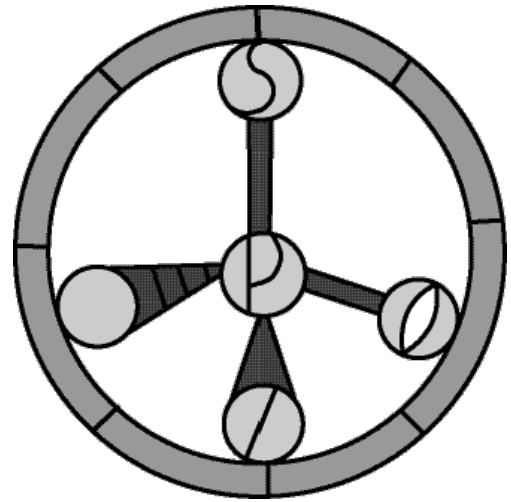


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General Information

Name Badges - Because several groups will be using the meeting and dining facilities, Steenbock participants are requested to wear the name badge during all the conference activities.

Registration - Pick Up of Conference Materials

Beginning on Thursday, May 29, 7:30 - 9:00 pm
(and continuing on Friday/Saturday) at the Pyle Center, 702 Langdon St.

Welcome Reception on Thursday, May 29 at 7:30-9:00

Speakers and registered participants are invited to attend an hors d'oeuvre reception in the Pyle Center.

Scientific Talks

All talks will be in the Pyle Center rooms 325-326.
Talks begin at 8:45 am on Friday and conclude at 12:15 pm on Sunday.

Lunch on Friday, Saturday & Sunday

Registration fee includes lunch in the Pyle Center main dining room. Please wear the name badge.

Poster Discussion Session

Friday from 2:00-6:00 pm. Posters can be set up beginning Friday morning and should be removed at 6:00 pm. Limited pins provided. Boards are numbered and reserved. See the reservation list in this booklet and by the boards.

Banquet honoring Perry Frey, Saturday, May 31, at Lowell Hall, 610 Langdon St.

Social Hour at 7:00. Dinner at 7:45. Please display the entree ticket to assist the server.

Emergency Messages

Pyle Center Conference Services (608) 262-1122.



Harry Steenbock (1886-1967)

A distinguished Professor of Biochemistry at the University of Wisconsin-Madison. His interests and contributions spanned many areas of nutrition and biochemistry. Of special note is his discovery of the conversion of carotenes to vitamin A, the production of vitamin D by ultraviolet irradiation, and his central participation in demonstrating that copper and iron cure nutritional anemia. The production of vitamin D by ultraviolet irradiation virtually abolished rickets, a widespread and debilitating disease. Professor Steenbock patented the irradiation process for producing vitamin D and conceived the idea of the Wisconsin Alumni Research Foundation, a nonprofit foundation which invests the proceeds from patents for support of research in the natural sciences at the University of Wisconsin-Madison. Professor Steenbock assigned his valuable patents to the Foundation, and revenue from them has provided uniquely flexible resources for fostering sustained scientific development on the Madison campus.

To honor Professor Steenbock, the Steenbock Endowment sponsors a symposium each year at the University of Wisconsin-Madison.

Symposium Organizers

Brian Fox

George Reed

Department of Biochemistry

University of Wisconsin-Madison

Janice Carberry

Symposium Coordinator

Symposium Sponsors

Monsanto

Merck

Cargill

GlaxoSmithKline

Boehringer Ingelheim

Promega Corporation

Eli Lilly

Abbott Labs

DuPont

Steenbock Endowment, UW-Madison

Previous Symposia

28th - Intracellular Protein and Lipid Traffic, Aug 12-14, 2000

27th - Adipocyte Biology and Hormone Signaling, 1999

26th - Enzymatic mechanisms, May 28-31, 1998

25th - Biosynthesis and function of metal clusters for enzymes, June 10-14, 1997

24th - Behavior and signaling in microorganisms, August 11-13, 1995

23rd - High Pressure Effects in Molecular Biophysics and Enzymology
May 15-19, 1994

22nd - Protein-nucleic acid interactions, May 22-26, 1993

21st - Cellular Communication in Plants, May 31-June 2, 1992

20th - Molecular biology of atherosclerosis, June 3-5, 1990

19th - Osteoporosis, June 5-8, 1989

18th - Hormones, thermogenesis, and obesity, June 12-16, 1988

17th - Current advances in vitamin K research, June 21-25, 1987

16th - RNA polymerase and the regulation of transcription, July 13-17, 1986

15th - Mechanisms of enzymatic reactions, June 30 - July 3, 1985

14th - Nitrogen fixation and CO₂ metabolism, June 17-22, 1984

13th - One hundred years of Agricultural Chemistry and Biochemistry at Wisconsin
August, 1983

12th - Biochemistry of metabolic processes, June 9-11, 1982

11th - Energy coupling in photosynthesis, July 6-8, 1981

10th - Osteoporosis--recent advances in pathogenesis and treatment, June 9-12, 1980

9th - Ribosomes, structure, function, and genetics, July 5-8, 1979

8th - Vitamin K metabolism and vitamin K-dependent proteins, June 10-13, 1979

7th - Nitrogen Fixation, June 12-16, 1978

6th - Isotope effects on enzyme-catalyzed reactions, June 4-5, 1976

5th - CO₂ metabolism and plant productivity, June 9-11, 1975

4th - Structure and conformation of nucleic acids and protein-nucleic acid interactions
June 16-19, 1974

3rd - Trace Element Metabolism in Animals, June 18-22, 1973

2nd - DNA Synthesis in Vitro, July 10-12, 1972

1st - The Fat Soluble Vitamins, 1969

Program

Welcome Reception and Talks at the Pyle Center, 702 Langdon Street, Madison, Wisconsin

Thursday, May 29

7:30-9:00 p.m. Pick-up registration materials at the **Welcome Reception** in the Alumni Lounge.

Friday, May 30 – Room 325

8:30 a.m. Coffee Break
8:45 **Opening remarks: (Brian Fox and George Reed)**
Session Chair: Wayne Frasch, Arizona State
9:00 **John Groves** - Molecular Probes of the Mechanisms of Heme and Non-Heme Oxygenases.
The Case for Oxygen Rebound
9:30 **John Markley** - Characterization of Iron-Sulfur Sites in Proteins through Combined NMR
Spectroscopy and Quantum Chemical Calculations
10-10:30 Coffee Break
10:30 **Squire Booker** -Stoichiometry, Configuration, and Function
of the Iron-Sulfur cluster(s) of E. coli Lipoyl Synthase
11:00 **John Richard** - Studies on the Mechanism for Catalysis of Proton Transfer at Carbon
11:30 **Rolf Thauer** - H₂ - forming methylene tetrahydromethanopterin dehydrogenase
from methanogenic archaea: surprises with no end
12:15-1:45 p.m. Lunch – Pyle dining room, lower level.
2:00-6:00 **Poster Session** – Rooms 225-226

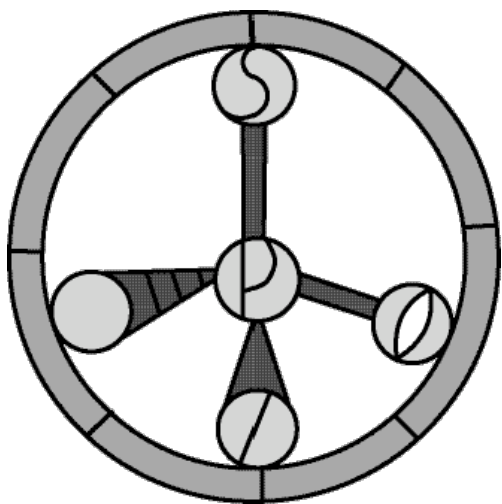
Saturday, May 31 – Room 325

8:30 a.m. Coffee
8:50 **Session Chair: Frank Raushel**, Texas A & M
9:00 **Rowena Matthews** - Shake, rattle and roll: controlling the conformational changes
necessary for methionine synthase catalysis
9:30 **Susan Taylor** - The Dynamics of Signaling by PKA
10-10:30 Coffee Break
10:30 **John Lipscomb** - Keeper of the Gate: Selective Methane Oxidation by Methane Monooxygenase
11:00 **Janos Retey** - Enzyme catalysis by radical and electrophile assisted substrate activation
11:30 **Dagmar Ringe** - Control of pyridoxal phosphate cofactor chemistry by the protein
12:15-1:45 p.m. Lunch – Pyle dining room, lower level.
1:50 **Session Chair: John Gerlt**, University of Illinois
2:00 **Wolfgang Buckel** - ATP induced electron transfer in clostridial 2-hydroxyglutaryl-CoA dehydratase
2:30 **Greg Petsko** - Structural Enzymology in Four Dimensions:
Direct Observation of Unstable Species in Enzyme-Catalyzed Reactions
3-3:30 Coffee Break
3:30 **Ben Liu** -Mechanistic Studies of Unusual Enzyme Catalyses
4:00 **Ruma Banerjee** - Redox Regulation of Homocysteine Metabolism
4:30-5 **Judith Klinman** - Investigations of the ethylene-forming enzyme, ACC Oxidase

7:00 **Social Hour** – Lowell Center, 610 Langdon St.
7:45 **Banquet - Honoring Perry Frey** Rowena Matthews speaker

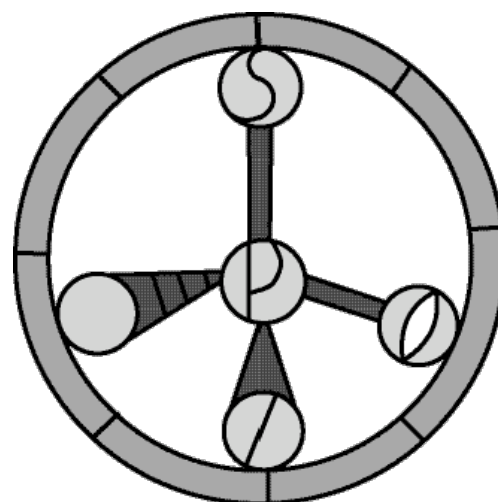
Sunday, June 1 – Room 325

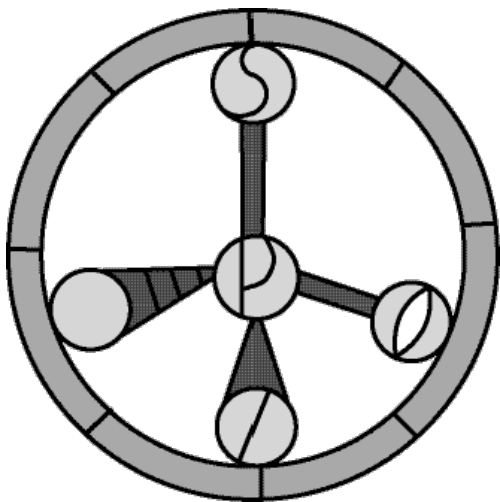
8:30 a.m. Coffee
8:50 **Session Chair: Ronald Raines**, University of Wisconsin-Madison
9:00 **W.W. Cleland** - The Use of Isotope Effects to Determine Enzymatic Mechanisms
9:30 **JoAnne Stubbe** - Radical Initiation in Class I Ribonucleotide Reductases
10-10:30 Coffee Break
11:00 **Chris Walsh** - Antibiotic Glycosyl Transferases
11:30 **Perry Frey** - From Adenosylcobalamin to Adenosylmethionine and Back
12:00 p.m. Closing remarks (**Fox/Reed**)
12:15 Lunch – boxed, eat in or 'to go'. Room to be announced.



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Judith Klinman	16	Investigations of the ethylene-forming enzyme, ACC Oxidase
John Lipscomb	17	Keeper of the Gate: Selective Methane Oxidation by Methane Monooxygenase
Hung-Wen Liu	18	Mechanistic Studies of Unusual Enzyme Catalyses
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The Reaction Mechanism and Regulation of Human Cystathionine beta-synthase
R. Banerjee, **Department of Biochemistry, University of Nebraska, Lincoln, NE 68588-0664, U.S.A**

Cystathionine β -synthase catalyzes the first step in the transsulfuration pathway that provides an avenue for conversion of the essential amino acid, methionine, to cysteine. In mammals, the enzyme displays a twin cofactor dependence on B₆ and heme, whereas the yeast enzyme requires only B₆. The overall reaction involves condensation of homocysteine and serine to give cystathionine. In this presentation, we will focus on three issues. (1) Kinetic analysis of the reaction catalyzed by yeast and human cystathionine β -synthase (2) The mechanism of redox sensing in human cystathionine β -synthase and (3) Regulation of cystathionine β -synthase activity.

The absence of the heme cofactor in the highly homologous yeast enzyme has facilitated elucidation of its kinetic mechanism by stopped-flow spectroscopy. The properties of the yeast enzyme will be compared to those of the full-length and heme-less variants of the human enzyme. Site directed mutagenesis has been employed to distinguish between the roles of the vicinal cysteines in the CXXC motif versus the heme in the redox responsiveness of the human enzyme under in vitro conditions. Under cell culture conditions we demonstrate differences in the response of human cystathionine β -synthase to oxidative stress imposed by exogenous bolus administration of peroxides versus stimulating endogenous ROS production. These studies provide insights into additional layers of regulation imposed on the human enzyme and its role in glutathione-dependent redox homeostasis.

Stoichiometry and Configuration of the Iron-Sulfur Clusters of *Escherichia coli* Lipoyl Synthase

Robert M. Cicchillo, Natasha M. Nesbitt, Camelia Gogonea, Matthew Souder,
Carsten Krebs, and Squire J. Booker*.

Department of Biochemistry and Molecular Biology, The Pennsylvania State
University, University Park, PA 16802

Lipoic acid (6,8 thioctic acid) is a covalently bound cofactor that functions in several multienzyme complexes that are involved in energy metabolism, as well as the glycine cleavage complex. Its importance is highlighted by the fact that many organisms have evolved at least two distinct pathways to govern its attachment to specific subunits of these complexes. In *Escherichia coli*, exogenously synthesized lipoic acid is imported into the cell and attached to conserved lysine residues of the relevant protein by an ATP-dependent lipoyl synthase. Lipoic acid also can be synthesized endogenously via a pathway that is an offshoot of fatty acid biosynthesis [Morris et al (1995) *J. Bacteriol.* **177**, 1]. Recent studies indicate that the sulfur insertion reaction in the endogenous pathway is catalyzed by a 36 kDa iron-sulfur cluster-containing protein termed lipoyl synthase (lipA), and that its substrate is octanoylated acyl carrier protein (ACP). In a second step, lipoyl transferase (lipB) catalyzes the transfer of the octanoyl group from ACP to the appropriate acceptor protein [Miller et al (2000) *Biochemistry* **39**, 15166].

We have cloned and isolated lipoyl synthase and lipoyl transferase, and have devised methods to assess their abilities to effect their respective reactions. We use a combination of site-specific mutagenesis, analytical chemistry, UV-visible spectroscopy, electron paramagnetic resonance spectroscopy, and Mössbauer spectroscopy to show that lipoyl synthase can accommodate two [4Fe-4S] clusters per polypeptide. We propose—analogously to the related protein, biotin synthase—that one cluster is used to generate a 5'-deoxyadenosyl radical from the reductive cleavage of AdoMet, while the other serves as a source of sulfur atoms to be inserted [Ugulava et al (2001) *Biochemistry* **40** 8343]. We show that other proteins are absolutely required to effect turnover, including lipoyl transferase and an appropriate acceptor protein, and that the transfer of the lipoyl group from lipoyl-ACP to the acceptor protein follows a sequential steady-state kinetic mechanism.

ATP induced electron transfer in clostridial 2-hydroxyglutaryl-CoA dehydratase

Marcus Hans, Marc Hetzel, Afsaneh Tamannei, Antonio J. Pierik,
and Wolfgang Buckel*

Fachbereich Biologie, Philipps-Universität, D-35032 Marburg, Germany.

The reversible *syn*-elimination of water from (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA is of considerable mechanistic interest, since the 3-*Si* proton has to be removed from the non-activated β -position ($pK = 40$), whereas a hydroxyl ion is expelled from the α -position adjacent to the electron withdrawing CoA-ester. The dehydratase from *Acidaminococcus fermentans* (Clostridiales) consists of two protein components A and D. The extremely oxygen sensitive homodimeric component A (2×27 kDa) contains one $[4Fe-4S]^{1+/2+}$ cluster between two α -helices, each from one subunit, and acts as an activator of the dehydration. Each subunit comprises an ADP/ATP-binding site related to actin [1]. The heterodimeric component D ($55 + 45$ kDa), the actual dehydratase, also carries one $[4Fe-4S]^{2+}$ cluster and exhibits 1.0 FMNH₂ and ca. 0.1 molybdenum. Component D from *Clostridium symbiosum* with an approximately 7-fold higher specific activity contains 2.0 $[4Fe-4S]^{2+}$, 1.0 FMNH₂ and 0.7 Mo. Component A is reduced by a 2 $[4Fe-4S]$ ferredoxin or by flavodoxin. In the presence of ATP the electron is further transferred to component D, in which Mo(VI) is reduced to Mo(V). The now oxidised component A hydrolyses ATP and returns to the resting state with bound ADP. It is postulated that the electron is transferred from Mo(V) further to the thiol ester of the substrate to yield a nucleophilic ketyl radical anion, which eliminates the adjacent hydroxyl group. The thereby formed enoxy radical ($pK = 14$) is deprotonated to the ketyl radical anion of the product glutaconyl-CoA. A further electron transfer back to Mo(VI) affords the product and completes the catalytic cycle. The regenerated Mo(V) is ready for the next turnover without further consumption of ATP; hence a single electron transfer suffices for many turnovers [2]. Remarkably, component D of the 2-hydroxyglutaryl-CoA dehydratase from *Fusobacterium nucleatum* contains a third smaller subunit (24 kDa) [3], but it has been shown recently that this enzyme lacks molybdenum or tungsten.

It should be noted that these enzymes resemble the phylogenetically unrelated nitrogenase system, in which similar to component A the iron protein catalyses an ATP-induced electron transfer to the FeMo-protein, where N₂ is reduced. Furthermore there is also an iron-only nitrogenase, which contains no molybdenum.

[1] Locher, K. P., Hans, M., Yeh, A. P., Buckel, W. & Rees, D. C. (2001) J. Mol. Biol. 307, 297-308.

[2] Hans, M., Bill, E., Cirpus, I., Pierik, A. J., Hetzel, M., Alber, D. & Buckel, W. (2002) Biochemistry, 41, 5873-5882.

[3] Klees, A.-G., Linder, D. & Buckel, W. (1992) Arch. Microbiol. 158, 294-301.

The Use of Isotope Effects to Determine Enzyme Mechanisms

W. Wallace Cleland*, Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, Madison, WI 53726.

Isotope effects are one of the most powerful kinetic tools for determining enzyme mechanisms. There are three methods of measurement. First, one can compare reciprocal plots with labeled and unlabeled substrates. The ratio of the slopes is the isotope effect on V/K , and the ratio of the vertical intercepts is the isotope effect on $V_{\max}$. This is the only way to determine $V_{\max}$ isotope effects, but is limited to isotope effects of 5% or greater. The second method is internal competition, where the labeled and unlabeled substrates are present at the same time and the change in their ratio in residual substrate or in product is used to calculate an isotope effect, which is that on V/K of the labeled reactant. This is the method used for tritium or ^{14}C , or with the natural abundances of ^{13}C , ^{15}N , or ^{18}O . The third method involves perturbations from equilibrium when a labeled substrate and corresponding unlabeled product are present at chemical equilibrium. This also gives just an isotope effect on V/K for the labeled reactant.

The chemistry is typically not fully rate limiting, so that the isotope effect on V/K is given by: $^x(V/K) = (^xk + c_f + c_r ^xK_{\text{eq}})/(1 + c_f + c_r)$ where x defines the isotope (D, T, 13, 15, 18 for deuterium, tritium, ^{13}C , ^{15}N , or ^{18}O), and $^x(V/K)$, ^xk and $^xK_{\text{eq}}$ are the observed isotope effect, the intrinsic one on the chemical step, and the isotope effect on the equilibrium constant. The constants c_f and c_r are commitments in forward and reverse directions, and are the ratio of the rate constant for the chemical reaction and the net rate constant for release from the enzyme of the varied substrate (direct comparison) or labeled substrate (internal competition and equilibrium perturbation) for c_f , or the first product released or the one involved in the perturbation for c_r . The intrinsic isotope effect, ^xk , can be estimated by comparing deuterium and tritium isotope effects on V/K , or by comparing the deuterium isotope effect with ^{13}C ones with deuterated and undeuterated substrates. Adding a secondary deuterium isotope effect and its effect on the ^{13}C one can give an exact solution for all intrinsic isotope effects and commitments. The effect of deuteration on a ^{13}C isotope effect allows one to tell if the two isotope effects are on the same or different steps.

Applications of these methods to several enzyme systems will be presented.

Supported by NIH grant GM18938.

From Adenosylcobalamin to Adenosylmethionine and Back

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A principal focus in this laboratory over the recent fifteen years has been on reactions of free radicals at enzymatic sites. Early successes in searching for free radicals in the reactions of lysine 2,3-aminomutase (LAM) and methane monooxygenase (MMO) led to the observation and structural characterization of eight free radicals as intermediates or suicide inactivation products at the active sites of LAM, lysine 5,6-aminomutase (5,6-LAM), and dioldehydrase (DDH). The tracks left by two radical intermediates in the reaction of MMO have also been observed as radical clock rearrangements.

LAM catalyzes the interconversion of L-lysine and L- α -lysine, the course of which is characteristic of adenosylcobalamin-dependent reactions. However, the coenzymes of LAM are S-adenosylmethionine (SAM), pyridoxal-5-phosphate (PLP) and a [4Fe–4S] cluster. As in B₁₂ reactions, the adenosyl moiety of SAM transiently becomes the 5'-deoxyadenosyl radical and carries out the hydrogen transfer process in a novel process for SAM. The 1,2-migration of the amino group takes place by a radical mechanism that is facilitated by the imine linkage in the lysine-PLP aldimine. In this way, PLP facilitates a radical reaction, a novel process for PLP. The cleavage of SAM to methionine and the 5'-deoxyadenosyl radical is brought about by the [4Fe–4S] cluster in a novel electron transfer process, which has been characterized by selenium XAS and ENDOR spectroscopy. The mechanism of the lysine rearrangement has been documented by the systematic characterization of four of the radical intermediates by EPR, ESEEM, and ENDOR spectroscopy.

5,6-LAM is a coenzyme B₁₂ and PLP dependent enzyme that catalyzes the interconversion of D-lysine and D-2,3-diaminohexanoate. The radical mechanism appears similar to that of LAM, except that coenzyme B₁₂ serves as the source of the 5'-deoxyadenosyl radical. Two radical intermediates have been identified by EPR and ENDOR spectroscopy.

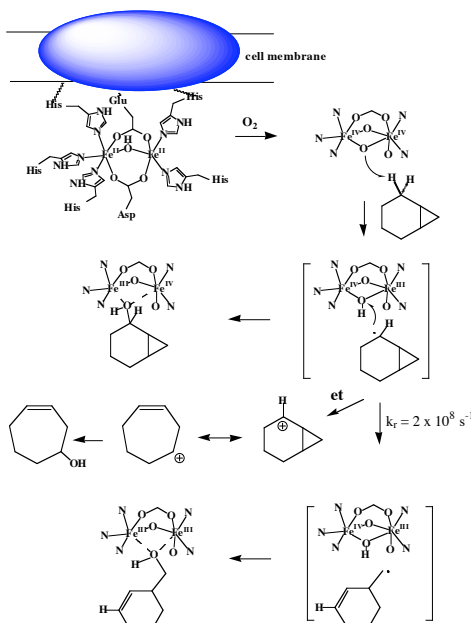
The transformation of 1,2-propanediol into propionaldehyde is catalyzed by DDH in a coenzyme B₁₂-dependent process that is initiated by the 5'-deoxyadenosyl radical. Glycolaldehyde and chloroacetaldehyde are suicide inactivators that lead to the cleavage of coenzyme B₁₂ to cob(II)alamin, 5'-deoxyadenosine, and a radical derived from the inactivator. This radical has been identified in both cases as the *cis*-ethanesemidione radical. In order to study the elusive 5'-deoxyadenosyl radical, a coenzyme B₁₂ analogue has been synthesized, 3'-[4'-anhydroadenosylcobalamin. The coenzyme analog is cleaved at the active site of DDH into the 3'-[4'-anhydro-5'-deoxyadenosyl radical and cob(II)alamin as a tightly coupled triplet state, which has been characterized by EPR spectroscopy. Supported by Grant No. DK28607 from the National Institute of Diabetes and Digestive and Kidney Diseases.

Molecular Probes of the Mechanisms of Heme and Non-heme Hydroxylases. The Case for Oxygen Rebound

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The soil organism *Pseudomonas oleovorans* TF4-1L (ATCC 29347) can grow on octane as its sole source of carbon. The monooxygenases AlkB and XylM from TF4-1L are dinuclear non-heme iron enzymes, similar to hemerythrin, which catalyze the terminal hydroxylation of simple alkanes (C₅-C₁₂) and aromatic hydrocarbons, respectively. These enzymes are wide-spread in nature and responsible for a major proportion of hydrocarbon oxidation in the environment. The diagnostic radical clock probes *trans*-2-phenyl-1-methylcyclopropane and norcarane have been used as diagnostic probes of the oxygenation mechanism of AlkB. Norcarane afforded a mixture of 2-norcaranols and 3-(hydroxymethyl)-cyclohexene (85:15), a ring-opened product characteristic of a substrate radical intermediate with a lifetime of approximately 1 ns. This is the first unambiguous evidence for an oxygen-rebound like mechanism in a non-heme diiron alkane hydroxylating enzyme. These results will be compared and contrasted to those obtained for cytochrome P450, xylene monooxygenase (XylM), toluene monooxygenase (T4mo), methane monooxygenase (sMMO), model systems, and as yet unidentified hydroxylases in whole organisms.



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We thank the National Science Foundation for funding through the Environmental Molecular Science Institute, CEBIC, at Princeton University, NSF9810248 and CHE9814301.

Probes of the Mechanism of the ACC Oxidase Reaction

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ACC (1-Aminocyclopropane-1-carboxylate) oxidase is a member of the large family of non-heme iron proteins that contain a 2-histidine-1-carboxylate iron-binding motif at their active sites. In general, these proteins control oxygen reactivity by preventing the binding and activation of molecular oxygen until both the substrate and electron donor are properly positioned within the active site. The majority of the proteins of this class use α -ketoglutarate as a two-electron donor, whereas ACC oxidase uses ascorbate as electron donor to produce ethylene, HCN and CO₂ as products. Three aspects of the ACC oxidase reaction will be addressed in this presentation: kinetic order for addition of ACC, ascorbate and O₂ to enzyme, the investigation of the uncoupling of O₂ consumption from product formation and the identification of substrates that generate aldehydes and ketones as products via a C-C bond cleavage process. Unique features of ACC oxidase will be highlighted in the context of other members of the 2-histidine-1-carboxylate class of iron binding proteins.

Keeper of the Gate: Selective Methane Oxidation by Methane Monooxygenase

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Methanotrophic bacteria oxidize CH₄ as their only source of carbon and energy. Thus, selective CH₄ oxidation in a sea of potential substrates with weaker C–H bonds poses a problem that these organisms must successfully address. The soluble form of MMO utilizes 3 components: a reductase (MMOR), a regulatory protein (MMOB) and a hydroxylase (MMOH). MMOH contains a dinuclear iron cluster where O₂ is activated by reductive O–O bond cleavage. MMOB accelerates the reaction of the [Fe(II)–Fe(II)] state of the cluster with O₂ by 1000-fold to initiate a catalytic cycle that has at least 6 transient intermediates. The reactive intermediate, termed **Q**, contains a unique bis μ -oxo-[Fe(IV)–Fe(IV)] cluster. NMR mapping of the MMOB surface that binds to MMOH allowed 4 mutants to be constructed that individually alter the rate constants of different steps in the catalytic cycle. One four-residue MMOB mutant termed “Quad” accelerates the reaction of **Q** with large substrates and the release of large products from the product complex, **T**. Based on this observation, we propose a model for the reaction in which MMOH acts as a molecular sieve for substrates, and MMOB gates substrates (hydrocarbon and O₂) into the buried MMOH active site by adjusting the “hole size” of the sieve. This idea could be directly tested due to two related observations. First, analysis of the activation parameters of the **Q** reaction showed that it occurs in two steps, nominally substrate binding and C–H bond cleavage. Second, a ²H KIE of 50 was found for CH₄ oxidation by **Q** while no KIE was observed for any other substrate. If MMOH is a molecular sieve, then binding may be limiting for all molecules larger than CH₄ and O₂, thereby eliminating the observed KIE. Accordingly, it was shown that when using the Quad mutant, a KIE of 2 was observed for the C₂H₆ reaction, presumably because the hole size of the sieve is enlarged so that binding is no longer rate limiting. Interestingly, the KIE for CH₄ is decreased to 6, perhaps because a major tunneling contribution to its reaction with **Q** is eliminated. Mutational analysis of the Quad mutant showed that T111 is the primary keeper of the gate (1). Cross-linking and mass-spec peptide sequencing showed that MMOB binds to MMOH on the surface above the diiron cluster. A model for the complex based on the cross-linked residues, places MMOB T111 between two MMOH helices that form one wall of the active site cavity, thereby providing the first structural insight into the mechanism of regulation by MMOB. [Supported by NIH GM-40466]. (1) See B. J. Brazeau and J. D. Lipscomb, *Biochemistry*, ASAP (2003) and references therein.

Mechanistic Studies of Unusual Enzyme Catalyses: Learning Nature's Strategies for Making Unusual Sugars

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Carbon-oxygen bond cleavage is one of the most common reactions in biological systems. Understandably, there is a wide range of structural variation in molecules that undergo this transformation. As expected, a diverse array of mechanisms has evolved in Nature to facilitate the C-O bond scission for these compounds. Most of the reaction mechanisms display a characteristic feature in that the reaction is initiated by the cleavage of a C-H bond prior to the breakage of the target C-O bond. Some of the enzymes catalyze such a transformation through substrate activation upon binding without the assistance of any cofactor. In contrast, other enzymes require organic coenzymes, in some cases even a highly reactive radical-generating cofactor, to break the C-H bond and to provide the necessary driving force for the reaction. Deoxy sugars are a group of compounds whose formation involves C-O bond cleavages. They play essential roles in many biological processes including cellular adhesion, cell-cell interactions, immune response, fertilization, and target recognition of toxins, antibiotics, and microorganisms. Our research on the biosynthesis of deoxy sugars has led to the discovery of a few unique mechanisms to achieve C-O bond cleavages at various positions of a hexose. It has been found that the immediate biosynthetic precursor of most deoxy hexoses is a nucleotidyl-diphospho-4-hexulose. The complexity of various deoxygenation modes is determined by the location of the C-O bond to be disrupted, that is whether it is α or β to the 4-keto group. The action mode of an α -deprotonation followed by a β -elimination is the choice for cleaving a C-O bond β to an activating keto group. However, in the cases where the C-O bond to be ruptured is at the α -position to a keto group, a more elaborate mechanism involving novel coenzyme B₆ dependent chemistry is adopted. This presentation will summarize our current knowledge on the deoxygenation mechanisms at C-3 and C-4 of a hexose for making the corresponding deoxy sugars. Insights into these enzymatic processes on the molecular level may be useful for the development of suitable strategies to control, mimic, or alter the formation of deoxy sugars.

Iron-Sulfur Centers in Proteins: NMR and Quantum Chemical Approaches toward Understanding Cofactor-Protein Interactions

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We have carried out detailed NMR investigations of clostridial rubredoxin, as an example of a [1Fe] protein, and one member of each of the four major classes of [2Fe-2S] ferredoxins: *Anabaena* PCC7120 vegetative ferredoxin (plant type), human ferredoxin (vertebrate type), *Aquifex aeolicus* ferredoxin (clostridial type), and the Rieske ferredoxin component from toluene 4-monooxygenase (Rieske type). Preliminary goals have been to resolve and assign signals from the cysteines ligating the iron atoms and from other residues in the loops near the cofactors of these proteins in their oxidized and reduced states and to determine their T_1 relaxation rates.¹⁻³ Owing to the slow electronic relaxation rates in these proteins, traditional ^1H -detected NMR experiments could not be used. The experimental strategy involved stable isotope labeling of the proteins (uniform labeling with ^{13}C and ^{15}N and selective labeling with ^2H , ^{13}C , and ^{15}N) combined with direct detection of ^2H , ^{13}C , and ^{15}N NMR signals.¹⁻⁴ Next, starting with available three-dimensional structural models of the oxidized states of each protein (plus a three-dimensional structure for reduced clostridial rubredoxin), hybrid density functional methods were used to calculate hyperfine shifts and T_1 relaxation rates for both oxidation states.¹ These theoretical values have been compared with experimental values. The results (both theoretical and experimental) are discussed in terms of the geometric structures of iron centers in these proteins, hydrogen bonds to the centers, and, in the case of [2Fe2S] ferredoxins, exchange coupling between the metal centers. With clostridial rubredoxin, the hyperfine ^{15}N and ^{13}C shifts of several single residue mutants with altered reduction potentials have been studied with the aim of discovering mechanisms by which primary sequence influences protein metal-site interactions.⁵ We are beginning use site-directed mutagenesis to test hypotheses concerning the structural origins of the observed differences in hyperfine shifts and relaxation rates in different classes of [2Fe-2S] ferredoxins.

Supported by NIH Grant GM R01 GM58667; T.E.M. is the recipient of an NIH Postdoctoral Fellowship.

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"Shake, rattle and roll: controlling the conformational changes necessary for methionine synthase catalysis"

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Cobalamin-dependent methionine synthase catalyzes methyl transfer from methyltetrahydrofolate to homocysteine; its B₁₂ prosthetic group serves as the acceptor of methyl groups from methyltetrahydrofolate and donates them to homocysteine to form methionine. The protein comprises four modules, which bind and activate homocysteine, methyltetrahydrofolate, B₁₂, and adenosylmethionine (AdoMet), respectively. The last module is required for reductive methylation to reactivate the protein when the B₁₂ cofactor is oxidized during turnover. The enzyme exists in solution in at least four conformations: conformations in which the B₁₂ bound to module 3 interacts with homocysteine, or methyltetrahydrofolate, or AdoMet, respectively, and a conformation in which the B₁₂ is capped by a four helix bundle in the same module and is inaccessible to substrates. We will discuss experiments designed to elucidate the effects of ligands on the ensemble of conformations.

Structural Enzymology in Four Dimensions: Direct Observation of Unstable Species in Enzyme-Catalyzed Reactions [^]

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The advent of X-ray diffraction techniques using synchrotron radiation has made it possible to collect protein crystal data sets on a rapid time-scale. Observing unstable species in enzyme reactions by this technique also requires that one overcome the problem of synchronization: it is necessary that all of the molecules in the crystal be at the same stage of the reaction at the same time. Thus the reaction in question must be initiated on a time-scale that is fast compared to its kinetics. For some biochemical events photochemistry or the diffusion of a gas is the most suitable trigger, but not all substrates are suitable for this. Often, individual intermediates will have to be stabilized by a combination of low temperatures and slow substrates or mutant enzymes. But with such methods, time-resolved protein crystallography can be used to study metastable species that can be trapped in the crystal under defined conditions for short periods of time. An entire reaction sequence then can be obtained as a series of individual steps, each of which is obtained from a separate experiment. We will describe the current methods and strategies for such studies in detail and present two dramatic examples of the application of time-resolved techniques: the structure and mechanism of the enzyme galactose mutarotase, carried out in collaboration with Jane Beebe and Perry Frey of the University of Wisconsin, and the high-resolution structures of all of the kinetically-significant intermediates in the reaction cycle of the hydroxylation of camphor by bacterial cytochrome P450, carried out in collaboration with Steve Sligar of the University of Illinois. In the first case, the structures of the enzyme and the enzyme-substrate complex have been obtained, the latter under equilibrium turnover conditions (Figure 1). These structures, plus some quantum mechanical calculations and work by our collaborators, shed light on the way the enzyme promotes efficient proton abstraction from a very weak carbon acid. In the second case, the structure of the activated oxygen intermediate of the P450 reaction has been obtained, along with those of the dioxygen intermediate and several other species on the pathway (Figure 2). These structures not only provide insights into the catalytic mechanism of this important metalloenzyme, they also illuminate fundamental questions about the role of the protein environment in catalysis and the structural basis for catalytic power, and they offer a paradigm for structural studies of other unstable enzyme reaction intermediates.

[^] Dedicated, with thanks and warmest wishes to Professor Perry Frey

Figure 1. Electron density of the substrate bound to the active site of mutarotase at 1.4 Å resolution.

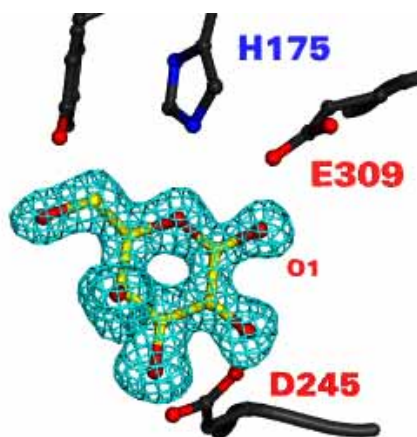
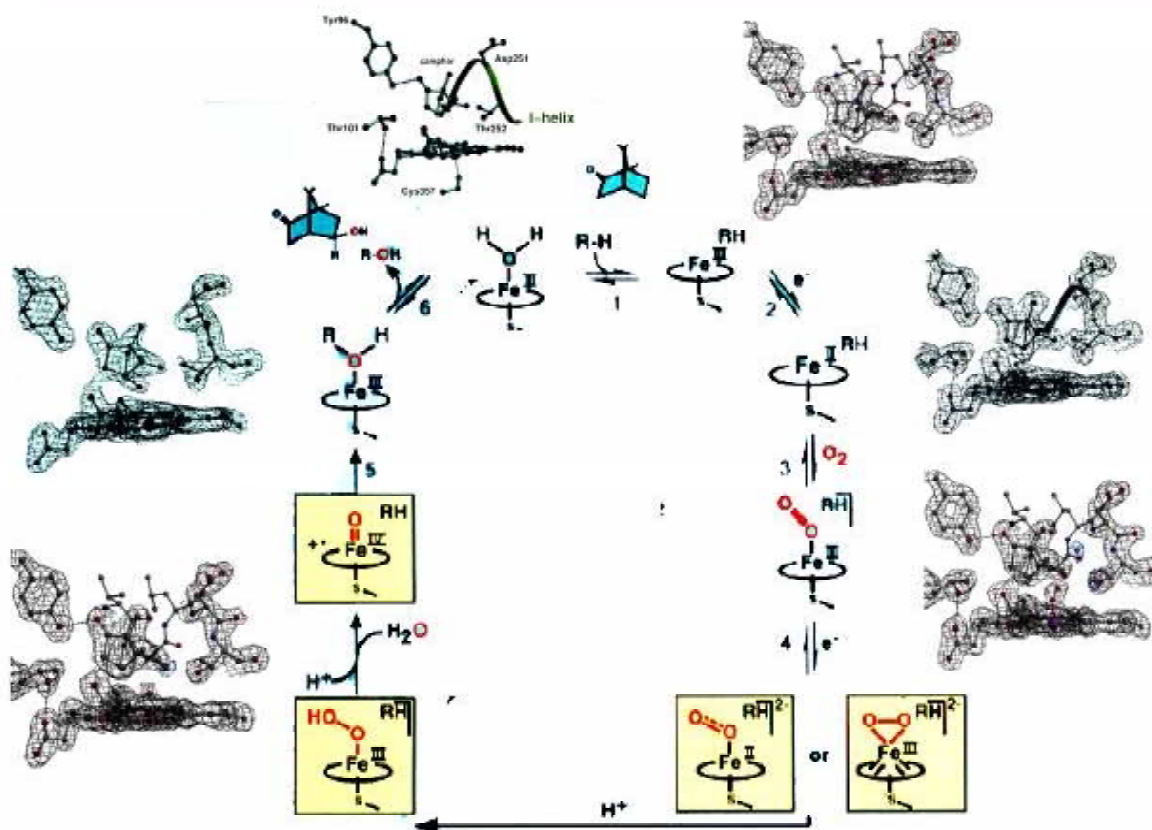


Figure 2. The catalytic cycle of cytochrome P450cam, with electron density maps showing the active site region for the structures of the unstable intermediates determined by time-resolved crystallography.



Enzyme catalysis by radical- and electrophile-assisted substrate activation

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According to the classical theory enzymes catalyse their reactions by stabilisation of the corresponding transition state, i. e. they bind the transition state stronger than the substrate. Another possibility of lowering the activation energy, however, is raising the ground state energy of the substrate to form an energy-rich species from which the reaction can occur. Such a substrate activation is especially important in radical and electrophilic reactions.

Among the most thoroughly investigated enzymatic radical reactions are those, in which coenzyme B₁₂ is the radical starter.

Homolysis of the cobalt-carbon bond generates the highly reactive 5'-deoxyadenosyl radical which, in turn, abstracts a hydrogen atom from the substrate thereby starting the radical reaction chain. One of the main roles of the enzyme is to prevent unspecific reactions of the transient radical species. In 1990 I coined the term "negative catalysis" for the latter.

One of the best enzymatic example in this respect is the rearrangement of methylmalonyl-CoA to succinyl-CoA. Here, the CoA-ester group migrates to the adjacent carbon atom, rendering the branched carbon chain to a straight one. Such a rearrangement is not characteristic for radicals, because they undergo other reactions much faster or dimerize. At the enzyme active site such reactions are ruled out mainly because of the isolation of the radical intermediates. As a consequence, the chemically unlikely, since slower, rearrangement takes place.

Such a situation can be, at least partially, imitated without an enzyme. If the methylmalonyl radical is generated by photolysis in zeolite, a seven times higher yield of the succinyl species is achieved than in hexane solution.

Of course, not only radicals, but other highly unstable molecules can be generated in a container and thereby significantly elongated their life times. Cyclobutadiene generated in the so-called hemicarcerand of Cram and coworkers is quite stable up to room temperature. Previously cyclobutadiene could be detected only at 4 Kelvin in an argon matrix.

To illustrate substrate activation by electrophiles, I would like to discuss two enzymatic examples. One of them is histidine ammonia-lyase (HAL) whose product urocanate is the first intermediate in the degradation of histidine. HAL occurs in bacteria, but also in animals and in our liver. Its lack is the cause for a severe genetic disease, called histidinaemia.

The other enzyme is phenylalanine ammonia-lyase (PAL). This is a very important plant enzyme whose product is cinnamate, the precursor of the phenylpropanoids, among those lignin, the flavanoids and coumarins. For this reason PAL is a target for herbicides which explains the interest of the agroindustry.

The elimination of ammonia from α -amino acids is chemically difficult because the acidity of the β -proton is very low. It has been believed for more than 30 years that HAL and PAL contain a catalytically essential dehydroalanine residue. Both enzymes are inactivated by nucleophilic reagents.

In collaboration with the group of G. Schulz in Freiburg (Germany) we could solve the 3D structure of HAL. Unexpectedly, the electrophilic prosthetic group turned out to be methylidene imidazolone (MIO) which is spontaneously formed by cyclization and dehydration of an inner tripeptide portion (Ala142Ser143Gly144). Obviously, the information necessary for the posttranslational modification is provided by the amino acid sequence of the precursor peptide. Although the 3D structure of PAL is not yet elucidated, we could show the presence of MIO by UV difference spectroscopy.

The active sites of both enzymes have been characterised and the corresponding amino acid residues exchanged by site-directed mutagenesis.

Formation and Stability of Carbanions in Water and at Enzymes

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Carbanions are reactive intermediates in a wide range of organic reactions in solution and at enzyme active sites. We have developed methods to determine or obtain accurate estimates of the rate and equilibrium constants for deprotonation of a wide variety of carbon acids to form the corresponding carbanions including the enolates of thioesters, esters, amides, alkane carboxylate anions, and α -amino acids and their derivatives in water. The relevance of these data to several questions will be discussed including: (1) At what point does the rate constant for protonation of simple carbanions in water approach the limiting value of a bond vibration? (2) What are the effects of ester, amide, carboxylate anion, ammonium cation and other electron-withdrawing α -substituents on the equilibrium constants and Marcus intrinsic barriers for protonation of carbanions. (3) What are the carbon acidities of simple amino acids, and the imperatives for enzymatic catalysis of their deprotonation to form amino acid enolates?

There is a great disparity between our knowledge of the structure and catalytic groups of enzymes that catalyze deprotonation of carbon and our understanding of the mechanism for stabilization of the high energy transition states of the corresponding nonenzymatic reactions. The results of studies on the mechanism of action of triosephosphate isomerase-catalyzed interconversion of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate will be presented which show that the majority of the catalytic rate acceleration for this enzyme is due to utilization of the binding energy of the nonreactive phosphate group of substrate to stabilize the transition state for transfer of an α -carbonyl hydrogen to the enzyme. Evidence will be presented that this enzymatic reaction occurs at an active site that is strongly shielded from interaction with solvent. It will be argued that a substantial catalytic rate acceleration can be obtained by sequestering the polar substrate for this reaction at a nonpolar enzyme active site.

**Hormone processing proteases:
structural determinants for the specificity of Kex2**

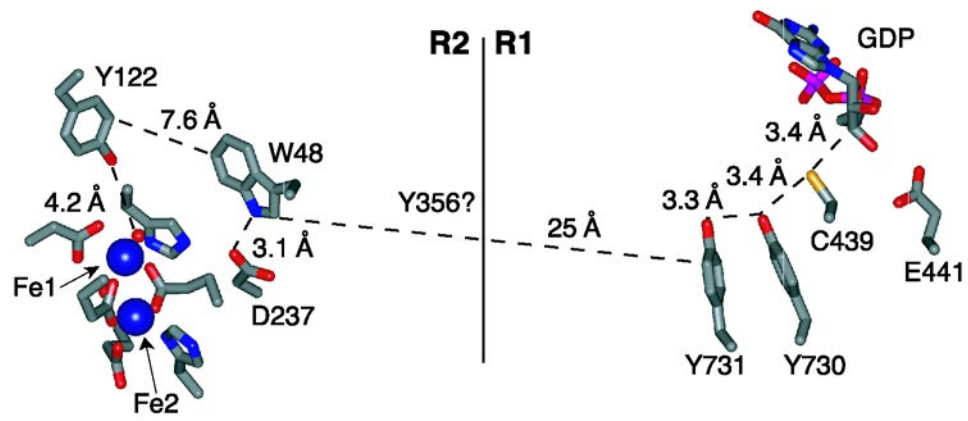
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Endoproteolysis of precursor proteins is an obligatory step in the synthesis of many biologically active proteins and peptides in eukaryotes. Typically, cleavage occurs at the carboxyl side of pairs of basic amino acid residues. The enzymes that carry out this cleavage belong to a family of multi-domain, membrane-bound, serine endopeptidases that include Kex2 from yeast, and furin and PACE-4 from higher eukaryotes. The proteolytic domain of these enzymes is similar in global fold to the subtilisin-like family of degradative enzymes. The extreme selectivity and unusual metal ion dependencies of these enzymes, however, distinguish these enzymes from the non-specific subtilisins. The role of a second domain, the P-domain in Kex2, is still undetermined. The three-dimensional structure of the prototype of these enzymes, Kex2 from yeast, has now been determined and will be discussed.

Toward Understanding the Mechanism of Radical Initiation by Long Range Proton Coupled Electron Transfer in the Class I Ribonucleotide Reductase

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E. coli ribonucleotide reductase (RNR) catalyzes the conversion of nucleoside diphosphates to deoxynucleoside diphosphates an essential step in DNA replication and repair. The enzyme is composed of two subunits: R1 and R2. R1 contains the active site for nucleotide reduction and the allosteric effector sites that regulate enzyme specificity and turnover rate. R2 contains the diferric-tyrosyl (Y•) radical cofactor that initiates nucleotide reduction by a proposed long range proton coupled electron transfer pathway (35 Å). This pathway is thought to involve specific amino acid radical intermediates (Y122 to W48 to Y356 within R2 to Y731 to Y730 to C439 within R1). In an effort to study radical initiation, R2 (375 amino acids) has been synthesized semi-synthetically. Residues 1-353 attached to an intein and a chitin binding domain was generated and purified using a chitin affinity column. This R2 is reconstituted with Fe^{2+} and O_2 to generate the diferric-Y• cofactor. Residues 354 to 375 of R2 were synthesized using solid phase peptide synthesis, where 354, a serine in the wild type (wt) R2 has been replaced by a cysteine. The peptide and the reconstituted R2 (1-353) are ligated and R2 is separated from truncated R2, by anion exchange chromatography. To investigate the radical initiation process, R2 with Y356 replaced with 3-nitrotyrosine. The pKa of the 3-nitrotyrosine (λ_{max} 430 nm for the phenolate) has been shown to be 7.8 in the presence of R1, CDP and TTP, in comparison with a pKa of 7.2 for this residue in the free peptide. Assays for catalytic activity reveal that the enzyme catalyzes formation of one to two deoxynucleotides at a rate $< 5 \times 10^{-4}$ that of the wt R2. Stopped flow UV/Vis spectroscopy on samples in which R1 and mutant R2 are mixed in the presence of CDP and TTP demonstrate a rapid biphasic increase in $A_{430 \text{ nm}}$ with k_{app} of 200 s^{-1} and 30^{-1} , both faster than the turnover of the wt RNR of 10 s^{-1} . This change occurs only in the presence of substrate and effector. These experiments provide the first demonstration of a kinetically competent conformational change triggered by nucleotides in the R1/R2 complex and that the pKa of a putative residue on the PCET pathway is minimally perturbed. In addition Y356 has been replaced with 2,3-difluorotyrosine and a pH rate profile of this protein determined. The profile is very similar to wt-R2 made by intein technology and shows that both enzymes can form dNDPs across the entire pH range. If the pKa of the 2,3-difluorotyrosine is not greatly perturbed in the R1R2 complex, as suggested by the 3-nitrotyrosine studies, the mechanism for radical initiation involving hydrogen atom transfer is not viable.



cAMP-Dependent Protein Kinase: Dynamic Properties of a Protein Kinase.

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cAMP-dependent protein kinase (PKA) is one of the simplest members of the protein kinase superfamily, one of the largest families encoded for by higher eukaryotic genomes. It is a highly dynamic protein that is poised to rapidly transfer the γ -phosphate of ATP to a protein substrate. The crystal structure of the C subunit in the presence of various substrates and inhibitors reveals not only the intramolecular network of interactions that extend throughout the molecule but also the intrinsic flexibility of the motions that allow for opening and closing of the active site cleft. Hydrogen/deuterium exchange coupled with mass spectrometry has allowed us to also map the dynamic features of the C subunit. In addition, point mutations show selective changes in this dynamic behavior and highlight the extended networks. Small angle Xray crystallography has confirmed that the holoenzyme in the presence of cAMP does not fully dissociate but instead is converted into a highly dynamic state where substrates can readily bind and thereby promote full dissociation. Quantum mechanical calculations of residues at the active site cleft predict a very rapid phosphoryl transfer process and highlight the critical role of Lys168 in mediating this process.

H₂-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) from methanogenic archaea: surprises with no end

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In many methanogenic archaea a hydrogenase is present that catalyzes the reversible dehydrogenation of methylenetetrahydromethanopterin to methenyltetrahydromethanopterin and H₂. The H₂-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) contains neither nickel nor iron-sulfur clusters and is therefore generally referred to as “metal-free” hydrogenase. Hmd harbours a low molecular mass cofactor of yet unknown structure. We report here that the enzyme was inactivated upon exposure to blue light (390-500 nm). Enzyme purified in the dark exhibited an absorbance spectrum, which mirrored its sensitivity towards light. In blue light the enzyme was bleached. The cofactor extracted from active Hmd was also light sensitive. It showed a similar UV/Vis spectrum above 340 nm as the active enzyme and was bleached upon exposure to light. Bleached cofactor no longer had the ability to reconstitute active Hmd from the apoenzyme. Inactivation and bleaching of Hmd in blue light prompted us to reinvestigate the presence of a transition metal in the enzyme. Maximally active Hmd prepared in the dark was found to contain approximately one mol of iron, which was released from the enzyme parallel to inactivation and bleaching. The cofactor preparation from active Hmd also contained iron, which could be separated from the cofactor by chromatography only after inactivation. The results indicate that Hmd could contain a functional transition metal after all.

Buurman, G., Shima, S. & Thauer, R.K. (2000) The metal-free hydrogenase from methanogenic archaea: Evidence for a bound cofactor. *FEBS Lett.* 485, 200-204.

Antibiotic Glycosyl transferases

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Many natural products with antibiotic activity are glycosylated at a late stage in the biosynthetic pathway by dedicated glycosyl transferases (Gtfs) encoded in the biosynthetic gene cluster. The sugar moieties added by such Gtfs are often deoxy or aminodeoxyhexoses also produced by enzymes encoded within the biosynthetic clusters. Manipulation of the sugars transferred to the aglycone portions of the antibiotics may allow reprogramming of the biosynthetic pathways for novel activity. Assay of the enzymes requires production of active Gtfs in heterologous hosts and the availability of both the aglycone substrates and the NDP-D-hexose and NDP-L-hexose substrates. To evaluate the specificity/permmissivity of such Gtfs we have examined Gtfs responsible for sequential transfer of D-glucose and then L-(epi)vancosamine to the crosslinked heptapeptide aglycone of the vancomycin and teicoplanin antibiotic scaffolds. We have also characterized the L-noviosyl transferase, NovM, involved in the biosynthesis of the aminocoumarin antibiotic novobiocin

New Substrates for β -Lactam-Recognizing Enzymes: Arylmalonamates

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Aryl malonamates are demonstrated to be novel substrates of a broad range of β -lactam recognizing enzymes. These compounds are isomers of the aryl phenaceturates which are well-known substrates of these enzymes, but the new compounds contain a retro-amide side chain. Several lines of evidence, including comparisons of steady state kinetic parameters between enzymes, and a detailed investigation of the methanolysis kinetics, solvent deuterium isotope effects, and pH-rate profile for turnover of a retro substrate by the *Enterobacter cloacae* P99 β -lactamase, suggested that the new substrates are likely to be hydrolyzed by the same chemical mechanisms as “normal” substrates. Molecular modeling indicated that the retro-amide group fits snugly into the active site of the P99 β -lactamase by hydrogen-bonding to the conserved lysine-67 residue. The retro-amide side chain may represent a lead to novel mechanism-based and transition state analogue inhibitors.

3-Deoxy-D-manno-Octulsonate 8-Phosphate Phosphatase from *Aquifex Aeolicus*

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The resistance to classic antibacterial agents, such as penicillin, is a major health problem facing the world today. The key 3-Deoxy-D-manno-octulsonate (KDO) pathway may lead to promising discoveries of new antibacterial drug agents that act in a completely novel manner as to avoid cross-resistance with current pharmaceuticals. The outer membrane of Gram-negative bacteria seems to be an attractive target for rational attempts to design antimicrobial agents effective solely against these bacteria, to whom this outer membrane is unique. It appears to be crucial to the survival of the bacteria as well as to its drug resistance and pathogenic potential. KDO is a unique eight-carbon sugar present in the lipopolysaccharide (LPS) layer, which is located on the surface of the outer membrane of Gram-negative bacteria. It provides a direct link between Lipid A and the growing polysaccharide chain, and the interruption of the synthesis or utilization of KDO has been shown to lead to cell cycle arrest due to an accumulation of Lipid A precursors. This makes the KDO pathway a very important target for antibacterial agents. KDO 8-phosphate (KDO 8-P) phosphatase catalyzes the hydrolysis of KDO 8-P to KDO and inorganic phosphate and is the last enzyme in the KDO pathway that has been recently identified. Inhibition of KDO 8-P phosphatase should lead to Gram-negative bacteria cellular breakdown, specifically targeting the LPS. The identification, overexpression, purification, and characterization of KDO 8-P phosphatase from *Aquifex aeolicus* will be presented.

^{13}C and ^{15}N Isotope Effects for the Conversion of Dihydroorotate to Carbamoyl-Aspartate (CA-Asp) Using Dihydroorotase (DHOase) from Hamster and the Thermophilic bacterium, *Bacillus caldolyticus*

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In the pyrimidine biosynthetic pathway carbamoyl-aspartate is converted to dihydroorotate by DHOase. This important mechanism was probed using primary and secondary isotope effects of the ring opening of dihydroorotate determined by $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ ratios using isotope ratio mass spectrometry (IRMS). The physiological reverse reaction was performed at three different temperatures (25, 37 and 45° for hamster DHOase; 37, 50, and 60 °C for *B. caldolyticus*) and the product CA-Asp was purified. The CA-Asp was hydrolyzed in concentrated HCl at 100 °C in a sealed vessel and the resultant NH_4Cl , CO_2 , and aspartate were isolated. The primary and secondary isotope effects for the ring opening of the dihydroorotate were determined from isotope ratio analysis of the N and C of the carbamyl group. The β carboxyl of the Asp was isolated by transamination to OAA and then decarboxylation. The $^{13}\text{C}/^{12}\text{C}$ ratio from the ensuing CO_2 was studied by IRMS yielding a second primary isotope effect value. The isotope effect values and their interpretation as to the mechanism of the ring opening of dihydroorotate will be discussed.

Kinetic Characterization of Lysine 2,3 Aminomutase with S-adenosyl-L-methionine Analogs

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Lysine 2,3 aminomutase from *Clostridium subterminale* SB4 catalyzes the interconversion of α - and β -lysine, which is the first step in a Stickland-type fermentation pathway that converts lysine into ammonia and acetyl-CoA. The mechanism of the reaction involves the formation of four organic radicals, initiating with the production of 5'-deoxyadenosine 5'-yl via a reductive cleavage of S-adenosyl-L-methionine. The electron is supplied by a $[4\text{Fe-4S}]^+$ cluster, which is held in the protein via three cysteines that reside in a CXXXCXXC motif, which is common to all enzymes within the Radical SAM superfamily. Studies on other members of the Radical SAM superfamily indicate that one of the irons of the cluster coordinates to the carboxylate and amino functionalities of AdoMet itself [Walsby et al (2002) *J. Am. Chem. Soc.* **124**, 11270; Cosper et al (2002) *J. Am. Chem. Soc.* **124**, 14006]. We have synthesized analogs of AdoMet, in which the amino group, the carboxylate group, or both groups are removed from the structure, to delineate the role in catalysis of this coordination. We use electron paramagnetic resonance spectroscopy in combination with activity assays in the presence of the substrate, lysine, or analogs of lysine to show the binding of the AdoMet analogs to the cluster and/or to the protein, as well as their ability to initiate the reaction.

Factors that influence conformational equilibria in the cobalamin-dependent methionine synthase

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Cobalamin-dependent methionine synthase (MetH) from *Escherichia coli* methylates homocysteine (Hcy) to form methionine using a methyl group that is derived from methyltetrahydrofolate ($\text{CH}_3\text{-H}_4\text{folate}$). The enzyme-bound cobalamin cofactor is alternately methylated by $\text{CH}_3\text{-H}_4\text{folate}$ and demethylated by Hcy and thus in the course of the catalytic cycle, the cobalamin cofactor alternates between the methyl cobalamin and cob(I)alamin forms. Cob(I)alamin is susceptible to oxidation to the catalytically inactive cob(II)alamin form. Reactivation of the protein requires reduction of cob(II)alamin to cob(I)alamin with an exogenously supplied electron, and methylation of the reduced cofactor by a methyl group from *S*-adenosyl-L-methionine (AdoMet). The cobalamin cofactor, Hcy, $\text{CH}_3\text{-H}_4\text{folate}$, and AdoMet are bound to and activated by separate regions of MetH thus requiring conformational changes to juxtapose each substrate-bearing region, in turn, with the cobalamin-binding region during the turnover cycle and during reductive methylation. We are interested in understanding the mechanisms by which MetH coordinates access to the cobalamin cofactor by the substrate bearing regions of the protein. This poster will present our recent studies on the relationship between cofactor environment, ligands and conformational changes in MetH.

Probing the effects of MMOB on the catalytic mechanism of the soluble methane monooxygenase

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Soluble methane monooxygenase (sMMO) from methanotrophic bacteria catalyzes the O₂-dependent hydroxylation of methane to methanol in the initial step of methane metabolism. The MMO system consists of 3 components; a hydroxylase (MMOH), an effector protein (MMOB), and a reductase (MMOR). MMOH is a dimer that contains an active site in each protomer. Each active site consists of a binuclear iron center and a hydrophobic pocket that is a putative substrate binding site. Transient kinetic studies have led to the discovery of several intermediates in the sMMO catalytic cycle, including a Fe(IV)-bis- μ -oxo species termed compound Q (**Q**) that is the intermediate that reacts with methane and other substrates. MMOR has been shown to shuttle electrons from NADH to the MMOH active site.

While the roles of MMOH and MMOR in catalysis are well-understood, a detailed understanding of the function of MMOB is lacking. Previous studies have established that MMOB affects the rates of interconversion of several intermediates in the sMMO catalytic cycle. In particular, a site-directed mutant form of MMOB (N107G/S109A/S110A/T111A) termed the Quad mutant was shown to affect the reaction between **Q** and substrates as well as the product release step. Interestingly, in the presence of the Quad mutant when large substrates such as nitrobenzene and furan were used, the rate of the **Q** reaction and the rate of product release increased. In contrast, the rate of the **Q** reaction with smaller substrates such as methane decreased. The fact that the effects of the Quad mutant on the **Q** reaction and product release were found to be dependent upon the size of the substrate suggests that MMOB has a role in regulating access of substrates to the sMMO active site in MMOH. The work presented in this poster focuses on understanding the origin of the effects of the Quad mutant on sMMO catalysis using transient kinetic techniques.

Catalytic Contributions of *Methylosinus trichosporium* OB3b Methane Monooxygenase Reductase

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Methane monooxygenase is a multi-component diiron enzyme that catalyzes the hydroxylation of methane to form methanol. Although extensive characterization of the three enzyme components (a diiron containing hydroxylase protein (MMOH), a cofactor-free effector protein (MMOB), and an iron-sulfur flavoprotein reductase (MMOR)) has been achieved upon isolation of protein from the host organism [1-3], numerous techniques (*e.g.* NMR and mutagenesis) are amenable only if a heterologous expression system is available. We describe a convenient system for producing recombinant *M. trichosporium* OB3b MMOR upon expression in *Escherichia coli*. This system has facilitated a site-directed mutagenesis study for probing the determinants of MMOH/MMOR interactions. Some of these amino acid substitutions lead to impaired activity of the MMO enzyme complex. Using a combination of steady-state and transient kinetics, we are able to monitor the build-up of the reactive enzyme intermediate, compound Q, as well as product formation. These data reveal that the identity of MMOR not only specifies the rate of intermolecular electron transfer, from NADH to MMOH, but may also affect the rate of product release from MMOH.

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Investigation of a Proposed Mechanism for UDP-Galactopyranose Mutase

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Abstract:

According to the World Health Organization, tuberculosis (TB) has made a huge resurgence in recent years, now infecting one-third of the world's population and killing 2 million people per year. The increased death toll is partially a result of the emergence of antibiotic resistant strains. Consequently, development of drugs working by a new mechanism of action has become increasingly important. UDP-galactopyranose mutase (UGM) is the enzyme responsible for the isomerization of UDP-galactopyranose (UDP-galp) into UDP-galactofuranose (UDP-galf) (Figure 1) during cell wall construction of a number of bacteria, protozoa and fungi. It is thought to be necessary for cell viability since it aids in protection of the cell's interior from foreign molecules. Although the mechanism of this sugar transformation is unknown, it has been determined that enzymatic activity is dependent on a fully reduced flavin adenine dinucleotide cofactor (FAD). We propose that FAD is directly involved in the sugar transformation and instead of performing a redox or structural role, it is acting as a nucleophile. Computational studies were carried out to examine the nucleophilicity of the flavin cofactor. To further assess the validity of this proposal, we plan to monitor enzymatic activity in the presence of several FAD analogs. Synthetic routes to both 1-deaza and 5-deazariboflavin (Figure 2) are known. However, both have proven to be difficult, dangerous and irreproducible. Thus, new synthetic pathways to both riboflavin analogs were designed and executed.

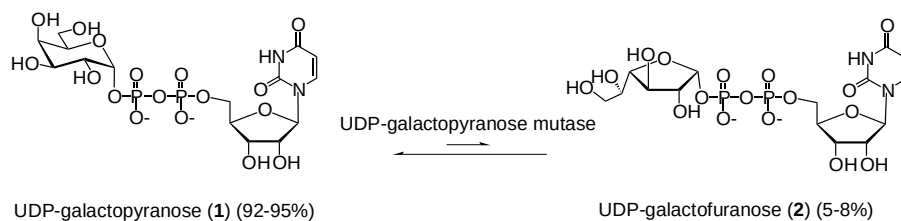


Figure 1. UGM provides gal_f for cell wall biosynthesis by catalyzing isomerization of the thermodynamically favored UDP-galactopyranose (UDP-galp, **1**, (92-95% present at equilibrium) and the disfavored UDP-galactofuranose (UDP-galf, **2** (5-8% present at equilibrium).

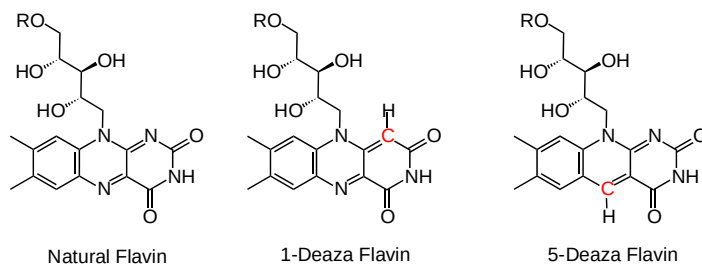


Figure 2. The flavin molecule has several potentially nucleophilic nitrogens, N1 and N5. We will study enzymatic activity using analogs lacking each of these potential nucleophilic centers by synthetically replacing each nitrogen with a carbon.

The Journey from Cloning to Density Functional/Molecular Mechanics Studies on *Bacillus subtilis* Oxalate Decarboxylase

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The *yvrK* gene product from *B. subtilis* catalyzes the mechanistically intriguing heterolytic carbon-carbon bond cleavage of oxalic acid to yield formic acid and carbon dioxide. Elucidation of the enzyme's structure by Anand, et al.¹ has opened the way for computational characterization of the electronic structure of the manganese centers that are the likely sites of catalysis. For calibration of our chosen density functional theory (DFT) method, the "simple" $\text{Mn}(\text{H}_2\text{O})_6^{2+}$ system was addressed. During our examination of spin state energies in this system, a procedure to achieve a reasonable approximation to a proper wavefunction was developed. Use of the procedure also solved SCF convergence problems, which are notorious in calculations on transition metal compounds.

Progress on more advanced model systems will be described. All have O/N ligation, as does the decarboxylase. $\text{Mn}^{\text{III}}(\text{Cl})(\text{salen})$ [salen =1,2-bis(salicylidene-imino)ethylene]² offers the challenge of spin-orbit coupling and tests the suitability of our methods for describing Mn^{III} systems. $\text{Mn}^{\text{II}}(\text{PAP})_2$ [PAP =2-(2-(phenylamino)phenyl)azopyridine]³ is a rare low-spin Mn^{II} complex; proper description of metal-ligand covalency and spin distribution will be important for reproduction of experimental data. N,N-bis(2-ethyl-5-methylimidazol-4-ylmethyl)amino-propane (biap) forms a Mn^{II} complex along with benzoic acid, $[\text{Mn}^{\text{II}}(\text{biap})(\text{C}_6\text{H}_5\text{COO})_2]$, that exhibits an EPR spectrum very similar to YvrK in the resting state.⁴ Finally, preliminary work on the YvrK system will be presented. It is hoped that this work will illustrate an effective confluence of experiment and computation applied to enzymatic mechanisms of catalysis.

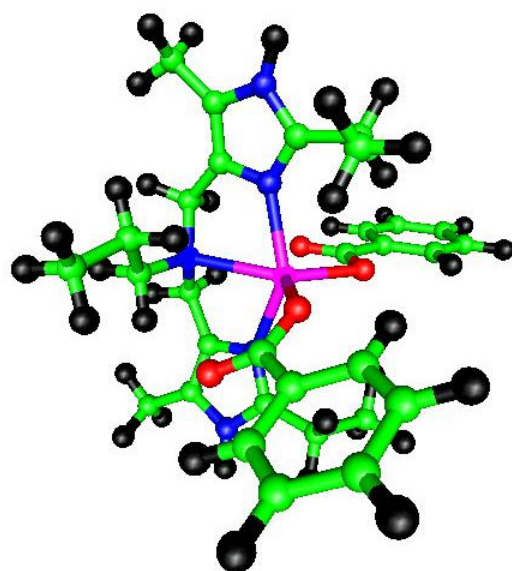
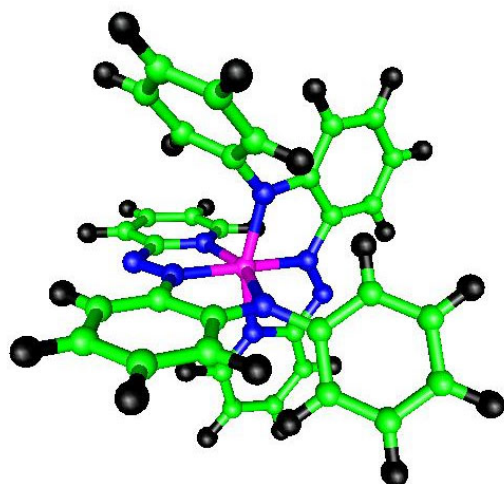
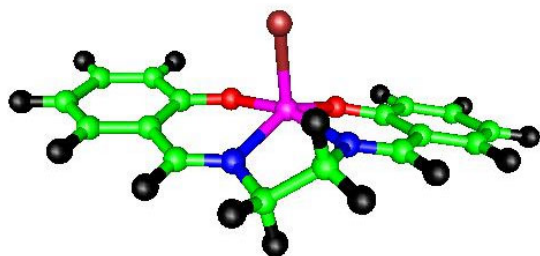
*Supported by NRSA DK61193 from the National Institutes of Health.

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Model complexes. Top, $\text{Mn}^{\text{III}}(\text{Cl})(\text{salen})$; middle, $\text{Mn}^{\text{II}}(\text{PAP})_2$; bottom, $\text{Mn}^{\text{II}}(\text{biap})(\text{C}_6\text{H}_5\text{COO})_2$. The atomic color mapping is Mn-magenta, Cl-rust, O-red, N-blue, C-green, and H-black.

Coordination and Mechanism of Reversible Cleavage of S-Adenosylmethionine by the [4Fe-4S] Center in Lysine 2,3-Aminomutase

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Lysine 2,3-aminomutase (LAM) catalyzes the interconversion of L-lysine and L-β-lysine by a radical mechanism initiated by the reversible, hemolytic scission of S-adenosylmethionine (SAM) into methionine and the 5'-deoxyadenosyl radical at the active site. The electron required for the cleavage of SAM derives from the [4Fe-4S]⁺ center associated with this enzyme. Selenium X-ray absorption spectroscopy in the reaction of Se-adenosyl-L-selenomethionine (SeSAM) in place of SAM shows that electron transfer from the [4Fe-4S] center occurs by an inner sphere mechanism culminating in direct ligation of selenomethionine to iron upon cleavage of SeSAM. Here we show through electron nuclear double resonance (ENDOR) spectroscopy that SAM binds to LAM by direct ligation to iron in the [4Fe-4S] center through the carboxylato and amine group of its L-methionine moiety. ENDOR spectroscopy was performed on [4Fe-4S]⁺-LAM (S=1/2) in the presence of SAM labeled at the carboxyl or the amino group of the methionine moiety with ¹⁷O or ¹⁵N (denoted [1+/¹⁷O-SAM] or [1+/¹⁵N-SAM]). The ENDOR spectrum from [1+/¹⁷O-SAM] shows a broad, asymmetric feature that is absent in the unlabeled sample. The signal corresponds to a hyperfine coupling of $A(^{17}\text{O}) = 11.4$ MHz at g2, which is assigned to the ν_+ branch of the ¹⁷O signal from the ¹⁷O-carboxylate of SAM. ENDOR spectrum of the unlabeled SAM ([1+/¹⁴N-SAM]) shows the ν_+ branch of the ¹⁴N signal, which disappears in the spectrum of [1+/¹⁵N-SAM] and is replaced by a well resolved ν_+ peak from a ¹⁵N with corresponding coupling $A(^{15}\text{N}) = 9.1$ MHz at g2. These large couplings require that the carboxylato O and the amino N of SAM are coordinated to the unique Fe of the cluster. ¹⁷O and ¹⁵N ENDOR data were also obtained for the interaction of SAM with the diamagnetic [4Fe-4S]²⁺ state of LAM, which is visualized through cryoreduction of the frozen [4Fe-4S]²⁺/SAM complex to the reduced state (denoted [2+/¹⁷O-SAM]_{red}) trapped in the structure of the oxidized state. ¹⁷O and ¹⁵N spectra from [2+/¹⁷O-SAM]_{red} are indistinguishable from the corresponding spectra from [1+/¹⁷O-SAM] and [1+/¹⁵N-SAM], showing that the interactions of SAM with the cluster and the active-site pocket are independent of the oxidation state of the enzyme. Based on the correlative ENDOR and XAS spectroscopic results, a mechanism for the cleavage of SAM through multiple ligation with the [4Fe-4S] center is presented.

**Biochemical and Physical Characterization of Lipoyl Synthase from
*Escherichia coli***

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Lipoic acid (6,8-thioctic acid) is an essential cofactor in several multienzyme complexes that are involved in energy metabolism, such as the pyruvate dehydrogenase complex, the 2-oxoketoglutarate dehydrogenase complex, the branched-chain oxo-acid dehydrogenase complex, and the glycine cleavage system. The biosynthesis of this cofactor involves the insertion of 2 sulfur atoms into two completely unactivated carbon atoms of an octanoyl chain esterified to the acyl carrier protein (ACP). The inertness of the substrate limits the possible mechanisms for sulfur incorporation, and should rule out polar processes. Lipoyl synthase, the protein thought to catalyze sulfur insertion, has been cloned and purified by immobilized metal affinity chromatography. This 36 kDa protein has sequence similarity to biotin synthase, another protein that catalyzes sulfur insertion into alkyl chains. Indeed, both lipoyl synthase and biotin synthase belong to a superfamily of enzymes that use S-adenosyl-L-methionine (AdoMet) and iron sulfur clusters to generate high-energy carbon-centered radicals that are intermediates in catalysis. Proteins that are members of this superfamily have a conserved iron sulfur cluster-binding motif consisting of cysteine residues in a CX₃CX₂C pattern. Lipoyl synthase deviates from other members in this class in that it contains an additional conserved set of cysteines lying in the motif CX₄CX₅C. We have shown, through site-directed mutagenesis, UV-visible spectroscopy, and Mössbauer spectroscopy, that both of these motifs can accommodate [4Fe-4S] clusters. We have developed an *in vitro* assay that monitors the reductive cleavage of S-adenosyl-L-methionine, and we show that lipoyl synthase activity requires other proteins and cofactors, suggesting that it functions as a member of a multiprotein complex.

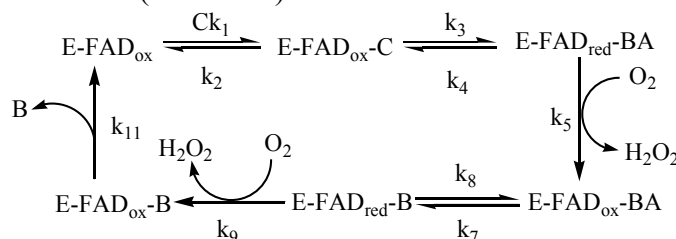
pH and Kinetic Isotope Effect Studies on Recombinant Choline Oxidase from *Arthrobacter globiformis*

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Choline oxidase (CHO; E.C. 1.1.3.17) catalyzes the four-electron oxidation of choline to glycine-betaine via two sequential FAD-dependent reactions in which betaine-aldehyde is formed as an intermediate. In both reactions oxygen acts as the primary electron acceptor with production of hydrogen peroxide. The enzyme is capable to accept either choline or betaine-aldehyde as substrate, allowing for the investigation of the reaction mechanism for both the conversion of choline to betaine-aldehyde and of betaine-aldehyde to glycine-betaine. Here, we report initial studies of the steady-state kinetic mechanism with either choline or betaine-aldehyde, and pH and deuterium kinetic isotope effect studies with [1,2-²H₄]-choline as substrate for recombinant CHO overexpressed in *Escherichia coli*.

The steady-state kinetic mechanism was determined at pH 6.5, 7 and 10 with choline or betaine-aldehyde as substrate to be sequential, suggesting that oxygen reacts with the reduced enzyme before release of betaine-aldehyde or glycine-betaine, respectively. With betaine-aldehyde, a γ -intercept was observed in a plot of 1/rate versus 1/[oxygen], consistent with a K_m value for betaine-aldehyde of 20 μ M or less. These kinetic data suggest that between pH 6.5 and 10 the aldehyde intermediate predominantly remains bound at the active site during turnover of the enzyme with choline (Scheme 1).



Scheme 1

The pH-profiles of the V and V/K_{choline} values increased to limiting values with increasing pH, indicating the presence of a catalytic base with a pK_a value of ~8 essential for catalysis. The ^D(V/K) values with [1,2-²H₄]-choline decreased from a limiting value of 11.8 at low pH to a limiting value of 4.2 at high pH, consistent with choline being a sticky substrate. The large ^D(V/K) values observed at low pH further suggest that CH bond cleavage of choline is fully rate-limiting for catalysis at low pH and almost fully irreversible.

(This study was supported in part by Grant PRF #37351-G4 from the American Chemical Society).

Was tyrosyl-tRNA synthetase a target of “chemical warfare” between primordial eukaryotes and bacteria?

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Sequence comparison suggests that there are differences between the catalytic mechanisms of bacterial and eukaryotic tyrosyl-tRNA synthetases. In particular, Lys 233 in *Bacillus stearothermophilus* tyrosyl-tRNA synthetase, which is the second lysine in the KMSKS signature sequence of the class I aminoacyl-tRNA synthetases, is replaced by either a serine or alanine residue in eukaryotic tyrosyl-tRNA synthetases (Ser 225 in human tyrosyl-tRNA synthetase). Kinetic analyses indicate that in human tyrosyl-tRNA synthetase, this conserved lysine is functionally replaced by a potassium ion. Replacing Ser 225 in human tyrosyl-tRNA synthetase with a lysine residue decreases the rate constant for tyrosine activation 10,000-fold. These results suggest that there is a selective advantage for eukaryotic tyrosyl-tRNA synthetases to maintain a catalytic mechanism that differs from that of their bacterial counterparts. One possible explanation for this observation is that tyrosyl-tRNA synthetase was a target of “chemical warfare” among the primordial eukaryotes and bacteria. This hypothesis is consistent with the recent discovery by GlaxoSmithKline Pharmaceuticals of a naturally occurring inhibitor that binds bacterial tyrosyl-tRNA synthetases 40,000-fold more tightly than it does the eukaryotic enzyme. We are currently analyzing the amino acid sequences of other aminoacyl-tRNA synthetases to determine whether additional members of this class of proteins are potential antibiotic targets.

Detection of Multiple Active Site Domain Motions in Transient-State Component Time Courses of the *Clostridium symbiosum* L-Glutamate Dehydrogenase-Catalyzed Oxidative Deamination Reaction

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ABSTRACT: We present a multiwavelength, transient-state kinetic study of the oxidative deamination reaction catalyzed by *Clostridium symbiosum* glutamate dehydrogenase (csGDH) producing the real-time reaction courses of spectroscopically resolved kinetically competent intermediate complexes. The results show striking differences from a corresponding transient-state study of the same reaction by the structurally homologous enzyme from beef liver (blGDH). In addition to the highly blue-shifted α -iminoglutarate and highly red-shifted carbinolamine complexes observed in both reactions, the csGDH reaction appeared to show the release of free NADH at a very early and mechanistically unlikely point in the reaction. Using lactic acid dehydrogenase as a “reporter” for free NADH, we show that the early portion of this signal reflects previously unobserved spectrally unshifted enzyme-bound NADH complexes. We provide experimental evidence to show that such spectrally anomalous complexes must represent forms of the known α -imino and α -carbinolamine complexes in which the active site cleft is open. This evidence includes isothermal calorimetric measurements and pH-jump experiments that show the existence of differing two-state transitions in blGDH and csGDH and locate active site domain motions at differing points in the transient-state time courses of the two enzyme reactions. We prove the kinetic competence of a new and more highly detailed mechanism for the csGDH reaction that involves the alternation of open and closed enzyme complexes as integral steps. These findings, supported by the available X-ray crystal structure data, suggest the existence of a programmed time course of protein domain motions coordinated with the classically considered chemical time course. This new viewpoint may be presumed to be applicable to enzyme reactions other than those of the α -amino acid dehydrogenases.

Identification of hydrogen bonds between the gamma subunit and the alpha or beta subunits in E. coli F1-ATPase that effect the rate of ATP hydrolysis.

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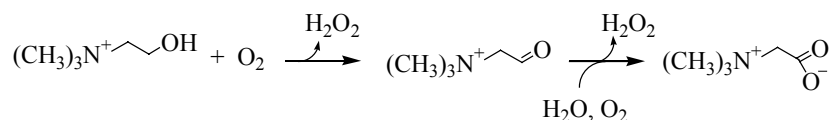
The F1-ATPase is a molecular motor in which each 120 degree rotation of the gamma subunit inside the core of alpha and beta subunits is driven by hydrolysis of an ATP. With each rotation event, the gamma subunit pauses after a 90 degree rotation at which point the helical coiled coil of this subunit is more tightly wound. Site-directed mutations to eliminate hydrogen bonds between that stabilize the more tightly wound coil were found to effect the rate of ATP hydrolysis. Removal of H-bonds from the gamma subunit C-terminus decreased the rate of ATPase while the same type of mutation at the N-terminus increased the ATPase activity. These results suggest that relaxation of the coiled coil provides some of the torque for rotation. Removal of H-bonds between the gamma subunit and the catch loop of the beta subunit dramatically decreased ATPase activity suggesting that this region is an essential link between the hydrolysis of ATP and the rotation of the gamma subunit.

Active Site Characterization of Recombinant Choline Oxidase

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Choline oxidase (CHO; E.C. 1.1.3.17) is an FAD-containing enzyme that catalyzes the oxidation of choline to glycine-betaine, with betaine-aldehyde as intermediate and molecular oxygen as primary electron acceptor (Scheme 1). From a chemical standpoint the enzyme is of interest because is one of a few flavin-dependent enzymes that carry out a four-electron oxidation of an alcohol to a carboxylate via an aldehyde intermediate utilizing covalently-bound FAD as the sole cofactor. Since many human pathogenic bacteria accumulate glycine-betaine to compensate for environmental stress, such as high osmolarity, water deficiency, and high or low temperature, the study of CHO is also of considerable interest for future drug development. Despite these reasons, minimal biochemical and mechanistic data are available for CHO to date. Here, we report biochemical and mechanistic studies on purified recombinant CHO expressed in *Escherichia coli* strain Rosetta(DE3)pLysS.



Scheme 1

MALDI-TOF mass spectrometry, gel filtration, and denaturation experiments showed that CHO is a dimer of identical subunits of 60.6 kDa, each monomer containing covalently-bound FAD. The UV-visible absorbance spectrum of CHO showed maxima at 372 nm and 453 nm, suggesting that the enzyme as purified contained a mixture of oxidized and anionic semiquinone flavin species. Aerobic treatment with dithionite of the enzyme as purified resulted in the formation of an air-stable anionic flavin semiquinone, with maximal absorbance at 373 nm and 489 nm. This enzyme-bound semiquinone flavin emitted light at 455 nm (with λ_{ex} at 373 nm). Steady state kinetic data indicated that the product of the reaction, glycine-betaine, was a competitive inhibitor with respect to choline. Kinetic data with choline and the substrate analogs N,N-dimethylethanolamine and 2-methylethanolamine showed that the V/K values for the substrate decreased monotonically with decreasing number of methyl groups, suggesting that hydrophobic interactions between the substrate quaternary amine and the enzyme play a significant role in catalysis. (This study was supported in part by Grant PRF #37351-G4 from the American Chemical Society).

Mechanism of Inactivation of Microsomal Stearoyl-CoA Desaturase by Sterculic Acid

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Stearoyl-CoA desaturase (SCD) is a non-heme diiron enzyme that catalyzes the O₂- and NAD(P)H-dependent desaturation of saturated fatty acyl-CoAs. One reaction catalyzed by SCD is the desaturation of stearoyl-CoA (Figure 1A) to produce oleoyl-CoA, which is important in maintaining cellular homeostasis.

Sterculic acid (8-(2-octyl-1-cyclopropenyl) octanoic acid, Figure 1B) is one of the cyclopropene fatty acids (CPFA) commonly found in the seed oils from plants of the order *Malvales*. In sterculic acid, a highly strained cyclopropene ring is located between carbons 9 and 10. The presence of this functional group has been associated with inhibition of SCD both *in vivo* and *in vitro*.

We previously determined that sterculic acid directly inhibited SCD activity in 3T3-L1 adipocytes without affecting the processes required for adipocyte differentiation, *scd* gene expression or SCD protein translation. Moreover, the cyclopropane fatty acid analog cyclo-9,10-18:0 (Figure 1C) failed to inhibit SCD, suggesting that the cyclopropenyl ring is an essential contributor to the inhibition of SCD.

In the present study, we used liver microsomes from mice fed a high-carbohydrate diet as an enriched source of SCD to study its inhibition by sterculic acid in more detail. Our results showed that the inactivation of SCD required catalysis as evidenced by the strict dependence for NADH. The inactivation rate was first-order with respect to time, the rate was dependent on the concentration of sterculic acid converted to the CoA derivative (sterculoyl-CoA) and the simultaneous presence of 18:0-CoA slowed the inactivation. Western blot analysis revealed that the electrophoretic mobility of the inactivated SCD protein was altered, and that ³H⁺ from solvent could be incorporated into the modified protein.

Taken together these results suggest that reaction with sterculoyl-CoA leads to a turnover-dependent inactivation of SCD that may also be associated with covalent modification. Potential mechanisms for inactivation are discussed in the framework of the emerging paradigm for diiron enzyme reactivity.

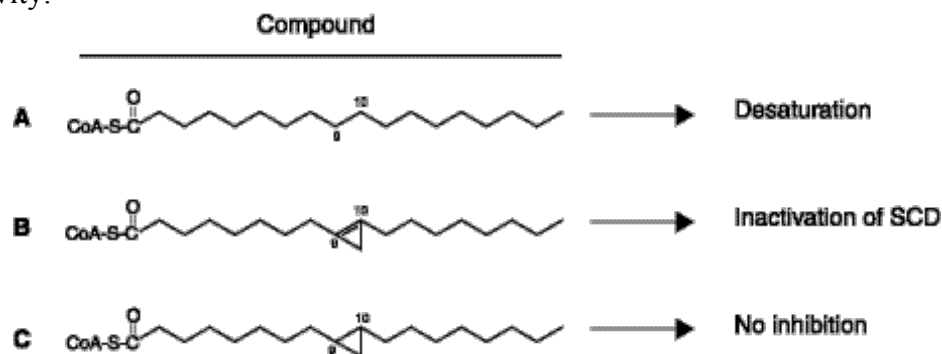


Figure 1. Chemical structures of the Coenzyme A derivatives of stearic acid (A), sterculic acid (B) and 9,10-cyclo-18:0 (C).

Seed specific expression of variants of anthranilate synthase in transgenic plants results in enhanced levels of the essential amino acid tryptophan

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Soybeans represent a valuable source of protein for both human and animal nutrition. Although present in large amounts, the protein in soybeans does not contain a balanced proportion of dietary essential amino acids. One strategy to increase the tryptophan content in plants is to make the tryptophan biosynthetic pathway less sensitive to end-product inhibition, which is controlled through anthranilate synthase (AS). Anthranilate synthase catalyzes the first committed step in the biosynthesis of tryptophan, and is typically found as a two subunit enzyme. The alpha subunit catalyzes the conversion of chorismate to anthranilate with ammonia as a co-substrate. The beta subunit is an amidotransferase that provides ammonia to the alpha subunit from glutamine. A gene previously identified from the C28 mutant maize line encoding a tryptophan feedback resistant AS alpha subunit was transformed into Arabidopsis and soybean for expression in the seed, and directed to the chloroplast using a transit peptide. The resulting seed from positively transformed plants had enhanced tryptophan levels in the range of 1000-4000 ppm compared to 100-200 ppm for non-transformed seed. In addition to the mutant maize alpha subunit, the AS gene of *Agrobacterium tumefaciens* (agro) was cloned through sequence homology, and expressed in *Escherichia coli*. The agro AS is unique for plant and microbial anthranilate synthases in that it contains both the chorismate conversion and glutamine amidotransferase activities on a single polypeptide. However, agro AS is also subject to feedback inhibition by tryptophan. Variants showing less sensitivity to feedback inhibition were rationally designed and constructed based on published structural information on a two subunit AS. The wild type agro AS gene was expressed in Arabidopsis and soybean seed, directed to the chloroplast, and showed tryptophan levels in the range of 2000-8000 ppm. Enzyme activities were 30-60 fold higher in transgenic seed than in controls. Transforming plants with one of the variant deregulated genes enhanced free tryptophan levels further, leading to tryptophan seed content of >10,000 ppm.

An Electrochemical Cell for Redox Titrations of Low-Potential Proteins

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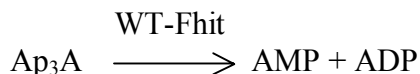
An electrochemical apparatus has been constructed for electrochemical titrations of low-potential proteins that can be analyzed by multiple methods; EPR and UV-Vis spectroscopy are the present designs. The apparatus is capable of very low potential reductions (-900 mV vs. Ag/AgCl electrode [$E^{\circ'} = -220$ mV vs. NHE]) and is designed with multiple interests in mind including ease of use, purging of oxygen, and sample transfer into the appropriate cell. Two small molecules, methyl viologen ($E^{\circ'} = -440$ mV vs. NHE) and 4,4'-dimethyl-1,1'-trimethylene-2,2'-bipyridyl ($E^{\circ'} = -648$ mV vs. NHE), were successfully reduced and their UV-Visible spectra obtained. They are currently in use for analysis of equipment and preliminary experiments. They are also possible candidates for mediators for two low-potential proteins that we are studying in the lab: lysine 2,3-aminomutase and UDP-galactose 4-epimerase. This equipment should be successful in electrically reducing low potential proteins and analyzing them through multiple means.

The Use of H96G-Fhit to Synthesize Dinucleotide Triphosphates and Their Analogues

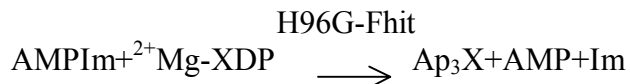
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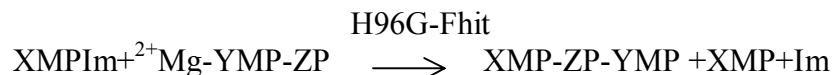
Fhit (fragile histidine triad) protein, encoded by the *FHIT* putative human tumor suppressor gene, catalyzes the hydrolysis of diadenosine 5',5'''-P¹,P³-triphosphate (Ap₃A) to give products AMP and ADP. The mechanism of this reaction is a double replacement mechanism through a covalent Fhit-AMP intermediate according to the information from stereochemical study (Abend A., Garrison P. N., Barnes L. D., and Frey P. A. (1999) *Biochemistry-US*, 38, 3668).



Our recent research revealed that both wild type Fhit and H96G-Fhit proteins can catalyze the hydrolysis of AMP-Imidazole to give AMP and imidazole, however, Ap₃A is not a substrate of the mutant. Most surprisingly, it was found that with the presence of high concentration of ²⁺Mg-XDP (X = A, C, G, U, or T) the mutant could transform AMPIm into Ap₃X in high yields. Because Ap₃X is not substrate of H96G-Fhit, the reaction proceeds in high yield of Ap₃X. Accordingly, Ap₃A, Ap₃U, Ap₃G, Ap₃C, Ap₃T were synthesized with yields of 76.3%, 73.4%, 63.1%, 74.6%, 66.9%, respectively.



To expand the use of this mutated enzyme, UMPIIm was investigated as a substrate of H96G-Fhit. The results showed that UMPIIm mimicked the role of AMPIm in the reaction with H96G-Fhit to synthesize Up₃X. So, it was believed that with GMPIm, CMPIm and TMPIm as substrates, Gp₃X, Cp₃X and Tp₃X could be synthesized by H96G-Fhit enzyme. Therefore, all of the Xp₃Y (X and Y are normal bases, same or different) type compounds can be synthesized by use of H96G-Fhit and different substrates. In the other hand, If XDP changed to its analogue, such as ADP to AMP-CP, all of XMP-ZP-YMP (X and Y are normal bases, and Z could be C, S, N, O etc.) compounds can be synthesized.

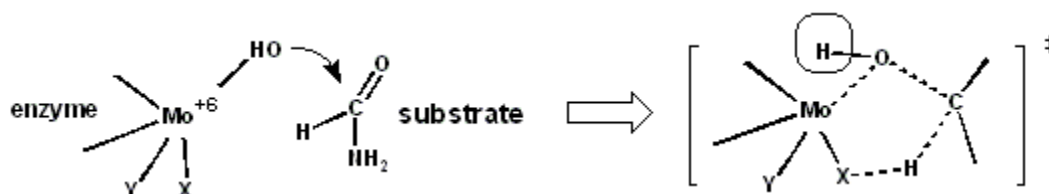


A Model for Dissociative Transition State at an Mo-pterin Active Site.

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In the xanthine oxidase class of Mo- and W-pterin oxidoreductases, experiment and molecular modeling identify a catalytically active oxygen ligand in all relevant oxidation states: $\text{Mo}^{+6} \rightarrow \text{Mo}^{+4} \rightarrow \text{Mo}^{+5}$. The degree of protonation of the ligand, Mo-OH_2 , Mo-OH , Mo-O , determines, among other things, the effectiveness of the non-specific initial nucleophilic attack of the enzyme on the substrate carbon atom:



First principles electronic structure calculations, using the Mo-enedithiolate : formamide model [Ilich & Hille, *J. Am. Chem. Soc.*, **2002**, 124, 6796; *J. Phys. Chem. B*, **1999**, 103, 5406], suggest a concerted deprotonation of the transition state and a lower reaction barrier due to the differences in solvation energies between the protonated monoanion Mo-OH and the deprotonated dianion Mo-O forms of the Mo-enedithiolate : formamide transition state. A coarse bracketing, based on calculated proton affinities, places the enzyme Mo-OH Broensted site in the transition state well below the pK_a of amino acid residues suggesting likely proton acceptors of the dissociative transition state.

Characterization of *Escherichia coli* Cyclopropane Fatty Acid Synthase Mechanism using Seleno- and Telluro- Analogs of S-Adenosyl-L-methionine as the Methylene Donor

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Cyclopropane fatty acid (CFA) synthases catalyze the formation of cyclopropane rings in the acyl chains of phospholipid unsaturated fatty acids. These modifications are found in a number of protozoa and bacteria, including *Mycobacterium tuberculosis*, *Escherichia coli*, and *Helicobacter pylori*. In *E. coli*, the modification has been proposed to protect the organism from acid shock. In slow-growing pathogenic species of mycobacteria such as *M. tuberculosis* and *M. leprae*, the modification is located on α -branched, long-chain ($\sim C_{70}$ - C_{90}) fatty acids called mycolic acids, which are covalently linked to the cell wall, and which aid the bacterium in resisting common antibiotics and chemotherapeutic agents. In *M. tuberculosis*, the modification is required for persistence and virulence, indicating that the enzyme that catalyzes its synthesis might prove to be a valuable drug target.

The CFA synthase reaction involves the formal transfer of a methylene group from the methyl moiety of S-adenosyl-L-methionine to an unactivated cis olefin of an unsaturated fatty acid, producing one equiv of S-adenosyl-L-homocysteine and H^+ per cyclopropane ring formed. The reaction is notable from the perspective of the two substrates involved. The enzyme itself is soluble, as is the S-adenosyl-L-methionine substrate; however, the fatty acid substrate is sequestered from the aqueous environment. Our lab has cloned and expressed the CFA synthase from *E. coli*, and has initiated a detailed kinetic analysis of the reaction. We have also synthesized the selenium and tellurium analogs of S-adenosyl-L-methionine and used these compounds as the methylene donors for CFA synthase to characterize the mechanism by which this novel reaction takes place.

Studies of the Interaction of *Streptomyces avermitilis* (4-hydroxyphenyl)pyruvate Dioxygenase with the Specific Inhibitor NTBC

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(4-Hydroxyphenyl)pyruvate dioxygenase (HPPD) is a non-heme Fe(II) enzyme that catalyzes the conversion of (4-Hydroxyphenyl)pyruvate (HPP) to homogentisate as part of the tyrosine catabolism pathway. Inhibition of HPPD by the triketone, NTBC, is used to treat Type I tyrosinemia, a rare but fatal defect in tyrosine catabolism. Although triketones have been used for many years as HPPD inhibitors for both medical and herbicidal purposes, the mechanism of inhibition is not well understood. The following work provides mechanistic insight into NTBC binding.

The tautomeric state of NTBC in solution at pH 7.0 has been verified as containing a single enol by NMR spectroscopy. NTBC binds to HPPD.Fe(II) as evidenced by a visible binding feature centered at 450 nm. The rate of binding of NTBC to HPPD.Fe(II) was measured on a stopped-flow spectrophotometer at 450nm and shown to have a hyperbolic dependence on NTBC concentration. ($K_{ntbc} = 1.25 \pm 0.08$ mM, $k_{limit} = 8.2 \pm 0.2$ s⁻¹) There is an isotope effect on the binding kinetics when D₂O is used as the solvent. ($k_h/k_d = 1.54$) It is therefore proposed that the Lewis-acid assisted conversion from iron(II) bound enol to enolate is the irreversible step k_{limit} .

Although the native enzyme without substrate reacts with molecular oxygen, HPPD.Fe(II).NTBC does not. The binding feature does not decrease over the course of two days when exposed to atmospheric oxygen. This means that not only does the HPPD.Fe(II).NTBC complex not oxidize, but also that the off rate for NTBC is essentially zero as any HPPD.Fe(II) would readily oxidize in the presence of oxygen. The NTBC bound enzyme also doesn't react with the oxygen mimic, NO. EPR spectroscopy has shown that only two percent of HPPD.Fe(II).NTBC forms an NO complex as compared to the holoenzyme.

***In vivo* and *in vitro* Assays for Inhibitors of Prolyl 4-Hydroxylase**

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Collagen is the most abundant protein in animals. The hydroxyl groups of the prevalent 4(*R*)-hydroxyproline residues contribute greatly to the conformational stability of collagen. These hydroxyl groups arise from the post-translational modification of proline residues by prolyl 4-hydroxylase (P4H), an α -ketoglutarate-dependent nonheme iron oxygenase. Inhibitors of P4H diminish collagen stability and could be used to treat scarring, fibrosis, and fibroproliferative disorders in humans. We have developed a sensitive *in vivo* assay for bioavailable inhibitors of P4H that uses the nematode *Caenorhabditis elegans*. A *C. elegans* mutant that lacks one of the two genes that encode P4H is viable, but its progeny die when exposed to low levels of P4H inhibitors. By using this assay to screen putative inhibitors. We have discovered a novel inhibitor that binds covalently to P4H. We have also developed an HPLC-based assay that utilizes recombinant P4H enzyme and a fluorescent tetrapeptide substrate analog. Finally, we have made progress in the production of active human P4H in *E. coli*.

Intramolecular Dephosphorylation and ERK Dimer Dissociation by MKP3

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The dual specificity mitogen-activated protein kinase phosphatase MKP3 has been shown to down-regulate mitogenic signaling through dephosphorylation of extracellular signal-regulated kinase (ERK). MKP3 consists of a noncatalytic N-terminal domain and a catalytic C-terminal domain. ERK binding to the N-terminal noncatalytic domain of MKP3 has been shown to increase catalytic activity of MKP3 up to 100-fold (1-4). Here, we investigated the fundamental question of how MKP3 dephosphorylates the physiological substrate pERK (phosphorylated at threonine 183 and tyrosine 185). We addressed the role of the N-terminal domain of MKP3, inter vs. intramolecular dephosphorylation of pERK, and stoichiometry of MKP3/pERK complex since pERK is known to be a dimer (5). Using a MKP3/VHR chimera protein (the N-terminal noncatalytic domain of MKP3 attached to VHR) and ERK mutants, we showed that the N-terminal domain of MKP3 increases the effective substrate concentration leading to an intramolecular dephosphorylation of pERK, that is, substrate pERK binds to the N-terminal domain of MKP3 and is dephosphorylated by the catalytic domain of the same MKP3. We also showed that MKP3 forms a heterodimer with pERK monomer using cross-linking studies and analytical ultracentrifugation.

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Taurine/ α -ketoglutarate Dioxygenase, a Nonheme Iron Hydroxylase, Self-hydroxylates a Tyrosine Residue in the Absence of Primary Substrate to Generate an Fe(III)-catecholate Complex

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Taurine/ α -ketoglutarate dioxygenase (TauD) belongs to a class of oxygen-activating, mononuclear nonheme iron enzymes which employ a 2-His-1-carboxylate motif to carry out a diverse range of metabolically important reactions that have environmental, pharmaceutical, and medical significance.^{1,2,3,4} Specifically, TauD catalyzes the hydroxylation of taurine (2-aminoethanesulfonic acid) to generate sulfite and aminoacetaldehyde in the presence of oxygen, α -ketoglutarate (α KG), and Fe(II).⁵ Initially, α KG displaces two water ligands as it chelates the ferrous center.⁶ Taurine then binds to the active site, promoting loss of the final water ligand to create a binding site for oxygen, which becomes activated for insertion into taurine.⁷ Taurine-free α KG-Fe(II)TauD reacts with oxygen to form a 550 nm species that has resonance Raman features characteristic of an Fe(III)-catecholate complex, which presumably arises from the self-hydroxylation of an active site tyrosine residue.⁸ Significantly, ¹⁸O studies reveal that the inserted oxygen atom derives from solvent rather than O₂, which lends credence for the involvement of an Fe(IV)=O intermediate. Interestingly, a 720 nm species arises from O₂- (or H₂O₂-) dependent self-hydroxylation of TauD in the absence of α KG but requiring its decarboxylated product succinate, and resonance Raman studies again confirm the generation of an Fe(III)-catecholate complex.⁹ Furthermore, the 550 nm and 720 nm species interconvert by the removal or addition of bound bicarbonate, suggesting that the α KG-derived CO₂ remains bound to the metal site as bicarbonate.

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Substrate Specificity of Transpeptidation Reactions of the *Streptomyces* R61

DD-peptidase

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Bacterial DD-peptidases are responsible for the final peptidoglycan cross linking step in bacterial cell wall biosynthesis. The ability of one of such enzyme, the DD-peptidase of *Streptomyces* R61, to perform transpeptidation was studied. The donor was a highly specific fragment of the cell wall precursor (glycyl-L- α -amino- ϵ -pimelyl-D-Ala-D-Ala) and a range of amino acids and peptides resembling the natural acceptor were tested for the acceptor role. Gly, D-Leu, D-norLeu, Gly-L-Ala and Gly-L-Phe acted as good acceptors. Surprisingly, hardly any transpeptidation product was observed with Gly-L-Met, Gly-L-norLeu, Gly- ϵ -N-Acetyl- L-Lysine and Glycyl-L- α -amino- ϵ -pimelyl-D-Ala-D-Ala itself. Also, no transpeptidation product was observed for D-Ala-L-Ala. The latter result implies that orientation of D-amino acids and Gly-L-amino acids in the acceptor site is different.

Probing the Roles of Active-Site Lysine Residues and N-terminal Pyroglutamate in Onconase, an Enzyme with Potent Antitumoral Activity

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Onconase, a member of the ribonuclease A (RNase A) superfamily from the Northern leopard frog, is currently in phase III clinical trials for the treatment of malignant mesothelioma (an asbestos related lung cancer). Similar to RNase A, onconase possesses a conserved active-site catalytic triad composed of His10, Lys31, and His97. The crystal structure of onconase suggests that two additional residues, Lys9 and a cyclized N-terminal glutamine residue (Pyr1; pyroglutamate), may play a role in catalysis. To test the role of Pyr1, Lys9, and Lys31, we replaced each residue with alanine, and determined the kinetic parameters of each variant with novel fluorogenic substrates. Binding assays with a non-hydrolyzable substrate analog indicate that Lys9 and Lys31 contribute little to substrate-binding affinity in the ground state ES complex. K9A and K31A onconase, however, displayed 10³-fold lower $k_{\text{cat}}/K_{\text{M}}$ values compared to wild-type, and a K9A/K31A double mutant enzyme exhibited more than 10⁴-fold reduction in catalytic activity. In addition, removing Lys9 and Lys31 from the active site eliminated the antitumoral activity of onconase. Substituting alanine for Pyr1 lowered the catalytic activity of onconase by 20-fold. Deletion of Pyr1 from a K9A variant enzyme did not further reduce catalytic activity, suggesting that the primary function of N-terminal pyroglutamate is to anchor Lys9 through a hydrogen bond, thereby positioning Lys9 for optimal catalysis.

CofE [F₄₂₀-0: L-glutamate γ -ligase (GDP-forming)] is involved in coenzyme F₄₂₀ biosynthesis in *Methanococcus jannaschii*

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The protein product of the *Methanococcus jannaschii* MJ0768 gene has been expressed in *Escherichia coli*, purified to homogeneity, and was shown to be involved in coenzyme F₄₂₀ biosynthesis. The protein catalyzes the GTP dependent addition of two L-glutamates to the L-lactyl phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (F₄₂₀-0) to form F₄₂₀-2. Since the reaction is the fifth step in the biosynthesis of coenzyme F₄₂₀, the enzyme has been designated as CofE, the product of the *cofE* gene (MJ0768). CofE, with an apparent molecular mass of 52 k Da, has no recognized sequence similarity to any previously characterized proteins. The enzyme has absolute requirement for a divalent metal ion, of which is best satisfied by Mn²⁺, and for a monovalent cation, of which K⁺ is the most effective. CofE catalyzes amide bonds formation with the cleavage of GTP to GDP and Pi, likely involving the activation of the free carboxylate group of F₄₂₀-0 to give an acyl phosphate intermediate. The reaction mechanism is proposed and compared to the members of the ATP-dependent amide bond ligase family.

Probing the Catalytic Reaction of Medium-chain Acyl-CoA Dehydrogenase with 2-Fluorooctanoyl-CoA

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Acyl-CoA dehydrogenases (ACDs) catalyze the first step of the β -oxidation cycle, in which saturated fatty acyl-CoA substrate is oxidized to trans-enoyl-CoA product. The catalysis is believed to be initiated by deprotonating the pro-R proton at the α -carbon of the fatty acid by the GLU376 at enzyme backbone and transferring the β -hydride to the flavin. It is not clear if these two steps are asynchronously concerted or step-wise.

We examine this seemingly thermodynamically unfavorable reaction with medium-chain acyl-CoA dehydrogenase isolated from pig kidney and artificial substrates. Fluorinated substrate analogs, where one of the α -protons is selectively replaced with a fluorine atom, are used to study the nature of the reaction mechanism. Proton exchange experiments, conducted by ^{19}F -NMR technique, are used to estimate the capability of the α -proton to be removed from each of the isomers, S-2-fluorooctanoyl-CoA and R-2-fluorooctanoyl-CoA. Results from redox chemistry are also presented. (Supported by NIH grant GM29344)

Cloning, Expression, and Purification of Acyl Carrier Protein and a Putative Soluble Diiron Fatty Acid Desaturase from *Mycobacterium Tuberculosis* H37Rv

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Mycolic acids are α -alkyl, β -hydroxyl fatty acids that comprise the “waxy” layer in the cell envelope of mycobacteria. The β -hydroxyl substituent of mycolic acid can contain up to 90 carbons and exhibit diverse functionalities such as desaturation, cyclopropanation, epoxidation, ketonization, hydroxylation and methoxylation. Currently, the desaturation steps, which likely precede insertion of other fatty acid modifications and chain elongation, have not yet been characterized. Analysis of the *M. tuberculosis* genome revealed three potential fatty acid desaturases. Two of these fatty acid desaturases, DesA1 and DesA2, share sequence similarity with the soluble diiron acyl-ACP desaturases, whereas the third desaturase, DesA3, is similar to the diiron integral membrane desaturases. DesA2, one of the two putative soluble fatty acid desaturases, has been cloned, expressed, and purified. Initial structural and spectroscopic characterization of this putative desaturase is currently underway. In addition, an acyl carrier protein, AcpM, which is the proposed fatty acid carrier during mycolic acid biosynthesis, has also been cloned, expressed and purified. Methods for synthesizing long-chain acyl-AcpM substrates to probe the specificity of DesA2 are currently being developed. These studies will increase our understanding of early steps in the biosynthesis of mycolic acids.

Funded by NIH GM50853

Biosynthesis of Pyrroloquinoline Quinone: Preliminary Characterization of PqqE from *Klebsiella pneumoniae*

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Pyrroloquinoline quinone (PQQ) is a redox-active coenzyme used by certain bacterial enzymes, such as methanol- and glucose dehydrogenases. The biosynthesis of PQQ is an intriguing process, whereby all carbon and nitrogen atoms of the cofactor are derived from two amino acids - tyrosine and glutamate. However, genetic evidence strongly suggests that a peptide containing highly conserved Glu and Tyr residues serves as a precursor for PQQ. The mechanism(s) by which the processing of this peptide yields the active cofactor is currently unknown. Our goal is to elucidate the enzymatic steps involved in PQQ biosynthesis, and we are currently working towards characterizing all the gene products in this pathway. There are six genes in the PQQ operon of *Klebsiella pneumoniae*, *pqqA-F*. This study describes the subcloning, expression, purification and preliminary spectroscopic characterization of PqqE. PqqE shares sequence homology with a recently discovered superfamily of enzymes that utilize *S*-adenosylmethionine and an iron-sulfur cluster to initiate radical chemistry.

Analysis of Radical Triplet States in Coenzyme-B₁₂ Dependent Enzymes

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Contribution from the Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53705 and Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

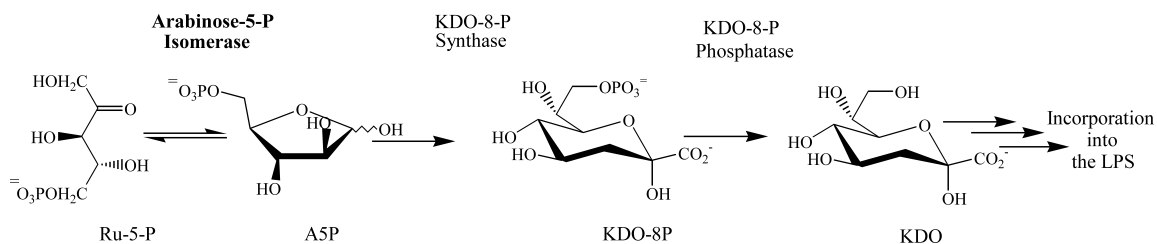
Abstract: The EPR spectra of strongly coupled triplet states in representative coenzyme B₁₂-dependent systems are analyzed. The experimental spectra are resolution enhanced using Fourier filtering methods and then simulated using a program that sets up and diagonalizes the generalized spin-Hamiltonian, obtaining the exact resonant frequencies and probabilities of the EPR transitions, without any assumptions of collinearity of the principle axis of interaction terms. A method for the acquisition of structural information from the EPR spectra through the decomposition of the electron-spin – electron-spin dipolar interaction tensor using data from electronic structure calculations and a simulated annealing algorithm is then presented and applied to the analysis of these spectra.

D-Arabinose-5-Phosphate Isomerase from *E. Coli*

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The rise in resistant pathogenic Gram negative bacterial strains necessitates the development of more potent antibiotics. With the observed increase in cross-resistance, there is a need for new antimicrobial agents with novel targets and thus no preexisting resistance. Enzymes in the biosynthetic pathway of 3-deoxy-D-manno-octulosonate (KDO) are attractive targets for the development of new Gram negative specific antibiotics. KDO is an essential component of the lipopolysaccharide (LPS) layer, which is located on the cellular surface of the outer membrane of virtually all Gram negative bacteria. The eight-carbon sugar provides a link between the outer membrane embedded lipid A and the O-antigen, a polysaccharide chain of varying composition which determines the antigenic specificity of the cell. Cells that are defective in KDO biosynthesis and hence have a compromised LPS are usually less pathogenic and more susceptible to antibiotics. D-Arabinose-5-phosphate (A5P) isomerase catalyzes the reversible isomerization of the pentose pathway metabolite D-ribulose-5-phosphate (Ru5P) to A5P, the first unique intermediate in the KDO biosynthetic pathway. A5P isomerase is the sole source of A5P in Gram negative bacteria and inhibition should lead to depletion of intracellular A5P pools, thus blocking the downstream production of KDO. It is reasonable to speculate that A5P isomerase inhibition will have similar cellular affects as direct KDO synthase inhibition, thus providing another viable Gram negative antibiotic target from the KDO pathway. The identification, overexpression, purification, and characterization of A5P isomerase from *Escherichia coli* will be presented.



A Genetic Selection System for Human Hexokinase Activity

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Hexokinase catalyzes the ATP-dependent phosphorylation of glucose in the first step of glycolysis. The open, twisted α/β scaffold of hexokinase is shared by a number of enzymes whose activities are coupled to ATP hydrolysis through protein conformational changes. Enzymes that belong to the hexokinase superfamily include actin, the hsc70 chaperone, O-sialoglycoproteases, and many sugar kinases. To evaluate the robustness of the hexokinase scaffold we have developed a genetic selection system for human hexokinase activity in *Escherichia coli*. Disruption of the bacterial genes encoding glucokinase and the phosphoenolpyruvate:carbohydrate phosphotransferase system yielded strain BM5340, which is incapable of utilizing glucose as a sole carbon source. Heterologous production of a passive glucose transporter from *Zymomonas mobilis* in combination with human hexokinase rescues the inability of strain BM5340 to grow on glucose minimal media. Our novel complementation scheme affords the selection of individual active hexokinase molecules from a library of more than 10 million distinct variants.

Demonstration of the *meta*-Hydroxylation of Toluene by Engineered Active Site Isoforms of Toluene 4-Monooxygenase

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The aerobic catabolism of toluene by soil microorganisms proceeds by initial reactions of iron-containing monooxygenases, dioxygenases, or benzylic hydroxylases. All of the known toluene monooxygenases are members of the soluble diiron hydroxylase superfamily, and naturally evolved isoforms with regioselectivity for *ortho*- and *para*-hydroxylation have been demonstrated (1, 2). For example, the natural toluene 2-monooxygenase from *Pseudomonas stutzeri* OX1 gives ~50% of *o*-cresol, while the natural toluene 4-monooxygenase from *Pseudomonas mendocina* KR1 gives 97% of *p*-cresol.

Since the methyl group provides an *ortho/para* directing substituent effect during electrophilic aromatic substitution, the characterization of an enzyme with high specificity for *meta*-hydroxylation of toluene could provide unique insight into the reaction mechanism and origin of regiospecificity in these enzymes (3).

Previous studies of a 10 kb DNA fragment from *Pseudomonas picketti* PK01 using a heterologous *Pseudomonas* expression host suggested the presence of a toluene 3-monooxygenase based on the accumulation of *m*-cresol in culture fluids (4). However, this presentation shows that expression of the subcloned 5.1 kb *tbu* gene cluster in an *Escherichia coli* host incapable of metabolizing cresol isomers or benzyl alcohol gave a product distribution of 82% *p*-cresol, 10% *m*-cresol, 4% *o*-cresol, and 4% benzyl alcohol. Thus the *tbu* gene cluster encodes a relaxed specificity *para*-monooxygenase instead of the previously reported *meta*-monooxygenase. Possible reasons for this faulty assignment are considered.

In the past few years, we have undertaken an extensive mapping of active site residues in T4moH (2) and found several active site positions that contribute to the regiospecificity of hydroxylation even as other catalytic parameters remain essentially unchanged (k_{cat} , k_{cat}/K_M , coupling). Thus the G103L isoform of T4moH is predominantly a toluene 2-monooxygenase (55% *o*-cresol) with regiospecificity, k_{cat} , and k_{cat}/K_M comparable to naturally occurring *ortho*-monooxygenases (2). Here we show that the G103L/F205I and I100P isoforms of T4moH function as toluene 3-monooxygenases, giving 59% and 68% of *m*-cresol, respectively. Based on these results, catalytically competent isoforms of T4moH hydroxylase that give *ortho*-, *para*-, and *meta*-hydroxylation of toluene have now been demonstrated. These results suggest relatively few residues near to the E104 ligand of canonical Fe_A serve as the locus for control of regiospecificity in aromatic hydroxylation.

Funded by NSF MCB 9733734.

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Catching Catalysis in the Act: Using Single Crystal Kinetics to Trap Reaction Intermediates.

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Methylamine dehydrogenase (MADH) is an $\alpha_2\beta_2$ heterotetramer containing a novel quinone cofactor, TTQ, derived from two modified tryptophan residues. It is expressed in response to methylamine, allowing certain bacteria to utilise methylamine as their sole carbon source. MADH catalyses the conversion of methylamine to formaldehyde and ammonia, leaving the TTQ cofactor in a 2e⁻ reduced state. To complete the catalytic cycle, MADH is reoxidised via two successive electron transfer (ET) events.

In the case of the *Paracoccus denitrificans* enzyme (PD-MADH) the physiologic ET chain involves the protein redox partners amicyanin (a blue-copper protein) and cytochrome *c551i*. Stable binary (PD-MADH/amicyanin) and ternary (PD-MADH/amicyanin/cytochrome *c551i*) catalytically competent complexes can be formed and crystallized, and their structures have been solved to better than 2.0 Å resolution in the laboratory of F. Scott Mathews (Washington University Medical School, St. Louis).

MADH (TTQ), amicyanin (Cu) and cytochrome *c551i* (Fe) have spectral features in the visible region that change during catalytic turnover, thus defining spectrally distinct intermediates that reflect the electron distribution in the complex.

Through a novel combination of single crystal visible microspectrophotometry, X-ray crystallography and freeze trapping, reaction intermediates of MADH in complex with the physiological redox partners in the crystalline state have been trapped.

This poster will present the methods used to monitor and trap reaction intermediates in the crystalline state, as well as some preliminary X-ray structural data.

Structural Studies of the Glycosyltransferase GtfA from the Antibiotic Chloroeremomycin Biosynthetic Pathway.

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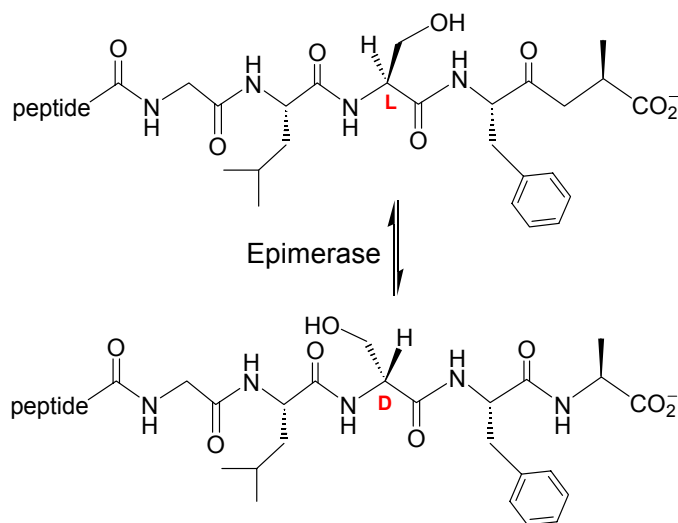
The bioactivity of vancomycin group glycopeptide antibiotics is dependent in part on the number, identity and position of sugar moieties added during biosynthesis. Each glycosylation step is performed by one in a series of homologous glycosyltransferases (GTFs), utilizing an NDP-hexose substrate as the sugar donor. Genetic manipulation of these GTFs to alter the kind of the sugar moiety transferred may allow for the design of new antibiotics with enhanced potency or novel activity. Among this family of enzymes, GtfA is responsible for the transfer of a 4-epi-vancosamine moiety to residue 6 of the heptapeptide scaffold during the final stages of chloroeremomycin biosynthesis. We have determined the X-ray crystal structure of GtfA, as well as of the ternary complex with its acceptor substrate and a dinucleotide ligand, at 2.8 Å resolution. As expected, GtfA is structurally homologous to the structure of GtfB, which transfers a D-glucose moiety to residue 4 of the heptapeptide scaffold [1]. However, the structure of GtfA clearly reveals the locations of the binding sites for the NDP-hexose and aglycone substrates, the conformational changes that accompany ligand binding, and the potential residues involved in catalysis and substrate recognition.

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Dehydroalanine-Based Inhibition of a Spider Venom Peptide Epimerase

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The venom of the funnel web spider *Agelenopsis aperta* contains two peptide toxins that share identical sequences and differ only by the stereochemistry at Ser-46. Interestingly, the venom also contains an enzyme responsible for interconverting these epimeric peptides.¹ Because enzymatic epimerization of peptides is otherwise unknown, the mechanism by which this occurs was investigated. Various substrate analogues have been synthesized, in which the functional groups about the epimerizable serine were modified.² One of these, containing chlorine in place of the hydroxyl, was found to undergo enzyme-catalyzed elimination of HCl to generate a dehydroalanine derivative. This dehydroalanine peptide was independently synthesized and found to be a potent inhibitor of the epimerase, exhibiting a sub-micromolar IC₅₀. These results support a deprotonation-reprotonation mechanism that proceeds through an enolate intermediate. The planarity of this intermediate is apparently mimicked by the *sp*² character of the α -carbon of the dehydroalanine, resulting in the observed inhibition.



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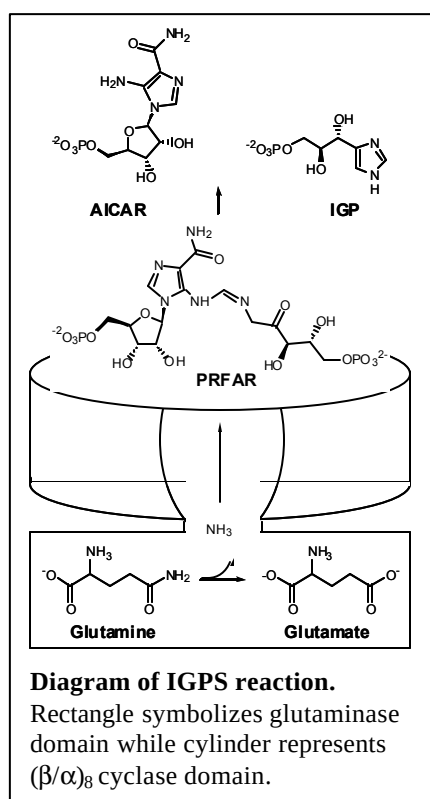
**IMIDAZOLE GLYCEROL PHOSPHATE SYNTHASE:
COMMUNICATION OF ACTIVE SITES THROUGH A $(\beta/\alpha)_8$ BARREL**

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Glutamine amidotransferases (GATs) utilize the amide nitrogen of glutamine to incorporate ammonia into various metabolites including nucleotides, amino acids, amino sugars. Imidazole glycerol phosphate synthase (IGPS) is a GAT in histidine biosynthesis that catalyzes two carbon-nitrogen ligations with *N*'-[(5-phosphoribulosyl)-formimino]-5-aminoimidazole-4-carboxamide ribonucleotide and an elimination reaction to form 5'-(5-aminoimidazole-4-carboxamide) ribonucleotide (AICAR) and imidazole glycerol phosphate (IGP). There are two active sites in the protein residing in distinct domains; one is the glutaminase and the second the nucleotide cyclase. Catalysis in the two active sites is a temporally-coupled event. The cyclase domain of IGPS is a $(\beta/\alpha)_8$ barrel motif which functions to transport ammonia 30 Å from the glutaminase domain to the nucleotide-binding site through the hydrophobic core of the barrel. *This is the first instance of a $(\beta/\alpha)_8$ barrel being used as a catalytic conduit.* Our focus in analysis of the structure-function of this GAT has been the protein interactions that signal the glutaminase event when PRFAR is bound.

The recent structure of IGPS in complex with PRFAR reveals key substrate-induced changes in the cyclase active site that are transmitted through the core beta strands to invariant residues at the domain interface.



The PRFAR binding residues, D245 and K258 on β 1 connect to R239 at the interface. T365, in the nucleotide phosphate-binding site on β 5 is proposed to transmit the substrate-binding signal to D359, a residue that forms an interdomain salt bridge to a residue in the glutaminase active site. These residues identified in the PRFAR binding site and at the domain interface were investigated by site-directed mutagenesis. The mutation K258A in the PRFAR active site disrupts the cyclase activity of the protein, while maintaining the glutaminase signal. At the subdomain interface, the β 1 residue R239, participates in an electrostatic gate to the hydrophobic core of the $(\beta/\alpha)_8$ barrel. The mutant R239A uncoupled the two half reactions to the degree that 122 molecules of glutamine are hydrolyzed for every PRFAR molecule; the cyclase activity is disrupted while glutaminase activity is still functional. The mutation D359A at the interface greatly decreased the glutamine-dependent activity of the protein, while ammonium-dependent activity was relatively unchanged. These data are consistent with the hypothesis that PRFAR binding confers a dual signal to the interface; one to trigger

glutamine hydrolysis through β 5 and D359, and a second signal down β 1 to R239 to open the electrostatic gate to allow ammonia passage to the PRFAR active site.

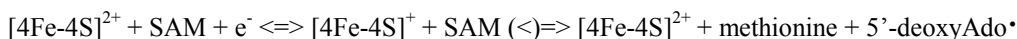
“Radical SAM proteins”: a superfamily? X-ray structure of biotin synthase gives new insights into the S-adenosylmethionine cleavage.

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and Catherine L. Drennan¹

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Over the last few years, several proteins have been shown to carry out radical based chemistry using a [4Fe-4S] cluster and S-adenosylmethionine (SAM) following the depicted reaction:



The resulting deoxyadenosyl radical is then involved in various different reactions such as unusual methylations, isomerizations, sulfur insertion, ring formation, anaerobic oxidation or protein radical formation. Most of these enzymes, such as biotin synthase (BioB), lipoate synthase (LipA), lysine-2,3-aminomutase (KAM) or the protein activases for pyruvate-formate lyase (Pfl) and class III anaerobic ribonucleotide reductase (protein β) were not recognized to be homologous until recently, even though they all exhibit a CxxxCxxC motif shown to be responsible of the coordination for the [4Fe-4S] cluster. However, a recent powerful bioinformatic study suggested that, all these proteins belong to the same family with a common fold called: “Radical SAM proteins”[1]. Over six hundred members were identified, and most of them are currently of unknown function. Nevertheless, BioB, LipA, KAM, Pfl activase and protein β are now the best biochemically and/or spectroscopically characterized members. Recent spectroscopic studies of KAM and Pfl activase suggest two similar but slightly different modes of SAM binding to the [4Fe-4S] cluster [2,3], and led to two different models for its cleavage.

Very recently, the x-ray structure of biotin synthase from *E. coli* was determined in the Drennan laboratory. It gives us new insights into the mechanism of this enzyme and more widely, on the SAM cleavage for all Radical SAM proteins. Based on this structure, we will also examine the definition of radical SAM proteins as a superfamily in addition to revisiting the metal/cluster content for some of these enzymes.

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[2] Cospér *et al.*, *Biochemistry* 2000, **39**, 15668-15673

[3] Walsby *et al.*, *J. Am. Chem. Soc.* 2002, **124**, 11270-11271

Isolation and Characterization of the Lipoyl Transferase from *Escherichia coli*

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Lipoic acid is a key component of several multienzyme complexes that are involved in primary energy metabolism. As a part of these complexes, lipoic acid is responsible for shuttling intermediates between the different enzyme subunits. Two pathways have been identified by which proteins can be lipoylated in *E. coli* [Morris, T.W., et al (1995) *J. Bact.* **177** (1), 1-10]. In the case of the endogenous pathway lipoic acid, in the form of lipoyl acyl carrier protein (ACP), is transferred to its apo-acceptor proteins by a 24 kDa protein designated lipoyl transferase. Under lipoate deficient conditions there is accumulation of octanoylated acceptor protein [Ali, S.T., et al (1990) *Mol. Microbiol.* **4** (6), 943-50], suggesting that octanoyl-ACP can serve as an alternate substrate for the lipoyl transferase. Although the enzymes responsible for transferring lipoic acid to its apo-acceptor proteins have been identified, the mechanism by which the acceptor proteins are lipoylated is not presently understood. The lipB gene, which encodes the lipoyl transferase, was cloned and a purification scheme was devised using hydrophobic, ionic exchange, and size exclusion chromatography to allow us to characterize the lipoyl transferase. An assay has also been developed to allow the determination of the steady-state kinetic parameters of the lipoyl transferase for lipoyl and octanoyl-ACP. Our data suggest that the reaction catalyzed by lipoyl transferase occurs via a sequential mechanism.

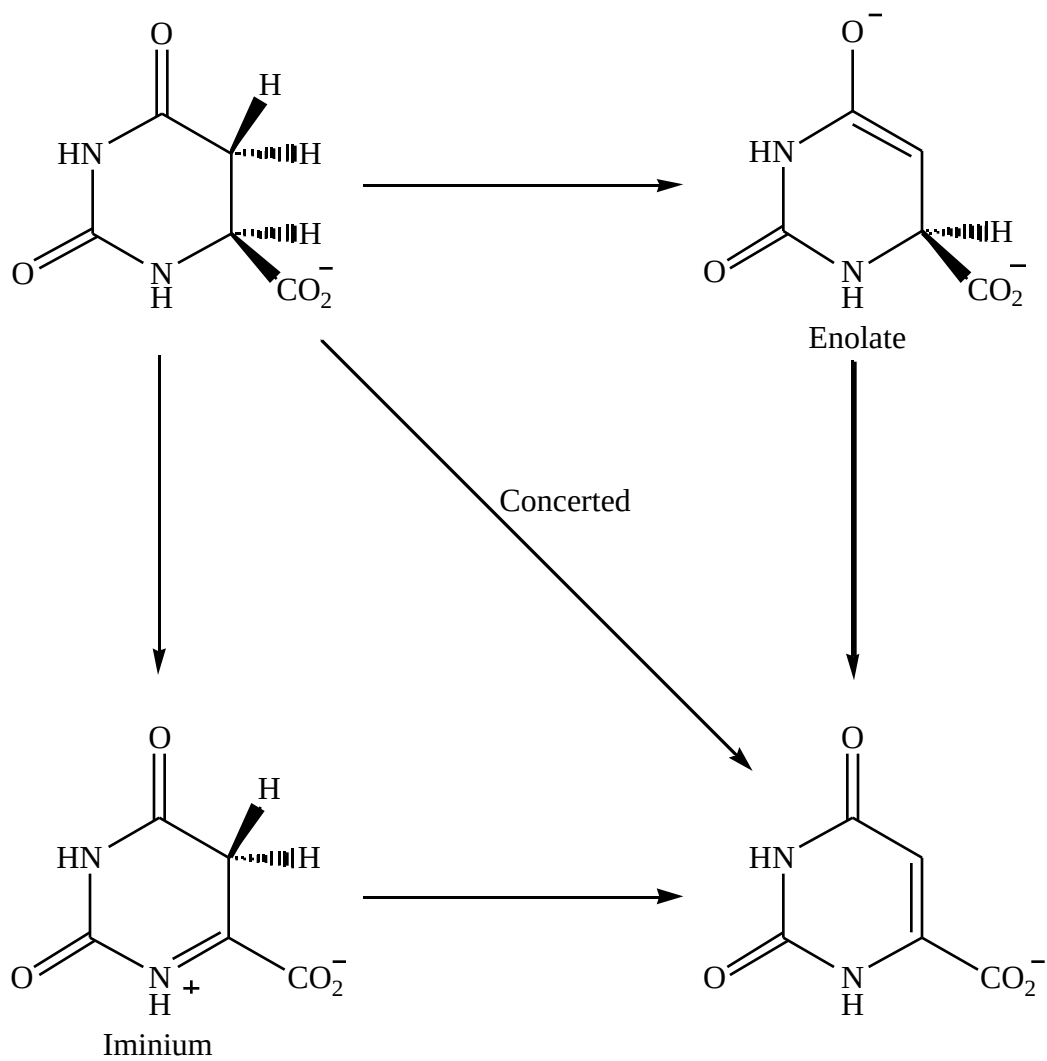
The Mechanism of Flavin Reduction in Class 2 Dihydroorotate Dehydrogenases

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The dihydroorotate dehydrogenases (DHODs) use an FMN prosthetic group to catalyze the conversion of dihydroorotate to orotate, the only redox reaction in pyrimidine biosynthesis. DHODs have been grouped on the basis of sequence similarity into the Class 1A, Class 1B, and Class 2 enzymes. Biochemical properties, such as cellular localization, subunit composition, and oxidizing substrate vary with class. The DHODs from most Gram-negative bacteria and most eukaryotes are Class 2 enzymes. These are membrane-bound and use ubiquinone as the physiological oxidant. We are studying the Class 2 enzymes from *E. coli* and *H. sapiens*. The structures of both of these enzymes have been determined by x-ray crystallography, revealing a high degree of structural similarity, especially at the active site. In both structures, orotate is held roughly parallel to the isoalloxazine of FMN by a ring of hydrogen-bonding residues (four asparagines and a threonine) and a salt-bridge to a lysine, and is covered with a protein loop holding a serine that serves as a base.

We are interested in determining the mechanism that DHODs from each class use to reduce the flavin. The oxidation of dihydroorotate requires the deprotonation of C5 of dihydroorotate and the transfer of a hydride equivalent from C6 of dihydroorotate to N5 of FMN. These two C-H bonds may be cleaved in a concerted or stepwise fashion. We have studied this in anaerobic stopped-flow experiments in the absence of oxidizing substrates, enabling us to directly measure intrinsic rate constants for flavin reduction using *protio*- or *deutero*-dihydroorotate. The kinetic isotope effects obtained with label at C5, C6, or C5 and C6 indicate that the reaction is stepwise, in contradiction to the result obtained for bovine liver DHOD by steady-state kinetics [Hines, V. & Johnston, M. (1989) *Biochemistry*, **28**, 1227-1234]. Our measurements of the pH dependences of the reduction rate constants for the *E. coli* and human enzymes show that a deprotonated group with an apparent pK_a of ~ 9.5 is needed for rapid flavin reduction. Two stepwise mechanisms are being considered: α -deprotonation by the base to form an enolate intermediate that reduces the flavin, or β -hydride abstraction by the flavin to form an iminium cation intermediate that loses a proton to the active site serine. While the enolate mechanism has been considered in the past, the iminium mechanism has not. However, the iminium mechanism may be more compatible with the poor acidity of dihydroorotate ($pK_a \sim 20-21$) and poor ability of serine to function as a base. The 1-carba-1-deaza analog of dihydroorotate (2,6-oxo-4-piperazine carboxylic acid) is a very poor substrate, consistent with the iminium mechanism. (Supported by NIH GM61087.)



Understanding Quinone Cofactor Biogenesis in Methylamine Dehydrogenase.

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Cofactors made from constitutive amino acids in proteins are now known to be relatively common. A number of these involve the generation of quinone cofactors, such as topa quinone in the copper-containing amine oxidases, and lysyltyrosyl quinone in lysyl oxidase. The biogenesis of the quinone cofactor tryptophan tryptophylquinone (TTQ) in methylamine dehydrogenase (MADH) involves the post-translational modification of two constitutive Trp residues in the β subunit (Trp ^{β 57} and Trp ^{β 108}). The modifications to generate TTQ are the addition of two oxygens to the indole ring of Trp ^{β 57}, and the formation of a covalent cross-link between C ϵ 3 of Trp ^{β 57} and C δ 1 of Trp ^{β 108}. The order in which these events occur is unknown. To investigate the role Trp ^{β 108} may play in this process, it was mutated to both a His (β W108H) and Cys (β W108C) residue. For each mutant, the majority of the protein that was isolated contained a biosynthetic intermediate with only one oxygen atom incorporated into Trp ^{β 57}. This biosynthetic intermediate was inactive and also exhibited weaker subunit-subunit interactions than native MADH. However, in each mutant preparation, a small percentage of the mutant enzyme was active and possessed a functional tryptophylquinone cofactor. In the case of β W108C, this cofactor may be identical to cysteine tryptophylquinone recently described in the bacterial quinohemoprotein amine dehydrogenase. In β W108H, the active cofactor is presumably a histidine tryptophylquinone, which has not been previously described, and represents the synthesis of a novel quinone protein cofactor.

We also will present data on the properties of *mauG*, a di heme protein absolutely required for the maturation of MADH, as well as preliminary data on the effects of mutations in *mauG* on the biogenesis of TTQ.

A Mechanistic Investigation of 4-Hydroxyphenylpyruvate Dioxygenase from *Streptomyces avermitilis* Using Substrate Analogs

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Moran

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4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27, HPPD) catalyzes the reaction of 4-hydroxyphenylpyruvate (HPP) to 2,5-dihydroxyphenylacetate (homogentisate, HG). HPPD belongs to the non-heme, Fe^{2+} -dependent, dioxygenase class of enzymes. 3-Fluoro-4-hydroxyphenylpyruvate (MFHPP) was enzymatically synthesized from 3-fluoro-DL-tyrosine using tyrosine aminotransferase (TAT). MFHPP was used to examine the catalytic mechanism of HPPD, by kinetic methods and product analysis. The reaction of MFHPP with HPPD gave rise to only one product, ruling out free rotation of the approximately isosteric aromatic ring in the active site. The product, 2,5-dihydroxy-4-fluorophenylacetate, was identified with ^1H NMR, ^{19}F NMR, EI mass spectrometry and was a substrate for homogentisate 1,2-dioxygenase (EC 1.13.11.15, HGD). MFHPP is a slow substrate for HPPD decreasing the V_{max} by 4 fold, allowing us to distinguish the accumulation of an additional intermediate, compared to the native substrate, using rapid mixing spectrophotometric methods. It is postulated that the hydroxyl group of HPP is necessary for catalysis via a hydrogen bonding interaction with HPPD since phenylpyruvic acid (PPA) and pyruvic acid (PYR) do not stimulate the enzyme to react with molecular oxygen, yet apparently bind to the active site in a similar manner. Binding constants were determined for PPA, HPP, and MFHPP with the $\text{HPPD}.\text{Fe}^{2+}$ complex. The Gibbs free energy of each analog was calculated and a $\Delta\Delta G$ values determined to ascertain the relative energetic effects of changing specific portions of the substrate.

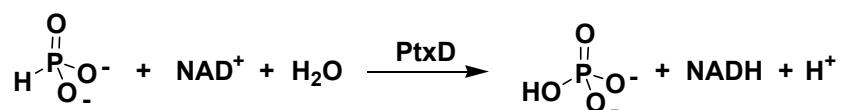
Phosphite Dehydrogenase: Mechanistic Studies of an Unusual Phosphoryl Transfer Reaction

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Phosphite Dehydrogenase (PtxD) oxidizes phosphite (hydrogen phosphonate) to phosphate while concomitantly reducing NAD^+ to NADH. This reaction is highly unusual in that it is the only enzymatic phosphorus redox chemistry observed in nature.

Based on sequence homology (23-49%) with the 2-hydroxyacid NAD^+ -dependent dehydrogenase family, three putative catalytic residues are proposed (Arg237, Glu266, His292). In an effort to determine the mechanism of this unusual reaction, the effects of protonation state on the rate of the reaction have been studied in both H_2O and D_2O . The effect of protonation state on binding ability has also been studied. It was observed that two distinct protonation states of active site residues are necessary for optimum binding and catalysis. Additionally, six mutants derived from site-directed mutagenesis of the putative catalytic residues have been characterized. Kinetic assays show that all His292 mutants are inactive while Arg237 and Glu266 are likely involved in phosphite binding.



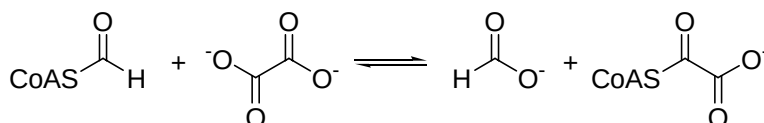
Structural and Mechanistic Studies of the Formyl-CoA Transferase From *Oxalobacter formigenes*

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Formyl-CoA transferase catalyses the transfer of CoA from formate to oxalate in the first step of oxalate degradation by *Oxalobacter formigenes*, an obligate anaerobe bacterium that is present in the intestinal flora, and which is implicated in oxalate homeostasis in humans and other mammals [1].



The gene encoding *Oxalobacter formigenes* formyl-CoA transferase has been cloned and sequenced [2], and the deduced primary structure places the enzyme in a novel class of coenzyme A transferases [3]. As part of efforts to characterize enzymes involved in oxalate catabolism [4], our group has developed expression and purification protocols that provide access to large quantities of *Oxalobacter formigenes* formyl-CoA transferase for use in structural and mechanistic studies. This poster will describe recent results from kinetic experiments that have set the scene for a detailed mechanistic understanding of this enzyme, and other members of this novel family of coenzyme A transferases.

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Reaction of Glycosylasparaginase with N^4 -(4'-Substituted Phenyl)-L-Asparagines

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Glycosylasparaginase catalyzes the hydrolysis of the *N*-glycosylic bond in β -*N*-acetylglucosaminyl-L-asparagine [(GlcNAc-)Asn] to give *N*-acetylglucosamine, aspartic acid and ammonium ion in the catabolism of N-linked glycoproteins. A deficiency or absence of activity of glycosylasparaginase gives rise to the most common inherited disorder of glycoprotein metabolism, aspartylglycosaminuria. In order to understand the mechanism of the enzyme in more detail, N^4 -(4'-substituted phenyl)-L-asparagines were synthesized and characterized, and their reaction with glycosylasparaginase from human amniotic fluid was studied to answer the question: Is the acylation step the rate-limiting step in the hydrolysis reaction? N^4 -(4'-Substituted phenyl)-L-asparagines, where 4' is H, Et, Br, NO₂, or OMe, were synthesized in a 4-step scheme; 4'-Et and Br were new compounds. Each N^4 -(4'-substituted phenyl)-L-asparagine was a substrate for the enzyme, and the kinetic parameters were dependent on the structure of the aniline. A Hammett plot of $\log(k_{\text{cat}})$ vs σ_p^- shows a nonlinear (biphasic) relationship, and indicates that acylation is the rate-determining step in the hydrolysis reaction. A Hammett plot of $\log(k_{\text{cat}}/K_M)$ vs σ_p^- shows a linear relationship, and indicates that upon binding the electron distribution of the substrate is perturbed toward the transition state. The data are evidence to indicate directly for the first time that acylation is the rate-determining step in the enzyme-catalyzed reaction of the natural substrate with formation of a covalent aspartyl-enzyme intermediate. A Brønsted plot of $\log(k_{\text{cat}}^X/k_{\text{cat}}^H)$ vs $\text{p}K_a$ indicates that the charge on the nitrogen atom of the leaving group anilines is a small, negative charge (-0.25) for electron-withdrawing substituents and a positive charge (+0.43) for electron-donating substituents. The free energy (incremental) change of binding ($\Delta\Delta G_b$) in the enzyme-substrate transition state complexes shows that substitution of a substituted phenyl group for the pyranosyl group in the natural substrate results in an overall loss of binding energy equivalent to a weak hydrogen bond, the magnitude of which is dependent on the substituent group. The data are consistent with a mechanism for glycosylasparaginase involving rapid formation of a tetrahedral structure upon substrate binding, and a rate-limiting breakdown of the tetrahedral structure to a covalent β -aspartyl-enzyme intermediate that is dependent on the electronic properties of the substituent group and on the degree of protonation of the leaving group in the transition state by a general acid.

Can Other Amino Acids Serve as Substrates for Lysine 2,3-aminomutase?

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Lysine 2,3-aminomutase (KAM, EC 5.4.3.2) from *Clostridium subterminale* SB4 catalyzes the stereospecific interconversion of L-lysine and L- β -lysine. This reaction proceeds through a radical based mechanism involving a 1,2-amino group shift facilitated by pyridoxal-5'-phosphate (PLP). KAM catalyzes the cleavage of an unactivated C-H bond of lysine by S-adenosyl methionine (SAM), the latter mediating hydrogen transfers between substrate and product. The product radical intermediate is observable by electron paramagnetic resonance (EPR) spectroscopy (Ballinger, M.D., Frey, P.A. and Reed, G.H., 1992, Biochemistry 31, 10782-1089). The current study evaluates the substrate specificity of KAM. Initial efforts were directed at detecting EPR signals from the product radical. Of the 17 amino acids tested to date, ten amino acids demonstrated strong to weak EPR radical signals whereas seven amino acids were found to inhibit the radical species formed in the presence of a low concentration of L-lysine (0.25 mM). Amino acids showing strong to moderate EPR signals were: L-alanine, DL-aspartate, L-asparagine, L-glutamate, L-cysteic acid, L-homocysteic acid, L-homoserine, L-homocysteine, and L-methionine. In this class, the EPR radical signal of L-alanine was potentiated by inclusion of either ethyl- or propylamine. Both L- and D-aspartate were approximately equally effective at generating the radical species suggesting a DL-aspartate racemase function for this enzyme if turnover occurred. The following amino acids were inhibitory to the formation of an L-lysine radical species: L-ornithine, L-2,4-diamino-n-butyrate, L-arginine, L-phenylalanine, L-glutamate, and L-histidine, and D-lysine. Turnover experiments were conducted by observing the enzymatic formation of β -amino acids that had been derivatized by phenylisothiocyanate and separated and measured by HPLC. KAM catalyzes the formation of β -alanine from L-alanine at a rate approximately 1000 fold less than L-lysine product formation. The rate is potentiated by the presence of ethyl- or propyl-amine. Formation of other β -amino acids from their respective L-amino acids has not been observed to date. Furthermore inter-conversion of D- and L- forms of aspartate has not been detected. Therefore KAM is able to accommodate a variety of amino acids containing basic, acidic, neutral, and polar groups at its active site and efficiently catalyze a partial reaction to a radical intermediate. However product formation is highly attenuated if at all possible. According to the proposed mechanism, the rate determining step is the abstraction of hydrogen from 5'-deoxyadenosine by the product radical. Binding of alternate amino acids may not allow for efficient hydrogen transfer at this step.

**Pathways for Decay of the Tryptophan Cation Radical Intermediate in
Oxygen Activation by Protein R2 of *Escherichia coli* Ribonucleotide
Reductase**

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The four-electron reduction of dioxygen to water at the carboxylate-bridged diiron(II) site of the R2 subunit of ribonucleotide reductase from *Escherichia coli* is balanced by oxidation of two Fe(II) ions to Fe(III), one-electron oxidation of tyrosine 122 (Y122) to the catalytically essential tyrosyl radical, and transfer of an “extra” electron from an exogenous source. In the transfer of the extra electron, a near surface tryptophan residue (W48) is transiently oxidized to W48 cation radical (W48⁺) by a kinetically masked two-electrons-oxidized adduct between the diiron cluster and oxygen. The W48⁺ may then be reduced by a facile one electron reductant (e.g. ascorbate, Fe(II)_{aq}, thiols, etc). Under these conditions, the last step in the reaction is the slow oxidation of Y122 by the Fe(IV)Fe(III) cluster **X** to produce the Y122• of the native protein. The mechanism of decay of the transient W48⁺ in the **absence** of a reductant has been examined by stopped-flow absorption experiments performed on R2-WT, R2-Y122F, R2-Y356F, and R2-Y122F/Y356F proteins under conditions of varying ionic strength. The results suggest that W48⁺ decays via three simultaneous pathways. The first pathway involves the formation of Y122 radical (Y122•) with a rate constant of 7 s⁻¹. The second pathway produces an unidentified product with a rate constant of 1 s⁻¹. The rate constants of W48⁺ decay by these two pathways are unaffected by ionic strength. The third pathway results in the formation of tyrosine 356 radical (Y356•) with a rate constant that can be expressed by the following equation: $k_{\text{obs}} = 29 \text{ s}^{-1} \times ([\text{NaCl}]/(0.24 \text{ M} + [\text{NaCl}]))$. One possible explanation for these observations is that Y356, which is located at the surface of the R2 subunit on the flexible C-terminus, becomes more ordered and at closer proximity with the W48 residue at higher ionic strengths, allowing for facile electron transfer between it and the W48⁺. The salt-dependent ordering of Y356 relative to W48 may have implications for the gating of electron transfer between the R1 and R2 subunits of the enzyme during ribonucleotide reduction.

Cloning and expression of myo-inositol oxygenase (*mio*) from the yeast *Cryptococcus neoformans*

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The oxidation of *myo*-inositol to D-glucuronate, the first dedicated step in *myo*-inositol catabolism, is catalysed by the enzyme myo-inositol oxygenase (MIO). This enzyme could be used in novel biosynthetic routes to produce vitamin C, D-glucaric acid, and D-glucurono- γ -lactone by fermentation or direct enzymatic conversion.. The 35 kDa enzyme was purified from the yeast *Cryptococcus terreus* by a combination of anion exchange chromatography, affinity chromatography, and 2-D gel purification. A peptide fingerprint of the purified protein was used to identify *mio* homologs and ESTs in tissues of fungi, higher plants, and in kidney tissues of mammals. Homologs of *mio* were cloned from human, rat, *Arabidopsis thaliana* and *Cryptococcus neoformans* cDNA libraries. Recombinant MIO was produced in yeast, bacterial and insect cells. Several groups have identified mammalian homologs of these genes that were expressed in the cortex of kidneys and found that repression was associated with acute renal failure, diabetic nephropathy and incorrect embryonic nephrogenesis. In plants, *mio* expression has been associated with environmental stresses and seedling development. Thus, in addition to its value in providing novel routes to fermentation products from D-glucose, myo-inositol oxygenase may be useful in the treatment of kidney disease and in the development of crops with improved agronomic traits.

A Proposal for the Catalytic Mechanism of UDP-Galactopyranose Mutase

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The flavoenzyme, uridine 5'-diphosphate (UDP)-galactopyranose mutase (UGM) catalyzes the interconversion of UDP-galactopyranose and UDP-galactofuranose. UGM is involved in the cell wall biosynthesis of various pathogenic bacteria, protozoa and fungi. Despite much interest in this important enzyme, a compelling chemical mechanism for the transformation is lacking. We propose a new chemical mechanism for the mutase which exploits the nucleophilic character of reduced anionic flavin. We hypothesize that a covalent flavin – galactose intermediate forms and that interconversion of the pyranose and furanose isomers occurs via a flavin-derived imine. The measured reduction potential of UGM suggests that the nucleophilicity of the reduced flavin in UGM is enhanced. The enzyme is inhibited in the presence of NaCNBH₃, which is consistent with the presence of an imine intermediate. Together the available data implicate the flavin as the transformative nucleophile.

**Statistical analysis of high-throughput production of *Arabidopsis* proteins in
*Escherichia coli***

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We have demonstrated protocols for high-throughput, large-scale production of proteins in *Escherichia coli* expression systems. Fermentations were conducted in terrific broth (TB) media with healthy inocula and antibiotics in inexpensive, two-liter disposable polyethylene terephthalate (PET) beverage bottles. Culture scale-up from transformed cells to inoculating the beverage bottles takes ~4 h. The cell production was optimized in 500 ml TB medium with an inducer and an induction time of 16 h at 20°C. The characteristics of cell growth, mass of cells, protein expression, and solubility of proteins are presented. The effects of parameters such as container vessel, volume, induction temperature, induction time, bacterial expression host, and characteristics of the expressed proteins on the results of cell production are evaluated. As compared with other bacterial expression systems, the pQE vector construct gave a statistically significant increase in the number of proteins expressed in the soluble fraction.

Application of Directed Evolution Technology to Optimize the Cocaine Hydrolase Activity of Human Butyrylcholinesterase

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In vivo, cocaine is slowly metabolized to inactive byproducts by the serum enzyme butyrylcholinesterase (BChE). Animal models suggest that exogenously administered BChE can confer some degree of protection against cocaine toxicity. Based on its therapeutic potential, this enzyme was chosen as a model to develop a system of functional optimization of complex proteins requiring mammalian expression. Focusing on residues lining the active site gorge of BChE, variant libraries were synthesized by codon-based mutagenesis. Libraries were transfected into human HEK 293T cells under conditions that resulted in the expression of a single protein variant per cell. Levels of BChE protein isolated from culture supernatants were normalized by saturating enzyme capture with an anti-BChE antibody, and assayed for cocaine hydrolase activity by measuring formation of metabolites from 3H-labeled cocaine. Primary screening of >10,000 variants and subsequent combinatorial analysis of beneficial mutations has led to the identification of variants exhibiting >100-fold increases in cocaine hydrolase activity. These results demonstrate the application of directed evolution technology in mammalian expression systems, enabling the engineering of complex proteins that require post-translational modifications unique to higher eukaryotic organisms. This research was supported by NIH SBIR grant #2R44GM060106-02A1.

Radical Formation in the Reaction of 4-Thialysine at the Active Site of Lysine 5,6-Aminomutase

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Lysine 5,6-aminomutase (5,6-LAM) catalyzes the interconversion of D-lysine and 2,5-diaminohexanoate (2,5-DAH) and of L- α -lysine and 3,5-diaminohexanoate (3,5-DAH) in the presence of adenosylcobalamin (AdoCbl) and pyridoxal 5'-phosphate (PLP). It is generally agreed that adenosylcobalamin functions as a radical initiator in enzymatic rearrangement reactions (Frey, P. A. 1990, *Chem. Rev.* **90**, 1343-1357). However, no radical intermediate is detected by EPR in the reaction of 5,6-LAM with a natural substrate, that is, D-lysine or L- α -lysine. Presumably, the radical intermediates are too short-lived to be detected. In order to study free radicals involved in the enzymatic mechanism, we synthesized the substrate analogue 4-thialysine, which could stabilize the free radical intermediate. In this report, we present the biochemical, spectrophotometric, and EPR spectral studies with substrate analogues, D-4-thialysine and L-4-thialysine. Our data support our proposed mechanism of action for lysine 5,6-aminomutase; that is, the process is initiated through hydrogen atom abstraction from C-5 of lysine by the 5'-deoxyadenosyl radical, which is derived by the homolysis of the Co—C bond of adenosylcobalamin.

Investigation of the Mechanism of Ethylene Formation by the Mononuclear Non-heme Iron Enzyme, ACC Oxidase

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1-Aminocyclopropane-1-carboxylate oxidase (ACC oxidase) catalyzes the last step in the biosynthetic pathway of the plant hormone, ethylene. This unusual reaction results in the oxidative ring cleavage of 1-aminocyclopropane carboxylate (ACC) into ethylene, cyanide, and CO₂, and requires ferrous ion, ascorbate, and molecular oxygen for catalysis. ACC oxidase belongs to a rapidly growing family of mononuclear non-heme iron enzymes that are characterized by a 2-histidine-1-carboxylic acid iron-binding motif. Within this class, a wide variety of reactions are catalyzed, such as hydroxylations, ring closures, ring expansions, and desaturations. The mechanistic understanding of how a class of enzymes with similar active sites can catalyze such a wide range of reactions is poor. Most of these reactions proceed by a sequential mechanism, making it difficult to separate out reductive and oxidative steps in the reaction cycle. We have examined the ability of ACC oxidase to catalyze both oxygen reduction and substrate turnover with a series of ACC analogs and have found that while some analogs gave product, several analogs were able to uncouple oxygen reduction from substrate oxidation. This result provides evidence that electron transfer can initially occur from ascorbate to activate oxygen, forming either an iron-peroxo or high valent iron-oxo species. Furthermore, the rate of oxygen turnover with several of the analogs decreased, indicating either a decrease in the rate-limiting step, or that a new step has become rate-limiting. We are currently using these analogs to look for potential iron-oxygen intermediates.

**Investigating the Function of an Essential NifS-like Protein from
*Synechocystis PCC 6803***

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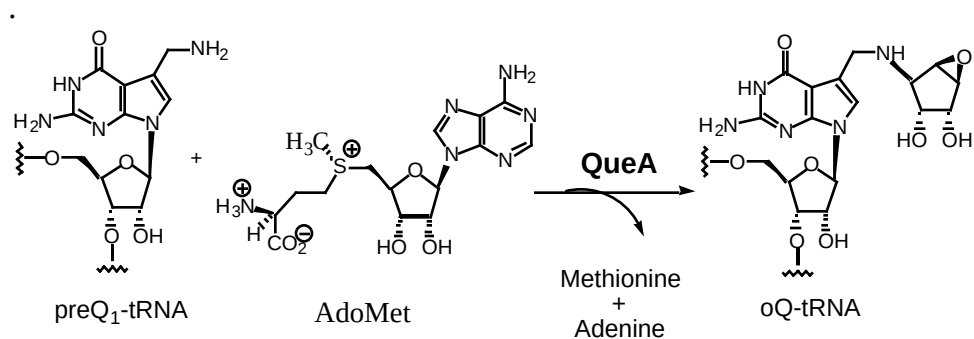
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Cysteine desulfurases designated NifS, IscS and SufS cleave L-cysteine to form L-alanine and an enzyme cysteinyl-persulfide species that *in vivo* is likely to transfer the sulfur directly to accessory factors prior to incorporation into a variety of organic and inorganic cofactors (such as molybdopterin, FeS clusters, etc). Gene disruption studies on the photosynthetic cyanobacterium *Synechocystis* PCC 6803 have shown that of the three IscS/SufS-like proteins encoded in its genome, only the sequence group II protein, Slr0077/SufS, is essential. The product of this essential gene Slr0077 has been over-expressed in *Escherichia coli*, purified to homogeneity, shown to bind pyridoxal-5'-phosphate and to catalyze cysteine desulfuration with dithiothreitol as co-substrate, and characterized in terms of its structure and kinetics. An enzyme-cysteinyl complex characterized by absorption maxima at 340 and 420 nm forms rapidly and decays sufficiently slowly to rate-limit the catalytic reaction. A spectrally indistinguishable but indefinitely stable adduct forms in the C372A variant protein, which lacks the presumptively nucleophilic cysteine residue. The two observations imply that the C-S bond-cleaving attack by the C372 thiol is rate limiting and much slower than the equivalent step in previously characterized group I desulfurase (Slr0387) from the same organism. The 1.8 Å crystal structure of the protein, which is very similar to that previously reported for *E.coli* SufS, shows that the loop on which C372 resides is well-ordered and shorter by 11 residues than the corresponding disordered loop of the group I NifS-like protein from *Thermotoga maritima*. The combined structural and kinetic data thus suggest that Slr0077/SufS have evolved expressly to be inefficient in the nucleophilic attack step, most likely for the purpose of regulation of cysteine cleavage by allosteric or other mechanisms.

Investigations of the Unprecedented Utilization of S-Adenosylmethionine as a Ribosyl Donor: Transfer RNA Modification by S-Adenosylmethionine:tRNA Ribosyltransferase-isomerase

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Posttranscriptional modification of transfer RNA encompasses a diverse range of chemistry from simple methylation of the base to hypermodification of the canonical bases by multiple enzymes. Queuosine, a case of the latter, is a 7-deazaguanosine derivative found in Bacteria and Eukaryotes in tRNAs specific for the amino acids tyrosine, histidine, aspartate, and asparagine. The de novo biosynthesis involves several unique enzymes, exemplified by the penultimate enzyme S-adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA). QueA catalyzes the unprecedented use of the ribosyl moiety of S-adenosylmethionine (AdoMet), which is transferred and isomerized to 7-aminomethyl-7-deazaguanine-containing tRNA (preQ₁-tRNA) to form the epoxycyclopentenediol of oQ-tRNA.



We have previously reported a chemical imperative for the use of AdoMet as a ribosyl donor and have developed an assay procedure that has allowed kinetic and mechanistic studies to be performed. Here we report our continued effort at characterizing QueA and understanding this mechanistically intriguing enzyme.

Mechanistic Study of Orotidine 5'-Monophosphate Decarboxylase by ^{13}C Isotope Effect

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Orotidine 5'-Monophosphate Decarboxylase (ODCase) has come under examination in recent years due to its enzymatic prowess. ODCase catalyzes the decarboxylation of OMP to UMP in the final *de novo* step of pyrimidine biosynthesis, with one of the highest known catalytic proficiencies that has been discovered for any enzyme ($\sim 10^{23} \text{ M}^{-1}$). An interesting attribute of such a high proficiency, is that it is reached without the involvement of any cofactor or coenzyme. (Miller and Wolfenden (2002) *Ann. Rev. Biochem.* 71:847-85) Over the past 30 years several mechanisms have been proposed for the decarboxylation of OMP. Of those proposed, three specific mechanisms have been given the most attention: covalent addition to C-5, nitrogen ylide, and carbene formation. Previous work from this lab has shown that the formation of the nitrogen ylide is unlikely, since there was no change in the bond order at N-1 during the rate-determining step. Additional studies have also taken support away from the covalent addition mechanism of catalysis. Researchers in these studies were able to show that C-5 does not change hybridization from sp^2 to sp^3 during the course of the reaction. (Rishavy and Cleland (2000) *Biochemistry* 39:4569-74) The direction of the research in this lab has been to understanding the nature of the carbene formation mechanism using ^{13}C isotopic studies on OMP, 4-deoxy-OMP, and 5-fluoro-OMP substrates with both wild type and a K49A mutant. We will report ^{13}C isotope effects for OMP and 4-deoxy-OMP wild type enzyme.

Crystal Structure of a Tetrameric GDP-D-mannose 4,6-dehydratase from a Bacterial GDP-D-rhamnose Biosynthetic Pathway.

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D-rhamnose is a rare 6-deoxy monosaccharide primarily found in the lipopolysaccharide of pathogenic bacteria, where it is involved in host-bacterium interactions and the establishment of infection. The biosynthetic pathway of D-rhamnose proceeds through two steps. GDP-D-mannose 4,6-dehydratase (GMD) first converts GDP-D-mannose to the 4-keto-6-deoxy intermediate, which is subsequently reduced to the nucleotide-activated D-rhamnose by a reductase. We have determined the crystal structure of a bacterial GMD in complex with NADPH and GDP. GMD belongs to the nucleotide-sugar modifying subfamily of the short-chain dehydrogenase/reductase (SDR) enzymes. SDRs are known to have homologous tertiary structures and share a conserved catalytic triad of Tyr-xxx-Lys and Ser/Thr. GMD has a bi-domain structure consistent with other related members of this subfamily. The larger N-terminal domain, the site of NADPH cofactor binding, consists of a Rossmann fold, a motif commonly associated with dinucleotide binding. The smaller C-terminal domain is responsible for substrate binding. GMD deviates from the typical homodimeric structures seen in most other related enzymes of this subfamily, as it is a tetramer like its plant homolog MUR1, a GDP-D-mannose 4,6-dehydratase isoform from *Arabidopsis thaliana*¹. At the tetramer interface, the cofactor binding sites are adjoined such that the adenosyl phosphate moieties of the bound NADPH molecules are within 8 Å. A short peptide segment forms a loop in this region that stretches into the neighboring monomer making not only protein-protein interactions, but also hydrogen bonding interactions with the neighboring cofactor. In addition, tetrameric contacts outside of this region are quite conserved across both the prokaryotic and eukaryotic GMDs. When the residues involved are not conserved, substitutions are conservative and expected to be compatible with tetramer formation. These observations suggest that the tetramer may be a more common and functionally important oligomeric state for GMDs than previously thought.

¹Mulichak, A.M. et al. (2002) *Biochemistry* 41, 15578-15589.

Identification of the genes encoding 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) synthase

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The genes and enzymes responsible for the biosynthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) moiety of coenzyme F₄₂₀ has been identified in *Mycobacterium smegmatis* (*cofGH*) and *Methanococcus jannaschii* (MJ445, MJ1346). The *cofGH* gene from *Mycobacterium smegmatis* and the *cofG* (MJ0446) and *cofH* (MJ1431) from *M. jannaschii* were each cloned and their respective gene products expressed in *E. coli*. *E. coli* cells containing the plasmid with the *cofGH* gene and the CofGH protein were found to produce FO whereas cells containing either *cofG* and the CofG protein or *cofH* and the CofH protein produced no FO. Most (> 90%) of the FO was found in the medium at a concentration of 0.2 μ M. Addition of tyrosine to the growth medium increased the amount of FO produced whereas the addition of methionine decreased the amount of FO produced. Incubation of the *E. coli* cell extract containing the CofGH protein with 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Compound 6), an intermediate in the biosynthesis of riboflavin, and 4-hydroxyphenylpyruvate the precursor of tyrosine, lead to the production of FO. Similar incubations of *E. coli* extracts containing either the CofG or CofH proteins only produced FO when the extracts were combined. The amount of FO produced was increased 50 fold by the simultaneous addition of S-adenosylmethionine (SAM), Fe²⁺ and sulfide to the extracts under anaerobic conditions. This information along with the clear presence of a SAM-radical motif in both the CofG and CofH homologous proteins indicate that they are members of the same SAM-radical group of enzymes. A radical based mechanism for the formation of FO is proposed.

Characterization of Homogentisate Phytyltransferase from *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana*

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Tocopherols are important nutrients for animals and humans and potent antioxidants in lipophilic cellular compartments. The homogentisate phytyltransferase (HPT) catalyses the first committed step in tocopherol biosynthesis, the prenylation of homogentisic acid (HGA) with phytyl pyrophosphate (PhyPP) and a decarboxylation reaction to form the tocopherol intermediate 2-methyl-6-phytylplastoquinol [1, 2, 3]. Overexpression of the *Arabidopsis* HPT gene in *Arabidopsis* resulted in a substantial increase in seed tocopherol levels [1]. In order to evaluate the relevance of the HPT for tocopherol pathway engineering, we studied HPT from *Synechocystis* sp. PCC 6803 (HPT_{Synco}) and *Arabidopsis thaliana* (HPT_{At}). Experiments for assay optimization revealed that a strict requirement for Mg²⁺ (0.3-4 mM). Pyridoxalphosphate is not required for HPT activity and diethylpyrocarbonate (DEPC) inhibits HPT completely at 0.5 mM. Analysis of substrate specificity revealed that both enzymes utilized PhyPP and geranylgeranylpyrophosphate (GGPP) as substrates. The HPT reaction product from GGPP presumably leads to the eventual formation of tocotrienols. The app V_{max} / K_m of the HPT_{Synco} for PhyPP is 7.7-fold greater than that for GGPP, suggesting that PhyPP is the preferred substrate of HPT_{Synco}. Isopentenylpyrophosphate (IPP) and geranylpyrophosphate (GPP) are not substrates for HPT at 100 μM. None of the downstream or upstream tocopherol intermediates showed any impact on HPT activity. Although initial experiments suggested that HPT is activated by protein kinase C, elaborate experiments showed no phosphorylation, and no activation of HPT by protein kinase C.

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The *cbiZ* gene in archaea encodes a new activity involved in the salvaging of the coenzyme B₁₂ intermediate cobinamide.

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Salvaging of the coenzyme B₁₂ intermediate cobinamide is conserved in cobamide producing bacteria. Because cobinamide is not an intermediate of the *de novo* cobamide pathway it must be converted to an utilizable molecule. In bacteria, this is achieved by the adenosylcobinamide kinase (CobU in *Salmonella enterica*), which converts adenosylcobinamide to the *de novo* intermediate adenosylcobinamide-phosphate. Because orthologs to CobU are absent in archaea, the questions of if and how archaea salvage cobinamide needed to be answered. This work reports the identification of an archaeal gene encoding a new amidohydrolase, which converts adenosylcobinamide to adenosylcobyric. At present, orthologs to the gene encoding this activity are limited to archaea. Genetic and nutritional analysis of the extremely halophilic archaeon *Halobacterium* sp. strain NRC-1 showed that this organism salvages cobinamide. It was shown that while a strain carrying a null allele of *cbiP* (cobyric acid synthase) was blocked for *de novo* synthesis of cobamides, it did respond to the addition of cobinamide to the growth medium. Cobinamide salvaging in *Halobacterium* required a new amidohydrolase activity, rather than a kinase as in bacteria. A strain carrying a null allele of *cbiB* (cobinamide-phosphate synthase) was blocked for both *de novo* cobamide biosynthesis and cobinamide salvaging, consistent with cobinamide entering the pathway after its conversion to cobyric acid. *Halobacterium* ORF Vng1583C (referred to as *cbiZ*) was shown to encode the amidohydrolase activity necessary for cobinamide salvaging in this archeon. A strain carrying a null allele of *cbiZ* had no effect on *de novo* cobamide biosynthesis, but blocked cobinamide salvaging. The *cbiZ* gene of *Methanosarcina mazei* restored cobamide biosynthesis in a *cobU* mutant of *S. enterica* grown under aerobic conditions where growth of the cell depended on cobinamide salvaging; the process required L-threonine-P decarboxylase activity (CobD in *S. enterica*), consistent with cobyric acid being the product of CbiZ. The *M. mazei* CbiZ protein was isolated. High-pressure liquid chromatography, mass spectrometry, UV-vis spectroscopy, and bioassay data showed that the product of the CbiZ reaction is cobyric acid. These data support the existence of an alternative pathway for salvaging cobinamide in prokaryotes.

^2H , ^{13}C , and ^{15}N Kinetic Isotope Effects on the Reaction of the Ammonia-Rescued K258A Mutant of Aspartate Aminotransferase

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Deuterium isotope effects at C2 of aspartate and heavy atom isotope effects at C2, C3, and the amino group of aspartate were determined for the reaction of the lysine-258 to alanine mutant of *Escherichia coli* rescued with exogenous ammonia. We were able to calculate an ^{15}N intrinsic isotope effect of 1.034. The intrinsic ^{13}C isotope effect at C3 is 1.0060 and the ^{13}C isotope effect at C2 is 1.0016. These isotope effects reveal that collapse of the carbinolamine (or gem-diamine) to give the final product is the rate-determining step in this system. Furthermore, these results indicate that lysine-258 is critical to the catalysis of the final breakdown to give product, and in fact this step is more strongly affected by mutation of lysine-258 than the deprotonation of the external aldimine.

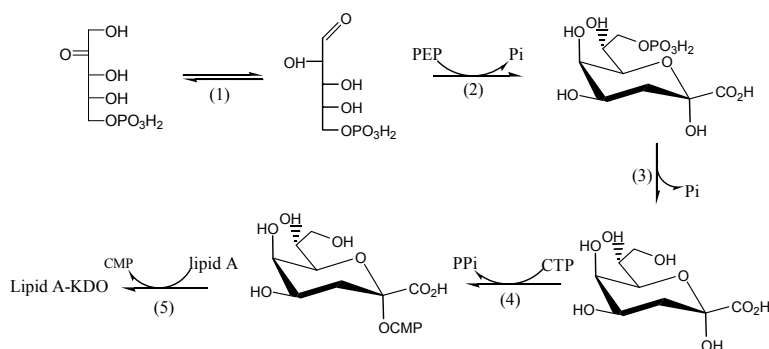
3-Deoxy-D-manno-octulosonate 8-phosphatase Phosphatase: a New Member of the HAD Hydrolase Superfamily

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3-Deoxy-D-*manno*-octulosonate (KDO) is an eight-carbon sugar that links the lipid A and polysaccharide moieties of the lipopolysaccharide region in Gram-negative bacteria. It has been demonstrated that an interruption in the biosynthesis of KDO leads to the accumulation of lipid A precursors, and subsequent arrest in cell growth. Thus, enzymes involved in KDO biosynthesis and/or its incorporation into lipid A are considered attractive targets for the design of novel antibiotics.

The biosynthesis and utilization of KDO involves five sequential enzymatic reactions that are catalyzed by (1) D-arabinose 5-phosphate isomerase, (2) 3-deoxy-D-*manno*-octulosonate 8-phosphate (KDO 8-P) synthase, (3) KDO 8-P phosphatase, (4) cytidine 5'-monophosphate-KDO synthetase, and (5) KDO transferase.



During the past two decades, the genes responsible for the expression of D-arabinose 5-phosphate isomerase (KpsF), KDO 8-P synthase (KdsA), cytidine 5'-monophosphate-KDO synthetase (KdsB), and KDO transferase (WaaA) have been identified and their respective enzymes have been studied extensively. KDO 8-P phosphatase, therefore, remains the last enzyme in the lipid A-KDO pathway for which a gene has not been assigned.

In the present work, for the first time, the gene for KDO 8-P phosphatase is identified. Amino acid sequence analysis indicates that KDO 8-P phosphatase is a member of the haloacid dehalogenase (HAD) hydrolase superfamily.

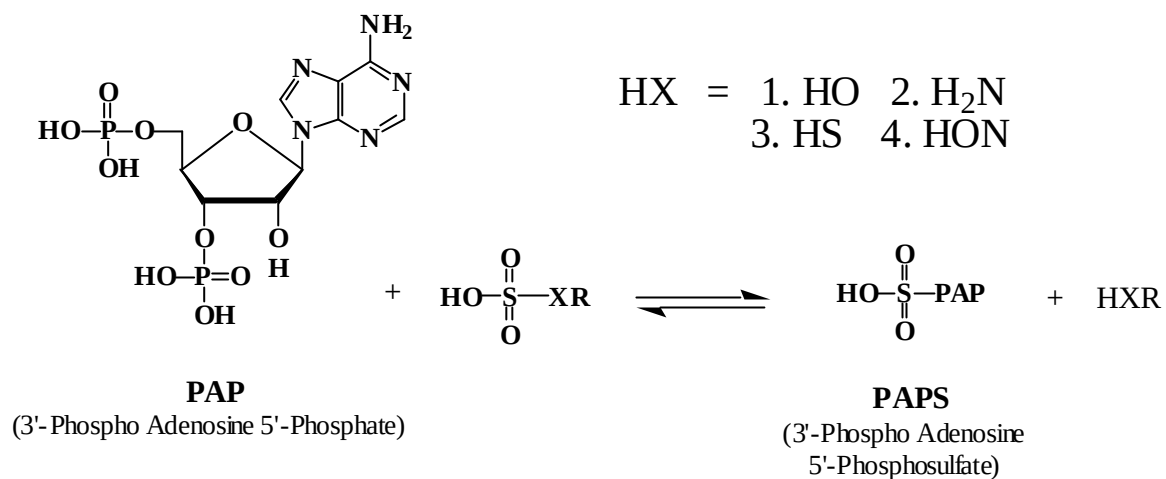
Nucleotide as Substrate, Inhibitor and Regulator of Sulfotransferase

Yuh-Shyong Yang

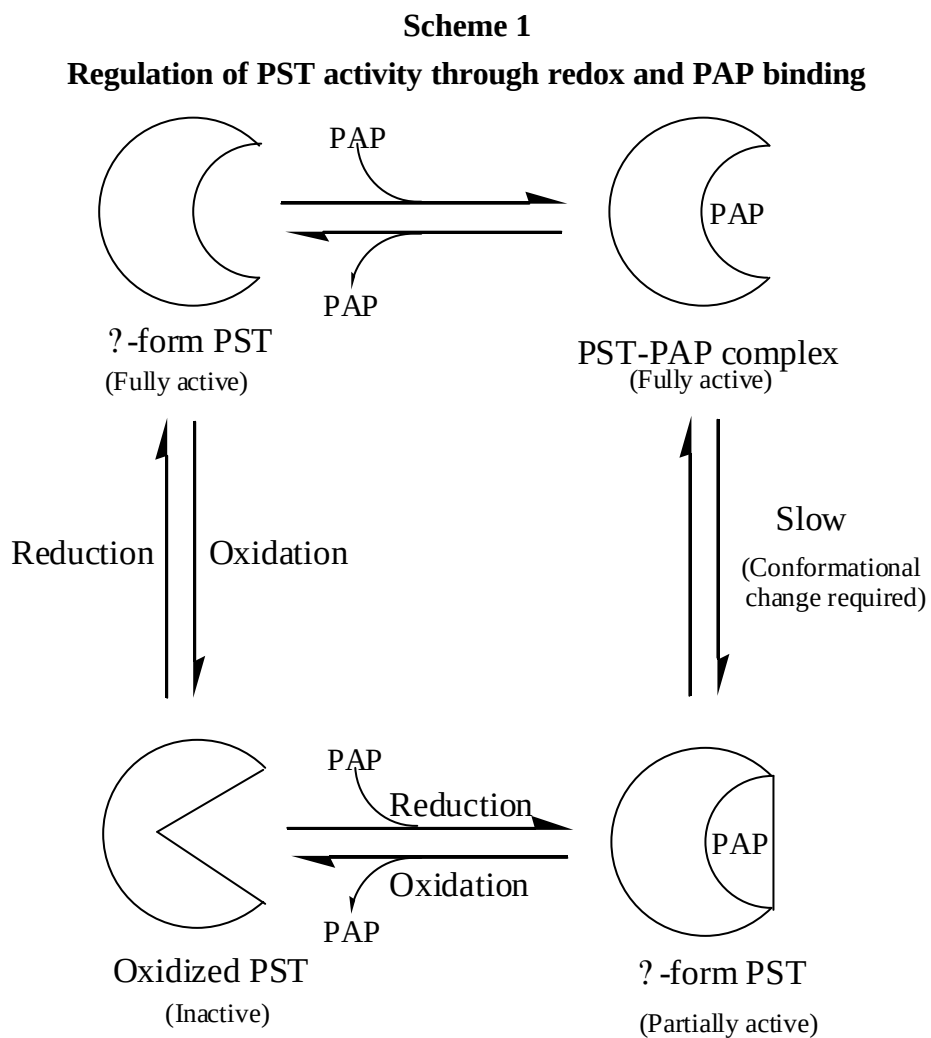
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Sulfotransferase catalyzes sulfuryl group transfer between nucleotide and a variety of nucleophiles that may be sugar, protein, xenobiotics and other small molecules (Reaction 1). A variety of nucleotides may serve as cosubstrate, cofactor, or inhibitor in the enzyme catalyzed sulfuryl group transfer reaction (1). Two forms of phenol sulfotransferase (PST) were obtained following the expression of a single cDNA. Only one form of PST contains a tightly bound nucleotide. Systematic analyses of the mutants of PST reveal that Ser134 is important for the regulation of nucleotide specificity between 3'-phosphoadenosine 5'-phosphate (PAP) and AMP (2). Although many nucleotides are known to tightly bind to PST, only PAP is found accumulated in recombinant PST. The combination of redox and PAP binding are required to interconvert the two PST forms. A mechanism for the formation of the two enzyme forms is proposed (Scheme 1). Site-directed mutagenesis reveals the redox regulation of the activity of phenol sulfotransferase is controlled by a flexible loop (amino acids residues 59 to 70 of PST). Our data shows that the mobility of this fragment was influenced by the charged amino acids and cysteine residue.

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Reaction 1 Sulfuryl group transfer in biological system



Electron Transfer in Tyrosyl Radical Formation in the R2 Subunit of Mouse Ribonucleotide Reductase

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Our previous studies on the mechanism of tyrosyl radical (Y177•) and μ -(oxo)diiron(III) cluster formation in the R2 subunit of ribonucleotide reductase from mouse established that an oxidized diiron intermediate spectroscopically similar to the well-characterized, formally Fe(III)Fe(IV) cluster **X** from the reaction of the *Escherichia coli* R2 protein precedes the Y177• in the reaction sequence and is probably the Y177 oxidant. In the *E. coli* R2 cofactor assembly reaction, cluster **X** formation proceeds by the transfer of an electron from the near-surface residue tryptophan 48 (W48) to the initial O₂ adduct to the buried diiron cluster. Substitution of W48 or its cognate in mouse R2 (W103) has been shown to disable electron transfer, but published data would suggest that the mechanisms of the electron-transfer-deficient variants of *E. coli* and mouse R2 differ markedly. To understand the basis for these differences, we have re-examined the mechanism of Y177• formation in two electron-transfer-deficient variants of mouse R2, R2-W103Y and R2-D266A. Preliminary results suggest, in contrast to previous proposals, that either addition of O₂ to the diiron(II) cluster becomes rate-limiting or that electron transfer is rate-limiting and O₂ addition is highly reversible in these variant proteins. Ongoing kinetics experiments will allow these possibilities to be distinguished.

Characterization of *Aeropyrum pernix* DAHP synthase

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The first committed step in the Shikimic acid pathway leading to the biosynthesis of aromatic amino acids, Phe, Trp, and Tyr, which are necessary for cellular functions in both plants and microorganisms, is an aldol-type condensation reaction between phosphoenolpyruvate and D-erythrose 4-phosphate. This condensation is catalyzed by the enzyme 3-deoxy-D-arabino-heptulsonate-7-phosphate (DAHP) synthase. The DAHP synthase (*aroG* gene) from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* (isolated from volcano vents in Kodakara-Jima Island, Japan) is the smallest DAHP synthase for which a sequence has been published. The orf is 831 base pairs coding for 276 amino acids (MW ~30 kDa). The main difference between the DAHP synthase amino acid sequence, is a major truncation of the N-terminal portion of the enzyme. This region is generally suspected to be the site for the allosteric feed back inhibition seen with most DAHP synthases by down stream products in the pathway.

The *aroG* gene from *A. pernix* was cloned into the pT7-7 expression vector for expression in *Escherichia coli* BL21(DE3) RIL. Results from the expression, purification and characterization of DAHP synthase from *A. pernix* along with data describing the thermal stability, metal requirements, and feedback inhibition of this enzyme will be presented.

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