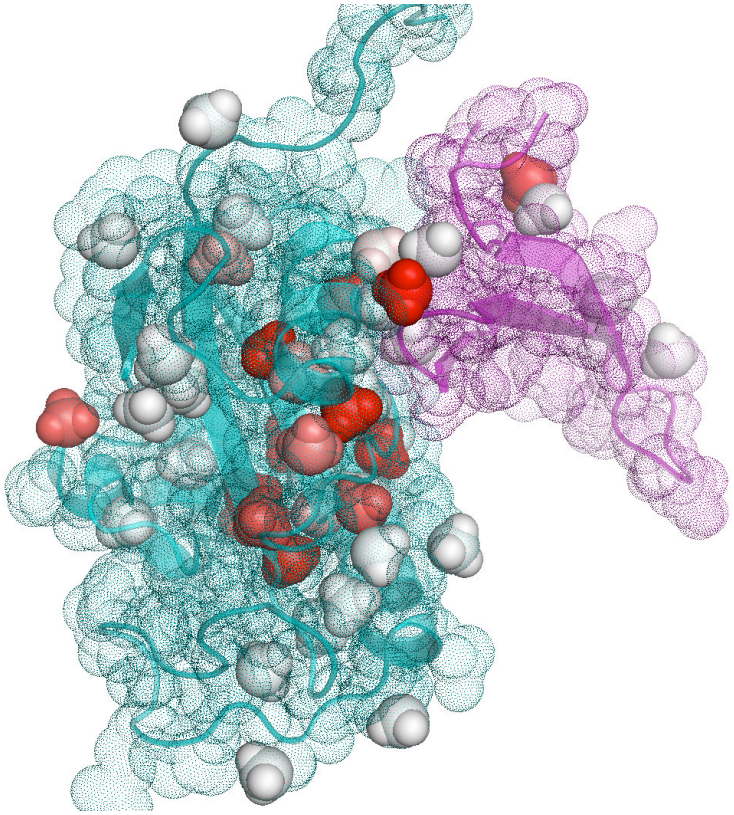
12th ANNUAL BIOCHEMISTRY RESEARCH FORUM

Biochemistry Group
Department of Chemistry and Biochemistry
University of Notre Dame
Notre Dame, Indiana



Swan Lake Resort
Plymouth, IN
May 3-4, 2007

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Holly Goodson and Anthony S. Serianni

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Cover Art

Courtesy of the Peng Lab

Cell cycle enzyme, human Pin1. Red spheres are methyl-bearing side chains that show the greatest loss of fast motion upon substrate binding. They connect the binding and catalytic sites of Pin1 and are evolutionarily conserved suggesting functional significance.

[Namanja, Peng, Zintsmaster, Elson, Shakour & Peng, *Structure* **2007** Mar15(3), 313-327.]

Previous Keynote Speakers

1996: Nicholas Paoni (Genentech)
1998: Les Kozak (Jackson Laboratory)
1999: Andy Mesecar (IU-Chicago)
2000: Thomas Kelly (Case Western)
2001: Kwok Yeung (Bayer)
2002: John Beals (Lilly)
2003: Dudley Strickland (Red Cross)
2004: Elizabeth Komives (UCSD)
2005: Erik Zuiderweg (Michigan)
2006: Lila Gierasch (U. Mass)

2007 Keynote Lecture

Professor Shelagh Ferguson-Miller

MSU Distinguished Professor and Chair
Department of Biochemistry and Molecular Biology
Michigan State University

Keynote Lecture Abstract

Cytochrome c oxidase is the key enzyme in energy transduction in aerobic organisms. As the terminal electron sink in the respiratory chain of microbes, plants and animals, it is one of the most critical and complex of the energy conversion enzymes in terms of subunit structure and metal content. A number of crystal structures have been obtained of cytochrome oxidase, but the mechanism of energy conversion remains unclear. Our lab has been engaged in a long term effort to dissect the structural and kinetic properties of cytochrome c oxidase (CcO) using the enzyme from *Rhodobacter sphaeroides (Rs)* as a model system. This simpler bacterial form is highly homologous to the mammalian enzyme and accessible by mutagenic, spectral, kinetic, computational and crystallographic techniques. In the past several years we have succeeded in refining methods of purification and crystallization, with the working hypothesis that some of the membrane lipid molecules are integral to the protein structure and their retention is important for maintaining the native form. To this end we have developed sensitive mass spectrometry techniques for assessing molecular homogeneity and lipid content. These studies have resulted in our being able to reproducibly crystallize native and mutant forms of *Rs*CcO at 2 Å resolution. The high resolution crystal structures reveal the presence of specific lipid binding sites that are conserved from bacteria through to mammals, indicating their importance in structure and function. At this resolution, water positions are well resolved and also observed to be conserved in their location. Computational studies support their role in proton pumping, acting as proton wells in addition to proton wires. These results are getting us closer to understanding the mechanism of coupling of electron transfer and proton translocation in CcO, leading to new insight into how rate and efficiency is controlled and how the process may lose (or gain) efficiency in disease, obesity and aging.

L. Qin, D. Mills, C. Hiser, A. Murphree, R. M. Garavito, S. Ferguson-Miller and J. Hosler (**2007**) "Crystallographic location and mutational analysis of Zn/Cd inhibitory sites, and the role of lipidic carboxylates in rescuing proton path mutants in cytochrome *c* oxidase", *Biochemistry***,** *in press*.

J. Xu, M.A. Sharpe, L. Qin, S. Ferguson-Miller and G.A. Voth (**2007**) "Storage of an Excess Proton in the Hydrogen-bonded Network of the D-pathway of Cytochrome *c* Oxidase:Identification of a Protonated Water Cluster", *J. Amer. Chem. Soc.*, *129*, 2910-3.

L. Qin, C. Hiser, A. Mulichak, R. M. Garavito, and S. Ferguson-Miller (**2006**) "Identification of Conserved Lipid/Detergent Binding Sites in a High-Resolution Structure of the Membrane Protein Cytochrome c Oxidase" *Proc. Natl. Acad. Sci. USA* (no 44, Oct 31)*103*, 16117-16122.

J. Hosler, S. Ferguson-Miller, and D. Mills (**2006**) "Energy Transduction: Proton Transfer through Respiratory Complexes", *Ann. Rev. Biochem***.** *75*, 165-187.

Program

THURSDAY AFTERNOON
Salon 3
Session Chair: H. Goodson

1:30 PM	WELCOME & INTRODUCTORY REMARKS (A. Serianni)
1:35 PM	INFORMATION ABOUT SWAN LAKE RESORT (Jeannette Teall)
1:40 PM	*Co-translational folding of GFP on an E. coli ribosome* K. Ugrinov & P.L. Clark
2:00 PM	*A FVII deficiency induces cardiac fibrosis resulting in diastolic dysfunction and is associated with regulation of extracellular matrix proteins* H. Xu, M. Sandoval-Cooper, D.L. Donahue, V.A. Ploplis & F. J. Castellino
2:20 PM	*Molecular evolution of bacterial actin-related proteins* B.J. Belin, M. Bartley & H.V. Goodson
2:40 PM	BREAK
3:00 PM	*Residual dynamics in Class I MHC/peptide complex and its immunological implications* F.K. Insaidoo & B.M. Baker
3:20 PM	*Identification and isolation of gene clusters involved in iron acquisition by P. mendocina ymp* J. Awaya & J. DuBois
3:40 PM	*Elucidation of the threitol biosynthetic pathway in U. ceramboides using ^{13}C NMR* K. Walters, Q. Pan, A.S. Serianni & J.G. Duman

4:00 - 6:30 PM OPEN TIME
Biochemistry faculty meeting

THURSDAY EVENING

6:30 – 7:30 PM DINNER - Golfer's Dining Room

7:30 – 7:45 PM OPEN TIME

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7:45 PM **KEYNOTE LECTURE** - Salon 3

Cytochrome c oxidase: A clean, oxygen-reducing, energy-transducing machine
Professor Shelagh Ferguson-Miller

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9:00 PM POSTER PRESENTATIONS / SOCIAL - Mezzanine A

FRIDAY MORNING
Salon 3
Session Chair: F.J. Castellino

8:00 - 9:00 AM BREAKFAST - Golfer's Dining Room

9:00 AM **GUEST SPEAKER**
Membrane recruitment and activation of cytosolic phospholipase $A_2\alpha$, the rate-limiting enzyme in eicosanoid biosynthesis
Dr. Robert V. Stahelin

9:40 AM Cloning, expression and kinetic studies of a bifunctional enzyme, AAC(3)-Ib/AAC(6')-Ib' from *Pseudomonas aeruginosa*
Choonkeun Kim, Adriel Villegas-Estrada, Dusan Hesek & Shahriar Mobashery

10:00 AM — *Subtype-selective antagonism in N-methyl-D aspartate receptor ion channels by synthetic conantokin peptides*
Z. Sheng, Q. Dai, M. Prorok & F.J. Castellino

10:20 AM — BREAK

10:40 AM — *Computational investigation of solvation dynamics of a DNA/drug complex using molecular dynamics simulations*
K. E. Furse, B. A. Lindquist & S. A. Corcelli

11:00 AM — *Sequence coded dynamics modulate recognition specificity in WW domains*
P. Peng, J.S. Zintsmaster, A.T. Namanja, G. Pasat, B. Wilson, K. Wilson & J. Peng

11:20 AM — *Regulation of apoptosis in breast cancer cells induced by cis-platin and L-PPMP*
R. Ma, P. Boyle, G. Crowford, M. Schmidt, Y. Cho, C. Thomas, S. Banerjee, J. Moskal & S. Basu

11:40 AM – 12:15 PM — OPEN TIME

12:15 – 1:35 PM — LUNCH - Golfer's Dining Room

FRIDAY AFTERNOON
Salon 3
Session Chair: P.W. Huber

1:35 PM — **GUEST SPEAKER**
Vector, pathogen, host: The infection trinity
Professor Mary Ann McDowell

2:15 – 3:00 PM — OPEN TIME

3:00 PM	*Sulindac treatment alters extracellular matrix remodeling in a colorectal cancer mouse model* H. G. Ahlers, S.A. Buechler, V.A. Ploplis & F.J. Castellino
3:20 PM	*Catalytic mechanism of penicillin-binding protein 5 of E. coli* W. Zhang, Q. Shi, S.O. Meroueh, S.B. Vakulenko & S. Mobashery
3:40 PM	BREAK
4:00 PM	Cross-reactivity of TCR A6 on haptenated HTLV-1 Tax_{11-19} peptides presented by HLA-A2 O.Y. Borbulevych & Brian Baker
4:20 PM	*Squaraine rotaxanes: Superior near-infrared fluorophores for in vitro and in vivo imaging* J.R. Johnson & B.D. Smith
4:40 PM	CLOSING REMARKS (H. Goodson)

ABSTRACTS: GUEST SPEAKERS

Membrane Recruitment and Activation of Cytosolic Phospholipase $A_2\alpha$, the Rate-Limiting Enzyme in Eicosanoid Biosynthesis

Robert V. Stahelin
Department of Biochemistry and Molecular Biology, Indiana University School of Medicine-South Bend and the Department of Chemistry and Biochemistry and Walther Center for Cancer Research, University of Notre Dame, Notre Dame, IN 46617

Research in the past decade has revealed that many cytosolic proteins are recruited to cellular membranes to form protein-protein and lipid-protein interactions during cell signaling and membrane trafficking. Typically, membrane recruitment of these proteins is mediated by a growing number of modular membrane-targeting domains that recognize their cognate lipid ligands. However, the mechanisms by which these domains and their host proteins are recruited to and interact with various cell membranes are only beginning to unravel with recent computational studies, in vitro membrane binding studies using model membranes, and cellular translocation studies using fluorescent protein-tagged proteins. The principal investigator's laboratory studies the dynamic processes of membrane recruitment and the biophysical basis for cellular membrane targeting and subsequent enzyme activation for a number of pharmacologically important enzymes. This talk will be geared towards the cellular activation mechanism of group IV cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$). $cPLA_2\alpha$ is the initial rate-limiting enzyme in eicosanoid biosynthesis in response to many inflammatory agonists and its cellular activation requires Ca^{2+}-depdendent membrane translocation of its N-terminal C2 domain. Cell-specific and agonist-dependent events coordinate translocation of $cPLA_2\alpha$ to the nuclear envelope, endoplasmic reticulum, and Golgi apparatus via this domain. At these membranes, $cPLA_2\alpha$ hydrolyzes membrane phospholipids to produce arachidonic acid, which initiates pathways leading to eicosanoid synthesis. Early studies on $cPLA_2\alpha$ demonstrated it had high affinity for zwitterionic phospholipids in the presence of Ca^{2+}. Subsequently, this lipid specificity was shown to induce its membrane translocation and biological activity in a variety of cell lines. Recently, we have demonstrated ceramide-1-phosphate (C1P) a new addition to a growing group of bioactive sphingolipids, and phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) are able to recruit $cPLA_2\alpha$ to the membrane and differentially influence $cPLA_2\alpha$ activity. Strikingly, this has suggested $cPLA_2\alpha$ to be activated by a number of lipid signals that require different Ca^{2+} levels for biological activity. The talk will focus on recent advances in understanding how $cPLA_2\alpha$ is recruited to the biological membrane in response to a variety of lipid signals and how these lipid signals affect the biological activity.

Vector, Pathogen, Host: The Infection Trinity

Mary Ann McDowell
Department of Biological Sciences, University of Notre Dame,
Notre Dame, IN 46556

Our research focuses on the immunobiology of vector-transmitted diseases. We focus on two vector-transmitted, intracellular parasites, *Leishmania* and *Plasmodium*. Projects in the laboratory are aimed at deciphering the intricate interactions between the vertebrate immune system, pathogens and vector components that lead to disease resistance or susceptibility. Our research utilizes methods in cell biology, immunology, and molecular biology; we employ a variety of model systems, ranging from *in vitro* culture systems to murine models to endemic human populations.

ABSTRACTS: ORAL PRESENTATIONS

Co-translational folding of GFP on an *E. coli* ribosome

Krastyu Ugrinov and Patricia L. Clark
University of Notre Dame, Department of Chemistry and Biochemistry
Notre Dame, Indiana 46556, USA

A growing amount of evidence suggests that the folding of nascent polypeptides may be influenced by the vectorial appearance of the polypeptide at the surface of the ribosome. Yet little is known about the conformational flexibility of nascent, ribosome-bound polypeptides. Our goal is to determine the effects of the ribosome on the conformations and dynamics of newly synthesized polypeptide chains. We are using green fluorescent protein (GFP) as a model for co-translational folding of β-sheet structures. The residues that make contacts in the native structure of GFP are often far apart in the primary sequence. This high 'contact order' renders GFP (and many other β-sheet proteins) prone to aggregation during folding, and suggests co-translational nascent chain conformations may be particularly important for productive folding. Experiments with ribosome-bound GFP chains have revealed that nascent chain conformational freedom increases with increasing chain length. Tests with ribosome-bound full length GFP show that GFP can fold to his native structure on the ribosomal surface. Complete folding is, however, only possible if the entire GFP sequence is exposed outside of the ribosome exit tunnel. Anisotropy measurements based on the intrinsic GFP chromophore show that the ribosome-bound GFP experience rotational freedom similar to that of free native protein.

A FVII deficiency induces cardiac fibrosis resulting in diastolic dysfunction and is associated with regulation of extracellular matrix proteins

Haifeng Xu, Mayra Sandoval-Cooper, Deborah L. Donahue, Victoria A. Ploplis and Francis J. Castellino

Department of Chemistry and Biochemistry, and the W. M. Keck Center for Transgene Research, University of Notre Dame, Notre Dame, Indiana 46556

Factor (F)VIIa/Tissue Factor (TF) pathway is the initiator of the coagulation cascade, leading to fibrin formation and coagulation protease signaling. Mice producing low levels (<1% of WT mice) of FVII ($FVII^{tTA/tTA}$) show spontaneous cardiac fibrosis at both 4 m and 12 m of age. Cardiac function was evaluated by a Vevo 770 High Resolution small animal high-frequency ultrasound system to image murine hearts non-invasively. There were no significant differences between groups at baseline for the hemodynamic and respiratory parameters: temperature and heart rate (HR). The end-systolic functions of $FVII^{tTA/tTA}$ mice heart were comparable to the WT control. However, there were significant changes in the end-diastolic parameters of $FVII^{tTA/tTA}$ hearts. The diameter at end-diastole in $FVII^{tTA/tTA}$ mice was smaller compared to WT mice, which was due to the stiffness of the interventricular wall. When viewed from parasternal short-axis, these mice had evidence of hypokinesis indicating the alteration of the elastic proterties of the LV wall. Correspondingly, the stroke volume, ejection fraction, fractional shortening, and cardiac output were lower in $FVII^{tTA/tTA}$ mice, compared to WT mice. Mitral valve inflow was compromised in $FVII^{tTA/tTA}$ mice as demonstrated by the reduced early/late, (E/A) filling ratio. We found that the spontaneous impaired cardiac function in $FVII^{tTA/tTA}$ mice was associated with an increased ratio of heart weight to body weight. The histological findings support a fibrosis pathology, as demonstrated by increased collagen content that results from spontaneous bleeding and leukocyte infiltration. We tested the hypothesis that the development of cardiac fibrosis is associated with regulation of inflammation and extracellular matrix proteins using Quantitative RT-PCR analysis. The results showed a higher TNF-α level in the hearts of 12 m $FVII^{tTA/tTA}$ mice compared to WT mice. The roles of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in cardiac fibrosis were also investigated. The mRNA levels of MMP-2, MMP-3, MMP-9, and MMP-12 in the hearts of $FVII^{tTA/tTA}$ mice were higher compared to WT mice while MMP-7, MMP-10, MMP-11, and MMP-13 remained similar between genotypes. The inhibitors of MMPs, TIMP-1 and TIMP-4 were found to be regulated in the hearts of $FVII^{tTA/tTA}$ mice compared to WT mice. The fibrinolytic pathway has been shown to regulate extracellular matrix directly or indirectly through MMPs. However, there was no significant difference in plasminogen activator inhibitor-1 (PAI-1) mRNA levels in the hearts of $FVII^{tTA/tTA}$ mice compared to WT mice. These data suggest that a FVII deficiency differentially regulates the expression level of inflammation and extracellular matrix proteins, which result in compromised cardiac function.

Bacterial actin-related proteins

Brittany J. Belin, Matt Bartley and Holly V. Goodson
Department of Chemistry and Biochemistry, University of Notre Dame,
Notre Dame, IN 46556

Recently it has become clear that many bacteria contain proteins related to actin, and that at least some of these proteins assemble into filaments that resemble actin both structurally and functionally. However, little is known about how many different actin-related proteins exist in bacteria or the phylogenetic distribution of these proteins. Even less is known the structure/function relationships in prokaryotic actin-like proteins. As a first step towards addressing these questions, we have identified all discernable actin-related proteins in all fully sequenced prokaryotic organisms. We find that three well-defined groups (MreB, FtsA, and the chaperone HSP 70) contain proteins from divergent organisms, suggesting that three groups represent ancient proteins with basic cell biological functions. However, ParM, a protein generally considered to be the third bacterial actin, appears to be unique to a small group of plasmids. We find that many proteins align with the actin superfamily at least as well as does ParM, suggesting that the number of bacterial actin-like proteins may be much higher than is presently appreciated, although many of these proteins appear to be restricted to small groups of organisms. To identify functionally important regions of the prokaryotic actins and address more specific questions about self-association, nucleotide hydrolysis, and protein-protein interaction interfaces, we are mapping sequence conservation within these groups onto representative crystal structures. These predictions can help direct and focus experimental structure-function analyses.

Residual dynamics in Class I MHC/peptide complex and its immunological implications

Francis K. Insaidoo and Brian M. Baker
Department of Chemistry & Biochemistry, University of Notre Dame,
Notre Dame, IN 46556

The complexes of antigenic peptides with class I histocompatibility complex (MHC I) molecules have been studied extensively via X-ray crystallography. For the class I MHC HLA-A2, crystallographic studies have revealed preferences for leucine or methionine at peptide position 2, whereas position _ prefers valine. These conserved residues serve as peptide anchors, contributing to tight peptide binding. However, crystallographic data provides little information on molecular dynamics. Fluorescence depolarization studies by Pöhlmann *et al*. have suggested that residual motion in peptides bound to the MHC I complex may influence immune recognition. In the case of the tumor associated antigen MART-1 peptide (AAGIGILTV), the presence of a suboptimal alanine position 2 has been implicated in poor T cell recognition. Although this may be due to the poor anchor residue, crystallographic studies suggest peptide dynamics may play a role.

To examine the potential for dynamics in the MART-1 peptide and anchor fixed variant when bound to HLA-A2, unrestrained molecular dynamics simulations were performed using AMBER 8. The simulations included the entire peptide/MHC complex with explicit water. The MART-1 peptide variant had a leucine substitution at position 2. These MART-1 variants are in use in clinical trials for the immunological treatment of melanoma. Starting with crystallographic structures, the two complexes (AAGIGILTV/HLA-A2 and A**L**GIGILTV/HLA-A2) were simulated for approximately 30ns following energy minimization. Specific peptide residues showing significant changes in crystal structures were examined extensively. Distance and dihedral angle calculations revealed that the more immunogenic, "anchor fixed" peptides are highly dynamic. Interestingly though, the native MART1-1 peptide (AAGIGILTV) complex exhibited less dynamics. The results from these simulations will be compared to crystallographic structures and NMR studies to further examine the effects of peptide motion on antigen recognition by the cellular immune system.

Identification and isolation of gene clusters involved in iron acquisition by *Pseudomonas mendocina* ymp

Jonathan Awaya and Jennifer DuBois
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Notre Dame, IN 46556

Iron is an essential bioelement. Its availability controls the distribution of life on earth as well as the viability of pathogens within the human body. However, much of the environmental iron is stored as in the unusable form of insoluble hydroxides. *Pseudomonas mendocina* ymp was isolated from the Yucca Mountain Site for long-term nuclear waste storage, and its ability to solubilize a variety of Fe-containing minerals under aerobic conditions has been previously measured. Using genomic and biochemical information from its better-characterized congeners as a guide, PCR and genetic hybridizations were used to probe this strain for genes important for Fe uptake. It was shown that *P. mendocina* contains a homolog to the ferric uptake receptor (*fur*) gene. Evidence for non-ribosomal peptide synthetase dependent siderophore production and for the presence of multiple TonB-dependent receptors was also obtained. Gene clustering patterns observed in other Pseudomonads suggested that hybridizing multiple probes to the same library could allow for the identification of one or more clusters of syntenic Fe-associated genes. Using this approach, two independent clusters were identified. One gene cluster revealed an open reading frame that encoded an ornithine monooxygenase (PvdA), which is involved in the first step of siderophore biosynthesis in other Pseudomonads. A *pvdA* promoter-*lacZ* fusion showed inducible β-galactosidase expression in iron-deplete media. A mutant within this gene also showed decreased amounts of siderophore production. Further characterizing and mapping of *P. mendocina* iron regulated genes is essential for future molecular-genetic studies of microbe-metal interactions.

Elucidation of the threitol biosynthesis pathway in *Upis ceramboides* using ^{13}C NMR

Kent Walters[1], Qingfeng Pan[2], Anthony S. Serianni[2] and John G. Duman[1]
[1] Department of Biological Sciences and [2] Department of Chemistry and Biochemistry,
University of Notre Dame, Notre Dame, IN 46556-5670

The Alaskan beetle *Upis ceramboides* freezes at –6 ˚C, but is capable of surviving down to –60 ˚C in midwinter. The accumulation of threitol is well correlated with the insect's ability to survive low temperature. Interestingly, *U. ceramboides* is the only animal known to accumulate threitol and the biosynthesis pathway is unknown. I hypothesize that threitol is produced from erythrose-4-phosphate originating in the pentose phosphate pathway. However, it is not understood how this pathway specifically produces threitol without accumulating erythritol and ribitol. I synthesized the metabolic intermediates D-[1-^{13}C]-erythrose-4-phophate (E4P) and D-[1-^{13}C]-threose-4-phophate (T4P) and added them to a crude homogenate at -2.5 ˚C. NMR was used to monitor the metabolism revealing that threitol was indeed produced from both E4P and T4P. Furthermore, it appears that erythritol is also produced, but does not accumulate because it is readily metabolized than threitol.

Cloning, expression and kinetic studies of a bifunctional enzyme, AAC(3)-Ib/AAC(6')-Ib' from *Pseudomonas aeruginosa*

Choonkeun Kim, Adriel Villegas-Estrada, Dusan Hesek and Shahriar Mobashery
Department of Chemistry and Biochemistry, University of Notre Dame,
Notre Dame, Indiana 46556

Aminoglycoside antibiotics are a large family of cationic amino sugars that exhibit broad antimicrobial spectra. Bacterial resistance to aminoglycosides, like other antibiotics, can be intrinsic or acquired due to the unregulated usage of the aminoglycosides in certain environments. Enzymatic modification of the aminoglycoside molecules is the most common mechanism for the acquired bacterial resistance to this class of antibiotics. Three different types of aminoglycoside-modifying enzymes have been described: the *O*-phosphotransferases (APHs), the *N*-acetyltransferases (AACs), and the *O*-nucleotidyltransferases (ANTs). Many individual aminoglycoside modifying enzymes can modify a wide range of the substrates. The emergence of bacteria with bifunctional enzymes that have two different modifying activities challenges the use of aminoglyclosides in clinical treatment because of these bacteria extremely wide aminoglycoside resistance. The kinetic mechanism of AAC(6')/APH(2") enzyme has been intensively investigated including an in depth analysis of the active site structure. Recently, we have investigated the kinetic mechanism of ANT(3")-Ii/AAC(6')-IId enzyme, the AAC(3)-Ib/AAC(6')-Ib' enzymes has however never been purified and studied. The positions of the acetylation have also remained unclear because the enzyme had never been studied but named based solely on the nucleotide sequence homology. For kinetic studies of each domain of AAC(3)-Ib/AAC(6')-Ib', *aac(3)-Ib* and *aac(6')-Ib'* gene segments were separately cloned into an expression vector, pET22b(+). The pH and substrate profiles of the enzyme were obtained from steady-state kinetics. In addition, the kinetic mechanisms of each domain of AAC(3)-Ib/AAC(6')-Ib' were determined by using the initial velocity patterns, dead-end and product inhibition, and viscosity effect analyses. The details of cloning, expression, and kinetic studies of this enzyme will be discussed.

Subtype-selective antagonism of *N*-methyl-D-aspartate receptor ion channels by synthetic conantokin peptides

Zhenyu Sheng, Qiuyun Dai, Mary Prorok and Francis J. Castellino
W.M. Keck Center for Transgene Research and the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556

Conantokin-G (con-G), conantokin-T (con-T), a truncated conantokin-R (con-R[1-17]), that functions the same as wild-type con-R, and variant sequences of con-T, were chemically synthesized and employed to investigate their selectivities as antagonists of glutamate/glycine-evoked ion currents in human embryonic kidney-293 cells expressing various combinations of NMDA receptor (NMDAR) subunits (NR), *viz.,* NR1a/2A, NR1a/2B, NR1b/2A and NR1b/2B. Con-G did not substantially affect ion flow into NR1a,b/NR2A-transfected cells, but potently inhibited cells expressing NR1a,b/NR2B, showing high NR2B selectivity. Con-T and con-R served as nonselective antagonists of all of four NMDAR subunit combinations. C-terminal truncation variants of the 21-residue con-T were synthesized and examined in this regard. While NMDAR ion channel antagonist activity, and the ability to adopt the Ca^{2+}-induced α-helical conformation, diminished as a function of shortening the COOH-terminus of con-T, NMDAR subtype selectivity was enhanced in the con-T[1-11], con-T[1-9], and con-T[1-8] variants toward NR2A, NR1b, and NR1b/2A, respectively. Receptor subtype selectivity was also obtained with Met-8 sequence variants of con-T. Con-T[M8A] and con-T[M8Q] displayed selectivity with NR2B-containing subunits, while con-T[M8E] showed enhanced activity toward NR1b-containing NMDAR subtypes. Of those studied, the most highly selective variant was con-T[M8I], which showed maximal NMDAR ion channel antagonism activity toward the NR1a/2A subtype. These studies demonstrate that it is possible to engineer NMDAR subtype antagonist specificity into con-T. Since the subunit composition of the NMDAR varies temporally and spatially in developing brain and in various disease states, conantokins with high subtype selectivities are potentially valuable drugs that may be used at specific stages of disease and in selected regions of the brain.

Computational investigation of solvation dynamics of a DNA/drug complex using molecular dynamics simulations

Kristina E. Furse, Beth A. Lindquist and Steven A. Corcelli
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Notre Dame, Indiana 46556

Drugs that target specific DNA sequences can disrupt replication and transcription processes, making them attractive for a broad range of antibiotic, antiviral (including anti-HIV) and anticancer therapies. Discovering new drugs of this type will require a detailed understanding of the mechanism of the recognition process and the factors that contribute to the free energy of binding. This free energy is affected not only by the interaction between the drug and DNA, but also by the solvent. The structural and dynamical properties of water molecules that are effectively bound at biological interfaces, such as the surface of DNA, differ substantially from bulk water. These more confined and ordered waters must reorganize to accommodate the drug and stabilize the resulting complex, dramatically impacting the kinetics as well as the thermodynamics of the recognition process. Understanding the role of dynamically ordered water in these interactions is vital to understanding molecular recognition and exploiting it in drug development strategies. We are currently using molecular dynamics (MD) simulations to investigate the role of water dynamics in the molecular recognition of a fluorescent drug molecule, Hoechst 33258 (H33258), by the minor groove of DNA. The fluorescence of the drug is strongly influenced by its environment, exhibiting a significant blue shift and increase in intensity when bound to DNA, relative to its behavior in aqueous solution. Recent experiments have shown that the timescale for the collective reorganization of water molecules at the DNA/water interface is more than an order of magnitude slower than in liquid water. We are using MD simulations to directly connect to these solvation dynamics experiments by calculating equilibrium normalized time correlation functions of the fluctuations in the difference in solvation energy for a vertical excitation when the drug is bound to DNA or free in solution. These correlation functions, $C(t)$, can be directly compared to experimental hydration correlation functions. The simulations will allow us to investigate the specific water properties responsible for the experimentally observed differences, as well as the relative contributions of water and DNA to the solvation response.

Sequence coded dynamics modulate recognition specificity in WW domains

Tao Peng, John S. Zintsmaster, Andrew T. Namanja, Gabriela Pasat, Brian Wilson, Kim Wilson and Jeff W. Peng
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556

The binding specificity of protein-recognition motifs generally is attributed to their distinctive chemical moieties in the constituent amino acid sequences. However, we show for a WW domain that the sequence crucial for specificity is an intrinsically flexible loop that partially rigidifies upon ligand docking. The critical residue, Arg17, in this loop undergoes dramatic changes in dynamics during ligand binding both in ps-ns and μs-ms time scale. A single-residue deletion in this loop simultaneously reduces loop flexibility and ligand binding affinity. Preliminary results of a mutant with the loop sequence swapped with an ortholog loop sequence also show similar flexibility in the loop. These results suggest that sequences of recognition motifs may reflect natural selection and exploitation of not only chemical properties but also dynamic modes that optimize specificity.

Regulation of apoptosis in breast cancer cells induced by *cis*-platin and L-PPMP

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Many tumor-associated antigens are characterized as cell surface glycolipids and glycoproteins. The overall objective of our study is to elucidate the regulation of glycolipid glycosyltransferases(GSL-GLTs) and the related signal transduction during apoptosis (induced by *cis*-platin and L-PPMP) in human breast carcinoma cells (SKBR-3, MCF-7, MDA-468). Abnormal expression or alteration of any particular GSL-GLTs may lead to a modified sequence attached to accumulated ceramide and affect their functions. Ceramide which is a apoptotic messenger to bind mitochondria resulting in release of cytochrome c (Glycoconj J. 2004;20(5):319-30). Little is known about the regulation of GSL-GLTs during apoptosis. We tested different GSL-GLT activities with the treatment of anti-cancer reagent L-PPMP (1 to 16μM) or *cis*-Platin (10 to 100μM). The enzyme inhibition is not due to the presence of any free inducers. The Golgi membranes were isolated and tested for GSL-GLT activities (GalT-4, GalT-5, SAT-2, SAT-3 and SAT-4). Sharp decrease of some of these GSL-GLT activities (GalT-4, GalT-5 and SAT-4) during apoptosis suggests that perhaps these enzymes are either inactivated or down-regulated in their expression. Different signal transduction pathways, such as MAPKs, NF-kB and caspases cascade, were founded to be involved in triggering apoptosis induced by L-PPMP or *cis*-Platin. The change of these signal cascades were found to be dependent on the drug and cell types, which implies the different signal protein patterns in the cancer cells could regulate individual responses to anti-cancer drugs. Deregulation of replication complexes in the presence of *cis*-platin was also observed (*Glycoconj J.* **2006** May; 23(3-4):175-87). Studies on gene expression using glyco-related microarrays are in progress with L-PPMP treated cell samples in collaboration with the laboratory of Prof. Joseph Moskal at Northwestern University.

Sulindac treatment alters extracellular matrix remodeling in a colorectal cancer mouse model

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Nonsteroidal anti-inflammatory drugs (NSAIDS), analgesics that inhibit the production of prostaglandins due to COX inhibition, have shown great potential as chemopreventive agents against cancer formation, especially against colorectal cancers. However, it benefits less than 50% of the cases, and the mechanism by which these drugs act, is not fully understood. Even though some of the effects are linked to COX-2 inhibition, it has been demonstrated that its chemopreventive actions can be independent of the production of eicosanoids. To address the mechanism by which NSAIDS reduce tumor burden, 80 day-old $APC^{Min/+}$ mice were treated with sulindac once a day, via gavage, for 6 days. The treated mice showed a tumor reduction of over 80% in comparison with the control group, which is consistent with previous reports. To analyze the genetic response to sulindac, the intestines were harvested after two days of treatment, when the number of adenomas started to decrease. RNA was extracted from crypt cells from adenomas undergoing change and dissected by laser capture microdissection (LCM). Using 430 A Mouse Expression GeneChip arrays, the expression of over 22,000 genes was measured. Among the genes with differential expression following sulindac treatment, Col1a2, Col6a3, Col5a2 and Col6a2 were upregulated and MMP7 was down-regulated. Real time RT-PCR was used to validate Col6a2 and Mmp7 expression. Confocal microscopy and immunofluorescence were used to identify type I, V and VI collagens and Mmp7 in the intestines and tumors of $APC^{Min/+}$ mice. The different collagens were localized between crypt cells of normal tissue. Within the tumors of untreated mice, collagen VI was present in low amounts, but was increased within the tumors of sulindac-treated mice. Mmp7 was present in 'hot spots' in the tumors of vehicle treated mice, but almost absent in sulindac treated mice. Extracellular matrix (ECM) remodeling is a crucial step for tumor growth and metastasis. Increase in metalloproteinases and degradation of collagens are a common events in different types of tumors. The current study demonstrates that sulindac partially reverses these features.

Catalytic mechanism of penicillin-binding protein 5 of *Escherichia Coli*

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Penicillin-binding proteins (PBPs) and β-lactamases are members of large families of bacterial enzymes. These enzymes undergo acylation at a serine residue with their respective substrates as the first step in their catalytic events. Penicillin-binding protein 5 (PBP 5) of *Escherichia coli* is known to perform a DD-carboxypeptidase reaction on the bacterial peptidoglycan, the major constituent of the cell wall. The roles of the active site residues Lys47 and Lys213 in the catalytic machinery of PBP 5 have been explored. By a sequence of site-directed mutagenesis and chemical modification, we introduced γ-thialysine at each of these positions individually. The pH dependence of k_{cat}/K_m and k_{cat} for the wild-type PBP 5 and for the two γ-thialysine mutant variants at positions 47 and 213 were evaluated. The pH optimum for the enzyme was at 9.5-10.5. The ascending limb to pH optimum is due to Lys47, hence this residue exists in the free-base form for catalysis. The descending limb from the pH optimum is contributed to by both Lys 213 and by a water coordinated to Lys47. These results have been interpreted as Lys47 playing a key role in proton transfer events in the course of catalysis during both the acylation and deacylation events. On the other hand, the findings for Lys213 argue for a protonated state at the pH optimum. Lys213 serves as an electrostatic anchor for the substrate.

Cross-reactivity of TCR A6 on haptenated HTLV-1 Tax_{11-19} peptides presented by HLA-A2

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Antigen recognition by the T cell receptor (TCR) is initiated by molecular contact between the TCR and the peptide/MHC complex, which leads to an intracellular signaling cascade resulting in a functional response by the T cell. Although antigen specificity is a distinct feature of the adaptive immune response, a growing number of evidences indicate that TCRs can bind and respond to structurally diverse antigens. Particularly, it has been found that the HTLV-1 Tax_{11-19}-specific TCR A6 can cross-reactively recognize several haptenated Tax_{11-19} peptides presented by the class I MHC molecule. In one of these haptenated peptides Tyr5 is substituted by lysine covalently attached to 3-indolyl-butyric acid (IBA). However the exact molecular mechanism underlying the cross-reactivity of A6 TCR has not been understood.

To unravel the structural details underlying molecular recognition of the haptenated peptide, we determined the crystal structures of MHC/Tax(Y5K-hapten) and its complex with TCR A6. The recognition involves cooperative conformational changes for both the peptide and TCR A6. Particularly, the IBA moiety of the haptenated peptide, which is highly flexible and disordered in the unbound state, becomes ordered in the complex and adopts a "folded" conformation allowing this side chain to occupy the same pocket in the A6 interface as occupied by Tyr5 in the native structure. Interestingly, the carbonyl-amide moiety of Lys5-IBA is highly complementary to the Arg95-Gly97 region of the A6 CDR3β loop, which might play an essential role in recognition. Furthermore, an accommodation of the bulky Lys5-IBA side chain also requires a considerable rearrangement of the CDR3β loop as well as a slight shift of Vα/Vβ domains of TCR A6.

Squaraine rotaxanes: Superior near-Infrared fluorophores for *in vitro* and *in vivo* imaging

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Recent breakthroughs in fluorescence microscopy are producing notable improvements in imaging resolution. These ongoing technical advances will have a major positive impact on the field of single molecule fluorescence methods, and help merge the sub-disciplines of cell and molecular biology. However, the full potential of these new high-resolution methods will only be achieved if extremely bright and highly stable luminescent probes are employed. NIR dyes are particularly attractive for fluorescence imaging of biomedical samples because there is very little undesired absorption and autofluorescence from common biomolecules, and diminished Rayleigh-Tyndall scattering of light. At present, the most popular organic near infrared (NIR) dyes for *in vivo* optical imaging are the cyanine series (Cy5, Cy5.5, Cy7). Although these fluorophores are the most common NIR dyes for *in vivo* bioimaging, they have moderate to poor photostability, undesired reactivity with nucleophiles, and propensity to self-quench upon aggregation. Our efforts have been devoted to squaraines, a promising family of NIR dyes that show desirable photophysical characteristics. Squaraines have been investigated previously as NIR probes for biomedical applications, but have many of the same issues of the previously mentioned NIR fluorophores. We have developed a novel and effective method of sterically protecting squaraines in a Leigh-type rotaxane such that these problems are eliminated, while the favorable photophysical properties are retained. The squaraine rotaxanes have major excitation and emission peaks at 643 and 667 respectively, making them compatible with common Cy5 optical filters. Additionally, their excitation peak is sufficiently narrow to allow multicolor imaging, their stability facilitates long term imaging procedures, and their structure is amenable to conjugation to a variety of targeting ligands. Here we show that squaraine rotaxanes are highly useful fluorophroes for *in vitro* and *in vivo* NIR optical imaging applications.

ABSTRACTS: POSTERS

Calorimetric characterization of TCR-peptide/MHC interactions

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The T cell receptor (TCR) is a cell-surface receptor responsible for recognizing peptide antigens "presented" by major histocompatibility complex (MHC) molecules. Recognition of antigenic peptides presented by a MHC is required for the initiation and propagation of a cellular immune response. We reported that the A6 TCR binds its Tax/HLA-A2 ligand with a favorable entropy change (at 25 °C) and a moderately negative heat capacity change. These observations were of interest given the unfavorable binding entropies and large heat capacity changes for other TCR-ligand interactions, which have been linked to TCR conformational changes. We performed a detailed calorimetric investigation of the thermodynamics of the A6–Tax/HLA-A2 interaction to further investigate the A6 TCR molecular recognition properties.

We observed that binding proceeds with a large shift in the pK_a of at least one ionizable group. Determination of the intrinsic binding thermodynamics required a global analysis of calorimetric data as a function of pH, buffer ionization enthalpy, and temperature. Intrinsically, the A6 TCR binds Tax/HLA-A2 at 25 °C with a very weak binding enthalpy change, large positive entropy change, and moderately negative heat capacity change. The protonation that is linked to binding occurs with a very large heat capacity change, suggesting that protonation might be coupled to an additional process.

We have started to examine a high-affinity A6 TCR mutant, A6c134, binding to Tax/HLA-A2. The ability to enhance the affinity of a T cell receptor for its cognate ligand allows for both biomedical applications and as a probe to investigate if the TCR binding affinity relates to signaling strength. Preliminary ITC data indicate that the A6c134 mutant binds Tax/HLA-A2 with a large favorable enthalpy change, an unfavorable entropy change, and an approximately 100-fold tighter affinity than wild-type A6. The overall thermodynamics are in stark contrast to the thermodynamic profile of A6 wild-type.

Kinetic characterization of an aminoglycoside phosphotransferase from *Enterococcus spp.*

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Aminoglycoside 2”-phosphotransferases (APH(2”)s) mediate high-level resistance to the aminoglycoside gentamicin in enterococci. The purification and mechanistic characterization of aminoglycoside (2”)-Ic phosphotransferase (APH(2”)-Ic) are described. APH(2”)-Ic phosphorylates only certain 4,6-disubstituted aminoglycosides with k_{cat} values of 0.5-1.0 s^{-1} and K_m values in the sub-micromolar range for all substrates but kanamycin A. The enzyme also exhibits a 200-fold preference for GTP over ATP and UTP as phosphate donor, a unique observation among all characterized APHs. Product and dead-end inhibition patterns indicated a random sequential Bi Bi mechanism and the solvent viscosity effect indicated that at pH 7.5 the release of GDP is rate-limiting. The pH-dependence of the catalytic rate constants, the solvent viscosity and kinetic isotope effects with kanamycin A are consistent with a change in the rate-limiting step from diffusion of product at pH > 7.5 to a chemical step at pH < 6.5. Kinetic solvent isotope data indicate that the APH(2”)-Ic-catalyzed phosphate transfer with GTP (the preferred phosphate donor) occurs through an associative-like transition state.

Phenotypic consequences of plasminogen activator inhibitor-1 Gene ablation on STAT1 activation and cell cycle progression in proliferating endothelial cells

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The enhanced proliferation observed in PAI-1-deficient endothelial cells (EC) is associated with Akt hyperactivation resulting in loss of growth control and resistance to apoptosis. Additionally, involvement of other signaling pathways that regulate cell proliferation was investigated. Evaluation of the STAT protein profile revealed that PAI-$1^{-/-}$ EC exhibited lower levels of STAT1, which is a negative regulator of cellular proliferation. The pro-proliferative STAT3 and STAT5 protein levels were similar in WT and PAI-$1^{-/-}$ cells. Furthermore, the activation status of STAT1 between WT and PAI-$1^{-/-}$ EC differs. The extent of phosphorylation of Ser727 within the STAT1 transcriptional activation domain is lower in PAI-$1^{-/-}$ EC. Immunofluorescent analyses of proliferating WT and PAI-$1^{-/-}$ EC demonstrate that the cellular distribution of STAT1(P-Tyr701), which is crucial for STAT1 dimerization and nuclear translocation, is different between the two cell types. In WT cells, STAT1(P-Tyr701) appears to be diffuse throughout the whole cell, including the nucleus. In contrast, in PAI-$1^{-/-}$ cells, STAT1(P-Tyr701) seems to be localized in the cytoplasm and plasma membrane. Additionally, it was observed that in WT cells STAT1 preferentially binds to Jak1, whereas in the PAI-$1^{-/-}$ EC, STAT1 is preferentially bound to Jak2. This differential binding may be responsible for the different activation states of STAT1. Cell cycle progression analysis demonstrated that a higher percentage of PAI-$1^{-/-}$ EC are in the S-phase compared to WT cells, which is likely a consequence of diminished STAT1 levels observed in the PAI-1^{-} deficient cells.

Structures of MART-$1_{26/27-35}$ peptide/HLA-A2 complexes reveal broad T cell cross-reactivity in the absence of structural homology

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Alterations to antigenic peptides that cause minor structural changes often result in substantial changes in immunogenicity, supporting the notion that TCRs can be exquisitely sensitive to antigen structure. Yet there are striking examples of TCR recognition of structurally dissimilar ligands. The resulting unpredictability of how T cells will respond to different antigens impacts our understanding of the physical bases for TCR specificity and cross-reactivity and the design and use of peptide variants for immunomodulation. In cancer immunotherapy, MART-1 protein derived epitopes and variants are widely used as clinical vaccines. Here we show that the overlapping MART-1_{27-35} and MART-1_{26-35} peptides and clinically-relevant variants adopt strikingly different conformations when bound to HLA-A2. Nevertheless, naturally arising MART-1-reactive T cells, which are among the most common in HLA-A2+ individuals, show broad T cell cross-reactivity towards these ligands. This finding, along with the observation that certain MART-1-specific T cells remain sensitive to very subtle structural differences, challenges our thinking about how structures of unligated peptide/MHC complexes should be best used when addressing questions of TCR specificity and cross-reactivity.

The effect of inflammation on adenoma development in the APC $^{MIN/+}$ mouse model

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Colon cancer is responsible for the deaths of over 60, 000 Americans annually. Much has been learned about this nefarious disease; however, the impact of inflammation on colon tumor development is only recently gaining attention. The $APC^{MIN/+}$ mouse is a model of an inherited form of colorectal cancer where patients develop a multitude of polyps in the colon. It is interesting to note that the phenotype of the $APC^{MIN/+}$ mouse is somewhat different than the human condition, since the mice develop adenomas of the small intestine, with fewer in the large intestine. The protein C (PC) pathway has been shown to play a role in inflammation and different aspects of tumor growth. At this time, it is unclear how the PC pathway affects tumor growth in a setting of chronic inflammation. Activated PC (APC) has been shown to not only regulate coagulation in the vasculature, but also affects inflammation, apoptosis, proliferation, and angiogenesis. APC inhibits apoptosis through upregulation of anti-apoptotic Bcl-2 and downregulation of p53, Bax, and caspases 3, 8, and 9, all through interactions with the endothelial protein C receptor (EPCR) and protease-activated receptor-1 (PAR-1).

A recent study demonstrated that $APC^{MIN/+}$ mice, administered 2% Dextran Sodium Sulphate (DSS) in their drinking water, rapidly developed large colonic tumors. In the current study, we administered 2% DSS for 1 week to $APC^{MIN/+}$ and $APC^{MIN/+}/EPCR^{-/-}$ mice, and monitored the number of tumors at 2 and 5 wk post-treatment. Both genotypes developed very large adenomas within 2 wk, but there was no significant difference in the number of adenomas per mouse. At 5 wk post treatment, significantly larger (diameter) tumors were found in $APC^{MIN/+}/EPCR^{-/-}$ mice.

We have examined inflammatory cell infiltration in adenomas using immunohistochemical methods. Preliminary results indicate an increased inflammatory cell infiltration in tumors of $APC^{MIN/+}/EPCR^{-/-}$ mice, which may be the result of enhanced cell adhesion molecule expression on inflammatory cells and tumor epithelium. Additionally, APC is known to affect the endothelial cell barrier integrity of vessels. In the absence of APC-mediated signaling, cells may be able to migrate from the vessels into the tumor more readily. Further investigations into these mechanisms of inflammatory cell invasion will help us to understand relationships between inflammation and tumor growth.

Influence of translation rate on co-translational folding intermediates

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Rare codons can slow protein synthesis by the ribosome. Yet modulation of translation rate via rare codon usage has been difficult to assess, as existing algorithms have focused either on the distribution of common codons, or the absolute usage of one codon versus all 63 others. To address this, we developed a novel algorithm to analyze rare codon usage. Application of this algorithm to all *E. coli* genes shows that, instead of a random distribution, rare codons often occur in clusters. These clusters accurately predict translation pause sites, which can be eliminated by synonymous codon substitution. Moreover, translation pauses occur at points known to influence protein folding. Application of this codon usage algorithm to mRNA sequences encoding a variety of beta-sheet structural topologies has been used to develop a hypothesis regarding the effects of translation pausing on co-translational structure formation.

The role of fibrinogen in a murine model of lethal endotoxemia

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Severe inflammation leads to haemostatic abnormalities, such as the development of microvascular thrombi. As a result, ischaemia-related downstream organ damage can occur. In the present study we show that mice with a total deficiency of fibrinogen present altered responses to challenge with Lipopolysaccharide (LPS). Survival was increased in these mice and results from histological findings indicate this improvement is correlated with a lack of fibrin deposition in organs and only a minor degree of ischemic damage. Neutrophils appeared early in lungs of challenged wild-type (Wt) mice, while similar infiltration in lungs of Fg deficient mice occurred at later times. This delayed response in Fg deficient mice was confirmed by studies that showed a strong dependence on Fg in binding of neutrophils to endothelial cells in the presence of LPS. In plasma, cytokines were elevated as indicators of the inflammatory process; nevertheless in fibrinogen deficient mice the levels were lower at early time points. The time course of MIP-2 expression correlated with the occurrence of pulmonary leakage after LPS challenge, which was delayed in Fg deficient mice. mRNA quantifications of several inflammation- and hemostasis-related gene products indicated that correlations existed between the cytokine levels in plasma and their mRNA expression in tissues. These results suggest that fibrinogen plays a central role as an early mediator in the crosstalk between activated coagulation and inflammation.

Identification of the residues and domains of human plasminogen implicated in the conformational transition during its activation

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Native plasminogen (Glu^{1}-Pg) is the circulating zymogen of the serine protease plasmin (Pm), the principal fibrinolytic enzyme in vertebrate organisms. Generation of Pm requires activator-catalyzed cleavage of the Arg^{561}-Val^{562} bond in Pg and proteolytic removal of the 77-residue, N-terminal activation peptide (AP). Lysine-binding kringle domains of Pg/Pm mediate the binding of these proteins to the surface of fibrin clots and a number of proteins. Pg can assume at least two major conformational states: a closed conformation, which is resistant to activation and is detected in the presence of Cl^-, and an open, activatable form, which is observed in the presence of lysine and lysine analogs, such as ε-amino caproic acid (EACA). Pg lacking the AP (Lys^{78}-Pg) exists in the open conformation, demonstrating that intramolecular interactions between the AP and the kringle domains of Pg enforce the closed form of the zymogen. To assess the contributions of specific domains involved in maintaining the closed form of Pg, we have generated a panel of recombinant Pg truncation mutants, which include AP contiguous with kringles 1-3, 1-4, and 1-5. Pg variants carrying single point mutations at select lysine residues in the AP have also been generated. Using analytical ultracentrifugation, we have determined the $S^0_{20,w}$ values of these constructs in the presence of Cl^- or EACA. Differential scanning calorimetry and secondary structure analysis using circular dichroism have been carried out to study the conformational stability of the generated constructs. The potential effects of the truncations and point mutations in the constructs and the conformational changes promoted by Cl^- or EACA have been studied using the same techniques. Our results suggest a critical, but not exclusive, role for kringle 5 and Lys residues 50 and 62 of the AP in the stabilization of the closed conformation of Glu^{1}-Pg.

Effects of an endothelial cell protein C receptor (EPCR) deficiency on inflammation in a murine model of asthma

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Asthma is a chronic pulmonary disease that affects over 150 million people worldwide and over 17 million in the US alone. In asthma, chronic inflammation causes an associated increase in airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. The pathophysiology of asthma is typically described as chronic inflammation in the airways, dominated primarily by the TH2 inflammatory response. This response is characterized by eosinophilic infiltration into the airway smooth muscle cells but also mast cells, macrophages, plasma cells, and neutrophils may be present. Another hallmark physiological characteristic of asthma is airway remodeling which encompasses structural changes in the airway wall as a result of the lung attempting to repair damage from the inflammatory response. To counter these effects, the protein C (PC) pathway, which controls the conversion of prothrombin to thrombin and the activation of PC to aPC, intervene by exerting anti-inflammatory and anticoagulant effects. In order for aPC to function, it interacts with Endothelial Cell Protein C Receptor (EPCR). EPCR is an endothelial transmembrane glycoprotein (EPCR) that is expressed on the luminal surface of the endothelium. When PC binds EPCR, it enhances the activation of PC to aPC by thrombin/thrombomodulin, by at least 20-fold. Therefore, it has been proposed that EPCR may contribute to the anti-inflammatory effects of aPC. The current study examined the role of EPCR in mice challenged with the inflammatory agent ovalbumin (OVA). $EPCR^{-/-}$ (EPCR very low-expressing mice) and wild-type (WT) mice were utilized for this study.

In this model of inflammation, while the Airway Hyperresponsiveness to OVA challenge in WT and $EPCR^{-/-}$ mice showed no differences, examination of bronchioalveolar lavage fluid (BALF), lung tissue, and circulating blood showed significantly higher levels of eosinophils in $EPCR^{-/-}$ mice versus WT mice, and significantly higher levels of eotaxin and IL-13. The TH2 cell response from isolated para-bronchial lymph nodes in $EPCR^{-/-}$ mice showed significantly higher IL-5 levels. BEAS-2B cells, a human epithelial cell line, when stimulated with BALF from WT and $EPCR^{-/-}$ mice, showed higher eotaxin mRNA production in the $EPCR^{-/-}$ mice. Therefore, the exacerbated eosinophilia in $EPCR^{-/-}$ mice indicates that a deficiency in EPCR increases the inflammatory response in a murine model of asthma.

Fluorescence anisotropy investigation of TAX/HLA-A2/TCR

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Cytotoxic T cells destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. The T cell receptor (TCR) is a molecule found on the surface of T cells responsible for recognizing antigens presented by major histocompatibility complex (MHC) proteins. MHC proteins are expressed on the surface of cells and display fragments of molecules from invading microbes or dysfunctional cells. Most mmeasurements of TCR-pMHC binding kinetics have been obtained through the utilization of surface plasmon resonance (SPR). Unlike pure solution methods, SPR does not allow for detailed resolution of kinetic mechanisms. Through the use of stopped flow fluorescence anisotropy, we hope to be able to obtain more accurate kinetic parameters to broaden our understanding of TCR-pMHC interactions. The stopped-flow fluorescence anisotropy measurements on the TCR-pMHC interactions are expected to reveal much about the recognition kinetic mechanism of the TCR to pMHC and further advance the knowledge of cross-reactivity and specification within TCRs. Towards these goals, Serine 195 has been engineered to a cysteine (S195C) which will allow for binding of the fluorophore to the surface of the protein. Fluorescence studies on the interaction will be completed using fluorescene (FAM) and 5-((((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid (IAEDANS) to label a free cysteine via disulfide bonding. Preliminary feasibility data are shown, demonstrating that using FAM and IAEDANS, the free cysteine can be labeled. Preliminary data shows that as the A6 concentration increases, a higher fraction of the analyte is bound is a larger protein-protein complex resulting in increased anisotropy (S195C-IAEDANS).

Interactions between EB1, Tubulin, and Microtubules: Implications for EB1 Function

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EB1 and other plus-end tracking proteins (+TIPs) act at crucial interfaces between microtubules and other subcellular structures. While it seems clear that +TIPs regulate microtubule dynamics, their role in greater cell function remains obscure. EB1 is the most ancient and conserved +TIP, and it binds to many other +TIPs. These observations suggest that EB1 is the core constituent of a +TIP network or "microtubule plus-end complex". To better understand the functions and activities of this network, we are investigating interactions between EB1, tubulin, and other +TIPs. Two hypotheses have directed this work: 1) EB1 tracks by an end-loading mechanism driven by its having higher affinity for tubulin than for microtubules (Folker MBOC 15:236a 2004); 2) EB1 binds its own tail in an autoinhibitory conformation released by binding of the tail to partners such as CLIP-170 (Hayashi MolCell 19:449 2005). While many of our experiments are consistent with these ideas, others reveal additional complexity. First, removal of the EB1 C-terminal tail (the part involved in autoinhibition) significantly increases the apparent affinity of EB1 for microtubules. However, it does not interfere with EB1 plus-end tracking in vivo. These observations are difficult to reconcile with a straightforward end-loading mechanism. Second, the EB1 tail-minus mutant binds microtubules with surprisingly high stoichiometry (~4EB1/tubulin dimer). This suggests that EB1 has more than one binding site on tubulin, or that EB1 dimers self-associate. Moreover, CLIP-170 and the EB1 tail mutant (which lacks the CAP-GLY binding site) can bind microtubules simultaneously. We are working on models for interaction between EB1, tubulin, and other partners that are consistent with these data. In particular, we are investigating the possibility that EB1 can form higher-order structures influenced by tubulin and/or other +TIPs.

Using molecular evolution to dissect the function of NPC2 (Niemann-Pick Type C2)

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Mutations in two genes (NPC1 and NPC2) cause defects in transport and processing of cholesterol and other lipids, resulting in the neurodegenerative disease Niemann-Pick Type C. Thus far, little is known about the physiologically relevant activities of these proteins or the precise mechanisms by which they cause disease. As a means to gain insight into these issues, we are studying the molecular evolution of the NPC1 and NPC2 proteins, and report here on our analysis of NPC2. We have identified the set of NPC2 proteins in vertebrate organisms, performed multiple sequence alignments and phylogenetic analysis, and used this information to calculate position-specific conservation scores. By mapping these scores onto the NPC2 crystal structure, we have identified evolutionarily constrained surfaces of the human NPC2 protein. This analysis has allowed us to identify three regions that are likely involved in functionally significant protein-protein interactions. Using similar approaches to study the multi-gene insect NPC2 family, we have identified several regions that are conserved within subfamilies but divergent between them, suggesting that these regions are involved in functional differentiation between the insect NPC2 subfamilies. Finally, we have identified in NPC2 a hydrophobic "knob" that is well conserved at the level of composition, but poorly conserved in primary sequence, suggesting that it may be involved in binding membranes. This analysis should provide the basis for directed experimental dissection of NPC2 function, which in the long term will hopefully lead to more effective NPC therapy.
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Methyl β-[1-^{13}C]GlcNAc-(1→2)-α-[2-^{13}C]mannopyranoside: Synthesis and conformational studies by NMR, MD, DFT and crystallography

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Complex-type human *N*-glycans contain GlcNAc, Man, Gal, NeuAc and/or Fuc residues connected by at least seven different O-glycosidic linkages. As part of ongoing studies to determine the effects of molecular context on oligosaccharide conformation and dynamics, efforts have been made in this laboratory to prepare ^{13}C-labeled disaccharides containing each of these biologically relevant linkages in isolation to provide the reference states required for studies of context. To date, most of these reference disaccharides have been prepared and investigated except for the α-NeuAc-(2→3)-β-Gal and β-GlcNAc-(1→2)-α-Man linkages. An efficient chemical route was developed to prepare the methyl glycoside of the latter disaccharide containing dual ^{13}C enrichment at the two carbons involved in the linkage. The latter labeling strategy allowed accurate measurements of the six trans-glycoside J_{CH} and J_{CC} sensitive to the two glycosidic torsion angles, ϕ and ψ. Concomitantly, DFT calculations were performed on model GlcNAc-Man structures to parameterize these J-couplings for use in quantitative analysis of the experimental data. Information on the dynamics of this linkage was obtained through solvated MD simulations using both CHARMM and AMBER, and an exhaustive survey of crystallographic databases gave statistical distributions of ϕ and ψ in diverse structures in the solid state. Results of these studies were compared to those obtained for the other 1→2-linkage found in *N*-glycans, namely, α-Man-(1→2)-α-Man. A long-range goal of this work is to develop trans-glycoside *J*-coupling parameterizations for all biologically relevant linkages, which would in turn permit reliable quantitative analysis of these couplings in any structures that contain them.

Pertactin β-helix folding mechanism suggests common themes for the secretion and folding of autotransporter proteins

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Many virulence factors secreted from pathogenic Gram-negative bacteria are autotransporter proteins. The final step of autotransporter secretion is C→N-terminal threading of the passenger domain through the outer membrane (OM), mediated by a cotranslated C-terminal porin domain. The native structure is formed only after this final secretion step, which requires neither ATP nor a proton gradient. Sequence analysis reveals that, despite size, sequence, and functional diversity among autotransporter passenger domains, >97% are predicted to form parallel β-helices, indicating this structural topology may be important for secretion. We report the folding behavior of pertactin, an autotransporter passenger domain from *Bordetella pertussis*. The pertactin β-helix folds reversibly in isolation, but folding is much slower than expected based on size and native-state topology. Surprisingly, pertactin is not prone to aggregation during folding, even though folding is extremely slow. Interestingly, equilibrium denaturation results in the formation of a partially folded structure, a stable core comprising the C-terminal half of the protein. Examination of the pertactin crystal structure does not reveal any obvious reason for the enhanced stability of the C terminus. *In vivo*, slow folding would prevent premature folding of the passenger domain in the periplasm, before OM secretion. Moreover, the extra stability of the C-terminal rungs of the β-helix might serve as a template for the formation of native protein during OM secretion; hence, vectorial folding of the β-helix could contribute to the energy-independent translocation mechanism. Coupled with the sequence analysis, these results suggest a general mechanism for autotransporter secretion. To further clarify the role of the pertactin C-terminus in folding and secretion, the stable core of the partially folded state has been crystallized. A high-quality electron density map of the stable core has been obtained and the crystal structure is currently being refined.

^{13}C-Labeled *N*-acetylneuraminic (sialic) acid: NMR and DFT studies of J_{HH}, J_{CH} and J_{CC} scalar couplings in protonated and ionized pyranoses

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^{13}C Isotopomers of *N*-acetylneuraminic (sialic) acid were prepared with single sites of enrichment at C1, C2 and C3 to facilitate experimental investigations of J_{HH}, J_{CH} and J_{CC} in this important biomolecule as a function of solution pH. ^{1}H and ^{13}C NMR spectra of these isotopomers in aqueous (H_2O and D_2O) solutions at pH/D 2.0 and 8.0 showed the presence of rapid exchange of $H3_{ax}$ with solvent protons/deuterium at pH 8.0 in both pyranose anomers at room temperature, followed by a slower exchange of $H3_{eq}$. Complementary *in vacuo* and solvated DFT calculations were conducted on SA structural mimics to assist in the interpretation of structural effects on the observed experimental couplings. $^{1}J_{C1,C2}$ was found to be sensitive to anomeric configuration and to solution pH, with smaller couplings observed in the anion. The latter effect appears related to the larger $r_{C1,C2}$ in the anion, and may be explained via a hyperconjugative mechanism. In addition to the previously reported dependence of $^{3}J_{C1,H3ax/eq}$ on anomeric conformation of SA, and to $^{1}J_{C1,C2}$, $^{2}J_{C2,H3ax/H3eq}$ also depend on anomeric configuration. Four-bond coupling between C2 and H7 in the β-pyranose was observed, analogous to $^{4}J_{C1,H6}$ recently reported in β-galactopyranosyl residues (Pan *et al.*, *J. Org. Chem.* **2005**, *70*, 7542-7549), supporting a C6-C7 bond torsion favoring that rotamer having H6 approximately anti to O7. Rotation of the C1-C2 bond in SA pyranoses modulates some *J*-couplings in the vicinity of the anomeric carbon more than others, and according to DFT data, preferred conformation about this bond in the pyranose forms differs. Importantly, a comparison of *in vacuo* and solvated DFT-computed *J*-couplings show that inclusion of solvent significantly improved the agreement with experimental couplings, especially for $^{1}J_{C1,C2}$. These results confirm that reliable calculated *J*-couplings in ionizable saccharides cannot be obtained without the inclusion of solvent in the calculation. These results have important implications for anticipated studies of trans-glycoside *J*-couplings involving SA linkages, whose conformational properties remain poorly understood.

Substrate recognition reduces side-chain flexibility for conserved hydrophobic residues in human PIN1

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Pin1 is an 18.2 kDa bi-modular signaling protein consisting of a docking WW domain that is flexibly linked to a catalytic peptidyl-prolyl isomerase (PPIase) domain. The Pin1-WW domain targets phospho-Ser/Thr-Pro motifs of multiple cell-signaling proteins while the Pin1-PPIase domain performs the *cis-trans* conversion reaction. Inhibition of Pin1 induces apoptosis of several cancer cell lines and may also contribute to neuronal death in Alzheimer's disease. As a result, Pin1 is an emerging therapeutic target for both cancer and Alzheimer's disease. Critically, structural data show conformational changes in Pin1 upon ligand binding. This represents the initial evidence for the link between Pin1 function and dynamics. To understand how conformational dynamics influence its binding and catalysis, we have employed Nuclear Magnetic Resonance (NMR) deuterium (^{2}D) and carbon (^{13}C) spin relaxation studies on *apo* and phospho-peptide substrate (Cdc25{EQPL[pT]PVTDL}) saturated Pin1. These NMR methods probe side-chain motions of Pin1's methyl bearing residues. Substrate interaction alters both microsecond-millisecond (μs-ms) and picosecond-nanosecond (ps-ns) side-chain dynamics. Alterations include: exacerbated chemical exchange processes suggesting intrinsic μs-ms motions for several methyl groups at or near the catalytic zone; and loss of ps-ns flexibility along an internal conduit of hydrophobic residues connecting the catalytic site with the inter-domain interface. These residues are conserved among Pin1 homologs; hence, their dynamics are likely important for the Pin1 mechanism. More importantly, these residues may constitute a dynamic path for modular allosteric regulation in Pin1.

Ref: Namanja AT, Peng T, Zintsmaster JS, Elson AC, Shakour MG, Peng JW. Substrate recognition reduces side-chain flexibility for conserved hydrophobic residues in human pin1. Structure. 2007 Mar;15(3):313-27.

Unanticipated complexities of a murine FXI total deficiency

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The zymogen serine-protease coagulation factor XI (FXI) is an essential player in the initiation of the intrinsic pathway of coagulation known as the contact system. A partial deficiency of this factor leads to localized bleeding in humans and other species, such as certain strains of cattle and dogs. In humans, the intriguing hallmark of this protease deficiency is the lack of a relationship between the levels of FXI in plasma and its capacity to form stable blood clots. This disparate relationship is exclusive to a FXI deficiency such that a deficiency of any other component of the contact system (e.g. prekallikrein, FXII, or High Molecular Weight Kininogen) displays no bleeding diathesis. Furthermore, mice with a total deficiency in FXI ($FXI^{-/-}$) crossbreed and develop with no apparent adverse phenotypes other than a slightly delayed plasma aPTT. However, a FXI deficiency in humans does not provide cardioprotective advantages like other deficiencies such as, FVIII and FIX. Additionally, reeexamination of FXI deficient mice at a young adult age (3 months-age) and later stages (9-12 months-age), using blood analyses, TEM, SEM, and histological approaches, revealed a series of vascular, hepatic, and blood abnormalities that uncovered novel complexities of the murine phenotype. These phenotypes provide new evidence for extended functions of FXI in blood, and may facilitate in explaining some uncorrelated observations related to its deficiency in other species.

Recognition of peptides by MHC and TCR molecules

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The immunogenicity of peptides presented in major histocompatibility complexes (MHCs) derives from both their stability in the MHC complex and from the ability of that peptide-MHC complex (pMHC) to be recognized by T-cell receptors (TCRs). Here we probe the determinants of TCR recognition of pMHC through incorporation of non-natural amino acids into the Tax 9 peptide, which is recognized by the A6 and B7 T cell receptors when presented by the class I MHC molecule HLA-A2. Tax9 is a nonomeric peptide with the sequence LLFGYPVYV. TCR binding affinity and kinetics were examined through surface plasmon resonance and the stability of the pMHC by circular dichroism. Of particular interest is how different positions in the Tax9 peptide contribute to affinity with different T-cell receptors. Tyrosine 5 of the peptide has been found to be extremely important for affinity with A6 and B7. With A6, substitutions of more electronegative atoms to the phenyl ring act to enhance affinity by decreasing k_{off} while substitutions of aliphatic moieties weaken affinity, with larger substitutions abrogating binding entirely. In contrast, B7 is much less tolerant of modifications to tyrosine 5. One of the principle changes in the Tax9 peptide upon either A6 or B7 TCR binding is a backbone shift away from the TCR at proline 6. The addition of a hydroxyl group to the proline ring was proposed to stabilize the peptide in the TCR-bound conformation. The substitution of trans-hydroxyproline at position 6 marginally increases stability of the pMHC, but has a deleterious effect on both A6 and B7 binding TCR affinity. It is unclear if this stems from a reduced ability of the TCR to recognize the TCR-bound peptide conformation or destabilizing effects from the hydroxyl group on the pMHC-TCR complex.

Folding and secretin studies of the PET autotransporter

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Pet is a monomeric 966 aa extracellular protein from *Escherichia coli*, composed of a large parallel β-helix domain and an N-terminal, globular protease domain. Pet is an autotransporter protein; >500 autotransporters have been identified in a wide variety of pathogenic gram-negative bacteria. Autotransporters are thought to facilitate their own transport across the outer membrane via a C-terminal β-porin domain (not present in the mature protein). Outer membrane secretion occurs in the absence of ATP or a proton gradient, leaving the driving force for efficient secretion unknown. Our laboratory has hypothesized that the folding of the autotransporter β-helix contributes to efficient secretion, and indeed, other laboratories have identified a wide range of mutations in the Pet β-helix domain that have negative effects on secretion. Pet is homologous to hemoglobin protease (26% identical and 43% similar), for which high-resolution structural information is available. We have purified Pet and inhibited its proteolytic activity in order to characterize its structure and folding properties. Far-UV circular dichroism spectroscopy confirms that Pet has significant β-sheet structure. Steady state fluorescence emission over a wide range of denaturant concentrations revealed that Pet adopts a stable, partially folded conformation at 1 M GdHCl. Pet refolds reversibly from the partially folded state to the native structure. There is also some evidence for reversible refolding from the unfolded state to the partially folded state; however, this reaction is very slow. Limited proteolytic digestion of the Pet partially folded state reveals a stable core fragment with a size of 25 kDa. The size of this stable core is very similar to the stable core identified for the non-homologous pertactin autotransporter, suggesting this structure may be a general feature of autotransporter proteins.

Involvement of coagulation Factor VII (FVII) and Tissue Factor in asthma

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Extravascular thrombin, fibrinogen, and fibrin have been found in the sputum of patients with asthma, and thrombin activity in bronchoalveolar fluid (BALF) has been shown to be increased by segmental antigen provocation in asthmatic patients. Recently, we showed that FXa is involved in airway remodeling. Specifically FXa induction of mucin production is present, allowing a hypothesis that locally activated coagulation cascades are important for the asthmatic response. Therefore, in this study, we attempted to clarify whether FVII/VIIa is associated with asthma since FVIIa is essential for the activation of FXa. A human mucus-producing cell line (NCI-H292), along with an anti-mucin (MUC5AC) antibody, was used to study FX or FVIIa-mediated production of mucus, in vitro. To evaluate the role of FVII/FVIIa in vivo, FVII low expressing mice ($FVII^{tta/tta}$) were used. Mice were immunized with ovalbumin (OVA)/alum and administered OVA, intranasally, 3 days/week for 2 weeks. mRNA from lung and bronchial fluid (BALF), airway hyperresponsiveness (AHR), and histological analyses were performed 1 day after the final challenge. In NCI-H292 cells, MUC5AC production was induced not only by FXa but also FX. FXa activity was detected in the supernates of FX-stimulated cells. These results suggest that FX was activated by FVIIa and tissue factor (TF) in the cell medium. FVII and TF mRNAs were detected in NCI-H292 cells, although it was not detected in another human epithelial cell line that does not produce mucin (BEAS-2B cells). Histological analysis demonstrated that FVII and TF were expressed in lung epithelial layers. In asthmatic mice, mucin production was decreased in $FVII^{tta/tta}$ mice and airway hyperresponsiveness was attenuated compared to WT mice. These results suggest that FVIIa/TF is associated with asthma by inducing mucin production and thereby exacerbating asthma.

Identification of XPHAX, a new mRNA that is localized to the animal hemisphere of *Xenopus* oocytes

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The protein VgRBP71 binds to a region of Vg1 mRNA that mediates its localization to the vegetal cortex of *Xenopus* oocytes. Homologues of VgRBP71 bind to the localization elements of other mRNAs in a variety of cell types, including fibroblasts, neurons, and oligodendrocytes. There is evidence that this family of proteins controls mRNA translation and stability. A consensus binding site for VgRBP71 was identified in a *Xenopus* expressed sequence tag (EST). Assembly of overlapping ESTs generated a theoretical transcript of 3674 nucleotides (nt); an actual RNA of 3,500 nt is detected in stage III through VI oocytes. The open reading frame of the mRNA encodes a 395-amino acid protein that has 69% identity with PHAX, a phosphoprotein involved in the nuclear export of snRNA. Much of the difference between PHAX and the putative *Xenopus* protein is due to an 85-amino acid addition at the N-terminal of the latter. The 3'-untranslated region (UTR) of the mRNA contains multiple E2 (WYCAC) and VM1 (YYUCU) motifs that are hallmarks of localized mRNAs. *In situ* hybridization experiments demonstrate that this mRNA is localized to the animal hemisphere. Since E2 and VM1 elements are also required for localization of mRNAs to the vegetal hemisphere, these results establish that these *cis*-acting sequences do not confer directionality. 3' RACE experiments have identified the end of the 3'-UTR in *Xenopus* oocytes. The 3'-UTR of the mRNA is different in oocytes and embryos, indicating that alternative nuclear polyadenylation removes the localization element from the mRNA in somatic cells. The open reading frame (ORF) of the mRNA has been cloned into an expression vector in order to produce antibody to the protein, which will allow for the identification of the stage of oogenesis when the protein is being expressed. Preliminary crosslinking experiments comparing the 3'-UTR of An3, another animal localizing mRNA, to the VLE of Vg1 shows a similar pattern of crosslinked proteins indicating that common trans-acting protein factors are involved in the localization of mRNAs to the two hemispheres.

Cloning of bla operon from *S. aureus*

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Staphylococcus aureus is an important human pathogen causing both community and hospital-associated infections. Staphylococcal resistance to β-lactam antibiotics is mediated by two mechanisms: (a) production of β-lactamase and (b) expression of an additional penicillin-binding protein (PBP), PBP2α. Expression of the β-lactamase, encoded by blaZ, is inducible. It is regulated by two genes, blaR1 and blaI, which are located immediately upstream and transcribed in the direction opposite that of blaZ. BlaR1 is an antibiotic sensor/signal transducer protein. In the presence of a β-lactam antibiotic it triggers a chain of events resulting in derepression of balZ. It was found that mechanisms of expression regulation for mecA encoding PBP2α and for balZ are very similar making these two pathways important targets for study. Our efforts toward cloning and expression of the entire bla operon and expression of BlaR1 with tags on both N- and C- termini in *S. aureus* is presented in this poster.

Characterization of chlorite dismutase: Understanding a novel enzymatic O_2 evolving reaction

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Chlorite (ClO_2^-) is widely used in pulp and paper bleaching plants and has recently been sited as a top 10 EPA contaminant of drinking water. Chlorite dismutase (Cld) is a bacterial heme enzyme catalyzing the disproportionation of chlorite (ClO_2^-) into chloride (Cl^-) and molecular oxygen (O_2). The Cld enzyme from *Dechloromonas aromatica* RCB has been cloned and expressed in high yields. The enzyme was determined to bind a heme b cofactor nearly stoichiometrically. Both product evolution (by oxygen electrode) and reactant depletion (by an iodometrically determined discontinuous assay) were monitored for the determination of initial rates of reaction. Under similar conditions both measuring techniques gave comparable rates of reaction and showed a first-order dependence on chlorite concentration. At pH 6.8 the $K_M = 210 \pm 20$ μM and $k_{cat} = (4.4 \pm 0.2) \times 10^5$ min^{-1} per heme were determined by construction of Michaelis-Menten plots. The stoichiometry of reaction of chlorite dismutation and oxygen evolution was determined to be 1:1 through comparison of the two measurement techniques. Using isotopically labeled water it was shown that both the O atoms in O_2 originate from ClO_2^-. No product inhibition by Cl^- was seen and future plans will address if O_2 acts as a product inhibitor as well. The enzyme concomitantly undergoes irreversible ClO_2^- dependent deactivation with a partitioning ratio of 5.0×10^4 turnovers per inactivation. Ongoing structural and functional characterizations are aimed at elucidating Cld's unique place among heme enzymes, and developing its technological potential for sensing and bioremediation.

Kinetic mechanism of enterococcal aminoglycoside phosphotransferase 2"-Ib

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The major mechanism of resistance to aminoglycosides in clinical bacterial isolates is the covalent modification of these antibiotics by enzymes produced by the bacteria. Aminoglycoside 2"-Ib phosphotransferase [APH(2")-Ib] produces resistance to several clinically important aminoglycosides in both gram-positive and gram-negative bacteria. Nuclear magnetic resonance analysis of the product of kanamycin A phosphorylation revealed that modification occurs at the 2"-hydroxyl of the aminoglycoside. APH(2")-Ib phosphorylates 4,6-disubstituted aminoglycosides with k_{cat}/K_m values of 10^5-10^7 $M^{-1}s^{-1}$, while 4,5-disubstituted antibiotics are not substrates for the enzyme. Initial velocity studies demonstrate that APH(2")-Ib operates by a sequential mechanism. Product and dead-end inhibition patterns indicate that binding of aminoglycoside antibiotic and ATP occurs in a random manner. These data, together with the results of solvent isotope- and viscosity effects studies, demonstrate that APH(2")-Ib follows the random bi-bi kinetic mechanism and substrate binding and/or product release could limit the rate of reaction.

Characterization of a novel protein, linking membrane and microtubule, "CLIPR-76"

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The microtubule cytoskeleton plays a central role in cell organization and membrane traffic in higher eukaryotes. Cytoplasmic linker proteins (CLIPs) have been implicated in both membrane-microtubule interactions and regulation of microtubule dynamics. We are studying two vertebrate-specific proteins related to CLIP-170. One of them, CLIPR-59, localizes to the Golgi apparatus and is involved in the control of early endosome/TGN transport (*JCB,* 156 (4), 631-642, **2002**). We are presently focusing on the second protein, CLIPR76. Multiple alternatively spliced isoforms of CLIPR-76 exist. All contain four ankyrin-like repeats at the N-terminus. One has three CAP-GLY microtubule-binding motifs, while others have two CAP-GLY motifs or one CAP-GLY motif and a highly hydrophobic C-terminus.

Serial deletion mutants of CLIPR-76 isoforms have been placed into mammalian and bacterial expression vectors to allow characterization of their domains *in vivo* and *in vitro*. Full length CLIPR76-1 and 3 colocalize with MTs, whereas CLIPR76-2 colocalizes only partially with MTs and at higher expression levels colocalizes better with acetylated MTs. Full-length CLIPR76-4 localizes to the endoplasmic reticulum (ER) and alters ER morphology at high expression levels. Detailed serial deletion analysis of CLIPR76-4 showed that the C-terminal hydrophobic tail is both necessary and sufficient for ER localization. Overexpression of CLIPR76-4 mutants missing the Ankyrin repeats strongly perturbs ER and ER-Golgi intermediate compartment (ERGIC) morphology, causing colocalization with microtubules. Preliminary data show that VSVG transport between ER and Golgi is inhibited in cells expressing these mutants. These data suggest a role for CLIPR76-4 in membrane transport, specifically ER-Golgi transport. Ongoing studies will further investigate the effects of CLIPR-76 and its fragments on membrane transport *in vivo* and on microtubule assembly *in vitro*.

Cloning, expression and characterization of a *K. pneumoniae* acetyltransferase involved in virulence

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Iron is an essential bio-element. Its availability controls the distribution of life on earth as well as the viability of pathogens within the human body. Acquisition of iron involves many discrete biochemical and in many cases enzymatic steps that are both critical for and specific to microbes, and therefore good antimicrobial targets. Increased resistance to existing antibiotics, coupled to growing demand for antimicrobial therapy, has generated a critical need for such new strategies for combating infectious disease. We are focusing on the IucB enzyme from a widespread hospital-associated pathogen, *Klebsiella pneumoniae*. IucB catalyzes the acetylation of N^6-hydroxylysine in the second step of the production of the iron-chelating hydroxamate portion of aerobactin, a siderophore and virulence factor. Because N^6-hydroxylysine N-acetyltransferase is small and monofunctional, it is an ideal target for determining structure-activity relationships. We have cloned the IucB gene from the host's virulence plasmid and re-engineered it for protein expression. We report our efforts toward producing and characterizing the enzyme, with the long-term goal of developing it as an inhibition target for antimicrobials.

Structure/function analysis of murine PAI-1

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Plasminogen Activator Inhibitor-1 (PAI-1) is the main physiological regulator of tissue-type plasminogen activator (tPA) in normal plasma. In addition to its critical function in fibrinolysis, PAI-1 has been implicated in other physiological and pathophysiological processes. Our previous studies indicated that the complex interactions traditionally associated with different PAI-1 functions apply to the murine system, thus demonstrating a commonality of subtle functions among different species and evolutionary conservation of this protein (Xu *et al., J. Biol. Chem.* **2004** Apr 23; 279(17): 17914-20). In an effort to separate these functions *in vivo*, gene knock-in mice with targeted mutations, within these domains, are under construction. The composition of the targeting vector includes the 5' and 3' flanking sequences containing the mutation, a *LoxP-Neo -LoxP* cassette, and a *CDA* expression cassette. After homologous recombination with the WT allele, the "floxed" cassette will be removed by crossing with Cre recombinase expressing transgenic mice. A cell culture-free system has been developed to examine the Cre-recombinase-mediated excision of the "floxed" element before the targeting vector was introduced to the embryonic cells. The targeting vectors were initially generated on the 129Sv/Ev background. They were then delivered into ES cells by electroporation and homologous recombination was screened by a PCR-based assay. Although we performed many steps to ensure the results from PCR-based screening assays, including restriction digesting and sequencing, it appeared that this assay still yielded false positive results. Therefore, despite many advantages that PCR-based screening assay offers, our results suggested that it is not a desirable screening method due to high chance of false positive. In order to bypass the lengthy backcrossing process from 129 background into C57BL/6 background, we are currently generating these knock-in mice using C57-derived ES cells, and screening the homologous recombination by Southern blotting. These mice will serve to further elucidate mechanisms associated with the observed phenotypes in PAI-1-deficient mice in a number of challenge models.

In vitro ^{13}C NMR studies of RNase A glycation using ^{13}C-labeled aldoses

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Protein amino groups react with reducing sugars such as D-glucose in vivo to form Schiff bases, which subsequently rearrange to produce stable, covalently bound Amadori adducts. These adducts subsequently degrade to produce reactive post-Amadori degradation products known as AGEs (advanced glycation end-products). Glycation reactions are promoted in diabetic patients due to elevated glucose concentrations, and many of the physiological complications caused by this disease are mediated by the effects of glycation on the structures and functions of long-lived proteins. To gain more insight into the chemistry of non-enzymatic glycation, ^{13}C NMR studies were conducted on a model protein, RNase A, which was reacted in vitro with a group of ^{13}C-labeled aldoses: D-[2-^{13}C]glucose, 6-*O*-methyl-D-[2-^{13}C]glucose, 3-*O*-methyl-D-[2-^{13}C]glucose, D-[2-^{13}C]ribose, D-[2-^{13}C]xylose, 5-*O*-methyl-D-[2-^{13}C]xylose, 3-deoxy-D-[2-^{13}C]glucose, and 2-deoxy-D-[1-^{13}C]ribose. These aldoses were selected to probe the glycation reaction pathway by either inhibiting/preventing enediol formation, affecting the equilibrium distribution of cyclic and acyclic forms of the Amadori intermediate, or promoting Schiff base formation without subsequent Amadori rearrangement. ^{13}C-Labeling at C2 allowed selective detection of the aldose C2 carbon above background, thus permitting the monitoring of Amadori formation and its chemical fate. Time-lapse ^{13}C{^{1}H} NMR spectra of glycated RNase glycated were collected over 1-2 months at 20 ^{o}C or 36 ^{o}C to follow the formation of labeled intermediates and end products and measure their rates of appearance or disappearance. Results show that aldose structure significantly influenced the course of the reaction. Labeled carbon signals were observed clustered near 210, 180, 130, 100, and 80 ppm, with signal intensities in these regions varying considerably with aldose reactant. In some cases, only Amadori signals were detected (~100 ppm), whereas in others signals were observed in all five regions over time, with ^{13}C label eventually accumulating at 130 ppm.

Parameterization of $^3J_{HCOH}$ and $^3J_{CCOH}$ spin-coupling constants in saccharides: Influence of terminal and internal electronegative substituents, and application to experimental studies of H-bonding in solution

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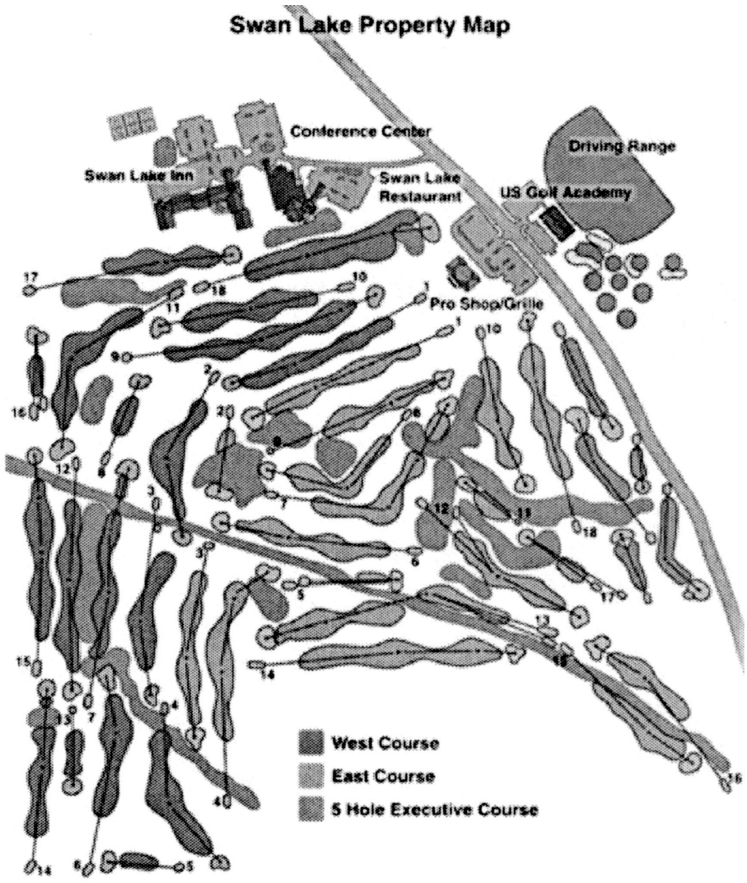
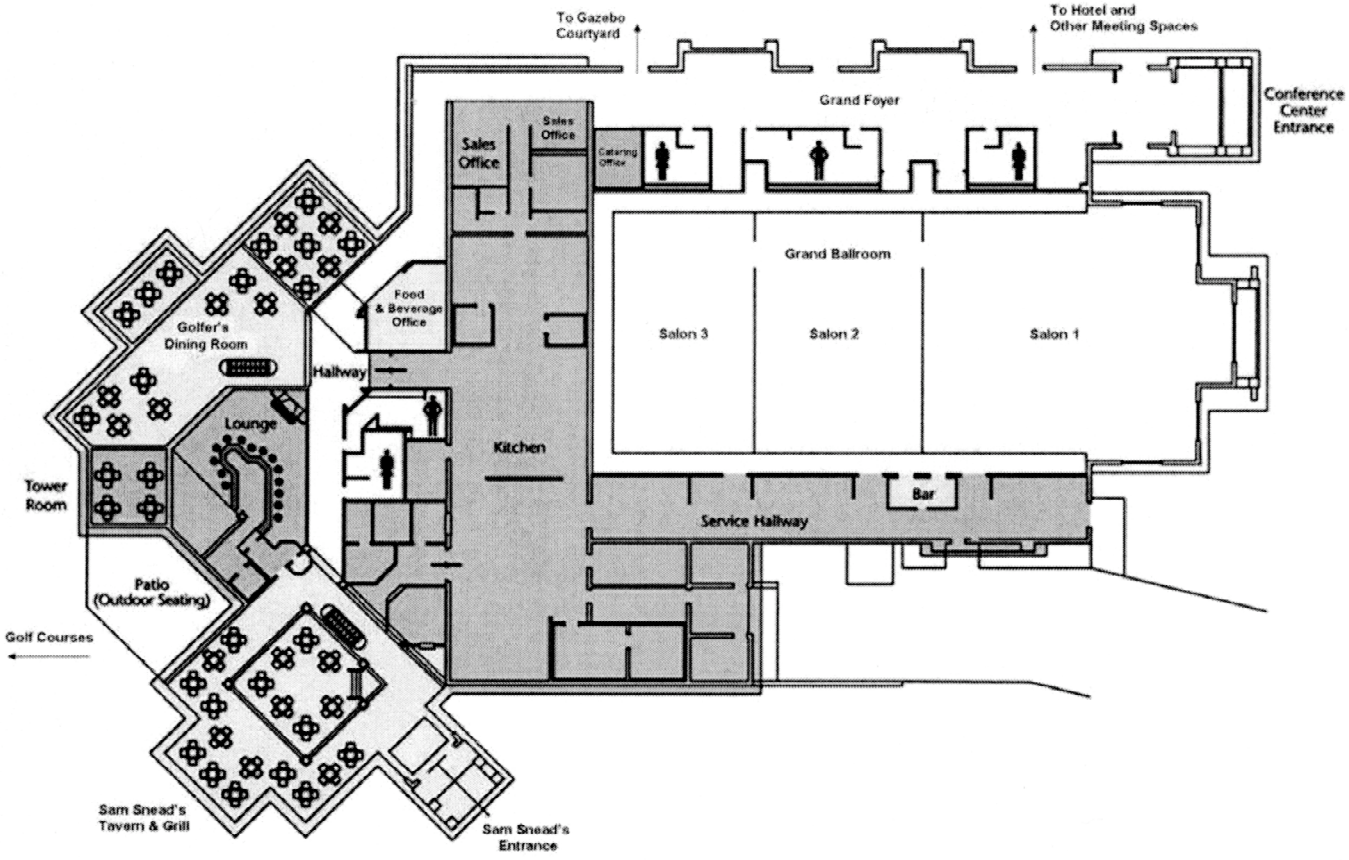
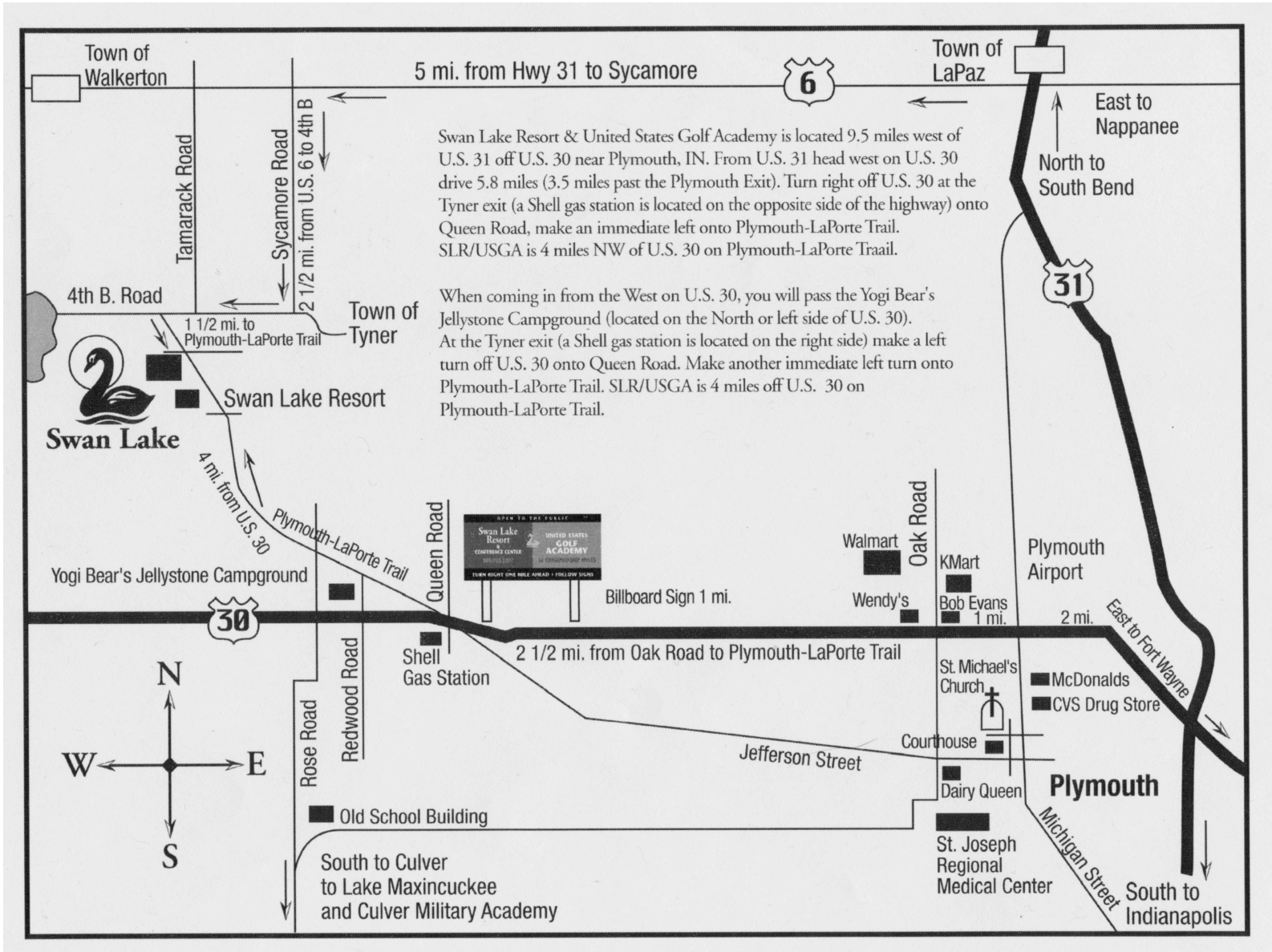
$^3J_{HCOH}$ and $^3J_{CCOH}$ were calculated using density functional theory (DFT) in model aldopyranoses and aldopyranosides and parameterized as a function of the H/C-C-O-H dihedral angle. Results support the use of a generalized Karplus equation to treat $^3J_{HCOH}$ involving all ring carbons except the anomeric carbon, where the internal O5 electronegative substituent influences the phase of the curve. While the CCOH torsion angle is the primary determinant, $^3J_{CCOH}$ also depends on the orientation of terminal substituents on the coupled carbon, and for $^3J_{C2,OH1}$, also on the presence of the internal electronegative (O5) substituent. Using equations pertinent to specific couplings, an analysis of experimental $^3J_{HCOH}$ and $^3J_{CCOH}$ in mono- and disaccharides showed some C-O torsion angles are influenced by molecular context and do not experience complete rotational averaging in solution. A strong bias in the H3-C3-O3-H torsion angle in the Glc residue in methyl-β-lactoside and other β-[1→4]-linked disaccharides, as discerned by quantitative interpretations of $^3J_{HCOH}$ and $^3J_{CCOH}$, favoring a *gauche* conformation indirectly suggests the presence of inter-residue H-bonding between $O3H_{Glc}$ and $O5_{Gal}$ in water-acetone solution and in DMSO, although the observed couplings cannot quantify the strength of this bonding. These results suggest that H-bonding between adjacent and/or remote residues may, in some instances, dictate/constrain glycosidic bond conformation and dynamics in simple and complex oligosaccharides in aqueous solution.


Swan Lake Property Map
Conference Center
Swan Lake Inn
Swan Lake Restaurant
Driving Range
US Golf Academy
Pro Shop/Grille
West Course
East Course
5 Hole Executive Course


To Gazebo Courtyard
To Hotel and Other Meeting Spaces
Grand Foyer
Conference Center Entrance
Sales Office
Sales Office
Catering Office
Grand Ballroom
Salon 3
Salon 2
Salon 1
Food & Beverage Office
Golfer's Dining Room
Hallway
Lounge
Kitchen
Tower Room
Bar
Service Hallway
Patio (Outdoor Seating)
Golf Courses
Sam Snead's Tavern & Grill
Sam Snead's Entrance


Town of Walkerton
5 mi. from Hwy 31 to Sycamore
6
Town of LaPaz
East to Nappanee
North to South Bend
31
Tamarack Road
Sycamore Road
2 1/2 mi. from U.S. 6 to 4th B
4th B. Road
1 1/2 mi. to Plymouth-LaPorte Trail
Town of Tyner
Swan Lake Resort & United States Golf Academy is located 9.5 miles west of U.S. 31 off U.S. 30 near Plymouth, IN. From U.S. 31 head west on U.S. 30 drive 5.8 miles (3.5 miles past the Plymouth Exit). Turn right off U.S. 30 at the Tyner exit (a Shell gas station is located on the opposite side of the highway) onto Queen Road, make an immediate left onto Plymouth-LaPorte Trail. SLR/USGA is 4 miles NW of U.S. 30 on Plymouth-LaPorte Traail.
When coming in from the West on U.S. 30, you will pass the Yogi Bear's Jellystone Campground (located on the North or left side of U.S. 30). At the Tyner exit (a Shell gas station is located on the right side) make a left turn off U.S. 30 onto Queen Road. Make another immediate left turn onto Plymouth-LaPorte Trail. SLR/USGA is 4 miles off U.S. 30 on Plymouth-LaPorte Trail.
Swan Lake
Swan Lake Resort
4 mi. from U.S. 30
Plymouth-LaPorte Trail
Queen Road
Swan Lake Resort & Conference Center
United States Golf Academy
Turn right one mile ahead • Follow signs
Billboard Sign 1 mi.
Walmart
Oak Road
KMart
Plymouth Airport
Yogi Bear's Jellystone Campground
Wendy's
Bob Evans 1 mi.
2 mi.
East to Fort Wayne
30
Shell Gas Station
2 1/2 mi. from Oak Road to Plymouth-LaPorte Trail
St. Michael's Church
McDonalds
CVS Drug Store
N
W
E
S
Rose Road
Redwood Road
Jefferson Street
Courthouse
Dairy Queen
Plymouth
Old School Building
St. Joseph Regional Medical Center
Michigan Street
South to Culver to Lake Maxincuckee and Culver Military Academy
South to Indianapolis