indicates that it functions as a PFL via the canonical PFL mechanism. Analysis of an *F. solaris* transcriptome data set[5] reveals that the genes encoding the fusion proteins are more highly expressed than the genes encoding the pyruvate dehydrogenase complex and pyruvate:ferredoxin oxidoreductases, suggesting that the fusion proteins could be major contributors to pyruvate metabolism even under aerobic culture conditions. By comparing the activities of the native fusion protein and the unfused variants, we find that the fusion shows higher activity than the unfused variants in more physiologically relevant concentration regimes. We propose that this fused domain architecture could be an adaptation that enables diatoms to exploit the oxygen-sensitive radical chemistry under fluctuating oxygen levels.

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Insight into the Mechanism of Flavin Reduction in *E. coli* Dihydroorotate Dehydrogenase

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Dihydroorotate dehydrogenases (DHODs) oxidize dihydroorotate to orotate in *de novo* pyrimidine biosynthesis. The scission of the two CH bonds of dihydroorotate to form the enone of orotate is superficially simple, yet there are subtle facets of mechanism and dynamics that control this reaction and appear to differ among the three phylogenetic classes of DHODs. For instance, the deuterium kinetic isotope effects determined by stopped-flow methods suggest that Class 1A DHODs cleave both CH bonds in a concerted reaction but that the Class 2 enzyme from *E. coli* cleave these bonds in steps[1, 2]. The order of bond-cleavage has not been determined. Other experiments suggest that the dynamic behavior of the Class 1A and Class 2 enzymes are linked to reactivity in different ways. Much remains unknown.

Several approaches adding insight into the reaction of the *E. coli* DHOD are presented here. Analogs of dihydroorotate, especially *carba-* *deaza-* analogs, suggest that the first step of dihydroorotate oxidation is hydride transfer to form an iminium intermediate rather than prereduction deprotonation for an enolate intermediate. While stopped-flow studies have demonstrated that orotate release from the reduced enzyme is excruciatingly slow[3] (and likewise for dihydroorotate), the stickiness of dihydroorotate to the oxidized enzyme was not known. We think it now is. A novel stopped-flow isotope- or analog-competition approach suggests that dihydroorotate binding to the oxidized enzyme is rapid equilibrium – quite slippery compared to reduced enzyme. These effects implicate an active site that is sensitive to the redox state of the flavin. Furthermore, locking-down orotate after flavin reduction requires that the oxidizing substrate ubiquinone react at a site different from the pyrimidine binding site. The ubiquinone binding site is almost certainly the hydrophobic tunnel that would allow ubiquinone to approach the 8-methyl edge of the flavin with only a tyrosine intervening. It isn't known whether ubiquinone-binding to this site alters the behavior of the pyrimidine site towards dihydroorotate or orotate. We don't think it does, based on initial studies using enzyme loaded with the ubiquinone analog 2-heptyl-4-(1H)-quinolone N-oxide.

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Fluorogenic Kinetics to Study CYP27A1 Drug Inhibition

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The human mitochondrial cytochrome P450 27A1 (CYP27A1) is responsible for the metabolism of cholesterol and vitamin D_3.[1] The enzyme's activity and its products have been linked to different hormonal cancers and neurodegenerative diseases.[2] This has generated great interest in studying the P450 to understand the protein's function within the context of human health. Kinetic assays with the enzyme's physiological substrates are intensive and timely; thus, even confirming active P450 after purification is tedious, let alone the screening of possible inhibitors or activators of the protein.

Here, we present a modified high-throughput method[3] that utilizes fluorogenic substrates, where, upon catalysis, non-fluorescent substrates are converted into fluorescent products. Enzyme activity was tested with several different substrates with different fluorophore backbones: fluorescein, cyanocoumarin, trifluoromethyl coumarin, and resorufin. The fluorogenic substrate, dimethyl fluorescein, DBF, was the best test substrate, being only ten-fold less catalytically efficient compared to vitamin D_3. This method enables more efficient testing of enzyme activity and inhibitor screening. We tested several commercially available drugs in the presence of DBF and the physiological substrate vitamin D_3. We found that IC_{50} values of tested drugs in the presence of DBF or vitamin D_3 were in the micromolar range and competitively inhibited CYP27A1 at similar drug concentrations. These results contrast with the inhibition of CYP27A1 with cholesterol as a substrate. For example, IC_{50} values for the drug, felodipine, were determined to be 14 ± 1 µM and 11 ± 1 µM in the presence of vitamin D_3 and DBF respectively. In contrast, when cholesterol is used as the reported substrate, the IC_{50} value was reported to be 0.94 µM.[2] The breast cancer drug, anastrozole, did not inhibit CYP27A1 in the presence of DBF or vitamin D_3. However, with cholesterol, CYP27A1 is inhibited by anastrozole with a reported IC_{50} = 2.4 µM.[4] This result suggests that CYP27A1 inhibition may be substrate dependent. In total, our results indicate the utility of DBF for screening of inhibitors and the need for further investigation of active site flexibility to aid in inhibitor design.

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Unlocking the Exit: Unconventional Product Release Mechanisms in Nonribosomal Peptide Synthetase (NRPS) Pathways

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A major class of natural products comprise peptide-based metabolites that are biosynthesized by multidomain and multi-modular proteins termed as Non-Ribosomal Peptide Synthetases (NRPSs). These NRPS proteins are primarily composed of adenylation, thiolation, and condensation domains, each performing a specific function in an assembly-line manner to generate peptide products. While the canonical enzymatic mechanisms of these NRPS domains are extensively studied, the chain release domains responsible for off-loading the peptide product remain poorly characterized. Our research aims to investigate the unconventional structural and enzymatic mechanisms underlying the chain release domains.

Herein, we present three investigations that elucidate the structural mechanisms of chain release domains in NRPS proteins. An unusual TE domain involved in biosynthesis of nocardicin exhibits the dual function: catalyzing a non-canonical epimerization reaction in addition to the canonical hydrolysis. The crystal structure of the isolated TE domain (NocTE) in complex with a substrate-mimic inhibitor was determined. Complemented with the functional assays, the structural analyses enabled us to propose a detailed enzymatic mechanism for this enigmatic epimerization process.[1] Another example is the TE domain in the biosynthesis of sulfazecin, a β-lactam antibiotic. The TE domain in this pathway facilitates an unprecedented β-lactam formation from a diaminopropionic acid residue (DAP). Structural elucidation of the TE domain (SulTE) uncovered unique features of its active site. Leveraging these insights, structure-guided genome mining was performed to identify three additional biosynthetic pathways with the potential to produce novel β-lactam natural products.[2] Furthermore, we determined the crystal structures of the adenylation domain activating DAP residue in sulfazecin biosynthesis. Substrate and inhibitor bound structures revealed the insights of substrate binding pocket and guided the mutagenesis efforts for engineering the adenylation domain to incorporate DAP analogs.[3]

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Conformational Disruption in Asparagine Synthetase Deficiency Linked Variant R48Q

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Asparagine synthetase deficiency (ASNSD) is a rare, severe neurometabolic disorder caused by mutations in asparagine synthetase (ASNS), a multidomain enzyme essential for asparagine biosynthesis.[1-3] Although numerous pathogenic ASNS variants have been identified in patients with profound neurodevelopmental impairments, the molecular mechanisms by which these mutations disrupt enzyme function remain enigmatic.[2-3] Here we show how a single missense mutation in ASNS can drive pathogenic loss of function by focusing on a clinically observed variant, R48Q. Steady-state kinetic analyses reveal that R48Q exhibits markedly impaired utilization of the physiologically relevant substrate glutamine, indicating a substantial catalytic defect. Importantly, these effects arise despite the overall structural integrity of the enzyme. To elucidate the structural basis for this impaired function, we utilized cryo-electron microscopy (Cryo-EM) to determine the structure of the R48Q variant, revealing alternative conformations within a highly conserved N-terminal flexible loop shared across Class II glutamine amidotransferases. More importantly, we note that this arginine residue is strictly conserved within this family of enzymes, likely playing a functional role in stabilizing the loop and its dynamics. Complementary 3D variability analysis and molecular dynamics simulations further show that R48Q perturbs loop mobility in a manner consistent with its catalytic deficiency. Together, these results demonstrate that R48Q exerts multiscale effects on ASNS function, structure, dynamics, establishing a mechanistic link between missense mutations in ASNS and the molecular etiology of ASNSD. This work provides a generalizable framework for uncovering how pathogenic variants perturb the balance between enzyme structure, function, and dynamics.

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Uncovering AKT Allostery to Drive Isoform-Selective Inhibitor Design

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Aberrant activation of AKT1 drives pathological angiogenesis in hereditary hemorrhagic telangiectasia (HHT) and vascular malformations (VMs), as well as tumor progression in oncology. Pan-AKT inhibitors lack isoform selectivity, leading to metabolic side effects such as hyperglycemia from AKT2 inhibition. This underscores the important need for AKT1-selective inhibitors to treat vascular anomalies and cancer while minimizing systemic toxicity.

To address this challenge, we leveraged Atavistik Bio's proprietary Atavistik Metabolite-Protein Screening (AMPS™) platform to uncover cryptic allosteric pocket features unique to AKT1. Structure-based drug design guided by these insights enabled development of reversible, allosteric AKT1-selective inhibitors. In vitro pharmacology characterization was central to this effort, integrating biochemical assays for potency and selectivity, cellular models to confirm isoform-specific signaling inhibition, and biophysical techniques such as SPR and crystallography to elucidate binding mechanisms. Importantly, SPR was employed to evaluate kinetic versus equilibrium selectivity, providing insights into how binding dynamics contribute to isoform specific inhibition. These assays also revealed the critical role of protein forms and dynamic conformational states in assessing allostery. Although evaluating the effects of inhibitors on cryptic pockets could be dependent on in vitro protein conformations, combining iterative assays in the design-make-test-analysis (DMTA cycle) with mechanistic studies helped enabled progression of lead compounds with sub-nanomolar AKT1 potency and >80-fold selectivity over AKT2.

This work demonstrates that decoding AKT allostery through comprehensive in vitro pharmacology and structural insights can drive the development of isoform-selective inhibitors to bring forward treatment options for diseases driven by dysregulated AKT signaling.

Radical SAM Maturase–Catalyzed Leaderless C-Terminal Modification Generates GLP-1-Mimetic Macrocycles

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Ribosomally synthesized and post-translationally modified peptides (RiPPs) constitute a rapidly expanding class of genetically encoded natural products distinguished by wide structural and functional diversity. Their biosynthesis typically occurs within dedicated gene clusters that encode both the precursor peptide and the maturase enzymes responsible for installing post-translational modifications that yield the final bioactive compound. Most RiPP precursors feature an N-terminal leader segment, which is believed to mediate recognition and binding by the corresponding maturase. Consistent with this model, structural analyses of RiPP enzymes frequently reveal N-terminal RiPP recognition elements (RREs) thought to engage these leader sequences. This work highlights the unusually broad substrate tolerance of PapB, a radical *S*-adenosyl-L-methionine (rSAM) RiPP maturase known for forming thio- and selenoether crosslinks across diverse peptide scaffolds. We show that PapB's promiscuity extends not only to sequence variability, but also to its ability to modify substrates that completely lack canonical leader regions. These results underscore the versatility of PapB, and related rSAM maturases, as powerful biocatalysts for generating macrocyclic peptides across a wide range of structural frameworks.

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Ornithine racemase uses a catalytic cysteine

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Salmonella enterica serover typhimurium expresses an ornithine racemase (OrnR), located in a small operon containing amino acid transporter homologues and D-ornithine decarboxylase[1]. This OrnR is part of a clade that has high identity (>75%) with other sequences in enterobacteria, but is distant (~40% identity) from another clade of OrnR found in anaerobic firmicutes and actinobacteria. OrnR is a member of the pyridoxal-5'-phosphate (PLP)-dependent alanine racemase (AlaR) superfamily (Fold III), but sequence alignments show that it lacks the catalytic Tyr acid/base of the other racemases; instead, it has a conserved Cys residue. OrnR shows hyperbolic kinetics in the L→D direction, but exhibits sigmoid kinetics in the D→L direction, with h=1.8. The structure of OrnR predicted by AlphaFold3 was complexed with PLP-DL-ornithine in silico, minimized, and subjected to molecular dynamics simulation. The resulting structure shows that SG of Cys-164 is in the active site, about 4 Å from the Cα of the external aldimine of L-ornithine (Figure 1), while the NZ of Lys-36 is located about 4 Å below Cα on the D-face. The C164A mutant enzyme has no measurable racemization activity, and does not exchange the α-H of L- or D-ornithine in D_2O, consistent with the function of Cys-164 as an acid/base catalyst for the racemization reaction. These data suggest a concerted acid/base catalysis mechanism for the racemization. These results show that OrnR is unique among racemases in the AlaR superfamily, in that it uses a catalytic Lys/Cys acid/base pair, rather than the canonical Lys/Tyr acid/base pair.

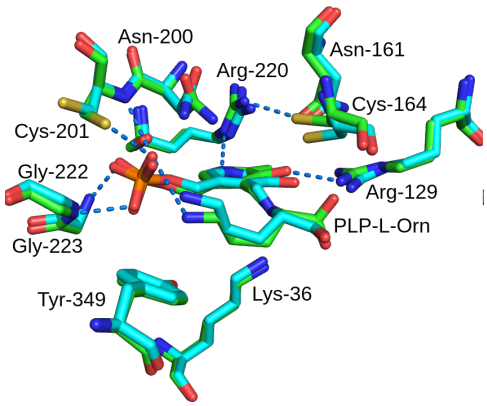

Figure 1: Active site of OrnR

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Biochemical analysis of a Prx5-group peroxiredoxin-glutaredoxin hybrid protein expressed by *Haemophilus parainfluenzae* species within the oral microbiome

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The Prx5 subgroup of peroxiredoxins (Prxs) is the most diverse among the six subgroups in terms of their mechanisms of reductive recycling and location of the resolving cysteine, if any. A subset of these, found in *Haemophilus influenzae* and other human pathogens (including *Yersinia pestis*, *Neisseria meningitidis* and *Vibrio cholerae*), are fused at their C-terminus with a glutaredoxin (Grx) domain homologous to *E. coli* Grx3 (designated PdgX). Together in the fusion protein, these domains catalyze the glutathione-dependent reduction of peroxides. *H. influenzae* and other species of *Haemophilus*, including *Haemophilus parainfluenzae*, form biofilms *in vivo* that contain PdgX. Bioinformatics of oral microbiome species support a protective role for *H. parainfluenzae* in humans to combat oxidative stress induced by chemoradiation therapy, and ensuing neutrophil infiltration and NET formation, that leads to oral mucositis, a painful and harmful side effect of head and neck cancer treatments. To analyze the biochemical properties of *Hp*PdgX, we have expressed and purified the intact PdgX protein and isolated Prx and Grx domains from *E. coli*. This has enabled us to assess the peroxide specificity of the multi-domain enzyme and biochemical properties of the individual domains. Glutathione-dependent peroxidase activity was also reconstituted using the isolated peroxiredoxin and glutaredoxin domains, and a model for the activities of the individual domains as well as the intact protein has been developed. Further work on variants of these proteins will be informed by the analysis of specific PdgX sequences from *H. parainfluenzae* isolates and their correlation with severity of oral mucositis after treatment to evaluate protective activity of variants, and their contribution to biofilm formation in the oral cavity.

Mechanistic Understanding of the Ureido Bond Forming Modular NPRS Enzymes Using Cryo-EM Analysis

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The non-ribosomal biological pathway to generate peptide natural products involves large, multi-modular, multi-domain enzymes that function as an assembly line. These enzymes, non-ribosomal peptide synthetases (NRPSs), use proteinogenic and non-proteinogenic amino acids to build small molecule peptides with therapeutic applications, including antibiotics and immune suppressants. These multi-modular/multi-domain enzymes are typically composed of repeating adenylation (A), thiolation (T), and condensation (C) domains, each with specific chemical functions. The A domain activates the amino acid substrate, which is then transferred to the T domain via a 4'-PP arm for delivery to the C domain. At the C domain, two amino acids are condensed, either through the typical head-to-tail amide linkage or through a less common head-to-head ureido linkage. To form a ureido, an extra carbonyl (incorporated from bicarbonate or carbon dioxide) links the amino groups of the substrates; however, the molecular mechanisms of this ureido-bond formation are not well understood. In this investigation, we aim to elucidate the molecular mechanism. Our study focuses on two ureido-bond-forming NRPSs with distinct domain architectures, ApnA and SylC, to identify shared and divergent features of their ureido bond formation mechanisms.

ApnA is a dimodular NRPS with an A_1–T_1–C–A_2–T_2–E domain architecture, where tyrosine and lysine condense in the C domain to form a ureido linkage. In contrast, the monomodular SylC (C–A–T) catalyzes the coupling of two valine residues to generate a ureido bond. Previous studies by the Walsh group proposed a mechanism for SylC involving Leuch's anhydride formation based on labeling experiments. For ApnA, our biochemical data suggest an alternative pathway proceeding through a lysine carbamate intermediate. Using cryo-EM, we have obtained a preliminary low-resolution (4.9 Å) map of the T_1–C–A_2–T_2 domains of ApnA, revealing domain organization and interdomain interactions. We have also obtained a high-resolution (2.9 Å) cryo-EM structure of the SylC dimer that revealed a unique dimer interface that has not been observed for any structurally characterized NRPS to date. We are currently creating point mutations based on this structure to probe the effects of dimerization on ureido formation and determine the active site residues necessary to form a ureido bond. Our biochemical and structural studies ultimately seek to elucidate the molecular mechanisms of ureido bond formation, the role of domain architecture within these mechanisms, and the residues that are responsible for determining the amide versus ureido-bond formation at the C domain.

Binding of Substrate to 2,4'-Dihydroxyacetophenone Dioxygenase is Largely Driven by Interactions Distal to the Catalytic Iron

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2,4'-dihydroxyacetophenone dioxygenase (DAD) catalyzes the oxidative cleavage of 2,4'-dihydroxyacetophenone (DHA) to form 4-hydroxybenzoic acid (4HB) and formic acid. Computational docking simulations suggest an important hydrogen bonding interaction between the 4'-OH group of DHA and the sidechain of aspartate-64 of DAD. We have investigated the significance of this interaction in the formation of the DAD-DHA complex through the generation of active-site mutants and substrate analogs. Specifically, we have prepared the D64L mutant of DAD and determined the values for k_{cat} and k_{cat}/K_M with DHA. In comparison to the wild-type reaction, the mutation resulted in an 85-fold decrease in the value of k_{cat} and a 9000-fold decrease in the value of k_{cat}/K_M. Similarly, when the substrate analog, 2-hydroxy-4'-methoxyacetophenone, was tested as a substrate for the wild-type enzyme, the reaction showed a 1600-fold drop in k_{cat}/K_M coupled with a more modest 15-fold decrease in k_{cat}. However, when the analog was tested against the D64L mutant, we observed partial recovery of the k_{cat}/K_M value showing a 30-fold increase over the DHA-D64L reaction (although still 300-fold lower than the wild-type reaction). Similar results were observed for the substrate analog, 2-hydroxy-4'-methylacetophenone. These results support direct interaction between the 4'-moiety of the substrate and the sidechain of residue 64. Specifically, polar/hydrogen bond interactions (4'-OH/aspartate-64) or nonpolar-nonpolar interactions (4'-methoxy/leucine-64) at these positions give magnitudes larger values for k_{cat}/K_M compared to mixed polar-nonpolar interactions (4'-OH/leucine-64 or 4'-methoxy/aspartate-64). These results support the direct interaction of aspartate-64 with the 4'-OH of the substrate as the primary driver for formation of the DAD-DHA complex.

Unwinding, Ratcheting, and Triplet Trimming: The Mechanism of an M9 Collagenase

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Collagen's tightly wound triple helix presents a formidable barrier to proteolysis. The pathogen *Hathewaya histolytica*secretes collagenase H (ColH), which processively unwinds collagen and trims it into tripeptides. We present atomic structures of ColH in two key states, a dynamic closed conformation bound to an intact triple helix and a rigid ratcheted conformation bound to unlaid strands, determined using cryo-EM and X-ray crystallography. These structures reveal how twisting between two domains generates shear stress and axial shortening, creating a three-residue bight in one strand while maintaining contact with the other two. Following hydrolysis, extension of this bight enables triplet-by-triplet advancement, with the intact strands serving as tracks. ColH thus converts collagen's geometric constraints into catalytic advantage. Importantly, bacterial collagenases employ a mechanism distinct from mammalian enzymes, illustrating divergent evolutionary strategies for collagen degradation. Together with mutagenesis data, these high-resolution structures provide a framework for understanding ColH's exceptional processivity and for developing selective inhibitors that target bacterial but not mammalian collagenases.

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Following Enzymes Through Complete Catalytic Cycles Using Next-Generation XFEL Capabilities

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Understanding how biology works—how enzymes reorganize, how proteins switch states, and how molecular interactions drive health and disease—is advanced by direct observation of structural dynamics as they unfold. The Linac Coherent Light Source (LCLS), the hard X-ray Free Electron Laser at SLAC National Accelerator Laboratory, has made this possible through serial femtosecond crystallography and ultrafast spectroscopy. These methods capture atomic-resolution snapshots of biomolecules on the femtosecond timescales of chemistry, assembling them into molecular movies that reveal catalytic mechanisms, conformational transitions, and short-lived intermediates with unprecedented clarity. This poster provides an overview of recent accelerator and instrument upgrades at LCLS and highlights the transformative potential of the upcoming LCLS-II High Energy (LCLS-II-HE) project. With its enhanced flux, extended energy range, and high repetition rate, LCLS-II-HE will enable access to states and length scales previously out of reach. These capabilities will allow researchers to follow enzymatic reactions through full catalytic cycles and generate molecular movies that directly connect structure, dynamics, and biological function.

Computational investigation of alliinase enantioselectivity toward (+)-alliin

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Alliinase (EC 4.4.1.4) catalyzes the conversion of alliin into allicin and preferentially accepts (+)-alliin over its (−)-enantiomer[1]. Crystal structures of garlic alliinase have been solved for several ground and late-intermediate states, including apo, internal aldimine, and aminoacrylate–PLP complexes. However, earlier transient states along the reaction pathway, such as the external aldimine with the natural substrate (+)-alliin, have not been directly observed because of their extremely short lifetimes. It is therefore crucial to complement these static structures with computational models of such "invisible" intermediates to understand how alliinase achieves its enantioselectivity and to explore possibility that its active site accommodates non-native substrates.

Here, we aim to establish an integrated computational framework combining Boltz_ext[2] (An AlphaFold3 [3] clone) with improved ligand stereochemistry, molecular dynamics (MD) simulations, and quantum chemical calculations to investigate how alliinase recognizes (+)-alliin and discriminates against its enantiomer. AlphaFold3-based modeling was used to generate enzyme–substrate complex models for (+)- and (−)-alliin, as well as sulfone and thiol analogues, providing initial structural hypotheses for how PLP and the chiral active site organize each substrate for quinonoid formation and subsequent β-elimination. MD simulations and QM(/MM) calculations are being set up to examine near-attack conformations, hydrogen-bond networks, and reaction energy profiles for each substrate.

In this poster, we will present current structural models and working hypotheses on how sulfur oxidation state and substrate chirality modulate active-site preorganization and reaction energies, and outline how our approach may guide future enzyme engineering toward accepting non-native sulfur chemotypes.

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Frozen in time: Uncovering the mystery of a 70-years-old drug leads to novel therapeutic strategy for glioblastoma

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Hydralazine (HYZ) is one of the oldest vasodilators with hydrazine (–$NHNH_2$) group. Developed ~70 years ago as an antimalarial, it unexpectedly lowered blood pressure in early trials and was serendipitously repurposed as an antihypertensive. Even today HYZ has secured its place on the World Health Organization's list of essential medicines for the clinical treatment of hypertensive crisis and (pre)eclampsia. Despite extensive study, HYZ's direct target(s) and mechanism of action were unclear. We pioneered reverse-polarity activity-based protein profiling (RP-ABPP) using hydrazine probes to tag electrophilic cofactors (e.g., metals, NAD, FAD) and post-translational functionalities proteome-wide, enabling discovery of active enzyme targets and quantification of inhibitor engagement[1].

Here we identify 2-aminoethanethiol dioxygenase (ADO)—an iron- and O_2-dependent enzyme and a key mediator of targeted protein degradation—as a selective HYZ target in cells and tissues[2]. Crystallography and RP-ABPP show that HYZ chelates ADO's metallocofactor in a bidentate fashion and can alkylate one of its ligands. The resultant inactivation stabilizes regulators of G-protein signaling (RGS4/5) that ADO normally marks for proteolysis, explaining the drug's vasodilatory activity and comporting with observations of diminished RGS levels in both clinical preeclampsia and a mouse model thereof. Its inhibition of ADO suggested use of HYZ against glioblastoma (GBM); indeed, a single dose robustly senesces cultured GBM cells. By establishing ADO as a nexus for GBM and preeclampsia and connecting it to HYZ, the results create opportunities for directed tailoring of the old drug for new therapies. In this presentation, I will overview the mechanism of action from biochemical viewpoints.

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Biosynthesis of RiPP Natural Products by Heme Oxygenase-like Domain Containing Oxidase

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Heme oxygenase–like domain-containing oxidases (HDOs) constitute a rapidly expanding enzyme family known to perform diverse chemistries using metal cofactors. The natural products generated through HDO-catalyzed transformations exhibit a wide range of biological, medicinal, and industrial relevant activities. To date, all characterized HDOs participate in small-molecule biosynthesis; however, recent genome-mining efforts have revealed that many HDOs co-localize with predicted ribosomally synthesized and post-translationally modified peptide (RiPP) gene clusters. This genomic association suggests an unexplored reservoir of chemical diversity and expands the potential for discovering novel enzymatic reactivities. Here, we elucidate the biosynthetic pathway of a RiPP natural product, with particular emphasis on the chemistry mediated by HDO enzymes. Moreover, we identify distinct differences in substrate recognition and processing compared with previously characterized HDO family members, highlighting new mechanistic diversity within this emerging enzyme class.

Autoinhibited Homodimer Architecture Enables Selective Acyl-ACP Recognition in FAA C Domains

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Non-ribosomal peptide synthetases (NRPSs) synthesize diverse bioactive natural products, yet the structural and mechanistic principles governing substrate recognition by condensation (C) domains remain poorly understood. A recently discovered NRPS-like pathway in the human gut microbiome produces fatty acid amides (FAAs), small molecules that modulate host G protein–coupled receptor (GPCR) signaling[1]. This pathway, encoded by *Coprococcus eutactus*, exhibits a minimalistic architecture in which each enzymatic domain functions as an independent protein and the system lacks an acceptor peptidyl carrier protein (PCP), raising fundamental questions about how its condensation domain (OaaC) engages its fatty-acyl donor acyl carrier protein (OaaACP). Using solution scattering and X-ray crystallography, we found that OaaC forms an atypical homodimer that occludes the canonical ACP-binding surface observed in multidomain NRPSs. To investigate how this homodimer interacts with its partner OaaACP, we performed mass photometry using apo-OaaACP (unmodified), holo-OaaACP (phosphopantetheinylated), and acyl-loaded OaaACP. Only the acyl-loaded state induced substantial OaaC dimer dissociation and the appearance of monomeric OaaC, revealing a **substrate-gated activation mechanism** in which the condensation domain becomes catalytically competent only when a loaded acyl-OaaACP is present. Complementary liquid chromatography–mass spectrometry (LC–MS) assays demonstrated that OaaC can utilize multiple physiologically relevant fatty acids, including lauric acid and palmitoleic acid, to generate FAAs with the preferred acceptor substrate 5-aminovaleric acid[1]. To contextualize this non-canonical system evolutionarily, we conducted a maximum-likelihood phylogenetic analysis of NRPS C domains and found that FAA-associated C domains form a distinct monophyletic clade adjacent to, but separate from, canonical starter C domains[2,3]. Multiple sequence alignment revealed conservation of a variant catalytic motif—**DHxxxDS**, rather than the canonical **HHxxxDG**—characterized by substitution of the fourth-position glycine with serine or alanine. This motif shift correlates with the use of small-molecule amine acceptors rather than PCP-tethered peptide substrates. Together, these findings establish that FAA condensation domains employ a substrate-dependent dimer-to-monomer switch and possess distinct evolutionary and catalytic features compared to canonical NRPS systems. These insights advance our understanding of how gut bacteria synthesize GPCR-active FAAs and provide a mechanistic foundation for engineering tailored FAA products with potential therapeutic applications.

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The First Transient State Analysis of Bilirubin Reductase from *Mediterraneibacter gnavus*

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Bilirubin Reductase (BilR) is a gut microbial enzyme that is reported to catalyze the reduction of unconjugated bilirubin to urobilinogen, an eight-electron reduction and a key metabolic step in heme catabolism. In the heme degradation pathway, heme is initially converted by heme oxygenase to biliverdin, which in turn is reduced by biliverdin reductase (BVR) to bilirubin. Bilirubin, after being secreted into the bile as its diglucuronide conjugate form it is deconjugated in the gut, becomes the substrate for BilR, and ultimately is reduced to generate urobilinogen for excretion[1]. The discovery of BilR is considered a target for the treatment of jaundice, inflammatory bowel disease, and kernicterus as bilirubin levels are significantly reduced in neonates and adults with these diseases[1, 2].There is no published crystal structure of BilR reported, however, AlphaFold-predicted models provide valuable insight. BilR shows homology to bacterial 2-4 dienoyl-CoA Reductase, a flavoenzyme containing an FMN, FAD and Fe_4S_4 cluster[3].The AlphaFold structure of BilR supports a similar arrangement in that the electron transfer chain from NADH to FAD, through the Fe_4S_4 cluster, to the FMN and finally to substrate. We have chosen to study BilR from *Mediterraneibacter gnavus*. Initial studies involved flavin cofactor determination, confirmed by UV-Vis spec and HPLC analysis. BilR contains a 1:1 ratio of FMN to FAD. The stereochemistry of the reductive half reaction was determined using NMR under anaerobic conditions. Solvent deuterium exchange during equilibrium hydride transfer reactions for NADH depleted signals for specific protons revealing Pro-S stereochemistry of the reaction for that substrate. Transient state kinetics under anaerobic conditions with NADH as the reductant shows that BilR can become 4-electron reduced and forms an E_{red}•NAD^+ complex that exhibits charge transfer absorption. Transient state single turnover analysis of BilR shows four phases. The first two phases show concomitant flavin reduction and accumulation of a charge transfer complex. The second two phases involve the oxidation of the flavin and reduction of bilirubin.

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Structure-guided engineering of a CoA synthetase for substrate scope expansion and selectivity

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Acyl- and aryl-CoA synthetases activate fatty acids and aromatic acids through conversion of their carboxylate moiety to a high-energy CoA-thioester. These enzymes operate through a two-step mechanism, in which ATP is used to adenylate the substrate, followed by a prominent C-terminal domain rotation to allow for the CoA-thioesterification. [1] Recent studies have demonstrated the biocatalytic utility of CoA synthetases in amide-bond formation and polyketide derivatization.[2–4] However, the substrate scope of these enzymes is typically limited to large and/or nonpolar substrates due to their native functions in fatty acid metabolism and aromatic degradation.

We have identified a thermophilic CoA Synthetase with high promiscuity toward a range of small, polar carboxylates including hydroxyacids with various substituents, such as ethers and thioethers, thiol groups, halogens and azides. We have demonstrated the utility of the resulting CoA-thioesters in downstream reactions including in biopolymers and enzymatic carbon-carbon bond formation.

To uncover the structural and mechanistic basis for the substrate promiscuity of this enzyme, we have employed a combination of X-ray crystallography, cryo-EM, and computational methods. Crystal structures and preliminary cryo-EM experiments with different bound hydroxyacids reveal a flexible binding mode for the carboxylate substrate, likely contributing to the permissive nature of the enzyme. These insights guided our ongoing rational and semi-rational engineering efforts to expand and control the substrate scope of the enzyme. Site-saturation mutagenesis of selected residues has allowed us to optimize activity and selectivity toward bulkier substrates, α-hydroxyacids, and β-amino acids, and has also revealed a possible role of a dynamic loop domain in controlling the substrate scope of the enzyme.

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Structural and mechanistic studies on bacterial sesterterpene synthases

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Terpenoids, compromising well over 100 000 compounds, make up the single largest group of natural products.[1] Many of which exhibit bioactivity and are of considerable interest as fragrances, flavorings and pharmaceuticals.[2, 3] Central to this chemical diversity are terpene synthases, which utilize achiral oligoprenyldiphosphates to generate complex oftentimes polycyclic compounds with multiple stereogenic centers.[4] A small subgroup of this family are sesterterpene synthases utilizing geranylfarnesyldiphosphate (C_{25}) as their primary substrate.[5] To gain deeper insights into substrate-binding modes and enzymatic mechanism, we investigated several bacterial sesterterpene cyclases. By crystallizing them with unreactive substrate analogues, the enzymes were trapped in a pre-catalytic state with ligands positioned in a productive conformation in line with the proposed cyclisation mechanism. These structures served as foundation for targeted mutagenesis of the active-site to pinpoint catalytically relevant residues and widen the product spectrum. This lead to mutants with significantly altered activity compared to the wildtype as well as the generation of new sesterterpene products. Among these were products with inverted stereochemistry, sesterterpene alcohols, and deprotonation products of intermediates formed throughout the catalysis, providing additional support for the previously proposed mechanism.[6,7]

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Glycan biosynthesis in *Helicobacter pullorum*

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Bacterial protein N-glycosylation in ε-proteobacteria is central to host interaction[1], yet biosynthetic pathways outside the canonical *Campylobacter* system are poorly defined. Here, the membrane-proximal N-linked glycan pathway from the emergent zoonotic pathogen *Helicobacter pullorum*[2] was reconstituted from purified enzymes using chemoenzymatically prepared undecaprenyl-linked substrates, which enabled unambiguous assignment of enzyme order and substrate specificity. *H. pullorum* PglC is unusually permissive and can transfer either phospho N,N'-diacetylbacillosamine or phospho N-acetylglucosamine to undecaprenyl phosphate, creating two potential membrane acceptors. Despite this upstream promiscuity, the downstream glycosyltransferase PglA acts as a specificity gate. In cells it extends only the undecaprenyl diphosphate N-acetylglucosamine intermediate, using N-acetylgalacturonic acid produced by the soluble dehydrogenase WbpO. Elongation by the subsequent membrane glycosyltransferases PglJ and PglL occurs only after the PglA product is amidated. This amidation is performed by the ATP-dependent amidotransferase WbpS, which uses glutamine as the nitrogen source and, unlike previously described bacterial pathways, modifies the lipid-linked oligosaccharide rather than the soluble nucleotide-activated sugar. Blocking amidation arrests the pathway at the trisaccharide stage, which explains earlier genetic observations that *H. pullorum* requires this step for full N-glycan elaboration and suggests an equivalent control point in related ε-proteobacteria.[3] Together, these data define lipid-linked amidation as the primary determinant of glycan extension in *H. pullorum*, establish PglA as the gatekeeper that determines downstream glycan composition, and identify a membrane-confined amide-forming step that can be exploited for anti-virulence targeting

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It's tough to unravel a DUF - exploring the role of the Domain of Unknown Function 2284 in vitamin B12 biosynthesis

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Vitamin B_{12} (cobalamin) is an essential cofactor involved in key metabolic processes in both humans and microorganisms. It comprises a corrin ring with a variable upper ligand (methyl, adenosyl, cyano) and a conserved lower ligand, 5,6-dimethylbenzimidazole (DMB)[1-2]. While the interconversion of upper ligands and their catalytic roles are mechanistically explored, the biosynthesis of the lower ligand, particularly under anaerobic conditions, remains mechanistically elusive.

In the anaerobic biosynthesis pathway, the *bza* operon orchestrates the formation of DMB [3]. One of the genes, *bzaC*, encodes a Domain of Unknown Function 2284 (DUF2284), whose specific role has not yet been defined. To elucidate its function, we employed an integrated approach involving bioinformatics, protein biochemistry, substrate synthesis, and spectroscopic characterization.

Bioinformatic analysis revealed the presence of DUF2284 predominantly existing in co-occurrence two other *bza* operon genes, suggesting a conserved role in DMB synthesis, however, it is also found outside the context of the *bza* operon in other organisms. We cloned, overexpressed, and purified several DUF2284 domain containing protein, which exhibited a brown color indicative of a metal center. UV–Vis and EPR spectroscopy confirmed the presence of an [Fe–S] cluster, and we are currently characterizing its specific type using these techniques.

To overcome the limited availability of native substrates, we synthesized various benzimidazolyl intermediates and their corresponding cobamides starting from simple aniline derivatives[4]. These enabled us to probe substrate specificity both *in vivo* and *in vitro*. We have also developed a KCN-free cyanation method for producing cobamide standards, facilitating downstream studies. Feeding assays using engineered *E. coli* strains demonstrated that several synthetic benzimidazoles function as effective lower ligands.

Ongoing complementation studies using DUF-containing and DUF-truncated BzaC constructs aim to elucidate the function of DUF2284. Preliminary results suggest a redox-dependent role in the transformation of specific biosynthetic intermediates.

In conclusion, this study not only advances our understanding of an uncharacterized domain but also showcases the power of chemical synthesis and biochemical tools in unraveling complex enzyme functions in cofactor biosynthesis.

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Structural Basis of PFK1 T-State Autoinhibition and Filament-Driven Glycolytic Compartmentalization

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Glycolysis is a central metabolic pathway that processes carbohydrates to generate energy as well as for biomass production. Regulated glucose metabolism is essential for cellular homeostasis and its dysregulation contributes to disparate diseases, including cancer. Flux through the glycolytic pathway is controlled by rate-limiting enzymes, one of the most important is phosphofructokinase-1 (PFK1), which catalyzes an irreversible phosphorylation reaction that commits glucose to breakdown in lower glycolysis. PFK1 is highly allosterically regulated. However, despite being studied for over 90 years, the molecular mechanisms enabling allosteric regulation of mammalian PFK1 remain poorly understood. To address this gap, we determined the first T-state structure of human liver PFK1 (PFKL).[1] The structure revealed that the transition from the inactive T-state to the active R-state involves a ~7° rigid-body rotation. Further, the previously unresolved C-terminal tail (CTT) folds across the catalytic and regulatory domains in the T-state, acting as a strap that stabilizes the inactive conformation. Consistent with this model, removal of the CTT increased affinity for sugar substrate, reduced ATP-mediated inhibition, and an elevated maximal velocity. Transition to the R-state stabilizes the formation of higher-order assemblies, allowing PFKL to assemble into elongated filaments in vitro and dynamic puncta in cells. We determined the structure of PFKL filaments and identified a key residue at the filament interface (Asn702) that, when mutated to Thr, abolishes filament formation without significantly altering catalytic activity or substrate affinity. In HepG2 cells, filament formation is required for the generation of biomolecular condensates, as wild-type FLAG-PFKL forms cytosolic condensates regulated by serum starvation, whereas FLAG-PFKL-N702T does not. In migrating MDA-MB-231 human breast cancer cells, catalytic activity and filament formation are required for PFKL recruitment to lamellipodia and for directional sensing of growth factor gradients, as both kinase-inactive (His199Tyr) and filament-incompetent (Asn702Thr) mutants fail to support these processes.[2] Together, these findings define the molecular mechanisms that regulate mammalian PFK1 activity and demonstrate how higher-order assembly organizes glycolysis at the mesoscale. Our results suggest that filamentation of PFK1 is a novel mechanism controlling localized glycolysis during cell migration.

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Structure and Mechanism of PhdC, a Prenylated Flavin Maturase

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Prenylated flavin mononucleotide (prFMN) is a modified flavin cofactor required by the UbiD family of (de)carboxylase enzymes[1]. While the reduced prFMNH_2 form is produced by the flavin prenyltransferase UbiX, the corresponding two electron oxidised prFMN^{iminium} form is required to support UbiD catalysis. Thus, oxidative maturation of prFMNH_2 is required which can be catalysed by UbiD[2]. However, heterologous (over)expression of UbiDs frequently leads to accumulation of the stable but non-active one-electron oxidized purple prFMN^{radical} species. A dedicated prFMN maturase enzyme (PhdC) from *Mycolicibacterium fortuitum* was recently identified as capable of catalysing the oxidative maturation of prFMN^{radical} to prFMN^{iminium}, thereby enabling an effective supply of active cofactor to the associated phenazine-1-carboxylate (de)carboxylase PhdA[3]. We report the crystal structure of PhdC in complex with flavin, revealing it is a distant member of the class I HpaC-like family of short-chain dimeric flavin reductases and demonstrate catalytic conversion of the prFMN^{radical} species to prFMN^{iminium} in the presence of oxygen or ferricyanide. Co-expression of PhdC or a distant homologue from *Priestia megaterium* (YclD) with the canonical UbiD from *E. coli* leads to activation of the latter, similar in effect to co-expression with the prFMNH_2-binding chaperone LpdD. Conserved Glu residues in the PhdC active site suggest catalysis occurs through C1' proton-abstraction coupled oxidation. This study thus provides both structural and mechanistic insight into the function of PhdC adding to the expanding repertoire of prFMN-binding proteins associated with the widespread UbiDX system.

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Temperature as a molecular switch for regioselectivity in CYP152 decarboxylases

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Peroxygenases of the CYP152 family catalyse fatty acid oxidation using hydrogen peroxide, bypassing canonical redox partners. Some members perform decarboxylation to generate terminal alkenes, key intermediates for renewable fuels, yet how CYP152 enzymes balance decarboxylation and hydroxylation remain unresolved. Here we identify a temperature-dependent switch in the peroxygenase from *Nosocomiicoccus massiliensis* (OleTNS) that governs regioselectivity. OleTNS displays a cold-adapted profile, producing alkenes with exclusive β-regioselectivity at low temperatures, a behavior not observed in other family members. Molecular dynamics and mutational analyses reveal that enhanced chemoselectivity arises from coordinated motions between the F–G loop and catalytic His85, promoting deeper substrate burial and favoring decarboxylation. At higher temperatures, this coordination is disrupted, weakening hydrogen-bonding networks and reducing alkene yield. The distinctive loop flexibility and charged surface characteristics of cold-adapted enzymes confer catalytic control, illustrating how structural dynamics and thermoadaptation shape P450 reactivity toward sustainable alkene production.

Mapping the Physiological and Proteomic Landscape of Mammalian LanCL Glutathione Transferases

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LanC-like (LanCL) enzymes are a conserved but understudied family of mammalian glutathione transferases. They are homologous to bacterial LanC cyclases involved in lanthipeptide biosynthesis. Previously, we reported that LanCLs catalyze C-glutathionylation of reactive dehydroalanine/dehydrobutyrine (Dha/Dhb) residues formed from phospho-Ser/Thr elimination, representing a rare protein-damage mitigation pathway in mammalian cells. Although we have uncovered LanCLs' enzymatic functions, their *in vivo* roles remain largely unclear. To delineate LanCLs' system-wide functions, we performed a comprehensive, multi-scale survey in LanCL-deficient mouse models, integrating whole-body physiological profiling, injury-induced muscle regeneration assays, and both global and targeted proteomics across multiple tissues. In this report, we demonstrate that LanCL-deficient mice exhibite broad physiological alterations across survival, growth, and tissue repair. In FVB mice, LanCL loss reduced overall survival, with knockout mice showing premature mortality compared to wild-type controls. LanCL deficiency resulted in modified muscle regeneration dynamics and altered immune responses to muscle injury. In LanCL knockout C57BL/6 mice, we observed altered growth patterns including differences in baseline weight and weight changes in responses to dietary challenge. We previously identified glutathionylated Dha/Dhb-containing peptides generated from endogenous phosphorylated kinases, indicating LanCLs act on physiologically relevant signaling proteins. In addition to mammalian kinases, our unbiased pulldown proteomic analysis revealed a diverse set of candidate LanCL-interacting proteins, suggesting a wider array of cellular substrates and providing a starting point for understanding the molecular context of LanCL activity. To directly assess LanCL-dependent modification, we are developing an isotopic labeling-assisted strategy capable of detecting LanCL-catalyzed C-glutathionylated residues. This method enables unambiguous identification of LanCL-catalyzed glutathionylation and provides a platform for mapping LanCL substrate specificity *in vivo*. Together, these findings give an initial system-level picture of LanCL function and provide practical tools for identifying LanCL-dependent protein-damage repair.

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The catalytic mechanism of de-ADP-ribosylation by the SARS-CoV-2 NSP3 macrodomain

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ADP-ribosylation is an ancient post-translational modification that regulates critical cellular processes across all domains of life. In eukaryotes, ADP-ribosylation plays essential roles in innate immune responses, while viruses remove ADP-ribose units to evade host detection. Macrodomains are evolutionarily conserved protein modules that recognize and remove ADP-ribose modifications, yet significant mechanistic and substrate diversity exists within this family, making the enzymatic mechanisms and features that confer substrate specificity poorly understood. We examined the substrate specificity and catalytic mechanism of SARS-CoV-2 Mac1 with X-ray crystallography, nuclear magnetic resonance spectroscopy, and targeted mass spectrometry. Structural analysis using a low activity Mac1 mutant captured the enzyme bound to C3"-linked substrate, while mass spectrometry experiments demonstrated that catalysis occurs at the C1" position, as evidenced by ^{18}O incorporation. To reconcile this apparent discrepancy, reactive quantum-based molecular dynamics simulations provided insights into ester migration, revealing the possibility that Mac1 initially binds the C3" isomer, followed by active site-mediated linkage migration to the catalytically competent C1" species prior to hydrolysis. These structural insights identify critical active site features that determine both substrate linkage specificity and distinguish catalytic macrodomain "erasers" from non-catalytic macrodomains that function solely as "readers" and lack hydrolytic activity.

Conference Menu

(conference menu subject to change)

Sunday January 4

Opening Reception

La Costa Garden Reception Station
Taco Central Reception Station
Sliders Reception Station

Monday January 5

Lunch – SoCal Barbecue

Starters
- Watermelon Salad with Tajin, feta, arugula
- Macaroni Salad
- Caesar Salad

Entrees
- Beef Burgers
- Fried Chicken with Hot Honey
- All-beef Hot Dogs
- Rosemary & Sea Salt Potato Wedges
- Lemon & Thyme Zucchini Steaks

Desserts
- Mini Apple and Pecan Pies

Evening Poster Social Session

Roasted Tomato & Ricotta Crostini
Organic Chicken and Mushroom Roulade
Mini New England Lobster Roll
Oriental Vegetable Spring Roll

Tuesday January 6

Lunch – Mediterranean Lunch Buffet

Starters
- Mezze Display, hummus, baba ghanoush, naan, dolmas, and vegetables
- Tabouli, arugula, bulgur wheat, heirloom tomatoes
- Fattoush, romaine, tomatoes, cucumbers, pita crisps and mint vinaigrette

Entrees
- Silan Chicken, date and honey glaze with lemon tzatziki
- Pan-Seared Skuna Bay Salmon, chickpea ragout
- Caramelized Cauliflower and artichoke hearts, harissa, fennel pollen
- Israeli couscous, chermoula
- Lemon & Thyme Zucchini Steaks

Desserts
- Baklava with pistachios
- Rose water panna cotta, mixed berry compote

Evening Poster Social Session

Shrimp & Mango Ceviche
Vegetarian Paella Risotto Balls
Lamb Lollipop with Jalapeño Peach Compote
Ancho Chicken Kebob with Spiced Pineapple Chutney

Wednesday January 7

Lunch – Santa Fe Buffet

Starters
- Southwest Caesar, cojita, dried cherries and poblano ranch
- Smoked black bean salad, roasted corn, green chilies, tomatoes
- Jicama slaw, cabbage, grilled pineapples, citrus & mango dressing

Entrees
- Chipotle Honey Grilled Flat Iron Steak
- Roasted Chicken Breast, sweet pepper mole
- Blackened Snapper, roasted corn pioco de gallo
- Calabacitas Stew, Zucchini, squash, tomatoes and onions
- Sonoran Ricew

Desserts
- Mini mole cakes
- Tres leches shots

Closing Banquet

Starters
- Asparagus Soup, crisp pancetta
- Quinoa Salad, raisins, herbs and lemon chive vinaigrette
- Baby Greens, grape tomotoes, cucumbers, and balsamic dressing
- Melon and Feta, toasted pstachios, frisée, olive oil and sweet chili

Entrees
- Layered Eggplant Lasagna
- Flat Iron Steak
- Pan Seared Chicken with herb jus
- Black Cod, warm beet and orange salad
- Rosemary & Garlic Mashed Potatoes
- Caramelized Cauliflower and Citrus Poached Broccoli

Desserts
- Vanilla bean panna cotta with berry compote
- Flourless chocolate cake with fruit foam

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