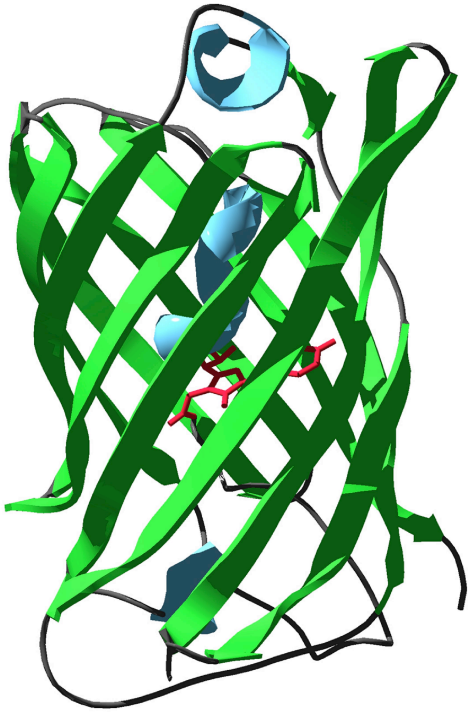
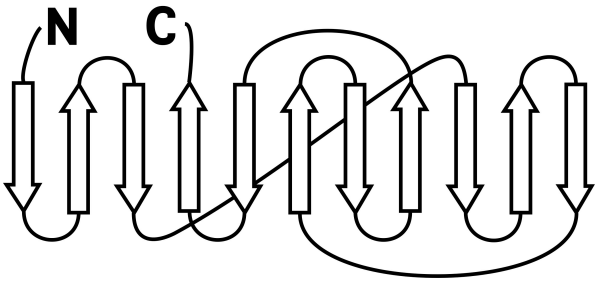
14th ANNUAL BIOCHEMISTRY RESEARCH FORUM

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





Swan Lake Resort
Plymouth, IN
May 22, 2009

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Cover Illustration

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Cover Legend. The 2008 Nobel Prize in Chemistry was awarded to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien for their discovery of Green Fluorescent Protein (GFP; PDB ID 1ema; *top*), and its development as a fluorescent protein tag for cell-based assays. Yet beyond its utility as a fluorescent reporter, GFP also provides a valuable model system for exploring the formation of β-sheet structure during folding. In native GFP, the polypeptide chain forms an 11-stranded β-barrel that wraps around the central α-helix, enabling the autocatalytic formation of the central imidazolinone chromophore (shown in *red*). A significant portion of the GFP β-barrel hydrogen-bonding network is formed by contacts between β-strands that are distant from one another in the GFP sequence (*bottom*). Discriminating between these native long-range contacts and alternative, non-native intermolecular interactions determines whether a given GFP polypeptide chain will fold (and fluoresce), versus misfold and aggregate.

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: Shelagh Ferguson-Miller (Michigan State)
2008: Christian R.H. Raetz (Duke)

♦ 2009 Keynote Lecture ♦

John L. Wang
Department of Biochemistry and Molecular Biology
Michigan State University

"Galectin-3: a Carbohydrate Binding Protein in the Cell Nucleus"

Abstract

Galectin-1 (Gal1) and galectin-3 (Gal3) are two members of a family of carbohydrate-binding proteins that exhibit dual localization, being found in both the intracellular (cytoplasm and nucleus) as well as the extracellular (cell surface and medium) compartments of cells. The mechanism of externalization appears to be unusual because there does not seem to be a typical signal sequence for secretion via the endomembrane pathway. Nevertheless, the literature on the galectins has been dominated by studies focused on their activity on the extracellular side, based on binding to cell surface glycoconjugates and adhesive glycoproteins of the extracellular matrix. On the other hand, Gal3 shuttles between the cytoplasm and nucleus inside cells and importin-mediated nuclear localization signal and exportin-1-mediated nuclear export signal have been identified on the polypeptide.

The laboratories of Dr. Ron Patterson (Microbiology) and John Wang (Biochemistry) at Michigan State University have been studying the intracellular activities of Gal1 and Gal3. Our interest in these proteins stemmed from the observation that they could be found in the nucleus, in the form of ribonucleoprotein complexes. Several key findings suggest that they are two of the many proteins involved in the splicing of pre-mRNA: (a) nuclear extracts derived from HeLa cells, capable of carrying out splicing of pre-mRNA in a cell-free assay, contained both Gal1 and Gal3; (b) depletion of both galectins from nuclear extract, either by antibody adsorption or by lactose affinity chromatography, resulted in concomitant loss of splicing activity; (c) either recombinant Gal1 or Gal3 was able to reconstitute splicing activity in a galectin-depleted extract; and (d) when a splicing reaction containing ^{32}P-labeled pre-mRNA is subjected to immunoprecipitation with either anti-Gal1 or anti-Gal3, radiolabeled RNA species corresponding to the starting substrate, intermediates of the splicing reaction, and the mature mRNA products of active spliceosomes are all co-precipitated with the specific galectin. Consistent with these observations, we have recently documented a mechanism by which Gal3 is assembled onto the forming spliceosome.

(1) Wang, J.L., Gray, R.M., Haudek, K.C. and Patterson, R.J. (**2004**) Nucleocytoplasmic lectins. *Biochim. Biophys. Acta* 1673: 75-93.

(2) Dagher, S.F., Wang, J.L. and Patterson, R.J. (**1995**) Identification of galectin-3 as a factor in pre-mRNA splicing. *Proc. Natl. Acad. Sci.* USA 92: 1213-1217.

(3) Wang, W., Park, J.W., Wang, J.L. and Patterson, R.J. (**2006**) Immunoprecipitation of spliceosomal RNAs by antisera to galectin-1 and galectin-3. *Nucleic Acids Res.* 34: 5166-5174.

(4) Gray, R.M., Davis, M.J., Ruby, K.M., Voss, P.G., Patterson, R.J. and Wang, J.L. (**2008**) "Distinct effects on splicing of two monoclonal antibodies directed against the amino-terminal domain of galectin-3," *Arch. Biochem. Biophys.* 475: 100-108.

Biography of John L. Wang

John Wang received his undergraduate degree in 1968 from Dartmouth College in Hanover, NH, where he majored in chemistry. He did his graduate work at The Rockefeller University in New York, NY and earned a Ph.D. degree in 1973. He stayed at The Rockefeller University, first as a Damon Runyon post-doctoral fellow (1973-75) and then as an Assistant Professor (1975-77). He then joined the faculty in the Department of Biochemistry at Michigan State University, where he has been Professor since 1985. In 1996, Michigan State University honored him with a Distinguished Faculty Award.

He has served the scientific community in several capacities. He was on the NIH Cell Biology and Physiology Study Section from 1988-1992, the American Cancer Society Peer Review Committee on Molecular and Cell Biology of Cancer from 1993 through 1998, and chairing the latter committee in 1998. He was on the editorial boards of the journal PLANTA (1990-1996) and The Journal of Biological Chemistry (1992-1997; 1998-2003). Most recently, he has served as a member of the Board of the Directors of the Van Andel Institute Graduate School, in which a distinctive graduate program in molecular, cellular, and genetic biology has been established.

Program

Friday Morning
Session Chair: Holly Goodson

7:30-8:30 Continental breakfast

8:30-8:40 Introduction and Orientation

8:40-9:00 Don D. Snedden, Dominic Vernon and Paul W. Huber
Examining the Role of E2 and VM1 Motifs in the Localization of mRNAs to the Animal Hemisphere of Xenopus Oocytes

9:00-9:20 Hector Guillen Ahlers, Steven Buechler, Mark Suckow, Victoria A. Ploplis and Francis J. Castellino
The Effects of Sulindac on a Colon Cancer Mouse Model and a Human Colon Cancer Cell Line

9:20-9:40 Kamlesh K. Gupta, Michelle V. Joyce, Aranda R.. Slabbekoorn, Zhiqing Zhu, Benjamin A. Paulson, Bill Boggess and Holly V. Goodson
Probing Interaction Between CLIP-170 and Microtubules

9:40-10:00 Xiaosong Hu, Ian Carmichael and Anthony S. Serianni
Amide CTI in Aqueous Solutions of N-Formyl-D-glucosamine and N-Acetyl-D-glucosamine: Chemical Equilibria and Exchange Kinetics

10:00-10:45 Mid-Morning Break

10:45-11:15 **Guest Speaker**
Prof. Joseph O'Tousa, Department of Biological Sciences, University of Notre Dame
Control of the Visual Response by Protein Phosphorylation

11:15-11:35 Kimberly A. Wilson, Tao Peng and Jeffrey W. Peng
Understanding the Sequence-dynamics-binding Affinity Relationship for WW Domains With Different Recognition Loop Sequences

11:35-11:55 Alexander G. White and Bradley D. Smith
Optical Imaging of Bacterial Infection in Murine Models Using a Novel Synthetic Near-infrared Fluorescent Probe

11:55-12:15 Francis K. Insaidoo, Oleg Y. Borbulevych and Brian Baker
Control of T-cell Receptor Recognition by Antigen Conformational Dynamics

12:15-2:15 Lunch and Recreation

Friday Afternoon
Session Chair: Paul W. Huber

2:15-2:45 **Guest Speaker**
Prof. Molly Duman Scheel, Indiana University School of Medicine – South Bend
Netrin and DCC: Axon Guidance Regulators at the Intersection of Nervous System Development and Cancer

2:45-3:05 Carrie S. Miller and Steven A. Corcelli
Carbon-Deuterium Vibrational Probes of Amino Acid Protonation State

3:05-3:25 Oleg Y. Borbulevych and Brian Baker
Conformational Heterogeneity of the CDR3b Lop Accounts for the Wide Spectrum of TCR A6 Cross-reactivity

3:25-3:45 Zhi Xu, Francis J. Castellino and Victoria A. Ploplis
A Deficiency of Plasminogen Activator Inhibitor-1 Results in Spontaneous Cardiac Fibrosis

3:45-4:05 Richard Besingi and Patricia Clark
Characterization of the VirG Autotransporter from Yersinia pestis

4:05-4:25 Kurt Piepenbrink and Brian Baker
Assessing the Physical Basis of TCR-MHC Recognition

4:25-5:30 Late Afternoon Break

Friday Evening

5:30-6:30 **Keynote Lecture**
Professor John Wang, Department of Biochemistry and Molecular Biology, Michigan State University
Galectin-3: A Carbohydrate Binding Protein in the Cell Nucleus

6:30-8:15 Poster Session and Reception

8:15-10:15 Dinner

10:15 Departure

ABSTRACTS: GUEST SPEAKERS

Control of the Visual Response by Protein Phosphorylation

Joseph E. O'Tousa[1], Alexander V. Kiselev[1], and Kirk L. Mecklenburg[2]
[1]Dept. of Biological Sciences, Univ. of Notre Dame, Notre Dame, IN, USA
[2]Dept. of Biology, Indiana University South Bend, South Bend, IN, USA

Many proteins active in the biochemical pathways underlying the photoreceptor responses are regulated by light-dependent phosphorylation. A well-studied example is rhodopsin phosphorylation, which together with arrestin proteins, is responsible for deactivation of rhodopsin and other G-protein-coupled receptors (GPCRs). We found that both monoclonal and polyclonal antibodies directed against the Rh1 C-terminal region are unable to bind if Rh1 has been phosphorylated within the last 17 amino acid residues of the C-terminal domain. We have used these reagents to study rhodopsin phosphorylation in mutant strains lacking arrestin. Our results show that arrestin is required for receptor phosphorylation. These results provide clear evidence that arrestin binding to the activated GPCRs is the first step of the deactivation process, and then the GPCR/arrestin complex is the substrate for GPCR kinase.

Rhodopsin is one of many photoreceptor proteins that are phosphorylated when photoreceptor cells respond to light. There are fewer examples of proteins that become dephosphorylated by light exposure. We have carried out a molecular analysis of *Drosophila* Retinophilin (RTP) and show that RTP is localized to the photosensitive rhabdomeric membranes and phosphorylated under dark conditions. To study the role of the protein, RTP mutants were created. These exhibit a high rate of spontaneous membrane depolarization events in dark conditions but show near normal light response kinetics. The results indicate RTP is required to suppress dark noise, most likely by regulating the membrane-associated activities of phototransduction signaling components. The role of RTP phosphorylation in these processes is now under investigation.

Netrin and DCC: Axon Guidance Regulators at the Intersection of Nervous System Development and Cancer

Molly Duman Scheel[1,2], Adrienne VanZomeren-Dohm[1], Paul Beach[3], and Wendy Simanton[1,3]
(1) Indiana University School of Medicine-South Bend at Notre Dame, South Bend, IN 46556 USA (2) University of Notre Dame, Department of Biological Sciences, Walther Cancer Institute, and Eck Institute for Global Health, Notre Dame, IN 46556 USA (3) Albion College, Biological Sciences Department, Albion, MI, USA

Cell growth and migration, important aspects of nervous system development and cancer, occur in the context of surrounding cells. It is therefore useful to study these processes in genetically tractable animal models in which a cellular context is maintained. The *Drosophila* imaginal discs are excellent systems in which to study cell growth and invasive migration, as it is possible to model human tumors through the generation of somatic clones of cells bearing multiple genetic lesions. This investigation takes advantage of these attributes to study Netrin (Net) and its receptor Deleted in Colorectal Cancer (DCC). These proteins, originally identified for their roles in nervous system development, have also been linked to cancer. However, the impacts of these signaling molecules on invasive cellular growth are not well understood. The impacts of modulating Net-DCC signaling on cellular growth were assayed through use of several standard *Drosophila* growth assays in the wing imaginal disc. Ectopic Net signaling was found to induce cellular growth and expression of several key growth regulators. These data, which were gathered in non-neural cells, illustrate that Net signaling can promote growth through induction of cellular growth regulators, and that these effects on growth are separable from the impacts of Net signaling on axon guidance. The impacts of loss of Net-DCC signaling were also assessed. Somatic clones lacking DCC display high levels of growth regulators, hyperproliferate, and exhibit characteristics of metastatic cells. Although loss of region 18q, which contains the human DCC gene, has been associated with many human cancers, these data are the first demonstration that loss of DCC can result in hyperproliferation and metastasis.

ABSTRACTS: ORAL PRESENTATIONS

Examining the Role of E2 and VM1 Motifs in the Localization of mRNAs to the Animal Hemisphere of *Xenopus* Oocytes

Don D. Snedden, Dominic Vernon, and Paul W. Huber
Department of Chemistry and Biochemistry, University of Notre Dame,
Notre Dame, IN 46556 USA

PHAX is a phosphoprotein that is required for the nuclear export of spliceosomal U snRNAs and intranuclear transport of U3 snoRNA. The 3'-untranslated region (UTR) of the mRNA encoding PHAX contains sequence elements that bind proteins involved in the localization of mRNAs to the vegetal hemisphere of *Xenopus* oocytes. These include E2 motifs that bind Vg1RBP/Vera, VM1 motifs that bind VgRBP60/hnRNP I, and a binding site for VgRBP71. *In situ* hybridization experiments establish that PHAX mRNA is indeed localized in oocytes; however, the mRNA accumulates at the opposite, animal, hemisphere. Examination of the 3'-UTRs of other mRNAs that are localized to the animal hemisphere, including An1 and An3, reveals that they also contain multiple E2 and VM1 motifs. UV-crosslinking experiments in oocyte extract reveal that mRNAs that move to different hemispheres of the oocyte bind an identical group of proteins, in accord with the presence of similar sequence motifs in the 3'-UTRs of all localized mRNAs examined. Mutation of E2 or VM1 motifs in Vg1 mRNA eliminates binding of Vg1RBP/Vera or VgRBP60/hnRNP I, respectively, and abolishes localization of the mRNA, establishing the requirement of these proteins for transport. Unexpectedly, elimination of these motifs from An1 does not effect localization of this mRNA. Crosslinking experiments are underway to determine how these mutations affect the profile of proteins that bind in the 3'-UTR of An1.

The Effects of Sulindac on a Colon Cancer Mouse Model and a Human Colon Cancer Cell Line

Hector Guillen Ahlers[1,2], Steven Buechler[4], Mark Suckow[3,5], Victoria A. Ploplis[1,2,3] and Francis J. Castellino[1,2,3]

[1]W. M. Keck Center for Transgene Research, [2]Department of Chemistry and Biochemistry, [3]Notre Dame Cancer Institute, [4]Department of Mathematics, and [5]Freimann Life Science Center, University of Notre Dame, Notre Dame, IN 46556 USA

Non-steroidal anti-inflammatory drugs (NSAIDs) have shown potential as chemo-preventive agents against cancer formation, especially colorectal cancers. However, the mechanisms by which these drugs act are not fully understood. In this study, $Apc^{Min/+}$ mice were treated with sulindac, a NSAID that functions as a Cyclooxygenase (COX) inhibitor. These mice demonstrated a tumor reduction of over 80%, consistent with previous reports. Gene microarray analyses of RNA from adenoma-derived dysplastic epithelial cells revealed that collagen genes, viz., *Col1a2, Col5a2, Col6a2,* and *Col6a3,* were upregulated, and matrilysin/metalloproteinase 7 (*Mmp7*) was downregulated, in sulindac-treated mice. Validation of the gene expression profile of the *Col6a2* subunit of collagen VI and of *Mmp7* was confirmed by RT-PCR. Confocal microscopy and immunofluorescence showed that collagen VI was present in low amounts within the tumors of nontreated mice and was enhanced within the tumors of sulindac-treated mice. Collagens I and V demonstrated similar patterns, but were not as prominent as collagen VI. Mmp7 was found in 'hot spot' areas within the tumors of $Apc^{Min/+}$ mice treated with the vehicle, but was greatly diminished in those treated with sulindac. Studies with $Apc^{Min/+}/Mmp7^{-/-}$ double-deficient mice demonstrated the reciprocal relationships of *Mmp7* expression and the levels of these three collagens *in vivo*. Gene expression in the human colon cancer cell line HT-29, showed that *MMP7* and *HMGB1* were downregulated after sulindac treatment, resembling what was observed in mice. Enzymatic assays demonstrated that sulindac treatment altered gelatinase activity. Activity-based protein profiling (ABPP) targeted to serine hydrolases and metallohydrolases revealed a sulindac effect on many of these members.

The results of this study demonstrated that sulindac is effective in increasing the expression of different collagens and decreasing the expression of *Mmp7*, and *Hmgb1*. These effects are also seen in the human colon cancer cell line HT-29 and may contribute to altered tumor burden in cancer patients undergoing NSAIDS treatments.

Probing Interaction Between CLIP-170 and Microtubules

Kamlesh K. Gupta, Michelle V. Joyce, Aranda R. Slabbekoorn, Zhiqing Zhu, Benjamin A. Paulson, Bill Boggess and Holly V. Goodson

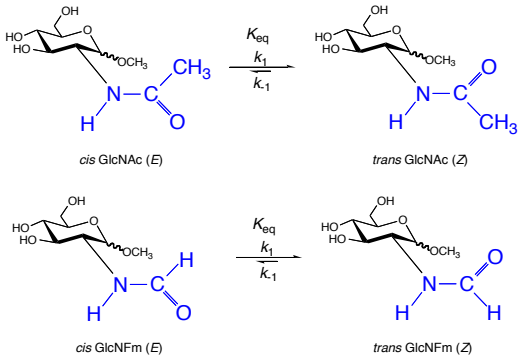
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA

CLIP-170 is the prototype microtubule (MT) plus-end tracking protein (+TIP) that dynamically localizes to the MT plus end and is involved in regulating MT dynamics. The mechanisms of these activities remain unclear because the CLIP-170-MT interaction is poorly understood, and also because the impact of interactions between different +TIPs remains inadequately defined. We are using a variety of biochemical and biophysical approaches to address these questions. First, we find that each CLIP-170 monomer has multiple (as many as five) MT binding sites: each CAP-Gly and serine-rich region has some affinity for MTs. It is generally understood that CLIP-170 binds to the C-terminal tail of α-tubulin, but the presence of multiple tubulin binding sites on CLIP-170 suggests that CLIP-170 might bind more than one region of tubulin. Using a combination of cross-linking and mass-spectrometry, we find that CLIP-170 binds to both α- and β-tubulin, and that it binds to sites in addition to the α-tubulin C-terminal tails. We provide evidence that these additional binding sites include the H12 helices of both α- and β-tubulin and are significant for CLIP-170 activity. These observations help to explain how CLIP-170 promotes MT polymerization and nucleation and provide insight into CLIP-170 function and plus-end tracking mechanism.

Amide CTI In Aqueous Solutions of *N*-Formyl-D-glucosamine and *N*-Acetyl-D-glucosamine: Chemical Equilibria and Exchange Kinetics

Xiaosong Hu,[1] Ian Carmichael[2] and Anthony S. Serianni[1]
[1]Department of Chemistry and Biochemistry, and [2]Radiation Laboratory, University of Notre Dame, Notre Dame, IN 46556 USA

Amide *cis*-*trans* isomerization (CTI) is an important exchange process in biological systems. In proteins, especially those that are proline-rich (*e.g.,* collagen), the configuration of Xaa-Pro peptide bonds influences the pathway of protein folding and determines the final protein tertiary structure. Amide *cis*-*trans* isomerization in glycobiology is less prominent than in proteobiology, largely because glycosidic linkages are involved in the assembly of oligo/polysaccharides rather than peptide bonds. However, some biologically important saccharides contain amide bonds as part of a side-chain substituent, most notably the *N*-acetyl side-chain in *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-neuraminic acid (Neu5Ac). While it is commonly held that amide configuration in GlcNAc monomers is exclusively *trans*, it is known that, in some glycoprotein X-ray structures, GlcNAc residues are observed with the *N*-acetyl side-chain in the *cis* configuration. In addition, examples have been reported where proteins bind GlcNAc-containing ligands in the *cis* form. These observations led us to ponder whether the amide *cis*–*trans* equilibrium can be detected and quantified in aqueous solutions of GlcNAc or GlcNAc-containing compounds. In this report, we describe the use of NMR approaches to detect and quantify *cis*-GlcNAc and *trans*-GlcNAc isomers in aqueous solution. Using saturation-transfer NMR methods, first-order rate constants and activation parameters for *cis*-*trans* isomerization have been measured and compared to those measured previously for peptide bonds. We show that the *cis*:*trans* equilibrium and exchange kinetics depend on GlcNAc anomeric configuration and side-chain structure (*N*-acetyl *vs* *N*-formyl), demonstrating the importance of local structure on this side-chain rearrangement.



Understanding the Sequence-dynamics-binding affinity Relationship for WW Domains with Different Recognition Loop Sequences

Kimberly A. Wilson, Tao Peng and Jeffrey W. Peng

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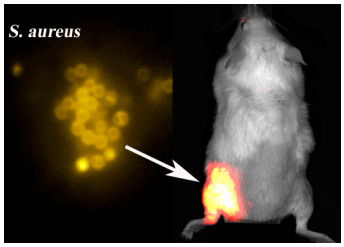
The WW domain from human peptidyl-prolyl isomerase PIN1 is a member of the family of proline binding modules mediating protein-protein interactions in signaling networks. Several of these interactions affect cancer, Alzheimer disease and Huntington disease. The PIN1-WW domain binds phopho-Ser/Thr-Pro motifs through loop I (16-Ser-Arg-Ser-Ser-Gly-Arg-21). Previous work from our lab demonstrated that a different loop I sequence (S19 deletion) causes a different binding affinity *and* different dynamics compared to the wild type. This suggests that different sequences encode for different binding related motions. We expanded on this hypothesis to include loop I mutations: 17-Lys-Ser-Lys-Lys-20, 17-Arg-Ser-His-Asn-20, and S19T.

Optical Imaging of Bacterial Infection in Murine Models using a Novel Synthetic Near-infrared Fluorescent Probe

Alexander G. White and Bradley D. Smith
Department of Chemistry and Biochemistry and the Notre Dame Integrated Imaging Facility, 251 Nieuwland Science Hall, University of Notre Dame, Notre Dame, IN 46556 USA

Recent advances in optical imaging of bacterial infection have been propelled by the invention of genetic methods that produce fluorescent and bioluminescent bacteria, and also the discovery of synthetic fluorescent probes that selectively target bacterial cell surfaces. Optical imaging is an effective method for conducting longitudinal studies of bacterial infection in small animals. It can be used to address questions in medical microbiology concerning migration and colonization and it is an attractive method for determining the efficacy of antibiotic therapies. We have discovered that synthetic zinc coordination complexes can target the anionic molecular structures on the bacterial surface. Furthermore, fluorescent conjugates of these complexes can very selectively target bacterial cells in the presence of healthy animal cells, and we have successfully imaged localized bacterial infection in living animals (*Bioconj. Chem*. **2008**, *19*, 686-692). Here we report a second-generation bacterial imaging probe that exhibits superior performance. We can detect localized murine infections of Gram-positive *Staphylococcus aureus* at levels of 10^6 CFU. The probe clearance time is only a few hours and it can be used to conduct longitudinal imaging studies that evaluate the efficacy of antibiotic drugs in living animals.



Control of T cell Receptor Recognition by Antigen Conformational Dynamics

Francis K. Insaidoo, Oleg Y. Borbulevych, and Brian M. Baker
Department of Chemistry & Biochemistry, 251 Nieuwland Science Hall, University of Notre Dame, Notre Dame, IN 46556 USA

In an immune response, activation of cytotoxic T cells depends on T cell receptor recognition of antigenic peptides stably bound and presented by class I MHC proteins. The stability of the MHC I peptide/protein complex depends on the sequence of the bound peptide. For the common allele HLA-A2, the optimum peptide sequence has leucine, methionine, or valine at the "anchor" positions of 2 and Ω. However, many poorly immunogenic viral and tumor antigens have alternative amino acids at the anchor positions and hence bind weakly to the MHC protein. The conventional approach for enhancing the immunogenicity of such antigens is to replace suboptimal anchors with optimal amino acids. This approach has most successfully been applied to the gp100 melanoma antigen, where replacement of a suboptimal threonine at position 2 with leucine or methionine significantly enhances peptide binding affinity and immunogenicity. However, in the case of the MART-1 melanoma antigen (AAGIGILTV), despite improving peptide binding 40-fold, replacement of the suboptimal alanine at position 2 with leucine (ALGIGILTV) completely abolishes T cell recognition. Although crystallographic structures indicated that the native and modified peptides adopt similar overall conformations in the peptide binding groove, analysis of multiple crystal forms suggested that the leucine substituted peptide is more dynamic than the native peptide, with the center of the peptide occupying multiple positions in different structures. The results of unrestrained molecular dynamics simulations at multiple temperatures tracked very closely with the crystallographic results, showing that the modified peptide samples all the conformations observed crystallographically. ^{15}N and ^{13}C FAST-HQSC NMR experiments on peptide/MHC complexes with the peptide labeled only at position 5 ($^{13}C/^{15}N$ glycine) confirmed the enhanced dynamics of the modified peptide, with multiple cross-peaks present for the modified peptide, but only a single cross-peak present for the native peptide. Collectively, these observations reveal that contrary to the usual considerations regarding peptide antigenicity, antigen dynamical properties can profoundly influence T cell recognition. Further, these observations demonstrate that modifications to peptide anchors can have dramatic unanticipated consequences such as increasing antigen molecular dynamics despite enhancing peptide binding affinity.

Carbon-Deuterium Vibrational Probes of Amino Acid Protonation State

Carrie S. Miller and Steven A. Corcelli
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA

The protonation state of titratable amino acid residues has profound effects on protein stability and function. Therefore, correctly determining the acid dissociation constant, p*K*a, of charged residues under physiological conditions is an important challenge. The general utility of site-specific carbon-deuterium (C–D) vibrational probes as reporters of the protonation state of arginine, aspartic acid, glutamic acid, and lysine amino acid side chains was examined using density functional theory (DFT) calculations. Substantial shifts were observed in the anharmonic vibrational frequencies of a $C–D_2$ probe placed immediately adjacent to the titratable group. Lysine exhibited the largest $C–D_2$ frequency shifts upon protonation, 44.9 cm^{-1} (symmetric stretch) and 69.5 cm^{-1} (asymmetric stretch). Furthermore, the predicted harmonic intensities of the $C–D_2$ probe vibrations were extraordinarily sensitive to protonation state of the nearby acidic or basic group. Accounting for this dramatic change in intensity is essential to the interpretation of an infrared (IR) absorption spectrum that contains the signature of both the neutral and charged states.

Conformational Heterogeneity of the CDR3β Loop Accounts for the Wide Spectrum of TCR A6 Cross-reactivity

Oleg Y. Borbulevych and Brian M. Baker
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556 USA

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. TCR antigen specificity is conferred by three hypervariable CDR loops of the α and β variable domains, which together generate a unique TCR binding surface. A paradox of the TCR is its inherent antigen cross-reactivity, now recognized as an essential feature of T cell biology. A fundamental question remaining to be answered is what determines the degree of cross-reactivity for a particular TCR?

The crystallographic structures of the A6 TCR represent the largest database of the same TCR bound to different pMHC ligands (12 structures with different pMHC molecules). Notably, A6 CDR3α has a similar conformation in all structures while CDR3β shows significant conformational variations.

We determined the crystal structure of unligated TCR A6 to 2.2 Å resolution and carried out 50ns molecular dynamics (MD) simulations of this receptor. Together this data allow us to illuminate the basis for cross-reactivity of the TCR A6.

In the free TCR structure, CDR3β adopts a distinct conformation which is different than that seen in any of the complexes. The CDR3β loop is highly disordered. Remarkably, in the MD simulations, CDR3β samples all conformations observed in the 12 A6-pMHC crystal structures, indicating that conformational selection from a broad CDR3β ensemble determines cross-reactivity.

A Deficiency of Plasminogen Activator Inhibitor-1 Results in Spontaneous Cardiac Fibrosis

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While it is relatively well established that decreased uPA activity prevents cardiac fibrosis, the role of its main inhibitor, plasminogen activator inhibitor-1 (PAI-1), remains controversial. In this study, we sought to determine the effects of a PAI-1 deficiency on the spontaneous development of cardiac fibrosis. Mice with a PAI-1 deficiency (PAI-$1^{-/-}$) spontaneously develop cardiac fibrosis as they age. Fibrosis is not localized within specific regions of the heart, but rather pervasive throughout the entire tissue. PAI-$1^{-/-}$ mice displayed significantly thickened left ventricular posterior walls and decreased left ventricular internal diameters compared to age-matched wild type (WT) mice, as determined by high-resolution echocardiography. A markedly reduced cardiac function was also observed in PAI-$1^{-/-}$ mice, as indicated by the reduction of left ventricular ejection fraction and fractional shortening. In an effort to mechanistically elucidate the role of PAI-1 in cardiac fibrosis, 12 week WT and PAI-$1^{-/-}$ mice were chosen to study the biological processes leading to cardiac fibrosis formation. Although no fibrosis was observed in both genotypes at this early age, PAI-$1^{-/-}$ hearts presented with elevated levels of inflammation, as indicated by increased expression of iNOS, iCAM, and KC. Macrophage infiltration into cardiac tissue was significantly increased in PAI-$1^{-/-}$ mice. To further investigate the effect of PAI-1 on leukocyte migration and infiltration, a thioglycollate-challenged peritonitis model was employed. More leukocytes migrated into the peritoneum of PAI-$1^{-/-}$ mice upon challenge than in WT mice, suggesting a pivotal role of PAI-1 in regulating leukocyte recruitment to a site of inflammation. PAI-$1^{-/-}$ hearts showed signs of vascular leakage and bleeding, as demonstrated by hemosiderin deposition, as well as increased level of extravasated Evans Blue dye in heart compared to WT mice. Furthermore, the expression levels of several proteases and inhibitors, including uPA, MMP2, and TIMP2, were significantly higher in PAI-$1^{-/-}$ hearts, suggesting active cardiac remodeling associated with the fibrotic pathology. A key fibrogenic cytokine, TGF-ß, was markedly enhanced in PAI-$1^{-/-}$ mice. Results from this study demonstrated that a lack of PAI-1 in the heart results in spontaneous cardiac fibrosis formation accompanied by restrictive cardiomyopathy and compromised cardiac function. The effects of a PAI-1 deficiency on cardiac fibrosis were mediated, at least in part, by increased local inflammation, enhanced leukocyte infiltration, and increased extracellular matrix remodeling, an environment conducive to accelerated fibrosis.

Characterization of the VirG autotransporter from *Yersinia pestis*

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VirG is a virulence-associated protein of *Yersinia pestis*, the causative agent of bubonic plague. It belongs to the family of autotransporter (AT) proteins, and like other ATs, it consists of a signal sequence that directs secretion across the inner membrane (IM) via the *sec* apparatus, a central passenger domain (mature virulence protein), and a C-terminal porin that inserts and forms a β-barrel in the outer membrane (OM), through which the passenger is transported to the exterior of the cell.

Like most other ATs, the VirG passenger is predicted to adopt α-helical structure, which we have proposed is important in AT biogenesis. Chemical unfolding studies of two unrelated ATs, pertactin (from *Bordetella pertussis*) and Pet from a pathogenic strain of *E. coli* have shown that the C-terminus of the passenger domain is more stable than the N-terminus, and this stable core is presumably important in transport across the OM. This study aims to identify whether VirG also has a stable core, and also to identify other structural characteristics common to ATs. Moreover, understanding the folding and secretion of VirG is important to its development as a drug target.

We PCR amplified the VirG gene from *Y. pestis* genomic DNA, and established an expression system in *E. coli*. The gene encodes a 131 kDa preproprotein. N-terminal sequence analysis identified the first eight residues of the VirG preproprotein, suggesting the signal sequence is not cleaved. OM purification of the transformed cells shows that VirG co-purifies with the OM. However, a small fraction (<10%) of the protein from OM preparations lacks the first 93 amino acids. Limited protease digestion of intact cells expressing VirG causes the disappearance of the VirG band even at very low protease concentrations. Hence, unlike pertactin and Pet, the VirG passenger domain does not seem to be resistant to protease digestion. We have also established expression systems for the VirG passenger domain, lacking both the predicted signal sequence and the C-terminal β-porin. The passenger domain has been purified, and the protein secondary structure and thermal stability determined by far-UV CD spectroscopy. Our preliminary results suggest that the purified protein is folded, with a characteristic spectrum of a β-sheet protein. Also, thermal denaturation suggests the VirG passenger unfolds in two transitions, similar to the behavior of pertactin and Pet.

Assessing the Physical Basis of TCR-MHC Recognition

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The ability of T-cell receptors to bind MHCs presenting antigenic peptides with a much stronger affinity than those presenting non-antigentic peptides has implied that TCR / peptide interactions may be more significant energetically than TCR / MHC interactions. Other studies have concluded that a small number of conserved TCR/pMHC contacts may be both neccesary and sufficient for recognition. Many of these models also presume that since, in the context of a single organism's restricted T-cell repertoire, the peptide is the determinant for immunopotency, TCR's interactions with the peptide must be more significant energetically than TCR/MHC interactions.

Here we probe the strength of various side-chain interactions between the pMHC and the TCR through the use of double-mutant cycles. In a double mutant cycle, by comparing the affinity of the wild type interaction, single alanine mutations of each of the target amino acids (eliminating the contacts between those side chains and the binding partner) and the double-mutant interaction when both amino acids are mutated to alanine, we can quantitatively measure the strength of the interaction between the two residue side-chains by subtracting the single mutant free energy changes from the wild type free energy change and adding the double mutant free energy change; $\Delta G_{int} = \Delta G\ (X_{wt}\text{-}Y_{wt}) - \Delta G\ (X_{Z\rightarrow A}\text{-}Y_{wt}) - \Delta G\ (X_{wt}\text{-}Y_{Z\rightarrow A}) + \Delta G\ (X_{Z\rightarrow A}\text{-}Y_{Z\rightarrow A})$.

Double-mutant cycles allow us to compare the contributions to affinity from contacts made by the germline CDR 1&2 loops and the variable CDR3 loops between the A6 TCR and the HLA-A2/tax9 pMHC. Although much work remains to be completed, we have found sidechain interactions of significant importance to binding (>1 kcal) in the periphery of the peptide and on the helices of the MHC, but so far have found no such contacts in the central region of the TCR/peptide interface. However some TCR/MHC contacts have been found to be of very large energetic importance to binding. This suggests that the A6-HLA-A2/tax9 interaction may be driven not by favorable CDR3/peptide interactions but by an absence of unfavorable interactions in the central region of the interface.

ABSTRACTS: POSTERS

1. Structural Adaptability of TCR and Peptide/MHC Binding Sites

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Selective recognition of a potential antigen by a T-cell is governed by the interactions of the T-cell receptor's complementarity determining regions (CDRs) with the MHC and its presented peptide. These CDR "loops" possess measurable degrees of flexibility, and it is suspected that the conformational sampling of a T-cell receptor (TCR) enables it to bind multiple ligands, thus expanding the TCR's antigen-recognizing repertoire. Examining the dynamics of CDR loops in their pre-binding state ("free" TCR) should reveal pertinent details of how several pMHCs are recognized by a single TCR. Recent studies have demonstrated that cross-reactivity may also be an effect of antigen-dependent dynamics, making it necessary to assess the structural adaptability of both sides of the TCR/pMHC binding interface to more completely model T-cell recognition. This study utilizes time-resolved fluorescence anisotropy (TRFA) to quantify the mobility of CDR loops and the peptide-presenting MHC platform. TRFA measurements require a fluorescent tag covalently bound to a mutated cysteine within the peptide sequence of interest. The fluorophore's orientation depolarizes over the span of its fluorescence lifetime, thereby reflecting the flexibility of the labeled region.

Specifically, the A6 TCR is cross-reactive with two pMHC complexes, HLA-A2/Tax and HLA-A2/Tel1p, making these ideal systems for dynamic studies. TRFA results for both pMHC complexes indicate that the central region of the presentation platform of HLA-A2 is more dynamic when Tel1p is bound. The higher degree of flexibility measured for the HLA-A2/Tel1p binding surface alludes to the structural adaptation the pMHC complex undergoes in order to be recognized by A6 TCR. Crystal structures of free and bound HLA-A2 with both Tax and Tel1p corroborate this binding mechanism: HLA-A2/Tel1p has a different binding solution than in its free state, whereas the Tax complex maintains its free conformation during recognition. Further studies on the A6 CDR loops will complement this binding system. TRFA measurements on a single-chain TCR variant, 2C, have also been collected and are in the preliminary stages of analysis.

2. Synthesis and NMR Studies of Mannose-Containing Di- and Trisaccharides

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Multiple ^{13}C isotopomers of di- and trisaccharides containing D-mannose (Man), representing nested fragments of high-mannose oligosaccharides commonly found covalently attached to human glycoproteins, were synthesized in 10-100 mg quantities using optimized chemical methods. Structures included α-Man-(1→3)-α-$ManOCH_3$, α-Man-(1→6)-α-$ManOCH_3$, α-Man-(1→2)-α-Man-(1→3)-α-$ManOCH_3$, and α-Man-(1→2)-α-Man-(1→6)-α-$ManOCH_3$. NMR studies of these labeled compounds at 500, 600 and 800 MHz yielded trans-glycoside J_{CC} and J_{CH} values (and other scalar couplings) within related structures, providing conformational information on their constituent linkages and information on the effect of molecular context in dictating preferred linkage conformation in solution. The effect of structural context was examined by comparing corresponding trans-glycoside *J*-couplings observed in the reference disaccharides to those in the trisaccharides (TriMan). NMR results were supplemented by the molecular dynamics simulations (MD) using AMBER and the *Glycam*04 force-field.

3. Understanding Cross-Reactivity Exhibited by MART-1 Specific TCRs

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The interaction of a T cell receptor (TCR) with a ligand bound to class I or II major histocompatibility complex molecules (pMHC) initiates T cell activation. We are interested in studying TCR binding specificity and cross-reactivity, focusing on epitopes from the MART-1 tumor protein presented by the class I MHC HLA A2. The MART-1 protein has two overlapping antigenic epitopes of particular interest: the 26-35 decamer and the 27-35 nonamer. These peptides have been shown in our previous work to adopt very different conformations when presented by HLA-A2; the decamer adopts a more bulged conformation compared to the nonamer, which adopts an extended structure. Nonetheless, many T cell clones cross-react with these two epitopes. To enhance TCR recognition, sub-optimal N-terminal anchor residue on the peptide was modified to a more optimal leucine. Sometimes modification of anchor residues have improved peptide and MHC interaction with each other and consequently improved TCR recognition. Interestingly, in our studies TCR cross-reactivity between the two native anchor-modified pMHC complexes was changed, where the altered nonameric peptide was no longer being recognized. Only the decameric variant showed interaction with most MART-1 recognizing TCRs. Despite the modified nonameric peptide binding to the MHC forty-times better than the native peptide, most TCRs showed no detectable interaction with this ligand. An exception to this observation was seen with a specific TCR, JKF6, which was able to cross-react with the nonamer and decamer, as well as their anchor-modified variants. Our binding results are supported by previously published data, which demonstrated that JKF6 cytotoxic T-lymphocytes were activated by both native nonamer and decamer, as well as their anchor-modified constituents, respectively. Here, we present surface plasmon resonance (SPR) data for the cross-reactive TCR JKF6, and, another classical MART-1 recognizing TCR, LAU444, interacting with the MART1 peptide and its variants. The results indicate that JKF6 TCR preferably interacts better with the native and anchor-modified nonameric peptide whereas LAU444 TCR can cross-react with both native MART-1 epitopes, but only the anchor-modified decameric ligand. Our data is beginning to illuminate how TCRs are able to cross-react with structurally dissimilar ligands, yet exhibit fine specificity between structurally comparable and almost chemically identical peptides.

4. Targeting Ebola Virus at the Plasma Membrane

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Ebola is a negative stranded RNA virus. Its pathogenesis is characterized by internal and external bleeding in primates as a result of coagulation abnormalities induced by the virus at the onset of the infection. Since its first discovery in 1979, no specific treatment or vaccines have been found. It is classified as biosafety level IV agent and therefore has the potential to be weaponized. While mechanistic details of the virus assembly process are lacking, recent evidence suggests that the two matrix proteins VP40 and VP24 play a crucial role in the budding of the virus. Generation of new virus involves a cascade of cellular events that recruits 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 studies suggest that VP40 and 24 bind lipid membranes with very high affinity and possess the ability to modify membrane structure, which is crucial for the egress of the virus. In this proposed research we seek to elucidate the mechanistic details of lipid interaction by Ebola matrix proteins and their mechanism of generating new viral particles. Specifically, we seek to determine (1) the membrane targeting and curvature mechanisms VP40 and VP24, (2) the interplay of F-actin targeting of V24 and its membrane curvature ability, and (3) the lipid dependent interaction properties of VP40 and human Nedd4 in the generation of Ebola VLPs. Methodologies to be employed in the studies include (a) an array of biophysical methods to analyze the interaction of matrix proteins with various membrane models. These include: monolayer, surface plasmon resonance, sedimentation and stop-flow analysis. (b**)** Cellular targeting and trafficking of the matrix proteins using fluorescent tags. (c**)** Structural investigation of the membrane curvature process with X-ray and fluorescent methods. (d**)** F-actin binding properties of VP24. Given the dearth of mechanistic details about Ebola replication, these studies are expected to elucidate the mechanism by which the Ebola virus interacts with the host plasma membrane and how new viruses are generated. This will provide clues to the development of therapeutic protocols for Ebola hemorrhagic virus.

5. Modular Synthesis of Biologically Active Phosphatidic Acid Probes and Diacylglycerol for Mapping Protein-lipid Interactions

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Cell membranes, a key participants in membrane trafficking and cell signaling activity are made up of 1000's of different lipids. Phosphatidic acid (PA) and Diacylglycerol (DAG) are important signaling lipids that play a role in a range of biological processes including both physiological and pathophysiological events. DAG and PA act as site-specific ligands for protein receptors in binding events that enforce membrane association and generally regulate both receptor function and subcellular localization. However, elucidation of the full scope of PA and DAG activities has proven difficult, primarily due to the lack of a consensus sequence among their effectors proteins. Thus, experimental approaches, such as those employing lipid probes, are necessary for characterizing interactions at the molecular level. In this work, we describe an efficient modular approach to the synthesis of a range of PA and DAG probes that employs a late stage introduction of reporter groups. Using this strategy, we exploited fluorescent and photo-affinity tags in the synthesis of active PA and DAG probes. These bifunctional probes containing both a photo-affinity moiety and an azide as a secondary handle for purification purposes. To discern the ability of these analogs to mimic the natural lipid in protein-binding properties, each compound was incorporated into vesicles for binding studies using known PA and DAG receptors, Protein Kinase C (PKC family). In these studies, each compound exhibited binding properties that were comparable to those of cellular PA and DAG, indicating their viability as probes for effectively studying the activities of second messenger in cellular processes.

6. Effect of Affinity Tags on EB1 Behavior In Vivo and In Vitro

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Microtubules (MTs) are components of the cytoskeleton, the network of proteinaceous fibers that endows the cell with structural integrity, motile properties, and internal organization. The cellular functions of MTs are highly dependent on their dynamic properties, which are regulated by a number of microtubule associated proteins (MAPs). The plus end tracking proteins (+TIPs) are a unique group of MAPs that preferentially localize to the MT plus end. EB1 (End Binding protein-1) is a highly conserved +TIP that appears to have a fundamental role in the regulation of MT dynamics, but the mechanisms of its +TIP behavior and activity are still poorly defined. Studies investigating these questions typically involve proteins that have affinity or localization tags. We have addressed this problem by assaying the behavior of untagged EB1 and EB1 fragments in vivo and in vitro. We find that the standard EB1-GFP fusion protein used to mark dynamic MTs interferes with CLIP-170 +TIP behavior, while untagged EB1 recruits CLIP-170 to MTs, suggesting that EB1-GFP might change the parameters it is being used to study. For the in vitro work, we use bacterially expressed EB1 purified by classical methods. We find that untagged EB1 and EB1 fragments behave differently from tagged EB1 proteins. In particular, EB1 proteins with N-terminal his-tags have much higher activity in polymerization assays than do untagged EB1 proteins. In addition, small amounts of his-tagged EB1 fragments can alter the behavior of untagged EB1 fragments present in the same assay. These observations call into question some interpretations based on work performed with tagged EB1 and EB1 fragments.

7. Conformational Properties of Ribosome-Bound GFP

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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. GFP is an excellent model for these studies because the residues that make contacts in the native structure are often far apart in the primary sequence. This high 'contact order' renders GFP (and many other β-sheet proteins) prone to aggregation during refolding *in vitro*, and suggests co-translational nascent chain conformations may be particularly important for productive folding. In this study, we have used fluorescence anisotropy as a powerful tool to provide information on the overall flexibility of polypeptide chains. For this purpose, we designed a series of GFP constructs with an N-terminal CCPGCC motif, which specifically binds the biarsenical fluorescein dye FlAsH. Experiments with ribosome-bound GFP chains have revealed that conformational freedom increases with nascent chain length. Moreover, ribosome-bound full length GFP can fold completely on the ribosomal surface. The complete folding is possible only if the entire GFP sequence is exposed on the ribosome. Conformational properties of nascent and ribosome-released polypeptide chains were evaluated.

8. Solution Structure of the Complex of VEK-30 and Plasminogen Kringle 2

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Plasminogen (Pg) is the zymogen precursor of plasmin (Pm), the major fibrinolytic protease in mammalian systems. The kringle domains of Pg and Pm mediate binding to effector molecules, which include C-terminal lysine and its analogues. The group A streptococcal surface protein (PAM), a major virulence factor, binds Pg with high affinity. A 30-residue internal polypeptide of PAM, VEK-30, represents the major binding determinant of the PAM/Pg interaction. While VEK-30 binds to Pg via its lysine binding sites, this peptide does not contain a C-terminal lysine residue, and, in addition, the binding of VEK-30 to Pg is highly specific for the kringle 2 domain of Pg ($K2_{Pg}$), despite the fact that $K1_{Pg}$, $K4_{Pg}$, and $K5_{Pg}$ interact more robustly with lysine analogues. We previously solved the X-ray crystal structure of the VEK-30/$K2_{Pg}$ complex, and showed that an internal "pseudo-lysine", comprised of positive (Arg17 and His18) and negative (Glu20) side-chains of VEK-30, inserts into the lysine binding site of $K2_{Pg}$. However, this binding modality does not explain the high affinity of the interaction, nor the specificity of VEK-30 for $K2_{Pg}$. We thus turned to determining the solution structure of the VEK-30/$K2_{Pg}$ complex, in order to obtain a more dynamical view of the interaction between these two molecules. The NMR-derived solution structure of the $mK2_{Pg}$/VEK-30 complex reveals that an intricate network of intermolecular hydrophobic and electrostatic interactions accounts for the high binding affinity of VEK-30 to $mK2_{Pg}$ and further demonstrates that Lys14, Arg17, His18 and Glu20 of VEK-30, with support from Asp7 and Glu9, are important for the binding interaction with $mK2_{Pg}$ in solution. Analysis of specific differences among the primary sequences of the five kringle domains provides additional insight into the specificity of VEK-30 for $K2_{Pg}$.

9. Coagulation Cascade Proteins in Asthmatic Responses

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Several studies have indicated that coagulation-related proteins are associated with asthma and we have reported that FXa and FVIIa influence asthmatic responses. In a chronic model of asthma, FXa inhibitor ameliorated airway hyperresponsiveness (AHR) by reducing airway remodeling, such as thickness of airway wall and collagen deposition, without affecting inflammatory cell infiltration to the lung. In low-FVII mice, AHR was ameliorated and inflammatory cell infiltration was also reduced. From these results, we speculate that locally-produced extrinsic coagulation factors are associated with asthma phenotypes. However, it is not known whether components of the intrinsic coagulation pathway are involved with asthmatic responses. Furthermore, there is no direct evidence relating downstream coagulation factors, such as thrombin or fibrin, to development and progression of asthma. In this study, we propose to clarify the association of these factors with asthma.

WT and FXII-deficient (FXII-/-) A/J mice were challenged with OVA intranasally for16 wk. The infiltration of inflammatory cells to the lung, as well as the AHR, were similar between WT and FXII(-/-) mice. On the other hand, when the thrombin inhibitor, hirudin was administered to the OVA-challenged A/J mice from 13 to 16 wk, and compared to vehicle-treated mice, there were no differences in the infiltration of inflammatory cells to the lung, but the AHR was ameliorated by hirudin. In histological analysis, the thickness of the airway smooth muscle and mucous layers were reduced and the OH-proline content of the whole lung was decreased by hirudin. In NCI-H292 cells, a human mucin-producing cell line, thrombin increased mucin (MUC5AC) production in a dose-dependent manner, and its effect was decreased by hirudin in a dose-dependently manner. Balb/c mice were challenged with house dust mite (HDM) for 12 wk to investigate the affects of a fibrin(ogen) deficiency (Fg-/-) on asthmatic responses. The infiltration of inflammatory cells to the lung, and the AHR were similar between WT and Fg(-/-) mice.

Taken collectively, our current and previous studies show that coagulation factors that can activate protease activating receptors (PARs) are associated with asthmatic responses, but the generation of fibrin is not an important factor in this regard.

10. Folding and Secretion of the *E. coli* Pet Autotransporter

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In Gram-negative bacteria, a wide range of virulence proteins are secreted via the autotransporter (AT) pathway. AT pre-proteins are synthesized with an N-terminal signal sequence which facilitates transport across the inner membrane, and a C-terminal β-barrel domain that acts as a pore for the mature virulence factor to cross the outer membrane, flanking the central passenger domain that forms the mature, secreted virulence factor. Intriguingly, there is no significant concentration of ATP in the periplasm, nor a proton gradient across the OM (outer membrane), so the energetic origin of efficient OM secretion of AT proteins is unknown. Yet more than 97% of AT proteins are predicted to contain right-handed parallel β-helical structure, and the three crystal structures available for AT passenger domains each contain a long right-handed parallel β-helix. Previous studies have shown that the passenger domain of pertactin, an AT from *Bordetella pertussis*, exhibits three-state folding and has a C-terminal stable core structure. Here, we show that Pet, an unrelated AT from *Escherichia coli*, also exhibits three-state unfolding and also has a C-terminal stable core structure. To test the requirement of the Pet β-helix on secretion, a series of passenger domain deletion mutants were made. A passenger domain consisting of only the stable core was efficiently secreted to wild type levels, while a construct with the protease domain fused to the stable core was secreted at lower levels than wild type, suggesting the stable core alone can promote efficient secretion of itself and the endogenous protease domain. Deletion of the stable core resulted in lower but still detectable levels of secreted protein, suggesting the stable core contributes to but is not required for OM secretion. Using protease digestion, we show that the passenger domain of the stable core deletion mutant is not correctly folded, suggesting the stable core may have a role in correct folding of the passenger domain. We are also interested in replacing the Pet protease domain with another protein to understand the constraints (size, stability, folding) of non-β-helical portions of the passenger domain. We have replaced the protease domain with dihydrofolate reductase (DHFR), a protein whose folding and stability are well characterized. The chimera containing the cysteine free, wild-type DHFR is secreted and the DHFR is active.

11. Can an Autotransporter Protein Truly Transport Itself Across a Lipid Bilayer?

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Autotransporter (AT) proteins are virulence factors in Gram-negative bacteria. They are synthesized with an N-terminal signal sequence, which is cleaved after secretion through the inner membrane, a central passenger domain (the mature protein), and a C-terminal porin domain. The porin domain is inserted in the outer membrane (OM) and the passenger domain is secreted through this pore. OM secretion does not require ATP nor a proton gradient, therefore the driving force for efficient secretion remains unknown, nor is it known what prevents premature folding of the passenger domain in the periplasm. There is an ongoing debate in the literature about the role, if any, of interactions between AT proteins and periplasmic chaperones and/or essential OM proteins like Omp85 on AT secretion. We are developing an *in vitro* system to test the autonomy of AT secretion, using the model AT pertactin from *Bordetella pertussis*. By mixing unfolded pertactin passenger+porin domains with lipid vesicles, we will test whether the purified protein is competent for secretion across a membrane. While this does not exclude the possibility of chaperone interactions and the participation of other proteins *in vivo*, it would show that ATs have the capacity to cross a membrane independently, strengthening the importance of β-sheet formation as a potential driving force for OM secretion. Vesicles consisting of PC, 10% PG, and 1% NBD-PC were made; the fluorophore NBD is distributed equally across the inner and outer membrane of the vesicles. Vesicle formation was confirmed by fluorescence microscopy and quenching experiments. We have successfully expressed the isolated pertactin porin domain, and refolded it into detergents and vesicles. We have also expressed the passenger + porin domain, a construct that is equivalent to the AT precursor in the periplasm. Initial experiments in the presence of vesicles suggest successful insertion and folding of the porin domain in the lipid environment and subsequent autocatalytic cleavage of the passenger domain, indicating that the *in vivo* situation is indeed mimicked to some extent. Furthermore, we designed a construct where the autocatalytic cleavage site was removed by a single amino acid mutation, without disrupting porin domain insertion and folding in the vesicle membrane. Future work will determine the extent of transport across the membrane.

12. Characterization of a Novel CLIP-170 Related Protein, CLIPR76

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The microtubule cytoskeleton plays critical roles in cell structure and intracellular transport. Cytoplasmic linker proteins (CLIPs) help to link microtubules with membrane bound organelles. We have undertaken a study of genes related to CLIP-170 (the founding CLIP) in hopes of expanding our knowledge of microtubule-membrane interactions. This presentation will focus on one of these, CLIPR76. The CLIPR76 transcript contains three C-terminal CAP-Gly motifs (instead of the usual one or two) and has ankyrin repeats at the N-terminus. When transiently expressed in tissue culture cells, CLIPR76 localizes to and bundles microtubules. However, the CLIPR76 gene is also alternatively spliced, producing at least three additional transcripts in humans. The smallest of these, CLIPR76-4, encodes a 37kD protein containing only one CAP-Gly motif followed by a unique hydrophobic C-terminus. This protein localizes to and reorganizes the ER when expressed at high levels, and the hydrophobic C-terminus is necessary and sufficient to target the protein to the ER. CLIPR76-4 is homologous to another CLIPR protein, CLIPR59. The hydrophobic C-terminal region of CLIPR59 mediates localization of this protein to Golgi and the critical residues responsible for the localization have been identified (Lallemand-Breitenbach *et al.*, 2004). It is intriguing that these proteins have similar hydrophobic tail regions that can specify localization to different organelles. Currently, we are investigating the C-terminal region of CLIPR76-4 in order to determine which amino acids of the hydrophobic tail are critical for the localization of CLIPR76-4 to the ER membrane. While CLIPR59 is expressed predominantly in brain, CLIPR76-4 is highly expressed in muscle and its expression level dramatically increases as muscle cells develop. siRNA knockdown and fluorescence microscopy techniques are currently being utilized in order to determine what role CLIPR76-4 plays in muscle development.

13. Mechanistic Studies of L-Ornithine N^5-monooxygenase, a Critical Step in Siderophore Biosynthesis and Fungal Iron Trafficking

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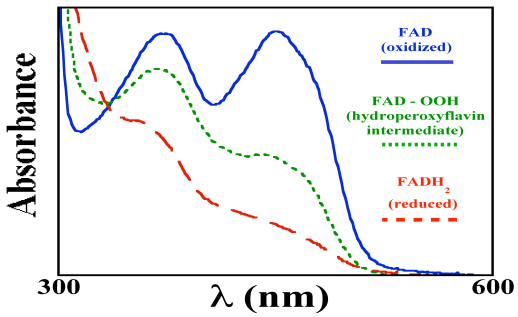
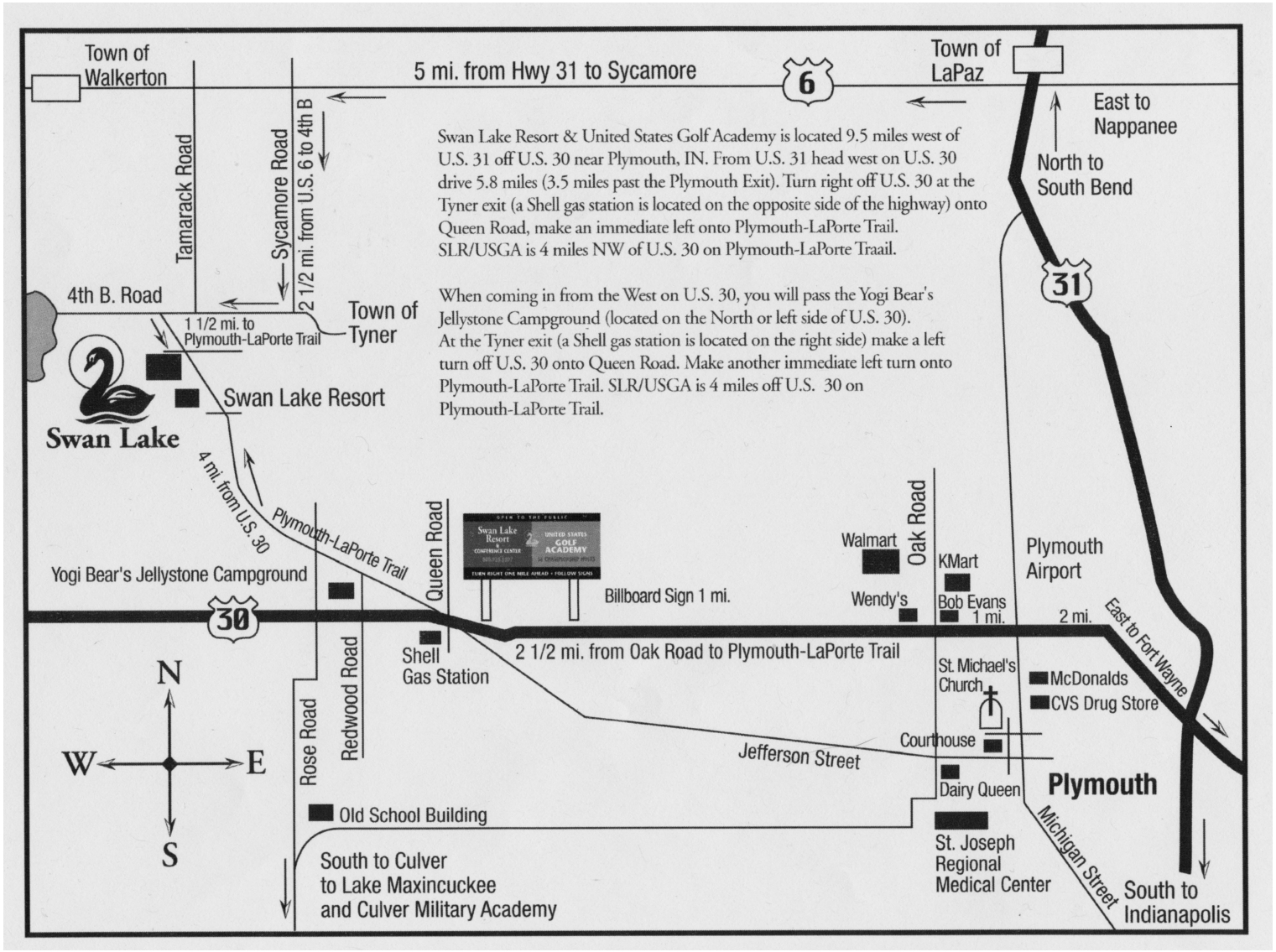
The FAD-containing enzyme L-ornithine N^5-monooxygenase (OMO) from *Aspergillus fumigates* is responsible for catalyzing the first committed step in the biosynthesis of siderophores. The siderophore product is required for the competitive uptake, import, and intracellular storage of iron by the pathogenic fungus. Complete synthesis of each compound requires hydroxylated ornithine produced via the monooxygenase to create the hydroxamic acid moiety necessary for iron chelation. The importance of the enzyme in fungal growth makes it a promising target for antifungal compounds, and a complete understanding of the enzyme reaction will have important implications in their design.

To begin to understand the details of the kinetic mechanism used by OMO, including the order of substrate addition and product release, a steady-state kinetic analysis is applied. OMO catalyzes a three-substrate reaction in which it activates molecular oxygen in the presence of NADPH forming a reactive hydroperoxyflavin intermediate capable of hydroxylation at the N^5 position of the amino acid derivative L-ornithine. It is able to perform the reaction efficiently without the production of harmful peroxide species and the unnecessary consumption of cellular reducing potential. Multiple enzyme families have the ability to catalyze such reactions using various control mechanisms, and OMO may represent a distinct class with a novel method of catalytic control.




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