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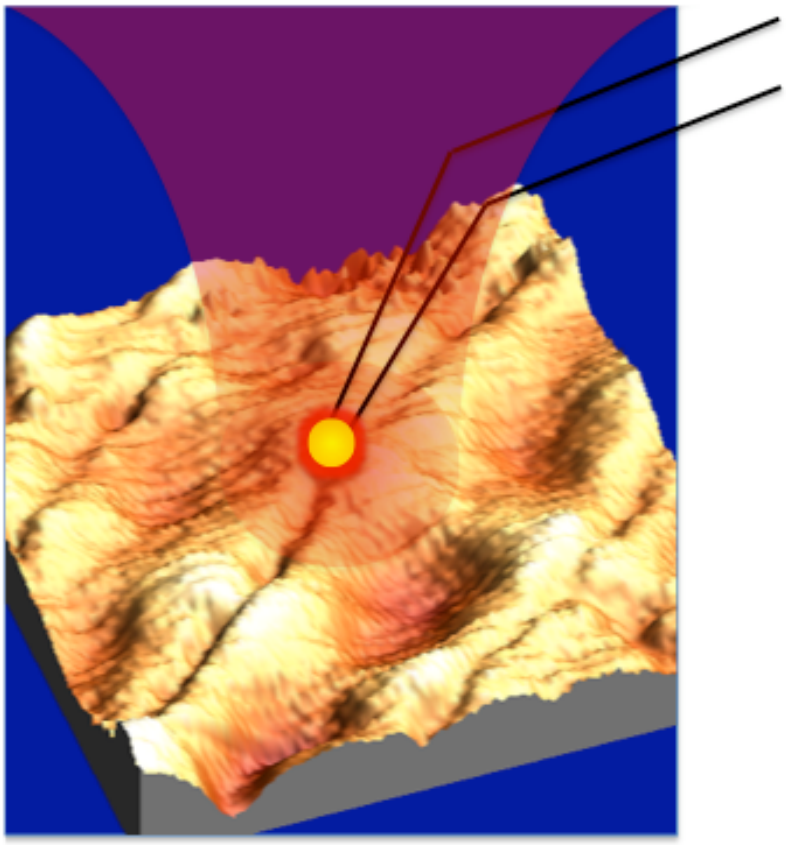

Swan Lake Resort
Plymouth, Indiana
June 1, 2011

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Cover Illustration

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Cover Legend. The plasma membrane of a rod photoreceptor cell is illustrated in the presence of a nanoparticle atomic force microscope tip. Excitation of the nanoparticle by a laser enables detection of biomolecules present in the cell membrane. The topography shown is that observed from an isolated and fixed rod photoreceptor cell. The nanoparticle shown is approximately 100 nm, consistent with our actual experiments.

Previous Keynote Speakers

1996: Nicholas Paoni (Genentech)
1998: Les Kozak (Jackson Laboratory)
1999: Andy Mesecar (IU-Chicago)
2000: Thomas Kelly (Case Western)
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2003: Dudley Strickland (Red Cross)
2004: Elizabeth Komives (UCSD)
2005: Erik Zuiderweg (Michigan)
2006: Lila Gierasch (U. Mass)
2007: Shelagh Ferguson-Miller (Michigan State)
2008: Christian R. H. Raetz (Duke)
2009: John L. Wang (Michigan State)
2010: Thomas A. Gerken (Case Western)

♦ 2011 Keynote Lecture ♦

John A. Gerlt

Professor of Biochemistry, Gutgsell Chair
School of Molecular and Cellular Biology
University of Illinois at Urbana-Champaign

Discovering and Predicting New Functions in the Enolase Superfamily

Abstract

Determining the functions of proteins encoded by sequenced genomes is a major challenge in biology. We are implementing an integrated sequence-structure-function strategy to facilitate functional assignment by predicting the substrate specificities of unknown proteins in the mechanistically diverse enolase superfamily. The reactions are initiated by abstraction of a proton from a carbon acid substrate to generate a Mg^{2+}-stabilized enolate intermediate. We are using three approaches: 1) operon context for unknowns encoded by bacterial genomes, 2) experimental screening of libraries of potential substrates, and 3) computational prediction by *in silico* docking of libraries of potential substrates to experimentally determined structures and homology models. This lecture will highlight functional assignments using these approaches. Our successes using computational prediction establish this approach as a viable strategy to facilitate functional assignment of unknown enzymes discovered in genome projects.
(Supported by 2R01GM071790 and 1U54GM093342).

Biography of John A. Gerlt

John Gerlt received his BS in Biochemistry from Michigan State University in 1969. He received his PhD in Biochemistry and Molecular Biology from Harvard in 1974 where he worked with Frank Westheimer on measuring the enthalpies of hydrolysis of phosphodiester models of 3',5'-cyclic AMP. Following one year of postdoctoral studies with Christian Anfinsen at NIH, John joined the Department of Chemistry at Yale University in 1975. In 1984, he moved to the Department of Chemistry and Biochemistry at the University of Maryland, College Park. Then, in 1994, John moved as Head to the Department of Biochemistry at the University of Illinois, Urbana-Champaign; in 2003 he "retired" from administration and was named Gutgsell Chair of Biochemistry. Since the late 1980s, John has focused on understanding the structural basis of divergent evolution of function in functionally diverse enzyme superfamilies and suprafamilies, including the enolase, enoyl-CoA hydratase, and RuBisCO superfamilies and the orotidine 5'-monophosphate decarboxylase suprafamily. As genome sequencing has become routine, John's attention has turned to devising multidisciplinary strategies for predicting the functions of unknown/uncharacterized enzymes discovered in genome projects. For more details, see *Nature Chemical Biology* **2007**, *8*, 486-491; *Structure* **2008**, *16*, 1668-1677; and *Biochemistry* **2009**, *48*, 11546-11558.

♦ 2011 Honorary Lecture ♦

Professor Thomas L. Nowak
Professor of Chemistry and Biochemistry,
University of Notre Dame

Phosphoenolpyruvate Carboxykinase Catalysis: A Retrospective

Abstract

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the reversible reaction of oxalacetate (*OAA*) and GTP and yields PEP, CO_2 and GDP. This is the committed step in gluconeogenesis in most species. This reaction has significant chemical homology to the reaction catalyzed by pyruvate kinase that gave stimulus to its investigation. PEPCK from mitochondria was purified and characterized. Kinetic studies demonstrate a dual divalent cation requirement and Mn^{2+} is the preferred metal that binds to the enzyme. Kinetic studies show a synergistic interaction of the Mn^{2+} and PEP to the enzyme but not with HCO_3^- or Mn-IDP. Pulsed NMR binding studies demonstrate the formation of the PEPCK-Mn complex with rapid access of H_2O to the metal. Each substrate binds to PEPCK-Mn and influences the environment around the metal. ^{31}P relaxation rate studies indicate that both PEP and nucleotide form second sphere complexes with the bound metal suggesting second sphere phosphoryl transfer. This proposal was supported by the formation of the exchange-inert PEPCK- Co^{3+} and PEPCK- Cr^{3+} complexes that were active. These metal-enzyme complexes allowed the investigation of the second metal site on the enzyme. The conformation of bound nucleotide on the enzyme was investigated by NOE studies. Chemical modification studies indicated an important but not active role of Cys in catalysis and active site functions for Arg, Lys and His. pH studies supported such roles. Subsequent x-ray structures of PEPCK-Mn, PEPCK-Mn-PEP and PEPCK-Mn-GDP complexes indicate that the structures, amino acid functions and chemical mechanism proposed from wet chemical studies are indeed correct.

Program

Wednesday Morning
Session Chair: Holly Goodson

8:30-9:00 Continental breakfast

9:00-9:10 Introduction and Orientation

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9:10-10:00 **Honorary Lecture**
Professor Thomas L. Nowak
Phosphoenolpyruvate Carboxykinase Catalysis: A Retrospective
Introduction by Professor Francis J. Castellino

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10:00-10:30 **Guest Lecture**
Professor Kevin T. Vaughan, Department of Biological Sciences, University of Notre Dame
A Hierarchy of Kinase-driven Interactions Required for Checkpoint Signaling During Mitosis

10:30-10:55 Mid-Morning Break

10:55-11:15 Jill S. Voreis and Holly Goodson
Characterization of a Novel CLIP-170 Related Protein, CLIPR76

11:15-11:35 Jennifer L. Starner-Kreinbrink, Allen Wayne Bryan Jr., Bonnie Berger, and Patricia Clark
Cap Structures Reduce β-Helix Aggregation Propensity

11:35-11:55 Michelle Bertke, Erliang Zeng, and Paul Huber
The Role of SUMOylation in Early Development of Xenopus laevis

11:55-12:15 William Hawse, Francis Insaidoo, Brian Gloor, Linda Nicholson, Jaroslav Zajicek, and Brian Baker
Resolving Intermediate Steps in the TCR-pMHC Binding Reaction

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12:15-2:15 Lunch and Recreation

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Wednesday Afternoon
Session Chair: Brian Baker

2:15-2:45 **Guest Lecture**
Professor Zachary Schultz, Department of Chemistry and Biochemistry, University of Notre Dame
Optical Spectroscopy of Biomolecules at the Nanoscale

2:45-3:05 Julie Chaney, Rory Carmichael, Scott Emrich, and Patricia L. Clark
Conservation of Rare Codon Clusters

3:05-3:25 Zhong Liang, Yueling Zhang, Vishwanatha K. Chandrahas, Victoria A. Ploplis, and Francis J. Castellino
Virulence Regulation in Pattern D Group A Streptococcus

3:25-3:45 Emmanuel Adu-Gyamfi and Robert V. Stahelin
Molecular Architecture of Ebola Virus Assembly

3:45-4:05 Rashna Balsara, Neill Li and Francis J. Castellino
Reversal of Excitotoxic Effects in Primary Neurons by Conantokin-G via N-Methyl-D-Aspartate Receptor Interaction

4:05-4:45 Late Afternoon Break

Wednesday Evening

4:45-5:45 **Keynote Lecture**
John A. Gerlt, Departments of Biochemistry and Chemistry, Institute for Genomic Biology, University of Illinois
Discovering and Predicting New Functions in the Enolase Superfamily

5:45-7:00 Poster Session and Reception

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7:15-9:15 Dinner and Presentation of Plaques

9:15 Departure

ABSTRACTS: GUEST LECTURES

A Hierarchy of Kinase-driven Interactions Required for Checkpoint Signaling During Mitosis

Kevin T. Vaughan
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Notre Dame, IN 46556

The spindle assembly checkpoint (SAC) is responsible for resisting anaphase onset during prometaphase but also for triggering anaphase onset once chromosome alignment has been achieved. The mechanisms that integrate these two questions into a single response are not known. The Vaughan laboratory has investigated phosphorylation of cytoplasmic dynein at kinetochores as an indicator of progression through chromosome alignment and as a sensor of chromosome alignment. Based on the identification of PP1g as a dynein phosphatase at metaphase, we used small molecule inhibitors and chemical genetics to identify mitotic dynein kinases. Inhibition of Plk1 with BTO-1 or analogue-sensitive Plk1 constructs ablated recruitment of phospho-dynein to kinetochores and induced errors in chromosome alignment during prometaphase. Dynein-binding proteins implicated in recruiting kinetochore dynein were not affected. In parallel, we assessed the requirement for Aurora B (AurB) in dynein recruitment, based on the antagonistic roles of PP1 phosphatases and AurB homologues in yeast. Inhibition of AurB blocked recruitment of phospho-dynein to kinetochores. However, the effects on phospho-dynein recruitment were indirect. Dynein, dynactin, spindly and *zw10* were each reduced after AurB inhibition, whereas zwint-1, Hec1 and Knl1 were not affected. Because the interaction between zwint-1 and *zw10* has been implicated in recruiting the *rod-zw10*-zwilch (RZZ) complex to kinetochores, we compared phosphorylation of zwint-1 and *zw10* using *in vitro* kinase assays. Zwint-1 but not *zw10* was phosphorylated by AurB, and a set of three novel AurB phosphorylation sites was mapped in zwint-1 by MS/MS analysis. A triple-A zwint-1 mutant blocked recruitment of the RZZ complex and all RZZ-dependent proteins to kinetochores and induced prometaphase arrest. A triple-E zwint-1 mutant overcame the effects of AurB inhibition on kinetochore assembly. However, the triple-E mutant blocked dynein-driven streaming of checkpoint proteins at metaphase, inducing metaphase arrest. These results suggest that phosphorylation of zwint-1 by AurB is required for assembly of a dynein-binding platform at kinetochores during prometaphase. However, dephosphorylation of zwint-1 at metaphase defines the boundary between stable and streaming proteins implicated in checkpoint silencing. These studies clarify the roles of Plk1 and AurB in the regulation of kinetochore dynein.

Optical Spectroscopy of Biomolecules at the Nanoscale

Zachary D. Schultz
Department of Chemistry and Biochemistry, University of Notre Dame,
Notre Dame IN 46656-4670

Optical microscopy of biological systems is useful for detecting various structures with varying chemical or structural contrasts. In the past, fluorescent tags have been useful as imaging probes of biomolecules. An alternative is to use optical properties of nanoparticles for contrast and detection. The local electromagnetic fields gained from the excitation of conduction band electronics of metal nanostructures can be used to enhance Raman scattering from molecules in close proximity. This effect, the electromagnetic enhancement responsible for surface-enhanced Raman scattering (SERS), thus provides a sensitive probe of chemical environments. We have coupled tip-enhanced Raman scattering (TERS) with nanoparticle probes to obtain chemical, structural, and spatial information simultaneously. In protein-ligand interactions, our results show signal enhancements from both the ligand, bound to a nanoparticle probe, and the target protein, thus demonstrating this environmental sensitivity. We are exploring these effects to distinguish the differences between the wild type and mutant proteins, as well as investigating intact cell membranes.

ABSTRACTS: ORAL PRESENTATIONS

Characterization of a Novel CLIP-170 Related Protein, CLIPR76

Jill S. Voreis and Holly Goodson
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA

The microtubule cytoskeleton plays a fundamental role in cellular processes ranging from cell division to cell motility. Numerous proteins regulate microtubule dynamics and interactions of microtubules with other cellular components. One group of these proteins is defined by the presence of a conserved CAP-Gly motif; these proteins have sometimes been called "CLIPRs" because they are related to the cytoplasmic linker protein CLIP-170 through these common CAP-Gly domains. Most CLIPR proteins share the ability to bind tubulin or microtubules through the CAP-Gly, but their other functions vary. We report here the characterization of a novel CAP-Gly containing protein, CLIPR76. In humans, the CLIPR76 gene is alternatively spliced and produces at least four splice isoforms, all of which contain ankyrin-like repeats at the N-terminus, followed by a different number of CAP-Gly domains. Three CLIPR76 isoforms localize to MTs and alter MT organization upon overexpression. The fourth (CLIPR76-4) localizes to the endoplasmic reticulum (ER) via its hydrophobic C-terminus, which has some similarity to that of the related Golgi-localized protein CLIPR-59. However, site-directed mutagenesis indicates that CLIPR76-4 and CLIPR59 target membranes by different mechanisms. CLIPR76-4 overexpression strongly alters the morphology of ER and ERGIC compartment and inhibits ER to Golgi transport, suggesting that CLIPR76-4 plays a role in early vesicular transport. While CLIPR-59 is expressed predominantly in brain, CLIPR76-4 is highly expressed in muscle and is induced early in muscle development. This CLIPR76-4 expression pattern is especially interesting because muscle development requires extensive remodeling of both the MT cytoskeleton and the ER. As noted above, CLIPR76-4 has both CAP-Gly domains capable of binding MTs and alters ER morphology when highly expressed. These observations make CLIPR76-4 a prime candidate for playing a critical role in the cellular re-organization that precedes complex tissue formation.

Cap Structures Reduce β-Helix Aggregation Propensity

Jennifer L. Starner-Kreinbrink[1], Allen Wayne Bryan Jr.[2], Bonnie Berger[2], and Patricia Clark[1]
[1]Department.of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 and [2]Department of Mathematics, Massachusetts Institute of Technology, Cambridge, MA 02139

Examination of β-sheets in protein crystal structures has revealed that edge β -strands are protected from solvent by a variety of mechanisms. Protection of the unpaired hydrogen bonds donors and acceptors in β -sheet edge strands could prevent intermolecular associations and therefore reduce aggregation of these proteins. We investigated the role of β-sheet capping mechanisms in the folding and aggregation properties of pertactin, an autotransporter protein from *Bordetella pertussis.* Pertactin, a right-handed β-helix protein, folds extremely slowly *in vitro*, yet is surprisingly resistant to aggregation. Examination of the pertactin crystal structure suggests two different strategies might prevent multimerization. The *N*-terminal rung of the pertactin β-helix contains three charged residues, which could act as a cap via electrostatic repulsion. Substitution of two of the three charged residues resulted in greater aggregation propensity and the formation of soluble oligomers. The pertactin C-terminus is capped by a specific non-β-helical structure, also observed at the *C*-terminus of many other β-helical proteins. This structure might prevent solvent exposure of both the hydrophobic core of the β-helix and its edge β-strands. Deletion of the *C*-terminal cap also led to increased aggregation and formation of soluble oligomeric structures, including fibers and ring-like structures, as observed by size exclusion chromatography and transmission electron microscopy. Limited protease digestion revealed that the *C*-terminus of the _C-terminal cap monomer is less stably folded and thus more susceptible to protease digestion than the *C*-terminus of wild type pertactin. We are currently creating a construct that lacks both the *C*- and *N*-terminal caps, to examine whether these mutations will lead to even greater aggregation and fiber formation. Results from these studies will allow us to determine to what extent pertactin, and by extension other β-helical proteins, employ capping mechanisms to reduce/prevent aggregation.

The Role of SUMOylation in Early Development of *Xenopus laevis*

Michelle Bertke, Erliang Zeng, and Paul Huber
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA

SUMOylation is a post-translational protein modification that occurs when the 11kDa protein, SUMO (small ubiquitin-related modifier), becomes covalently attached to a target protein in order to control the activity of that protein. Conjugation of SUMO to its target protein has many varied consequences. In the case of transcription factors, DNA binding, subnuclear localization, and transcriptional activation activity can be affected. The goal of this work is to determine whether SUMOylation plays a significant role in changing patterns of gene expression during early development. SUMOylation activity in *Xenopus* embryos was knocked down by expression of the adenovirus protein, Gam1, through the microinjection of mRNA encoding the protein into one-cell embryos. Gam1 binds directly to the E1 SUMO-activating enzyme and triggers degradation of its SAE1 subunit, thereby inactivating the SUMOylation pathway. Embryos injected with mRNA survive and display subtle, but reproducible, developmental defects. We collected total embryonic RNA samples from Gam1 and ddH_2O injected embryos from three different time points during embryogenesis. These were analyzed on Affymetrix microarrays in order to determined genes whose expression pattern changed significantly ($p<0.05$) at each of the three time points. The entire data set was also analyzed for genes whose expression patterns changed identically across all three time points (ie/ up regulated, up regulated, down regulated). Eight clusters were constructed using common expression patterns and these gene lists were used for further analysis. The differentially expressed genes ($p<0.05$) from each time point were analyzed to identify the transcription factors that were significantly enriched within the datasets. The cluster gene lists were analyzed in order to determine the potential reasons underlying their co-expression patterns by determining the GeneGo processes most enriched in each of the eight clusters. It appears that these processes are distinct for each cluster and could account for their association.

Resolving Intermediate Steps in the TCR-pMHC Binding Reaction

William Hawse[1], Francis Insaidoo[1], Brian Gloor[1], Linda Nicholson[2],
Jaroslav Zajicek[1], and Brian Baker[1]
[1]Department of Chemistry and Biochemistry, University of Notre Dame, IN and
[2]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY

T-Cells are the mediators of the cellular immune response to pathogens and cancer. The T-cell response to antigens is initiated by binding of the T cell Receptor (TCR) to a peptide-major histocompatibility complex (pMHC), where pMHC binding stimulates T-cell effector functions. A hallmark of TCRs is their propensity to cross-react with multiple pMHC complexes, which is functionally important for mounting an immune response to both pathogenic and tumor antigens. While the importance of TCR cross-reactivity is well established, the molecular mechanisms underlying TCR cross-reactivity are ill defined. To understand how TCRs cross react with multiple ligands, new methods have to be employed that are capable of resolving the kinetics and binding mechanisms of the TCR-pMHC interaction at atomic resolution. *The goal of this work is to define at the molecular level how TCRs bind to pMHC.* To understand the TCR-pMHC binding reaction at the atomic level, we performed NMR titrations between a TCR and its cognate pMHC and used line shape analysis to determine the kinetics and binding mechanism of this reaction. This powerful method has allowed us to determine that the regions of the TCR contacting the antigen undergo an induced fit binding mechanism and form a long lived binding intermediate with the antigen. Surprisingly, regions of the TCR that contact the MHC use a conformational selection mechanism to bind MHC. During the binding reaction the MHC molecule behaves as a rigid platform, while the presented antigen is quite dynamic and readily adopts multiple conformations. Our data indicate that the TCR can bind to at least two distinct conformations of the antigen, demonstrating that there is a melding between the TCR and antigen to reach the ground state complex. Together, the results from this work give us the first atomic snap shots of intermediate steps in the TCR-pMHC binding reaction and report the first directly observed TCR-pMHC binding intermediate. This work provides key mechanistic insight that impacts our understanding of the immune response to pathogens and cancer and illuminates principles that will help us design TCRs to viciously recognize cancer antigens that can be used in T-cell based cancer immunotherapies.

Conservation of Rare Codon Clusters

Julie Chaney, Rory Carmichael, Scott Emrich, and Patricia L. Clark
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA

The genetic code is degenerate: some synonymous codons occur more often than others, and rare codons are associated with slower translation and lower translational accuracy. Surprisingly, however, rare codons form clusters in both prokaryotic and eukaryotic ORFeomes at levels far greater than predicted by random chance, suggesting that rare codon clusters may serve a functional role. It has been suggested that rare codon clusters may promote co-translational protein folding by modulating the rate of protein synthesis. If rare codon clusters are functionally significant, then the rareness of a coding sequence is expected to be conserved through evolution. To determine if rare codon clusters are conserved, clusters of homologous proteins from bacteria were identified and aligned. The %MinMax algorithm was used to quantify coding sequence rareness and locate rare codon clusters. The resulting alignments were analyzed to calculate the probability of conservation. Preliminary results show that rare codon clusters are conserved in bacteria, with >10% of homolog clusters analyzed having a conserved rare codon cluster ($p \leq 10^{-5}$ that a rare codon cluster occurred in the same positions in the homologs by chance). Currently clusters of homologous eukaryotic and archaeal proteins are being analyzed for conservation of rare codons to determine if this phenomenon occurs across all three domains of life.

Virulence Regulation in Pattern D Group A Streptococcus

Zhong Liang, Yueling Zhang, Vishwanatha K. Chandrahas, Victoria A. Ploplis, and Francis J. Castellino
The W. M. Keck Center for Transgene Research, and the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana, USA, 46556

The virulence of the strict human pathogen, *Streptococcus pyogenes*, a Group A streptococcus (GAS), is in large part determined by M or M-like (e.g., PAM) proteins and streptokinase (SK) in the bacterium and a functional fibrinolytic system in the host. The underlying mechanisms of the roles played by these proteins in GAS virulence are still uncertain. Expression of M or M-like proteins is tightly regulated by the Mga response regulator. Mga also controls expression of genes involved in GAS adhesion and immune evasion. SK activates human plasminogen (hPg) to plasmin (hPm), which assembles as a protease on the bacterial surface. Although SK is secreted by all strains of GAS, it exhibits structural diversity; which gives it alternative functions in streptococcal diseases, as well as tissue specificity. We hypothesize that deletion/alteration of *skb* or *pam*, in the skin trophic GAS strain, AP53, will lead to altered virulence. We also hypothesize that deletion of *mga* will alter GAS virulence. In this study, we have generated *skb*, *pam*, and *mga* deletion isogenic AP53 strains and tested their virulence in a hPg transgenic mouse model. Our results suggest that SK and PAM are virulence-determinants in this pattern D GAS strain. Real time PCR analysis of the AP53/_*mga* genome indicated that Mga controls the expression of the *pam*, *fcr*, *enn*, *scpA*, and *fbp* genes. Lethal infections by AP53 are attenuated by deletion of the *mga*, pam, and *skb* genes. These studies further elucidate an understanding of the role of SK and Mga in GAS virulence.

Molecular Architecture of Ebola Virus Assembly

Emmanuel Adu-Gyamfi[1] and Robert V. Stahelin[1,2]
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Ebola is a negative stranded RNA virus. Its pathogenesis is characterized by internal and external bleeding in primates due to coagulation abnormalities induced by the virus at the onset of the infection. Since its first discovery in 1976, no specific treatment or vaccines have been found. Ebola is classified as bio-safety level IV agent and therefore has the potential to be used as a biological weapon. While mechanistic details of the virus assembly process are lacking, recent evidence suggest that the major matrix protein of the virus; VP40 plays a crucial role in virus budding from the plasma membrane. Generation of new virus involves a cascade of cellular events that recruit the viral genome, the matrix proteins and subsequent acquisition of the viral envelope from the host cell. The new virus or virus like particle (VLP) forms at a bud site at the inner leaflet of the plasma membrane and can serve as a primary therapeutic target for inhibiting Ebola virus replication. Preliminary results demonstrate that VP40 and 24 bind lipid membranes with nanomolar affinity and possess the ability to modify membrane structure. These proteins remodel the plasma membrane by inducing curvature changes, which are required for the egress of the newly formed virus. The goal of the project is to elucidate the mechanistic details of VP40 assembly on the plasma membrane using an interdisciplinary approach. Specifically, we have employed in vitro lipid binding and curvature assays with cellular scanning and single molecule microscopy to investigate the basis of VP40 lipid binding, membrane bending and viral egress. Results from this study will be key to understanding the general principles governing the remodeling of membrane by matrix proteins from lipid enveloped viruses such as Ebola and HIV.

Reversal of Excitotoxic Effects in Primary Neurons by Conantokin-G via *N*-Methyl-D-Aspartate Receptor Interaction

Rashna Balsara, Neill Li, and Francis J. Castellino
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N-Methyl-D-aspartate receptors (NMDARs) are ion-gated channel receptors composed of two NR1 and two NR2 subunits and are crucial for synaptic transmission, learning, and memory. Aberrant activation of these receptors is observed in several neuropathies, such as ischemic stroke, Alzheimer's disease, and Parkinson's disease. Activation of synaptically localized NMDARs promotes neuronal survival, whereas activation of extrasynaptic NMDARs leads to neuronal excitoxicity. Conantokins, are small peptides rich in gamma-carboxyglutamic acid residues and act as selective antagonists of NMDARs. Conantokin-G (con-G) is specific for the NR2B subunits and conantokin-T (con-T) has broad subunit specificity. Con-G, con-T, and an established NR2B-specific inhibitor, ifenprodil, were used to study their effects on primary rat hippocampal neurons that were differentially activated for synaptic or extrasynaptic NMDARs. Phosphorylation of signaling molecules ERK1/2 and CREB, mitochondrial survival, and actin organization were evaluated. Results demonstrated that neurons with pharmacologically activated extrasynaptic NMDARs, con-G, in particular enhanced activation of ERK1/2 and CREB molecules. Con-G also blocked extrasynaptic NMDAR-mediated loss of mitochondrial function. Moreover, disorganization of neuronal actin following extrasynaptic NMDAR activation was abrogated by con-G. Overall, con-G had noticeably enhanced beneficial effects than con-T or ifenprodil. It was further confirmed that NR2B subunits are critical for extrasynaptic activation, as a total lack of NR2B subunits abrogated the detrimental effects of extrasynaptic activation on phosphorylation of ERK1/2 and CREB, mitochondrial survival, and actin organization. In conclusion it can be stated that conantokin- and ifenprodil-mediated antagonism of NMDARs regulates neuronal calcium influx and subsequent downstream signaling complexes influencing neuron survival.

ABSTRACTS: POSTERS

1. Tau Binds Different Microtubule Conformations and Aggregates at the Microtubule Surface

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Tau is a neuronal microtubule (MT) associated protein found abnormally aggregated into plaques in the brains of Alzheimer's patients. Tau's normal cellular function is to stabilize MTs, which are cylindrical, intracellular tubes composed of tubulin subunits. MTs can dynamically grow (polymerize into long filaments) and shrink (depolymerize/ fall apart) in order to perform many critical cellular functions, such as chromosomal separation during mitosis and intracellular cargo transportation. MT dynamics is regulated by MT-associated proteins including Tau. Based on the various experimental methods and reported affinities of Tau for MTs found in the literature, we hypothesized that the Tau-Tau interactions which occur in Alzheimer's disease might also occur under normal conditions, resulting in different binding behaviors of Tau for MTs at different Tau concentrations. Using strategic MT binding assays, we found that the apparent affinity of Tau for MTs depends on the concentration of Tau, suggesting that Tau-Tau interactions may contribute to normal Tau function. Additionally, to better understand the MT stabilizing mechanism of Tau, we are also testing the sensitivity of Tau for different tubulin/MT conformations. Our analyses thus far show that Tau binds to GMPCPP-MTs with relatively strong affinity, though more weakly than it binds to Taxol-stabilized GDP-MTs. GMPCPP-MTs are believed to mimic the conformation of the GTP cap at the dynamic MT plus end, while Taxol-stabilized GDP-MTs represent the conformation of the MT lattice. Preliminary data also show that Tau binds to Dolastatin-10 tubulin rings. This is significant in elucidating that Tau has a separate binding site on the inner wall of the MT. We are currently following up on this hypothesis with TEM studies. The observations that Tau binds GMPCPP-MTs and Dolastatin-10 tubulin rings suggest that Tau may play a role in MT polymerization by incorporating itself into the inner framework of the MT starting at the dynamic plus end.

2. Importance of Position 170 in the Inhibition of GES-Type β-Lactamases by Clavulanic Acid

Hilary Frase, Marta Toth, Matthew M. Champion, Nuno T. Antunes, and Sergei B. Vakulenko
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Notre Dame, IN 46556 USA

Bacterial resistance to β-lactam antibiotics (penicillins, cephalosporins, carbapenems, etc.) is commonly the result of the production of _-lactamases. The emergence of β-lactamases capable of turning over carbapenem antibiotics is of great concern, as these are often considered the last resort antibiotics in the treatment of life-threatening infections. β-Lactamases of the GES-family are extended-spectrum enzymes that include members that have acquired carbapenemase activity through a single amino acid substitution at position 170. We investigated inhibition of the GES-1, -2, and -5 β-lactamases by the clinically important β-lactamase inhibitor clavulanic acid. While GES-1 and -5 are susceptible to inhibition by clavulanic acid, GES-2 shows the greatest susceptibility. This is the only variant to possess the canonical asparagine at position 170. The enzyme with asparagine, as opposed to glycine (GES-1) or serine (GES-5) then leads to a higher affinity for clavulanic acid (K_i = 5 μM), a higher rate constant for inhibition, and a lower partition ratio ($r \approx 20$). Asparagine at position 170 also results in the formation of stable complexes, such as a cross-linked species and a hydrated aldehyde. In contrast, serine at position 170 leads to formation of a long-lived *trans*-enamine species. These studies provide new insight into the importance of the residue at position 170 in determining the susceptibility of GES enzymes to clavulanic acid.

3. A Comparative Study of Different MART-1 Specific TCRs Interacting With Structurally Distinct Antigens

Moushumi Hossain, Sujatha Santhanagopolan, and Brian M. Baker
Department of Chemistry and Biochemistry, University of Notre Dame
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T-cells mediate the cellular immune response against foreign pathogens and transformed cancer cells. T-cells are activated by binding to antigens presented by the major histocomapatibility complex (pMHC). Though T cells are sensitive to specific antigens, T cell receptors (TCR) can cross-react with multiple pMHCs. T cell cross-reactivity is important for the maintenance of TCR repertoire, initiation of immune responses, and can also cause organ rejection. Though TCR cross-reactivity is well appreciated, the molecular mechanisms of TCR-pMHC cross-reactivity are poorly understood. Our goal is to investigate the physical mechanisms different TCRs use to cross-react with multiple ligands. We are addressing this question by characterizing the binding affinities between different TCRs derived from T cells that recognize the melanoma-associated MART-$1_{26/27\text{-}35}$ antigens presented by the class I MHC HLA-A2.. The 27-35 MART-1 antigen is a nonamer, AAGIGILTV (AAG), whereas the 26-35 antigen is a decamer, EAAGIGILTV (EAA). The two antigens differ from each other by only one amino acid, yet they adopt strikingly different conformations when bound to HLA-A2, making this an ideal system to study cross reactivity.

Here, we present surface plasmon resonance (SPR) data for several TCRs recognizing the MART-1 antigens and single amino acid variants. Through the introduction of alanine substitutions, our binding data is beginning to illuminate how different TCRs are able to cross-react with the structurally diverse MART-1 nonamer and decamer. Along with highlighting aspects of TCR specificity and cross-reactivity, we can help in the development of immunological anticancer therapeutic strategies. As the MART-$1_{26/27\text{-}35}$ epitopes are widely used in experimental, immunological therapies for melanoma, our studies could provide guidance for the design of improved cancer vaccine candidates. Hence, by studying T cell recognition of MART-1 antigens, we can address not only basic questions related to TCR cross-reactivity, but also questions related to cancer immunology.

4. Molecular Description of How Different MART-1 Antigenic Peptides Influence T cell Receptor Recognition

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T-cells are integral in mediating cellular immune responses against foreign pathogens and cancerous cells, and are activated by antigenic peptide-major histocomapatibility complex (pMHC). Though T cells are sensitive for specific antigens, many T cell receptors (TCR) can cross-react with multiple antigenic peptides. T cell cross-reactivity is important for initiation of immune responses against pathogens and tumors. Though, TCR cross-reactivity is well appreciated; the molecular mechanisms of TCR-pMHC interactions are poorly understood. We want to provide a molecular description of TCR specificity and cross-reactivity by structural characterization of a melanoma specific TCR bound to different MART-1/HLA-A2 ligands. MART-1 melanoma tumor protein is presented by the class I MHC, HLA-A2. The MART-1/HLA-A2 antigens: a nonamer, AAGIGILTV (AAG) and a decamer, EAAGIGILTV (EAA), differ only by one amino acid, and adopt strikingly different conformations when bound. Therefore, making this system ideal to study cross reactivity. Here we present X-ray crystallographic structures for a highly cross-reactive MART-1 specific TCR recognizing structurally distinct MART-1 ligands.

5. Peptide-dependent Tuning of the pMHC Local Backbone Dynamics

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The T-cell receptor (TCR) is responsible for recognizing antigens presented by the major histocompatability complex (MHC) on the surface of diseased cells. The ability for the TCR to cross-react with multiple pMHCs is a hallmark of the cellular immune response, which allows a single TCR to recognize multiple antigens. One model for TCR cross-reactivity involves conformational changes within the TCR complementary determining region (CDR) loops, while another model involves conformational changes in both the peptide and the MHC. Peptide specific tuning of the dynamics of the MHC could facilitate the adoption of altered structures in the binding site, which could facilitate cross-reactivity. Peptide dependent dynamics can also influence the entropic cost of binding the TCR.
To address how different peptides alter the dynamics of a single MHC, we have measured the dynamics at key positions in HLA-A2 bound to different ligands using fluorescence anisotropy. To assess the changes in flexibility, we engineered a cysteine mutant at 8 different solvent exposed positions that line the binding surface for the TCR that is comprised of the _ 1 (D61, K68, Q72, V76) and _2 (K146, H151, R157, R169) helicies of HLA-A2. The peptides used in this study are Tax 9 (LLFGYPVYV), Tel1p (MLWGYLQYV), gp100 T2M (IMDQVPFSV), ELA (ELAGIGILTV) and FluM1 (GILGFVFTL). Steady-state fluorescence anisotropy measurements demonstrated that the overall flexibility on the MHC peptide binding groove varies dependent on the peptide presented on the MHC complex. These exciting results demonstrate that the bound peptide can alter the dynamics of MHC, which could influence the binding with TCR. More generally, these results demonstrate that subtle changes in a ligand can alter the dynamics of a binding partner.

6. Construction of a Stable Single Chain T-cell Receptor for Site-specific Analysis of the Binding Mechanism to Peptide-MHC Complexes

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The interaction between a T-cell receptor (TCR) and a peptide presented by an MHC molecule forms a complex that initiates a cellular immune response. The body deploys a large number of diverse TCRs that are responsible for recognizing both foreign and self peptides and initiating a signaling cascade that will result in appropriate action. The complete mechanism by which an individual TCR recognizes both foreign peptides and a self MHC molecule is still unknown. The A6 human TCR has been evaluated for its binding affinity with a variety of peptide-MHC complexes; additionally, the thermodynamic contributions to the binding of A6 with the Tax9 peptide have been well characterized. To further examine the site-specific details of the A6 binding profile, line-shape analysis of NMR titration experiments will be used to observe the behavior of specific residues on A6. In order to achieve this, a single chain fragment of A6 containing only the variable domains connected by a flexible linker was engineered by yeast display. Modifications to this single chain construct (scA6) have resulted in a stable TCR that displays only a slightly weaker affinity for binding to well-characterized peptides. Further evaluation of the scA6 TCR will provide details to help fully characterize the specific binding mechanism that the A6 TCR makes with a peptide MHC complex.

7. Characterizing the Role of *S. cerevisiae* ARP4

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Actin is a fundamental component of the cytoskeleton that has been implicated in critical roles for an ever increasing number of processes, ranging from mitosis to muscle contraction. Although mostly studied in the context of the cytoplasm, it is now evident that actin is also present in the nucleus along with several actin-related proteins (ARPs). Of particular interest is ARP4, the closest relative of actin that has been found in the nucleus. ARP4 is a known subunit of the ATP-dependent chromatin remodeling complexes Swr1 and INO80, as well as the histone acetyltransferase complex, NuA4. What remains unknown is the role ARP4 plays in the assembly and function of these multi-subunit complexes, or whether it might even have more interacting partners.

In order to address these questions we are taking an approach that combines bioinformatics, molecular biology, and biochemistry. We hypothesize that Arp4p contains a set of conserved surface residues that are likely functionally significant and involved in protein-ligand interactions. To test the functionality of these amino acids, we have made site-directed mutants in a *S. cerevisiae* model system. Thus far, we have conducted phenotypic tests on eight mutants and obtained encouraging preliminary results for conditional lethality and temperature sensitivity in four of these eight mutants. We hope that these mutations will help us establish how various parts of ARP4 are involved in interactions with the multiple complexes in which it participates. The use of tandem-affinity purification (TAP)-tagged Arp4p (and site-directed mutants) has provided a biochemical means for the purification of Arp4p and bound ligands, which are currently in the process of being identified through tandem mass spectrometry.

8. Investigating the Secretion of an Autotransporter Passenger Domain

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Autotransporter proteins are virulence factors secreted from Gram negative bacteria. They consist of N-terminal signal sequence that is cleaved after translocation across the inner membrane. The C-terminal porin domain forms a 12-stranded β-barrel in the outer membrane. The 100 kDa central passenger domain is transported out of the outer membrane via Type Va secretion in a proton gradient and ATP independent mechanism. There are a few crystal structures available of the porin and passenger domains, but these do not provide insight into the mechanistic details of the role of porin domain in transport of the passenger domain. Pertactin is an autotransporter protein that is secreted from *B. pertussis.* Our lab has shown that the pertactin passenger domain undergoes transport from C to N terminal direction and has suggested contribution of the vectorial transport and folding of the β-helical passenger domain (Junker *et al.*, 2009). This was demonstrated by creating stalled secretion intermediate of the pertactin passenger domain by engineering double cysteine mutants and stalling in the oxidizing environment. As the role of the porin domain in the transport is essential, yet unclear, a crystal structure of the porin domain with the stalled passenger domain will provide the necessary missing information. Here, we attempt to purify this stalled secretion intermediate from the outer membrane for crystallization.

9. Characterizing the Interaction Between EB1 and Microtubules

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EB1 is a highly conserved microtubule binding protein classified as a plus end tracking protein (+ TIP) that localizes to the microtubule plus end and regulates microtubule dynamics. Microtubules are made up of tubulin dimers that form a cylindrical, closed sheet conformation that contains a longitudinal "seam" at the point of closure. Microtubules are dynamic and can continuously grow (polymerize) and shrink (depolymerize) primarily at their plus ends. This dynamic process is necessary for microtubule function that includes several cellular processes such as separating the chromosomes during cell division and transporting different cargos throughout the cell. The regulation of microtubule growth and shrinkage is controlled by a group of + TIPs that network together. EB1 is the master regulator in the +TIP network that helps to polymerize the microtubule plus end. The interactions between EB1 and microtubules are not fully understood, in particular the mechanism by which EB1 regulates the plus end of the microtubule. Previous electron microscopy studies indicate that EB1 binds to the "seam" rather than to the microtubule body. However, some of our data conflict with this idea. Moreover, while it is true that the published results on EB1-microtubule binding are consistent with "seam" binding, they also appear to be consistent with weak binding along the microtubule body. To resolve this issue, we are using a combination of mathematical modeling and experimental procedures. More specifically, we are using MTBindingSim to predict the binding behavior of EB1 to microtubules under different binding models, and then testing these models through experiments. Preliminary data suggests EB1 does not bind strongly to the seam although complete verification requires further analysis. These results will help establish how EB1 interacts with microtubules, which in turn is essential for fully understanding how cells regulate the dynamics of the microtubule plus end.

10. Co-translation Folding Monitored by Isotopic Labeling and H/D Exchange

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Amide hydrogens in proteins are in continuous exchange with the hydrogens in solution. Hydrogen-deuterium exchange experiments take advantage of this: proteins are placed in D_2O and H-D exchange is monitored by mass spectroscopy or NMR. Rates of exchange vary greatly depending on solvent accessibility and whether the hydrogen is involved in hydrogen bonds or not. Hence amide hydrogen exchange rates can provide useful information regarding protein conformation and dynamics.

Proteins fold co-translationally as they are synthesized by the ribosome, but little is known about the *in vivo* conformations these nascent polypeptide chains adopt while still attached to the ribosome or how these conformations affect subsequent folding steps. Current methods, such as NMR, suffer from large sample requirements and impose limits on protein size. We plan to measure the extent of folding of ribosome-bound nascent chains of Green Fluorescent Protein, a β-sheet protein, by monitoring hydrogen-deuterium exchange monitored by mass spectroscopy.

Preliminary *in vitro* results using natively folded GFP have shown that GFP can be subjected to H/D exchange and this exchange can be viewed with MALDI-TOF MS.

11. The *N*-terminus of the VirG Autotransporter Destabilizes the Entire Passenger *in vitro:* Implications for *in vivo* Secretion

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VirG is a virulence-associated protein of *Yersinia pestis*, the causative agent of bubonic plague. VirG belongs to the autotransporter (AT) family of virulence proteins, and like most other ATs, the mature extracellular (passenger) domain is predicted to adopt β-helical structure, which we have proposed is important for AT biogenesis. Previous studies in our lab with two other ATs, pertactin from *B. pertussis* and Pet from a pathogenic strain of *E. coli,* have shown that the C-terminal β-helical portion of the passenger domain adopts a stable structure that is resistant to chemical and thermal denaturation, and extracellular folding of this domain could serve as a driving force for outer membrane OM) secretion. This study aims to identify the features of the VirG passenger domain that are important for its biogenesis, including what keeps the passenger domain unfolded during its transit across the periplasm prior to OM secretion. We established a VirG expression system in *E. coli* and purified two VirG passenger domain constructs. Our preliminary results suggest that the shorter C-terminal passenger domain construct is more resistant to chemical and heat denaturation, and is more resistant to protease digestion, than the longer construct with an *N*-terminal extension. The N-terminal portion of the passenger domain could therefore be preventing premature folding of the entire passenger domain while in the periplasm maintaining a conformation compatible with OM secretion.

12. The Molecular Basis of Ceramide-1-Phosphate Recognition by Peripheral Proteins

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The sphingolipid ceramide-1-phosphate (C1P) plays a critical role in the cellular signaling that mediates inflammation, cell proliferation and phagocytosis. C1P has been shown to increase the activity of cytosolic phospholipase A_2 _ ($cPLA_2$_) as well regulate its translocation to cellular membranes, a process that promotes inflammation through the production of arachidonic acid. For the first time, the conformational changes that result from C1P binding to the C2 domain of $cPLA_2$_ have been mapped structurally with NMR. Subsequently, this novel-binding site was confirmed with *in vitro* biophysical and biochemical analysis as well as molecular dynamics simulations. After elucidating the specificity of this interaction, we sought to discover other C1P binding proteins in the proteome. In searching the proteome for conserved C1P binding sites, the $p47^{phox}$ subunit of the NADPH oxidase complex was identified as a potential C1P binding protein. Preliminary *in vitro* data confirms that $p47^{phox}$ binds C1P in the presence of $PI(3,4)P_2$ producing an increased association when the two target lipids are present together. These studies have implicated C1P to be vital for targeting proteins to specific membranes. Future work will experimentally test the ability of other proteins to bind C1P to gain an understanding of the characteristics of C1P binding sites.

13. The Influences of T-Cell Receptor Dynamics on pMHC Recognition

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The T-cell-mediated immune response is initiated by the T cell receptor (TCR) protein's recognition of foreign or aberrant peptide/MHC ligands. It is inconclusive how this interaction ultimately activates the T cell, despite the exhaustive investigations of the TCR-pMHC complex formation. A stout library of TCR and pMHC structures, both bound and unbound, has been constructed in order to characterize the contact surfaces and structural adaptability of the proteins at the interface of the complex. Crystal structures, however, fall short in describing the level of conformational dynamics that each protein inherently possesses at their respective binding surfaces, so any conclusions drawn thus far regarding the contributions of pre-existing flexibility towards TCR recognition from a structural standpoint are largely speculative and lack fundamental evidence.

The pre-existing dynamics of the __ TCR A6 were therefore determined using both time-resolved fluorescence anisotropy and explicit-solvent molecular dynamics (MD) simulations. Based on the conformational sampling of the receptor's complementarity-determining regions (CDRs), the inner loops of the TCR binding surface (CDR3_ and CDR3_) demonstrate dynamic features essential to pMHC cross-reactivity. By superimposing the MD snapshots of the A6 TCR onto the ternary structure of A6 (bound to its native pMHC ligand, Tax-HLA-A2), it has been revealed that clashes with the pMHC ligand dictates the conformational selection of the A6 CDR3 loops, ultimately influencing antigen recognition. Thus, in the context of both free and bound TCR-pMHC complexes, measured CDR loop dynamics have helped us elucidate the means by which the A6 receptor may recognize chemically and conformationally distinct antigenic peptides via inherent flexibility.

14. Expression and Purification of the Cytoplasmic Domain of BlaR1

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The BlaR1 protein has been implicated in the mechanism of ß-lactam resistance of *Staphylococcus aureus*. It is a membrane protein that includes four transmembrane segments (TM1-4) connected by loops (L1-L3). The L3 loop is located in the cytoplasm and is described as a metalloprotease. Upon activation, this domain of BlaR1 is responsible for the degradation and subsequent removal of the BlaI repressor of the *bla* operon, leading to transcription of the *blaZ* gene and production of β-lactamase, culminating in antibiotic resistance. Currently, the L3 loop has yet to be isolated as a pure, soluble protein. In an effort to recover a significant amount of soluble L3 protein for crystallographic studies, we have designed two constructs of this cytoplasmic loop (cytBlaR), with the addition of a solubility tag to aid in expression and purification. A combination of affinity and size-exclusion chromatography is used to purify cytBlaR from the protein tag. In the present study, we use Western Blot analysis monitor the degradation of purified BlaI as a measure of activity of the purified cytBlaR protein.

15. Inhibitors for Bacterial Cell-Wall Recycling

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Bacterial cell wall is recycled in the normal course of growth of many Gram-negative bacteria. The recycled muropeptide components are processed during the growth and maturation of the cell wall, and in response to damage by antibiotics. Peptidoglycan recycling in the periplasm commences with the catalytic action of the lytic transglycosylases on the recovered muropeptides. These enzymes catalyze the non-hydrolytic fragmentation of the glycosidic bond between the *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues of the peptidoglycan-derived muropeptides. This unusual fragmentation gives the NAG-1,6-anhydromuramyl as a product. Following internalization through the AmpG permease. An early cytoplasmic event of muropeptide recycling is the hydrolytic action of the NagZ glycosylase, which removes NAG from NAG-1,6-anhydromuramyl to produce compound 1,6-anhydromuramyl. Subsequent reaction of the protease AmpD removes the peptide segment from both compounds. The distinct reactions of lytic transglycosylase(s) and NagZ—one is a non-hydrolytic transglycosylase and the other a hydrolytic glycosidase—are nonetheless proposed to go through conformationally distinct oxocarbenium species in the key step of their respective reactions. The oxocarbenium species in the former entraps the C6 hydroxyl group as a nucleophile, whereas a different oxocarbenium species in the latter is captured by a water molecule. Here we evaluate some piperidine iminosaccharides, inspired by the structures of the NAG and the NAM moieties found in the bacterial peptidoglycan, as possible mimics of oxocarbenium species generated in the course of the reactions by these two enzymes. Although the subjects of iminosaccharide properties, conformations and bioactivities have been reviewed, we report herein their syntheses and their inhibitory properties against purified recombinant NagZ of Pseudomonas aeruginosa and the lytic transglycosylase MltB of Escherichia coli.

16. MTBindingSim: A Program to Simulate Protein Binding

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We have developed a program, MTBindingSim, to help researchers model protein binding. In particular, MTBindingSim was written with modeling protein binding to microtubules and other polymeric systems. Microtubules are an essential part of the cell cytoskeleton, responsible for the maintenance of cell architecture and polarity, cargo transport, cell movement, and cell division. MTs exhibit continuous growing and shrinking, a behavior known as dynamic instability. In addition, the MTs are regulated by MT-associated proteins (MAPs). In order to understand MAP function and mechanisms, it is important for experimentalists to fundamentally understand the binding interactions between MTs and MAPs. Many MAPs exhibit binding behavior that cannot be explained by simple first order binding, and several models have been proposed to account for the experimentally observed binding data. MTBindingSim simulates binding curves for MAPs binding to MTs using various binding models. The purpose of this program is to help researchers develop intuition regarding protein binding behavior in different binding models as well as to assist researchers in designing binding experiments that will be able to distinguish between these. In addition to its MT-specific applications, MTBidningSim also can be used to help students understand simple binding intreactions and how they relate to more complex interactions. MTBindingSim also can be used to investigate the binding of proteins to polymeric systems other than the MT. MTBindingSim is freely distributed using Google Code under the GNU General Public License.

17. Metabolism of 5-Fluorouracil, Deoxyuridine and dUTP in Isolated Brain Mitochondria: Implications for Current Treatment Options in Glioma

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Incorporation of dUTP into mtDNA can lead to mutagenesis and apoptosis, especially as base excision repair (BER) is known to be inefficient in brain mtDNA. These investigations aim to establish the activity of the enzymes necessary for regulation of dUTP pools in brain mitochondria: dUTPase, thymidine phosphorylase, and thymidine synthase. We also investigate the metabolism of 5-flourouricil (5FU), the metabolite of which, 5-FdUMP, functions as a dUMP analogue.

Mitochondria were isolated from freshly removed brains from adult Harlan Sprague Dawley rats. Mitochondrial intactness was determined by measuring the respiratory control ratio (RCR) and only preparations with RCR values over 5 were used. Mitochondria were incubated at a final concentration of 4 mg protein /ml in media with labeled and unlabeled deoxynucleosides and deoxynucleoside analogs. Samples of the mitochondrial incubation were removed at specific time points and combined with an equal volume of 10% trichloroacetic acid to lyse mitochondria and precipitate the protein and nucleic acids. This mixture was placed on ice, centrifuged, and the supernatant extract neutralized by addition of AG-11A8 resin. Labeled deoxynucleosides and phosphorylated products in the filtered extracts were analyzed and quantitated by HPLC on an Alltech nucleoside-nucleotide reverse phase column coupled to an inline UV monitor and liquid scintillation counter as previously described.2 Peaks were identified by comparison to standards.

Isolated rat brain mitochondria were able to transport [3H]-dU across the inner membrane into the matrix. And phosphorylate it to [3H]-dUMP. Of note, there is no evidence of conversion of dUMP to TMP or any other phosphorylated products. There was no breakdown of dU to uracil. Isolated rat brain mitochondria were able to transport [3H]-5FU across the inner membrane and phosphorylate it to [3H]- 5-FUMP, 5-FUDP, and 5-FUTP. There was no evidence of conversion to 5-FdUrd or 5-FdUMP. Isolated rat brain mitochondria were able to transport labeled dUTP across the inner membrane into the matrix. dUTP was rapidly and nearly completely dephosphorylated to dUMP with subsequent de-phosphorylation to dU. 5-FU did not appear to compete with thymidine phosphorylation as it had no significant effect on the mitochondrial conversion of [3H]-thymidine to [3H]-TTP.

It is well known that dNTP levels must be carefully regulated to support normal mitochondrial DNA replication. Incorporation of dUTP for TTP into mitochondrial DNA is mutagenic. We demonstrated in isolated brain mitochondria that [3H]-dU was slowly converted to dUMP, but no other phosphorylated forms were detected. Further, we demonstrated the presence of a highly active and specific dUTPase that plays a critical role in removing dUTP should it be formed. It is not yet clear if [3H]-dUMP can be further phosphorylated in isolated brain mitochondria, since the very active dUTPase would quickly de-phosphorylate any [3H]-dUTP made. There was no evidence of thymidine phosphorylase or thymidylate synthase activity in brain mitochondria. Work continues to determine the activity of these enzymes in brain cytosol. Lastly, we demonstrated that 5-FU is

phosphorylated to 5-FUTP, and could cause mutagenesis by incorporation into RNA. This may suggest a more likely mechanism for neurotoxicity than thymidine depletion.

18. Molecular and Biophysical Investigation of Golgi-Localized Protein FAPP1 with PI4P and Novel PI4P Analogs

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ADP-ribosylation factor 1 (Arf1) and four phosphate adaptor protein (FAPP1) are known to co-localize at the trans-golgi network and be essential for the process of membrane trafficking from the Golgi to the plasma membrane. Arf1 is a 21 kDa protein known to act in the recruitment of coat proteins and the coating of cargo vesicles by interacting with coatomer and clathrin-adaptor complex proteins. FAPP1, a 34 kDa protein harboring a phosphotidyl-4-phosphate (PI4P)-binding PH domain has recently been shown to alter membrane shape in a PI4P-dependent manner. Though crystal structures are available for both individual proteins, their structural and biophysical interaction has not been elucidated. In collaboration with the Kutateladze lab we have investigated the structural interaction between FAPP1 and Arf1 using X-ray, NMR, and biophysical analysis. In addition, we have investigated the interaction of FAPP1 with novel, metabolically stable analogs of PI4P. Results demonstrate a high affinity interaction between the active form of Arf1 and the PH domain of FAPP1, and between FAPP1 and the three PI4P analogs.

19. Mitochondrial DNA Depletion Syndrome: Is Organ Specificity and Susceptibility Determined at the Gene Transcription Level?

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Mitochondrial DNA (mtDNA) depletion syndrome (MDS) is characterized by a quantitative reduction of mtDNA in a tissue specific manner. Nucleoside metabolic irregularities have long been implicated in MDS. Further nucleoside metabolic complications associated with long-term treatment with nucleoside reverse transcriptase inhibitors (NRTIs) such as the thymidine analogue AZT (3'-azido-3' deoxythymidine; zidovudine) also affects some of the vital tissue such as heart and kidney. Although mitochondrial DNA depletion has been suggested to be the mechanism of the toxicity resulting in the metabolic complications, it is still unknown why the toxicity of NRTI is tissue specific. The current investigation was performed to determine mRNA levels of the enzymes involved in nucleoside metabolism both in normal adult male rat tissues and in rats treated with an acute dose of AZT (200 mg/kg b.w) given over a 24h period as 3 doses of 50, 50 and 100 mg/kg b.w. at 12h intervals. RT-PCR analysis revealed significant differences in the mRNA levels of the enzymes involved in *de novo* synthesis, nucleoside salvage, nucleoside degradation and nucleoside transportation among the normal adult rat tissues *viz.,* heart, liver, spleen, kidney, lung and brain. The results were further corroborated by microarray analysis of total mRNA from rat heart, liver and muscle tissues. Further, tissue mRNA levels of heart and liver were compared to that with AZT treated rat heart and liver using RT-PCR studies. There was a significant increase (2.3 fold) in ribonucleoside reductase subunit 1 mRNA and a significant 0.5 fold decrease in the nucleoside transporter protein 1 mRNA expression in heart upon treatment with AZT for 24h. Liver mRNA levels however did not show any significant alterations due to AZT treatment for 24h. Further, heart and liver RNA samples were subjected to microarray analysis and the heart sample array presented a similar trend corroborating our RT-PCR results. There was a moderate increase in the mRNA levels of the enzymes involved in the *de novo* pathway, namely ribonucleotide reductase and thymidylate synthase. As AZT has been shown to decrease TTP pools in heart, heart may respond to the acute NRTI toxicity by increasing the *de novo* pathway in order to compensate for the loss of TTP pools in such tissues. The results compiled from the above study suggest that differences in mRNA expression of these enzymes may be the reason for organ specific pathophysiology of MDS.

20. Crystal Structures of Bacterial Peptidoglycan Amidase AmpD and an Unprecedented Activation Mechanism

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AmpD is a cytoplasmic peptidoglycan (PG) amidase involved in bacterial cell-wall recycling and in induction of b-lactamase a key enzyme of β-lactam antibiotic resistance. AmpD belongs to the amidase_2 family that includes zinc-dependent amidases and the peptidoglycan-recognition proteins (PGRPs), highly conserved pattern-recognition molecules of the immune system. Crystal structures of *Citrobacter freundii* AmpD were solved for the apoenzyme, for the holoenzyme at two different pH values, and for the complex with the reaction products, providing insights into the PG recognition and the catalytic process. These structures are significantly different compared to the previously reported NMR structure for the same protein. The NMR structure does not possess an accessible active site and shows the protein in what is propose herein as an inactive "closed" conformation. The transition of the protein from this inactive conformation to the active "open" conformation, as seen in the X-ray structures, was studied by molecular dynamics simulations, which revealed large conformational rearrangements (as much as 17 Å) in four specific regions representing one third of the entire protein. It is proposed that the large conformational change that would take the inactive NMR structure to the active X-ray structure represents an unprecedented mechanism for activation of AmpD. Analysis is presented to argue that this activation mechanism might be representative of a regulatory process for other intracellular members of the bacterial amidase_2 family of enzymes.

21. Arrestin is Required for Activation of Drosophila Rh1 Rhodopsin Kinase

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To elucidate the role of light-driven posttranslational modifications of *Drosophila* Rh1 rhodopsin on receptor cycling, membrane internalization and cell viability, we have developed and characterized reagents to follow *in vivo* light-driven rhodopsin phosphorylation. The phosphorylation of Rh1 rhodopsin happens within minutes of illumination and requires the presence of either arrestin-1 or arrestin-2. During white light illumination, the photoactivated Rh1 cycles between arrestin-bound and arrestin-unbound states. Saturating blue light (< 440 nm) produces a system in which approximately 30% of total Rh1 is in arrestin-bound state, while the remaining 70% of rhodopsin does not interact with arrestin. Red light (> 610 nm) does not activate Rh1 and thus all rhodopsin molecules do not form a complex with arrestin. Illumination by white or blue light of the same intensity resulted in almost complete phosphorylation of Rh1, while red light Illumination failed to phosphorylate Rh1. Together these results show that rhodopsin kinase activity is dependent on arrestin-Rh1 interaction. Activated rhodopsin kinase is able to phosphorylate the photo-activated Rh1 molecule in the arrestin-bound and arrestin-unbound states.

Arrestin binding and receptor phosphorylation are common elements of the deactivation process in various G protein-coupled receptors (GPCR) including rhodopsin. The current prevailing view is that rhodopsin phosphorylation is necessary for recruiting arrestin. Now our results show that arrestin is required for receptor phosphorylation. We propose that the sequence of molecular events in the GPCR deactivation process is: 1) arrestin binding to stimulated receptor; 2) GPCR/arrestin complex activates GPCR kinase; 3) activated kinase phosphorylates stimulated receptors in the arrestin-bound and arrestin-unbound states.

22. Time-Dependence of Activation of the *bla* System in *Methicillin-Resistant Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a globally important pathogen that is resistant to virtually all commercially available β-lactam antibiotics. The BlaR1 β-lactam sensor/signal transducer protein of MRSA has been implicated primarily in induction of β-lactamase expression (*bla* operon). BlaR1 fragmentation has been described as an event that occurs after induction of MRSA by β-lactam antibiotics. In addition, it was shown that BlaR1 proteolysis is required for activation of the cytoplasmic domain of the protein. Here we analyzed the time course of induction of the *bla* system in different MRSA strains, and we show the existence of antibiotic-related variations in the duration and extent of the activation of the system. We also present data that show that BlaR1 fragmentation occurs in non-induced cells as well, and in slightly different positions in different MRSA strains and in recombinant BlaR1 expressed in *E. coli*, which suggests the presence of a mobile and proteolysis-prone region in the cytoplasmic domain. Our results hence suggest that BlaR1 fragmentation could be adventitious, and not necessarily part of the activation process. We have also documented that in certain MRSA strains, BlaR1 sheds its β-lactam sensor domain to the medium upon exposure to antibiotics. This shedding in evolution of class D β-lactamases from a progenitor penicillin-binding protein in an evolutionary time frame has been presumed.

23. Purification and Crystallization of PBP2a from Methicilin-Resistant *Staphylococcus aureus*

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The Gram-positive bacterium *Staphylococcus aureus* is a leading cause of hospital- and community-associated infections. Of particular concern is the growing prevalence of methicillin-resistant *S. aureus* (MRSA) in both hospital- and community-associated infections. Staphylococci have two primary mechanisms for resistance to β-lactam antibiotics: the expression of PC1 β-lactamase, an enzyme that hydrolyzes the β-lactam ring, thus rendering the antibiotic inactive, and the acquisition of a gene encoding a modified penicillin-binding protein (PBP), known as PBP 2a, found in MRSA and coagulase-negative staphylococci. PBP 2a is intrinsically resistant to inhibition by _-lactams. PBP 2a remains active in the presence of concentrations of β-lactam antibiotics that inhibit most PBP enzymes, thus substituting for their functions in cell wall synthesis and allowing growth in the presence of the β-lactam inhibitors. In this report, we describe a three-step purification of a soluble form of PBP 2a (PBP 2a') to homogeneity using anion-exchange, cation-exchange, and size-exclusion chromatography. Purified PBP2a' was a 74-kDa monomeric protein, that retained the ability to be irreversibly acylated by β-lactam antibiotics, as was shown using the reporter chromogenic β-lactam nitrocefin. Purified PBP2a' was crystallized, which diffracted at low resolution. Unexpectedly, mass spectrometry analysis of the protein in the crystals showed the presence of a truncated protein (amino acids 122-668). In order to improve the quality of the crystals for structure determination, and eventually co-crystallization with inhibitors, we constructed two truncated versions of PBP2a': one identical to the truncated protein observed in the crystals, and a smaller version in which a predicted mobile loop was removed (amino acids 138-668). The purification of these new truncated proteins is under way in the lab.

24. Plasminogen Regulates Early Events in a Type-IIa Familial Hypercholesterolemia Murine Model of Atherosclerosis

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Components of the fibrinolytic system have been identified as major contributors in the developing plaque (early stages), as well as plaque instability (late stages). Plasminogen (Pg) activators are overexpressed in the atherosclerotic wall and macrophage-targeted overexpression of urokinase-type plasminogen activator (uPA) in mice has been shown to accelerate the disease process. Additionally, plasmin (Pm) modifies oxidized-LDL (oxLDL) by proteolysis, and this modified lipoprotein is more readily taken up by macrophages, thus leading to fatty streak formation during the early stages of plaque development. Pm can also stimulate SMC proliferation, resulting in progression of neointimal formation. The goal of this study was to employ a novel mouse model of spontaneous human type-IIa familial hypercholesterolemia (FH) that serves as an additional model of LDL-cholesterol (LDL-C)-driven human atherosclerosis. These mice possess a double-targeted deletion of the low-density lipoprotein receptor *(Ldlr)* gene and the apoB editing gene present in mouse liver *(Apobec)* ($L^{-/-}/A^{-/-}$). A second line of mice was generated that had an additional deficiency of Pg ($L^{-/-}/A^{-/-}/Pg^{-/-}$) in order to determine the impact of Pg in an LDL-driven model of atherosclerosis. For these studies, 12 and 36 week mice were analyzed. Cholesterol levels were significantly elevated in blood from $L^{-/-}/A^{-/-}/Pg^{-/-}$ mice versus $L^{-/-}/A^{-/-}$ mice at both timepoints. Additionally, circulating leukocytes, especially neutrophils and monocytes were enhanced in $L^{-/-}/A^{-/-}/Pg^{-/-}$ mice versus $L^{-/-}/A^{-/-}$ mice. The LDL obtained from the plasma of $L^{-/-}/A^{-/-}/Pg^{-/-}$ mice was not efficiently taken up by macrophages from these mice indicating compositional differences of these particles. mRNA for select inflammatory markers, i.e., IL6, and adhesion molecules, i.e.,VCAM and ICAM were diminished in the heart tissue from $L^{-/-}/A^{-/-}/Pg^{-/-}$ mice versus $L^{-/-}/A^{-/-}$ mice which corresponded to attenuated plaque formation, the result of diminished smooth muscle and inflammatory cells in the tissue. Results from these studies indicate that uptake of LDL by macrophages and migration and proliferation of cells associated with the atherosclerotic plaque are regulated by the plasminogen system.

25. Investigating Conserved Motions in Proteins: Defining the "Signaling Conduit" in Pin1

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Human Pin1 is a peptidyl-prolyl isomerase composed of a catalytic isomerase (PPIase) domain that is flexibly linked to a WW domain. Pin1 recognizes phospho Ser/Thr-Pro segments in signaling proteins regulating the cell cycle, and thus serves as both a potential cancer and Alzheimer's disease target. Our work using Nuclear Magnetic Resonance (NMR) has demonstrated that substrate-induced inter-domain interactions can stimulate changes in internal conformational dynamics, generating a "conduit" for catalytic activity. Residues displaying a change in side-chain flexibility upon substrate binding are highly conserved (≥90%) as observed through bioinformatic analysis, strongly suggesting that their dynamics is pertinent to the Pin1 mechanism. To test this notion, we have begun dynamics-function studies of Pin1 mutants that include site-substitutions of the conserved residues. Here we present our initial results on the M130A mutant, which includes an analysis of its backbone dynamics by ^{15}N NMR spin relaxation and methyl-side chain dynamics by ^{2}H relaxation. Similarities and differences with wild-type are compared, along with the implications for flexibility-function correlations in Pin1.

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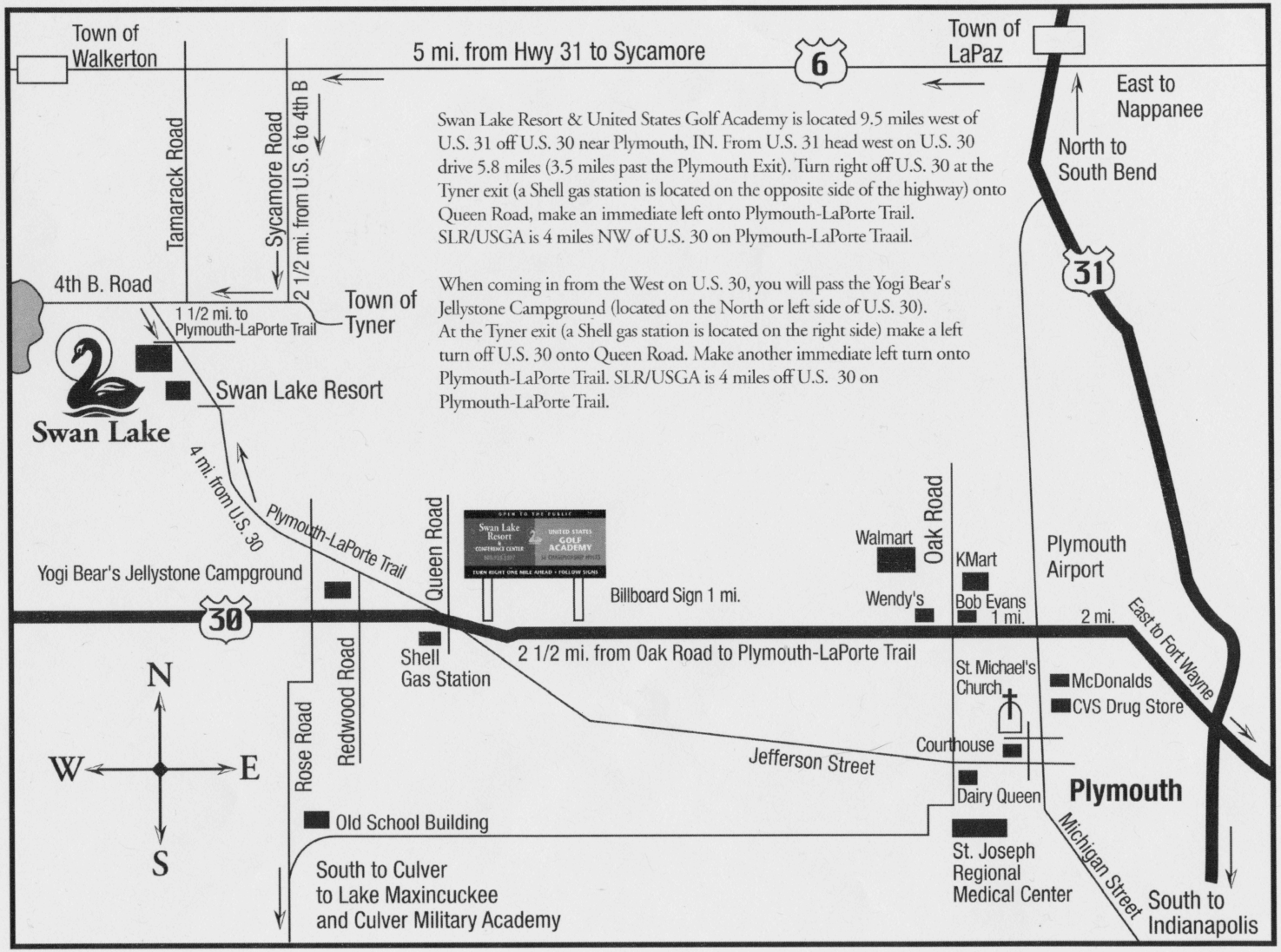
Town of Walkerton
5 mi. from Hwy 31 to Sycamore
6
Town of LaPaz
East to Nappanee
North to South Bend
Tamarack Road
Sycamore Road
2 1/2 mi. from U.S. 6 to 4th B
Swan Lake Resort & United States Golf Academy is located 9.5 miles west of U.S. 31 off U.S. 30 near Plymouth, IN. From U.S. 31 head west on U.S. 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.
4th B. Road
1 1/2 mi. to Plymouth-LaPorte Trail
Town of Tyner
31
Swan Lake Resort
Swan Lake
4 mi. from U.S. 30
Plymouth-LaPorte Trail
Queen Road
Yogi Bear's Jellystone Campground
Billboard Sign 1 mi.
Walmart
Oak Road
KMart
Plymouth Airport
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