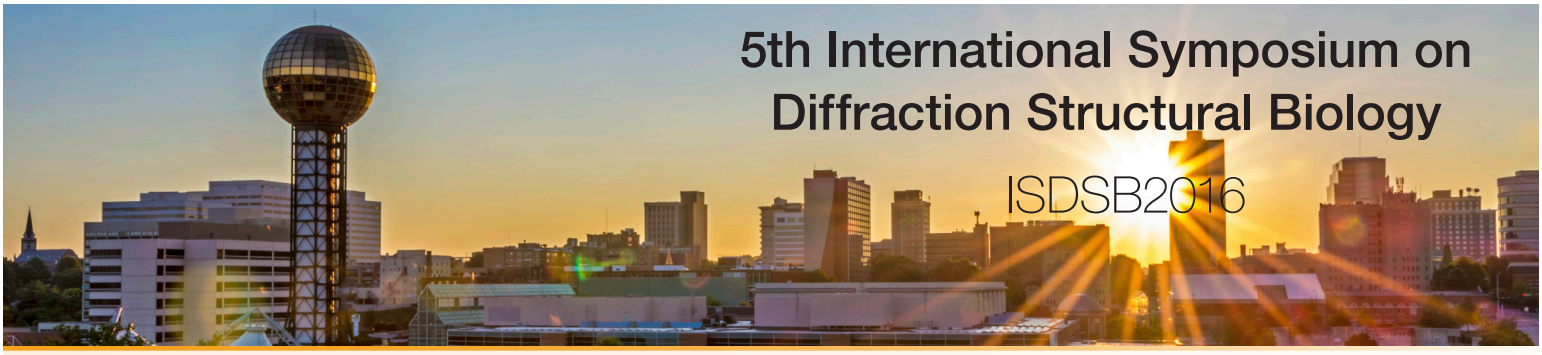

5th International Symposium on Diffraction Structural Biology

ISDSB2016

August 7–10, 2016
Knoxville, Tennessee, USA

This symposium will report on the latest discoveries in structural biology using X-ray, neutron, and electron diffraction, and complementary techniques such as small-angle scattering, nuclear magnetic resonance spectroscopy, electron microscopy, and advanced computational approaches. Scientific insights at the molecular level allow structure guided drug discovery and protein engineering for improved biocatalysis. This basic research is important for supporting applications in the pharmaceutical industry and for new, sustainable, and environmentally friendly approaches to the production of chemical reagents and biofuels.

Discoveries in structural biology are accelerated by the availability of advanced research user facilities and new methods and technologies. In this symposium, we will cover the latest developments in electron, photon, and neutron sources and in associated methods and instrumentation.

Covered Topics

Bioenergy
Drug Design
Enzyme Mechanism and Allostery
Macromolecular Complexes
Membranes
Membrane Proteins
Sources and Facilities

Workshops

Saturday, August 6, 2016

Neutron Protein Crystallography
Lectures and practical instruction on neutron structure refinement using PHENIX. Overview of MaNDi and IMAGINE neutron diffractometers at Oak Ridge National Laboratory.

Small-Angle Neutron Scattering
Lectures and tutorials will introduce basic small angle scattering techniques and related computational tools with emphasis on how to integrate molecular simulations with experiments.

Tours of International User Facilities at Oak Ridge National Laboratory

- High Flux Isotope Reactor
- Spallation Neutron Source
- Titan supercomputer

Executive Committee

Paul Langan, Oak Ridge National Laboratory
Trevor Forsyth, Institut Laue-Langevin
John Helliwell, University of Manchester
Jack Johnson, Scripps Research Institute
Sean McSweeney, Brookhaven National Laboratory
Atsushi Nakagawa, Osaka University
Noriyoshi Sakabe, Emeritus Professor of KEK
Takashi Yamane, Emeritus Professor of Nagoya University
Nori Yasuoaka, Emeritus Professor of Himeji Institute of Technology

Organizers

Paul Langan
Ava Ianni
Volker Urban

For questions contact: conf_reg@ornl.gov

For more information, visit: conference.sns.gov/event/2

16-G00176/jdh

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FOREWARD

The aim of this symposium is to report on the latest discoveries in structural biology using X-ray, neutron, and electron diffraction, and complementary techniques such as small-angle scattering, nuclear magnetic resonance spectroscopy, electron microscopy, and advanced computational approaches. Scientific insights at the molecular level allow structure guided drug discovery and protein engineering for improved biocatalysis. This basic research is important for supporting applications in the pharmaceutical industry and for new, sustainable, and environmentally friendly approaches to the production of chemical reagents and biofuels. Discoveries in structural biology are accelerated by the availability of advanced research user facilities and new methods and technologies. In this symposium, we will cover the latest developments in electron, photon and neutron sources and associated methods and instrumentation. The symposium will include tours of the two advanced neutron scattering research facilities, the High Flux Isotope Reactor (HFIR) and the Spallation Neutron Source (SNS), and the TITAN supercomputer operated by Oak Ridge National Laboratory. Neutron scattering facilities are vital and unique tools for studying biological systems because the neutron's fundamental physical properties can provide information about biological systems that is unattainable by other means. Neutrons are most powerfully applied when combined with complementary experimental techniques that use photons and electrons. In addition, computer simulations using high performance computing enable prediction and interpretation of neutron scattering data from systems that are too complex for analytical theory.

Session topics include:

Bioenergy

Drug Design

Enzyme Mechanism and Allostery

Macromolecular Complexes

Membranes and Membrane Proteins

New Instruments and Methods

Sources and Facilities

Executive Committee

Paul Langan, Oak Ridge National Laboratory

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Jack Johnson, Scripps Research Institute

Sean McSweeney, Brookhaven National Laboratory

Atsushi Nakagawa, Osaka University

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Xiaolin Cheng

Leighton Coates

Matthew Cuneo

Ava Ianni

Andrey Kovalevsky

Flora Meilleur

Dean Myles

Dean Myles

Hugh O'Neill

Jerry Parks

Loukas Petridis

Shuo Qian

Volker Urban

Program

Sunday, August 7, 2016

Time	Event
10:30 a.m. - 12:00 p.m.	**Registration** Hilton Downtown Knoxville, 2nd Floor Lobby
12:00 - 12:30 p.m.	**Opening Ceremony – Lunch provided** **Paul Langan, Oak Ridge National Laboratory**
	Plenary Lecture
12:30 - 1:15 p.m.	**Jack Johnson, Scripps Research Institute** *"Structural Studies of Virus Particle Maturation: An Experimental Laboratory for Large-Scale Macromolecular Dynamics"*
1:15 - 1:45 p.m.	**Break**
Session 1: Sources and Facilities **Chair: Volker Urban**	
1:45 - 2:10 p.m.	**Jean Jakoncic, National Synchrotron Light Source II** *"NSLS-II Biomedical Beamlines for Micro-Crystallography, FMX, and for Highly Automated Crystallography, AMX: New Opportunities for Advanced Data Collections"*
2:10 - 2:35 p.m.	**Nadia Zatsepin, Arizona State University** *"Serial Femtosecond Crystallography at LCLS: The First 5 Years"*
2:35 - 3:00 p.m.	**Atsushi Nakagawa, Osaka University, Japan** *"SPring-8 BL44XU, A Beamline for Large Biological Macromolecular Assemblies"*
3:00 - 3:25 p.m.	**Richard Gillilan, Cornell University** *"CHESS, CHESS-U, and the Future: Modeling Signal AND Noise on BioSAXS Beamlines"*
3:25 - 3:50 p.m.	**Paul Langan, Oak Ridge National Laboratory** *"Opportunities for Biology at the New and Improved Oak Ridge Neutron Sources"*
3:50 p.m.	**Adjourn**

Monday, August 8, 2016

Time	Event
9:00 - 9:45 a.m.	**Plenary Lecture** **Michael Crowley, National Renewable Energy Laboratory (NREL)** *"Cellulose and Diffraction: Connecting Molecular Structure to Measurements"*
9:45 - 10:15 a.m.	**Break**
Session 2: Drug Design **Chair: Jerry Parks**	
10:15 - 10:45 a.m.	**Jeremy Smith, University of Tennessee** *"Proteins: Forever Aging?"*
10:45 - 11:10 a.m.	**Irene Weber, Georgia State University** *"Protein Crystallography for Tackling the Problem of HIV Drug Resistance"*
11:10 - 11:35 a.m.	**Mayank Aggarwal, Oak Ridge National Laboratory** *"Mapping the H-Bonding Patters in Human Carbonic Anhydrase II Complexed with Clinical Drugs"*
11:35 a.m. - 12:00 p.m.	**Jerry Parks, Oak Ridge National Laboratory** *"Discovery of Inhibitors of Multidrug Efflux Pumps in Gram-Negative Bacteria"*
12:00 - 1:30 p.m.	**Conference Photos** **Lunch on your own**
Session 3: New Instruments and Methods **Chair: Matt Cuneo**	
1:30 - 1:55 p.m.	**Paul Adams, Lawrence Berkeley National Laboratory** *"Computational Methods for Neutron Crystallography in Phenix"*
1:55 - 2:20 p.m.	**Wah Chiu, Baylor College of Medicine** *"CryoEM of Molecular Machine with Variable Conformations of Its Components"*
2:20 - 2:40 p.m.	**Ichiro Tanaka, Ibaraki University, Japan** *"Cryoprotectant-Free High-Pressure Freezing and Dynamic Nuclear Polarization for More Sensitive Detection of Hydrogen in Neutron Protein Crystallography"*
2:40 - 3:00 p.m.	**Flora Meilleur, North Carolina State University** *"IMAGINE, New Science and Capabilities at HFIR"*
3:00 - 3:30 p.m.	**Break**
Session 4: Bioenergy **Chair: Hugh O'Neill**	
3:30 - 4:00 p.m.	**Jochen Zimmer, University of Virginia, School of Medicine** *"Crystallographic Snapshots of a Polysaccharide Secretion Machinery"*
4:00 - 4:30 p.m.	**Yoshiki Higuchi, University of Hyogo** *"Structural Studies of [NiFe]-hydrogenases"*

4:30 - 4:45 p.m.	**William Brad O'Dell, North Carolina State University** *"Oxygen Species at the Active Site of a Fungal Polysaccharide Monooxygenase"*
4:45 - 5:00 p.m.	**Nayomi Plaza, University of Wisconsin** *"Understanding Moisture-Induced Swelling of Wood Nanostructure Using SANS"*
5:00 - 7:00 p.m.	**Poster Session** **Refreshments will be provided**
7:00 p.m.	**Adjourn** **Dinner on your own**

Tuesday, August 9, 2016

Time	**Event**
	Plenary Lecture
9:00 - 9:45 a.m.	**Peter Moody, Leicester University** *"Combining Cryo-Neutron & X-ray Crystallography with Single Crystal Spectroscopy to Catch Peroxidase Intermediates"*
9:45 - 10:15 a.m.	**Break**
Session 5: Macromolecular Complexes **Chair: Loukas Petridis**	
10:15 - 10:45 a.m.	**Frank Gabel, Institut de Biologie Structurale, Grenoble, France** *"SANS, NMR and Crystallography: A Powerful Combination to Study Challenging Protein-RNA Complexes"*
10:45 - 11:10 a.m.	**Bret Freudenthal, University of Kansas Medical Center** *"Molecular Snapshots of DNA Damage Processing"*
11:10 - 11:35 a.m.	**Matthew Cuneo, Oak Ridge National Laboratory** *"An Additional Allosteric Switch in ABC Transport"*
11:35 a.m. - 12:00 p.m.	**Venu Vandavasi, Oak Ridge National Laboratory** *"How Many Cellulose Synthases in the Cellulose Synthesis Complex?"*
12:00 - 1:30 p.m.	**Lunch on your own**
1:30 - 5:30 p.m.	**ORNL Tours** • TITAN • SNS • HFIR
	Banquet and Awards Ceremony – By ticket only
7:00 - 9:00 p.m.	**Ken Herwig, Oak Ridge National Laboratory** *"Prospects at the Oak Ridge National Laboratory Spallation Neutron Source Second Target Station"*

Wednesday, August 10

Time	Event
	Plenary Lecture
9:00 - 9:45 a.m.	**Greg Hura, Lawrence Berkeley National Laboratory** *"Combining SAXS and Crystallography to Build Intuition in Functional Macromolecular Networks and Engineering"*
9:45 - 10:15 a.m.	**Break**
Session 6: Enzyme Mechanism and Allostery **Chair: Andrey Kovalevsky**	
10:15 - 10:45 a.m.	**Walter Chazin, Vanderbilt University** *"How Does Human DNA Primase Count?"*
10:45 - 11:10 a.m.	**Donald Ronning, University of Toledo** *"Redefining Our Understanding of Nucleosidase Mechanisms One Proton at a Time"*
11:10 - 11:35 a.m.	**Robert Phillips, University of Georgia** *"Structure of the Trytophan Indole-layase-Oxindolylalanine Complex"*
11:35 a.m. - 12:00 p.m.	**Yota Fukuda, Osaka University, Japan** *"New Hot Topics on Copper Nitrite Reductases"*
12:00 - 1:30 p.m.	**Lunch on your own**
Session 7: Membrane Proteins **Chair: Flora Meilleur**	
1:30 - 2:00 p.m.	**Chuck Sanders, Vanderbilt University** *"The Amyloid Precursor Protein C99 Domain Binds Cholesterol and Undergoes a Structural Change When Reconstituted into Raft-Like Model Membranes"*
2:00 - 2:30 p.m.	**Geoffrey Chang, University of California, San Diego** *"Transporter: Structure, Function, and Application"*
2:30 - 3:00 p.m.	**Ella Mihailescu, University of Maryland** *"Neutron Diffraction Reveals Conformation and Interactions of a Voltage-Sensor Toxin with Lipid Membranes"*
3:00 - 3:30 p.m.	**Break**
Session 8: Membranes **Chair: Shuo Qian**	
3:30 - 4:00 p.m.	**John Katsaras, Oak Ridge National Laboratory** *"Lateral Membrane Organization in Model Systems and Live Bacteria"*
4:00 - 4:30 p.m.	**Michael Wiener, University of Virginia, School of Medicine** *"Functional Recognition of Membrane-Bound Substrates by the Integral Membrane Protein Protease Ste24p"*

4:30 - 5:00 p.m.	**Fred Heberle, University of Tennessee** *"Toward a Better Plasma Membrane Model: Probing Lipid Bilayer Asymmetry with SANS"*
5:00 p.m.	**Closing** **Paul Langan, Oak Ridge National Laboratory**

ABSTRACTS

Oral Contributions

Structural Studies of Virus Particle Maturation: An Experimental Laboratory for Large-Scale Macromolecular Dynamics

Jack Johnson

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Nudaurelia Capensis Omega Virus (NWV) is a eukaryotic, quasi-equivalent virus, with a T=4 surface lattice, where maturation is dramatic (a change in particle size of 100Å) and is novel in that it can be investigated in vitro. We characterized the kinetics of morphological change, structures of maturation intermediates, an associated auto-catalytic cleavage, and demonstrated that regions of NWV subunit folding are maturation-dependent and occur at rates determined by their quasi-equivalent position in the capsid.

NSLS-II Biomedical Beamlines for Micro-Crystallography, FMX, and for Highly Automated Crystallography, AMX: New Opportunities for Advanced Data Collections

Jean Jakoncic

National Syncfrotron Light Source II

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The two new beamlines at the National Synchrotron Light Source-II, for highly Automated Macromolecular Crystallography (AMX) and for Frontier Macromolecular Crystallography (FMX) will begin user operation in 2016. The low emittance of the NSLS-II storage ring is the basis for providing previously unattainable beam parameters to address current and new challenges in crystallography. With a flux of 0.5 – 1×1013 ph/s at 1 Å, and beam sizes from 1 – 20 μm (FMX) and 4 – 100 μm (AMX), the new beamlines' dose rates are up to two orders of magnitude higher than those of the current brightest MX beamlines. They will cover a wavelength range from 0.4 Å (FMX) and 0.7 Å (AMX) to 2.5 Å. A focus in designing the beamlines lay on supporting a broad range of structure determination methods.

The highly flexible design of the experimental stations and of the beam control will support serial crystallography on micron- and sub-micron sized crystals, structure determination of complexes in large unit cells, rapid sample screening using advanced automation and room temperature data collection. The associated wide variety of samples includes frozen crystals in standard loops and meshes, single crystals or up to 5 acoustically deposited crystals, crystals in SBS trays, specialized sample holders such as silicon nitride membranes, micro-fluidic- or LCP-plates and jets.

Serial Femtosecond Crystallography at LCLS: The First 5 Years

Nadia Zatsepin

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The advent of hard X-ray free-electron lasers (XFELs) has enabled the determination of high-resolution structures from microscopic crystals of important biological targets, at room temperature, in native conditions, without radiation damage. In serial femtosecond crystallography (SFX), structure factors are obtained from thousands of nano/microcrystals typically delivered in a continuous liquid or lipidic stream, in random orientations. Some highlights from the rapid development of SFX over the first 5+ years include the determination of high-resolution structures of a number of photosynthetic membrane proteins, G protein-coupled receptors, from nanocrystals grown in vivo in cells, as well as ultrafast (fs) dynamics probed by pump-probe SFX with time resolution inaccessible at synchrotrons. SFX at XFELs has also enabled novel approaches to phasing crystallographic data. This talk will describe the unique opportunities and challenges in XFEL SFX at LCLS and new XFELs coming online around the world.

SPring-8 BL44XU, A Beamline for Large Biological Macromolecular Assemblies

Atsushi Nakagawa

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4 RIKEN SPring-8 Center
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X-ray crystal structure analysis is one of the most powerful and most efficient techniques to reveal the structure of biological macromolecules at atomic resolution.
The beamline BL44XU, named the beamline for macromolecular assemblies, at SPring-8 is managed by the Institute for Protein Research of Osaka University since 1999. This beamline is designed for high precision diffraction data measurement from large biological macromolecular assemblies. X-ray diffraction from biological macromolecular assemblies crystal is generally weak and closely-spaced because of its large unit cell. Therefore, high brilliance and paralleled synchrotron radiation and high performance detector with large area are required for diffraction data collection. The light source of this beamline is a SPring-8 standard type in-vacuum undulator. X-ray is monochromatized by a liquid-nitrogen-cooling Si double-crystal monochromator and focused (or collimated) by Rhodium coated horizontal and vertical mirrors. Pinhole system is used to define various beam shapes and sizes to support diverse crystal shapes and sizes and measurement condition. Recent upgrade of the beamline resulted more than 10 times stronger beam (6.6x1012 photons/sec after 50 micrometer pinhole) than before upgrading.
The goniometer is controlled by a high-speed air-bearing goniostat with a small sphere-ofconfusions (< 1 μm) (Kohzu Precision Co., Ltd., Japan). A crystal spindle axis can be aligned from 0 to 10 degrees by a μ-angle axis that is perpendicular to the horizontal plane to get more freedom of crystal geometry and reduce the blind region in reciprocal space.
The high efficiency back-illuminated CCD detector, Rayonix MX300-HE is mounted on the bench with the wide crystal-to-detector distance of 75~1200 mm with vertical offset of 0~150 mm and 2θ angle of 0~15 degrees. It is capable to collect up to 3.7 Å resolution data from a crystal with unit cell length of 2000 Å. In addition, ultra-low resolution (> 400Å) data can be collected by changing the position of the beamstop. The beamstop can be moved from 16 to 99 mm from the sample position to the downstream direction. Ultra high-resolution data (< 0.56 Å) also can be collected with this system.
A crystal can be cooled to 90K by nitrogen gas or 30K by helium gas using a cryo-stream system (Cryo Industries of America, Inc., USA).

The beamline operation software BSS (Beamline Scheduling Software) and a sample auto-changer SPACE (SPring-8 Precise Automatic Cryo-sample Exchanger) are installed to unify user operation throughout protein crystallography beamlines in the SPring-8. Sample storage of a SPACE enables to set 8 uni-pucks for more than 12 hours beam time. We will present details, results and the ongoing plan to upgrade BL44XU.

Signal and noise: modelling data quality in biological small angle solution scattering

Richard E Gillilan

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R.E. Gillilan, C. Wang, D. Bougie, and Y. Lin
MacCHESS, CLASSE, Cornell University, Ithaca NY
Dept. of Physics, Barnard College, NY

The challenges of frontier problems in structural biology often extend beyond the doors of the sample preparation laboratory. Tough problems in biology can translate into small sample volumes, high dilution, poor contrast, short timescales and other difficulties for biological small angle solution scattering. Researchers need to know if their samples will yield good enough signal to draw scientific conclusions: can they tell the difference between conformation A and conformation B? Is the sample monodisperse? What is the physiological oligomeric state? Will the data yield a meaningful and interpretable shape? The effective use of scarce resources, such as synchrotrons, requires that users screen their samples for viability and be able to justify to reviewers that their experiment is feasible. Furthermore, as facilities upgrade to more powerful sources, it is desirable to model and understand how increased flux and beam quality impact structural biology under realistic experimental conditions. This talk will introduce our strategy for estimating signal-to-noise levels of biological samples in x-ray solution scattering. First-principles calculations are validated against measurements made at several CHESS beamlines at various energies. Factors such as buffer modeling, windows materials, instrumental background scatter, detector response, as well as sample type are considered. A publicly-accessible online version of the simulations will also be presented. Implications for beamline design and future experiments will be discussed.

Opportunities for Biology at the New and Improved Oak Ridge Neutron Sources

Paul Langan

Oak Ridge National Laboratory

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Frontier challenges in biological research increasingly require predictive understanding of complex multi-component systems from combining several experimental approaches to inform multi-scale computer models and simulations. Complementary experimental approaches used include electron microscopy, mass spectrometry, X-ray scattering, and NMR. Neutron scattering has great potential to be developed as another vital tool to provide elusive unique information that cannot be provided by other tools. Researchers are now gaining access to new instrumentation on the world's most intense neutron beam lines at the Spallation Neutron Source (SNS) and the High Flux Isotope Reactor (HFIR) operated by Oak Ridge National Laboratory (ORNL). These developments provide an unprecedented opportunity that we propose to exploit by developing and broadening the use of neutrons in biological research by leveraging deuterium (D) labeling and modeling and simulation using the high performance (HP) computing. In this talk I will present an overview of neutron facilities at ORNL, and give examples of their growing application in biology. Plans for a second target station at SNS will also be discussed, illustrating for the audience the additional future impact neutrons can have on structural biology research.

Cellulose and diffraction: Connecting molecular structure to measurements

Michael Crowley

National Renewable Energy Laboratory (NREL)

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The elusive structure of cellulose has been largely solved by Langan and colleagues, mainly for large algal and bacterial cellulose fibers. There is still much to be determined about the structures and behaviors of the smaller fibers in land plants which make up the bulk of the biomass on the planet, especially the biomass targeted for conversion to renewable fuels and materials. We have worked with experimentalists to connect the measurements of fiber diffraction, solid state NMR, AFM, and TEM to make connections between molecular structure in measurements and observations. Our results show how shape, surface characteristics, and other molecular features affect those measurements and add to both our understanding of cellulose nano fibrils and our interpretation of measurements.

Proteins: Forever Aging?

Jeremy Smith

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Protein Crystallography for Tackling the Problem of HIV Drug Resistance

Irene Weber

Georgia State University

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Drug resistance is a severe challenge for successful treatment of HIV/AIDS patients since the virus evolves rapidly to evade therapy by mutating the drug targets. To tackle this problem, we are studying structures and activities of variants of the virally encoded protease and assisting in development of new antiviral inhibitors with increased potency. Highly resistant variants of HIV protease from clinical isolates exhibit different sets of about 20 mutations and several orders of magnitude worse binding affinity for existing drugs. Our analysis of the extremely resistant PR20 and selected other variants has led to novel antiviral inhibitors with higher affinity than the current drug, darunavir, for resistant HIV.

This research is supported by the National Institutes of Health award GM062920

Mapping the H-bonding Patters in Human Carbonic Anhydrase II Complexed with Clinical Drugs

Mayank Aggarwal

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Carbonic anhydrases (CAs; EC 4.2.1.1) catalyze the interconversion of CO2 and HCO3-, and their inhibitors have long been used as diuretics and a therapeutic treatment of many disorders such as glaucoma and epilepsy. Presented and discussed are the jointly refined X-ray/neutron structures of human CA II in complex with 4 clinically used CA drugs - Acetazolamide (AZM), Methazolamide (MZM), Brinzolamide (BZM), and Ethoxzolamide (EZM). Using molecular dynamics, we also discuss the preferential binding modes of BZM, changes in hydrogen bonding that take place upon the binding of these drugs, and plausible reasons behind comparable binding constants of AZM/MZM despite differential water displacement by them. in the active site of the enzyme. This study also reports the first perdeuterated neutron structure of a carbonic anhydrase refined to a resolution of 1.8 A.

Discovery of Inhibitors of Multidrug Efflux Pumps in Gram-Negative Bacteria

Jerry Parks

Oak Ridge National Laboratory

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Antibiotic resistance is a major threat to human health. Multidrug efflux pumps are major contributors to resistance in Gram-negative bacteria. We used a combination of experimental screening and computational ensemble docking to discover four efflux pump inhibitors that target the periplasmic membrane fusion protein AcrA, a component of the AcrAB-TolC efflux pump in E. coli. These inhibitors potentiate the activity of existing antibiotics, providing a promising path for reviving the activities of antibiotics in resistant bacteria.

Computational Methods for Neutron Crystallography in Phenix

Paul Adams

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Neutron crystallography, in contrast to X-ray crystallography, is capable of accurately locating hydrogen atoms, providing information on the protonation of amino acids, nucleic acids and small molecules, the identity of solvent molecules, and the details of hydrogen bonds. The current development of next generation spallation neutron sources is accelerating the use of neutron for structural studies. This has driven a need for improved computational methods for neutron crystallography. We have therefore implemented a number of new approaches, in the Phenix program, to help the neutron diffraction research community. These include methods for improving density maps for model building and structure completion, automated water addition, and the refinement of atomic models against both neutron and X-ray diffraction data. These methods will be briefly presented along with examples of their impact on neutron crystallographic results.

CryoEM of Molecular Machine with Variable Conformations of Its Components

Wah Chiu

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Electron cryo-microscopy (cryoEM) is in the midst of a revolutionary advance in resolving the biological structures of molecular machines previously either difficult or impossible to attain, at atomic resolutions. We owe this achievement partly to direct electron detectors capable of recording electron images of frozen, hydrated molecular machines with enhanced signal to noise ratio at all spatial frequencies. In addition, the availability of many software packages for 3D reconstruction, structure classification, and modeling, contributed freely from many academic labs, has facilitated structure solution. In our Center, we have solved cryoEM structures of molecular machines, including viruses, chaperonins and membrane proteins, to the level where either partial or full-atom models of the protein components can be derived from our cryoEM maps. Some of these molecular machines have complex and variable conformations in their protein components in each single particle. Examples will be presented illustrating how we derive and validate structures of these variable protein conformations, and extract meaningful biochemical knowledge from their structures.

Cryoprotectant-Free High-Pressure Freezing And Dynamic Nuclear Polarization For More Sensitive Detection of Hydrogen In Neutron Protein Crystallography

Ichiro Tanaka

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1 College of Eng., Ibaraki Univ., 2 Frontier Res. Ctr., Ibaraki Univ., 3 RRI, Kyoto Univ. , 4 Faculty of Sci., Yamagata Univ.

Neutron protein crystallography (NPC) can more easily find hydrogen in macromolecules, but it might be difficult to detect hydrogen atoms involved in chemical reactions in the living body by conventional neutron methods because they have low occupancies. In order to improve the hydrogen detection sensitivity by neutron, dynamic nuclear polarization (DNP) of hydrogen in protein is expected to gain hydrogen detection sensitivity about eight times larger than the normal NPC [1]. Several technical difficulties, however, should be overcome to realize the DNP method in NPC; (1) freezing a large protein single crystal and (2) obtaining higher proton polarization rate of protein sample doped with proper concentration of a radical molecule under low temperature (~1K) and high magnetic field (~3T). Here we report the current status of these developments.

To freeze larger crystals certainly, high-pressure freezing method has been employed. The highest pressure is 200 MPa. As a result, lysozyme single crystals grown with only NaCl (no cryoprotectant (soaking)) in sodium acetate buffer, the maximum size of which was about 0.8 mm in three edges, could be successfully frozen, and diffracted up to 1.17 Å with synchrotron source under 100 K. The resolution limit was comparable to those of small flash-frozen lysozyme crystals using cryoprotectant. If even another protein single crystal could be also frozen under high-pressure without cryoprotectant, this freezing method might be also very useful in synchrotron radiation experiment.

To perform DNP experiment on protein, many small lysozyme single crystals (about 300 mg) co-crystallized with a soluble radical were used. The concentration of the radical in the protein single crystal in the same batch was determined by ESR. Under the condition of 2.5 T magnetic field and 0.5 K in temperature, the maximum proton polarization ratio was estimated to be 26 %.

Reference:

[1] I. Tanaka, K. Kusaka, T. Chatake, N. Niimura: J. Synchrotron Rad. 20 (2013) 958.

IMAGINE, New Science and Capabilities at HFIR

Flora Meilleur

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IMAGINE is a new high intensity, quasi-Laue neutron crystallography beam line developed at the High Flux Isotope Reactor (HFIR) at Oak Ridge National Laboratory (ORNL). IMAGINE is a state-of-the-art facility for neutron-diffraction analysis of advanced materials and macromolecules. IMAGINE is especially suited to pinpoint individual hydrogen atoms in protein structures, enabling neutron protein structures to be determined at or near atomic resolutions (1.5 Å) from crystals with volume < 1 mm3 and with unit cell edge of < 100 Å. Beam line features include novel elliptical focusing mirrors that deliver neutrons into a 2.0 x 3.2 mm2 focal spot at the sample position with full width vertical and horizontal divergence of 0.5° and 0.6°, respectively, and variable short and long wavelength cutoff optics that provide automated exchange between multiple wavelength configurations

We will give an overview of the IMAGINE beam line at the HFIR, and present recent results from the instrument.

The acquisition and installation of IMAGINE at the HFIR was funded by the National Science Foundation (NSF) under award No. 0922719.

ORNL is managed by UT-Battelle, LLC, for the U.S. Department of Energy under Contract DE-AC05-00OR22725.

Crystallographic snapshots of a polysaccharide secretion machinery

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Under certain conditions, all biopolymers, including polypeptides, polysaccharides and nucleic acids, must be translocated across at least one membrane to reach their site of biological function. Cellulose is a linear glucose polymer synthesized and secreted by a membrane-integrated cellulose synthase. Biochemistry and X-ray crystallography with the catalytically-active bacterial cellulose synthase BcsA-B complex reveals structural snapshots of a complete cellulose biosynthesis cycle, from enzyme activation to substrate binding and polymer translocation. Substrate and product-bound structures of BcsA provide the basis for substrate recognition and demonstrate the stepwise elongation of cellulose. Furthermore, the structural snapshots show that BcsA translocates cellulose via a ratcheting mechanism involving a “finger helix” that contacts the polymer's terminal glucose. Cooperating with BcsA's gating loop, the finger helix moves ‘up’ and ‘down’ in response to substrate binding and polymer elongation, respectively, thereby pushing the elongated polymer into BcsA’s transmembrane channel. Reversible tethering of the finger helix and biochemical analyses support a ratcheting mechanism for cellulose translocation.

Structural Studies of [NiFe]-hydrogenases

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[NiFe]-hydrogenases are classified into at least four groups (Group 1 to 4). “Standard” [NiFe]-hydrogenases in Group 1 are reversibly inactivated by O2, producing two inactive forms, Ni-A and Ni-B. Ni-A requires a prolonged reactivation time, whereas Ni-B can be immediately reactivated with H2.

Some H2-oxidizing bacteria have an O2-tolerant [NiFe]-hydrogenase in Group 1 which forms only Ni-B even in the oxidized form and shows the catalytic activity even at ambient O2 concentration. The Fe-S cluster proximal to the active site of O2-tolerant [NiFe]-hydrogenase is not an ordinary [4Fe-4S]-4Cys, but a [4Fe-3S]-6Cys type. This [4Fe-3S]-6Cys shows structural changes upon redox of the enzyme. The original structure of this cluster found in the reduced form was recovered from ferricyanide -oxidized form by H2-reduction, indicating that it has a key role in preventing the formation of the Ni-A form.

Cytoplasmic NAD+-reducing [NiFe]-hydrogenases in Group 3d catalyze bidirectional oxidation of H2 with NAD+. The enzyme from H. thermolteolus TH-1 is composed of two heterodimeric catalytic subcomplexies, HoxHY (hydrogenase unit) and HoxFU (diaphorase unit). The sequences of the NAD+-reducing [NiFe]-hydrogenases have an extensive similarity to the peripheral subunits of the respiratory complex I, suggesting that the evolution of the catalytic building blocks of the energy metabolic machineries in the cells.

In this symposium, the recent progress on studies of the O2-tolerant and NAD+-reducing [NiFe]-hydrogenases will be presented.

Oxygen species at the active site of a fungal polysaccharide monooxygenase

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Fungal polysaccharide monooxygenases (PMOs) are extracellular enzymes that break glycosidic bonds at the surfaces of crystalline carbohydrates, primarily cellulose, and enhance the susceptibility of the substrate to further enzymatic degradation. PMOs require input of two electrons and one oxygen molecule to achieve hydroxylation of one carbon in the glycosidic bond which leads to spontaneous bond cleavage. We will present and discuss new structures of Neurospora crassa polysaccharide monooxygenase 2 obtained by high resolution X-ray cryo-crystallography and room temperature neutron protein crystallography.

Understanding Moisture-Induced Swelling of Wood Nanostructure Using SANS

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Combining cryo-neutron & x-ray crystallography with single crystal spectroscopy to catch peroxidase intermediates

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X-ray crystallography has given us unprecedented insight into the mechanisms of enzyme reactions. However, the method has important limitations. It does not show hydrogen atom positions reliably, even at sub-angstrom resolution. Furthermore, X-rays are ionizing and strongly reducing (due to the photoelectric effect). This especially complicates the interpretation of redox enzyme structures. Neutron crystallography can place hydrogen (as Deuterium) at modest resolutions and the thermal neutrons used do not perturb the chemistry. However, neutron crystallography has its own problems.

The example of the heme peroxidases will be discussed, showing how spectroscopic monitoring of intermediate formation and destruction has allowed the cryo-trapping of the intermediates Compound I and Compound II and the determination of these structures with combined neutron and X-ray methods has shown the nature of the ferryl heme and active site, revising the accepted enzyme mechanism.

SANS, NMR and crystallography: a powerful combination to study challenging protein-RNA complexes

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I will present an overview over recently published work on large and challenging multi-subunit protein-RNA complexes by a combination of small-angle neutron scattering (SANS), crystallography and NMR. The examples presented will include the ternary protein-RNA complex Sxl-Unr-RNA which is a key player in gene regulation and sexual development [1], and the 400 kDa Box C/D complex which regulates the assembly and function of ribosomes by chemically modifying (methylating) rRNA [2].

In all cases SANS, in combination with deuteration, was essential to determine the three-dimensional structures of the complexes by defining the respective positions of the individual protein and RNA partners within the complexes and by providing complementary structural restraints with respect to the other techniques used (crystallography and NMR).

In the light of these recent examples on important systems in structural molecular biology I will elaborate on the state-of-the-art of combining SANS (and SAXS) information with structural restraints from complementary biophysical and biochemical techniques.

Finally, I propose to discuss future perspectives of such hybrid approaches and requirements to apply them on a number of important and challenging molecular biology systems involved in genome and proteome regulation in biological systems of biomedical relevance.

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Molecular Snapshots of DNA Damage Processing

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The accurate repair of the genome is vital to genomic stability throughout nature. DNA abnormalities (lesions) generated by either endogenous or environmental stressors promote multiple deleterious human health outcomes ranging from cancer to premature aging. Since DNA repair involves multi-protein complexes it is essential to understand how these proteins process toxic DNA intermediates in a larger co-complex. This molecular understanding is a pre-requisite for rationale biological intervention. The primary defense mechanism during the repair of oxidative DNA damage is Base Excision Repair (BER). BER utilizes a multi-protein complex that facilitates the channeling (i.e., direct transfer) of toxic DNA intermediates between repair proteins. This process involves (a) the removal of the damaged base by a DNA glycosylase, (b) the 5' phosphodiester backbone cleavage by Ape1 generating a nick substrate, (c) pol β mediated removal of the 5'-deoxyribose phosphate blocking group and subsequent 1-nt gap filling, and (d) the ligation of the repaired DNA by a DNA ligase. The structural architecture during this direct transfer remains poorly understood. The utilization of neutron studies will help to elucidate how the key co-complexes during DNA repair are formed. Preliminary results will be presented that support the utilization of neutrons to elucidate this key biological question.

An additional allosteric switch in ABC transport

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The ligand-induced conformational changes of periplasmic binding proteins (PBP) play a key role in the acquisition of metabolites in ATP binding cassette (ABC) transport systems. The conformational change alters the molecular envelope of the PBP, allowing for differential recognition of the ligand occupancy state of the PBP by the ABC transporter. In many systems the conformational change of PBPs is insufficient in minimizing ATP hydrolysis that is not coupled to metabolite transport. Additionally, owing to the large protein:protein interaction surface formed between the PBP and the ABC transporter and the similar molecular envelope the apo protein and the ligand bound protein present, the apo proteins can still interact with sufficient affinity to stimulate ATP hydrolysis. These scenarios lead to little discrimination of the ligand occupancy of the PBP and higher levels of ATP hydrolysis that is not coupled to the metabolic reward of ligand transport. Here we demonstrate an additional state of the PBP that is also allosterically regulated by the ligand occupancy of the PBP, and in-turn allows for further differentiation the apo PBP molecular envelop. These results suggest a new paradigm where the PBP not only provides fuel for ATP production, but also plays an active role in ensuring the hydrolysis of the cellular ATP pools are coupled to transport of the high energy compounds that are also used to maintain cellular ATP levels.

How Many Cellulose Synthases in the Cellulose Synthesis Complex?

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Cellulose is a vital component of plant cell walls and constitutes the Earth's largest store of biorenewable material for cellulosic ethanol, yet the molecular mechanism of its synthesis by plants is poorly understood. The plant cellulose synthesis complex (CSC), also called a 'rosette' because of its hexameric appearance in electron microscope images, is a large multi-subunit transmembrane protein complex responsible for synthesis of cellulose chains and their assembly into microfibrils. The number of cellulose synthase (CESA) proteins in the CSC and the number of cellulose chains in a microfibril have been debated for years. This work reports analysis of CSCs obtained using refined methods in freeze facture transmission microscopy (FF-TEM) and structural studies of a recombinant cytosolic domain of plant CESA in solution. Together, these studies provide new insights into the structure and oligomeric state of CESAs and the probable number of cellulose chains in the microfibril. Class averaging of the FF-TEM images of individual lobes in the CSC combined with computational modeling of the transmembrane helical (TMH) region of CESA revealed each lobe of the CSC likely contains three CESAs. Analysis of the individual lobes in the FF-TEM images indicates that the lobe-lobe interactions are relatively weak suggesting the forces that hold the CSC together must be due to protein-protein interactions in the cytosolic portion of CESA and/or glucan chain interactions near the plasma membrane surface. Small-angle neutron and X-ray scattering were used to study the solution structure of the cytosolic domain of Arabidopsis CESA1 (CESAcatD). Ab initio models for CESAcatD derived from the SANS and SAXS data provide the first experimental evidence to support the self-assembly of CESAcatD monomers into stable homotrimer complexes. By combining small-angle scattering data with computational approaches, possible configurations for the arrangement of CESAcatD monomers in the homotrimer were obtained. Several candidate trimers were identified with monomers oriented such that that newly synthesized cellulose chains project towards the cell membrane. In these models, the class specific region is found at the periphery of the complex and the plant-conserved region forms the base of the trimer. Comparison of the FF-TEM images with the sizes of a TMH trimer and a CESACatD trimer provides compelling evidence that each lobe of a CSC contains three CESAs. The combined data from the two studies refutes the long-standing model of 36 CESAs within the rosette CSC and strongly supports the 'hexamer of trimers' model for rosette CSC that synthesizes an 18-chain cellulose microfibril as the fundamental product of cellulose synthesis in plants.

Prospects at the Oak Ridge National Laboratory Spallation Neutron Source Second Target Station

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From its beginning the Oak Ridge National Laboratory Spallation Neutron Source (SNS) was designed to enable a significant increase in accelerator power and to host a second target station. The upgrade of SNS is comprised of two major elements: a Proton Power Upgrade (PPU) that doubles the power of the accelerator complex and the construction of a Second Target Station (STS) optimized for the production of long-wavelength neutrons. STS will be optimized for highest cold neutron peak brightness of the neutron pulses emitted from the moderators. It will take advantage of the existing SNS accelerator complex and use the accumulator ring to provide sharp proton pulses to a solid, rotating tungsten target. The combination of a compact neutron production zone, optimized moderator placement, and compact moderator geometry will produce pulses having 20x the peak brightness of the comparable moderators on the SNS first target station. Operating at a low repetition frequency to access a large wavelength band in each pulse, STS will provide unique capabilities to the neutron scattering community. This talk will introduce the STS concept and discuss its current status.

Combining SAXS and crystallography to build intuition in functional macromolecular networks and engineering

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Biological macromolecules function by adopting conformationally-distinct states through processes such as phosphorylation, nucleotide binding, ATP hydrolysis, ligand binding, complex formation, or combinatorial post-translational modification. Many of these conformational states may be biologically important, but relatively few are observed and compared by current structural techniques. Crystallography is precise but low throughput, typically captures one low-energy conformation, and often requires truncations or mutations. SAXS experiments can probe the solution state under any condition, provide information at resolutions sufficient to distinguish conformational states, characterize flexible macromolecules and screen in high throughput. Moreover SAXS can be accurately calculated from atomic resolution models forming a valuable complementarity between crystallography and SAXS. Here we present several examples of the application of SAXS which significantly enhance our understanding of biological networks beyond what either a high resolution structure or informatics analysis can yield.

For macromolecular engineering the same combinatorial challenges exist. Because our understanding of the chemical and structural implications of even one point mutation is limited, techniques are required with rapid throughput to build intuition. Moreover crystallography typically provides perspective in one stochastic solvent condition whereas engineered molecules may be designed for many solvent environments. A crystal structure of one construct combined with computational modelling and SAXS data on mutations and in varying solution conditions can provides remarkable insights for engineering. We will describe experimental and analytical frameworks that have aided in engineering projects with applications in human health and commercial chemical synthesis.

How does Human DNA primase count?

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Faithful copying of our genome requires the coordinated action of multi-domain proteins operating within dynamic multi-protein machines that operate at the replication fork. DNA primase plays a central role in the initiation of DNA synthesis on both the leading and lagging strand templates because it is the only polymerase capable of de novo synthesis on a naked ssDNA template. Primase also liberates the template ssDNA from the eukaryotic ssDNA-binding protein replication protein A before synthesizing the first 8-10 nucleotides of the primer and handing off the substrate to polymerase α for primer extension.

While high-resolution crystal structures are available for domain fragments and full-length primase, none of these reveal the active configuration of the enzyme or how it functions. We are particularly intrigued by, and motivated to define, the structural mechanism of primer length counting, i.e. how and why primase is able to count 8-10 nucleotides and then hand off the substrate to polymerase α. In this presentation, I will summarize our progress using an integrated X-ray crystallography, NMR, X-ray scattering and computation modeling approach to determine how primase functions. I will also briefly present provocative new data suggesting that DNA charge transport driven by the 4Fe-S cluster of primase can be used as a molecular switching mechanism to control primer length counting and hand off of the primed substrate to polymerase α.

Redefining our understanding of nucleosidase mechanisms one proton at a time

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5'-Methylthioadenosine nucleosidase (MTAN) catalyzes the hydrolysis of the N-ribosidic bond of a variety of adenosine-containing metabolites. Substrate binding of the adenosyl moiety is mediated almost exclusively by hydrogen bonds and the proposed catalytic mechanism requires multiple proton transfer events. This talk will present three co-refined neutron/X-ray crystal structures of wild-type Helicobacter pylori MTAN co-crystallized with S-adenosylhomocysteine (SAH), Formycin A (FMA), and p-ClPh-Thio-DADMe-ImmA as well as one neutron/X-ray crystal structure of an inactive variant (HpMTAN-D198N) co-crystallized with SAH. These results support the proposal of an enzyme mechanism that differs from that which is currently accepted. In addition, the described neutron structures highlight active site features that promote transition state stabilization and highlight slight differences in the interactions between the enzyme and different types of transition state analogs that partially explain the large range of binding affinities to MTAN.

Structure of the Tryptophan Indole-lyase-Oxindolylalanine Complex

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Tryptophan indole-lyase (TIL) is a bacterial enzyme that catalyzes the reversible formation of indole and ammonium pyruvate from L-tryptophan. Indole produced by TIL is a signaling molecule that regulates a number of physiological processes in E. coli, including biofilm formation, plasmid stability, and antibiotic resistance. Oxindolyl-L-alanine (OIA) is a potent inhibitor of TIL, with Ki value of about 5 μM, and crystals of TIL from Proteus vulgaris were shown previously to form a stable quinonoid complex upon soaking with OIA. We have now determined the structure of the complex of P. vulgaris TIL with OIA at 2.2 Å. The ligand is present at ~100% occupancy and forms a quinonoid complex with the pyridoxal-5'-phosphate (PLP) cofactor. A hydrogen bond forms between the NH of the inhibitor and Asp-133. The small domain moves ~7 Å to close the active site, allowing His-458 to donate a hydrogen bond to Asp-133. This then brings Phe-459 into close steric contact with the aromatic ring of OIA. Mutation of this Phe to Ala results in a 300-fold decrease in kcat and kcat/Km for L-Trp, with much less effect on the reaction of other β-elimination substrates. A model of the L-Trp quinonoid complex with PLP shows that there would be a severe clash of Phe-459 and the benzene ring of the substrate, which would result in bending of the substrate aromatic ring out of plane with the Cβ-C" bond, allowing formation of the hydrogen bond of the indole NH with Asp-133. This bending of the aromatic ring out of plane moves the substrate upward on the reaction coordinate toward the transition state, thus reducing the activation energy and accelerating the enzymatic reaction.

New hot topics on copper nitrite reductases

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Copper nitrite reductase (CuNIR), a crystallographic target from long ago, is a key enzyme in the denitrification pathway and catalyzes one electron reduction of nitrite to nitric oxide. Although the reaction mechanism of nitrite reduction by CuNIR has been enthusiastically investigated with crystallography, conventional techniques have failed to visualize some reaction intermediates because of inevitable radiation damages; therefore, proposed mechanisms are controversial. By using ultra-bright and ultra-short pulses generated by X-ray free electron lasers, we have determined high resolution damage-free structures of CuNIR with and without nitrite [1, 2]. Based on those new data, we will discuss an updated mechanism of nitrite reduction. Besides, we want to shed light on a hidden face of CuNIR as an oxygen reductase. Although CuNIR has been known to reduce dioxygen to hydrogen peroxide for more than 30 years [3], the reaction has been seldom discussed contrary to that of nitrite reduction. We have recently focused on the phenomenon and tried to reveal its mechanism with crystallography. New crystal structures that we determined showed a diatomic molecule located on the catalytic copper [4]. It can be assigned as dioxygen in a rare side-on mode. We will discuss the reaction mechanism of dioxygen reduction and compare CuNIR with other copper-containing enzymes that utilize dioxygen molecules to perform biologically important reactions.

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The Amyloid Precursor Protein C99 Domain Binds Cholesterol and Undergoes a Structural Change When Reconstituted into Raft-Like Model Membranes

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It is known that elevated cholesterol promotes amyloidogenesis, but the mechanism has not previously been clear. In this study the structure of C99, the transmembrane C-terminal domain of the amyloid precursor protein (APP) was determined using NMR spectroscopy and found to have several surprising features to provide insight into how this protein is proteolytically processed in the pathway to formation of amyloid-β, which is closely associated with the etiology of Alzheimer's disease. It was also discovered that C99 is a cholesterol binding protein. We have recently discovered that while the structure of C99 seems to be remarkably tolerant of change in lipid composition in conventional bicelles, it appears to undergo a structural change in novel bicelles that have a lipid raft-like composition, suggesting its conformation is perturbed upon association with lipid rafts.

Transporter: Structure, Function, and Application

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Transporters mediate the passage of metabolites and toxins across cell membranes. One such transporter, P-glycoprotein (P-gp), is pharmacologically significant. We present new P-gp co-crystal structures with a series of rationally designed ligands. We observed that the binding of certain ligands, including an ATP hydrolysis stimulator, produces a large conformational change to a transmembrane helix, positioned to potentially transmit a signal to the nucleotide-binding domains. We also describe a new ligand-binding site on the surface of P-gp facing the inner leaflet of the membrane, providing vital insights regarding the entry mechanism of hydrophobic drugs and lipids, into P-gp. These results represent significant advances in our understanding of how P-gp and related transporters bind and export a plethora of metabolites, antibiotics, and clinically-approved and pipeline drugs. We also present P-gp structure with oceanic pollutant compounds and studies with a-beta peptide, extending the work to areas of environment/health and the brain. Finally, we discuss a useful technology for the rapid discovery of binders (antibodies) useful as biomarkers and for therapeutic development of transporters.

Neutron diffraction reveals conformation and interactions of a voltage-sensor toxin with lipid membranes

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Tarantula toxins are a particularly interesting class of protein toxins that have been found to target all three families of voltage-activated cation channels, stretch-activated cation channels, as well as ligand-gated ion channels as diverse as acid-sensing ion channels (ASIC) and transient receptor potential (TRP) channels. Although tarantula toxins have been shown to partition into membranes, and the membrane is thought to play an important role in their activity, the structural interactions between these toxins and lipid membranes are poorly understood. Here, we use neutron diffraction and solid-state NMR to investigate the interactions between a voltage sensor toxin (VSTx1) from the venom of Grammostola spatulata and lipid bilayers (1). Using deuterium labels at specific positions in the VSTx1 we localize the toxin in the membrane and reveal its perturbatory effects on membrane structure. We couple this study with our previous neutron diffraction results on the conformation of voltage-sensors in membranes (2), and show that the toxin orients such that the surface of the toxin that mediates binding to voltage sensors is ideally positioned within the lipid bilayer to favor complex formation between the toxin and the voltage sensor.

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Lateral Membrane Organization in Model Systems and Live Bacteria

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Biomembranes are the active boundary between cells and their surroundings. They are sophisticated and dynamic machines that perform a diverse array of functions, including selective transport, localization, communication and recognition, to name a few. It is also widely accepted that the plasma membrane is laterally heterogeneous, containing nanoscopic regions enriched in certain types of lipids – these lipids have different physical properties from those that surround them. In biology, these functional lipid domains are commonly referred to as “rafts”, and are thought to participate in a range of membrane processes including viral entry and exit from cells. Yet, despite their central role in biology, lipid rafts have yet to be observed in the membrane of a living system. So why is it the case that these structures have eluded detection? In recent years, we have used small angle neutron scattering (SANS) and neutron spin echo to study nanoscopic lipid domains in model membrane systems [1-3] and more recently, in the Gram-positive bacterium Bacillus subtilis. Data from these studies will be presented, including the “visualization” of the membrane in live bacteria by SANS.

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Ste24p: A Membrane-Bound Protease with Novel Structure, Provocative Mechanism, and Emergent Biology

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Ste24p is an integral membrane protein protease, originally discovered in yeast genetic screens via its role in processing of the yeast mating pheromone a-factor. It is a zinc metalloprotease, containing the canonical HExxH zinc-binding motif. The human ortholog of Ste24p, ZMPSTE24, processes prelamin A, an intermediate filament protein that is a component of the nuclear lamin. Mutations in ZMPSTE24 that reduce proteolytic activity, or mutations in prelamin A that impede its processing by ZMPSTE24, give rise to genetic diseases of accelerated aging (progerias). Additionally, some of the side-effects, particularly lipodystrophy, of HIV drug therapy are likely to arise from off-target effects of HIV protease inhibitor drugs upon ZMPSTE24. Up until quite recently (2016), Ste24p (ZMPSTE24) was characterized as a so-called “CaaX protease,” where CaaX stands for cysteine-aliphatic-aliphatic-anything and refers to the C-terminal tetrapeptide repeat that is recognized and cleaved in CaaX protease substrates. Moreover, prenylation of the C of the CaaX motif was also considered to be required. New results in the literature suggest significantly broader roles for Ste24p/ZMPSTE24; among them are clearance of clogged translocons of misfolded/aggregated protein chains and the ability to cleave substrates completely lacking an isoprenyl group. In this presentation, I review the current and emergent literature on Ste24p/ZMPSTE24, present our previously-published crystal structure of a yeast ortholog and the model of mechanism that emerged from analysis of this unique membrane protein structure, and present unpublished results on our functional characterization of this fascinating enzyme. (Supported by NIH Grant R01 GM108612)

Toward a Better Plasma Membrane Model: Probing Lipid Bilayer Asymmetry with SANS

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Poster Contributions

An effective deuterium exchange method for a neutron structure analysis by use of unfolding-refolding processes

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It is important in the structural analysis of protein tertiary structures to determine the positions of hydrogen atoms and solvent water. The catalytic sites of enzymes include specific hydrogen atoms that are essential to their catalytic mechanisms. Neutron diffraction is a powerful technique to determine the positions of hydrogen atoms in a protein molecule with high precision, although it is necessary to crystallize the deuterated protein because of decreasing incoherent scattering from the many hydrogen atoms in a protein molecule. Deuterated protein is archived by use of an over-expression of native or genetically modified proteins in Escherichia coli with deuterated reagents and D2O, but the deuterium is very expensive. Therefore a simple H/D exchange technique is desirable for this purpose. A method of hydrogen/deuterium (H/D) exchange with an unfolding/refolding process has been applied to hen egg-white lysozyme (HWL). When an unfolded protein is refolded in a deuterium solution, the refolded molecule has the same H/D ratio for surface and core residues, and the total deuterium content is increased, and accurate evaluation of its deuteration was estimated by time-of-flight mass spectroscopy. Addition of a small amount of acid or base to protein solutions with heating effectively increased the number of deuterium up to more than 20% of that of all hydrogen atoms, and refolded structures were determined by X-ray structure analysis at high resolution. Refolded HWL had increased deuterium in its protein core and its structure was fully preserved among treated or non-treated proteins.

Flanking Mismatches Alter the Structure and Function of the APE1/DNA Complex

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DNA damage resulting from environmental stress threatens genomic stability and consequently leads to adverse human health outcomes. The Base Excision Repair (BER) pathway is a key defense mechanism against DNA damage and involves the removal and subsequent repair of DNA lesions. The first step in BER is the removal of the damaged base by a DNA glycosylase and subsequent generation of an abasic site. This abasic site is processed by human apurinic/apyrimidinic endonuclease 1 (APE1). APE1 cleaves the DNA sugar-phosphate backbone at the phosphodiester bond 5' of the abasic site generating a 5’ nicked substrate for subsequent repair. Previous structural and kinetic studies with APE1 have represented ideal conditions in which canonical Watson-Crick base pairing flanks the abasic site. The present study uses structural and kinetic approaches to examine the effect of various 5’ mismatches on the APE1/abasic DNA binary complex. Interestingly, unique Hoogsteen base pairing is observed between purines and wobble base pairing is often observed with pyrimidines. The base pairing properties appear to be effected by both the nature of the base and the strain imposed by the residues that form the active site of APE1.

Developing semi-synthetic composite materials for investigating cellulose and matrix polymer interactions during pretreatment

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Plant cell walls comprise of three main components cellulose, hemicellulose and lignin. The spatial arrangement of these components with respect to each other and changes among them during pretreatment of biomass are not clear. This is due to the complex and polymeric nature of plant cell walls. In order to understand the interactions of the plant cell wall components during pretreatment, we are developing semi-synthetic composite materials comprising of binary combinations of hemicellulose, lignin, and cellulose. In this study, hemicellulose-cellulose composites were prepared by synthesizing bacterial cellulose from Acetobacter xylinus in presence of glucomannan or xyloglucan dissolved in the growth media. Quantitative saccharification was carried out which show xyloglucan and glucomannan being incorporated in the xyloglucan-cellulose and glucomannan- cellulose composites respectively. In-situ small angle neutron scattering (SANS) was used to study morphological changes during dilute acid pretreatment. The cellulose in the composite material was deuterated to provide contrast between it and the hemicellulose. SANS measurements were performed in 45% D2O solvent, the contrast match point for protiated hemicellulose, making it possible to extract size and shape information of cellulose. The samples were heated to 180 ˚C at 5 ˚C/min rate, held at this temperature for 5 mins, before cooling to 25˚C. SANS profiles were recorded at 1 min intervals. Through the course of pretreatment not much change was observed on cellulose-only sample. However, in presence of xyloglucan and glucomannan (results not shown), the surface morphology and particle size change. These changes between the cellulose only and composites are seen at the start of pretreatment but they become more pronounced as temperature rise from 120 to 180 ˚C. After pretreatment the composite sample is more entangled than before pretreatment with significant changes in the high Q region. X-ray diffraction showed a significant decrease in the crystal size and crystallinity of cellulose synthesized in presence of hemicelluloses. Analysis of the changes in cellulose structure and morphology supported by X-ray diffraction and small angle neutron scattering will be presented.

TRAIL-R 2 Superoligomerization Induced by Human Monoclonal Agonistic Antibody KMTR2

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The fully human monoclonal antibody KMTR2 acts as a strong direct agonist for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2), which is capable of inducing apoptotic cell death without cross-linking. To investigate the mechanism of direct agonistic activity induced by KMTR2, the crystal structure of the extracellular region of TRAIL-R2 and a Fab fragment derived from KMTR2 (KMTR2-Fab) was determined to 2.1 Å resolution. Two KMTR2-Fabs assembled with the complementarity-determining region 2 of the light chain via two-fold crystallographic symmetry, suggesting that the KMTR2-Fab assembly tended to enhance TRAIL-R2 oligomerization. A single mutation at Asn53 to Arg located at the two-fold interface in the KMTR2 resulted in a loss of its apoptotic activity, although it retained its antigen-binding activity. These results indicate that the strong agonistic activity, such as apoptotic signaling and tumor regression, induced by KMTR2 is attributed to TRAIL-R2 superoligomerization induced by the interdimerization of KMTR2

High-resolution crystal structures of the hemi-binding domain of porcine cytochrome b_5

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Mammalian microsomal cytochrome b5 has multiple electron-transfer partners that functions in various electron-transfer reactions, such as lipid unsaturation, cholesterol synthesis and drug metabolism. Mammalian microsomal cytochrome b5 consists of 134 residues, in which the N-terminal ~100 residues contain the heme-binding region located at the cytoplasmic side of the endoplasmic reticulum membrane and the C-terminal ~30 residues contain the membrane-anchoring region and the signal sequence targeting the endoplasmic reticulum membrane. Four crystal structures of the solubilized heme-binding domain of cytochrome b5 from porcine liver were determined at sub-angstrom resolution (0.76-0.95 Å) in two crystal forms for both the oxidized and reduced states. The high-resolution structures clearly displayed the electron density of H atoms in some amino-acid residues and the heme molecule. Unrestrained refinement of the bond lengths revealed that the protonation states of the heme propionate group may be involved in stabilization of the heme redox properties. The heme Fe coordination geometry did not show significant differences between the oxidized and reduced structures. However, structural differences between oxidized and reduced states were observed in the hydrogen-bond network around the axial ligand His68. The hydrogen-bond network could be important for maintaining the redox states of the heme group.

Structural determination of DNA polymerase complex from Nanoarchaeum equitans using segmental labelling and small-angle scattering techniques

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Insights into how individual domains in multidomain proteins interact can be obtained by employing small-angle neutron scattering (SANS). The use of neutrons provides the advantage to selectively highlight the structure of the functional region within a protein. This is accomplished by combining segmental labeling and contrast variation SANS. Here, we present our progress in applying this combined approach to study the changes in the structural conformation of Nanoarchaeum equitans family (Neq) DNA polymerase required to perform DNA repair. The B-type DNA polymerase Neq DNA polymerase is encoded by two separate genes: the large gene encodes the N-terminal region (Neq L) and the small gene encodes the C-terminal region (Neq S). NeqL and NeqS contains a natural split mini intein sequence that when heated to 80 °C triggers a trans-splicing reaction. This reaction causes the two extein regions to fuse and form the complete Neq DNA polymerase. In this work, using the natural protein trans-splicing process, we have successfully performed deuterium labeling of N- and C- terminal regions of form heterodimer Neq DNA polymerase. We overexpressed NeqL and NeqS gene products separately in E.coli and then purified them using affinity chromatography. NeqL and NeqS were overexpressed as soluble and insoluble proteins respectively, but NeqS becomes soluble in the presence of NeqL. In-vitro heat treatment, similar to the natural process, was used to drive the protein trans-splicing reaction to form the intact segmentally labeled Neq DNA polymerase. The low-resolution structure of the soluble NeqL subunit and contrast variation study of the heterodimer Neq DNA polymerase will be presented. The experience gained with this system will be transferable to other protein complex systems requiring segmental labeling and advanced SANS contrast variation studies.

Understanding Trichoderma reesiCel7A Mechanism of Cellulose Depolymerization through Crystallography

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Cel7A is a cellobiohydrolase responsible for the break down of cellulose into cellobiose. The efficiency of Cel7A to hydrolyze crystalline cellulose makes this enzyme key to converting biomass into biofuels. Trichoderma reesei Cel7A is comprised of a large catalytic domain (CD), a flexible linker, and a small carbohydrate-binding module (CBM). Cellulose is threaded through the long tunnel-like active site of the CD. Once the tunnel is loaded, the cellulose strand is hydrolyzed and cellobiose is released from the exit site. The tunnel of the CD is enclosed by a series of loops. We have captured previously unobserved tunnel loop conformations in a crystal structure. These findings suggest that tunnel loop movements are coordinated and play a role in the processivity cycle.

Joint X-ray/Neutron Structures of the Helicobacter pylori 5'-methylthioadenosine Nucleosidase Describe Proton Sharing and Protonation States

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5'-Methylthioadenosine nucleosidase (MTAN) catalyzes the hydrolysis of the N-ribosidic bond of a variety of adenosine-containing metabolites. In Helicobacter pylori, MTAN hydrolyzes 6-amino-6-deoxyfutalosine in the second step of the alternative menaquinone biosynthetic pathway. Because of this vital role, H. pylori MTAN (HpMTAN) is an intriguing drug target for the development of H. pylori-specific antibiotics. The proposed catalytic reaction of HpMTAN progresses through a SN1 mechanism requiring multiple proton transfer events. Of particular interest is the protonation state of residue D198, which possesses a pKa above 8.1 and functions as a general acid to initiate the enzymatic reaction. In this study we present three co-refined X-ray/neutron crystal structures of wild-type HpMTAN co-crystallized with S-adenosylhomocysteine (SAH), Formycin A (FMA), and p-ClPh-Thio-DADMe-ImmA as well as one X-ray/neutron crystal structure of an inactive variant (HpMTAN-D198N) co-crystallized with SAH. The X-ray/neutron structures allow for the visualization of exchangeable hydrogen atom positions that play direct roles in substrate recognition, transition state stabilization, and promoting enzyme turnover. These results provide support for the proposed catalytic mechanism of D198 pKa elevation through the unexpected sharing of a deuterium ion between atom N7 of the adenyl moiety and possessing unconventional hydrogen bond geometry. Additionally, the neutron structures highlight the active site features that promote transition state stabilization but highlight slight differences in these interactions that result in very different binding affinities.

DFT/MM Simulations of the L-arabinose-Xylose Isonerase Complex with MG^{2+} and Cd^{2+}

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Xylose Isomerase (XI) is an important industrial enzyme that finds several uses in production of biofuels from lignocellulosic biomass and as a way to make precursors of different antiviral and anti-cancer drugs. Though not ideal for many of these processes, structures of XI co-crystallized with L-arabinose and different metal ions provides a basis for enzyme redesign efforts. XI is a sugar isomerase that binds two Mg2+ ions in the active site. The joint x-ray/neutron (XN) structure of XI co-crystallized with L-arabinose and Cd2+ reveals a water molecule bound to M2 and a neutral Lys289 residue located near O1 of L-arabinose (Langan et al., 2014). Simulations of XI are challenging due to extensive charge transfer effects between the metal ions and coordinating residues. These challenges can be met with hybrid Density Functional Theory (DFT)/Molecular Mechanical (MM) methods. Simulations of a solvated XI-L-arabinose model containing either Cd2+ or Mg2+ with DFT/MM methods indicate that Lys289 can easily reorient to be positioned for proton transfer and ring opening while forming direct hydrogen bonding interactions with active site Asp257, consistent with Ni2+ structure. In addition and in contrast with the XI-glucose structure, Glu181 forms a low barrier hydrogen bond (LBHB) with L-arabinose as indicated by several proton transfers between the two over the course of the DFT/MM simulation. Interestingly, ordered water molecules in the active site can lead to inhibitory, non-productive proton transfer pathways or productive ones that facilitate ring opening. These results provide insight into XI redesign strategies geared towards increasing the binding of L-arabinose without adversely affecting the catalytic rate.

Neutron diffractometer for protein crystallography covering crystals with large unit cell volume at J-PARC

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The structural information of hydrogen atoms and hydration waters obtained by neutron protein crystallography is expected to contribute to elucidation of protein function mechanism as well as improvement and modification of its function. However, many proteins, especially membrane proteins and protein complexes, have larger molecular weight and then unit cells of their crystals have larger volume, which is out of range of measurable unit cell volume for conventional diffractometers. Since interests of structural biology community are increasing in such proteins, our group had designed the diffractometer which can cover crystals with large unit cell volume (target lattice length: 250 Å). This diffractometer is dedicated for protein single crystals and has been proposed to be installed at J-PARC. This proposal was accepted by Neutron Instrument Program Review Committee of J-PARC in September 2012 .

Larger unit cell volume causes a problem to separate spots closer to each other in spatial as well as time dimension in diffraction images. Therefore, our proposed diffractometer adopts longer camera distance (L2 = 800mm). In order to cover large neutron detecting area due to long camera distance, novel large-area detector (larger than 300mm × 300mm) with a spatial resolution of better than 2.5mm is under development based on the detector adopted for the diffractometer, SENJU (BL18) at J-PARC. In addition, decoupled hydrogen moderator is selected as neutron source which has shorter pulse width. Under the conditions that L1 is 33.5m, beam divergence 0.4° and crystal edge size 2mm, this diffractometer is estimated to afford the resolution ($\Delta d/d$) of 1% at the middle and high 2θ-S angles and be able to resolve spots diffracted from crystals with a lattice length of 220 Å in each axis at d-space of 2.0 Å. To increase measurement efficiency more than 40 detectors plan to be installed, providing the total solid angle coverage of larger than 33%. Ellipsoidal and curved shape were introduced in the vertical (m-value is 1.8 in maximum) and the horizontal design (m-value is 3.0 in maximum) of the guide design, respectively, which provide maximum beam divergence of 0.8° in vertical and 0.6° in horizontal, respectively, at the sample position. According to ray-tracing simulation by McStas code, this guide design provides neutron transport efficiency of 96% in vertical and 88% in horizontal, respectively, and, as a result, neutron flux at the sample position is estimated to be 5×10^7/cm^2/s in the wavelength range of 1.5 Å - 5.6 Å (first frame).

SANS Study of Moisture-Induced Changes in Chemically Treated Wood Cell Walls

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Wood-based products can be a sustainable, and more environmentally friendly alternative to traditional construction materials because of their lesser contribution to air and water pollution. Yet because wood is hygroscopic, and an integral component in these products is the adhesive; moisture-induced swelling in the cell walls near the wood-adhesive bondlines can lead to durability and performance issues. Unfortunately, researchers working towards improving the moisture-durability of forest products are hindered by an incomplete understanding of the nanoscale mechanisms that lead to moisture-induced swelling.

Because of its ability to probe structural changes in the range of 1-100 nm caused by either chemical infiltration or moisture uptake while controlling the relative humidity (RH) in situ, we use small angle neutron scattering (SANS) to learn why treatments such as acetylation or phenol formaldehyde result in forest products with improved durability. Tangential-longitudinal latewood loblolly pine samples (0.5mm thick) were acetylated or treated with phenol formaldehyde. Both deuterated and hydrogenated chemicals were used. Then they were tested using SANS and a custom-built RH chamber at the Oak Ridge National Laboratory at the EQ-SANS and Bio-SANS beamlines.

Using SANS, we are able to detect (1) an increase in the cellulose elementary fibril spacing with RH and (2) differences between chemical treatments, suggesting that both treatments are able to prevent moisture-inducing swelling by targeting different nanoscale features.

Tension wood provides insight into structural changes in biomass resulting from chemical pretreatment

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http://www.ornl.gov/science-discovery/clean-energy/research-areas/systems-biology/bioenergy/dynamic-visualization-of-lignocellulose

Project Goals: Lignocellulosic biomass comprises the vast majority of biomass on Earth and has the potential to play a major role in generation of renewable biofuels if cost-effective conversion can be achieved. Largely composed of plant cell walls, it is a complex biological composite material that is recalcitrant to the structural deconstruction and enzymatic hydrolysis into sugars that is necessary for fermentation to bioethanol. The Scientific Focus Area in Biofuels is developing "Dynamic Visualization of Lignocellulose Degradation by Integration of Neutron Scattering Imaging and Computer Simulation" for multiple-length scale, real-time imaging of biomass during pretreatment and enzymatic hydrolysis. This is providing fundamental information about the structure and deconstruction of plant cell walls that is needed to drive improvements in the conversion of renewable lignocellulosic biomass to biofuels.

Plant cell walls comprise the bulk of lignocellulosic biomass. Therefore, a greater understanding of the chemistry, architecture, physical and mechanical properties of cell walls is essential to improve biomass-based biofuel production. Here we propose to understand biomass properties underlying recalcitrance to enzyme-based sugar (ethanol substrate) release. Towards this end, the spatio-temporal progression in chemical and anatomical changes that occur in growing plants under tension stress will be investigated. An earlier neutron diffraction study of hydrogenated and partially deuterated poplar tension wood found that, unlike fibers from the ramie plant, the characteristic diffraction peaks were not present. The authors postulated that the contribution of laterally aligned microfibrils complicated the signal from the majority of the microfibrils, which are longitudinally aligned, but did not further investigate the tension wood structure. Here, we report structural studies of tension wood using small-angle neutron scattering at the Bio-SANS instrument.1 We studied a similar type of system namely tension wood in which external force such as a bending stress causes overexpression of cellulose in the stressed side (tension) while the other side is called as the opposite. We used intact microtomed samples of normal and tension wood that resulted in anisotropic scattering. Figure shows the scattering profiles; a crystalline core (Rg = 10 ~ 11 Å) is observed for normal wood.2 While, the tension side showed evidence of different levels of associations between elementary fibrils, forming different aggregate sizes. Most interestingly, these aggregates are multiples of the elementary fibril. This propensity to associate due to the application of mechanical stress (also called tension) produces identical effect on the plant nanostructure as observed during most thermochemical pretreatments, especially dilute acid pretreatment.

Reference

1. The development of cell was partially funded by the Oak Ridge National Laboratory's Center for Structural Molecular Biology (CSMB), which is supported by the Office of Biological and Environmental

Research, using facilities supported by the U.S. Department of Energy, managed by UT-Battelle, LLC under Contract No. DE-AC05-00OR22725.

2. S.V. Pingali et al., Biomacromolecules 2010, 11, 2329-2335.

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Highly resistant HIV-1 proteases and strategies for their inhibition

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About 37 million people are infected with HIV. The infection is treated via drugs that inhibit several targets such as the viral protease. Inhibition of HIV-1 protease has been successful so far, however, the virus has developed resistance to all 9 FDA approved clinical protease inhibitors. We are investigating highly resistant variants of HIV-1 protease with multiple mutations as well as alternative strategies of inhibition. One clinically isolated mutant has 20 mutations conferring extreme resistance and several orders of magnitude lower binding affinity for all FDA approved protease inhibitors. In addition to studying clinical inhibitors, we are also researching novel inhibitors derived from the scaffold of the potent drug darunavir. Protease-inhibitor interactions in the X-ray structures provide insight into the rational development of the next-generation of inhibitors that target infection with resistant HIV-1.

Application of profile fitting method to neutron time-of-flight protein single crystal diffraction data collected at the IBiX

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iBIX is a time-of-flight neutron single-crystal diffractometer for elucidating mainly the hydrogen, protonation and hydration structures of biological macromolecules. iBIX is installed at BL03 at the Materials and Life Science Experimental Facility of J-PARC in Japan. The diffractometer was installed on a coupled moderator which has more intense peak and integrated intensity but more asymmetric and wider pulse shape than a decoupled and poisoned moderators.

Intensities of the reflections from a protein crystal are relatively weak and some weak reflections are buried under the error of strong background by strong incoherent scattering of hydrogen atoms in protein crystals. Thus, the methods to determine accurate integrated intensities of weak reflections are essential

for protein neutron structural analysis. So, we tried to find appropriate fitting function, develop profile fitting algorithm for integration method and apply it to full set neutron TOF protein single crystal diffraction data by using iBIX.

As pulsed neutron shape is asymmetric, asymmetric fitting function must be used in profile fitting method. In order to determine proper fitting function, 4 asymmetric functions were evaluated using strong intensity peaks of neutron diffraction data from ribonuclease A collected at iBIX. It was shown that all 4 asymmetric functions fit well to strong intensity peaks and significant differences were not found. In order to reduce calculation time and the number of parameters, Gaussian convolved with two back-to-back exponentials was selected as a most suitable fitting function (Fig. 1). We developed test program and applied it to full set ribonuclease A and α-thrombin neutron diffraction data. Intensity statistics were calculated and joint refinements of neutron and X-ray data were carried out. In order to evaluate algorithm utility, intensity and refinement data statistics were compared to those of summation integration by using same integration region, same reflections and same initial refinement model.

In this work, we could demonstrate that profile fitting method is applicable to comparatively weak and high background TOF neutron protein single crystal diffraction data and improve data statistics.

In the future, acceralator power of J-PARC will be increased to 1MW and we will be able to collect single crystal neutron diffraction data with larger unit cell, including membrane proteins. Since iBIX was designed to measure samples with their unit cell up to around 135 Å. Problems caused by overlapping of adjacent peaks are expected. In order to solve the problems, we are trying to apply the profile fitting technique to separate overlapped peaks in the TOF direction.

New Capabilities at the Oak Ridge National Lab Center for Structural Molecular Biology

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The Bio-SANS instrument at the High-Flux Isotope Reactor is ideally suited for studies of biomacromolecules including proteins, DNA/RNA, lipid membranes and hierarchical complexes. A high-angle detector bank to be installed early Summer 2016 will increase the accessible scattering q-range 15-fold, critical for time-resolved studies of hierarchical and large biological systems. Advancements in sample environment capabilities include an improved pressure cell, a multi-position sample holder with rotational (tumbling) capability, a humidity-controlled chamber, and a flow cell. Furthermore, grazing-incidence SANS in conjunction with the humidity chamber is currently being commissioned. The sample area has also been reconfigured to provide flexibility in mounting a wider range of sample environments. Recent implementation of automated batch-processing for data reduction through open source software (MantidPlot) enhances the efficiency of the neutron scattering user experiments. In support of Bio-SANS user experiments requiring deuterium labeling for contrast variation, the Bio-Deuteration Laboratory located at the Spallation Neutron Source features a parallel minibioreactor system for methods development and a new preparative-scale bioreactor system that allow high-density cell growth with precise control and monitoring of dissolved oxygen, pH, agitation, and feeding rates.

Visualizing the Bohr effect in hemoglobin: neutron structure of equine cyanomet-hemoglobin in the R-state and comparison with human deoxy-hemoglobin in the T-state

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Hemoglobin (Hb) is one of the best-characterized macromolecules and is regarded as an ideal model for the studies of protein evolution, cooperativity, and allostery. The release and uptake of protons by hemoglobin is known as the "Bohr effect" and ionizable residues influenced by this effect are known as "Bohr groups". From a structural point of view, the transition from R- to T-state alters the local environments of potential Bohr groups. As expected, small changes in relative positions shift the pKas of ionizable residues altering the proton affinity of a particular Bohr group. Neutron crystallography provides direct visual evidence of the atomic positions of deuterium-exchanged hydrogens, enabling the accurate determination of the protonation/deuteration state of hydrated biomolecules. Comparison of two neutron structures of hemoglobins, human deoxy Hb (T-state) and equine cyanomet Hb (R-state), offers a direct observation of histidine residues likely to contribute to the Bohr effect. Previous studies have shown that the T-state N-terminal and C-terminal salt bridges appear to have partial instead of primary overall contribution. Four conserved histidine residues (αHis72[EF1], αHis103[G10], αHis89[FG1], αHis112[G19], and βHis97[FG4]) can become protonated/deuterated from R to T while two histidine residues (αHis20[B1] and βHis117[G19]) can lose a proton/deuteron. Interestingly, this is the first time that αHis103[G10], an α1:β1 interface contact, has a definitive structural linkage to the Bohr effect where NMR, H/D exchange, and calculations anticipate no differences.

A Structural, Computational, and Inhibitory Study of *Mycobacterium tuberculosis* Ag85C Covalently Modified by Ebselen Derivatives

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Mycobacterium tuberculosis (M.tb) is an ancient, yet curable disease still plaguing modern society. Due to lengthy, complicated, and expensive treatment regiments, multiple and total drug resistant strains of the infectious disease have been discovered and are increasing in number. A push to discover and develop novel antibiotics to combat drug resistant strains of M.tb is underway. One such effort led to the recent in vitro discovery of ebselen as a potent inhibitor of the M.tb Antigen 85 complex (Ag85). The Ag85 complex is composed of three homologous enzymes (Ag85A, Ag85B, and Ag85C), and plays an essential role in the biosynthesis of cell wall components unique to mycobacteria and closely related bacteria. Further in vitro experiments revealed a unique mode of inhibition through the covalent modification of a conserved, solvent accessible Cys209 residue, causing an allosteric change to helix α9. This structural change disrupts the active site by repositioning a catalytically relevant Glu228 away from the active site. X-ray crystallographic studies of Ag85C covalently modified by ebselen were conducted; however, complete electron density was lacking for the entire ebselen molecule. In an effort to optimize the original lead molecule, a small library of ebselen derivatives was synthesized. Two bulkier ebselen derivatives were selected for x-ray crystallographic studies, one containing an azide group the other possessing an adamantyl moiety. M.tb Ag85C was successfully co-crystallized in the presence of both azido and adamantyl ebselen derivatives, resulting in 2.01 and 1.30 angstrom resolution structures, respectively. Both structures displayed continuous electron difference density for the covalent linkage between the sulfur of Cys209 and the selenium from the ebselen derivative as anticipated. Additionally, electron density can be observed for all atoms of the adamantyl derivative as well as a vast majority for the azido derivative, allowing for the assessment of interactions that dictate or favor orientation of the ebselen derivatives upon covalent modification of Cys209. Additionally, a larger shift in helix α9 away from the active site was observed in both structures resulting in the repositioning of residue side chains and a greater disordering of a loop region that acts as a lid to the enzyme active site when compared to the previous Ag85C ebselen structure (PDB: 4QDU). Aside from these apparent structural changes, a notable solvent channel was observed in both structures, spanning from the active site nucleophile Ser124 to the covalently modified Cys209. These two recent structural studies of Ag85C modified by both azido and adamantyl ebselen derivatives provide insight on new strategies in the development of future Ag85 inhibitors with greater specificity.

Inhibitor development against aspartate semialdehyde dehydrogenase: X-ray crystallography, docking and molecular dynamics approaches

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Aspartate semialdehyde dehydrogenase (ASADH) functions at a critical junction in the aspartate biosynthetic pathway and represents a validated target for antimicrobial drug design. This enzyme catalyzes the NADPH-dependent reductive dephosphorylation of β-aspartyl phosphate to produce the key intermediate aspartate semialdehyde. Production of this intermediate represents the first committed step for the biosynthesis of essential amino acids in fungi and in bacteria. The absence of this enzyme in humans and other mammals will allow selective targeting of pathogenic microorganisms. We have accumulated significant structural and mechanistic information about the bacterial ASADHs, but have had only limited knowledge of their fungal counterparts. We have recently determined the structure of new fungal forms of ASADHs from Cryptococcus neoformans and Aspergillus fumigatus. The overall structure of these fungal ASADHs are similar to its bacterial orthologs, but with some critical differences both in biological assembly and in secondary structural features that can potentially be exploited for the development of species-selective drugs with selective toxicity against only certain infectious fungal organisms. In addition, ASADHs from two new fungi Histoplasma capsulatum and Blastomyces dermatitidis have now been cloned, purified and kinetically characterized. Both apo-enzyme and ligand-bound diffraction quality crystals of BdASADH have been obtained. Unlike the dimeric bacterial ASADHs, native PAGE gel has shown that fungal forms of ASADH assembly as a tetramer. A customized fragment library has been screened against several fungal ASADHs and initial hits have been identified with inhibition constants (Ki) in the low millimolar range. However, the ASADHs obtained from these fungal species shows different inhibition selectivity when compared to this enzyme from either bacterial or other fungal species. Shape based screening, high throughput docking and molecular dynamics studies with the CnASADH and AfASADH structure are being used to guide the design and development of more potent inhibitors. The long term goal of this project is to develop drugs that selectively target these fungal enzymes and block the aspartate biosynthetic pathway. This work has been supported by funding from the NIH (AI077720).

GlgE: the addition of maltose to an a-glucan through an S_N1 mechanism

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In the last few years, the number of Multiply Drug Resistant Tuberculosis (MDR-TB) cases continue to rise and Totally Drug Resistant Tuberculosis (TDR-TB) have emerged. Because of this, new drugs and drug targets to treat TB are urgently required. GlgE, the enzyme involved in the unconventional pathway responsible for the conversion of trehalose to α-glucan, was revealed to be an interesting drug target. Not only is it absent from the human proteome, but loss of this enzymatic activity has the intriguing ability to induce rapid cell death in Mycobacterium tuberculosis. GlgE catalyzes the addition of maltose to α-1,4-glucan via an SN1 mechanism. Using the homologue Streptomyces coelicolor GlgEI to gain better resolution, crystal structures of the protein in complex with an early dissociative transition state mimic as well as a late dissociative transition state mimic have been resolved at 3.2 and 2.5 Å, respectively. A third structure has also been obtained showing the last step of the reaction using maltotriose as substrate. This structure, obtained at 2.8 Å, shows the maltotriose bound in the active site revealing the location and potential residues responsible for positioning the glucan to which maltose will extend. Finally, an assay based on the reverse mechanism of GlgE has also been developed afford determination of the Ki values of the transition state mimics as well as for future inhibitor screening efforts.

Mutation of putative nucleophile Glu303 has a differential effect on kinetics and structure of the human ABO(H) blood group glycosyltransferases GTA and GTB

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Glycosyltransferases (GTs) are fundamentally important enzymes, yet the mechanism by which roughly half of them function remains unknown. GTs catalyze glycan transfer from a donor substrate, typically a nucleotide sugar, to a target acceptor, to generate an assortment of products, among them polysaccharides, glycolipids, and glycopeptides.

One class of GTs, the 'retaining' enzymes, performs glycan synthesis with net retention of product stereochemistry with respect to the donor sugar. For these GTs the mechanism of glycosyltransfer is debated, and the main candidates are double displacement with a covalent intermediate and SNi-like with an oxocarbenium ion-like intermediate (see mechanism schematic).

Retaining GTs GTA and GTB, which catalyze the final step in the synthesis of the human ABO(H) blood group A and B antigens, serve as an excellent model for mechanistic investigation. Early GTA/GTB structures suggested that active site residue Glu303 acts as the catalytic nucleophile in the double displacement mechanism and more recently, mass spectrometry studies with GTA/GTB E303C mutants yielded evidence of a covalent glycosyl-enzyme adduct. To determine the role of Glu303, we generated point mutants E303C, E303D, E303Q, and E303A for kinetic characterization and structure determination via X-ray crystallography.

Although the enzymes differ in only 4 'critical' amino acids, remarkably each mutation has a different effect on GTA compared to GTB; GTA E303C and E303D mutants retained 5 and 13 % of wild-type activity, while the corresponding GTB mutants had 25 and 1 % of wild-type activity. The crystal structures revealed a range of disorder for two interpenetrating hairpin turns (a 'double turn' motif; residues 298-303) in the active site (see figure). Our results indicate that residue 303 provides a strong local dipole, which may be more critical for catalysis than maintaining the stability of the double turn; although E303C and E303D mutants retained activity, they had disrupted active site architectures. Conversely, the GTB E303Q mutant had no activity but maintained double-turn integrity.

Based on our structures, Glu303 is positioned to act as a nucleophile in a double displacement mechanism but could also stabilize the oxocarbenium ion-like intermediate of a dissociative mechanism, and it is still unclear which is in effect. Although these structures do not distinguish between the proposed retaining mechanisms, they suggest that both double displacement and SNi-like mechanisms are feasible, which is consistent with recent quantum mechanics and molecular mechanics studies. Our findings also reveal a lack of correlation among closely related GTs GTA, GTB, α3GalT, LgtC, and BoGT6a, given that

mutation of the putative nucleophile of each of these enzymes does not have a consistent effect on double turn stability and catalysis. It may be the case that retaining GTs, even those within the same family, do not utilize a conserved mechanism. To further examine these possibilities we are undertaking an alternative approach, one that can distinguish features invisible to X-ray crystallography. This will involve neutron diffraction and structural NMR methods, which will identify the protonation state and dynamics of key active site residues, including Glu303, and resolve the ongoing mechanistic debate.

Increasing terpene content in foliage of Eucalyptus for specialty biofuels

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Terpenes are the most prominent secondary product produced by plants. Their extensive range in structures and functionality, along with highest energy density and affordable conversion makes them quite attractive proposition for further utilization into bio fuels sector. These compounds are stored in a wide variety of storage units like trichomes, idioblasts and sub-dermal ducts. In Eucalyptus species such specialized structures are known as oil glands, which contain volatile and non-volatile terpenes. These oil glands are located in the sub-dermal tissue and therefore their isolation has been difficult which compromised the integrity of the sample for characterization. In this work, we report the progress achieved in isolation and characterization of the oil glands. We employed two approaches: (1) Study of isolated oil glands; and (2) Use of models systems to mimic formation of oil glands internal architecture. In the former approach, an existing protocol was modified to extract oil glands with minimal mesophyll debris. These cleaner oil glands were characterized using confocal microscopy and resulted in significantly better clarity in the micron-level dimension of the oil glands. As part of the latter approach, the interaction energetics in the formation of supra-molecular structures with volatile and non-volatile terpenes were investigated by employing small angle neutron scattering (SANS) studies. SANS data obtained for the non-volatile/volatile system of Geranyl b-D-glucoside, GDG and α-pinene, αP were modeled to a spherical model with Schulz polydispersity in the sphere radius. The results of the model fitting established that GDG prefers to associate in aqueous solution to form micelles. On addition of αP, these GDG micelles subsequently transforms into nano αP-droplets by incorporating αP in its interior. Unraveling the energetics in the supra-molecular structures will provide the foundation for a scientifically inspired approach in genetic up-regulation of jet-fuel targeted terpenes as well as down-regulation of non-beneficial terpenes with minimal effect on the integrity of the oil gland structure and functionality.

In-Vivo Probing Lateral Organization of Lipid Membranes using SANS

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Lipid rafts are phase-separated nanoscopic domains within cellular membranes and are believed to be associated with a wide range of cellular functions such as interactions with pathogens, signal transduction, cell recognition, etc. Though lipid rafts have been visualized with lipid-soluble dyes or fluorescent protein tags, perturbing the membrane using these methods may introduce artifacts and controversy remains regarding the existence of lipid rafts in general. Another potential way to detect lipid rafts is via small-angle neutron scattering (SANS) through selectively deuterated samples. We chose the model Gram-positive bacterium, Bacillus subtilis for our studies. Initial experiments were conducted to establish contrast-match points by adapting the organism to grow in a high D2O background. Next, by using a combination of genetics, microbiology and biochemical techniques a fatty acid obligate organism was created and hydrogenated fatty acids were supplied exogenously to these cultures grown in high D2O. SANS experiments on these samples showed that the cellular membrane could be resolved using this technique. Finally, current work is focusing on using differentially labeled fatty acids to introduce lateral contrast in the membrane which, in theory, will allow phase separated nanoscopic domains, or lipid rafts, to be detected in intact cells using SANS techniques.

Analysis of the Catalytic Mechanism of Copper Amine Oxidase from *Arthrobacter Globiformis*

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Copper amine oxidase catalyzes oxidative deamination of various primary amines to the corresponding aldehydes with concomitant production of ammonia and hydrogen peroxide. The enzyme contains a covalently-bound quinone cofactor, topaquinone (TPQ), which is produced by post-translational modification of a specific Tyr residue contained in its precursor protein in the presence of Cu(II) ion and dioxygen. The catalytic reaction of amine oxidase proceeds by a Ping-Pong bi-ter mechanism, consisting of reductive and oxidative half-reactions [1]. In the former reductive half-reaction, the substrate amine reduces the TPQ to an aminoresorcinol form (TPQamr) that is in equilibrium with a semiquinone radical (TPQsq) via an intramolecular electron transfer to the copper ion.

To elucidate the structure-based reaction mechanism of copper amine oxidase, the reductive half-reaction catalyzed by the enzyme from Arthrobacter globiformis (AGAO) was analyzed by soaking of the crystals with its substrate, phenylethylamine, anaerobically. The crystals were frozen at appropriate time intervals to trap the reaction intermediates transiently formed in the crystals [2]. Before X-ray data collection, the crystals were subjected to single-crystal microspectrometry for monitoring the absorption spectrum of TPQ that reflects its chemical structure. As a result, the active center structure shifted the equilibrium toward TPQsq in an "on-copper" conformation, in which the 4-OH group ligated axially to the copper center, which was probably reduced to Cu(I). When the crystals were soaked with substrate in the presence of halide ions, which act as uncompetitive and noncompetitive inhibitors with respect to the amine substrate and dioxygen, respectively, the equilibrium in the crystals shifted toward the "off-copper" conformation of TPQamr [3]. In addition, concerted conformational changes of gate residues at the entrance of the substrate channel were observed with the progress of reductive half-reaction. These findings demonstrate that the enzyme undergoes large conformational changes during the reductive half-reaction.

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Neutron Crystallographic Studies of HIV-1 Protease: Drug Binding, Drug Resistance, and Proton Transfer

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HIV-1 protease is an essential target for the design of antiviral inhibitors to treat HIV/AIDS, and is considered a paradigm for the success of the structure-guided drug design. Current clinical drugs, however, are prone to the development of drug resistance that thwarts the treatment efforts. We are studying HIV-1 protease structure, dynamics, function and the effects of drug resistant mutations on these properties using innovative biophysical methods, including macromolecular neutron crystallography and neutron scattering, and computation, including QM/MM and MD simulations. Several room-temperature neutron structures of wild-type and drug resistant variant HIV-1 protease have been obtained in complex with clinical inhibitors amprenavir and darunavir. The structures provide direct determination of hydrogen atom positions in the enzyme active site, affording an atomic-level picture of drug-enzyme interactions. The structures reveal fewer drug-enzyme interactions than was inferred from 100K X-ray structures, and demonstrate significant differences between the contacts observed at low and room temperatures. In addition, a two-proton transfer occurring in the protease catalytic site due to long-range electrostatic effects of surface residues changing their protonation states at different pH values was captured for the first time by neutron crystallography. The proton transfer mechanism and pH effect are supported by QM/MM calculations. The structural information obtained from the neutron structures may be valuable for the design of improved protease inhibitors. Neutrons therefore represent a superb probe to obtain structural details for intermolecular noncovalent interactions and proton transfer reactions in biological systems at a truly atomic level.

Neutron Diffraction Studies of a HAD Superfamily Model Enzyme, KDN9DD

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The haloalkanoate dehalogenase superfamily (HADSF) is a large superfamily (~120,000 nonredundant sequences) of mostly phosphoryl transfer enzymes that are involved in diverse biological processes enzymes are found in all three domains of life, and share a similar nucleophilic and general acid/base catalytic mechanism involving two conserved aspartate residues. The enzyme 2-keto-3-deoxy-D-glycero-D-galacto-nonic acid-9-phosphate (KDN9P) phosphatase (KDN9PP) is a member of the HAD superfamily that dephosphorylates KDN9P, producing 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN). KDN9PP serves as an ideal model enzyme for further elucidating the HAD superfamily catalytic mechanism through neutron studies (i) because its tetrameric state allows for only the catalytically competent, closed, solvent excluded form to crystallize (ii) because it can be crystallized at a range of pH values which allows for elucidation of protonation states of the catalytic residues and (iii) because there are already high resolution X-ray crystal structures (~1.1 Å) of KDN9PP available. We have determined a 2.3 Å neutron structure of KDN9PP at pH 8.5 from Bacteroides thetaiotaomicron as well as a 2.3 Å neutron structure of unliganded KDN9PP at pH 6.5 collected at Oak Ridge National Labs (on IMAGINE). Both structures show that the general acid/base catalyst is deprotonated. These structures support the hypothesis that the aspartate residue is deprotonated in the unliganded form of the enzyme at optimum pH and then protonated upon substrate binding and catalysis. Future work aims to obtain a neutron structure of KDN9PP cocrystallized with the transition state analog complex (KDN9P and orthovanadate) at pH 6.5 to provide further support for the proposed mechanism.

Making an Aquaporin Water-tight: The Structural Basis of Selectivity of Plant Nodulin 26-Intrinsic Proteins

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The evolution of land plants led to an amplification and diversification of the aquaporin superfamily of membrane channels. Among the subfamilies of plant specific aquaporin-like changes are the nodulin-26 intrinsic proteins (NIPs) which are multifunctional transporters of water, ammonia, glycerol and metalloid nutrients that participate in a number of osmoregulatory and metabolic functions. NIPs share the canonical hourglass fold of the aquaporin family, but possess substitutions within the aromatic arginine (ar/R) selectivity filter. The nine proteins of the NIP subfamily in the model plant Arabidopsis thaliana can be subdivided into two ar/R subgroups: the NIP subgroup I, which form an aquaglyceroporins that are permeated by glycerol, water and ammonia, and the NIP subgroup II, which form metalloid transporters which are lack aquaporin activity and are essentially "water tight". These two NIP pore familie differ principally by the substitution of a conserved alanine (NIP subgroup II) for a conserved tryptophan (NIP subgroup I) in the helix 2 position (H2) of the ar/R filter. Based on transport analyses and molecular dynamics simulation, a model is proposed through which the alanine substitution results in both the selectivity for critical metalloid nutrients such as boric acid while simultaneously restricting water flow through the ar/R selectivity filter. A mechanism involving two different rotameric states of the conserved arginine residue in this selectivity region is proposed to responsible for the water-tight character of the pore. (Supported in part by NSF grant 1121465).

Crystallographic Studies of Human Acetylcholinesterase Reactivation by Oximes

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Human acetylcholinesterase (hAChE) is responsible for degrading neurotransmitter acetylcholine at synapses of the nervous system. Organophosphate (OP) nerve agents and pesticides inactivate hAChE through chemical modifications of the enzyme's active site leading to a life-threatening increase of acetylcholine (ACh) concentration. Exposure to OPs may be fatal if not treated. The current generation of antidotes is not highly efficient, the rates of reactivation are far slower than the catalytic rate of ACh hydrolysis.

Insights into the molecular structures of Torpedo and mammalian AChEs reveal the underlying limitations in enhancing reactivation rates but limited in their ability to resolve positions of hydrogens important for understanding of proton transfer in reactivation of OP inhibited AChEs. The only experimental method that can provide this information is macromolecular neutron crystallography.

In preparation for the neutron diffraction studies we have obtained several new X-ray structures of hAChE at 100 K in complex with oxime reactivators and ligands. hAChE forms well defined crystallographic dimers in reversible complexes with oximes 2PAM and RS2-170B (at 2.5 Å and 2.15 Å resolutions, respectively) and inhibitors BW284c51 and 9-aminoacridine (at 2.35 Å and 2.5 Å resolutions, respectively). In addition we have determined the first room temperature structure of hAChE:BW284c51 complex at 3.2 Å. hAChE in these complexes forms crystals in previously unseen unit cell (a=b=124.3, c=129.1 Å; space group P31) amenable to neutron crystallography. Detailed analysis of the reactivators and ligand interactions with the enzyme will be presented. (Supported by Grant 1U01NS083451 from NINDS)

Protonation States and pK_as in Xylanase II, Combining Neutron Crystallography and Molecular Simulation

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Glycoside hydrolase (GH) enzymes apply acid/base chemistry to catalyze the decomposition of complex carbohydrates. These ubiquitous enzymes accept protons from solvent and donate them to substrates at close to neutral pH by modulating the pKa values of key side chains during catalysis. However, it is not known how the catalytic acid residue acquires a proton and transfers it efficiently to the substrate. To better understand GH chemistry, we used macromolecular neutron crystallography to directly determine protonation and ionization states of the active site residues of a family 11 GH at multiple pD (pD = pH + 0.4) values. The general acid glutamate (Glu) cycles between two conformations, upward and downward, but is protonated only in the downward orientation. We performed continuum electrostatics calculations to estimate the pKa values of the catalytic Glu residues in both the apo- and substrate-bound states of the enzyme. The calculated pKa of the Glu increases substantially when the side chain moves downward. The energy barrier required to rotate the catalytic Glu residue back to the upward conformation, where it can protonate the glycosidic oxygen of the substrate, is 4.3 kcal/mol according to free energy simulations. These findings shed light on the initial stage of the glycoside hydrolysis reaction in which molecular motion enables the general acid catalyst to obtain a proton from the bulk solvent and deliver it to the glycosidic oxygen.

Structural and Dynamical Insights into Substrate Recognition by Maltose-binding Proteins in *Thermotoga Maritima*

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Bacterial periplasmic maltose-binding proteins (MBPs) are involved in the transportation of myriad sugar entities, with varied specificity, through an associated ABC transport system. The thermophile, Thermotoga maritima was found to have two closely related maltose transport operons (MalE1 and MalE2), with each containing an MBP. More recently, a third maltose transport operon was identified in T. maritima. Published literature indicated a stark difference between substrate recognition and binding by these three MBPs, nonetheless there is no clear evidence at the amino acid level that suggests a preferential binding and a mechanistic insight into this selective binding-mode. We have employed X-ray crystallography, small-angle X-ray scattering (SAXS), computational modeling and simulations to characterize the structural basis for substrate selection by different MBPs. We have performed SAXS on both apo- and ligand-bound proteins and our initial data suggests a change in the overall conformation of protein when it binds to the ligand. We are also crystallizing MalE1, MalE2 and MalE3 in ligand-bound and ligand-free form. Additionally, we have collected X-ray diffraction datasets for MalE2 with maltotetreose and MalE3 with maltose. Also, we performed a 200nanosecond computer simulation to compare the dynamics of the MalE1 in presence and in absence of ligand. The simulations have been analyzed to identify the relative sampling of different conformational states associated with the apo and ligand-bound protein. The generated datasets clearly indicate a significant restriction in dynamics and conformational sampling of protein once it is bound to the substrate molecule. This evidently bolsters our hypothesis of differential-binding of substrates depending on the size of the binding pocket and the overall dynamics of the protein, which varies significantly between the three MBPs.

X-ray and neutron crystallographic investigations into the multiple functions of Dehaloperoxidase

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The relationship between substrate binding and enzymatic function has been well established for monofunctional systems, however a similar understanding for multifunctional enzymes is lacking by comparison. Our chosen platform for the elaboration of the structural features and other determinants that impart specific discrete functions to heme proteins (and more generally to all proteins) is the enzyme Dehaloperoxidase (DHP), the coelomic hemoglobin from the marine worm Amphitrite ornata. DHP is the first globin to possess a biologically relevant peroxidase activity, and in the past three years additional activities (peroxygenase, oxidase, and oxygenase) have also been identified by our laboratory. Thus, five different functions have been found to be performed by the single heme active site of DHP. In an attempt to explain this functional coexistance, a systematic investigation of substrate binding sites for DHP is presented here. Specifically, experimentally and computationally obtained peroxygenase substrate binding sites are discussed in regard to the published peroxidase substrate binding sites. In addition, a detailed analysis of mono-, di-, and tri-halogenated (Cl, Br) phenolic binding sites is presented in correlation to their respective enzymatic functions. The comparison of ligand binding sites between inhibitors and substrates (both peroxidase and peroxygenase) will provide insight on the structural consequence of this functional coexistance in an effort to demonstrate the factors that help DHP differentiate between peroxidase and peroxygenase activities. Additionally, in regard to subtle details, DHP does possess structurally intermediate aspects between globin and peroxidase systems (i.e. heme Fe-distal His distances, proximal His rotation angle). In an effort to further understand these structural consequences, neutron diffraction data was collected on the ferric state of DHP. Neutron diffraction data provide insight on H-bonding networks, enzymatic and residual protonation sites. This analysis, in comparison to the available myoglobin and cytochrome c peroxidase neutron structures, could enhance our understanding of these subtle structural aspects on the multifunctional nature of DHP.

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