Poster Abstract 1

THE ROLE OF BASE-STACKING DURING DNA POLYMERIZATION

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The molecular contributions of hydrogen-bonding, base-stacking, electrostatic interactions, and shape complementarity toward DNA synthesis have been evaluated by monitoring the insertion of normal and modified nucleosides opposite thymine (correct DNA synthesis) or opposite the pro-mutagenic abasic DNA lesion (translesion DNA synthesis). Similar to that of correct DNA synthesis, normal dNMP insertion opposite an abasic site is limited by the conformational change preceding chemistry. However, the rate of the conformational change is surprisingly dependent upon the dNMP to be inserted (dAMP> dGMP>>dCMP, dTMP), suggesting that the dynamics of insertion are dependent upon the relative stability of the dNMP:abasic site mispair. Of the panel of modified analogs tested, two were distinctive as they displayed unique behavior. Specifically, 8-oxo-dATP was efficiently inserted opposite thymine but not opposite the abasic site. By contrast, 5-nitro-1-indolyl-2'-deoxyribose-5'-triphosphate (5-NI) was unique as it was efficiently inserted opposite an abasic site. Results of a series of transient kinetic studies yield a k_{pol} of 126 +/- 7 sec^{-1} and a K_d value of 18 +/- 3 μM for 5-NI insertion opposite an abasic site. These values rival those measured for correct insertion and suggest that base-stacking contributions play a significant role for polymerization efficiency. The dynamics of base-stacking were further evaluated by monitoring the insertion of 2-aminopurine-2'-deoxyribose-5'-triphosphate (2-AP) opposite the DNA lesion. A series of steady-state and transient kinetic analysis indicate that 2-AP is "stacked" in an intrahelical conformation. Comparison of the rate constants obtained through fluorescence quenching versus primer-elongation suggest that base-stacking occurs *after* phosphoryl transfer. The collective results are discussed in a model correlating base-stacking with fidelity and enzyme translocation.

Poster Abstract 2

STUDIES ON THE PROPAGATION OF GENETIC ERRORS BY DNA REPLICATION

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Inappropriate modification of DNA can initiate disease by altering the genetic message of an individual. Although various repair pathways exist to correct DNA damage, it is the inappropriate action of the DNA polymerase when it encounters damaged DNA that propagates these potential genetic errors. During processive DNA replication, the "lifetime" of the DNA polymerase on nucleic acid is increased through association with its respective processivity factor. This increased "lifetime" at a DNA lesion may contribute significantly toward mutagenesis by enhancing the propagation of the genetic error. In this report, we evaluate the effects of enhanced processivity on the dynamics of both precise and translesion DNA replication using the bacteriophage T4 replication system as a model. We demonstrate that the T4 replicase (polymerase and processivity factors) can perform efficient, processive replication using unmodified DNA as the substrate, but it cannot extend beyond a template abasic site. Furthermore, the inability of the replicase composed of an exonuclease-deficient polymerase to extend beyond the abasic lesion indicates that molecular processes involved in DNA polymerization activity play the predominant role in preventing extension beyond certain DNA lesions. The complex, however, remains stably associated at the site of DNA damage. Idle turnover, the partitioning of the complex between the exonuclease and polymerase active sites, may take place during this increased association. We are characterizing the idle turnover process in the replicase system to determine its role in the maintenance of DNA fidelity and as a signal for repair or recombination.

Poster Abstract 3

EVIDENCE FOR GLUTAMATE-72 AS A LIGAND TO THE DIVALENT CATION ACTIVATOR OF HMG-COA LYASE

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HMG-CoA lyase catalyzes the cleavage of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) to form acetyl-CoA and acetoacetate. The enzyme requires a divalent cation (e.g. Mg^{2+}, Mn^{2+}) and sulfhydryl reagents (e.g. DTT) for activity. To identify amino acids that may support acid/base catalysis or ligation of the activator cation, a survey of invariant acidic residues was conducted using site-directed mutagenesis. The survey focused on acidic residues that are invariant not only in HMG-CoA lyase but also in the entire family of HMG-CoA lyase related enzymes. Of several mutants characterized, E72A was the most notable. A reduction in V_m of almost 200-fold is observed ($V_{m\,WT}$ = 190 U/mg; $V_{m\,E72A}$ = 1 U/mg). K_m for divalent cation substantially increases (for Mg^{2+}, $K_{m\,WT}$ = 233 μM; $K_{m\,E72A}$ = 5200 μM; for Mn^{2+}, $K_{m\,WT}$ = 0.2 μM; $K_{m\,E72A}$ = 43 μM). The possible effect on cation binding was more directly addressed by ESR measurements on binary Mn^{2+}-enzyme complexes. Scatchard analysis indicates that E72A stoichiometrically binds Mn^{2+} with a $K_{D\,Mn}$ (23 μM) that is elevated by an order of magnitude in comparison with the comparable $K_{D\,Mn}$ (1.6 μM) measured for wild-type enzyme. These data suggest a role for E72 in ligation of the divalent cation activator of HMG-CoA lyase.
(Supported by NIH DK21491 and a fellowship (RT) from the American Heart Assn.)

Poster Abstract 4

CRYSTAL STRUCTURE OF PCOC, A METHIONINE RICH COPPER RESISTANCE PROTEIN FROM ESCHERICHIA COLI.

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PcoC is a soluble periplasmic protein encoded by the plasmid-born *pco* copper resistance operon of *Escherichia coli*. Like PcoA, a multicopper oxidase encoded in the same locus and its chromosomal homolog CueO, PcoC contains unusual methionine rich sequences. The functions of PcoC and of these conserved methionine rich sequences are not known. Similar methionine motifs are also observed in eukaryotic copper transport proteins and have been proposed to bind copper, but there are no precedents for such metal binding sites in structurally characterized proteins. The high resolution structures of apo PcoC, determined for both the native and selenomethionine containing protein reveal a seven stranded β barrel with the methionines unexpectedly housed on a solvent exposed loop. Several potential metal binding sites can be discerned by comparing the structures to spectroscopic data reported for copper-loaded PcoC. These structures provide possible insights into the role PcoC plays in copper resistance.

Poster Abstract 5

THE HETERODIMERIC COMPLEX BETWEEN SUPEROXIDE DISMUTASE AND ITS COPPER CHAPERONE

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The disproportionation of superoxide to hydrogen peroxide and dioxygen by copper, zinc superoxide dismutase (SOD1) is a crucial reaction in cellular antioxidant defense. Activation of SOD1 in vivo requires a metallochaperone (CCS) that inserts the copper ion cofactor. The structure of CCS has been determined to 1.8 ≈ resolution. To understand the mechanism of metal ion insertion, protein-protein complex formation between CCS and SOD1 was investigated using a variety of biophysical and biochemical techniques including gel filtration chromatography, analytical ultracentrifugation, dynamic light scattering, and chemical crosslinking. These data indicate that a heterodimer is formed between CCS and SOD1 for metal ion delivery. Recently, the crystal structure of this complex has been solved to 2.9 ≈ resolution. Complex formation is mediated by the conserved dimerization interface present in both the chaperone and enzyme. Striking conformational rearrangements are observed in CCS and SOD1 upon complex formation, and the functionally essential C-terminal domain of CCS is well positioned to play a key role in the metal ion transfer mechanism. The structure provides important new insights into the molecular mechanisms of SOD1 activation by CCS.

Poster Abstract 6

CRYSTAL STRUCTURE OF THE PROLINE DEHYDROGENASE AND DNA-BINDING DOMAINS OF THE MULTIFUNCTIONAL PUTA FLAVOPROTEIN

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The PutA flavoprotein from *Escherichia coli* plays multiple roles in proline catabolism by functioning as a membrane-associated bi-functional enzyme and a transcriptional repressor of proline utilization genes. As an enzyme, PutA catalyzes the two-step oxidation of proline to glutamate via the intermediate to Δ^1-pyrroline-5-carboxylate (P5C). As a regulatory protein, PutA represses the divergent transcript of its own gene and the gene for a proline transporter. In a larger context, the human homologue of the PutA proline dehydrogenase (PRODH) domain plays critical roles in p53-mediated apoptosis and schizophrenia. We report the 2.0 Å crystal structure of a 669-residue truncated form of PutA (PutA669) that exhibits two of the three activities of PutA: the PRODH and DNA-binding activities. The structure was determined using multiple isomorphous replacement and anomalous scattering based on mercury and halide derivatives. The PutA669 structure is the first of a PutA protein and the first structure of a PRODH enzyme from any organism; thus it serves as the prototype for FAD-dependent PRODH enzymes. The structure is a domain-swapped dimer with each subunit comprising three domains: a helical dimerization arm, a helix-turn-helix DNA-binding domain, and a beta/alpha barrel PRODH domain with bound lactate inhibitor. Our work provides insight into the mechanism of proline oxidation to P5C, and reveals a novel structural setting for the helix-turn-helix motif.

Poster Abstract 7

INVESTIGATION OF REDOX REGULATED CALCINEURIN CATALYTICAL ACTIVITY

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Calcineurin, a ubiquitous protein found in all mammalian tissues but most abundantly in neuronal tissues, is also the target of the immunosuppressant drugs cyclosporin A (CsA) and FK506. Calcineurin is classified as an Fe-Zn member of serine/threonine protein phosphatases containing dinuclear metal active centers. The enzymatic activity of calcineurin is found to be both Ca^{2+}/calmodulin and Fe oxidation state dependent, in which the latter indicates a redox-regulated signal transduction response to intracelluar redox potential changes caused by the generation of reactive oxygen species such as hydrogen peroxide. The specific activities for both reduced and oxidized calcineurin have been measured using phospho-RII peptide, a 19 residue peptide phosphorylated on serine that corresponds to the phosphorylation site of regulatory RII subunit of protein kinase A. The redox state was followed by EPR spectrometry. The results indicate a correlation between specific activity and redox state, with activity increasing upon the decrease of the concentration of oxidized enzyme form identified by EPR spectroscopy. The results for reduced and oxidized enzyme activities treated by different oxidants and reductants will be provided.

Poster Abstract 8

INVESTIGATING THERMODYNAMIC REGULATION IN HUMAN SHORT-CHAIN ACYL-COA DEHYDROGENASE

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Acyl-CoA dehydrogenases (ACDs) are a family of flavoproteins that catalyze the first step in the β-oxidation cycle, producing up to 40% of the total human energy requirement. Previous work with bacterial short-chain (bSCAD) and mammalian medium-chain (MCAD) ACDs has shown that these enzymes are specifically modulated upon binding of the substrate/product couple, allowing the reaction to proceed in a thermodynamically favorable manner[1]. Although bSCAD and MCAD are mechanistically comparable, there are differences in their redox properties that may be attributed to chain-length specificity or to variations occurring between bacterial and mammalian systems[2]. In order to explore these differences, human SCAD (hSCAD) has recently been cloned and expressed. Using spectroelectrochemistry, the redox properties of hSCAD have been characterized and indicate that the enzyme is regulated to a greater extent than other ACDs. Specifically, the S/P couple midpoint potential shifts -28 mV when it binds to enzyme. This shift is twice as large as that seen when the same ligand binds to bSCAD and four times larger than that with mammalian MCAD[2,3,4]. The kinetically dead hSCAD mutant (E368Q) has also been generated and purified to further study the effects of substrate and product binding. Electrochemical studies with these two hSCAD enzymes will be presented and compared to other ACD systems.

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Poster Abstract 9

INVOLVEMENT OF PROTEIN AND SUBSTRATE DYNAMICS IN CATALYSIS BY LIVER ALCOHOL DEHYDROGENASES

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The roles of protein and substrate dynamics in enzymatic catalysis need to be determined. Amino acid residues at the active site may participate by binding and orienting the substrates and by contributing to the dynamics of formation of the transition state. We examined the involvement of protein and substrate dynamics in catalysis by horse liver alcohol dehydrogenase (ADH) by site-directed mutagenesis, steady-state and transient kinetics, x-ray crystallography and ^{19}F-NMR studies. Characterization of alcohol dehydrogenases with amino acid substitutions in the nicotinamide binding site (Thr-178, Val-203 and Val-292) suggest these residues contribute to the rates of hydride transfer and to the energetics of the conformational change that occurs upon coenzyme binding. The temperature dependencies of reactions catalyzed by the V292S enzyme are best explained by the hydride transfer occurring through hydrogen tunneling that is facilitated by the motions of the protein and/or substrates. The structures of the wild-type ADH-NAD^+-iodopyrazole and V292T ADH-NAD^+-pyrazole complexes show that C4 of the nicotinamide ring forms a partial covalent bond with a nitrogen of the pyrazole and adopts a boat conformation, which is postulated to be relevant for hydride transfer. The V292T enzyme has a two-fold slower rate constant for hydride transfer and more than a 30-fold decrease in affinity of conenzyme, as compared to the wild-type enzyme, but the reasons are not evident from the protein structure. The F93A substitution in the substrate binding site decreases the rate constants for hydride transfer of benzyl alcohol oxidation and benzaldehyde reduction, but it does not have significant affects on the overall structure of the protein. ^{19}F-NMR studies on 2,3,4,5,6-pentafluorobenzyl alcohol bound to wild-type and F93A enzymes show the fluorobenzyl ring rotates considerable faster in the F93A enzyme as compared to the wild-type enzyme. The increased mobility appears to decrease the probability that the substrate is preorganized for hydride transfer and reduces the rates at which hydride transfer occurs. NMR and x-ray studies show that 2,3-difluorobenzyl alcohol can bind in two alternative conformations. These studies suggest the motions of the protein and substrates contribute to catalysis by alcohol dehydrogenase. (Supported by NIH grants T32 GM08365 and AA00279)

Poster Abstract 10

INSULIN-MIMESIS OF VANADYL CHELATES INVOLVES TYROSINE PHOSPHORYLATION AT THE RECEPTOR LEVEL

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Insulin elicits a spectrum of reactions by binding to its cell surface receptor. To assign target enzymes underlying the insulin-mimetic effect of the vanadyl (VO^{2+}) cation, we have compared the influence of $VOSO_4$ with that of *bis*(acetylacetonato)oxovana-dium(IV) [$VO(acac)_2$], using 3T3-L1 adipocytes. Treatment of cells with 0.25 mM $VO(acac)_2$ in the presence of 0.25 mM BSA caused an 8-fold increase in glycogen synthesis. 0.25 mM $VOSO_4$ caused a 2-fold increase compared to a maximal 20-fold increase by 10 nM insulin. $VO(acac)_2$ markedly increased glycogen accumulation stimulated by 0.1 and 1 nM insulin but caused no further increase at maximal insulin concentration. In agreement, 0.25 mM $VO(acac)_2$ potentiated phosphorylation of signaling molecules Akt and GSK-3 at submaximal insulin concentrations but showed no further increase at 10 nM insulin. $VOSO_4$ had no significant effect. Treatment of 3T3-L1 adipocytes with 0.25 mM $VO(acac)_2$ for 5 min caused significant stimulation of tyrosine phosphorylation of the insulin receptor (IR) and the insulin receptor substrate IRS-1. In contrast to published reports that insulin mimesis by VO^{2+} involves only post-receptor signaling pathways, our results provide evidence that IR phos-phorylation also occurs. These results cumulatively indicate that $VO(acac)_2$ is a potent insulin mimetic agent in 3T3-L1 adipocytes, extending our earlier studies [*J. Biol. Chem.* **2002**, *277*, 12215-12220]. The synergistic effects of $VO(acac)_2$ and insulin may explain why insulin-mimesis of VO^{2+} compounds is observed in diabetic laboratory animals only. Our results provide strong support to the notion of development of chelates of VO^{2+} for therapy of type II diabetes. (Supported by NIH DK57599 and the American Diabetes Association.)

Poster Abstract 11

THE CATALYTIC AND STRUCTURAL ROLE OF THE METAL ION IN dUTP PYROPHOSPHATASE

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The metal ion dependence of the catalytic activity of recombinant *E. coli* dUTP pyrophosphatase (dUTPase), an essential enzyme for preventing the incorporation of uracil into DNA, has been investigated by steady-state kinetic methods and electron paramagnetic resonance (EPR) spectroscopy. In the absence of added divalent metal ions, the enzyme was associated with values of $4.5 \pm 0.1\ s^{-1}$ and $0.49 \pm 0.1 \times 10^6\ M^{-1}s^{-1}$ for k_{cat} and k_{cat}/K_M, respectively. These values were increased to $14.7 \pm 2.2\ s^{-1}$ and $25.1 \pm 7.4 \times 10^6\ M^{-1}s^{-1}$ in the presence of Mg^{2+}. Identical kinetic behavior was observed for Mn^{2+}, while a 2-fold increase in k_{cat} and 20-fold increase in K_M was observed for the vanadyl (VO^{2+}) cation. EPR studies showed that VO^{2+} was bound specifically by the enzyme in the presence of dUDP, a competitive inhibitor. Binding of VO^{2+} was competitive with Mg^{2+}. Comparable steady-state kinetic parameters were observed for dUTP hydrolysis in presence of *bis*(acetylacetonato)oxovanadium(IV). EPR studies demonstrated that the VO^{2+}-chelate was bound by the enzyme in the presence of dUDP, forming a ternary enzyme complex in which VO^{2+} retained at least one coordinated acetylacetonate ligand. On the basis of molecular modeling, a binding site for a catalytically active divalent metal ion is identified that is consistent with all kinetic data and with stabilization of an ordered conformation of C-terminal amino acid residues in the presence of a dUTP inhibitor analog. These results require complete alteration of all previous suggestions for the catalytic and structural role of Mg^{2+} in dUTPase.

Poster Abstract 12

EFFECTS OF FLUORINE SUBSTITUTION ON THE MECHANISM OF MEDIUM-CHAIN ACYL-CoA DEHYDROGENASE

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The β−oxidation cycle produces up to 40% of the energy needed by the human body. Medium-chain acyl-CoA dehydrogenase (MCAD), a flavoprotein, is one of the enzymes that catalyze the first step in this process. Deficiencies in MCAD have been linked to Sudden Infant Death Syndrome (SIDS) and other metabolic disorders. Redox studies of ACDs have provided insight into the overall energetics of the reaction and have revealed that substrate/product binding plays a key role in the regulation of fatty acid metabolism[1]. Relatively little is known about the thermodynamic contribution of the E/S complex to regulation due to its transient nature. In order to better understand the catalytic mechanism of MCAD, 2-fluoroacyl-CoA (*S* and *R* isomers) will be used as a slow substrate and a reversible inhibitor[2]. The proposed fluorine substitution will attempt to slow the reaction mechanism and identify the mechanism as either an asymmetric concerted reaction or a stepwise reaction with a distinct intermediate. Stopped-flow kinetics and ^{19}F NMR will be utilized with the *S*-isomer to follow the rate of flavin reduction and to observe the proposed intermediate. The *R*-isomer will be studied using spectroelectrochemical methodology to investigate the effects of substrate binding and Raman spectroscopy to explore electron rearrangement. Proposed redox characterization and experiments will be presented. (Supported by NIH Grant GM29344)

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Poster Abstract 13

MEASURING INTERACTIONS BETWEEN THE Ser70Ala MUTANT OF SHV-1 AND TAZOBACTAM USING NON-RESONANCE RAMAN DIFFERENCE SPECTROSCOPY

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Many researchers have studied the irreversible products of inhibition of several Class A β-lactamases, yet the precise structural elements that define a clinically effective β-lactamase inhibitor remain elusive. We wish to delineate the details of initial inhibitor binding using Raman spectroscopy to study the interaction of tazobactam and SHV β-lactamases. The unreactive S70A mutant of SHV β-lactamase was produced by site-directed mutagenesis and purified using preparative isoelectric focusing and HPLC. Non-resonance Raman spectra of tazobactam, S70A, and S70A plus tazobactam (1:1) in phosphate buffer pH 7.4 were obtained at 647 nm. The difference spectra were generated and examined from 1900 to 600 cm^{-1}. The spectra in the 1780 cm^{-1} region reveal a broadened C=O peak in the bound tazobactam. Peaks at 1400 cm^{-1} and 1385 cm^{-1} are assigned to the tazobactam's COO^- group. Other spectral changes are also observed.

Conclusions: The first Raman spectrum of a Michaelis complex of tazobactam and SHV is reported. The data suggest that the lactam C=O is not strongly polarized in the active site. The presence of two COO^- features for the bound tazobactam indicate that this group may bind at two distinct sites.

Poster Abstract 14

NMR CONFORMATIONAL STUDIES OF RESTRICTION ENZYME-LIGAND INTERACTIONS

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Restriction enzymes are ideal models for understanding metal ion-dependent protein-DNA interactions. Of particular interest are conformational changes associated with enzyme interactions with DNAs of various sequences and metal ion cofactors. Using the Mg(II)-dependent *Pvu*II endonuclease as a model system (2x18 kD), we previously established the feasibility of using biosynthetic fluorine incorporation and ^{19}F NMR spectroscopy as a probe of restriction enzyme conformation. We now turn to two goals: 1) Assessing the utility of isotopic labeling and multidimensional NMR methods for *Pvu*II endonuclease, a system which is large by NMR standards. 2) Characterizing the magnitude of enzyme and DNA solution conformational changes using this powerful technique. ^{1}H-^{15}N HSQC experiments with uniformly ^{15}N-labelled *Pvu*II endonuclease reveal series of interesting enzyme conformational changes. The introduction of Ca(II), which supports DNA binding but not turnover, results in a substantial backbone conformational response which may account for differences between Mg(II)-supported and Ca(II)-supported function. The further addition of an oligonucleotide containing the 5'-CAGCTG-3' recognition sequence results in dramatic improvements in linewidth and increases in chemical shift dispersion, producing spectra of quality approaching that needed for triple resonance experiments needed for sequence-specific resonance assignments. In an effort to characterize the structural differences between enzyme complexes with specific (target) and nonspecific DNAs, HSQC and ^{31}P NMR spectra of the two complexes were compared in the presence of Ca(II). Specific complexes are structurally distinct from nonspecific systems; interestingly, DNA backbone distortions are only observed in the former case. Spectral comparison of complexes formed in the presence and absence of metal ions is in progress.

Poster Abstract 15

SITE-DIRECTED SPIN LABELING OF *Vibrio proteolyticus* AMINOPEPTIDASE: TOWARDS FURTHER UNDERSTANDING OF SUBSTRATE BINDING

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Metalloaminopeptidases are implicated in a number of pathologies and are molecular targets for anti-tumor, anti-angiogenesis, anti-HIV and immunomodulatory drugs. A wide variety of functionalities exhibit potent inhibitory action against aminopeptidases due to reaction at their metal centers. However, the clinical efficacy of these inhibitors is limited by the low specificities towards metallohydrolases. The aminopeptidase from *Vibrio proteolyticus* (VAP) exhibits a preference for bulky hydrophobic groups in the P1 and P1' positions. VAP contains a hydrophobic patch adjacent to the di-metal active site and this has been implicated in substrate binding and specificity. We are currently examining binding of substrates and analogues to this patch using EPR spectroscopy of spin-labeled probe molecules in complexation with site-directed spin-labeled (SDSLd) VAP. We have constructed, expressed and purified soluble, active SDSLd variants of VAP and synthesized labeled substrate analogs. We present data indicating specific labeling of VAP and substrate analogs by the methanethiosulfonate radical (MTSL). Initial studies of the binding of the labeled competitive inhibitor HO-Leu-Trp-Phe-Cys-MTSL to MTSL-labeled VAP variant S246C are described. These and similar data can be analyzed in terms of a spin Hamiltonian

$$\mathbf{H} = \mathbf{g}_1\beta\mathbf{S}_1\mathbf{H}_0 + \mathbf{g}_2\beta\mathbf{S}_2\mathbf{H}_0 + \mathbf{S}_1\mathbf{A}_1\mathbf{I}_1 + \mathbf{S}_2\mathbf{A}_2\mathbf{I}_2 - \omega_n(I_1 + I_2) + \mathbf{J}\mathbf{S}_1\mathbf{S}_2.$$

Interspin distance information resides in the dominant dipolar component, **D,** of **J** and examination of multiple variants therefore yields direct structural information by triangulation.

Poster Abstract 16

THE PLASMID-BASED RECONSTRUCTION OF THE AVR AND DES DEOXYSUGAR GENES FOR EXPRESSION IN A HETEROLOGOUS STRAIN TO ALTER THE STRUCTURES OF THE AVERMECTINS BY COMBINATORIAL BIOSYNTHESIS

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Some of the most important antibiotic and antitumor agents contain modified deoxyhexoses as essential components for biological activity of these clinically important natural products. Variations in the biological activity of glycosides can be obtained by construction of novel gene combinations of deoxysugar biosynthesis genes. This has been most efficiently achieved by using plasmid vectors and a host in which the introduced genes are well expressed. Two *avr* and *des* gene cassettes responsible for biosynthesis and attachment of TDP-L-oleandrose and D-desosamine to the avermectin and picromycin aglycones respectively were used in this work. Versions of *avr* (Δ*avrH, ΔavrFH, ΔavrGIH, ΔavrFGIH*) and *des* (*des I, II, III, IV, VI, VII, R; Δdes I,II; Δdes VI,R; Δdes I, II, VI, R*) genes sets were combined after introducing plasmids with different *avr* and *des* gene sets into *Streptomyces lividans* strain. Transformants having third plasmid with *ery BIII* gene from mycarose pathway were also obtained. Bioconversion experiments were carried out in medium to which avermectin A1a aglycone was added and the extracts of transformants were analyzed by HPLC followed by mass spectrometry. Proposed novel mono- and disaccharide avermectin A1a derivatives having deoxysugar parts that structurally differ from L-oleanderose were produced by transformants carrying modified versions of *avr* gene set and des gene set with deletions in *desI, II, or des I, II, VI, R* genes as well as transformants carrying additionally *eryBIII* genes.

Poster Abstract 17

THERMODYNAMIC MODULATION OF MEDIUM CHAIN ACYL-COA DEHYDROGENASE AND ITS LIGANDS

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All mammals store excess energy as fat in adipose tissue for later use. The acyl-CoA dehydrogenases (ACDs), a family of non-membrane-bound mitochondrial enzymes, catalyze the first step of beta-oxidation, the primary means through which adipose energy stores are accessed. It is well established that ACDs are activated by substrate/product binding to accept electrons from their substrates, but the source of this activation energy has not been identified. In this study spectrally active alternative substrates and products were used to elucidate the ligand's role in activation. The thermodynamic effects of enzyme-ligand binding on the ligands themselves were studied by combining spectroelectrochemical techniques and "pseudo"-substrates and products with spectrally distinct redox states. These experiments demonstrated that while the substrate/product couple is activated for electron transfer, likely via polarization, the extent of activation is significantly less than anticipated. Other factors such as active site desolvation, pK_a changes, and charge changes must also play significant roles in ACD activation.

Poster Abstract 18

SUBSTRATE BINDING INDUCES A COOPERATIVE CONFORMATIONAL CHANGE IN THE 12S SUBUNIT OF TRANSCARBOXYLASE: RAMAN CRYSTALLOGRAPHIC EVIDENCE

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The 12S subunit of transcarboxylase is a 338,000 Dalton hexamer that transfers carboxlylate from methylmalonyl-CoA (MM-CoA) to biotin. Here, Raman difference microscopy is used to study the binding of substrate and the product, and their analogs, to single crystals of 12S. Crystalline ligand-protein complexes were formed by co-crystallization or by soaking in/soaking out method. Raman difference spectra were obtained by subtracting the spectrum of the apo-crystal from that of a crystal with the substrate or product bound. Raman difference spectra from crystals with the substrate bound are dominated by bands from the protein's amide bonds and aromatic side chain residues. In contract, Raman different spectra involving the product, propionyl-CoA, are dominated by modes from the ligand. These results show that substrate binding triggers a conformational change in 12S, whereas product binding does not. The conformational change involves an increase in the amount of α-helix since markers for this secondary structure are prominent in the difference spectra of the substrate complex. Most crystal samples had 6 MM-CoAs per haxamer although a few, from different soaking experiments, contained only 1-2. However, both sets of crystals showed the same degree of protein conformational change indicating that the change induced by the substrate is cooperative. This effect allowed us to record the Raman spectrum of bound MM-CoA without interference from protein modes, the Raman spectrum of a 12S crystal containing 2 MM-CoA ligands per hexamer was subtracted from the Raman spectrum of a 12S crystal containing 6 MM-CoA ligands per hexamer. The conformational change is reversible and can be controlled by soaking out or soaking in the ligand, using either concentrated ammonium sulfate solutions or the solutions used in the crystallization trials. Malonyl-CoA also binds to 12S crystals and brings about conformational changes identical to those seen for MM-CoA; in addition, butyryl-CoA binds and behaves in a similar manner to propionyl-CoA. These data implicate that $-COO^-$ group on MM-CoA as the agent bringing about the cooperative conformational change in 12S.

Poster Abstract 19

CHARACTERIZATION OF RADICAL INTERMEDIATES IN THE REACTION OF LYSINE 5,6-AMINOMUTASE

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Lysine 5,6-aminomutase (5,6-LAM) catalyzes the interconversion of lysine/L-β-lysine and 2,5-DAH/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 and natural substrate, i.e. lysine or L-β-lysine. We proposed that the radical intermediates are too short-lived to be detected is likely to be the reason. In order to study free radical involved enzymatic mechanism of action, we synthesized the substrate analogue, which could stabilize the free radical intermediate. In this report, we present the biochemical, spectrophotometric, and EPR spectral studies with substrate analogues, 4-thia-D-lysine and 4-thia-L-lysine. Our data supports our proposed mechanism of action for lysine 5,6-aminomutase, i.e., 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.

Poster Abstract 20

A COMMON MECHANISM UNDERLYING PROMISCUOUS INHIBITORS FROM VIRTUAL AND HIGH-THROUGHPUT SCREENING

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High-throughput and virtual screening are widely used to discover novel leads for drug design. On examination, many screening hits appear non-drug-like: they act noncompetitively, show little relationship between structure and activity, and have poor selectivity. Despite the common occurrence of such inhibitors, no known mechanism explains their peculiar behavior. To investigate this problem, 45 diverse screening hits were studied. Of these, 35 were shown to inhibit several unrelated model enzymes. These 35 compounds showed time-dependent, reversible inhibition that was very sensitive to enzyme concentration and ionic strength. By light scattering and electron microscopy, these compounds formed aggregates of 30 to 400 nm diameter. These large aggregates appear to be the active inhibitory species. Unexpectedly, aggregate formation may explain the activity of many nonspecific enzyme inhibitors, which are widespread in drug-discovery screening databases. Preliminary data suggest that this mechanism may also account for nonspecific inhibition by some widely used kinase inhibitors.

Poster Abstract 21

SPECTROSCOPIC CHARACTERIZATION OF THE STABLE CYANO-BRIDGE DINUCLEAR IRON-CLUSTER FORMED UPON OXIDATION OF SUPEROXIDE REDUCTASES FROM *TREPONEMA PALLIDUM* AND *DESULFOVIBRIO VULGARIS* with $K_3Fe(CN)_6$

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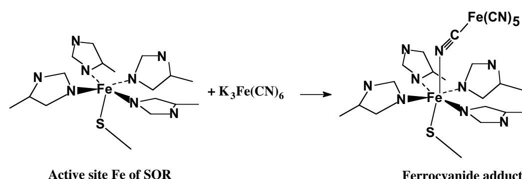
Superoxide reductases are implicated in the original defense of anaerobes against oxidative stress by catalyzing the monovalent reduction of superoxide anion to hydrogen peroxide. We report here the first spectroscopic evidence for the formation of a dinuclear cyano-bridged adduct after $K_3Fe(CN)_6$ oxidation of the superoxide reductases neelaredoxin from *Treponema pallidum* and desulfoferrodoxin from *Desulfovibrio vulgaris*. Oxidation with $K_3Fe(CN)_6$ is associated with the appearance of an absorbance in the near IR with λ_{max} at 1020 nm, coupled with an increase of the iron content by almost 2-fold. FTIR confirms a ferrocyanide adduct, with CN-stretching vibrations observed at 2095 cm^{-1}, 2025-2030 cm^{-1}, and 2047 cm^{-1}. Molecular ions observed by ESI mass spectrometry are in agreement with the adduction of 1 or 2 $Fe(CN)_6$ to the dimeric protein (MH^+ + 211.9). Interestingly, low temperature EPR spectra revealed structural heterogeneity in the coordination environment of the active site Fe ion. As isolated Neelaredoxin exists as a a mixture of Fe^{2+} and Fe^{3+} forms, with the latter exhibiting an EPR spectrum with a major axial species with E/D ~ 0.05 and a second minor rhombic species with E/D = 1/3. The intensity of the axial species increases upon oxidation with Na_2IrCl_6 but oxidation by the physiological substrate superoxide or by the product H_2O_2 produces yet another rhombic species with E/D = 0.25. Given the likely 6-coordinate geometry of the active site Fe^{3+} ion in the ferrocyanide adduct, we propose that the rhombic EPR species can serve as a model of a hexacoordinate form of the active site.



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Abreviations : FTIR: Fourier transform infrared spectroscopy; ESI-MS: Electrospray ionization mass spectrometry; EPR: electronic paramagnetic resonance.

Poster Abstract 22

OVEREXPRESSION, PURIFICATION AND PROPERTIES OF SHIKIMATE KINASE I (*AROK*) FROM *AQUIFEX AEOLICUS*

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Shikimate kinase (EC2.7.1.71) catalyzes the ATP-dependent phosphorylation of shikimic acid to yield shikimate 3-phosphate. This is the fifth step in shikimate pathway which is responsible for the biosynthesis of aromatic amino acids in bacteria as well as in plants. In *E. Coli*, phosphorylation of shikimic acid is carried out by two isozymes: shikimate kinase I, encoded by the *aroK* gene. and shikimate kinase II. encoded by the *aroL* gene. The *aroL*-encoded enzyme appears to play a major role in the biosynthesis of the aromatic amino acids. Expression of the *aroL* gene is controlled by both the TyrR and TrpR proteins and the amino acids tyrosine and tryptophan. The role and/or function of shikimate kinase I is less clear. Expression of the *aroK* gene appears to be constitutive and the enzyme has much lower affinity for shikimate than does the the *aroL* gene product.

The *aroK* gene from the hyperthermophilic bacterium *Aquifex aeolicus* was cloned into the pT7-7 vector for expression in *Escherichia coli*. Results from the expression, purification and characterization of Shikimate Kinase I from *A. aeolicus* along with data describing the thermostability, metal requirements, and feedback inhibition of this enzyme will be presented.

Poster Abstract 23

THERMOTOGA MARITIMA 3-DEOXY-D-*ARABINO*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE: THE ANCESTRAL EUBACTERIAL DAHP SYNTHASE?

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The enzyme 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to form DAHP and inorganic phosphate. The formation of DAHP is the first committed step in the Shikimate pathway leading to the production of the aromatic amino acids and other biologically important compounds in microorganisms and plants. Based on phylogenetic analysis, the DAHP synthase family has been divided into two classes: Class I and Class II. It is unclear what property leads to the distinstion between the Class I and Class II enzymes.

Phylogenetic analyses have placed two hyperthermophiles, *Aquifex aeolicus* and *Thermotoga maritima,* basal to all other bacteria. However, no DAHP synthase gene can be found in the genomic DNA sequence of *A. aeolicus* based on annotated genome and homology searches, thus the *T. maritima* DAHP synthase represents the earliest known diverging bacterial DAHP synthase. Studies on the DAHP synthase from *T. maritima* should provide better understanding of the ramification of the two classes of DAHP synthases and the evolution of the aldol-type condensation catalyzed by this enzyme family.

Results from the expression, purification and characterization of DAHP synthase from *T. maritima* along with data describing the thermostability, metal requirements, and feedback inhibition of this enzyme will be presented.

Poster Abstract 24

THE OVEREXPRESSION OF 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE FROM THE HYPERTHERMOPHILE *AEROPYRUM PERNIX*

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The first committed step in the Shikimic acid pathway leading to the biosynthesis of aromatic amino acids, Phe, Try, and Typ, 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*-heptulosonate 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 a smallest DAHP synthase for which a sequence has been published. The orf is 831 base pairs coding for 276 amino acids (MW 30 kDal). The main difference between the DAHP synthase amino acid sequence is a major truncation in 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 product 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 thermostability, metal requirements, and feedback inhibition of this enzyme will be presented.

Poster Abstract 25

D-ARABINOSE-5-PHOSPHATE ISOMERASE FROM *E. COLI*

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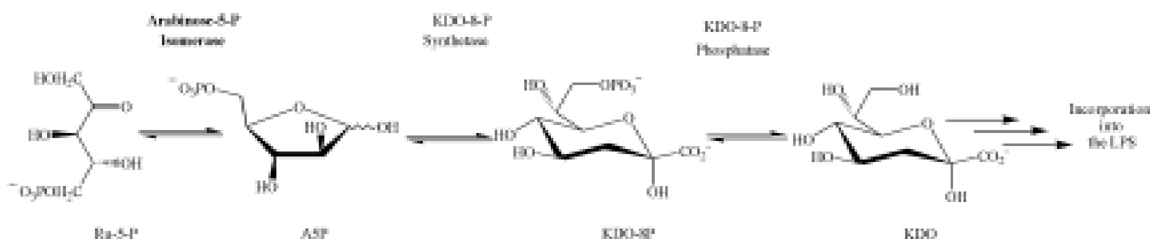
The rise in resistant pathogenic Gram negative bacterial strains necessitates the development of more potent antibiotics. With the observed increase in cross-resistance, there is a need for new antimicrobial agents with novel targets and thus no preexisting resistance. Enzymes in the biosynthetic pathway 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 (*kspF*, EC 5.3.1.13) catalyzes the reversible aldol-ketol 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 synthetase 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.

Poster Abstract 26

INHIBITION OF LIVER ALCOHOL DEHYDROGENASES AND ETHANOL METABOLISM

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Several formamides and sulfoxides were tested as inhibitors of purified horse, human, and mouse liver alcohol dehydrogenases and of ethanol metabolism in mice. These compounds are unreactive analogs of aldehydes, bind to the enzyme-NADH complex and are uncompetitive inhibitors of alcohol dehydrogenases with respect to ethanol as substrate. Uncompetitive inhibitors are advantageous for therapeutic purposes since the inhibition is not overcome at higher concentrations of alcohol, as it is with competitive inhibitors, such as 4-methyl pyrazole. Lengthening (up to 8 carbons) or branching of the alkyl substituent increases the inhibitory potency because of tighter binding in the hydrophobic substrate-binding pocket of the alcohol dehydrogenases. The structure of horse liver enzyme complexed with NADH and (*R*)-*N*-1-methylhexylformamide was determined by X-ray crystallography at 1.6 Å resolution. In the complex, the carbonyl oxygen of the inhibitor binds to the catalytic zinc and forms a hydrogen bond to the hydroxyl group of Ser-48. The amide group of (*R*)-*N*-1-methylhexylformamide is best modeled in the *cis* conformation, the *trans* isomer does not fit into the density. Some of the inhibitors were tested *in vivo,* and the metabolic data were fitted to numerically integrated equations for the complete mechanisms with kinetic simulation (KINSIM/FITSIM) in order to estimate *in vivo* inhibition constants (K_i) and the rates of metabolism of inhibitors. The uncompetitive inhibition constants for 3-butylthiolane 1-oxide, *N*-isopropyl, *N*-neopentyl, *N*-1-methylhexyl, and *N*-cyclohexyl formamides for mouse liver alcohol dehydrogenase *in vitro* were 0.4, 6.1, 7.6, 0.43, and 2.5 μM, respectively, whereas *in vivo* the values were 17, 110, 127, 100 and 29 μM. The differences in the values may be due to bioavailability of the compounds. Since high concentrations of alcohol do not prevent the inhibitory effects of these compounds, *N*-isopropylformamide, may be particularly useful for preventing poisoning by methanol or ethylene glycol. (This work is supported by NIH grant AA00279.)

Poster Abstract 27

INVESTIGATION OF THE REDOX-DEPENDENT CONFORMATIONAL CHANGE OF THE PUTA FLAVOPROTIEN FROM *ESCHERICHIA COLI*

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The PutA flavoprotein from *Escherichia coli* catalyzes the $4e^-$ oxidation of proline to glutamate using separate proline dehydogenase and P5C dehydrogenase active sites. It also transcriptionally regulates expression of the proline utilization regulon. PutA undergoes a conformational change upon substrate binding, which causes PutA to switch from a transcriptional repressor to a membrane bound dehydrogenase. Cofactor reduction and substrate binding were investigated separately to determine their roles in the conformational change. We observed that chemical and electrochemical reduction of the FAD in PutA causes the same conformational change as proline binding. A controlled potentiometric digest showed the reduction potential for the conformational change (-58 mV, in 50mM potassium phosphate, 10% Glycerol, pH7.5) is near that of PutA bound FAD. Substrate analogues, which bind to proline dehydrogenase active site but do not promote electron transfer to the FAD, were used to evaluate the proline binding effect on the conformational change. Spectroscopic and electrochemical properties of PutA complexed to the proline analogue (s)-tetrahydrofuroic acid were characterized. A dissociation constant of 0.28mM for TFA with PutA was determined by spectra titration. A midpoint potential of –88 mV (in 50mM potassium phosphate, 10% Glycerol, pH7.5) for the analogue-bound PutA was determined by potentiometric titration. Surprisingly, analogue binding causes a different conformational change in PutA than that of proline binding and FAD reduction. Reduction of analogue bound PutA, however, results in the proteolytic pattern observed with proline. Our observations indicate that the conformational change is redox dependent.

Poster Abstract 28

IN SEARCH OF THE PUTA DNA BINDING DOMAIN

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PutA, a bifunctional protein comprised of 1320 amino acids, acts both as a dehydrogenase (PHD) and a transcriptional repressor of the *putA* and *putP* (proline transporter) genes. By changing its position from the *put* control DNA, where it works as a transcriptional repressor, to the membrane, where functions as a PDH, PutA regulates proline metabolism. From the X-ray crystal structure of a truncated PutA protein containing residues 1-669(PutA669), a helix-turn-helix (HTH) putative binding motif was identified. PutA constructs, which contained this domain, were prepared and analyzed. Some of the PutA constructs were difficult to solubilize. However, low temperature for cell growth and/or induction and the use of urea during dialysis improved the solubility of the PutA proteins. Gel shift assays demonstrated that the DNA binding domain is within the first 261 amino acids at the N-terminal domain, which includes the HTH domain. Site-directed mutagenesis of some basic residues (R230, R234, K238) in the recognition helix of HTH domain was performed to test their role in PutA-DNA interactions. PutA669R230A and PutA669R234A showed no difference in DNA binding compared with wild type PutA. Another interesting phenomenon is that without a small N-terminal domain (residue 1-85), PutA does not bind *put* control DNA. This result suggests that a small N-terminal domain may play a key role in PutA conformational changes that occur during DNA binding, or may even be the actual DNA binding

Poster Abstract 29

ANIONIC SUBSTITUTES FOR CATALYTICALLY IMPORTANT ASPARTIC ACIDS IN PHOSPHORIBULOKINASE

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Mutagenic substitution of D42 and D169 with neutral residues alanine and asparagine identified these two aspartic acids as catalytically significant residues in PRK; rates diminished by 10^5 and 10^4, respectively. X-ray structural studies confirmed the location of these residues in the catalytic site and suggested a role for D42 as the catalytic base which deprotonates the C1 hydroxyl of Ru5P; the role for D169 is less clear. In order to further explore the importance of anionic groups at these positions, substitutions with glutamic acid (D42E and D169E) and cysteine (D42C and D169C) were evaluated. Mutants responded similarly to WT in fluorescent binding studies with TNPATP and the allosteric effector NADH. For D42E and D42C, V_{max} decreased by factors of 135 and 220, whereas for D169E and D169C the comparable factors were 39 and 26. Thus, regardless of the type of substitution, changes at D42 more profoundly affect catalytic rate than at D169. Precedent with enzymes in which cysteine replaces an acidic residue suggests that oxidation of the thiolate to a sulfinate can convert low activity cysteine mutants into enzymes with improved activity. Periodate oxidation of cysteine-free PRK results in a decrease in activity. In contrast, treatment of D42C and D169 increases activity by 5 and 7-fold.Thus, for efficient catalysis, PRK requires anionic character in the side chains of residues 42 and 169, but substantial structural and chemical variability is tolerated.

Poster Abstract 30

IN VITRO GLYCOSYLATION OF SECONDARY METABOLITES

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While it is known that the sugar ligands of pharmaceutically important metabolites often define their corresponding biological activity, efficient methods to alter these essential carbohydrate ligands are still lacking. This project now focuses on an approach to accomplish this goal while also providing invaluable mechanistic and structural information on a critical, but poorly understood, enzyme class of glycosyltransferases. Specifically, our studies are designed to exploit structure/function-based protein engineering to generate a *in vitro* nucleotidylyl transferase/glycosyltransferase system which will provide a library of potentially new bioactive metabolites not accessible via conventional organic synthesis. The selected *in vitro* approaches include the *Salmonella rml*A-encoded α-D-glucose-1-phosphate thymidylyltransferase (Ep) and glycosyltransferases which are involved in the biosynthesis of secondary metabolites of actinomycetes, e.g. Vancomycin (*A. orientalis*), Rebeccamycin (*S. aerocolonigenes*), Avermectin (*S. avermitilis*), Novobiocin (*S. spheroides*), Daunorubicin (*S. peucetius*), Calicheamicin (*M. echinospora*), Erythromycin (*S. erythrea*), Megalomicin A (*M. megalomicea*) and Staurosporine (*S. staurosporeus*). This work presents the extension of our nucleotide sugar library by the use of Ep as well as the first successful approaches for the *in vitro* glycosylation by recombinant glycosyltransferases.

Poster Abstract 31

PRIMARY AND SECONDARY H/T *V/K* KIE STUDIES WITH wt *E. coli* DIYHDROFOLATE REDUCTASE

KELLI MARKHAM and Amnon Kohen, University of Iowa, Department of Chemistry, Iowa City, IA, 52242

Dihydrofolate reductase (DHFR) is an important enzyme involved in the metabolic production of thymine for DNA synthesis. As a result, this small, flexible enzyme is often targeted for anti-microbial or chemotherapeutic agents. A better understanding of its mechanism is needed to further medicinal studies, and it is also an ideal model system to advance the general scientific knowledge about enzyme catalysis.

Competitive *V/K* KIEs are being used as a tool to study the chemical step, cf. the hydride transfer step, of the reaction catalyzed by DHFR. These KIEs are determined by product analysis via HPLC and LSC. The substrates needed for primary (1˚) and secondary (2˚) H/T analysis are [*R*-^{3}H]-NADPH, [*S*-^{3}H]-NADPH and [^{14}C-Ad]-NADPH and their enzymatic synthesis will be described.

Thus far, 1˚ and 2˚ *V/K* KIEs have been measured under saturation of substrates at pH 9.0, where the chemistry is more rate limiting. Preliminary data suggests a 1° KIE of 4.87±0.35 and a 2° KIE of 1.12±0.02 for wt *E.coli* DHFR. 2˚ KIE data can provide insight into various mechanistic issues such as the location of the transition state or to distinguish between an associative or dissociative mechanism. D/T *V/K* KIE studies are in progress. This data, in conjunction with the H/T data will facilitate the calculation of the Swain-Schaad relationship to determine the extent of hydrogen tunneling.

Experimental H/D/T competitive KIEs measured for this enzyme will verify or contradict the current theoretical dogma surrounding DHFR's mechanism. Future studies with mutants of DHFR with altered dynamics will provide a relationship between this enzyme's catalytic ability and protein dynamics. This experimental work will provide much needed mechanistic insight and has many implications, not only for DHFR, but also for other nicotinamide dependent enzymes and enzymology in general.

Poster Abstract 32

IDENTIFICATION AND CHARACTERIZATION OF C-GLYCOSYLTRANSFERASE FROM HEDAMYCIN BIOSYNTHETIC PATHWAY

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Secondary metabolites produced from various Actinomycetes still provide the most abundant leads for medicine in the pharmaceutical industry. Most of these agents contain polyketide rings decked with various sugar moieties. The carbohydrate ligands have been shown to be important for specificity and activity of these metabolites. The aryl-*C*-glycoside antibiotics hedamycin produced by *Streptomyces griseoruber* exhibits both and an *ortho*- and *para*-*C*-glycosyl attachment to an aromatic aglycon. In order to characterize nature's mechanism of *C*-glycosylation, we have cloned the hedamycin biosynthetic gene cluster containing the glycosyltransferase, along with the polyketide synthase and sugar biosynthetic genes. A Keto Synthase disruption mutant is constructed to confirm the importance of the cloned genes for the production of hedamycin in the producing strain. Furthermore, heterologous expression of the cloned cluster in *S. Lividans K4-114* leads to the production of new compounds in the transformed strain. Future work will focus on mechanism of the recombinant C-glycosyltransferase and ultimately, the C-glycosyltransferase genes will be utilized to bioengineer novel compounds *in vitro* and *in vivo* by mixing a combinatorial selection of sugars and various aglycons.

Poster Abstract 33

NOVEL OXIDASES AND HALOGENASES REQUIRED FOR CALICHEAMICIN BIOSYNTHESIS

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Calicheamicin is an enediyne antitumor antibiotic with remarkable reactivity whose complex structure results in the site specific oxidative double strand scission of targeted DNA. Among the variety of proteins involved in the biosynthetic pathway of calicheamicin are two P450 oxidases (CalS, CalW) and a halogenase (CalT). Initial studies of theses proteins include overexpression and purification of each protein and activity studies using an assortment of substrates. All three proteins have been expressed in *E. coli*, though CalS and CalW show low expression. The current growth conditions for CalT result primarily in an insoluble protein. Purification of these proteins can be achieved using nickel chelate chromatography with since they contain a polyhistidine tag. CalS and CalW show purification to near homogeneity. This work will allow determination of the location of action for each protein. In addition, closer inspection of the reactions could reveal insight on P450 specificity in biosynthesis and understanding of the mechanisms involved, particularly in the case of the halogenase. A better understanding of the role of these proteins in the biosynthesis of calicheamicin will help forge the construction of new enediyne-based therapeutics.

Poster Abstract 34

SPECTROELECTROCHEMICAL STUDIES OF THE DIIRON CENTERS OF STEAROLY-ACYL CARRIER PROTEIN Δ^9 DESATURASE

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Stearoyl-acyl carrier protein (stearoyl-ACP) Δ^9 desaturase (Δ^9D) is a diiron enzyme that catalyzes the insertion of a cis double bond between the 9 and 10 position of the stearoyl-ACP to convert into oleoyl-ACP. Each subunit of homodimeric resting Δ9D contains a nonheme diferric cluster, while $4e^-$ reduction produces the diferrous active form of the enzyme. The diiron centers of Δ^9D are studied by spectroelectrochemical methods in HEPES buffer (pH 7.5) with 5% glycerol. Coulometric reductive titrations of the resting diferric clusters following the absorption at 356 nm (ε = 8400 M^{-1} cm^{-1}) reveals $4e^-$ reduction per homo-dimer. No obvious difference is observed between the reduction of the diiron center of each subunit. Potential metric titrations of Δ^9D are being carried out to obtain the midpoint reduction potential (E_m). Mediator dyes including 2.6-dichloroindophenol (E_m = +256 mV vs SHE), phenazine methasulfate (E_m = +100 mV vs SHE), pyocyanine (E_m = -8 mV vs SHE), and indigo disulfonate (E_m = –84 mV vs SHE) were selected to cover a wide range of redox potentials. E_m of Δ^9D in the range of +20 to +80 mV vs SHE (standard hydrogen electrode) is obtained from selective mediator dye co-reductions. The determination of the exact midpoint reduction potential of resting Δ^9D by Nernst plot is underway. The regulations of substrate/carrier protein binding on the redox potential of the diiron center are also being pursued.

Poster Abstract 35

CONTINUOUS PATHS FOR PROTEIN CONFORMATIONAL CHANGES

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We have applied the Conjugate Peak Refinement method (S. Fischer & M. Karplus, *Chem. Phys. Let.* (1992) 194:252) to calculate the paths for several important conformational changes in proteins. In each case the simulations provide continuous, energetically plausible paths. The nature of the conformational changes vary from relatively simple ones, such as the loop movement in lactate dehydrogenase, to complex motions, like the allosteric mechanism of hemoglobin. Simulations will be presented for myoglobin, nitrophorin, adenylate kinase, lactate dehydrogenase, HIV protease and the subunits of hemoglobin. Using the Conjugate Peak Refinement method to study substrate binding will be discussed, using the example of oxygen binding to myoglobin. In this case, the simulations can distinguish between energetically reasonable and unreasonable paths of oxygen getting into the heme pocket. The tetrameric nature of hemoglobin is required for the allosteric transition, but it also demands changes in the conformations of the individual subunits. By comparing the simulated paths for each subunit in the presence and absence of the other three subunits, we can see what restrictions are placed on the conformational change by the tetrameric nature of the protein.

Poster Abstract 36

INVESTIGATIONS OF ISONIAZID ACTIVATION BY HIGH VALENT IRON-OXO AND OXYFERROUS INTERMEDIATES OF KATG

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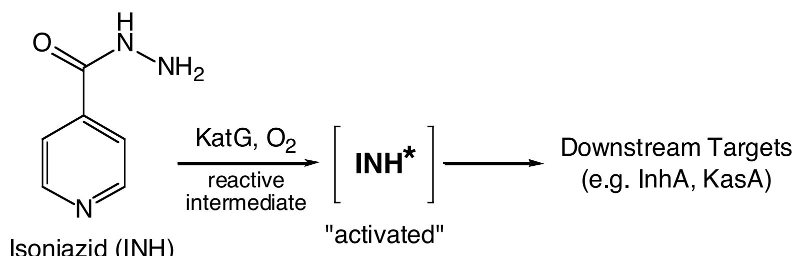
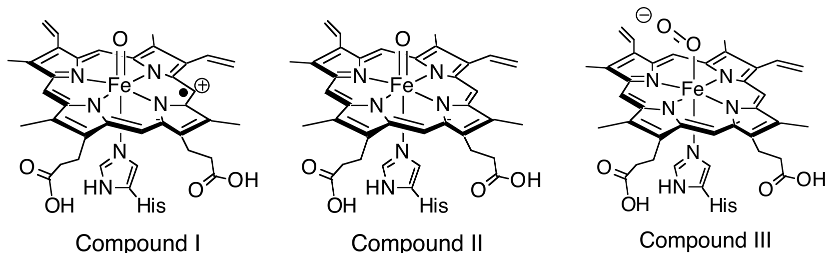
Isoniazid (INH) is a frontline anitbiotic used in the treatment of tuberculosis. As a prodrug, INH requires activation (via oxidation) to an as-of-yet undetermined form by the enzyme KatG, a hemoprotein possessing catalase-peroxidase, Mn^{2+}-peroxidase, peroxynitritase and cytochrome P450 oxygenase activities. Downstream targets of this activated form of isoniazid include InhA and KasA, two enzymes which are involved in the synthesis of mycolic acids that are constituents of the mycobacterium cell wall.

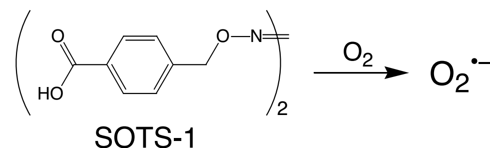


The reactive intermediates of KatG which have been proposed to be capable of oxidizing INH are either the high-valent iron-oxo compounds I/II, or oxyferrous (compound III) KatG. To help further establish the mechanism by which INH is activated by KatG, our efforts have focussed on the generation and characterization of these reactive species by means of low-temperature spectroscopy, as well as by more traditional biochemical studies.



Significant differences in the reactivities of wild-type KatG and its S315T mutant, whose serine to threonine point mutation confers isoniazid resistance to *Mycobacterium tuberculosis*, have been observed in the formation of compound I. In contrast to wild type KatG, no reaction is observed upon addition of *m*-CPBA to KatG S315T, indicating that this peroxide is unable to oxidize KatG S31T to compound I. However, both *tert*-butyl hydroperoxide and PAA give rise to compound I formation with the S315T and the wild-type enzymes, and further studies involving the constitutional isomers of *m*-CPBA are in progress to determine the origin of this effect and map conformational flexibility in the active site of both enzymes.

Furthermore, recent work with the superoxide thermal source SOTS-1 has confirmed an earlier study which was able to correlate differences in INH susceptibility by KatG WT versus the S315T mutant to superoxide by invoking an oxyferrous intermediate. Details of these investigations will also be presented.



Poster Abstract 37

MOLECULAR CLONING, LOCALIZATION AND FUNCTIONAL EXPRESSION OF HYDROXYISOURATE HYDROLASE, A NOVEL ENZYME OF THE UREIDE PATHWAY

ANIRUDDHA RAYCHAUDHURI and Peter A. Tipton. Department of Biochemistry, University of Missouri, Columbia, MO 65211

Allantoin and allantoate, collectively referred to as the ureides, are the most efficient nitrogen transport molecules in tropical legumes. Purine oxidation is the major route for ureide biogenesis, and the enzymes that carry out the conversion of IMP to allantoin and allantoate constitute the so-called ureide pathway. Here, we report the molecular cloning and functional expression of a novel gene of the ureide pathway that encodes for hydroxyisourate hydrolase (HIUHase). HIUHase catalyzes the hydrolysis of hydroxyisourate, the true product of the urate oxidase reaction. The cDNA is 1,690 bp in length and codes for a precursor protein comprising a 33-residue transit peptide and a 512-residue mature protein of 54 kD. The cDNA encoding the mature protein was overexpressed in *Escherichia coli*. Immunogold studies demonstrated the localization of the enzyme in the peroxisomes, which is also the locale of the other ureide metabolizing enzymes. HIUHase protein and gene expression is coordinate with urate oxidase expression. HIUHase has considerable homology with family 1 β-glucosidase proteins from bacteria, plants and animals. E197 and E406 were identified as potential catalytic residues on the basis of sequence homology with β-glucosidases. The E197A and E406A mutant proteins were prepared and found to be devoid of catalytic activity. Basic kinetic studies of the enzyme and the functional role of both the residues, probed by kinetic studies will be presented.

Poster Abstract 38

CHARACTERIZATION OF THE SELF-RESISTANCE MECHANISM TOWARDS THE ENEDIYNE ANTITUMOR MOLECULE CALICHEAMICIN IN *MICROMONOSPORA*

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Calicheamicinγ_1^I (CLM), from soil-bacterium *Micromonospora echinospora* spp. *calichensis*, is the most prominent member of the enediyne family of antibiotics, and its unprecedented molecular architecture in conjunction with its superb biological activity and therapeutic value brand CLM an excellent target for the study of natural product biosynthesis. Its biological activity results in site-specific oxidative double-strand scission of DNA as targeted through the minor groove binding of its aryltetrasaccharide moiety. As Such, CLM has displayed potencies >8000-fold higher than that of adriamycin. Despite this profound toxicity, this molecule is a natural product; therefore a resistance mechanism must exist which prevents the host microbe from succumbing to its own toxic metabolite. We report the discovery and characterization of a gene (*cal*C) encoding a protein (CalC) that specifically confers resistance to CLM. This work represents the first cloning and characterization of a resistance mediator to any known enediyne antibiotic, and in using a unique assay based on fluorescence resonance electron transfer (FRET), we have elucidated the mechanism with which CalC affords protection to the genomic DNA of *Micromonospora*. By acting as a 1,4-benzyl diradical quencher, CalC is able to detoxify activated CLM, resulting in site-specific oxidative cleavage (*i.e*. proteolysis) in the place of DNA. This intracellular detoxification, which is believed to act as a “failsafe” mechanism to ensure genomic integrity, provides an unprecedented self-resistance mechanism within the repertoire of known antibiotic producing microorganisms.

Poster Abstract 39

pH-DEPENDENCE OF THE KINETIC ISOTOPE EFFECT ON DIHYDROOROTATE DEHYDROGENASE USING DIHYDROOXONATE AS A SUBSTRATE

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The flavo-enzyme dihydroorotate dehydrogenase from *Lactoccocus lactis* (DHODA) oxidizes dihydroorotate (DHO) to orotate. This oxidation is the fourth and only reduction/oxidation step in biological pyrimidine synthesis. DHO oxidation by DHODA occurs by the transfer of a hydride from DHO to flavin mononucleotide (FMN) and the transfer of a proton from carbon-5 of DHO to the base Cys 130. Subsequently, orotate dissociates from the reduced enzyme. Dihydrooxonate (DHOx) is a synthetic analog of DHO where carbon-5 has been replaced by a nitrogen atom. DHOx is oxidized to oxonate by DHODA through a supposedly similar mechanism. However, since the atom at position 5 in the two substrates differ, the rate constant for some steps in the oxidation reaction may differ for DHO vs. DHOx. For example, the atom at position 5 must be deprotonated; however, the electronegativity of the atom at position 5 will greatly influence the ease of deprotonation. Using stopped-flow spectrophotometry, the rate of reduction was monitored at 456 nm as the enzyme reacted with DHOx. Kinetic isotope effects were determined over a range of pH values from 6 to 9.5.

Poster Abstract 40

SALICYLATE INHIBITS THE HUMAN FLAVOENZYME DIHYDROOROTATE DEHYDROGENASE

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Dihydroorotate Dehydrogenase (DHOD) is a flavoenzyme catalyzing the oxidation of dihydroorotate to orotate in the fourth step of *de novo* pyrimidine biosynthesis. Pyrimidine biosynthesis is a necessary metabolic function for proper cell growth and replication. This makes DHOD a target for specific inhibition, which could be useful in cases of malaria, cancer or rheumatoid arthritis. Salicylate, the hydrolysis product of aspirin, has been found to inhibit human DHOD, as well as other Class 2 DHOD's, which are membrane bound proteins found in eukaryotes and some prokaryotes such as *E.coli*. In an effort to study the mechanism of inhibition caused by salicylate, the reductive and oxidative half reactions were studied using rapid reaction methods, both with and without salicylate. It was found that salicylate does not inhibit the reductive half-reaction, which involves the conversion of dihydroorotate to orotate while reducing the flavin. Enzyme monitored turnover experiments showed that salicylate is uncompetitive with DHO. Additional evidence from binding experiments showed that salicylate does not bind at the DHO/Orotate site. In the oxidative half-reaction, a quinone acts as an oxidizing agent. This half-reaction was found to be inhibited by salicylate.

Poster Abstract 41

DIHYDROURIDINE IN TRNA IS SYNTHESIZED BY A FLAVOENZYME

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One of the most common post-transcriptional base modification in tRNA is the conversion of uridine to 5,6-dihydrouridine, found in the D-loop. Recently, the enzyme responsible for formation of dihydrouridine in tRNA, dihydruridine synthase (DUS), has been identified. *S. cerevisiae* has 4 different types of DUS. DUS2 was found to utilize pre-$tRNA^{Leu}$ and pre-$tRNA^{Tyr}$ as a substrate, but not pre-$tRNA^{Phe}$ [Xing, F., Martzen, M.R., and Phizicky, E.M. (2002). RNA **8**;370-381].

We have begun to characterize the N-terminal Histidine tagged DUS2 of *S. cerevisiae*, which has a molecular weight of 45.3 kDa. Absorbance spectra reveal peaks at 457 nm and 370 nm, indicating the presence of a flavin cofactor, found to be FMN by TLC analysis. The extinction coefficient at 456 nm was found to be 10600 $M^{-1} cm^{-1}$, and the pI was found to be 9. In the absence of oxygen, NADPH rapidly reduces DUS2 as evidenced by the change in the flavin spectrum. NADH also reduces the enzyme, but at a slower rate, indicating that NADPH is the physiological substrate. Both NADH and NADPH were found to stereospecifically reduce DUS2 with the *pro-R* hydrogen on C4 of the pyridine ring. Our data and analogies with dihydroorotate dehydrogenase and dihydropyrimidine dehydrogenase suggest a chemical mechanism for dihydrouridine synthesis: enzyme-bound FMN is first reduced by NAD(P)H; after NAD(P) release, the flavin hydroquinone reduces the α,β-unsaturated carbonyl moiety of uridyl-tRNA, forming dihydrouridine.

Poster Abstract 42

MECHANISTIC STUDIES OF THYMIDYLATE SYNTHASE USING KINETIC ISOTOPE EFFECTS

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Thymidylate Synthase (TS; EC 2.1.1.45) catalyzes the reductive methylation of Uracil (dUMP) to 2'- deoxythymidylate-5'- monophosphate (dTMP). This enzyme is an important target of chemotherapeutic and antibiotic drugs that eventually lead to cell death. The mechanism of this enzyme is of great interest because it catalyzes 2 independent reactions where the cofactor N^5, N^{10} -Methylene 5,6,7,8-tetrahydrofolate serves both as a methylene donor and a hydride (H^-) donor in the reaction. We are developing tools to study the chemical mechanism of this enzyme by measuring intrinsic kinetic isotope effects (KIEs). Our experimental procedures might also detect quantum mechanical H-tunneling that has not been studied for this enzyme previously. In order to measure the KIEs, we are synthesizing isotopically labeled *R*- [6- ^{x}H] methylene tetrahydrofolate (where **X**= H, D, T). Reverse phase HPLC is used to monitor and purify the labeled compounds. With these labeled substrates and [^{14}C] dUMP, mechanistic studies are designed to enhance our understanding of the TS catalyzed reaction's mechanism. Such understanding, in turn, should lead to the development of new approaches to cancer related chemotherapeutic and antibiotic drugs.

Poster Abstract 43

UNDERSTANDING THE MECHANISM OF OLIGOSACCHARYLTRANSFERASE: DEVELOPMENT OF A RAPID ASSAY AND SYNTHESIS OF ISOTOPICALLY LABELED SUBSTRATES

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Oligosaccharyltransferase (OST) catalyzes the co-translational en bloc transfer of dolichylpyrophosphate oligosaccharides to an asparagine residue found in the sequon Asn-Xaa-Thr/Ser of growing peptides as they exit the ribosomal complex and cross the E.R. membrane. The method most commonly used to monitor this reaction, involving multiple extractions and HPLC, is extremely time consuming. We have developed a rapid assay based on use of a biotinylated peptide as the acceptor substrate, and dolichylpyrophosphate [^{3}H]chitobiose as the donor for the OST reaction[1]. This allows for separation (avidin-agarose chromatography) and identification (scintillation counting) of only the biotinylated glycopeptide product of the OST-catalyzed reaction. This new assay method yields fast and highly reproducible results. We also present the *de novo* design and synthesis of dolichylpyrophosphate chitobiose analogues that will enable us to use our new biotin capture assay to probe for possible α-secondary kinetic isotope effects in the OST-catalyzed reaction.

This study was supported by funds from the American Foundation for Pharmaceutical Education, the Pharamceutical Sciences Training Program (NIH training grant, GM 07767), the College of Pharmacy and the Hans & Ella McCollum Valteich Research Endowment Fund.

1. Srinivasan, A., Coward, J.K., A biotin capture assay for oligosaccharyltransferase. *Anal. Biochem.*, 2002, 306; 328-335.

Poster Abstract 44

DEPENDENCY OF DNA AND PROTEIN INTERACTIONS ON COFACTORS: TAKING ADVANTAGE OF METAL CATIONS AS PROBES FOR SITE SPECIFIC BINDING AND HYDROLYSIS

LORI BOWEN and Cynthia Dupureur, University of Missouri St. Louis, Department of Chemistry and Biochemistry, St. Louis, MO 63121

A fundamental question from nature concerns the ability of nucleic acid associated enzymes to recognize and hydrolyze specific phosphodiester bonds. Divalent metal cations often play an important role for these processes. Using *PvuII* restriction endonuclease (RE) as a model system, the interaction between protein and DNA in the presence of divalent metal cations is being investigated. Magnesium (II) is commonly associated with enzymes to facilitate target recognition and/or hydrolysis and is the preferred metal for *PvuII* RE activity. It has been shown that Ca(II) has the ability to promote specific *PvuII* RE-DNA binding (6000 fold over that of metal free conditions) while inhibiting hydrolysis. What properties of divalent metal cations make them suitable for promoting both recognition and catalysis? Nitrocellulose filter binding, fluorescence anisotropy, and single turnover kinetics are being used to study the effect of a variety of Ca(II) mimics and divalent metal cations on *PvuII* RE-DNA binding and phosphodiester hydrolysis. Preliminary results show that *PvuII* RE-DNA binding is most greatly enhanced in the presence of Ca(II) with a Kd of 50 pM while Zn(II) has a much smaller effect with a Kd of >250 nM. Binding enhancement is as follows with Ca(II) having the largest effect: Ca(II)>Tb(III)>Eu(III)>Ba(II)>Cd(II)~Co(II) ~Sr(II)>Zn(II). Phosphodiester hydrolysis in the presence of these metals has thus far been negligible with $k>1 day^{-1}$.

Poster Abstract 45

A PREDICTIVE MODEL OF STRONG HYDROGEN BONDING IN PROTEINS: THE $N^{\delta 1}$-H-$O^{\delta 1}$ HYDROGEN BOND IN SERINE PROTEASES

JAN H. JENSEN and Pablo A. Molina, University of Iowa, Department of Chemistry, Iowa City, IA 52242-1294

We present the first theoretical model of a strong hydrogen bond (HB) in an enzyme (the $N^{\delta 1}$-H-$O^{\delta 1}$ HB in low-pH α-chymotrypsin) that satisfactorily reproduces the experimentally observed proton chemical shift and H/D fractionation factor. The validated model is used to predict additional properties for this HB (the NH bond length, the proton-triton effect on the chemical shielding, the ratio of vibrational stretch frequencies: ν_{AH}/ν_{AD}) that are consistent with experimental values obtained for related systems.

SITE DIRECTED MUTAGENESIS STUDIES OF THE PROLINE DEHYDROGENASE ACTIVE SITE IN PUTA FROM *ESCHERICHIA COLI*

BEREVAN BABAN[1], Jack Tenner[2] and Donald F. Becker[1]. [1]Department of Chemistry and Biochemistry, University of Missouri-St. Louis, St. Louis, MO 63121. [2]Department of Chemistry, University of Missouri-Columbia, Columbia, MO 65211.

The PutA flavoprotein from *Escherichia coli* is a multifunctional enzyme that catalyzes the two-step conversion of proline to glutamate. In the first enzymatic step, the oxidation of proline to pyrroline-5-carboxylate is coupled with the reduction of tightly associated FAD. In the second reaction, the oxidation of pyrroline-5-carboxylate to glutamate is coupled with reduction of NAD. PutA is also a DNA binding protein that regulates the transcription of the proline utilization regulon that contains the *putA* and *putP* (proline transporter) genes. PutA is comprised of 1320 amino acid and purifies as a homodimer of 293 kDa. Recently, the crystal structure of a truncated PutA protein containing the proline dehydrogenase (PDH) (261-612) and DNA binding domains (139-260) was solved. On the bases of this structure, we engineered a PutA protein comprised of residues 85-601 ($PutA_{85-601}$) in order to study the known structural form of PutA and the mechanism of proline oxidation.

$PutA_{85-601}$ was purified to homogeneity and was shown to have PDH specific activity 5-fold higher than PutA. Unexpectedly binding of $PutA_{85-601}$ to the *put* control DNA of the *put* regulon was not observed in gel-shift assays. The reduction potential of $PutA_{85-601}$ is ~ -65 mV (vs. SHE, pH 7.0) which is similar to that reported previously for PutA (E_m = - 76 mV, pH 7.5) (4). In the three-dimensional structure of PutA, PDH active site residues Asp370, Tyr540, and Arg431 appear to play an important role in the catalytic mechanism of proline oxidation. The hydrogen-bonding network between these residues helps position the substrate (proline) relative to the FAD cofactor and may be involved in general base catalysis of proline oxidation. Arg431 interacts directly with the N5 position of the FAD indicating it is important for FAD binding and stabilization of reduced FAD. To test the role of these residues we mutated the wild type $PutA_{85-601}$ at residues 370, 431, 540 as follows: D370A, R431A, Y540A, Y540F, and a double mutant D370A, Y540F. The kinetic parameters K_m and k_{cat}, and the reduction potential were determined for each mutant. From our preliminary characterization of the $PutA_{85-601}$ site–directed mutants, we conclude residues D370 and Y540 are not involved in a general base mechanism and that they primarily serve to help form the proline-binding site.

Poster Abstract 47

INVESTIGATING THE CATALYTIC PROCESSIVITY OF HUMAN FOLYLPOLY-γ-GLUTAMATE SYNTHETASE

JOHN TOMSHO, Laurel Royer, and James K. Coward, Departments of Medicinal Chemistry and Chemistry, University of Michigan

Folylpoly -γ -glutamate synthetase (FPGS) is the enzyme responsible for catalyzing the formation of poly(γ-glutamyl) folates and anti-folates, the kinetically preferred substrates or inhibitors for many folate-dependent enzymes. These enzymes catalyze one-carbon transfer reactions that are responsible for the biosynthesis of certain amino acids and nucleotides making them indispensable for cellular growth and repair. Also, the poly(γ-glutamyl) folates are retained within the cell to a greater extent. Mechanistic studies of this enzyme may therefore play a part in designing better anti-folate chemotherapy drugs. The question of how the enzyme adds multiple glutamate residues onto the folate substrate to yield the polyglutamate products remains to be elucidated. Pulse-chase kinetic experiments have been proposed to address the processivity of FPGS.

The combination of human FPGS and 5,10-dideaza tetrahydrofolic acid (DDATHF) has been chosen as the enzyme-substrate pair. It has been shown that the folate products of this reaction undergo several glutamate additions. DDATHF, [^{3}H]-DDATHF, and [^{14}C]-DDATHF have been synthesized via the 5,10-dideaza tetrahydropteroyl azide. This route gives access not only to the radiolabeled folate substrates but also to different chain-length polyglutamates and FPGS inhibitors with the same heterocycle. Currently, a continuous, spectrophotometric assay that monitors ATP hydrolysis has been employed as a method to study the non-productive ATPase activity of FPGS. A non-continuous, radioactivity-based assay that measures glutamate incorporation into the folate polyglutamate has been re-established. Finally, an HPLC/scintillation counting system has been developed for the separation of the folate polyglutamates based on chain-length followed by quantitation.

This work is supported by grants from the National Institutes of Health (CA28097) (JT), Chemistry-Biology Interface Training Grant (GM08597) (JT), American Foundation for Pharmaceutical Education Fellowship (JT), University of Michigan College of Pharmacy Fred W. Lyons Fellowship (JT), and the University of Michigan Horace H. Rackham School of Graduate Studies Summer Research Opportunity Program (LR).

Poster Abstract 48

CALCINEURIN: A PEROXIDE-SPECIFIC SENSOR IN T-LYMPHOCYTES

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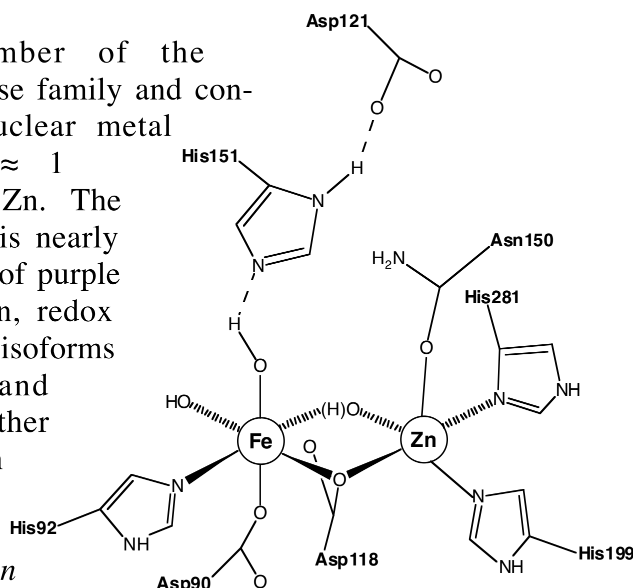
Calcineurin is a member of the serine/threonine phosphatase family and contains an active site dinuclear metal cluster consisting of ≈ 1 equivalent each of Fe and Zn. The active site of calcineurin is nearly identical to the active site of purple acid phosphatase; a diiron, redox sensitive enzyme. Metalloisoforms of mammalian PAP and calcineurin, containing either dinuclear Fe-Fe or Fe-Zn metal clusters are active only in the mixed valent oxidation *in vitro* (Fe^{3+}-Fe^{2+} and Fe^{3+}-Zn^{2+}, respectively).



Calcineurin activity in T-lymphocytes has been shown to be sensitive to H_2O_2 (IC_{50} ≈ 30 μM), yet insensitive to superoxide (1 mM paraquat) and hypochlorite (600 μM). Sensitivity to H_2O_2 and hypochlorite *in vivo* correlates with the half-life of each species in cell medium. The intracellular redox potential is unaffected by H_2O_2 (100 μM) or hypochlorite (600 μM), indicating that H_2O_2 inhibits calcineurin via a direct mechanism that does not involve a change in the cytosolic redox potential. In contrast, calcineurin activity in cell lysate is inhibited by these oxidants *in vitro*: superoxide (60 μM), hypochlorite (IC_{50} ≈ 150 μM), and H_2O_2 (IC_{50} ≈ 70 μM). Calcineurin inactivation in brain lysate follows very rapid kinetics (inactivation within 10 seconds of H_2O_2 addition). H_2O_2 inactivation of calcineurin is reversible in T-lymphocyte lysate using DTT alone, while in rat brain lysate, reversibility requires both DTT and Fe^{2+}, implicating that the enzyme may exist in different metal/redox-sensitive states in different tissues. Redox titration experiments were performed utilizing mono-Fe, Fe-Zn, and Fe-Fe metalloisoforms of λ protein phosphatase (λPP), a bacterial enzyme with an identical metal coordination and mechanism to those found in calcineurin. The titration results indicate that all metalloisoforms of λPP have an $E^{o\prime} \geq +130$ mV. By analogy, this suggests that calcineurin exists in its reduced state (Fe^{2+}-Zn^{2+} or Fe^{2+}-Fe^{2+}) in the cytoplasm (E = – 260 mV).

Poster Abstract 49

INVESTIGATION OF ISONIAZID ACTIVATION BY *MYCOBACTERIUM TUBERCULOSIS* KATG: HOW IS ISONICOTINAMIDE FORMED?

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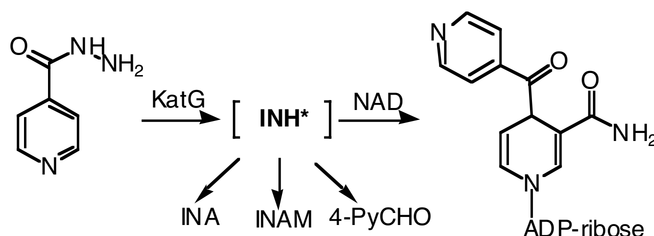
Isoniazid (INH) is a front line antibiotic for the treatment of tubuerculosis. It is believed that *in vivo*, isoniazid is oxidized into its active form by KatG (catalase-peroxidase) followed by reaction with NAD^+ or NADH to form an INH-NAD adduct. The latter is an inhibitor of the enoyl acyl carrier protein reductase, InhA, a key component in the biosynthesis of mycolic acids, long-chain fatty acids that are an intergral part of the mycobacterial cell wall. However, the identity of the intermediate and the chemical pathway of activation is not yet understood or well illustrated. Kinetic data indicates that the INH-NAD adduct is formed in the active site of InhA while other studies have suggested that the adduct is formed before binding to InhA. As these contradictory conclusions are derived from different experimental conditions, different mechanisms or different intermediates are possible.



The metabolites of isoniazid by KatG (*in vitro*) are isonicotinic acid (INA), isonicotinamide (INAM), and 4-pyridinecarboxaldehyde. INA is the major metabolite but the formation of INAM has yet to be accounted for. There is debate whether the amide reaction product is generated from splitting of the N—N bond of INH through a possible disproportonation of the INH hydrazide moiety or whether its formation is generated by coupling of the isonicotinoyl radical with ammonia. Supportive evidence for either is lacking.

We present evidence for the formation of ammonia in the INH activation reaction by KatG, and that the amount of NH_3 is greater than that of INAM. We propose that the formation of NH_3 is the result of diimide disproportonation, generating hydrazine and N_2, with the former being reduced by another equivalent of diimide to generate NH_3. Another possibility, that INH is reduced by diimide to give rise to INAM, is less likely given the greaer stoichiometry of NH_3 to INAM. The investigation of reduction of INH by chemically generated diimide is under way. The measurement of hydrazine will be discussed. Hammett linear relationship of substituent effect made possible by wide substrate specificity of KatG will also be discussed.

Poster Abstract 50

MECHANISM OF ACTIVATION OF CTP:PHOSPHOCHOLINE CYTIDYLTRANSFERASE BY LIPIDS

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CTP:phosphocholine cytidyltransferase (CCT) is an enzyme found in eukaryotic cells. It catalyzes the formation of CDP-choline from CTP and phosphocholine, which is important for the biosynthesis of phosphatidylcholine. CCT exists in two forms: free floating within the cell and attached to the cell membrane. The latter is the activated form and is the focus of the study. We are investigating what amino acids are responsible for CCT binding to the cell membrane, using site-directed mutagenesis. Using *C. elegans* CCT, previous work has identified amino acids 246-266 as critical for lipid activation. This region is thought to be an amphipathic α-helix. We have mutated amino acids 246, 249, 256, 260 and 264, which are of the hydrophobic face of the α-helix. His-tagged forms of the mutant enzymes are produced in Sf9 insect cells, purified using metal affinity chromatography and analyzed for ability to be activated by lipids.

Poster Abstract 51

HETEROLOGOUS EXPRESSION AND PURIFICATION OF SPAB INVOLVED IN SUBTILIN BIOSYNTHESIS

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Lantibiotic peptides contain thioether bridges termed lanthionines that are putatively generated by dehydration of Ser and Thr residues followed by Michael addition of cysteine residues within the peptide. The LanB and LanC proteins have been proposed to catalyze the dehydration and formation of the thioether rings, respectively. We report here the first heterologous overexpression in *Escherichia coli* of SpaB, the putative dehydratase for subtilin. Sequence analysis of *spaB* revealed several nucleotide differences with current gene database entries. The solubility of SpaB was increased dramatically when co-expressed with GroEL/ES, and soluble His_6-tagged SpaB was purified. The oligomerization state of SpaB was confirmed to be at least a dimer. Antibodies were raised against the purified SpaB protein and utilized in interaction studies with SpaC (the putative cyclase). The physical interaction between SpaB and SpaC was observed by coimmunoprecipitation experiments. SpaS the putative substrate for SpaB was overexpressed in *E. coli* as an intein fusion protein, and after cleavage, the peptide was obtained in good yield. RPLC purification of SpaS was complicated by the presence of a mixture of disulfides and was remedied by pre-treating the sample with a strong reducing agent. The intein expression system was employed to generate SpaS analogues incorporating a biotin tag. These modified substrates may prove useful to understand the mechanistic steps in lantibiotic maturation.

Poster Abstract 52

METAL SITES IN YEAST ENOLASE

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Yeast enolase catalyzes the reversible trans dehydration of 2-phospho-D-glycerate (2-PGA) to form phosphoenolpyruvate (PEP) and water. Enolase has an absolute requirement for divalent metals for enzymatic activity. With Mg^{2+} and Mn^{2+} as activators, there are three metal sites per subunit. Apoenolase binds one metal per subunit and in the presence of the substrate, a second metal binds. At higher metal concentrations, a third metal binds and is inhibitory. Crystallographic and spectroscopic data give conflicting information regarding the location and function of metal site two. At low pH (<6.5) there is no inhibition by Mg^{2+} and Mn^{2+} and there are two metal sites per subunit. Fenton chemistry with the protein has been used to locate the metal sites. Radicals are generated and can promote cleavage of the amide bonds in the vicinity of the site of generation. Fenton chemistry is catalyzed by specific transition metals (e.g. Fe^{2+} & Cu^{2+}). Cu^{2+} and Fe^{2+} both activate yeast enolase. At pH 7.5, treatment of enolase with Fe^{2+} and ascorbate or with Cu^{2+} and ascorbate leads to a time dependent inactivation of enolase. The presence of substrate protects against Fe^{2+}-catalyzed Fenton chemistry but not Cu^{2+}-catalyzed inactivation. Similar results are observed at pH 6.2. SDS-page demonstrates loss of protein by Cu^{2+} inactivation but neither Fe^{2+} nor Cu^{2+} show clear evidence of selective cleavage. The results indicate that the generation of Fenton chemistry at the metal sites induces protein damage ergo inactivation but little selective cleavage of the protein is detected.

Poster Abstract 53

A STRUCTURAL BASIS FOR CHEMOPREVENTIVE ACTIVITY OF RESVERATROL-A NATURAL PRODUCT OF WINE

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Non-steroidal antiinflammatory drugs block the cyclooxygenase activity of prostaglandin-H synthase, also known as cyclooxygenase, the enzyme that mediates biosynthesis of eicosanoids from arachidonic acid. Two enzyme isoforms have been identified: COX-1, the constitutive form, and COX-2, the inducible one. Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a phytoalexin found predominantly in grapes, has shown to have antiinflammatory and cancer chemopreventive activity by inhibiting COX activity.

Using the crystal structures of COX-1/resveratrol (determinated by our laboratory) and COX-2/flurbiprofen complexes, we performed receptor docking studies of resveratrol using Dock 4.0.1. The results show that resveratrol binds more tightly to COX-1 than to the COX-2. The 50 top Dock solutions indicate that the resveratrol/COX-1 complex is stabilized by a H-bond between an OH group of the ligand and the aminoacidic residues Ser530 and Arg120. Our results also show that resveratrol is unable to penetrate the extra space of a side-pocket in the active site of COX-2 that is not present in COX-1, and that the interaction with Arg513 is absent, diminishing the selectivity toward COX-2.

In conclusion, our results provide new insights into the binding mode of resveratrol to the two COX isozymes, and should contribute to the design of new selective compounds.

Poster Abstract 54

CHELATION OF THE AMINO ACID MOIETY OF S-ADENOSYLMETHIONINE TO THE UNIQUE IRON SITE OF THE [4FE-4S] CLUSTER OF PYRUVATE FORMATE-LYASE ACTIVATING ENZYME

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Pyruvate formate-lyase activating enzyme (PFL-AE) generates a catalytically essential radical on pyruvate formate-lyase (PFL) and converts S-adenosyl-methionine (AdoMet) to methionine and 5'-deoxyadenosine. Like other members of the Fe-S/AdoMet family of enzymes, PFL-AE is thought to function via generation of AdoMet-derived deoxyadenosyl radical upon the interaction of the catalytically active $[4Fe\text{-}4S]^{+}$ cluster with AdoMet. All of the members of the Fe-S/AdoMet family of enzymes also appear to have a unique iron site, based on the three-cysteine cluster binding motif. However, the role of this unique iron site in the [4Fe-4S] cluster has been elusive. In order to probe the mechanism by which the Fe-S cluster interacts with AdoMet and to determine the role of the unique iron site in the generation of a deoxyadenosyl radical intermediate, we have utilized 35-GHz pulsed electron-nuclear double resonance (ENDOR) studies of the $[4Fe\text{-}4S]^{+}$ cluster of PFL-AE in complex with isotopically labeled AdoMets to show that the unique iron serves to anchor the AdoMet for catalysis.

Poster Abstract 55

CLONING AND EXPRESSION OF DROSOPHILA CCT ISOFORMS I AND II

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The enzyme CTP: phosphocholine cytidylyltransferase (CT), a member of the CDP-choline pathway, results in the biosynthesis of phosphatidylcholine (PC). PC is a major component of eukaryotic cell membranes and a precursor to vital components of signal transduction pathways such as diacylglycerol and phosphatidic acid. This enzyme is of two forms in *Drosophila melanogaster*, and recently, the same has been proven true in humans. Using the Bac-to-bac baculovirus system with Sf9 insect cells, the protein products of both the histag and non-histag forms of isoform I have been produced and purified. Enzymatic assays indicate that both forms are lipid activated and of similar activity. The histag form displays K_m values of 0.25 mM for phosphocholine and 0.30 mM for CTP and a v_{max} value of approximately 1250 nmol/min/mg. Work in progress includes investigating the activation role of other lipids and the cloning and expression of isoform II.

Poster Abstract 56

ENLIGHTENING FEATURES OF THE HIGH RESOLUTION STRUCTURE OF BENZOYLFORMATE DECARBOXYLASE

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Benzoylformate decarboxylase (BFD) is a thiamin diphosphate-dependent enzyme that catalyzes the nonoxidative conversion of benzoylformate into benzaldehyde and carbon dioxide. The overall architecture of BFD resembles those of related enzymes, and residues that bind the cofactor or metal ions are extremely well conserved. Surprisingly, many of the residues in the active site are not conserved among any of the known structures of ThDP-dependent enzymes. Therefore, some of the current challenges are the understanding of the roles of the active-site residues and the evolution of the enzyme reaction mechanism. We solved a series of mutant structures, with and without substrates and inhibitors, to get an idea about the roles of the active-site residues. In some mutant structures, a water molecule replaces an atom of the missing side chain and makes similar hydrogen bonds. The structure of BFD with bicarbonate bound in the active site gives us a hint of the importance of the “forgotten” product of this reaction, carbon dioxide. The various positions of Ser 26 in the structures suggest that it would help in the removal of the carboxyl group. An interesting property of BFD is that it is able to crystallize in different space groups under the same conditions. The change of the conformation of Phe 464 in different space groups may modulate the introduction of substrates and retention of intermediates. The high resolution of the current structures, to 1.0 Å, has provided us a more detailed understanding of BFD and its bound molecules.

Poster Abstract 57

PRELIMINARY STRUCTURAL INVESTIGATIONS OF WILD-TYPE AND MUTANT GLUTARYL-COA DEHYDROGENASE WITH INHIBITORS/PRODUCT

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Glutaryl-CoA dehydrogenase (GCD), one of the eight known acyl-CoA dehydrogenases, is an enzyme involved in lysine and tryptophan catabolism. The initiation of this catalytic reaction where glutaryl-CoA is converted to its trans-enoyl form, glutaconyl-CoA involves the abstraction of an α-proton by the catalytic residue, Glu370, with a corresponding hydride transfer to the FAD cofactor. GCD is subsequently re-oxidized by a series of two, 1-electron transfers to electron transfer flavoprotein.

X-ray structural investigations of wild-type GCD complexed with inhibitors 4-nitrobutaryl-CoA and 3-thiaglutaryl-CoA and the mutant GCD(E370Q) with glutaconyl-CoA have been performed. The structures have been solved by molecular replacement method using the wild-type GCD structure as the starting model and the inhibitors/product have been located in the active site by difference Fourier methods. The structures studied here provide an insight into the catalytic mechanism of GCD utilizing inhibitors, substrate analog and product.

Poster Abstract 58

CRYSTALLOGRAPHIC ANALYSIS OF *E. COLI* 2, 4 - DIENOYL COA REDUCTASE

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Bacterial 2, 4 - dienoyl CoA reductase (DCR) is an iron-sulfur flavoprotein required for the β-oxidation of unsaturated fatty acids with double bonds at even numbered positions. DCR has been crystallized and analyzed by x-ray crystallography. The crystals belong to orthorhombic space group, $P2_12_12_1$; with unit cell dimensions 66.3 x 108.0 x 111.2 Å, and diffract to 2.2 Å. An EXAFS scan of the native crystal form confirms the presence of iron within the enzyme, and has allowed MAD data to be collected at the iron absorption edge. The MAD data have been combined with in-house x-ray data to provide partial phase information. Diffraction data from lead, mercury, and osmium derivatives have also been collected. The iron – sulfur cluster has been located within the asymmetric unit, and the protein – solvent boundary has been traced for solvent flattening procedures. There is one protein molecule per asymmetric unit, with currently 70% of the amino acids traced.

Sequence analysis shows DCR to share 26% identity with trimethylamine dehydrogenase (TMD). The current polypeptide trace shows a TIM barrel domain, with FMN and the 4 Fe – 4 S cluster non covalently attached to the enzyme in a similar manner to TMD. Parts of a second and third domain appear to overlap the medium and small domains of TMD, and give a total Cα *r.m.s.* deviation of 1.62 Å. Further efforts are underway to complete the structure.

Poster Abstract 59

ACCELERATING STRUCTURE-BASED DRUG DESIGN OF FACTOR Xa VIA ENGINEERED BOVINE TRYPSIN

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Trypsin and Factor Xa (FXa) belong to the Serine protease family. They share high degrees of sequence (36%) and structural similarity. We are attempting to accelerate the rate of structure-based drug design of selective FXa inhibitors by transforming the S4 site of bovine trypsin that is easy to crystallize, into the S4 site of FXa that is difficult to crystallize. Single and double mutations of Leucine 99 (L99Y) and Glutamine 175 (Q175F) in bovine trypsin have been designed to mimic S4 specificity pocket of FXa. Kinetic characterization of these mutants indicate that the single mutant (Q175F) has a 7-fold increase in specificity (determined by Vmax/Km) and a 2-fold higher activity towards FXa specific substrates. Crystal structures of both Q175F and L99Y with the active site inhibited by benzamidine have been determined to 1.35è and 1.38è resolution to gain further insight into designing FXa selective inhibitors.

Poster Abstract 60

TRANSIENT-STATE KINETIC CHARACTERIZATION AND THE IDENTIFICATION OF INTERMEDIATES OF GDP-MANNOSE DEHYDROGENASE FROM *PSEUDOMONAS AERUGINOSA*

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The biosynthesis of alginate by the human pathogen *Pseudomonas aeruginosa* requires a multi-enzyme pathway whose committed step is carried out by GDP-mannose dehydrogenase (GMD). GMD catalyzes the four electron oxidation of GDP-mannose to GDP-mannuronic acid, utilizing two equivalents of NAD^+ as cofactors. It is believed that in the first step of the catalytic cycle the substrate is oxidized to an aldehyde. The subsequent fate of this aldehyde intermediate, however, is not fully resolved. One possibility would be attack by a water molecule forming a tetrahedral intermediate that is ultimately oxidized to form the acid product. A second possibility would be the formation of a tetrahedral intermediate that is covalently bound to the enzyme. This intermediate would form as a result of a nucleophilic attack by an active site residue, such as a cysteine, and would then undergo a second oxidation step to form product. Kinetic characterization of GMD has been undertaken in an attempt to identify the intermediates of the catalyzed reaction and distinguish between the two possible fates of the aldehyde intermediate. Rapid chemical quench experiments using radiolabeled GDP-mannose have indicated the formation of both a noncovalent and a covalent, enzyme bound intermediate. These species may represent the aldehyde and/or the two possible tetrahedral intermediates of the reaction. Further characterization of these intermediates in conjunction with the identification of potential active site nucleophiles will not only reveal the chemical mechanism of GMD but also lay the groundwork for the design of inhibitors to the alginate biosynthetic pathway.

Poster Abstract 61

ELUCIDATING THE TIMING MECHANISM OF THE ATP-DEPENDENT PEPTIDE CLEAVAGE REACTION BY LON PROTEASE

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Lon protease, also known as protease La, belongs to a class of ATP-dependent serine protease functioning in living cells to degrade misfolded/nonsense proteins as well as certain short-lived regulatory proteins. Homologs of *lon* have since been found the cytoplasm of prokaryotes and in the mitochondria of eukaryotes. In *E. coli*, Lon functions as a homotetramer, and each monomeric subunit contains an ATPase and a peptidase site. Optimal polypeptide degradation activity of Lon is maintained by the kinetic coordination of the ATPase, the peptidase and putative allosteric protein binding sites.

Using fluorescence resonance energy transfer techniques, we have shown that ATP is more effective than AMPPNP in mediating the cleavage of an unstructured 11 amino acid long fluorescent peptide (S1), whose sequence was designed to mimic a physiological substrate of *E. coli* Lon. Because S1 is degraded by E. coli Lon with the same ATP-dependency as protein substrates, this defined peptide substrate provides us with the opportunity to kinetically examine the contribution of ATP hydrolysis and ADP inhibition in Lon-mediated hydrolysis of an amide bond.

The timing of ATP hydrolysis with respect to peptide bond cleavage was monitored by pre-steady state kinetic techniques. The time courses for ATP hydrolysis and S1 peptide cleavage were monitored by the rapid quench and the stop flow apparatus respectively. The pre-steady rate constants for these microscopic events were determined from the pre-steady state time courses. A minimal kinetic mechanism that accounts for the ATP-dependency in Lon mediated peptide bond cleavage is proposed.

Poster Abstract 62

FUNCTIONAL DISSECTION OF PURR OPERATOR SITE: COMPRISED OF TWO PURBOXES

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The *Bacillus subtilis pur* operon encodes the ten enzymes for *de novo* synthesis of IMP. Two regulatory elements are required for the regulation of pur operon, the purine repressor (PurR) and a DNA operator for repressor (Weng, M., Nagy, P.L., and Zalkin, H. (1985) *Proc. Natl. Acad. Sci.* 92, 7455-7459). Transcription of the operon is initiated 242 nt upstream of the operon (Ebbole, D., and Zalkin, H. (1987) *J. Biol. Chem.* 262, 8274-8287). PurR binding to the operator site is blocked by PRPP. In the related bacterium *Lactococcus lactis*, the "GAAC" motif occurs within "PurBox" sequences in a 13-nt (AWWWCCGAACWWT) consensus region (Kilstrup *et al* (1998) *J. Bacteriol.* 180, 3900-3906). The PurBoxes in *B. subtilis* are arranged as a pseudo-palindrome (5'-GAAC-N_{24}-GTTC-3') beginning at position -75 relative to the transcriptional start site. (Shin, B.S., Stein, A., and Zalkin, H. (1997) *J. Bacteriol.* 179, 7394-7402).

We have investigated the interaction of the PurR repressor with a series of shortened upstream DNA fragments to determine the minimum length and specificity elements of the operator. The PurBoxes are necessary but not sufficient for the PurR-DNA interaction. The apparent K_d value for PurR binding to the full-length control region (207 bp, -182 to +25) was 6 nM. The length of the operator-DNA profoundly influences PurR binding. A DNA oligomer of 92 bp (-96 to -5) had a K_d value of 18 nM. Mutation of the downstream PurBox to produce a strict palindromic PuRBox increased PurR affinity three fold and have cooperative binding nature. This suggested that upstream PurBox has tight and the downstream PuRBox has loose binding affinity for PuR. Surprisingly, PRPP can't knock off the PurR from the operator having two tight binding PurBoxes. The effect of PRPP in the PurR-DNA complex is found only when operator DNA contains both tight and loose binding PuRBoxes.

Poster Abstract 63

A MECHANISTIC INVESTIGATION OF 4–HYDROXYPHENYLPYRUVATE DIOXYGENASE FROM *STREPTOMYCES AVERMITILIS* USING SUBSTRATE ANALOGS

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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, despite free rotation of the approximately isosteric aromatic ring. The product, 2,5–dihydroxy–4–fluorophenylacetate, was identified with ^{1}H 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 hydoxyl group of HPP is necessary for catalysis, via a hydrogen bonding interaction with HPPD since phenylpyruvic acid (PPA) and *p*–aminophenylpyruvic acid (PAPPA) do not stimulate the enzyme to react with molecular oxygen, yet apparently bind to the active site in a similar manner.

Poster Abstract 64

MECHANISTIC STUDIES OF YEAST T298C PYRUVATE KINASE

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Pyruvate kinase (PK) catalyzes the phosphorylation of ADP by phosphoenolpyruvate (PEP) followed by protonation of enolpyruvate. X-ray diffraction of muscle and yeast PK indicates T298 (yeast numbering) as the putative proton donor. The protonation step by yeast PK was investigated by the construction, purification and study of T298C. T298C shows 20% k_{cat} and increased $K_{m,PEP}$ relative to wild type (WT) PK. T298C is kinetically similar to WT with Mn^{2+} as the divalent activator. With Mg^{2+}, T298C also requires the activator fructose-1,6-bisphosphate (FBP) for activity. A shift of $pK_{a,2}$ from 6.4 to 5.5 is observed with T298C from k_{cat} vs. pH studies. A pH dependence of the rate constant of inactivation by iodoacetate of wild type and T298C YPK indicates an intrinsic pK_a of 8.2 for C298. Fluorescence binding studies reveal altered K_d values for Mn^{2+} and for FBP to all T298C complexes. Successive fluorescence emission spectra of the T298C-Mg-PEP-MgADP complex indicate that MgADP and PEP bind to T298C-Mg. Lack of catalytic activity by T298C with Mg^{2+} is not because of the loss of substrate binding. Proton inventory studies reveal solvent isotope effects on k_{cat} and $k_{cat}/K_{m,PEP}$. Fractionation factors are metal dependent and are 0.2-0.7. The data are consistent with water from a channel into the active site as the ultimate proton donor in PK; one water is bound to the active site metal. T298C influences the activity of this water.

Research supported by the Chemistry & Biochemistry Department

Poster Abstract 65

CRYSTALLIZATION AND KINETIC STUDY OF 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE FROM *STREPTOMYCES AVERMITILIS*

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The second step of the tyrosine catabolism pathway is the conversion of 4-hydroxyphenylpyruvate to homogensitate by the FeII-dependent enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27). Although several plausible mechanisms have been proposed for this enzyme, no direct evidence has yet been presented for any of the reaction coordinate intermediates. The initial goal of our research is to study HPPD using steady state and pre-steady state methods to find evidence for the accumulation of intermediates. We have observed the steady-state isotope effect of the deuterated substrate with the HPPD, by synthesizing ring deuterated HPP from deuterated tyrosine, using tyrosine amino transferase (TAT).

We proposed to mutate conserved residues in the active site of HPPD in order to gain additional mechanistic insights. However, in the absence of the crystal structure of the HPPD 4-Hydroxyphenylpyruvate complex it is difficult to define a clear rationale for making site directed mutants and even more problematic to interpret the observations seen with them. Thus, a concurrent goal has been to structurally characterize HPPD in complex with its substrate, 4-hydroxyphenylpyruvate.

HPPD is the target of specific inhibitors that are used both as herbicides and in the treatment of type 1 tyrosinemia. Although NTBC was the first effective drug used therapeutically for type 1 tyrosinemia, the mode of action is still unknown. Therefore, we hope to also crystallize the enzyme-inhibitor complex to elucidate its mechanism of action.

Poster Abstract 66

AMMONIA TRANSFER IN IMIDAZOLE GLYCEROL PHOSPHATE SYNTHASE: COMMUNICATION BETWEEN TWO ACTIVE SITES 30Å APART

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Glutamine amidotransferases (GATs) catalyze the incorporation of ammonia into a wide variety of molecules. In the histidine biosynthetic pathway, the GAT imidazole glycerol phosphate synthase (IGPS) utilizes the amide nitrogen of glutamine as a source of ammonia and catalyzes both carbon-nitrogen ligation and an elimination reaction to form 5'-(5-aminoimidazole-4-carboxamide) ribonucleotide (AICAR) and imidazole glycerol phosphate (IGP). The active sites in the protein exist in distinct domains, one having glutaminase activity while the cyclase domain binds an unusual nucleotide, N^1-[(5-phosphoribulosyl)-formimino]-5-aminoimidazole-4-carboxamide ribonucleotide, and catalyzes the amination reaction. The catalysis of the two active sites is coupled such that glutamine hydrolysis is stimulated 5800-fold by addition of PRFAR. The cyclase domain of IGPS is an α/β 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. The structure of IGPS complexed with PRFAR reveals key protein-small molecule interactions in the cyclase active site. At the interface of the two domains, highly conserved residues are shown to have essential communication roles between the two active sites. Mutation of K258 in the PRFAR active site to alanine appears to disrupt the cyclase activity of the protein, while maintaining the glutaminase signal. The two activities are uncoupled such that 43 molecules of glutamine are hydrolyzed for every molecule of PRFAR. The residue R239 located at the bottom of the α/β barrel participates in an electrostatic gate to the hydrophobic tunnel. Mutation to an alanine at this position resulted in uncoupling where 122 molecules of glutamine were hydrolyzed for every PRFAR molecule. Conservative mutations at these two sites restore the coupling of the two reactions.

COUPLING OF PROTON RELEASE AND NAD^+ ASSOCIATION TO HORSE LIVER ALCOHOL DEHYDROGENASE

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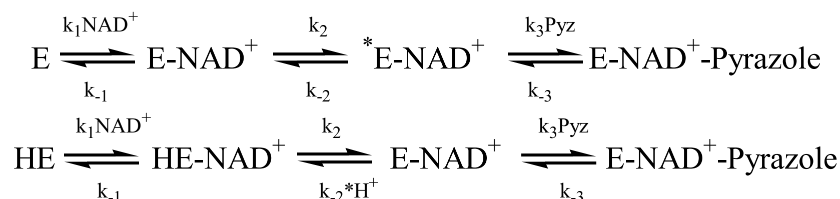
The overall rate of NAD^+ association to horse liver alcohol dehydrogenase (ADH) is limited by the isomerization of the binary complex from an open to a closed conformation, which is important for installation of the proton relay system. His-51 acts as an active site base by facilitating proton transfer from the Zn-bound ligand to solvent via a hydrogen-bonding network. The strong pH dependence of the rates for NAD^+ association to ADH suggests that proton transfer may be required for isomerization and may control the overall reaction. Transient kinetics of proton release during NAD^+ association to the wild-type and H51Q enzymes were determined in order to evaluate the roles of Zn-bound water and facilitated proton transfer. Different pH indicators in unbuffered solutions were used to monitor proton release, and pyrazole was used to trap the enzyme-NAD^+ complex as the ternary complex. Proton release and formation of the enzyme-NAD^+-pyrazole complex were saturable processes with respect to NAD^+ concentrations in reactions of both enzymes. In the wild-type enzyme, the macroscopic rate constants (k_{lim} and k_{lim}/K_{NAD}) for proton release matched those for NAD^+ binding, as did the rate constants for the H51Q enzyme that lacks the active site histidine. However, the observed proton release kinetics in the wild-type enzyme, were much faster and exhibited a different pH profile than in the reaction of the H51Q enzyme. The stoichiometry of the protons released per active site of the wild-type enzyme exhibited a single pK, whereas a bell-shaped profile was observed for the reaction of H51Q enzyme. The transient data for reaction of the H51Q enzyme at each pH value are described well by the mechanism where diffusion-limited NAD^+ association is followed by either a slow isomerization or a proton release step. The results suggest that the overall NAD^+ association to ADH is controlled by deprotonation of the Zn-bound water, which is facilitated by His-51. (Supported by USPHS grant AA00279)



Poster Abstract 68

PROBES OF *THERMUS AQUATICUS* DNA POLYMERASE FIDELITY: AZOLE CARBOXAMIDE NUCLEOBASES

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Azole carboxamide nucleobases (ACNs) have been designed to serve as mechanistic probes of *Thermus aquaticus* (*Taq*) DNA polymerase fidelity. The ACNs are 5-membered isosteric heterocycles that differ in electronic distributions while preserving hydrogen bonding properties of natural bases. ACNs were incorporated in the DNA template as well as synthesized as triphosphates for steady state kinetic studies with *Taq* polymerase. In single-base incorporation experiments using the four natural triphosphates, ACNs (in template strand) showed different levels of degeneracy in pairing with natural bases. In the context of the DNA polymerase catalysis, the observed difference in V/K values caused by subtle changes in the structure suggests an important role of electronics in selection of a base at the polymerase active site. We further studied the incorporation azole carboxamide triphosphates (dACTs) opposite the natural base template. The synthetic route for these dACTs involved transformation of the deoxyribonucleoside to diphosphate by selective tosylation at the 5′-OH, nucleophilic displacement of the tosylate by inorganic pyrophosphate, and enzymatic transformation of the diphosphate to the triphosphate. The V/K values for incorporation opposite the natural template base were consistent with the relative values observed in template studies. Primer extension studies with ACNs in the primer and template strand are also reported. Presence of the artificial base appears to influence the extension beyond the site of incorporation, suggesting the influence of molecular features of ACNs on recognition during processive synthesis by *Taq* polymerase.

Poster Abstract 69

REVISED NUCLEOTIDE SEQUENCE OF THE MITOCHONDRIAL FORM OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

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Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the first critical step in gluconeogenesis, the process of remaking glucose during starvation. The eventual goal of our project is to use recombinant DNA technology to synthesize the mitochondrial form of PEPCK from chicken liver in insect cells using the pFASTBAC vector. A preliminary step of this investigation was to sequence the cDNA PEPCK to ensure correct encoding of the protein after expression. The published cDNA sequence was obtained by manual dideoxynuclotide sequencing using urea-gel electrophoresis and consists of the composite nucleotide sequence of three separate clones needed to overlap the entire length of the 3.6 kb mRNA for PEPCK. Automated DNA sequencing utilizes fluorescently labeled nucleotides to make new DNA fragments that are separated by capillary electrophoresis. Automated sequencing was employed on the cDNA clone and resulted in two major deviations from the published sequence. The improved automated sequencing recognized one area where significant compression of a G-C rich region had occurred during the gel electrophoresis, hiding three nucleotides. A second difference demonstrated an improper insertion in a G-C rich region, presumably the result of an older reverse transcriptase. Reverse-transcriptase polymerase chain reaction was utilized to eliminate the insertion. Following the removal of the insertion, introduction of the new PEPCK cDNA into pFASTBAC is currently in progress. Future work will involve expression in insect cells and subsequent characterization of the PEPCK product.

Poster Abstract 70

CHARACTERIZATION OF THE CYCLASES INVOLVED IN LANTIBIOTIC BIOSYNTHESIS, SPAC AND NISC

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Lantibiotics are peptide-derived antimicrobial agents that are ribosomally synthesized and post-translationally modified by a multienzyme complex to their biologically active forms. Nisin has been commercially used as a food preservative, while other lantibiotics show promising activity against bacterial infections. The post-translational modifications are believed to be carried out by a multienzyme complex. At present the enzymes catalyzing the formation of the lantibiotic signature structural motifs, dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine (Ln), and methyllanthionine (MeLn), are not well characterized. In an effort to gain insight into the mechanism by which lantibiotics are biosynthesized, the cyclase enzymes involved in the synthesis of nisin and subtilin (NisC and SpaC, respectively) have been cloned, expressed, and purified. Both proteins exist as monomers in solution and contain a stoichiometric zinc atom. EXAFS analysis suggests that two cysteines coordinate to the metal and possibly two histidines. The metal may function to activate the cysteine thiol of the peptide substrate toward intramolecular Michael addition to the dehydroalanine and dehydrobutyrine residues in the peptide. Sequence alignments of the LanC enzymes reveal four conserved residues, two cysteines and two histidines. Mutants of SpaC replacing the conserved cysteines with alanines have been generated and are being characterized to determine if these residues are involved in metal binding.

Poster Abstract 71

A PROTON SHUTTLE MECHANISM FOR EXTRADIOL DIOXYGENASES REVEALED BY HIGH RESOLUTION CRYSTAL STRUCTURES AND KINETIC CHARACTERIZATION OF SITE-DIRECTED MUTANTS

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The oxidative cleavage of ortho-dihydroxy aromatic compounds by extradiol and intradiol dioxygenases is essential in microbial degradation of naturally occurring and man-made aromatic molecules. Intradiol dioxygenases catalyze the aromatic ring-fission between the hydroxyl groups. Extradiol dioxygenases catalyze the cleavage of C-C bond adjacent to one of hydroxyl groups. Despite extensive crystallographic and spectroscopic studies on these enzymes, the detailed mechanism of aromatic ring cleavage reaction has not been determined. We have initiated investigations into the kinetic, chemical and structural mechanisms of 2,4,5-trixydroxytoluene dioxygenase (THT-DO) from *Burkholderia sp*. strain DNT. THT-DO is the terminal enzyme in the pathway for biodegradation of 2,4-dinitrotoluene in *Burkholderia sp*. strain DNT. It utilizes a non-heme Fe (II) as cofactor, and O_2 as a second substrate and catalyzes the extradiol cleavage of 2,4,5-trixydroxytoluene. The crystal structures of native THT-DO and a complex with the inhibitor 4-nitrocatechol suggest that Y261, which is 3 Å from the active site metal, acts as a base via a proton shuttle with H252. We propose that H206, located near molecular oxygen, acts as an acid in the mechanism. To elucidate the mechanism of extradiol ring-fission catalysis, we have constructed and characterized kinetically the point mutants Y261F, H252A and H206A of THT-DO. We have determined the high-resolution x-ray crystal structures of apo-H252A (1.5 Å), apo-Y261F (1.35 Å), and the 4-nitrocatechol bound form of Y261F (1.2 Å). These structures show that the active site is similar to that of wild-type enzyme. Mutation of H252 to an alanine produces a “hole” that gets filled by a water molecule in the position of the imidazole ring. Preliminary kinetic data indicate that mutations of these residues reduce significantly the catalytic activity of the enzyme.

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 (equation 1).

This reaction is highly unusual in that it is the only enzymatic phosphorous redox chemistry observed in nature. Based on sequence homology (23-49%) with the 2-hydroxyacid dehydrogenase family, three putative catalytic residues are proposed (Arg 237, Glu266, His292). In an effort to determine the mechanism of this unusual reaction, isotope effects and inhibition investigations, and site-directed mutagenesis studies have been carried out. The dependence of the reaction on the protonation state of the enzyme-substrate complex has been determined in both H_2O and D_2O. The enzyme was found to be inhibited by iodoacetamide, suggesting a possible role for cysteine in catalysis. Six mutants have been characterized. Kinetic assays show that H292N, H292F, H292K, and R237L are all inactive. R237K displayed a 3000-fold reduced V_{max} but the V_{max} of E266Q was unperturbed with respect to wild type PtxD. The K_m of the two substrates decreased in both mutants with respect to wild type (WT: $K_{m,\ phosphite}$ = 54 μM and $K_{m,\ NAD}$=53 μM; R237K: $K_{m,\ phosphite}$ = 6.9 mM and $K_{m,\ NAD}$ = 1.4 mM; E266Q: $K_{m},\ _{phosphite}$ = 5.1 mM and $K_{m,\ NAD}$ = 1mM).

Poster Abstract 73

A CALORIMETRIC APPROACH TO THE ALLOSTERIC TRANSITIONS OF METHYLGLYOXAL SYNTHASE

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Methylglyoxal synthase (MGS) is an allosterically regulated enzyme important in the methylglyoxal bypass of the lower half of the glycolytic pathway. This bypass is presumed to be important in the recovery of microorganisms from a state of starvation. The allosteric regulation is unusual in that a product of the reaction, phosphate, is the effector. We are using isothermal titration calorimetry and enzyme kinetics to understand the binding of phosphate and other inhibitors and the transition between the T and R states. Unlike wild type MGS, the H98Q enzyme is stable in the absence of phosphate. This variant of MGS therefore allows the collection of binding isotherms over a full range of phosphate concentrations. It is also stable over a range of temperatures. At some temperatures including 25°C, the H98Q variant appears to exist in the "T" state. Surprisingly, the binding of phosphate at 25°C has been found to be endothermic. Binding isotherms between 10° and 50°C with phosphate and the intermediate analog, phosphoglycolate, will be presented.

Poster Abstract 74

KINETIC AND STRUCTURAL STUDIES OF GMP SYNTHETASE

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The glutamine amidotransferase GMP synthetase (GMPS) catalyzes the last step of *de novo* guanine nucleotide biosynthesis, the amination of XMP to generate GMP. The goal of our work with GMPS has been to more fully characterize the function of the enzyme using kinetic and crystallographic methods. Using stopped-flow techniques, we have observed rapid events on *E. coli* GMPS when mixed with various substrates. Mixing of the enzyme with its nucleotide substrates leads to an absorbance drop, likely due to formation of an adenyl-XMP intermediate. Attempts to identify this intermediate after quenching are in progress. Monitoring intrinsic tryptophan fluorescence of the same process reveals a fluorescence intensity drop with a rate similar to that of the absorbance process, implying that charging of the XMP by adenylation is coupled to a large-scale conformational change. We are also pursuing crystallographic evidence for an alternative conformation. Rapid mixing of glutamine with GMPS, pre-charged with its nucleotide substrates, produces an absorbance rise, which we predict is due to amination of the adenyl-XMP intermediate to form GMP. The rates of these processes are faster than the rate of steady-state turnover, consistent with a slow step occurring outside of binding and chemistry. Our current view of GMPS catalysis pictures nucleotide binding and activation concomitant with a large conformational change that creates a tunnel for ammonia passage, followed then by amide cleavage and transfer.

Poster Abstract 75

AN ALGORITHM TO SAMPLE RECEPTOR FLEXIBILITY IN MOLECULAR DOCKING

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Treating receptor flexibility in molecular docking holds promise for discovering novel ligands that do not fit the receptor when it is held rigid. It poses a major challenge for current database docking algorithms.

We devised a method to sample an ensemble of receptor conformations using grid-based energy functions. Database screens were performed against several binding sites that exhibit flexibility upon ligand binding (aldose reductase, thymidylate synthase, T4 lysozyme mutant L99A). The effect of considering receptor conformational energy was investigated.

Compared to docking every receptor conformation in the ensemble sequentially, the new algorithm gives the same result but is significantly faster. When receptor conformational energy is considered, sampling receptor flexibility improves the enrichment of known ligands, reduces the deviation of the predicted ligand-binding mode from the corresponding experimental structure, and increases the diversity of the top ranked molecules. This is not the case when the conformational energy is ignored. To test the method, a conformational ensemble was calculated for the cavity of T4 lysozyme mutant L99A/M102Q based on the observed conformations of the mutant L99A in complex with different ligands. This ensemble was docked to predict novel ligands of L99A/M102Q which can fit the cavity only if it changes conformation. The predictions were tested by thermal denaturation upshift experiment. Efforts to determine the structures of the complexes are underway.

We conclude that receptor conformational energy should be accounted for when receptor flexibility is explored. The proposed method allows efficient sampling of receptor conformations in database docking.

SYNTHESIS AND CHARACTERIZATION OF 1-INDOLYL-2'-DEOXYRIBOSE-5'-TRIPHOSPHATE: A TOOL TO EVALUATE THE MOLECULAR MECHANISM OF DNA POLYMERIZATION FIDELITY

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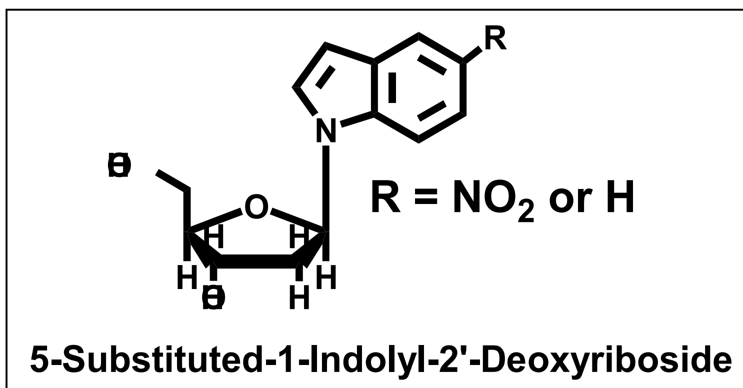
The fidelity of DNA polymerization has been historically attributed to the formation of Watson-Crick hydrogen bonds formed between the incoming nucleoside triphosphate with the complementary template nucleobase. Studies monitoring the insertion of 5-nitro-1-indolyl-2'-deoxyribose-5'-triphosphate (5-NI) indicate that this novel nucleoside can be inserted opposite any of the four natural nucleobase. Surprisingly, the insertion of 5-NI opposite an abasic site (devoid of any hydrogen bonding capabilities) is 1000-fold more efficient compared to that for insertion opposite natural nucleobases. These data suggest that base-stacking as opposed to hydrogen-bonding plays the predominant role in DNA polymerization efficiency. To further evaluate this mechanism, a series of 5-substituted 1-indolyl-2'-deoxyribose-5'-triphosphate analogs are being synthesized and tested for their ability to be inserted opposite natural and modified nucleobases. In this presentation, the synthesis and characterization of 1-indolyl-2'-deoxyribose-5'-triphosphate is discussed. Kinetic characterization measuring the insertion of this non-hydrogen bonding molecule during correct and translesion DNA replication is likewise provided.



5-Substituted-1-Indolyl-2'-Deoxyriboside

Poster Abstract 77

PURIFICATION AND CHARACTERIZATION OF TRUNCATED PUTA PROTEINS

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The proline utilization flavoprotein, PutA, from *Escherichia coli*, is a peripherally membrane-bound enzyme that catalyzes the conversion of proline to glutamate via a two-step oxidation process. PutA contains two catalytic domains: a proline dehydrogenase domain where proline is oxidized to Δ^1-pyrroline-5-carboxylate and a P5C dehydrogenase domain where P5C is oxidized to glutamate using NAD^+. PutA is also a repressor of the *put* regulon. Proline availability has been shown to control whether PutA acts as a transcriptional repressor or as a membrane-bound proline dehydrogenase. In the absence of proline, it represses *put* gene expression. In the presence of proline, PutA binds to the membrane to catalyze the oxidation of proline to glutamate. In order to understand the structure –function relationship in PutA the following deletion constructs of PutA were made; PutA55-1320, PutA137-1320 and PutA1-1230. These constructs were studied for their properties and functions such as DNA-binding, membrane-binding, proline dehydrogenase activity, P5C dehydrogenase activity and oligomeric state. The characterization of these constructs has revealed new insights into the DNA-binding and membrane-binding functions of PutA.

Poster Abstract 78

SUBSTRATE BINDING TO 5S SUBUNIT OF RANSCARBOXYLASE ENZYME BY RAMAN CYSTALLOGRAPHY

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Transcarboxylase (TC) from *Propionibacterium shermanii* is a complex biotin-containing enzyme composed of 30 polypeptides of three different types: a central hexameric 12S subunit to which 6 outer dimeric 5S subunits are attached through 12 biotinyl 1.3S subunit. The enzyme catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate, forming oxalacetate and propionyl-CoA(figure 1). The focus of the study is on the 5S subunit. The structure of 5S has not been reported yet. The aim of our study is to complement the structural study of 5S in the crystal form, defining and comparing in detail the chemistry of the substrates bound to its active site in both crystal and solution forms. Studying the detailed chemical changes in the substrates and protein upon pyruvate binding to the 5S subunit by Raman difference spectroscopy both in the crystal and solution forms: The X-ray structure of the free 5S subunit is being studied by Vivien Yee's group and compared to the structure of the protein bound to substrate, to identify any conformational change upon substrate binding. Raman difference spectroscopy is used both in the crystal and in the solution phases to study the enzymatic mechanism for the second transcarboxylase carboxyl transfer half reaction, the vibrational modes for the $–COO^-$ and C=O entity of pyruvate is identified using ^{13}C and ^{18}O substitution with the aim of probing the chemistry of $–COO^-$ and C=O groups in the 5S active site. Insight into the catalytic mechanism for the second transcarboxylase half reaction (figure1) is provided by Raman difference spectroscopy of the crystal of 5S complexed to pyruvate. In addition , use of the deuterated, $–CD_3$, form of pyruvate will permit the modes of the methyl group to be studied in a spectral window, and shifts in these vibrations upon binding will provide insight into the interaction between the methyl group and the active site.

Poster Abstract 79

EXPRESSION, CHARACTERIZATION AND EVOLUTION OF THE SUGAR KINASE GALK

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In vitro glycorandomization technology is dependent upon the ability to rapidly synthesize sugar phosphates. While less complicated than the synthesis of an NDP-sugar counterpart, the synthesis of sugar remains a fairly challenging and tedious process. Thus, novel methods for sugar phosphate synthesis would significantly enhance glycorandomization capabilities. There is a small class of enzymes, exemplified by the enzyme GalK, which phosphorylate at the anomeric position. This project now focuses on characterization of GalK with a particular focus on substrate specificity. The work already done includes overexpression, purification of GalK and activity assay with Galactose and other analogs. Also, high throughput assay methods were developed. This work provides the foundation from which we will pursue both structure-based enzyme engineering and directed evolution of GalK toward a more promiscuous sugar kinase for glycorandomization

Poster Abstract 80

ACTIVE SITE CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* PHOSPHOMANNOMUTASE/PHOSPHOGLUCOMUTASE

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Phosphomannomutase/phosphoglucomutase (PMM/PGM) catalyzes the formation of mannose 1-phosphate and glucose 1-phosphate from the corresponding 6-phospho-sugars in the bacteria *P. aeruginosa*. Mannose 1-phosphate is crucial for the biosynthesis of the polysaccharide alginate that forms an antibiotic resistant capsule around the bacteria, and glucose 1-phosphate is required in the biosynthesis of lipopolysaccharides. There are currently no effective antibiotics for the treatment of *P. aeruginosa* infections. Since alginate and lipopolysaccharide contribute to the virulence of the bacteria, PMM/PGM is an excellent target for drug development. Characterizing the roles of active site residues will help us understand the catalytic mechanism of PMM/PGM.

PMM/PGM is phosphorylated at Ser108. In the catalytic reaction the phosphoryl group is transfered from the serine to the substrate at the 1-position forming a 1,6-bisphosphorylated intermediate. The intermediate reorients to allow transfer of the phosphoryl group from the 6-position of the intermediate back to Ser108 to regenerate phosphorylated enzyme and the product sugar phosphorylated at the 1-position. The multiple phosphoryl transfers in the overall reaction require general acid/base catalysis . Inspection of the active site reveals Lys118 and several histidine residues appropriately positioned to function as general acid/base catalysts. Indeed, V_{max} values in the K118L, H109Q, and H329N mutants were 4%, 7%, and 6% of wild-type, respectively. Surprisingly, V_{max} in the S108A mutant enzyme was 11% that of wild-type, suggesting that another nearby residue may be phosphorylated in the absence of Ser108. We are in the process of making double mutations with S108 and His308, His329, and His109. Characterizing these double mutants should help us determine whether one of the His residues can be phosphorylated to support the catalytic reaction in the absence of Ser108.